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1	ECAR	European College of Animal Reproduction	2
2	ECAWBM	European College of Animal Welfare and Behavioural Medicine	1
3	ECAAH	European College of Aquatic Animal Health	2
4	ECBHM	European College of Bovine Health Management	3
5	ECEIM	European College of Equine Internal Medicine	0
6	ECLAM	European College of Laboratory Animal Medicine	0
7	ECPHM	European College of Porcine Health Management	3
8	EPVS	European College of Poultry Veterinary Science	3
9	ECSRHM	European College of Small Ruminant Health Management	10
10	ECVAA	European College of Veterinary Anaesthesia and Analgesia	1
11	ECVCN	European College of Veterinary Comparative Nutrition	0
12	ECVCP	European College of Veterinary Clinical Pathology	1
13	ECVD	European College of Veterinary Dermatology	3
14	ECVDI	European College of Veterinary Diagnostic Imaging	1
15	ECVECC	European College of Veterinary Emergency and Critical Care	0
16	ECVIM-ca	European College of Veterinary Internal Medicine--companion animals	0
17	ECVN	European College of Veterinary Neurology	0
18	ECVO	European College of Veterinary Ophthalmology	0
19	ECVP	European College of Veterinary Pathology	0
20	ECVPH	European College of Veterinary Public Health	7
21	ECVPT	European College of Veterinary Pharmacology and Toxicology	1
22	ECZM	European College of Zoological Medicine	1
23	ECVS	European College of Veterinary Surgery	0
24	EVDC	European Veterinary Dentistry College	0
25	EVPC	European Veterinary Parasitology College	4
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History of the Hellenic Veterinary Medical Society

The Hellenic Veterinary Medical Society (HVMS) is one of the oldest Scientific Societies in our Country. It was founded in 1924 and its first scientific journal was published in 1926. Prompter, Founder and Animating Spirit of HVMS was the General Ioannis D. Petridis (1870-1947), first President and for many years Honorary President of the HVMS. Among the 49 founding members of the HVMS there was also the memorable professor Konstantinos Livadas, the founder of the Veterinary School of the Aristotelian University in Thessaloniki. In spite of the disagreements, the HVMS contributed greatly to the foundation of Veterinary School.

During that time there was only one Scientific Society in Greece, the Medical Society of Athens, which was founded in 1835 and published its first scientific journal in 1922. The HVMS dealt not only with scientific but also with professional topics, like the establishment of the invoices for the veterinarians' payment, taxes, insurance etc. Also, at that period, the accession of the Veterinary Branch in the Hygienists' Pension and Self Insurance Treasury (TSAY) was achieved.

The first post-war assembly of the HVMS took place in the private medical office of Petros Kiappe, on Peta Street in Athens. With its post-war first president Konstantinos Melanidis, the HVMS has been working by implementing its old memorandum of association and has been located in the premises of the Veterinary Microbiological Institute of Votanicos, from where all members of the Governing Board and the Editorial Board of the Journal of the HVMS, were coming from. There, the first «nucleus» of the Library of the HVMS, has been created. That is the reason, this second period of the HVMS successor of the «Petridis period», used to be called «Votanikos period, 1944-1965».

Because HVMS's income was very small, it will remain homeless for many years. Looking for a meeting place the HVMS will find positive response from several services and societies (State Veterinary Offices, Greek Chemical Society, Hellenic Agricultural Society, Medical Society of Athens, Institute of Agricultural Studies, State Veterinary Service of Athens, National Organization of Greek Handwork), which during the following years are going to offer its premises, while in the mid 1958 and for a short period, depending on its financing capabilities, the HVMS will rent its own room.

In 1944, the HVMS writes down its first post-war Member Book and in 1948 has already acquired its first 74 regular members. Also, HVMS is actively working with scientific subjects during regular meetings and public seminars, analyzing current veterinary issues, members' proposals and so on. On 29th May 1947 Mr Petridis presented in the Academy of Athens an issue for veterinary science and its contribution to the progress of the agricultural production and safeguard of Public Health. Also, it should be pointed out, that because there was no professional body, the HVMS is also dealing with issues related to the execution of the veterinary profession.

Furthermore, the role of the HVMS has been determinative on the decision making of the Ministry of Agriculture on veterinary legislation, on the organization of the Veterinary Service in the Ministry of Agriculture as well as on livestock topics. In the decade of 30s the Supreme Veterinary Advisory Council was created mainly dealing with scientific issues and other aims like promotion, publicity and consolidation of the veterinary science and the veterinary profession in our country and internationally.

The Hellenic Veterinary Medical Society publishes a quarterly scientific journal called Journal of the Hellenic Veterinary Medical Society (J Hellenic Vet Med Soc), as well as other scientific publications, organizes Congresses, Symposiums, Meetings, Lectures etc and generally and almost exclusively it has undertaken for life the Continuing Education of the Greek veterinarians and the students of the two Veterinary Schools.

Nowadays, the Hellenic Veterinary Medical Society is governed by a 9 member Governing Board which is elected every 3 years and has 3 branches:

- **Branch of Companion Animals**
- **Branch of Food Hygiene and Public Health**
- **Branch for Farm Animals**

The HVMS collaborates with the Supreme Educational Foundations, the Technological Educational Institutes, the Veterinary Services, and the Veterinary Associations as well as with Scientific Societies and the Greek and Foreign Chambers.

- **The HVMS is member of the:**
- **Worldwide Veterinary Society**
- **Worldwide Veterinary Society for Companion Animals**
- **Federation of European Veterinary Societies for Companion Animals (founding member)**
- **Veterinary Society of the Balkan and the Black Sea (founding member)**

The HVMS has a total of 1220 members many of which have been distinguished in the scientific field (University Professors, Researchers), in the Public Administration, in the Army as well as in the Professional Veterinary Societies and Chambers, in Greece and abroad.

Since 29 May 2001, having signed the contract and since 15 December 2002 the date on which the official opening celebration took place, the Hellenic Veterinary Medical Society is housed in its private premises in a beautiful and majestic one-floor apartment, on the 7th floor of a building in the centre of Athens at 158, Patission street, of 265m2 area, including main lobby (14m^2), secretary (13m^2), lecture room (91m^2), the President's office (22m^2), the Governing Board meeting room & library (44m^2), the kitchen (18m^2), two big baths, a storage room and a large veranda. Al the actions performed for possessing this new private office for the HVMS were performed during the presidency of Dr Theodoros Cl. Ananiadis and the following Governing Board:

President	: Theodoros Cl. Ananiadis†
Vice-President	: Veniamin Albalas
General Secretary	: Athanassios E. Tyrpenou
Spec. Secretary	: Konstantinos Chandras
Treasurer	: Olga Sabatakou
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The Board of Directors and the Editorial Board of the Journal of the Hellenic Veterinary Medical Society, warmly thank the reviewers that substantially contributed in the successful publication of the 68th volume 2017 of the J Hellenic Vet Med Soc, the names of which are sited below in alphabetical order:

Το Διοικητικό Συμβούλιο και η Συντακτική Επιτροπή του Περιοδικού της Ελληνικής Κτηνιατρικής Εταιρείας, ευχαριστούν θερμά τους κριτές που συνέβαλαν ουσιαστικά στην επιτυχή έκδοση του 68ου τόμου 2017 του ΠΕΚΕ, τα ονόματα των οποίων παραθέτουμε στον παρακάτω πίνακα:

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Review article
Ανασκόπηση

Conjunctival cytology assessment in dogs and cats. Sampling, diagnostic techniques and findings

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Η κυτταρολογική εξέταση του επιπεφυκότα στον σκύλο και τη γάτα: δειγματοληψία, διαγνωστικές τεχνικές και ευρήματα

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ABSTRACT. The conjunctiva provides a physical and physiological barrier against microorganisms and foreign bodies and also contributes to the ocular immunological defense. It constitutes a straightforward and accessible tissue for sampling and examination. Sampling indications include: changes in color, surface irregularities, thickening, or masses, ocular discharge and the identification of infectious organisms. Samples for conjunctival evaluation may be collected with exfoliative or abrasive techniques, aspiration, impression and conjunctival biopsy. The most commonly used and clinically useful laboratory methods for the assessment of conjunctival specimens are: microscopic examination of cytological preparations, culture and susceptibility testing, live virus isolation, polymerase chain reaction, direct immunofluorescent antigen test and histopathological examination for snip biopsies. Findings like inflammatory or neoplastic cells, cellular alterations, inclusion bodies and microorganisms, offer valuable information not only for localized ocular disorders, but for systemic diseases as well.

Keywords: conjunctiva, sampling techniques, diagnostic methods, cytology, findings, dog, cat

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ΠΕΡΙΛΗΨΗ. Ο επιπεφυκότας λειτουργεί ως φυσικός και φυσιολογικός φραγμός έναντι μικροοργανισμών και ξένων σωμάτων, συμμετέχοντας ταυτόχρονα στην ανοσολογική άμυνα του οφθαλμού. Αποτελεί ένα εύκολα προσβάσιμο βιολογικό υλικό για δειγματοληψία και εξέταση. Η συλλογή του κυτταρολογικού υλικού συνιστάται σε παρουσία οφθαλμικού εκκρίματος, ερυθρότητας ή μάζας καθώς και σε πιθανή εντόπιση παθογόνων παραγόντων. Στις τεχνικές συλλογής, ανάλογα με τις εξετάσεις που θα ακολουθήσουν και τις πιθανές επιπλοκές, περιλαμβάνονται κυρίως τα ξέσματα, η παρακέντηση με λεπτή βελόνα, τα εντυπώματα και η λήψη ιστοτεμαχίου. Οι διαγνωστικές μέθοδοι που συνήθως εφαρμόζονται είναι η μικροσκοπική εξέταση του ληφθέντος κυτταρικού υλικού, η καλλιέργεια και το αντιβιόγραμμα, η δοκιμή απομόνωσης ιού, οι μοριακές τεχνικές (PCR), η δοκιμή άμεσου ανοσοφθορισμού για την ανίχνευση αντιγόνου καθώς και η ιστοπαθολογική εξέταση των ιστοτεμαχίων. Τα ευρήματα των εξετάσεων αυτών όπως η παρουσία φλεγμονικών, νεοπλασματικών ή άλλων μη τυπικών κυττάρων, ενδοκυτταρικών έγκλειστων αλλά και η ανίχνευση παθογόνων μικροοργανισμών, συνεισφέρουν στη διάγνωση όχι μόνο παθολογικών καταστάσεων του οφθαλμού αλλά και συστημικών νοσημάτων.

Λέξεις ευρετηρίασης: επιπεφυκότας, τεχνικές δειγματοληψίας, διαγνωστικές μέθοδοι, κυτταρολογία, ευρήματα, σκύλος, γάτα

INTRODUCTION

The conjunctiva is the thin, semi-transparent mucous membrane covering the eyelids (palpebral conjunctiva), the globe (bulbar conjunctiva) and the entire third eyelid (nictitating conjunctiva). It is variably pigmented and normally appears smooth and moist. Bright, red blood vessels are apparent in non-pigmented areas, indicative of its prolific vascular supply (Maggs, 2008).

The conjunctiva plays a significant role in preventing the desiccation of the cornea and in increasing the mobility of the eyelids and the globe. In addition, it constitutes a straightforward, accessible tissue for sampling and examination, as well as a convenient site for administration of medications (Bauer et al., 1996). Interestingly enough, even though the conjunctiva is the most exposed of all the mucous membranes in the body, it does not stand unprotected. The only lymphatic drainage of the eye is situated in the conjunctiva. On top of that, beneath the upper and lower eyelids lies the conjunctival sac, where mucin is produced. Mucin provides a physical and physiological barrier against microorganisms and foreign bodies (Samuelson et al., 1984) by trapping and disposing both debris and bacteria and by providing a medium for adherence of immunoglobulins (i.e., immunoglobulin A) and microbicidal lysozymes (Nichols et al., 1983). This latter function is essential, considering that conjunctival sacs house considerable microbial flora, including many potential pathogens (Samuelson et al., 1984).

SAMPLING INDICATIONS

There are several clinical manifestations suggesting that sampling of the conjunctiva should be attempted. These include: changes in color (attributed to hyperemia, anemia, icterus or melanosis), any surface irregularities, thickening, or masses, inadequate or excessive surface moistness, conjunctival edema (chemosis), subconjunctival hemorrhage or emphysema and ocular pain (blepharospasm, rubbing) (Maggs, 2008). In addition, among the primary goals when obtaining cytological samples are the characterization of an ocular discharge (serous, mucoid or purulent), the assessment of inflammatory or neoplastic cells and the identification of infectious organisms involving these surface tissues. Finally, collection and evaluation of conjunctiva cells is encouraged in severe, progressive or recurrent conjunctival lesions and in those cases that are resistant to empirical treatment (Young, 2014).

SAMPLING TECHNIQUES

Samples for conjunctival evaluation may be collected with exfoliative or abrasive techniques, aspira-

tion and impression cytology. These methods should supply material in adequate amounts for assessment, preserve morphologic integrity and not be uncomfortable or painful to the animal. Additional requirements are operational simplicity and minimum induced-trauma (Bolzan et al., 2005).

The three techniques routinely employed when collecting surface cells are sampling with swabs, spatulas and cytology brushes. A comparison among these reveals their relative advantages and disadvantages, as shown in Table 1 (Bauer et al., 1996; Willis et al., 1997; Maggs, 2008).

Table 1. + = poor, +++ = good

Characteristic	Swab	Spatula	Cytobrush
Total Cellularity	+	+++	+++
Cellular Integrity	+++	+	+++
Cellular Distribution	+++	+	+++

Cytological assessment of the conjunctiva is preferably made from freshly derived cells. Therefore, the ocular surface should be rinsed to remove mucus and debris that often conceal the primary lesion. Prior to removing the external debris contained in the conjunctival sac, it is suggested that imprints are made in case this material holds diagnostically essential information (Young, 2014).

Swabs

Surface samples are collected by gently rolling a sterile swab across the conjunctival fornix (Fig.1). This is a simple technique and topical anaesthesia is rarely required since it is well tolerated by patients. The number of harvested cells tends to be insufficient for a thorough cytological assessment. However, cellular integrity is well preserved and cells are spread in an even monolayer (Bauer et al., 1996, Willis et al., 1997).

This technique is commonly employed for collection of microbial samples. Pre-moistened swabs, either with proper culture media or sterile saline, are more likely to yield viable organisms. Care should be taken to avoid contact of the swab with the lid margin or facial skin in order to minimize the risk of contamination (Maggs, 2008).

Spatulas

Scrapings performed with spatula produce highly cellular samples. However, the cells may clump together, making microscopic examination more difficult (Bauer et al., 1996; Willis et al., 1997). The technique involves conjunctival scraping performed gently with a flat, round-tipped spatula so as not to abrade surface cells that may be diagnostically important (Young, 2014). Conjunctival scrapings are best performed using a Kimura platinum spatula or alternatively, the blunt end of a scalpel blade (the edge closest to the scalpel blade handle) (Bauer et al., 1996; Willis et al., 1997; Bolzan et al., 2005). Swift scraping movements in the same direction until a small drop of fluid accumulates on the edge of the instrument, will harvest enough cellular material for assessment (Fig. 2). Caution is advised, so as not to rupture the globe due to manipulation during the scraping procedure. If the entire conjunctival surface is involved, sampling from the lower eyelid is preferred for convenience reasons.

Collection of conjunctival samples may require administration of topical anaesthetic and when needed, sufficient physical or chemical restraint to avoid any injury to the eye. Due to the highly vascular nature of the conjunctiva, a relatively prolonged application of a topical local anaesthetic may become necessary. This is accomplished by applying a cotton-tipped applicator soaked in proxymetacaine or proparacaine to the conjunctival surface for 20-30 seconds. After 1–2 min, a cotton-wool tip may be applied on the medial canthus to absorb any excess of anaesthetic and the inferior tear lake (Bolzan et al., 2005).

Cytobrushes

It has been described that nylon-bristled cytobrushes for collection of conjunctival cytology specimens

from veterinary patients, form more even monolayers and result in superior cell quality and yield, when compared to swab samples (Bauer et al., 1996; Willis et al., 1997; Perazzi et al., 2017). They do tend to be less cellular than those acquired by scraping but this technique is superior in safety and patient tolerance. The brush is carefully rolled over the palpebral conjunctiva after pulling down the lower eyelid (Fig. 3). A topical anaesthetic may be applied prior to sampling to ensure patient compliance.

Impression

Impression allows the obtainment of conjunctival epithelium components with a good preservation of morphologic features. However, it will not offer clinically significant advantages over scrapings or cytological brush samples, both of which should collect a more satisfactory number of cells from deeper in the epithelium and the superficial stroma. This technique concerns cells that exfoliate with ease, therefore it is better suited when investigating superficial conjunctival disease (Bolzan et al., 2005; Perazzi et al., 2017). Besides using a clean glass slide, conjunctival imprints employing filter strips have been reported in dogs (Young, 2014). The cellulose acetate filter paper in particular, is pressed firmly against the area to be sampled and then peeled away so that exfoliated epithelial cells and surface inflammatory cells are examined (Fig. 4).

Fine needle aspiration

Fine-needle aspiration is an essential method for assessing conjunctival masses. The technique, identical to the one used for skin masses at other sites, provides an excellent yield from lesions that shed cells relatively freely, especially round cell neoplasms, granulomas and abscesses. The risk of ocular penetration is avoided with adequate physical (or less commonly, chemical) restraint and by ensuring that the needle is always directed away from the globe.

Biopsy

When standard diagnostics are unrewarding, conjunctival biopsy may be performed on tissues that are too deep to be sampled with the aforementioned cytological methods, or when tissue architecture, rather than individual cellular morphology, is considered to be of value diagnostically. Good samples for histopathological evaluation offer greater amounts and often better preserved cells than does cytology and are more likely to lead to an accurate diagnosis (Young, 2014).

The area of conjunctiva to be sampled is anaesthetized, the eyelid is everted and delicately elevated using a fine-toothed forceps. A small snip biopsy of conjunctiva and subconjunctiva is then resected from its base using small tenotomy scissors (Maggs, 2008). Ocular tissues are very delicate and require smooth handling during the procedure. Normally, hemorrhage is minimal and no sutures are required. Gentle pressure may be applied to the conjunctival wound, that is usually healed without complications. An impression smear of the sample prior to fixation, may offer valuable diagnostic information until results from the histopathological examination become available (Young, 2014).

DIAGNOSTIC METHODS

Microscopic examination is one of the most important and cost-effective laboratory procedures that are often underutilized during initial diagnostic investigations. Collected samples may be suspended in a sterile solution for testing by polymerase chain reaction (PCR), assessed with direct immunofluorescent staining of a conjunctival scraping or submitted for culture. Scrapings and fluid aspirates can also be applied in a sterile manner to a pre-moistened swab, for microbiological assessment.

Microscopic examination

Exudative or exfoliative features of conjunctival specimens may supply essential data for a more informed diagnosis. Bacterial, fungal, viral, allergic, degenerative or neoplastic diseases could be determined by cytological evaluation of the conjunctiva (Naib et al., 1967; Young, 2014). Specifically microscopic examination of smears, scrapings, imprints and aspirates may assist in determining any cellular alterations and inclusion bodies and often permits direct observation of organisms, their number and morphology, as well as associated host cellular responses (Maggs, 2008). This information may be

used as a basis for initiating an appropriate treatment plan and assessing the clinical significance of subsequent culture results.

Once collected, samples are gently spread, as thinly as possible, on to a clean microscope slide. The aim is to create a monolayer of cells on the slide, with minimal disruption of cellular morphology. Air-dried slides are then stained appropriately for thorough assessment: modified Wright-Giemsa stains are used for rapid, overall screenings, while Gram stains are often selected for easier detection of smaller organisms, such as bacteria.

Normal findings

Cytological examination of specimens from normal conjunctiva reveals sheets of non-keratinized epithelial cells with large, round, homogeneous nuclei and abundant cytoplasm, possibly with melanin granules (depending on coat color) (Lavach et al., 1977; Maggs, 2008). The inner epithelial layer of the eyelid is composed of pseudostratified columnar epithelium and interspersed goblet cells. (Fig. 5 and Fig. 6) The bulbar conjunctiva is composed of stratified squamous epithelium. In most conjunctival samples, nucleated squamous cells are more numerous than columnar cells, and they appear round to cuboidal in shape (Young, 2014). Keratinized epithelial cells are uncommon. Occasionally bacteria may be seen, mainly of the gram-positive type (Lavach et al., 1977). Some white blood cells can also be seen, but basophils and eosinophils are always abnormal. The conjunctival fornix contains lymphoid tissue, however, without clinical signs of conjunctivitis the observation of lymphocytes or plasma cells among epithelial cells, is of little diagnostic importance (Young, 2014). Other routine structures found on conjunctival cytology include mucin strands or plugs.

Abnormal findings

Normal, non-keratinized epithelial cells may become keratinized following prolonged exposure associated with ectropion and lagophthalmos. Keratinization may also occur with keratoconjunctivitis sicca (KCS), vitamin A deficiency, and irradiation. An increase in the number of goblet cell occurs with KCS, chronic conjunctivitis, and vitamin A deficiency (Murphy, 1988). Chronicity also causes the epithelium to proliferate, creating folds that give it a "velvety" appearance (Maggs, 2008). An atypical cell population (other than nonkeratinized epithelial cells) with or without mitotic features may suggest neoplastic infiltration. However, chronically, multinucleated giant cells are considered a nonspecific change (Lavach et al., 1977).

Degenerative and non-degenerative neutrophils indicate acute infections (Fig 7), especially of bacterial or viral origin (Maggs, 2008). In chronic disease, neutrophils remain the predominant cell type, with an increased number of mononuclear cells (Lavach et al., 1977; Murphy, 1988). Eosinophils and/or mast cells are also indicative of eosinophilic conjunctivitis/keratoconjunctivitis, particularly when they exceed the number routinely seen in a normal peripheral blood smear. Eosinophils are also detected in parasitic infestations and allergic or immune-mediated conjunctivitis, especially in cats. Plasma cells and/or an abnormal population of lymphocytes, are more typical of reactive hyperplasia, allergic, or chronic conjunctivitis (Maggs, 2008). Plasma cells are characteristic of plasma-cell conjunctivitis. Amorphous, fibrillar hyaline-like material is commonly found in lymphocytic conjunctivitis.

Observed bacteria are often large or small cocci and less frequently rods. The dilemma is determining whether they are of primary importance or simply opportunistic.

Cytology of an ocular discharge can assist in distinguishing simple mucous from purulent material, which contains numerous bacteria and neutrophils. A serous ocular discharge in particular is due to an increase in tear production and often related to superficial irritation of the conjunctiva or cornea. Stimulation to the goblet cells may result in exudates containing mucous, which characteristically causes cells to be aligned in rows on the smear. A purulent discharge often indicates a bacterial infection (Maggs, 2008). More specifically, the neutrophilic exudate of canine conjunctivitis often contains bacteria, regardless of the primary cause. On the other hand, the exudate of feline neutrophilic conjunctivitis rarely contains bacteria. When it does, it should be considered a clinically significant finding (Young, 2014).

Fungi are commonly recovered from the eyelids and conjunctiva of normal animals, and they are believed not to be permanent floral residents of the ocular surface but evidence of random environmental exposure. Fungal hyphae stain as linear septate structures with parallel walls, branching at various angles. The presence of fruiting bodies (conidiophores) could allow speciation, while fungal detection and identification can also be achieved by fungal culture or genetic sequencing (Sparagano and Foggett, 2009). Fungal conjunctivitis is very rare in the dogs.

Even though viruses, *Mycoplasma spp*. and *Chlamydiophila spp*. are too small to be detected by means of traditional light microscopy, occasionally, distinctive inclusion bodies may be discerned, especially in acute infections (Maggs, 2008).

Canine distemper inclusion bodies (Fig. 9) may be found in the conjunctival epithelial cells after approximately six days of infection and are seen more frequently in cells originating from the nictitating membrane. However, these inclusions are scarce and are rarely discovered. Therefore, a search for them is of limited diagnostic value (Young and Taylor 2006; Young, 2014).

Feline Herpesvirus (FHV-1) infection is a common cause of feline neutrophilic conjunctivitis. Multinucleate epithelial cells may be found, but intranuclear inclusion bodies are seen rarely, if ever, cytologically (Young, 2014).

Mycoplasma spp. may be seen as clusters of small indistinct basophilic 'dots' on routinely stained smears, over the flattened surface of squamous epithelial cells or between cells (Young, 2014). There have been studies claiming cytological examination is less reliable in the diagnosis of mycoplasmosis (Hillstrom et al., 2012).

Chlamydiophila felis, an obligate intracellular organism, causes mainly conjunctivitis. The diagnosis may be confirmed by identifying intracytoplasmic inclusion bodies during the acute phase of the disease. These basophilic to slightly purple elementary intracytoplasmic bodies are found in the cytoplasm of squamous epithelial cells while they may also appear as aggregates of coccoid basophilic bodies (elementary bodies) (Hillstrom et al., 2012). In chronic conjunctivitis, intracytoplasmic organisms are present only infrequently (Hoover et al., 1978; Nasisse et al., 1993).

It should be mentioned, that inclusion bodies are not frequently discerned, and failure to detect them does not prove that these organisms are not present. Furthermore, caution is advised when differentiating such inclusions bodies from intracytoplasmic melanin granules (Maggs, 2008) (Fig. 8) while also taking into consideration that in animals treated with topical ophthalmic ointments (particularly neomycin), epithelial cells may possibly contain dense basophilic homogeneous cytoplasmic inclusions (Streeten and Streeten, 1985).

The tumor types associated with the conjunctiva are similar to those that involve the eyelids, and include the following: papilloma, sebaceous adenoma, apocrine (basal cell) adenoma or trichoblastoma, squamous cell carcinoma, histiocytoma, lymphoma, mast cell tumor, melanoma, lipoma and others (Fife et al., 2011).

Culture and susceptibility (sensitivity) testing

Microbial flora in the conjunctival sac can be divided into resident and opportunistic pathogenic organisms. Resident bacterial populations are usually isolated from bacteriologic samples of the canine conjunctiva in large numbers. They consist of non-invasive organisms that play an important homeostatic role by competing with pathogenic species for space and nutrients and also by secretion of active substances that limit their ability to colonize the ocular surface. It follows that indiscriminate use or long-term application of antimicrobials and/or corticosteroids may disrupt this balance and predispose to over-growth of pathogens (Gerding and Kakoma, 1990; Maggs, 2008; Wang et al., 2008).

Bacteriological samples should preferably be collected prior to the start of antibiotic administration; however, organisms that persevere in spite of the antimicrobial treatment are also relevant. Similarly, sampling for bacterial culture should precede the application of topical anaesthetics, due to the inhibitory preservatives they contain. On the other hand, it has been reported that it is unlikely these anaesthetic preparations may alter cultures in a clinically relevant way (Champagne and Pickett, 1995).

Results of cytological examination and bacterial culture have been compared, and found to be complementary (Massa et al., 1999). In all cases, better results are expected when sufficient material is available for assessment. Refrigeration, not freezing, of the sample will maintain the number of viable organisms when a delay in testing is anticipated. Bacteria, chlamydiae, mycoplasmas, fungi and viruses have different culture requirements. Swab type, transport medium and storage and transport conditions are factors that should be taken into consideration. For instance, *Chlamydiophila* and *Mycoplasma* require specific transport medium, as these are obligate intracellular organisms. This involves close communication between the examiner and the associated laboratory to which the sample will be sent. On top of that, the clinician should make certain that the laboratory is equipped to test antibiotics that are applied topically, since these are not routinely included in all test panels.

Cultures of normal flora tend to be represented by more than one isolate, and usually appear in light growth, often only in enrichment media. Nevertheless, culture results must be carefully interpreted because differentiation of pathogens and normal flora may often prove difficult.

Bacteria can be cultured from the conjunctival sac of about 40%– 90% of normal dogs. Gram-positive aerobes are the most commonly cultured, with *Staphylococcus spp., Bacillus spp., Corynebacterium spp.,* and *Streptococcus spp*. predominating. Predominant gram-negative isolates recovered from the conjunctival sac in 7%–8% of normal dogs are *Acinetobacter sp., Neisseria sp., Moraxella sp., Pseudomonas sp.,* and *Escherichia coli* (Gerding and Kakoma, 1990; Whitley 2000; Thangamuthu and Rathore, 2002; Prado et al., 2005; Wang et al., 2008).

Bacterial cultures from normal cats' eyes tend to yield organisms approximately half as frequently as those from dogs' eyes.Bacteria cultured from the conjunctival sac of 4%–67% normal cats are principally gram positive: *Staphylococcus sp., Corynebacterium sp., Streptococcus sp.,* and *Bacillus sp*. Predominant gram-negative isolates are *Pseudomonas sp., Chlamydiophila felis, Mycoplasma sp.,* and *Parachlamydia acanthamoebae* (Espinola and Lilenbaum, 1996; Di Francesco et al., 2004; Richter et al., 2010).

Anaerobes are rarely isolated, and susceptibility testing of anaerobic isolates is not commonly performed and may only be required with aspirates and deeper biopsies, particularly from orbital masses.

Fungal culture

Despite the fact that the normal ocular surface is home to a wide range of both commensal and transient fungal populations, detection of these organisms in a diseased eye may prompt the clinician to consider treatment with an appropriate antifungal agent. Fungal involvement is otherwise indicated when an appropriate antibacterial treatment has failed to produce the anticipated results, or when the bacterial flora has been altered, following a systemic or local immunosuppression or prolonged use of antimicrobial drugs. Material harvested by conjunctival aspirates, deep biopsies, swabs or scrapings can be submitted for fungal culture at specialist laboratories, but tends to be expensive.

The ubiquitous free-living saprophytic fungi that are most commonly found on the conjunctival surface of normal dogs and cats are *Penicillium sp., Cladosporium sp, Aspergillus sp, Alternaria sp, Fusarium sp.* and related species (Whitley, 2002; Prado et al., 2005; Wang et al., 2008).

Live virus isolation (VI)

This method confirms the presence of live virus in a collected sample. It is considered unsuitable for in-clinic use, as it is technically demanding and labor-intensive. The virus replicates on specific cell lines resulting in characteristic cytopathic effect on the cells. The most frequent ophthalmic application for viral culture or virus isolation has been the diagnosis of FHV-I (Young, 2014).VI is a sensitive and specific technique, as long as the viruses are not labile and the sample transport and cultural conditions are optimal. Swabs are collected from the conjunctival surface and then transported in viral and chlamydial transport medium (VCTM). Regarding herpesviruses particularly, it is essential to refrain from calcium alginate swabs and stains such as fluorescein and rose Bengal, due to their inhibitory

effect. Instead, use of Dacron or cotton-tipped swabs is advised. Furthermore, it should be noted that there is a possibility that the use of topical anesthetics prior to sampling may reduce sensitivity (Storey et al., 2002). The diagnosis of FHV-1 infection can be confirmed by virus isolation, however identification of FHV-1 DNA using PCR is the most sensitive and specific technique even though it poses some difficulty because of the relatively high frequency of normal cats reported to test positive for FHV-1 (Stiles et al., 1997).

Polymerase Chain Reaction (PCR)

PCR does not require the presence of viable organisms since it detects even minute quantities of DNA. However, this is similarly considered an unsuitable method for in-clinic use because it is technically demanding and it requires great care at all stages of collection, transport and testing, to guard against contamination. It should be noted that the quality of the produced results is relative to the quality of the laboratory.

Conjunctival samples are principally submitted for PCR testing to diagnose *Chlamydiophila felis*, *Mycoplasma spp*. and FHV-1. These are obligate intracellular pathogens, therefore highly cellular samples are more likely to yield positive results. Plain swabs may be used and samples can be suspended in sterile phosphate buffered saline when forwarded for testing.

The use of non invasive sampling, such as collection of conjunctival swabs as a diagnostic tool for the detection of *Leishmania* sp. DNA through PCR has recently been studied and the results showed that the technique is a sensitive and practical method and represents a good option for an early and simple diagno-



Fig 1. Sample collection by sterile swab



Fig 2. Conjunctival scraping



Fig 3. Collection of conjunctival cells by cytobrush

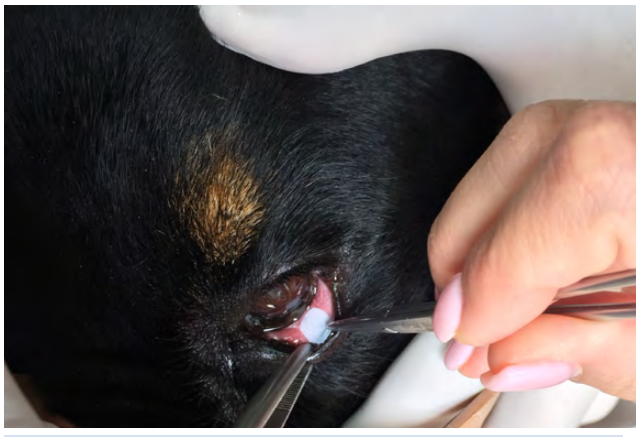


Fig 4. Use of cellulose acetate filter paper for sampling

sis of canine *Leishmania* infection in asymptomatic animals, for regular screenings of dogs and for monitoring relapses in drug-treated dogs (Lombardo et al., 2012; Geisweid et al., 2013).

The conjunctiva tends to contain relatively large numbers of bacteria, as well as fungal organisms, often part of the commensal flora of the ocular surface. Since bacteria can usually be readily cultured and standard PCR cannot distinguish transient flora from the one involved in pathogenesis of disease, PCR has infrequent application in their detection.

Direct Immunofluorescent Antigen Test

Direct Immunofluorescent Antigen testing is a diagnostic aid that can be performed on conjunctival tissue to confirm a viral or chlamydial infection (Maggs, 2008). The technique involves addition of a fluorescently labeled antibody to an air-dried cytological preparation (Fig. 5). The most common agents diagnosed with Direct Immunofluorescent Antigen Test are FHV-1, canine distemper virus (Athanasiou et al, 2018), adenovirus and *Chlamydiophila felis*. False negatives occur when an adequate sample is not obtained. Furthermore, because most of these tests use fluorescein-conjugated antibody to detect FHV-1 antigen within the submitted tissue, topical fluorescein should be avoided prior to collection.

CONCLUSION

In conclusion, sampling of the conjunctiva should be considered as an essential, non-invasive procedure that produces specimens allowing multiple diagnostic approaches and offering valuable information not only for localized ocular disorders, but for systemic diseases as well.

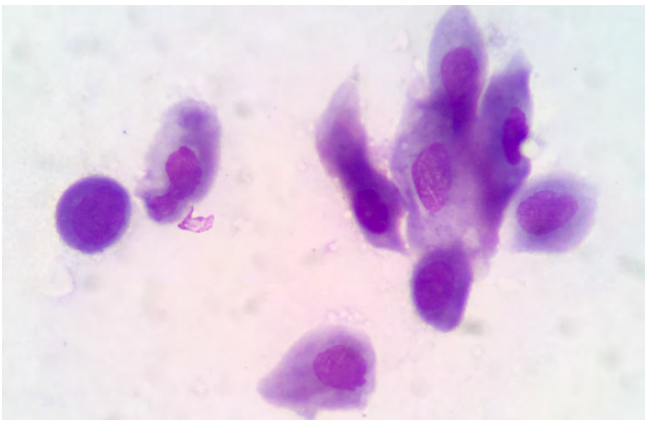


Fig 5. Pseudostratified columnar epithelial cells

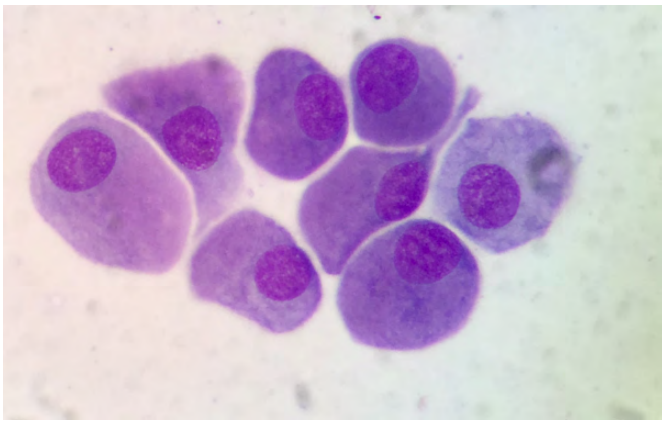


Fig 6. Conjunctiva goblet cells

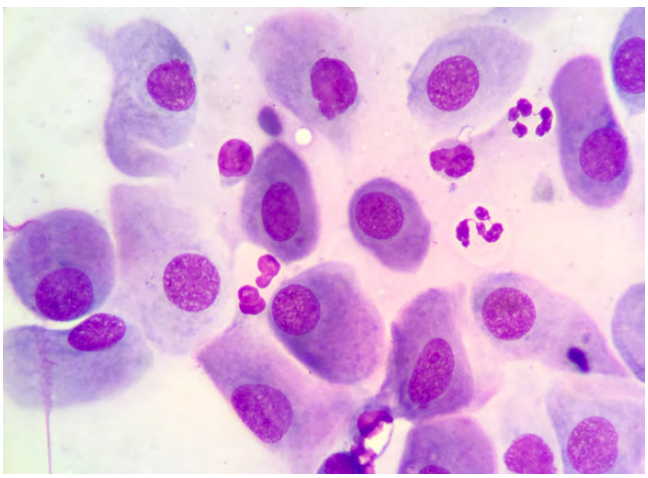


Fig 7. Presence of neutrophils in conjunctival cytological preparation

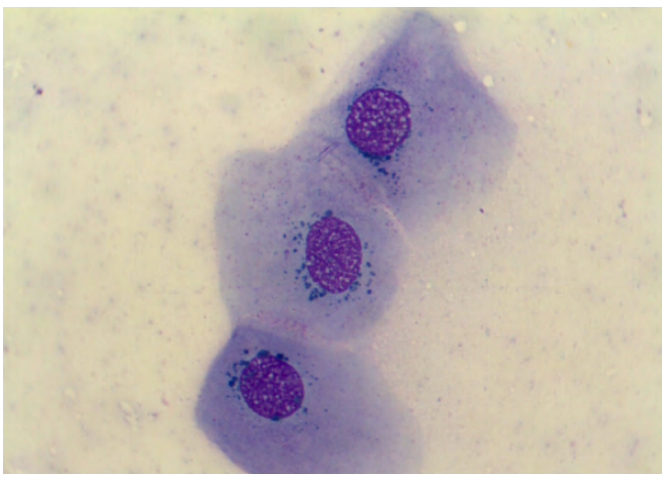


Fig 8. Melanin granules as seen in conjunctival epithelial cells

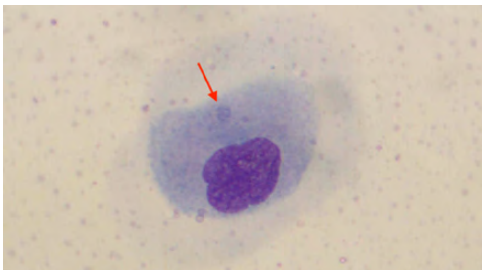


Fig 9. Goblet cell with an inclusion body in a conjunctival sample of a dog suspected of distemper virus infection

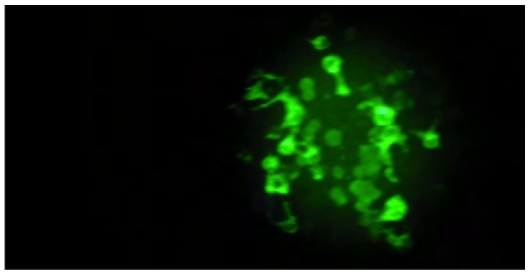


Fig 10. Antigen fluorescence of a distemper positive conjunctival sample due to labeled antibody to an air-dried cytological preparation

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Review article
Ανασκόπηση

Botanicals: a natural approach to control ascaridiosis in poultry

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Φυτοβιοτικά: Νέα φυσική προσέγγιση αντιμετώπισης της ασκαριδίωσης των πτηνών

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ABSTRACT. Parasites (protozoa, helminthes, arthropods) represent a main threat for poultry worldwide. Among helminthes, nematodes constitute the most important group of parasites of poultry. The nematode *Ascaridia galli*, the cause of ascaridiosis in poultry, is one of the most important and prevalent parasites, resulting in serious economic losses, associated with the treatment cost, the decreased feed efficiency, and the poor egg and meat production. During the past few decades the indiscriminate use of anthelmintic drugs has generated several cases of resistance in helminthes in poultry, situation which is coupled with the severity of residues in poultry products. For this reason, nowadays attention has been drawn to the use of botanicals in poultry diet, due to their anthelmintic properties. Furthermore, the dietary use eco-friend-

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ly of these plant derived substances compared to conventional synthetic anthelmintic drugs is considered as a natural and ecofriendly approach by the consumers. The focus of the present review is to recapitulate the studies, both *in vivo* and *in vitro*, that have demonstrated the anthelmintic efficacy of various dietary botanicals in controlling poultry ascaridiosis.

Keywords: Helminthes, *Ascaridia galli*, Botanicals, Poultry.

ΠΕΡΙΛΗΨΗ. Τα παράσιτα (πρωτόζωα, έλμινθες, αρθρόποδα) αποτελούν σημαντική απειλή για τα πτηνά παγκοσμίως. Μεταξύ των ελμίνθων, τα νηματώδη συνιστούν τη σπουδαιότερη ομάδα παρασίτων των πτηνών. Το νηματώδες *Ascaridia galli*, αίτιο της ασκαριδίωσης των πτηνών, είναι ένα από τα πλέον σημαντικά και συχνά παράσιτα, προκαλώντας σοβαρές οικονομικές απώλειες, που συνδέονται με το κόστος θεραπείας, τη μειωμένη αξιοποίηση της τροφής και τη μείωση της παραγωγής αυγών και κρέατος. Κατά τη διάρκεια των τελευταίων δεκαετιών, η αλόγιστη χρήση ανθελμινθικών φαρμάκων οδήγησε στην ανάπτυξη ανθεκτικότητας σε κάποια ανθελμινθικά που χρησιμοποιήθηκαν σε πτηνά, γεγονός που σχετίζεται με την παρουσία καταλοίπων στα προϊόντα των πτηνών. Για το λόγο αυτό, τα τελευταία χρόνια το ενδιαφέρον των ερευνητών έχει στραφεί στη χρήση φυτοβιοτικών στη διατροφή των πτηνών εξαιτίας των ανθελμινθικών ιδιοτήτων τους. Επιπλέον, η χρήση αυτών των φυτικής προέλευσης ουσιών σε σύγκριση με τα συμβατικά συνθετικά ανθελμινθικά φάρμακα θεωρείται ως μια φυσική και φιλική προς το περιβάλλον προσέγγιση από τους καταναλωτές. Σκοπός της παρούσας ανασκόπησης είναι η παράθεση των ερευνών, τόσο *in vivo* όσο και *in vitro*, που παρουσιάζουν την ανθελμινθική αποτελεσματικότητα διαφόρων φυτοβιοτικών στον έλεγχο της ασκαριδίωσης των πτηνών.

Λέξεις ευρετηρίασης: Έλμινθες, *Ascaridia galli*, Φυτοβιοτικά, Πτηνά.

INTRODUCTION

Parasitism with protozoa, helminthes and arthropods remains a main threat for poultry worldwide inducing heavy production losses in animals (Ahmad et al., 2013). Between helminthes, like Roundworms (Nematodes), Tapeworms (Cestodes) and Flukes (Trematodes), nematodes are the most important ones (Rafi et al., 2011). *Ascaridia galli* (*A. galli*), *Heterakis gallinarum* (*H. gallinarum*) and *Capillaria* spp. are the most common roundworms of poultry, with *A. galli* being the most prevalent (Kaufman et al., 2011). Researchers' interest in relation to alternative control measures has been focused mainly on *A. galli*. This parasite is responsible for clinical and subclinical parasitism. In heavily infected poultry the clinical signs include droopiness, diarrhea and hemorrhages. Also, during heavy infestation birds may show signs of decreased weight gain and retarded growth, due to damaged integrity of the intestinal mucosa and subsequent impaired nutrient utilization (Das et al., 2010). In more severe cases and especially in young birds, intestinal blockage may occur, leading to death (Abdelqader et al., 2008). *A. galli* infections result in serious economic losses, usually associated with treatment cost, decreased feed efficiency and poor egg and meat production (Martin-Pacho et al., 2005). Another very debilitating factor resulting in economic losses is the ability of *A. galli* eggs to act as vectors for transmission of fatal bacterial infectious organisms, such as *Salmonella enterica* and *Escherichia coli* (Permin et al., 2006).

During the past few decades, novel research on the transmission of helminthes has enabled scientists to develop efficient products for their control. However, the indiscriminate use of antiparasitic drugs in poultry has generated cases of resistance to conventional drugs, especially piperazine and benzimidazoles, such as fenbendazole and albendazole (Abdelqader et al., 2012; Yazwinski et al., 2013). This situation, coupled with the severity of the associated risks of chemical residues in poultry products and the high cost of treatment compliance in endemic regions, necessitates further efforts into the discovery of novel drugs from either natural or synthetic sources (Anthony et al., 2005).

For sustainable control of *A. galli* different approaches have been employed such as, nutrition of host animal (Das et al., 2010), utilization of genetic resistance (Kaufman et al., 2011), biological control (Braga et al., 2011), and the use of plants with promising anthelmintic activity (Anthony et al., 2005).

Attention has been drawn to the screening of botanicals for their anthelmintic properties (Anthony et al., 2005). Accordingly, the knowledge of traditional herbal remedies is scientifically examined in order to find new plants having potent broad spectrum anthelmintic activity with less toxicity (Mali and Mehta, 2008). Compared to conventional synthetic anthelmintic drugs, plant derived products are considered natural and eco-friendly. Moreover, many such products are certified as GRAS (Generally Recognized As Safe) by the FDA (Food and Drug Administration) and therefore could be ideal candidates as feed additives (Brenes and Roura, 2010; Christaki et al., 2012). The use of phytogenic bioactive compounds for poultry nematode control is increasing in different commercial production systems and it has a reduced impact on environment (George et al., 2009). Furthermore, the active components of plants are compounds with great structural diversity and low molecular weight. These components can be active against many biological processes of the parasites and this diversity can preclude the development of anthelmintic resistance (Tariq et al., 2009).

Aim of this review is to summarize the current knowledge regarding the use of plant derived substances to control *A. galli* parasitism in poultry. Under this effort their anthelmintic activities and various proposed modes of action are discussed.

BOTANICALS

Recently, strong research interest is focused on botanicals (or phytogenics or phytobiotics). Botanicals are made from plants, algae, fungi or lichens (European Food Safety Authority, 2009b). Currently, they are under examination for their various bioactive activities: improving feed intake and flavour; stimulating the secretion of digestive enzymes; increasing gastric and intestinal motility; endocrine stimulation; anticoccidial and other antiparasitic activities; antimicrobial, anti-viral, immune-stimulating, anti-inflammatory and antioxidative activity. The main active substances of botanicals are considered to be the plant secondary metabolites. Botanicals vary greatly due to the enormous variety of different plants used, the different methods used in their preparation, and their formulations (Christaki et al., 2012; Bozkurt et al., 2013).

Traditionally, the easiest way to prepare botanicals is to separate the plant part that contains the active substances (seeds, leaves, bark, etc) and then dry and grind it to powder (Christaki et al., 2012; Bozkurt et al., 2013). Nevertheless, the research focus is now on the separation and identification of their active ingredients, using gas chromatography and mass spectrometry (Brenes and Roura, 2010).

Some of the main botanical extracts are essential oils and oleoresins. Essential oils (volatile oils; ethereal oils; aetherolea) are aromatic oily liquids that originate from plants. Essential oils can be complex mixtures of many secondary plant metabolites, mainly low boiling terpenes (examples: linalool, geraniol, borneol, menthol, thujanol, citronnillol, α-terpineol), phenols (examples: thymol, carvacrol, eugenol, gaiacol), aromatic aldehydes (examples: cinnamaldehyde, cuminal and phellandral), and their alcohol, aldehyde or ester derivatives. (Brenes and Roura, 2010; Christaki et al., 2012).

Oleoresins are naturally occurring mixtures of essential oils and resins. They can be obtained from plants by extraction with a nonaqueous solvent (alcohol, ether, or acetone), followed by the removal of the solvent through evaporation. They contain volatile and non-volatile plant constituents (McCloud, 2010).

Botanicals can be produced from single plants or they can be mixtures from different plants. The idea of using mixtures is to exploit possible synergistic effects, so as to maximize the bioactive effects of different secondary metabolites and to minimize the concentrations required to achieve a particular effect (Kirkpinar et al., 2011).

The solvent of the extract also plays an important role in the activity of the botanical. Different solvents have variable physical properties such as polarity, which can affect the solubility and the activity of the plant metabolites, when the extract is ingested by the parasite or comes in contact with its surface (transcu-

ticular absorption), especially under *in vitro* experiments (Ahmad et al., 2013; Kaingu et al., 2013).

ACTIVE SUBSTANCES OF BOTANICALS WITH ANTHELMINTIC ACTIVITIES

As already mentioned, the active substances of botanicals are plant metabolites that are synthesized by plants throughout their life cycle. They are distinguished in primary and secondary metabolites (Hrcova and Velebny, 2013; Marin et al., 2015).

Plant primary metabolites such as carbohydrates, lipids, proteins and nucleic acids, are the main compounds of basic metabolic pathways and also precursors for the synthesis of the plant secondary metabolites (Hrcova and Velebny, 2013; Marin et al., 2015).

Plant secondary metabolites are organic compounds synthesized by plants that have important functions for the plant, mediating interaction with other plants or organisms; for example protection against microbial or insect attack or attraction of pollinators and seed-dispersing. Plant secondary metabolites are often colored, fragrant or flavorful compounds. Based on their biosynthetic origin and chemical structure they be divided into three wide groups: terpenes (or terpenoids), phenolics (or phenols or phenolic compounds) and nitrogen-containing compounds (Hrcova and Velebny, 2013).

MODES OF ACTION OF BOTANICALS AGAINST HELMINTHES

Various modes of action have been suggested for the botanicals although it is possible that other mechanisms are not sufficiently identified yet.

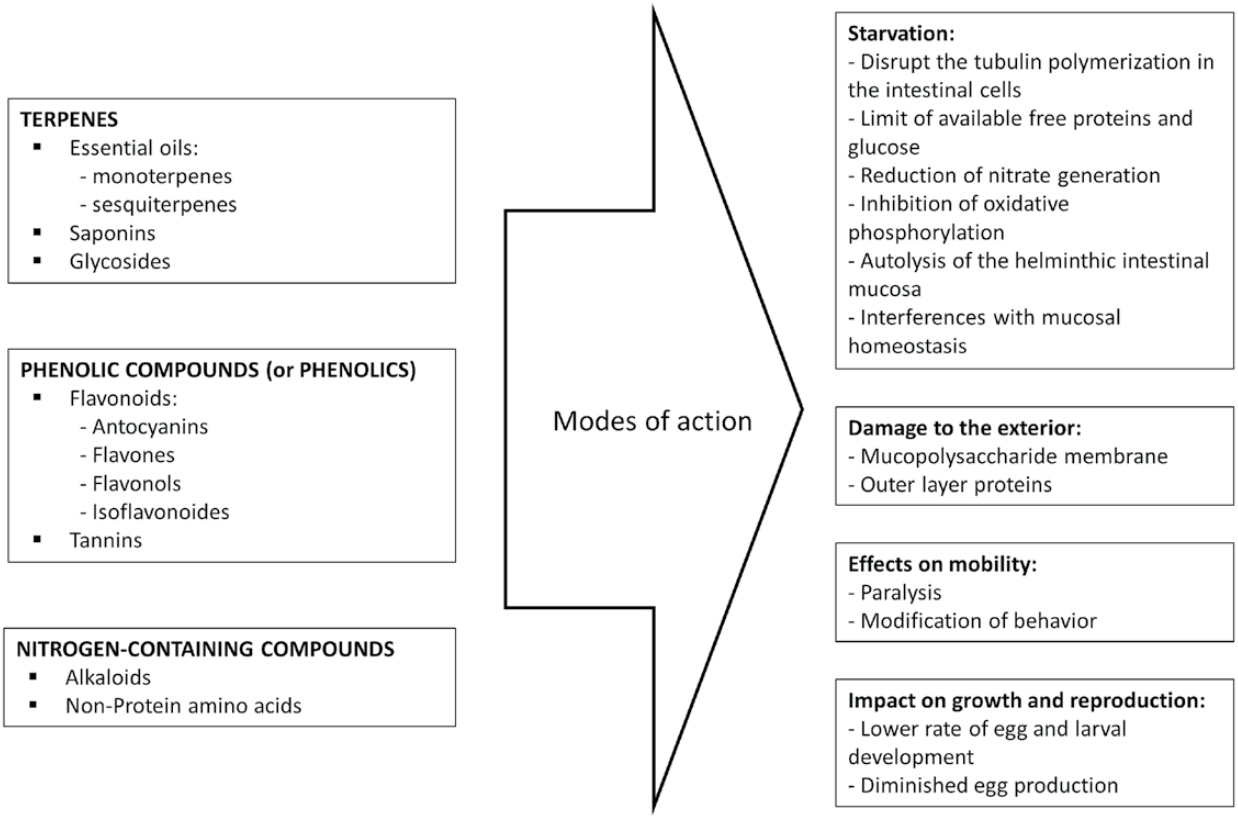
1. Antelminthic activity of plant secondary metabolites

An overview of plant secondary metabolites effects against helminthes is presented in Figure 1.

1.1. Starvation

Some monoterpenes, like ascaridole (from plant

Fig 1. Plant secondary metabolites and their modes of action against helminthes



Chenopodium ambrosioides), can disrupt the tubulin polymerization in the intestinal cells of the parasites, which leads to their degeneration and death (Wink, 2012; Jain et al., 2013). Ascaridole is effective against helminthes and has been used since the 1900s, but today its use has been limited, as it is considered mutagenic and poisonous. Also, some plant secondary metabolites separately or jointly can block glucose uptake by the parasite (Jain et al., 2013).

Tannins have the capacity to bind free protein in the digestive tract, thus limiting the nutrient availability and possibly resulting in larval starvation (Jain et al., 2013). Another possible action of tannins is the decrease in gastrointestinal metabolism directly through inhibition of oxidative phosphorylation, again leading to larval death (Athanasiadou et al., 2001; Kateregga et al., 2014). Tannins and flavonoids could inhibit energy production in the parasite cells by blocking phosphorylation reactions (Sharma and Prasad, 2014). Tannins may also bind to free protein in the gastrointestinal tract, as well as to proteins of the cuticle of parasite, thus limiting the ability to absorb nutrients. Condensed tannin ingested by the larvae of nematodes can bind to their intestinal mucosa and cause autolysis (Schultz, 1989; Athanasiadou et al., 2001).

Steroidal alkaloids and oligoglycosides can limit the amount of sugars, such as sucrose that reach the small intestine from the stomach, as well as affect the generation of nitrates (Borba et al., 2010). These effects modify the local conditions in the intestine, making them unfavorable for the development of intestinal parasites (Borba et al., 2010).

Likewise, saponins can restrict feed intake, limiting the available nutrients for the helminthes and possibly causing their death (Kateregga et al., 2014).

Also, it has been hypothesized that some plant extracts induce an inflammatory response in the gastric and intestinal mucosal of the host, which disrupts the local homeostasis that is necessary for the development of the helminthes (Borba et al., 2010). In addition, it has been suggested that some plant secondary metabolites play an important role in the regulation of the carbohydrate metabolism (Bazh and El-Bahy, 2013). In this case, it is speculated that the branch point of phosphoenolpyruvate carboxykinase/pyruvate kinase forms the basis of the anthelmintic attack by the plant derived components (Bazh and El-Bahy, 2013).

1.2. Damage to the helminth cuticle

Any damage to the mucopolysaccharide membrane of the parasite results in movement restriction and possibly in paralysis (Chandrashekhar et al., 2008; Jain et al., 2013). The binding effect of tannins on the cuticle results in the loss of its flexibility due to hydrogen bonding. This type of reactivity may lead to increased toughness of the cuticle and hence the helminthes become immobile and nonfunctional. Larvae are then unable to burrow into the mucosal lining of the small intestine of the host, and are subsequently eliminated from the host (Salhan et al., 2011; Jain et al., 2013).

1.3. Effects on mobility

Alkaloids may act on central nervous system of the parasite and cause paralysis of helminthes (Roy et al., 2010; Jain et al., 2013). Alkaloids pelletierine from *Punica granatum* (Lythraceae) and arecoline from *Areca catechu* (Arecaceae), target acetylcholine receptors as competitive antagonists and can cause paralysis of the helminthes (Wink, 2012). The anthelmintic activity of alkaloids has been demonstrated in two rat nematodes; *Strongyloides ratti* and *Strongyloides venezuelensis* (Kateregga et al., 2014). Paralyzed parasites in the digestive tract are unable to remain adhered on the intestinal wall of the host and are removed through peristalsis. It is also possible that these parasites starve to death (Wink, 2012).

Some terpenes, such as thymol and carvacrol, are neurotoxic to nematodes and interact with Ser-2, a *Caenorhabditis elegans* tyramine receptor (Lei et al., 2010; Kaplan et al., 2014). In nematodes, tyramine is believed to play a role in foraging behavior and pharyngeal pumping (Rex et al., 2004). Tyramine is a nonpeptidic hormone that can only be found in invertebrates and thus it is considered an important substance in antiparasitic research (Klowden, 2007).

Phloroglucinols, such as aspidin, deaspidin, and filixic acid, which can be found in *Dryopteris filix-mas* (*Dryopteridaceae*) can paralyze helminthes, and this effect is more pronounced on cestodes (Murthy et al., 2011; Wink, 2012).

1.4. Impact on growth and reproduction

It is possible that, tannins directly or indirectly reduce the number of hatching eggs, the rate of larvae development and the number of eggs produced from adult parasites (Athanasiadou et al., 2001; Van Krimpen et al., 2010), via not sufficiently identified mechanisms. It has been proposed that substances with hormonal effect, such as triterpenes, disrupt the reproductive cycle of the parasite. These effects have been investigated for some plants, for example for the genus *Artemisia*, against the nematodes *Ascaris suum* of pigs, as well as *Toxocara* spp. of carnivores and the cestodes *Moniezia* spp. of ruminants (Githiori, 2004; Van Krimpen et al., 2010; Acton, 2012).

It is possible that the secondary metabolites affect helminthes variously depending on different stages of their development. Also, a main factor that contributes to the anthelmintic activity of the secondary metabolites is the conditions in the digestive tract. For example, the formation and disassociation of complexes between proteins and tannins is greatly affected by the *pH*. Complexes between condensed tannins and protein remain stable in *pH* between 5 and 7, but they disassociate in *pH* higher or lower than the above (Athanasiadou et al., 2001). Also, the presence of surfactants, such as bile acids, has been reported to be important for the disassociation of tannin–protein complexes.

Further research is required in order to identify additional modes of action of botanicals against helminthes of poultry, as well as the possible interactions between major and minor bioactive components.

2. Additional beneficial activities of plant secondary metabolites

Botanicals can have additional effects that benefit poultry health and performance such as antimicrobial activity (Christaki et al., 2012), immunomodulatory effects (Anthony et al., 2005), antioxidant activity (Christaki et al., 2012), anti-inflammatory properties (Borba et al., 2010) and appetite and digestion enhancing effects (Borba et al., 2010).

IN VIVO AND *IN VITRO* STUDIES

Several up-to-date studies have demonstrated the anthelmintic efficacy of different botanicals and most of them evaluate the effect of plants against *A. galli* in chicken (*Gallus gallus domesticus*). It has been reported that botanicals exert similar anthelmintic activity both *in vivo* and *in vitro*, usually in a concentration and time-dependent manner (Alawa et al., 2003; Adedapo et al., 2007). In a number of studies, the anthelmintic effects of botanicals against *A. galli* in poultry were compared to conventional antiparasitic drugs, such as albendazole, levamisole, piperazine, etc, suggesting that botanicals can partially or totally substitute those reference drugs (Akhtar and Riffat 1985, Al-Harbi 2011, Bazh and El-Bahy 2013). It is notable that in most cases the efficacy of botanicals was adequate, although not up to par, with the anthelmintic drugs. Since testing biological activity under *in vivo* conditions has several difficulties, such as the inherent features of animals and self-cure phenomenon, most of the studies refer to *in vitro* screenings of the anthelmintic efficacy of different botanicals (Sandoval-Castro et al., 2012).

1. *In vivo* studies

Under *in vivo* studies when botanicals were supplemented, either in the water or in the feed, they depressed the fecal egg count of *Ascaridia galli* as well as reduced the adult worm burden in parasitized poultry (Table 1).

In a study with Lohmann Leghorn chicks a mixture of ethanol extracts from orange (*Citrus x sinensis*), lemon (*Citrus x lemon*), and mandarin (*Citrus reticulata*), was added in the feed (at 300, 600 or 1200 mg/kg of b.w.) and a significant dose dependent reduction in fecal egg output and parasitic worm burden was recorded (Abdelqader et al., 2012). Moreover, *Melia azedarach* fruit (powder at 20 mg/kg of b.w. or equivalent amounts of water extract, methanol extract or ethanol extract) were found to inhibit *A. galli* egg development in chickens infected with the parasite (Akhtar and Riffat, 1985). Likewise, it was shown that *A. galli* challenged cockerels exhibited a dose dependent reduction in fecal egg count, when treated with graded doses of ethanolic extract (100, 200 and 400 mg / kg b.w.) from the bark of *Piliostigma thonningii* (Asuzu and Onu, 1994). In addition, it has been demonstrated that *Caesalpinia crista,* known as karanjwa, when administered to broilers as seed powder (at 30, 40, and 50 mg/kg of b.w. or as equivalent

Table 1. *In vivo* anthelmintic activities of various botanicals against *Ascaridia galli*.

Plant name	Plant part used	Examined form and dosage	Source of *A. galli* worms	Effect against *A. galli*	References
Citrus x sinensis *Citrus x lemon* *Citrus reticulata*	Peels Peels Peels	Dietary addition of mixtures at 300, 600 and 1200 mg/kg of body weight	Chickens	Fecal egg reduction; Worm motility inhibition	(Abdelqader et al., 2012)
Melia azedarach	Fruit	Fruit powder at 20 mg/kg; Equivalent water extract, methanol extract, ethanol extract	Chickens	Egg development inhibition	(Akhtar and Riffat, 1985)
Piliostigma thonningii	Bark	Ethanolic extracts at 100, 200 and 400 mg / kg body weight	Chickens	Fecal egg reduction	(Asuzu and Onu, 1994)
Caesalpinia crista	Seeds	Powder and methanolic extracts at 30, 40 and 50 mg/kg body weight	Chickens	Fecal egg reduction	(Javed et al., 1994)
Tephrosia vogelli *Vernonia amygdalina*	Leaves Leaves	Water extracts. Doses not mentioned	Chickens	Fecal egg reduction; Reduction of adult worms population	(Siamba et al., 2007)
Punica granatum	Peel	Dry peel at 0.5, 1.0 and 1.5 g/kg of body weight	Laying hens	Fecal egg reduction Increased packed cell volume, total serum proteins, body weight	(Sabri, 2013)
Azadirachta indica	Leaves	Aqueous extract at 200 mg / kg of body weight	Chickens	Increased bird body weight; Parasite death	(Khokon et al., 2014)

amounts of water and methanol extracts) reduced *A. galli* egg numbers in chicken faeces, analogously to piperazine (Javed et al., 1994). Also, the extracts of *Tephrosia vogelli* and *Vernonia amygdalina* (doses not mentioned) not only significantly depressed fecal egg output, but also reduced the adult worm population in *A. galli* parasitized poultry (Siamba et al., 2007). Another study reported that *Punica granatum* dry peel, orally administered to infected hens at 0.5, 1.0 and 1.5 g/kg of b.w., reduced fecal egg count, analogously to levamisole, while increasing hematocrit (packed cell volume, PCV), total serum proteins and body weight (Sabri, 2013). Furthermore, neem (*Azadirachta indica*) leaves extract was supplemented to chickens at 200 mg/kg of b.w., leading to a significant increase in body weight (Khokon et al., 2014).

2. *In vitro* studies

Researchers have evaluated different plant extracts under *in vitro* conditions against *A. galli* collected from freshly slaughtered poultry. The commonly observed anthelmintic effects included paralysis and death, as well as inhibition of egg and larvae development (Table 2).

Bazh and El-Bahy (2013) revealed that when living worms were incubated at 37 °C in media containing ginger (*Zingiber officinale*) and curcumin (*Curcuma longa* L) methanolic extracts at three concentration levels (25, 50 and 100 mg/ml), their physical activity (spontaneous movement) as well as their survival was reduced in a concentration and time dependent manner. Likewise, Lal et al. (1976) demonstrated

that extracts from *Carica papaya* seeds (alcohol extract at 25 mg/ml*), Sapindus trifoliatum* fruit pericarp (alcohol extract at 10 mg/ml)*, Butea frondosa* seeds (alcohol extract at 200 mg/ml) and *Momordica charantia* fresh juice (alcohol extract at 100 mg/ml) caused paralysis and death of *A. galli* procured from fowls. Additionally, crude alcohol and aqueous extracts of seeds of *Cleome viscosa* exhibited considerable dose-dependent antiparasitic results (10, 50, 100 mg/ml) against *A. galli* worms (Mali et al., 2007). Kosalge and Fursule (2009) recorded the paralysis and death of *A. galli* and *Raillietina* spp. when administering the aqueous extract of *Thespesia lampas* roots at concentrations of 10, 20 and 50 mg/ml, and thus proposed that this extract can be used effectively as an anthelmintic. Another similar study showed that the methanolic extract (10, 25, 50 mg/ml) of *Cymbopogon citratus* leaves displayed better antihelmintic efficacy against *A. galli* in terms of paralysis and death, than the aqueous extract (10, 25, 50 mg/ml) of the same plant (Gore et al., 2010). Al-Harbi (2011) comparing aqueous solution of dried *Artemisia abssinthium* leaves and powder suspension of dried *Lepidium sativium* seeds with levamisole against *A. galli*, found that all of them have the same effectiveness against this parasite, causing paralysis and subsequently death after a period of exposure.

Kundu et al. (2012) revealed the broad wormicidal (paralysis and death) *in vitro* activity of *Cassia alata*, *Cassia angustifolia* and *Cassia occidentalis* (crude ethanol extracts at 10, 20 and 40 mg/ml) against various parasites (trematode *Catatropis* spp., cestode *Raillietina tetragona* and nematode *H. gallinarum*) collected from domestic fowl. The observed effects could be attributed to the large amount of alkaloids, flavonoids, glycosides, tannins that these plants are known to contain (Hossain et al., 2012). Another study revealed similar effects for the ethanolic extract of the leaves of *Eupatorium triplinerve* (50 and 100 mg/ml) and of the rhisome of *Alpinia galangal* (100 mg/ml) against *A. galli*. The activity of these plants was comparable to albendazole and can be attributed for *A. galangal* to the many flavonoids that it contains, such as kaempferide, kaemperol, galangin and alpinin, whereas for the crude extracts of *E. triplinerve* to the presence of phenolic compounds and coumarins (Charles et al., 1992; Subash et al., 2012). Likewise, it was demonstrated that various concentrations of crude hydroalcoholic extracts (25 and 50 mg/ml) and aqueous extract (50 mg/ml) from *Mentha longifolia* leaves resulted in concentration and time dependent paralysis and death of *A. galli* (Ahmad et al., 2013). Also, aqueous (25, 50, 100 mg/ml) and ethanol extracts (10, 25, 50 mg/ml) of *Azadirachta indica* leaves, *Carica papaya* seeds and *Momordica charantia* bark, caused cessation of motility and increased mortality of *A. galli* (Shah Alam et al., 2014), comparable to the effects of levamisole and piperazine. Moreover, it has been reported that the extracts of *Tephrosia vogelli* and *Vernonia amygdalina* (doses not mentioned) can inhibit larvae mobility (Siamba et al., 2007).

In another study, *A. galli* treated with different concentrations (5, 10, 20 mg/ml) of *Acacia oxyphylla* methanol extract demonstrated extensive structural alterations, such as rupture of the ovaries and deformity on the egg membranes, detachment of the cuticle, disintegration of the muscular layers of the nematode and subsequent death (Lalchhandama, 2008). The wormicidal *in vitro* potential of leaves and fruits of aqueous extracts of *Sesbania grandiflora* and *Solanum torvum* (10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, and 130 mg/ 100 ml) was proven against *A. galli* from laying hens (Jothi Karumari et al., 2014). Furthermore, Kateregga et al. (2014) revealed that the use of *Cassia occidentalis* methanolic leaf extract (8, 12, 16, 20 and 24 mg/ml) caused the death of *H. gallinarum* and *A. galli*. The main phytochemicals of *C. occidentalis* leaves are saponins, flavonoids, terpenes, sterols, alkaloids and tannins (Muyibi et al., 2000). Likewise, another study demonstrated that a mixture of ethanol extracts from orange (*Citrus x sinensis*), lemon (*Citrus x lemon*), and mandarin (*Citrus reticulata*) at 50 mg/ml has potential wormicidal properties against *A. galli in vitro* (Abdelqader et al., 2012). Similarly, neem (*Azadirachta indica*) leaves aqueous extract, in a range of concentrations (1, 2, 4 and 20 mg/ml), presented sufficient anthelmintic effect, causing death of *A.galli* worms (Khokon et al., 2014). In addition, Kaushik et al. (1981) evaluated 11 plants extracts (*Amomum aromaticum* root and rhisome, *Ammora wallichii* stem, *Anthocephalus indicus* stem and bark, *Calamintha umberosa* plant, *Dalbergia latifolia* stem

Table 2. *In vitro* anthelmintic activity of various botanicals against *Ascaridia galli* from freshly slaughtered poultry.

Plant name	Plant part used	Examined form and dosage	Effect against *A. galli*	References
Zingiber officinale *Curcuma longa* L.	Root Root	Methanolic extracts at 25, 50 and 100 mg/ml.	Paralysis; Death	(Bazh and El-Bahy, 2013)
Carica papaya, *Sapindus trifoliatum* *Butea frondosa* *Momordica charantia*	Seeds Fruit pericarp Seeds Fresh juice	Alcohol extract at 25 mg/ml Alcohol extract at 10 mg/ml Alcohol extract at 200 mg/ml Alcohol extract at 100 mg/ml	Paralysis; Death	(Lal et al., 1976)
Cleome viscosa	Seeds	Crude alcohol and aqueous extracts at 10, 50 and 100 mg/ml	Paralysis; Death	(Mali et al., 2007)
Thespesia lampas	Roots	Aqueous extracts at 10, 20 and 50 mg/ml.	Paralysis; Death	(Kosalge and Fursule, 2009)
Cymbopogon citrates	Leaves	Methanolic and aqueous extracts 10, 25 and 50 mg/ml	Paralysis; Death	(Gore et al., 2010)
Artemisia abssinthium *Lepidium satirium*	Leaves Seeds	Aqueous solution and powdered suspension. Dose not mentioned	Paralysis; Death	(Al-Harbi, 2011)
Cassia alata *Cassia angustifolia* *Cassia occidentalis*	Leaves	Crude ethanol extracts at 10, 20 and 40 mg/ml	Paralysis; Death	(Kundu et al., 2012)
Eupatorium triplinerve *Alpinia galangal*	Leaves Rhisome	Ethanolic extracts at 50 and 100 mg/ml	Paralysis; Death	(Subash et al., 2012)
Mentha longifolia	Leaves	Crude hydroalcoholic extracts at 25 and 50 mg/ml; Crude aqueous extract at 50 mg/ml	Paralysis; Death	(Ahmad et al., 2013)
Azadirachta indica *Carica papaya* *Momordica charantia*	Leaves Seeds Bark	Aqueous extracts at 25, 50 and 100 mg/ml; Ethanol extracts at 10, 25 and 50 mg/ml	Paralysis; Death	(Shah Alam et al., 2014)
Tephrosia vogelli *Vernonia amygdalina*	Leaves Leaves	Water extracts. Doses not mentioned	Paralysis	(Siamba et al., 2007)
Acacia oxyphylla	Bark	Ethanol extracts at 5, 10 and 20 mg/ml	Body structural alterations; Death	(Lalchhandama, 2008)
Sesbania grandiflora *Solanium torrum*	Leaves, fruit Leaves, fruit	Aqueous extracts at 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120 and 130 mg/ 100 ml	Death	(Jothi Karumari et al., 2014)
Cassia occidentalis	Leaves	Methanolic extracts at 8, 12, 16, 20 and 24 mg/ml	Death	(Kateregga et al., 2014)
Citrus x sinensis *Citrus x lemon* *Citrus reticulata*	Peels Peels Peels	Mixture of ethanol extracts of the three plants at 50 mg/ml	Death	(Abdelqader et al., 2012)
Azadirachta indica	Leaves	Aqueous extracts at 1, 2, 4 and 20 mg/ml.	Death	(Khokon et al., 2014)
Amomum aromaticum *Ammora wallichii* *Anthocephalus indicus* *Calamintha umberosa,* *Dalbergia latifolia* *Datura quercifolia* *Datura metel* *Ficus religiosa* *Sentia myrtina* *Sumplocos crataegoides*	Root, rhizome Stem Stem, bark Plant Stem, bark Fruit Plant Stem, bark Plant Leaves	–	Death	(Kaushik et al., 1981)
Azadirachta indica *Carica papaya* *Momordica charantia* *Polygonum hydropiper* *Swietenia macrophylla*	Leaves Leaves Leaves Leaves Leaves	Fresh juice at 5%, 10% and 20%; Aqueous extract at 1%, 2% and 4%; Ethanol extract at 1%, 2% and 4%; Methanol extract at 1%, 2% and 4%; Powder at 10% and 20%	Inhibition of egg development	(Islam et al., 2008)
Aloe secundiflora	Leaves	Hexane, ethylacetate, acetone, methanol, and chloroform extracts at 5, 10, 20, 40 and 50 mg/ml	Inhibition of larvae development	(Kaingu et al., 2013)

and bark, *Datura quercifolia* fruit, *Datura metel* plant, *Ficus religiosa* stem and bark, *Sentia myrtina* plant, and *Sumplocos crataegoides* leaves) which were all proven lethal to *A. galli*.

Some *in vitro* studies reported other anthelmintic effects of botanicals against *A. galli*, such as inhibition of egg and larvae development. Particularly, fresh juice (at 5, 10 and 20%), aqueous extract (at 1, 2 and 4%), ethanol extract (at 1, 2 and 4%), methanol extract (at 1, 2 and 4%), and powder (at 10 and 20%) of leaves of *Azadirachta indica, Carica papaya*, *Momordica charantia*, *Polygonum hydropiper*, and *Swietenia macrophylla* inhibited the development of *A. galli* eggs, with *C. papaya* showing the highest efficacy 92% when the concentration was 4% (Islam et al., 2008). Another experiment using larvae development assays of *A. galli* revealed that different types of extracts (hexane; ethylacetate; acetone; methanol; chloroform) of *Aloe secundiflora* at 5, 10, 20, 40 and 50 mg/ml have inhibitory effects on the parasite's development (Kaingu et al., 2013).

CONSTRAINTS OF USING BOTANICALS AS ANTHELMINTIC AGENTS

One important problem with botanicals is the difficulty to characterize and standardize their ingredients and composition. Many factors can influence the chemical composition of the plants, such as species, subspecies, geographical location, harvesting and the collected part, such as seeds, leaf, root or bark (Christaki et al., 2012; Bozkurt et al., 2013). Also, the processing technique (cold expression, steam or alcohol distillation, extraction with non-aqueous solvents, etc.) can modify the active substances and associated compounds in the final product (Tariq et al., 2009; Windisch et al., 2009).

Another important consideration is that botanicals may also have adverse or toxic side effects for the treated animals. It has been demonstrated that plant substances, which interfere with parasite development, such as steroidal alkaloids, may also exhibit toxic effects on animal tissues. These effects include mutagenicity, embryotoxicity, hepatotoxicity, central nervous system symptoms, cardiac arrhythmia, etc (European Food Safety Authority, 2009a; Borba et al., 2010). Therefore, further studies are required to evaluate plant substances with possible detrimental effects for the animal, as well as to carefully quantify the optimal beneficial doses, versus the potential harmful ones (Wu et al., 2004; Bozkurt et al., 2013). Additionally, residue studies should be required before botanicals can be safely integrated in poultry management system.

CONCLUSION

The diversity of botanicals provides an important source of bioactive compounds, which may lead to potential new candidates remedies of natural origin against ascaridiosis of chickens. Keeping in view the economic importance of the parasitic infections in the development of profitable poultry industry, the anthelmintic properties of botanicals represent a very promising alternative solution to overcome current treatment inadequacies.

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Research article
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The Effect of Lyophilized Pomegranate Extract on Epididymal Sperm Quality, Oxidative Stress and Spermatogenic Cell Density in Rabbits

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ABSTRACT. The aim of this study was to investigate the effect of lyophilized pomegranate extract on spermatological features, pathology of testes and total antioxidant/oxidant status in rabbits. Adult male rabbits were divided into four groups containing six rabbits each. For 8 weeks, Group I received standard diet and 1 ml % 0.5 carboxymethyl cellulose (CMC), Group II received 25 mg/kg/day pomegranate extract + 1 ml % 0.5 carboxymethyl cellulose (CMC), Group III received 50 mg/kg/day pomegranate extract + 1 ml % 0.5 CMC and Group IV received 100 mg/kg/day pomegranate extract + 1 ml % 0.5 CMC. Rabbits were sacrificed by using xylazine 5 mg/kg+ketamine 35 mg/kg anaesthesia and were euthanized by 150 mg / kg intraperitoneal thiopental sodium at the end of the eighth week. Spermatozoon motility, abnormal sperm rate, sperm membrane integrity, total antioxidant/oxidant level and spermatogenic cell density were investigated. All analyses were done only once at the end of study period. Data were compared by analysis of variance (ANOVA) and the degree of significance was set at ($p<0.05$). Sperm motility and membrane integrity increased significantly ($p<0.05$) in groups II, III and IV; abnormal sperm rate decreased significantly ($p<0.05$) in groups III, IV; total oxidant status decreased significantly ($p<0.05$) in group IV in comparison to the control group. Seminiferous tubule diameter increased significantly ($p<0.05$) in all groups compared to the control group. Germinal cell layer thickness significantly increased ($p<0.05$) in group IV compared to the control group. Results of this study suggest that 50 mg/kg/day + 1 ml % 0.5 CMC and 100 mg/kg/day + 1 ml % 0.5 CMC improve sperm parameters in rabbits.

Keywords: Oxidative stress, Pomegranate extract, Rabbit, Sperm characteristics, Spermatogenic cell density

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INTRODUCTION

As a member of the Punicaceae family, pomegranate has been known since the ancient era. It is emphasized in religious scripture as the symbol of abundance, productivity and chance, while its Latin name is Punica granatum which is the derived from Pomum (apple) and Granatus (seedy). Pomegranate (Punica granatum) is used for protection against diseases as folk medicine in the Middle East (Gurib-Fakim, 2006), and 52% of total mass of pomegranate is edible, of which 78% juice and 22% is seed (Türk et al., 2008a).

Pomegranate has rich content comprising phenolic compounds and flavonoids (composition of anthocyanins, catechins and other flavonoids) and tannin (punicic acid, punicalagin, gallic acid, ellagic acid) (Afaq et al., 2005). Pomegranate seeds are rich in multiple unsaturated fatty acids, vitamins, minerals, polyphenols and polysaccharides. Eighty percent of pomegranate seed oil consists of punicic acid, phytoestrogens and estrone. Moreover, there are great amounts of polyphenols in pomegranate juice (Yılmaz, 2010, Teixeira et al., 2013).

In comparison to other parts of the fruit, pomegranate peel has higher antimicrobial properties (Tehranifar et al., 2011). Polyphenols, anthocyanins mines and ellagitan are the main antioxidants in its juice (Gil et al., 2000). Ninety two percent of antioxidants in pomegranate are ellagitannins, which are concentrated in the peel. Punicalagin is a basic ellagitannin which decomposes into ellagic acid and other small polyphenols in vivo (Seeram et al., 2004). Pomegranate's capacity of iron reduction and activity of free radical-scavenging is three times higher than those in usage of red wine and green tea for antioxidant purposes (Fuhrman et al., 2000). Furthermore, pomegranate content of antioxidant is higher than those of orange juice, blueberry, grape, and grapefruit (Seeram et al., 2006).

Pomegranate has recently become popular in usage for prevention of cancer (Afaq et al., 2005, Lansky et al., 2005)the search for novel agent(s, antiproliferative and apoptotic purposes (Seeram et al., 2005), and as an inhibitor of HIV-I and a microbicide (Neurath et al., 2005). Moreover, many studies have reported that pomegranate and its by-products have a strong activity of free radical scavenging, as well as effective antioxidant properties (De Nigris et al., 2005, Rosenblat et al., 2006). Reactive oxygen species (ROS) are highly oxidized reactive free radical compounds. ROS production in organs like the testes is a commonplace physiological event, whereas increased synthesis of ROS brings about DNA damage in cells and oxidation (Sikka, 1996). Antioxidants usually fight against lipid peroxidation and formation of ROS, minimize DNA damage and cellular lysis, as well as repairing sperm functions (Garcia-Perez et al., 2009).

Extract of pomegranate has been demonstrated to inhibit cancer cell growth in mice with prostate cancer, as well as inducing apoptosis in such cells (Malik and Mukhtar 2006). Singh et al. (2002) reported using model systems of DPPH and β-carotene-linoleate that methanol extract in pomegranate seeds had much lower antioxidant power than the peel. According to Tzulker et al. (2007) the homogenates taken from the entire fruit carried about 20 times higher antioxidant activity than shown in aril juices. Ahmed et al. (2014) reported that pomegranate extract showed natural protective instrument behavior in brain injury induced by ischemia/reperfusion in rats due to its effects as an anti-inflammatory, antioxidant, anti-apoptotic and ATP replenishing agent. In this study, pomegranate extract, an effective antioxidant, was orally administered to rabbits for 8 weeks, and the effects of spermatological parameters, oxidative stress parameters, and germ cell density were analyzed.

MATERIALS AND METHODS

Pomegranate Extract and Chemicals

The extract was prepared in Gazi University Faculty of Pharmacy, Department of Pharmacognosy. The fresh fruit was squeezed using a fruit processor. The resulting pomegranate juice was frozen at -80 oC and powdered by drying in a lyophilizer. The extract was given to experimental animals in three different doses. All chemicals supplied the Sigma-Aldrich Corporation (St. Louis, MO, USA).

Animals and the Design of the Experiment

This study used 2-4 kg on average, five-month-old twenty-four healthy adult male New Zealand White (NZW) rabbit bucks. Steps of the experiments were agreed by Afyon Kocatepe University's Committee of Animal Care and Use (2015-49533702/53) and they

complied with the codes of laboratory animal usage and care published by the National Institute of Health. The rabbits were held in galvanized wire cages separately with a light cycle of 12/12 day/night, temperature in the range of 18 to 25˚C and they were provided free access to ad-libitum feeding and fresh water. Four groups containing 6 rabbits each were randomly formed. Group I was given a standard diet and 1 ml % 0.5 carboxymethyl cellulose (CMC), Group II was fed with 25 mg/kg/day pomegranate extract + 1 ml % 0.5 carboxymethyl cellulose (CMC), Group III received 50 mg/kg/day pomegranate extract + 1 ml % 0.5 CMC and Group IV was provided 100 mg/kg/day pomegranate extract + 1 ml % 0.5 CMC. This period of administration (8 weeks) was required to reveal the effects of pomegranate extract on production of sperm as rabbits require a period of 40 to 50 days for the completion of spermatogenic cycle together with spermiogenesis, meiosis, as well as spermatocytogenesis.

Collection of the Sample

The rabbits were put down with 35 mg ketamine and 5 mg xylazine per kg for anesthesia and 150 mg/kg for euthanasia at the end of the period of treatment. For histological analyses, the first testis tissue was fixed in Bouin's solution, followed by the other testis tissue stored at -20 ºC until biochemical evaluations for each rabbit.

Evaluation of Epididymal Sperm Parameters

The percentage of forward progressive sperm motility was evaluated using a phase contrast microscope with heated stage as described by Sönmez et al (2005). For this process, a slide was placed on a phase contrast microscope with a heated stage warmed up to 37 ºC and then several droplets of Tris buffer solution [0.3 m Tris (hydroxymethyl) aminomethane, 0.027 m glucose, 0.1 m citric acid] were dropped on the slide, a very small droplet of fluid obtained from left cauda epididymis with a pipette was added to the Tris buffer solution and mixed by a cover-slip. The percentage of forward progressive sperm motility was evaluated visually at 200 x and 400 x magnification. Motility estimations were performed by three different fields in each sample. The mean of three successive estimations was used as the final motility score.

The osmotic resistance was assessed by modified cumulative analysis of hypoosmotic eosin staining test (HE-test), using the eosin exclusion test and the Hypoosmotic Swelling Test (HOST) (Ducci et al., 2002, Fukui et al., 2004, Mansour, 2009). Semen samples were diluted 1:10 (v/v) in 100 mOsm fructose solution including 1% (w/v) eosin-Y, and were incubated in a water bath at 35ºC for 30 minutes. The sperm suspension smears were prepared by 10 µL of mixed samples, and 100 spermatozoa were observed in each slide under a phase contrast microscope at 400× magnification and they were classified into four types (Type I: tail swollen and head white, HOS+/E-; Type II: tail non-swollen and head white, HOS-/E-; Type III: tail swollen and head red, HOS+/E+; Type IV: tail non-swollen and head red, HOS-/E+) according to staining status of sperm head and curling of the sperm tail in smears.

Morphologically abnormal acrosomes were estimated on a wet mount slide using 2 - 3 semen drops thinned in Hancock's solution (Hancock, 1952) using a phase contrast microscope (Olympus CX 31, Olympus Optical Co., Ltd., Japan) and spermatozoa ratios were recorded.

Biochemical Studies

Measurement of Total Antioxidant Status in Epididymal Rabbit Sperm

Seminal plasma was separated from ejaculates by centrifugation at 5000 rpm for 10 min. The supernatants were transferred into Eppendorf tubes, recentrifuged 5000 rpm 10 min. to eliminate the remaining cells and the supernatant was stored at -20 ºC before being assayed. The status of the serum total antioxidant capacity (TAC) was determined using a new colorimetric measurement method which is automated (Erel, 2004). This technique was founded on the decolorizing of color characteristics of a ABTS (2,2'-azino-bis[3-ethylbenzothiazo-line-6-sulfonic acid]) radical cation with higher stability by antioxidants. The assay has exceptional values of accuracy by less than 3%. The method's results were stated in units of mmol Trolox equivalent/L.

Measurement of Total Oxidant Status in Epididymal Rabbit Sperm

Seminal plasma was obtained and treated as it is

above mentioned. The status of the serum total oxidant capacity (TOC) was determined by a colorimetric measurement method which is automated (Erel, 2005). In the usage of this technique, the ferrous ion-o-dianisidine complex was oxidized into ferric ion by oxidants in the sample. The reaction of oxidation was improved by glycerol molecules, which are richly existing in the medium of reaction. The ferric ion and xylenol orange gave rise to a colored complex in the acidic medium. The spectrophotometrically measurable color intensity was in relation with oxidant molecules' total amount in the sample. Hydrogen peroxide was used for the calibration of the assay and the results were stated in units of micromolar hydrogen peroxide equivalent per liter (mmol H2O2 Equivalent/L).

Histologic Examination

For determination of changes in spermatogenic cell density, Bouin's solution was used to fix testis tissues for 48 h, sorted ethanol concentrations were used for dehydration, the tissues were fixed in paraffin wax, cut at 5 mm thicknesses and stained using Mayer's hematoxylin and eosin (H&E). For each section, ten seminiferous tubules (ST) were examined randomly and their thickness of germinal cell layer (starting from the lumen of the tubule to the basal membrane) and diameters were determined by an ocular micrometer under a light microscope, followed by the calculation of the mean ST size and germinal cell layer thickness.

Statistical Analysis

The data are demonstrated as mean and SEM (standard error of means). The significant level was chosen as $p < 0.05$. Tests of post hoc Tukey-HSD and one-way ANOVA (analysis of variance) were used to calculate the variances amongst the groups by all characteristics of sperm, biochemical parameters, as well as histological findings. The SPSS (Version 10.0; SPSS/PC, Chicago, IL) package software was used to carry out all the analyses.

RESULTS

Epididymal Sperm Characteristics

Table 1 presents the outcomes of different dosages of pomegranate extract on epididymal sperm motility, abnormal sperm rate, acrosome reaction rate and the hypoosmotic eosin staining test (HE-test). Group II, III and IV dosages of pomegranate extract increased sperm motility significantly ($p < 0.05$), while pome-

Table 1. Mean (± S.E.) spermatological parameters in epididymal rabbit sperm.

Groups	Motility (%)	Abnormal Sperm Rate (%)	Acrosome Rate (%)	H+/E- (%)	H-/E- (%)	H+/E+ (%)	H-/E+ (%)
Control	70±3.65^{b}	26.5±4.61^{a}	28.6±3.62^{ab}	64.2±2.12^{b}	30.3±2.56^{a}	2.3±0.49^{a}	3.2±0.79^{a}
SD+25 mg/kg/day	84±2.44^{a}	23.1±3.08^{ab}	33.1±4.29^{a}	72.6±1.24^{a}	25.2±1.62^{a}	0.6±0.40^{b}	1.6±0.24^{b}
SD+50 mg/kg/day	86±2.23^{a}	16.7±2.11^{b}	25.1±1.15^{ab}	72.8±2.94^{a}	25.3±2.77^{a}	1.0±0.25^{b}	0.8±0.47^{b}
SD+100 mg/kg/day	84±3.26^{a}	15.4±1.84^{b}	23.2±2.35^{b}	71.5±1.60^{a}	27.5±1.83^{a}	0.7±0.33^{b}	0.2±0.16^{b}

Different superscripts (a and b) within the same column showed significant differences among the groups. ($p < 0.05$)
HOS+/E-: tail swollen and head white; HOS-/E-: tail non-swollen and head white; HOS+/E+: tail swollen and head red; HOS-/E+: tail non-swollen and head red.
SD: Standard Diet

granate extract in both group III and IV decreased the ratio of abnormal sperms significantly in contrast to the control group. In contrast to the control group, all doses of pomegranate extract provided significant growth in the H+/E-($p < 0.05$) and significant decreases in H+/E+ and H-/E+ ($p < 0.05$).

Total Antioxidant and Oxidant Status Level

Table 2 shows the outcomes provided by pomegranate extract in different doses on epidydimal sperm total antioxidant and oxidant status level. The total oxidant status level in the rabbits treated with group IV decreased in comparison to all other groups ($p < 0.05$). Degrees of total antioxidant status for the rabbits treated with all doses of pomegranate extract were like those of the control group.

Spermatogenic Cell Density

Table 3 demonstrates the influences of different dosages of pomegranate extract on epidydimal sperm seminiferous tubule diameter and germinal cell layer thickness. In comparison to the control group, all doses provided significant growth in seminiferous tubule diameter and only the dosage of 100 mg/kg pomegranate extract per day increased the thickness of germinal cell layer significantly ($p < 0.05$) (Fig. 1).

DISCUSSION

In this study, daily pomegranate extract consumption for 8 weeks in different doses brought about increase in sperm motility, functional membrane integrity, seminiferous tubule diameter and germinal cell layer thickness. On the other hand, it decreased the abnormal sperm ratio and total oxidation level in NZW rabbits.

Various inquiries have revealed that oxidative stress occurring in the seminal fluid, that is, reactive oxygen species (ROS) in excess over antioxidant levels, reduces quality of sperm in two ways: 1) Some ROS acting as free radicals harm the cell membrane of sperms by lowering motility of sperm, 2) the sperm DNA may be damaged by the free radicals (Tremellen, 2008). The balance between the male reproductive tract's antioxidant defense system and ROS production is disturbed by oxidative stress, which causes defective sperm function to rise (Aitken and Sawyer, 2003). As there is an abundant amount of polyphenols in the fruit in P.

Table 2. Mean (± S.E.) total antioxidant/oxidant status in epidydimal rabbit sperm

Groups	TAS (mmolTrolox Equiv./L)	TOS (μmol H2O2 Equiv./L)
Control	0.8±0.09[a]	56.4±2.97[a]
SD+25 mg/kg/day	1.02±0.06[a]	48.5±3.32[a]
SD+50 mg/kg/day	1.06±0.07[a]	48.7±2.72[a]
SD+100 mg/kg/day	0.86±0.11[a]	34.4±2.12[b]

Different superscripts (a and b) within the same column showed significant differences among the groups. ($p < 0.05$)
TAS: Total antioxidant status TOS: Total oxidant status SD: Standard Diet

Table 3. Mean (± S.E.) spermatological cell density in rabbit testis tissue

Groups	Diameter of ST (μm)	GCLT(μm)
Control	152.70±4.43[b]	33,20±1,03[b]
SD+25 mg/kg/day	182.80±6.80[a]	34,26±0,91[b]
SD+50 mg/kg/day	184.50±5.65[a]	35,34±0,53[b]
SD+100 mg/kg/day	191.90±7.31[a]	41,04±1,50[a]

Different superscripts (a and b) within the same column showed significant differences among the groups. ($p < 0.05$)
STs: seminiferous tubules GCLT: Germinal cell layer thickness SD: Standard Diet

Granatum which can act directly or indirectly as antioxidants based on their chemical structure, it expresses antioxidant activity. As the concentrations in the system are not high enough to have any direct and significant antioxidant outcome in vivo, the main concern is on the availability of polyphenols in consideration of their direct antioxidant effects. Hence, the inhibitive influence of polyphenols against, for instance, oxidative stress possibly comes from their ability to trigger the system of endogenous antioxidant defense; so, they work indirectly as antioxidants. By phase-2 enzymes, ortho-phenol groups containing polyphenols present in the fruit of pomegranate may be metabolized into electrophilic orthoquinones, which are crucial for inducing the system of endogenous antioxidant defense (Dinkova-Kostova and Talalay, 2008). Antioxidant

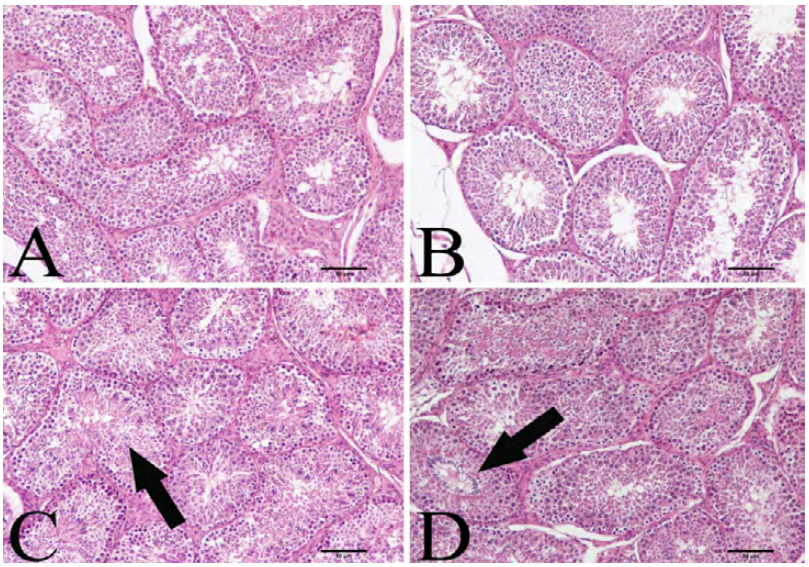


Fig 1. The effect of pomegranate extract in testis of rabbits. Representative figures were stained with Mayer's hematoxylin and eosin (H&E). A: Control group and 1 ml % 0.5 carboxymethyl cellulose (CMC) B: animals treated with 25 mg/kg/day pomegranate extract + 1 ml % 0.5 carboxymethyl cellulose (CMC) C: animals treated with 50 mg/kg/day pomegranate extract + 1 ml % 0.5 CMC D: animals treated with 100 mg/kg/day pomegranate extract + 1 ml % 0.5 CMC.

characteristics of the most widespread among P. granatum's polyphenols are accordingly considered to be led by their indirect antioxidant functions. Ellagic acid, which is a potent scavenger of free radicals (O2 − and OH), inhibits oxidative damage on DNA (Türk et al., 2008b). It is thought that pomegranate has higher antioxidant activity than most other plant species (Thring et al., 2009). Pomegranate's antioxidant activity and ascorbic acid's ROS-quenching activity are close to each other. Although it is widely accepted that antioxidant mechanisms in biological matrices are highly complicated and several different factors may take a role, the functioning mechanism put in motion by antioxidant activity of these compounds has not yet been understood completely (Çam et al., 2009). According to Madrigal-Carballo et al. (2009), as phenolic hydroxyl groups rapidly provide hydrogen to reduction agents, polyphenolic molecules in pomegranate sustain redox reactions. Hussen and Arrack (2014) reported pomegranate peel extract to be effective in protection of rabbits against carbon tetrachloride (CCl4) toxicity by their antioxidant activity. In our study, improvements observed in total oxidant status may be explained by deterrence of excessive creation of free radicals formed by spermatozoa, with the help of the pomegranate extract's antioxidant properties.

Pomegranate juice consumption caused rise in sperm motility, functional membrane integrity, width of seminiferous tubule and thickness of germinal cell layer. On the other hand, it decreased the abnormal sperm ratio and total oxidation level in comparison to the control group. This finding agrees with those of many scholars. According to the study by Türk et al. (2008b), consumption of pomegranate juice in rats caused an increase in epididymal motility of sperm, seminiferous tubule diameter and thickness of germinal cell layer, while it reduced the rate of abnormal sperms in when compared with the control group. Similarly, Türk et al. (2010) suggested that ellagic acid has a defensive influence against testicular and spermatozoal toxicity brought about by cyclosporine A. This protecting effect of ellagic acid appears to be somehow related to curtailment of oxidative stress. Hence, after tissue transplantation and in autoimmune diseases, ellagic acid may be used in association with cyclosporine A to enhance cyclosporine A-induced injuries in terms of quality of sperms and oxidative stress parameters. Mansour et al. (2013) reported that administering P. Granatum extract to rats for 6 weeks lowered abnormal sperm rate and raised epididymal sperm concentration and sperm motility led to reduction in the amount of malondialdehyde, a by-product of lipid peroxidation, which means that antioxidant effect prevented lipid peroxidation in the spermatozoa membrane. In a rabbit study of Fayed et al. (2012), it was reported that a supplementary diet with pomegranate peel in different concentrations (0.5%, 1.0% and 1.5%) for eight weeks raised semen volume, motility and total number of spermatozoa, while the morphological abnormal and dead/live sperm ratio were decreased. On the other hand, in human the daily consumption of tablets of P. granatum extract for 3 months resulted in a significant increase of the sperm motility and volume (Fedder et al., 2014). According to Leiva et al. (2011), daily administration of 500 mg/kg pomegranate extract to rats exposed to lead poisoning for

35 days reversed the effects poisoning and increased sperm count and motility. Zeweil et al. (2013) showed in male rats exposed to temperature stress that, different concentrations of pomegranate peel extract added to diets of rats for 8 weeks increased sperm count, sperm motility and the amount of fructose in seminal plasma, while the rate of dead spermatozoon was decreased. Guo et al. (2008) stated that as pomegranate juice is consumed, plasma antioxidant capacity rises. Lowering malondialdehyde levels means decreasing lipid peroxidation in seminal plasma, evidenced by the rise in seminal plasma total lipids with pomegranate treatments. In this study, enhancements found in density of spermatogenic cells and quality of sperm can be referred to deterrence of too much formation of free radicals generated by spermatozoa, with the help of the antioxidant properties of pomegranate extract.

CONCLUSION

The results of study support that the daily consumption of pomegranate extract for eight weeks caused increased spermatogenic cell density, epididymal sperm motility, membrane integrity and decreased abnormal sperm rate in male rabbits. The total oxidant status level in the rabbits treated with 100 mg/kg/day of Pomegranate extract was decreased when compared to the control group. Thus, it can be say that Pomegranate extract at concentrations of 50 up to 100 mg/kg/day of body weight, is beneficial improving the epididymal sperm parameters in rabbits.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Research article
Ερευνητικό άρθρο

Cytotoxicity of Contrast Media Iohexol on IL-1beta stimulated Bovine Chondrocytes

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ABSTRACT. The aim of this study was to determine the cytotoxic and metabolic effects of iohexol on cultured bovine chondrocytes in clinical dose. Chondrocytes were exposed to 50%, 25% and 12.5% iohexol and 50% mannitol for 2 hours. Cell proliferation, apoptosis and necrosis were analyzed. Real time PCR was performed for aggrecan, collagen type I and II gene expression. Cells in alginate beads stimulated by interleukin-1β (IL-1β) were analyzed for cytotoxicity. MTT assay showed that 50% iohexol inhibited the proliferation of cells at 2 hours culture period. Propidium iodide results showed significantly higher dead cells at 50% iohexol compared to control, however PCR results revealed that chondrogenic gene were not affected. Cells in alginate beads stimulated with IL-1β showed significantly higher percentage of dead cells at 50% iohexol exposure ($p<0.05$). These results suggested that iohexol has a cytotoxicity on chondrocytes and this cytotoxic effect possibly increased in inflammatory joint diseases.

Keywords: Alginate beads, Chondrocytes, Chondrogenic gene, Cytotoxicity, Iohexol

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INTRODUCTION

The diagnostic imaging technology among various animal diagnostic methods makes it possible to examine the internal organs in a non-invasive manner, thereby making it available as an important diagnostic method. In order to uplift the efficiency of evaluation in imaging diagnosis, contrast media were developed and applied to the gastrointestinal tract, the urinary system, blood vessels, pancreas and biliary duct and joint. While CT and X-ray utilizing a contrast medium are applied to examine the overall shape of joint cartilage, the degree of destruction, ligaments and so forth less frequently than MRI system in the joints, still they are widely used in the veterinary medicine in particular (De Rycke et al., 2015; Hong et al., 2010; Van Vynckt et al., 2013).

Iodized contrast media for CT and X-ray diagnosing are divided largely into ionic contrast media and non-ionic contrast media, and chemically into monomer and dimmer. Side effects are also reported on the cardiovascular system, neurological system, renal system such as vascular endothelial cell damage, blood clots, hypotension, hypersensitive reaction and shocks, while the common contrast media for clinical use are evaluated as highly safe (Bettmann et al., 1997; Katayama et al., 1990; Margulies et al., 1991; Nyman et al., 1980). High osmotic pressure and chemical toxicity of contrast media can also play an important role in causing cellular toxicity (Heinrich et al., 2005; Wang et al., 1998; Zhang et al., 2000). However, the pathogenesis of contrast media-induced side effects is not clearly understood.

The contrast medium injected into the joint is quickly absorbed into the blood vessel through the synovial tissue and excreted through the kidney, and is not observed in the joint after 3 hours of injection (Edwards et al., 2007). Iohexol, a non-ionic contrast medium having low osmotic pressure, shows a high glomerulus filtration rate and is a safe contrast medium with little direct side effects on the tissue and cells, compared with other iodized contrast media, thus typically making it available for use in both humans and animals in clinical fields (Barkin et al., 1991). However, it is reported that iohexol with known safety is toxic for vascular endothelium cells (Takatsuki et al., 2004; Zhang et al., 2000) and renal cells (Duan et al., 2006; Gong et al., 2010; Lee et al., 2006). While numerous reports on the effects of iohexol on several types of cells are made available recently, research reports on its effects on chondrocytes are not to be found in the medical area as well as in the veterinary science. Therefore, this study will try to elucidate the effect of iohexol on cellular toxicity and metabolism in clinically applicable concentrations with bovine chondrocytes.

MATERIALS AND METHODS

Isolation and culture of bovine chondrocytes, and measurement of osmotic pressures

Bovine chondrocytes were isolated from six-month-old Holstein calf that died accidentally, without suffering from any osteoarthritic disease. Full thickness articular cartilages were aseptically collected from the distal femoral condyle and digested with collagenase type I (0.1%) (Welgene, Daegu, South Korea) for 18 h at 37◦C in a shaking water bath. After isolation, the cells were cultured with high glucose DMEM with phenol red (Dulbecco's modified eagle's medium; Welgene, Daegu, South Korea) containing 10% FBS (Fetal bovine serum; Welgene, Daegu, South Korea) at 37◦C in 95% air and 5% CO2 humidified atmosphere. Culture medium was changed two times per week. After the cells were confluent, they were trypsinized with 0.25% trypsin-EDTA (Welgene, Daegu, South Korea) and used for this study. The cellular survival rate was evaluated with Trypan blue staining during cell culture. Osmotic pressures of Iohexol (Omnipaque 300; GE Healthcare, Cork, Ireland) and mannitol (Daehan D-Mannitol 20%; Daehan Pharm Co., Korea) were measured by using the micro-osnometer.

MTT assay

To measure the proliferation of chondrocytes, an MTT assay was conducted. Bovine chondrocytes were set at 2~4×10^5 cells/ml, added to 96well plate 100 µl each and cultured in the incubator at 37°C and 5% CO_2. After they were cultured for 24 hours, medium was changed into isotonic saline, 50%, 25%, 12.5% iohexol and a 50% mannitol and incubated for 2 hours. The medium were removed, and all of the wells were added with 100 µl of DMEM without phenol red (Welgene, Daegu, South Korea) and 10 µl of MTT

solution (final concentration of MTT: 0.5 mg/ml). After 2 hours incubation, the 96-well plate was emptied and 50 µl of dimethyl sulfoxide (DMSO; Junsei Chemical Co. Ltd, Japan) added to each well to dissolve the formazan crystals. The optical density (OD) was measured at 540 nm using a spectrophotometer (Emax, Molecular Devices, Sunnyvale, CA, USA). The optical densities were converted into percentages using the following formula: Cell viability (%) = (OD of test/ OD of control-isotonic saline) x 100. This process was repeated 4 times.

Double stain of propidium iodide and hoechst 33258

Bovine chondrocytes were set at 2~4×105 cells/ml, cultured in 6 well plate at 37°C and 5% CO2. After they were cultured for 24 hours, medium was replaced with 50%, 25%, and 12.5% iohexol and a 50% mannitol diluted from commercial solution and cultured for 2 hours. The cells were trypsinized for 5 minutes with 0.25% Trypsin-EDTA and separated, cautiously mixed with the final concentration set at 5 µg/ml Hoechst 33258 (H33258; Sigma, St. Louis, MO, USA), and cultured for 15 minutes. To observe the necrotic cells, 20 µg/ml Propidium Iodide (PI) was added to the mixture, and observed with a UV filter of a fluorescent microscope. More than 200 cells were observed from various angles and results were documented.

RNA extraction and real time PCR

For reverse transcription PCR (RT-PCR), total RNA was extracted from the 1~2×10^5 cells using easy-blue (iNtRON, Seoul, Korea) and first-strand cDNA was synthesized by Maxime RT Premix Kit (iNtRON, Seoul, Korea). Primers which were designed previously to detect specific mRNA are described in Tabel 1. The PCR conditons were set for one minute at 95°C, for 5 seconds at 95°C, for 15 seconds at 55°C. The PCR products were electrophoresed on 1% agarose gel and stained with ethidium bromide. The amount of cDNA was measured by reactions of 1 µl cDNA template with 10 µl of 2 x SYBR premix Ex Taq (TaKaRa Bio Inc.) and 10 pmol of the specific primers. Forty cycles reaction was carried out at 95°C for 15 seconds, 58°C for 15 seconds, and 72°C for 30 seconds. The amount of product was measured of the intensity of fluorescence and the mRNA expression level of target genes was normalized by β-actin.

Three-dimensional culture of chondrocytes in alginate beads

The effect of iohexol on inflammation chondrocytes were evaluated with alginate beads culture. Chondrocytes and alginate solution were prepared at a final concentration of 5 × 10^6 cells/ml and 1.2% alginic acid sodium salt. A bead-shaped alginate gel was completed in 102 mM calcium chloride solution by using 200 µl pipette tips. After washing beads, those were cultured for 2 weeks in 10% FBS (Welgene, Daegu, South Korea) containing DMEM. The inflammation cell model was cultured in DMEM with 10 ng/ml recombinant canine interleukin 1-β (IL-1β; R&D Systems, Abingdon, UK) for 24 hours. Beads were divided into 4 groups and were cultured; 1) culture in normal medium, 2) culture in 50% iohexol for 2 hours, 3) culture in IL-1β for 24 h, 4) culture in IL-1β for 24 hours and then 50% iohexol for 2 hours. An evaluation of cell toxicity was conducted utilizing a double dying method of PI and H33258 three times.

STATISTICAL ANALYSIS

Evaluation of the cell proliferation rate and the results of a real-time PCR were expressed in an average ± deviation, each value being compared and evaluated by using one-way analysis of variance (ANOVA) and post hoc Dunnett test. In case the P value was less than 0.05, it was considered as statistically significant.

RESULTS

Osmotic pressures

In evaluating the effects of the contrast medium on chondrocytes, mannitol was selected for comparisons of high osmotic pressures to evaluate the presence or absence of the effects of osmotic pressures of the contrast medium itself on the cells. The osmotic pressure of the undiluted solution of the contrast medium reached 885 mOsm/kg and the undiluted solution of mannitol pointed to 1013 mOsm/kg. The 50% mannitol (828 mOsm/kg) selected for the com-

Table 1. Primer sequences used in RT-PCR and Real time PCR.

Gene	Primer sequence	Product size (bp)	Accession number
Collagen type I	F 5'TGCTGGCCAACCATGCCTCT	120	AB008683
	R 5'CGACATCATTGGATCCTTGCAG		
Collagen type II	F 5'ATCCATTGCAAACCCAAAGG	147	X02420
	R 5'CCAGTTCAGGTCTCTTAGAG		
Aggrecan	F 5'CACTGTTACCGCCACTTCCC	303	U76615
	R 5'GACATCGTTCCACTCGCCCT		
β-Actin	F 5'CGCACCACTGGCATTGTCAT	227	K00622
	R 5'TCCAAGGCGACGTAGCAGAG		

parative group showed an osmotic pressure higher than any other iohexal concentration which is lower than 50% iohexol (592 mOsm/kg).

Cell proliferation

After stimulating cells by isotonic saline solution, 50%, 25%, 12.5% iohexal and 50% mannitol for 2 hours, differences were observed under the microscope. While the cells cultured in saline solution maintained the way they grew on the normal medium, it was observed that those cells stimulated by 25% iohexol for 2 hours declined in the cell proliferation depending on concentration. The cells stimulated by 50% iohexal were not clearly observed due to the inability of microscopes to focus due to the contrast medium properties. The MTT results showed outcomes similar to those observed under the microscope. The results of MTT assay showed that 50% iohexol inhibited significantly the proliferation of bovine chondrocytes at 2 hours culturing (p=0.030). The cell viability stood at 85.9±5.6 at 50% iohexol, 91.8±4.6 at 25% iohexal, 95.1±3.9 at 12.5% iohexal, and 94.9±3.3 at 50% mannitol (Figure 1).

Apoptosis of chondrocytes

The dead cells was observable since the nucleus was dyed in the red by PI, and apoptosis was also observable since the nucleus was dyed in the blue by H33258, and it was confirmed through the observation of the nucleus with respect to whether it is condensed or made amorphous (Figure 2). The number of dead cells dyed by PI was observed as significantly high in the 50% iohexol group, compared with isotonic saline solution ($p<0.05$). The dead cell rate was pointed to 17.9±12.3 in 50% iohexol, 7.8±3.8 in 25% iohexol, 6.3±3.9 in 12.5% iohexal, 5.6±2.6 in 50%

Table 2. The percentage of dead cells stained with propidium iodide.

Isotonic saline	Iohexol			Mannitol
	50%	25%	12.5%	50%
2.0±0.6	17.9±12.3*	7.8±3.8	6.3±3.9	5.6±2.6

*Data are reported as means ± SD. * The percentage of dead cells stained with propidium iodide is significantly higher at 50% iohexol compared to isotonic saline group ($p<0.05$)*

Fig 1. MTT results expressed as a percentage of control absorbance. The results of MTT assay showed that 50% iohexol inhibited significantly the proliferation of bovine chondrocytes at 2 hours culturing (p=0.030).

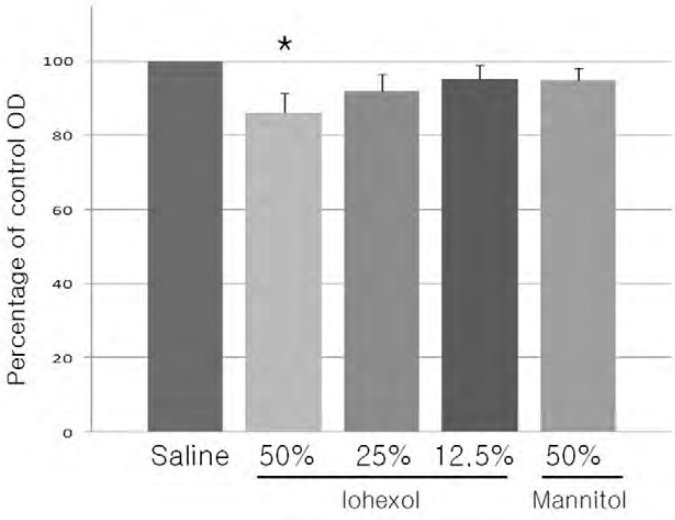
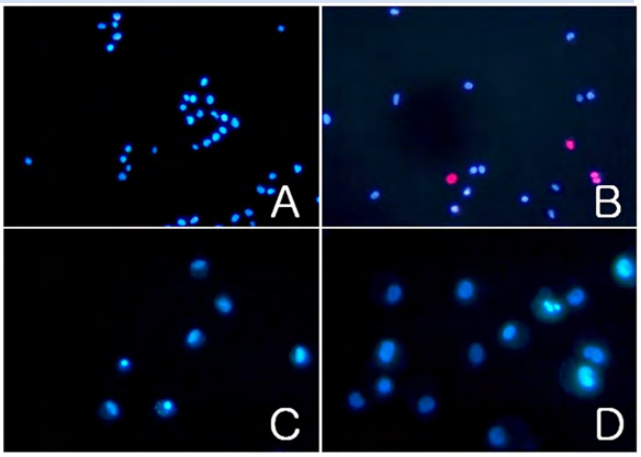


Fig 2. Dead cells stained with propidium iodide are observed more frequently in 50% iohexol (B) than saline (A). No significant iohexol-induced apoptotic nuclei are not observed in hoechst stained cells for 2 hours, however those are observed in cells at 12 hours culturing (C, D).



mannitol and 2.0±0.6 in saline solution, respectively (Table 2). With respect to the observation of apoptotic nucleus showing abnormal forms of nucleus such as condensation, collapse due to contrast media, they were not frequently observed in all the groups during 2 hours culture period. While numerous apoptotic nucleus were observed in the cells stimulated by the contrast medium for 12 hours.

Comparison of gene expression

To evaluate the effects of exposure to the contrast medium on the metabolism of cells, RT-PCR with respect to mRNAs of collagen type II and Aggrecan, particular indicators of chondrocytes, and mRNA of Collagen type I, a degenerative indicator of chondrocytes, was conducted. All the genes amplified by compounded primer were observed in expected molecular weight in PCR conducted 35 times. It was confirmed that in the cells cultured used for this experiment, mRNAs of Collagen type II and Aggrecan were strongly manifested normally. The results of evaluations of the effects of iohexol stimulation on the quantity of the chondrogenic genes through a quantitatively real-time PCR three times repetitively show that collagen type I and II increased and Aggrecan decreased, while no significant differences were observed (Figure 3).

Cytotoxicity evaluation of cells stimulated by IL-1β

Unlike monolayer culture, cells were cultured with their original form maintained in alginate beads, and proliferated cells were observed as the culturing time elapsed. When IL-1β was stimulated for 24 hours at a 10 ng/ml concentration, cell proliferation was observed to be effectively suppressed. It was observed that 15.4% of the dead cells were dyed on PI when the cells were stimulated in a 50% iohexol, compared with the control group where about 7.9% of the cells were dyed. Stimulation by IL-1β for 24 hours caused 21.6% cell death. However stimulation by a 50% iohexal for 2 hours after that by IL-1β for 24 hours caused significantly higher percent of dead cells (31.3%) compared to those of other experimental conditions ($p<0.05$) (Table 3).

DISCUSSION

Noticeable strides have been made in the diagnostic imaging with the aid of image technology through the application of contrast media in the veterinary science as well as human medical science. The use of contrast media, a non-invasive approach, made it possible to conduct precise diagnoses in internal and surgical medicine. It is true that X-ray and CT are less frequently used for joint cartilage disorders, rather

Table 3. The effect of 50% iohexol on IL-1β stimulated chondrocytes cultured in alginate beads.

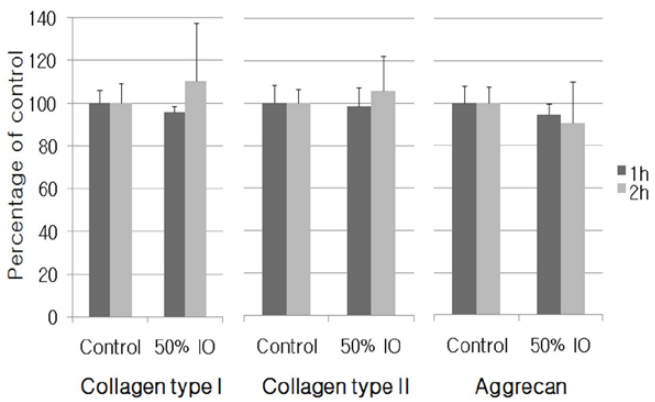
Control	50% Iohexol	10 ng/ml IL-1β	10 ng/ml IL-1β + 50% Iohexol
7.9±4.3	15.4±7.1	21.6±6.3	31.3±6.6*

Data are reported as means ± SD of the percent of dead cells. Cells were cultured with 10 ng/ml IL-1β for 24 hours and/or 50% Iohexol for 2 hours. * The percentage of dead cells stained with propidium iodide is significantly higher at 10 ng/ml IL-1β + 50% iohexol group compared to control group ($p<0.05$).

than new diagnostic approaches by means of image technology such ultrasonography and MRI technology. However, where such new approaches are hardly available, X-ray and CT approaches are generally applied. Therefore, the development of safer contrast media is steadily under way.

This study evaluated the effects of iohexol generally used in conducting arthrography on the chondrocytes of joint cartilage. It was confirmed that iohexol causes chondrocytes death. It has been reported that iodinated contrast media can induce apoptosis of endothelial cells even with brief exposure of 15 minutes (Zhang et al., 2000). Iohexol was also found to cause apoptosis of neutrophis following a short exposure (Fanning et al., 2002). Much of the contrast medium in the joint was absorbed through the synovia within 3 hours of injection (Edwards et al., 2007). Therefore a toxicity evaluation of long-time stimulation to the cells was not conducted in this study, because it is clinically insignificant to evaluate it. Inhibitory effects on cell growth are seen when exposed to iohexol having a low osmotic pressure compared with mannitol with high osmotic pressure. Minor inhibitory effect of osmotic pressure on chondrocytes was observed here. The effects of contrast media on the metabolism of cells were confirmed by means of molecular biological techniques. This experiment indicates that, during the stimulation for 2 hours, a time span when the contrast medium exists clinically in the joint, any effect of iohexol on the cell metabolism was hardly observed.

Fig 3. Real time PCR was performed to measure expression of collagen type I, collagen type II and aggrecan mRNAs according to factor iohexol stimulation. The mean and standard deviation are shown. All mRNAs were not affected by 50% iohexol exposure.



It is reported that substances inducing inflammation factors such as IL-1β, IL-6 and TGF-α that are secreted in abundance in synovial cells of animals suffering from arthritis affect the proliferation and metabolism of chondrocytes (Blanco et al., 1995; Fiorito et al., 2005; Zhou et al., 2008). To evaluate the toxicity of contrast media for chondrocytes in the patients with arthritis, model cells inducing inflammation by stimulating the cells cultured on a 3-dimension alginate beads with IL-1β 10 ng/ml were completed. IL-1β-stimulated cells induced 21.6±6.3% cell death, to a significant extent, exposure of IL-1β-stimulated cells to contrast media induced a 31.3±6.6%, indicating approximately a 10% increase in the cell death. This finding indicates that a high cell death rate of the IL-1β-stimulated cells is seemingly attributable to the fact that cells in inflammatory joints declined in their resistance to outside stimulation. In a normal joint cartilage, chondrocytes were surrounded by plenty of extracellular matrix and contrast medium injected into the joint was absorbed through the synovia within 3

hours. However the arthrography with contrast medium is applied for cases of the abnormal joint such as arthritis or cartilage damages. In such cases, cells are expected to be affected easily by the contrast medium. Cell toxicity of the contrast media could supposedly be reduced through the process of removing and washing off contrast media in the joint as early as possible after arthrography has been performed.

There are limitations in this study about the cytotoxicity of iohexol on chondrocytes. All in vitro data was obtained with cells of only one species. Moreover, it is rare to apply arthrography to bovine joint diseases. The differentiation potential of chondrocytes varies between animal species (Giannoni et al., 2005). Additional in vitro and in vivo studies are needed to determine the cytotoxicity of contrast media more clearly.

CONCLUSIONS

This study evaluated the cell toxicity of iohexol as a contrast medium, widely used in veterinary science that utilizes bovine chondrocytes and its effects on cell metabolism. This study confirmed a significant decline in the cell proliferation at 50% iohexol. Moreover cells stimulated with IL-1β showed higher percentage of dead cells at 50% iohexol exposure. In a normal joint cartilage, the cells were surrounded by plenty of extracellular matrix and the contrast medium injected into the joint was absorbed through the synovia within 3 hours of injection. However, the contrast medium is used in cases with the abnormal joint such as arthritis or cartilage defects. In such cases, where chondrocytes are frequently exposed to contrast media, the cells exposed to them are expected to be affected by the contrast medium. In conclusion, iohexol has a cytotoxicity on chondrocytes and the inhibitory effect possibly increased in inflammatory joint conditions. The repeated arthrography with iohexol should be performed with an awareness of cytotoxic effect, especially among patients that have suffered from cartilage damages and arthritis.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Significance of selected biochemical markers in predicting the outcome of schistosomiasis

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ABSTRACT. This study aimed to correlate the histopathological changes in mice liver with alterations either in liver tissue antioxidants enzymes (catalase and reduced glutathione (GSH)), oxidative stress marker (malondialdehyde (MDA)) or in serum liver function parameters (Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (T.P.) and albumin) to predict the outcome of schistosomiasis. Fourty male Swiss albino mice were used in this study and infected with *Schistosoma mansoni* for 2, 4, 6 and 8 weeks (8 mice for each group), while, the uninfected mice were used as negative control. Liver tissue antioxidants enzymes, oxidative stress marker and serum liver function parameters were determined in coincide with the liver tissue histopathological changes. All selected biochemical makers showed a strong significant positive correlation ($p < 0.05$) with liver histopathology score except serum albumin and liver tissue catalase enzyme. The last two parameters exhibited negative correlation with liver histopathology score. These results revealed that the more increase in the level of AST, ALT, T.P. and globulin in serum or liver tissue MDA and GSH indicating severs histopathological changed into the affected liver and hopeless prognosis is expected. On contrary, the increase in albumin level in serum or catalase level in liver tissue of affected patient/animal demonstrating mild liver histopathological changes. Subsequently, good prognosis and response to anti-schistosomal treatment will be predictable. This study opens the way to predict the outcome of schistosomiasis through easy and rapid biochemical test. Therefore, other studies are required to apply such correlation with other biochemical parameters especially that synthesized into the liver.

Keywords: *S. mansoni*; Mice; Oxidative stress; Histopathology.

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INTRODUCTION

Schistosomes are blood flukes, inhabit the portal blood system of many mammalian species and considered the causative agent of the second most important human parasitic disease in the world following malaria (Despommier et al., 2000).

Schistosomiasis is a serious parasitic disease causing a severe impairment in the liver functions in approximately 10 % of infected persons and affecting more than 200 million people in tropics and subtropics with 97 % of them living in Africa (Steinmann et al., 2006). It is usually characterized by an unnoticed acute phase, followed by liver fibrosis at chronic and advanced stages (Cheever et al., 2002). The chronic and debilitating nature of the disease has resulted in great losses in public health and economic productivity in developing countries (Fenwick et al., 2003). Due to the chronic nature of this disease, predicting its outcome is urgently required.

Schistosoma mansoni (*S. mansoni*) infection is characterized by the embolization of eggs from the intestine to the liver through the portal system. Next, most pathology is attributed to the host reaction to the eggs (Abdallahi et al., 1999). The toxic egg material destroys the host tissue cells and the antigenic material stimulates the development of strong inflammatory reactions around the egg. At the site of inflammation, oxidative stress occurs and leads to the generation of free radicals and the reduction of endogenous antioxidants (Abdallahi et al., 1999). Therefore, the present study was carried out to correlate the liver histopathological changes with the antioxidant enzymes, oxidative stress marker responses in *S. mansoni* infected mice. That will help in rapidly and easily predicting the outcome of this disease under field condition.

MATERIAL AND METHODS

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Animals

40 male Swiss albino mice (aged between 6-8 weeks) were bred and maintained at the experimental animal research unit of the *Schistosoma* biological supply program at Theodor Bilharz Research Institute (TBRI), Giza, Egypt). Mice were kept on a standard commercial pellet diet (El-Kahira company for oils and soap) and provided with water ad libitum in an air- conditioned animal house at 20-22°C. The animal experiments were conducted at the TBRI animal unit in accordance with international, ethical guidelines after approval of the institutional ethical committee of TBRI.

Experimental infection of mice with *S. mansoni*

Animals were infected with the Egyptian strain of *S. mansoni* (80 ± 10 cercariae/mouse) using the body immersion technique according to the method described by Liang et al. (1987).

Experimental design and blood samples collection

Mice were infected with *S. mansoni* for 2, 4, 6 and 8 weeks and the uninfected mice served as a control (eight mice for each group). Blood samples were collected from each mouse by cardiac puncture. Serum of each mouse was separated by centrifugation (1500 xg for 10 min) and kept frozen at −80°C until use. The experiment was performed twice.

Tissue homogenate

The liver was homogenized as previously described by Jatsa et al. (2015). Briefly, the liver lobe was collected from each mouse and homogenized in Tris–Hcl 50 mM buffer. Next, the homogenates were centrifuged at 3500 rpm for 25 min at 4 °C and supernatants were stored at −80 °C for the determination of oxidative stress biomarkers.

Liver function test

Total protein (T.P.) and albumin were measured spectrophotometrically using commercial test kits (Biodiagnostic, Cairo, Egypt) according to standard methods. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using commercial test kits (Biodiagnostic, Cairo, Egypt) according to Murray (1984).

Antioxidant enzymes and oxidative stress marker determination

Malondialdehyde (MDA) level, reduced gluta-

thione (GSH) and catalase enzymes activities were measured spectrophotometrically using commercial test kits (Biodiagnostic, Cairo, Egypt) according to (Beutler et al., 1963; Yoshioka et al., 1979, and Aebi, 1984), respectively.

Histopathological examination and scoring

Specimens from liver were collected and fixed in 10% buffered formalin and processed to paraffin blocks. Sections of 5-µm thickness were prepared from all specimens and stained by Haematoxylin and Eosin stain for microscopical examination (Teixeiral et al., 1996). The scoring was performed independently as fellows; no change in liver tissue, portal veins and blood vessels = 0, mild congestion in blood vessels with absence of worm, necrosis or fibrosis = 1, mild hydropic degeneration = 2, infiltration with inflammatory cells = 3, presence of *Schistosoma mansoni* worm in portal vein = 4, severe hydropic degeneration = 5, presence of coagulative necrosis = 6, presence of hepatic granuloma = 7, hepatic fibrosis = 8, hepatic fibrosis and bile duct hyperplasia = 9.

Faecal Examination

Faecal samples were collected from each mouse and examined for *S. mansoni* egg by sedimentation method as previously described (Katz et al., 1972).

Touchdown PCR

The 121-bp tandem repeats DNA sequence unit of *S. mansoni* described previously (Hamburger et al., 1991) was selected for our experiments. Primers for the touchdown PCR were 5′-CCGACCAACCGTTCTATGAA-3′ and 5′-CCCACGCTCTCGCAAATAAT-3′.The expected length of the product of the amplification was 92bp. *Schistosoma*-infected mouse sera were used directly as templates without a DNA extraction step. Human serum sample was included as negative control. Touchdown PCR was performed by using a GeneAmp PCR System 2700 (Applied Biosystems, CA, USA). A two-step cycle was applied in the touchdown PCR; i.e. a denaturing step and an annealing step. The annealing temperature (60 °C) was gradually lowered (1 °C after each cycle) to 50 °C. Fourty-cycle amplification was then performed with an annealing temperature of 50 °C. The PCR products were Acquiring to FAM fluorescence. By sequencing of the cloned amplification product, it was verified to be identical to the part of the 121-bp highly repeated DNA sequence.

Statistical analysis

Data analysis was performed using SPSS version 16.0 (SPSS). Mean values and standard deviation for each assessed variable were calculated. Statistical differences between examined groups were performed using one-way ANOVA with *post hoc* Duncan multiple comparison test. Differences between means at $p < 0.05$ were considered significant. Non-parametric correlation test (Kendall's tau-b and Spearman's rho correlation tests were used to test relation between liver histopathology score and selected biochemical variables. It assesses how well the relationship between variables can be described using a monotonic function.

RESULTS

Effect of *S. mansoni* infection on the liver function parameters, antioxidant enzymes and oxidative stress marker

In view of evaluating the impact of *S. mansoni* infection on the liver function, some parameters that known to be indicators of liver injuries were measured in the mice serum. ALT, AST activities and T.P. levels were increased significantly ($p < 0.05$) at 4, 6 and 8 weeks post-infection in comparison with control group (Table 1). On the other hand, there was a significant decrease ($p < 0.05$) in albumin level starting from 6 weeks post-infection (P.I.) (Table 1).

GSH and catalase have a great role in protecting the cells against oxidative stress. Our results revealed that GSH activity significantly increased ($p < 0.05$) in comparison with control with increasing the period of infection starting from 4 weeks P.I. (Table 2). On contrary, a significant decrease ($p < 0.05$) in the catalase activity was observed at 4, 6 and 8 weeks P.I. (Table 2).

MDA is the most important free radical that produced as sequel to lipid peroxidation process. Therefore, determination its level is an important indicator to the cellular oxidative destruction. MDA

Table 1. Effect of *S. mansoni* infection on mice serum ALT, AST activities, total proteins and albumin levels.

Groups	ALT (U/L)	AST (U/L)	T.P. (g/l)	Albumin (g/l)	Globulin (g/l)
Healthy mice	30.10 ± 2.80 ^{d}	31.60 ± 3.40 ^{d}	58 ± 1.40 ^{c}	48.50 ± 3.06 ^{a}	9.50 ± 1.7^{d}
2 weeks P.I.	29.80 ± 0.50 ^{d}	33.20 ± 1.10 ^{d}	56.90 ± 1.10 ^{c}	46.70 ± 1.49 ^{a}	10.20 ± 0.41^{d}
4 weeks P.I.	84 ± 4.10 ^{b}	140.20 ± 6.50 ^{b}	65.80 ± 3.20 ^{b}	46.40 ± 2.40 ^{a}	19.43 ± 0.88^{c}
6 weeks P.I.	94.50 ± 2.20 ^{a}	178 ± 7.80 ^{a}	69.20 ± 3.20 ^{b}	32.60 ± 2.50 ^{b}	36.69 ± 0.73^{b}
8 weeks P.I.	78.80 ± 3.30 ^{c}	128.70 ± 8.70 ^{c}	87.20 ± 2.90 ^{a}	26.80 ± 3.11 ^{c}	60.41± 0.24^{a}

a, b, c, d Variables with different superscript letters in the same column means significantly different at P < 0.05. Each value represents the mean ± S.D. for two experiments. P.I.; post infection, T.P.; Total protein, ALT; Alanine aminotransferase, AST; Aspartate aminotransferase.

Table 2. Effect of *S. mansoni* infection on the level of mice liver tissue antioxidant enzymes and oxidative stress marker.

Groups	Catalase (U/g)	GSH (mg/g)	MDA (mg/g)
Healthy mice	1.05 ± 0.10^{a}	12.30 ± 1.40 ^{d}	0.29 ± 0.10 ^{d}
2 weeks P.I.	1.06 ± 0.08 ^{a}	12.60 ± 1.07 ^{d}	0.52 ±0.04 ^{c}
4 weeks P.I.	0.48 ± 0.041 ^{b}	20.90 ± 2.10 ^{c}	0.66 ±0.06 ^{b}
6 weeks P.I.	0.38 ± 0.036 ^{c}	54.10 ± 6.04 ^{b}	1.47 ±0.19 ^{a}
8 weeks P.I.	0.26 ± 0.027^{d}	87.90 ± 6.90 ^{a}	1.82 ± 0.07 ^{a}

a, b, c, d Variables with different superscript letters in the same column means significantly different at P < 0.05. Each value represents the mean ± S.D. for two experiments. P.I.; post infection, GSH; Reduced glutathione, MDA; Malondialdehyde.

activity was increased significantly ($p < 0.05$) with increasing the period of infection (Table 2). Thus, MDA is the most sensitive biochemical parameter to the oxidative damage resulting from *S. mansoni* infection.

Effect of *S. mansoni* infection on the liver tissue histopathology

Two weeks infection by *S. mansoni* resulted in hydropic degeneration besides mild congested blood vessels in the mice hepatic tissues (Fig. 1a). Two weeks later, the immature *Schistosoma* worms were observed in portal vein (Fig. 1b). Subsequently, the portal areas became infiltrated with inflammatory cells mainly eosinophil, congestion was observed at the portal hepatic vessels and severe hydropic degeneration was seen at the hepatocytes (Fig. 1c). Two weeks later, *Schistosoma* eggs were observed in portal vein and the portal areas were infiltrated with eosinophil and round cells. Moreover, focal areas of coagulative necrosis infiltrated with eosinophils were seen adjacent to portal vein (Fig. 1d). In addition, young egg granuloma consisted from single or multiple mature or immature eggs surrounded with inflammatory cells mainly eosinophils were seen replacing the hepatic parenchyma (Fig. 1e). Two months post infection, the hepatic parenchyma were focally replaced with young or old hepatic granuloma (Fig. 1f). Caseous necrosis and fibrous tissue infiltrated with eosinophils and macrophages were surrounded this granuloma (Fig. 1g) and some egg nodules were completely replaced with mature fibrous tissue (Fig. 1h). Moreover, hyperplasia of bile ducts was seen in the portal area with congestion of hepatic blood vessels and presence of inflammatory cells (Fig. 1i).

Correlation of selected biochemical markers with liver tissue histopathological changes

All selected biochemical makers showed a strong significant positive correlation ($p < 0.05$) with liver histopathology score except serum albumin and liver tissue catalase enzyme. The last two parameters exhibited negative correlation with liver histopathology score (Tables 3 and 4). These results revealed that the more increase in the level of AST, ALT, T.P. and globulin in serum or liver tissue MDA and GSH indicating severs histopathological changed into the

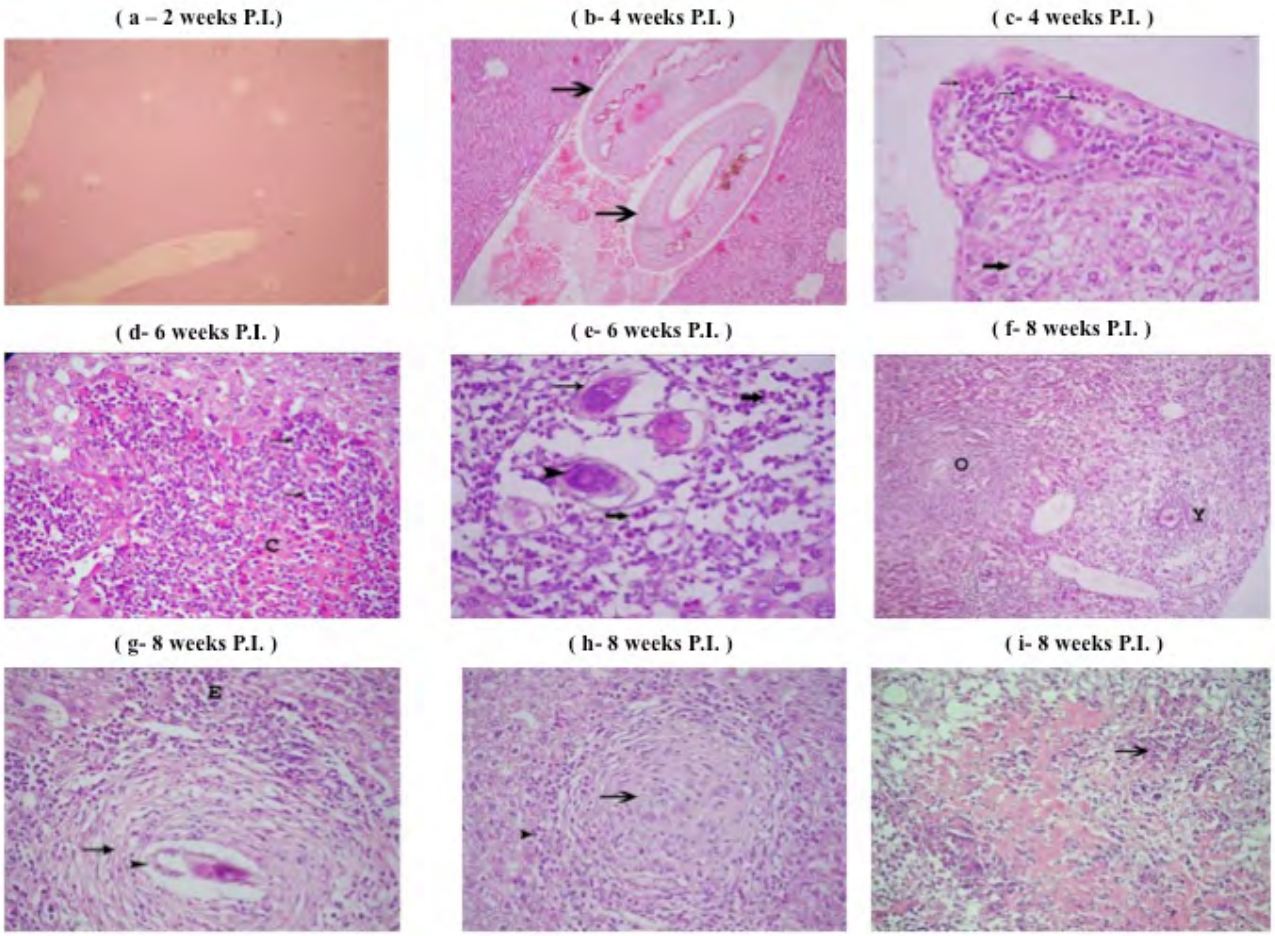


Fig 1. Histopathology of mice liver infected with *S. mansoni*. (a) Hydropic degeneration besides mild congested blood vessels were shown in the mice hepatic tissues (b) The immature *Schistosoma* worm was observed in portal vein (arrows) (c) The portal areas became infiltrated with inflammatory cells mainly eosinophil (small arrows) and severe hydropic degeneration was seen at the hepatocytes (large arrows). (d) The portal areas were infiltrated with eosinophil and round cells (arrows). Also, focal areas of coagulative necrosis infiltrated with eosinophil were seen adjacent to portal vein (C; coagulative necrosis). (e) Young egg granuloma surrounded with inflammatory cells mainly eosinophil were seen replacing the hepatic parenchyma (arrows). (f) The hepatic parenchyma was focally replaced with young or old hepatic granuloma (Y; young granuloma, O; old granuloma). (g) Caseous necrosis or fibrous tissue infiltrated with eosinophil's and macrophages were seen surrounded the old hepatic granuloma (arrow) (h) Mature fibrous tissue were shown replaced the egg nodules completely (arrows). (i) The portal areas showed hyperplasia of bile ducts besides congestion of hepatic blood vessels and inflammatory cells (arrow). P.I.; post infection.

affected liver and hopeless prognosis is expected. On contrary, the increase in albumin level in serum or catalase level in liver tissue of affected patient/animal demonstrating mild liver histopathological changes. Subsequently, good prognosis and response to anti-schistosomal treatment will be expected.

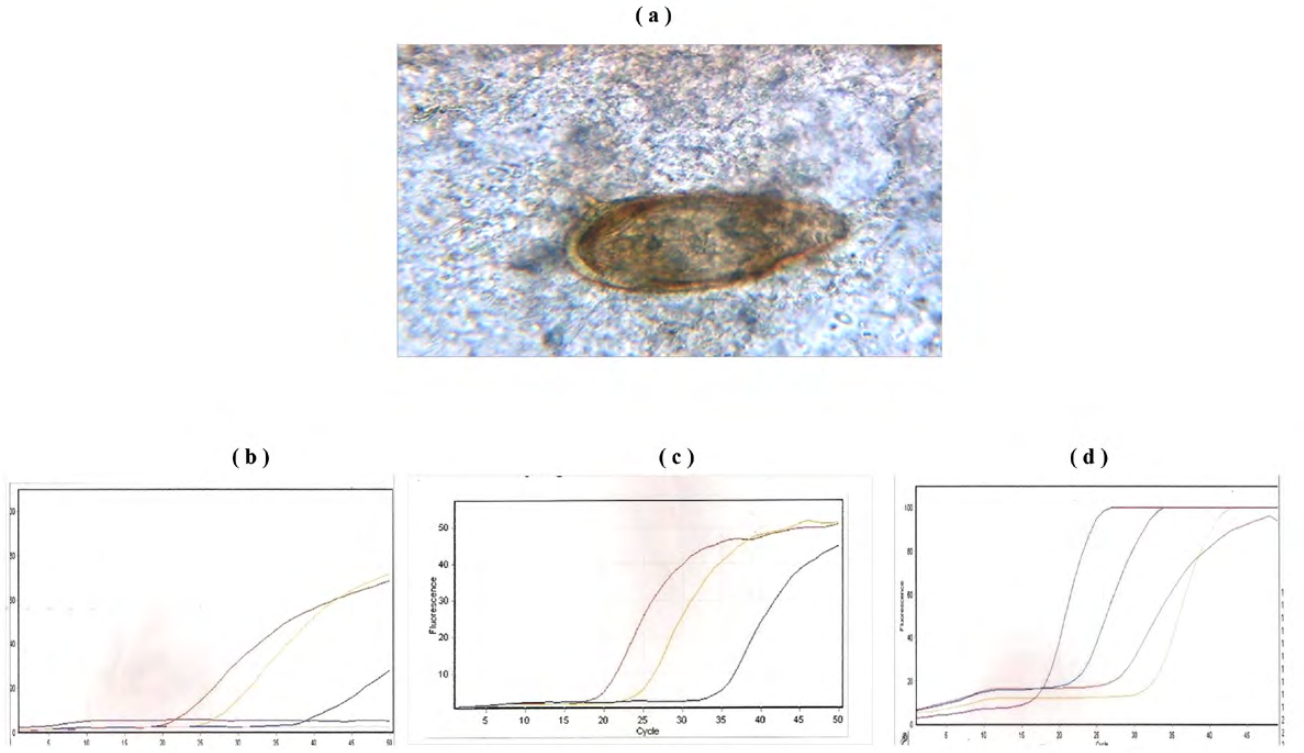
Confirmation of S. mansoni infection

In this study, 2 tests; faecal examination and touch-down PCR were used for confirmation the infection by *S. mansoni* in the infected mice. The *S. mansoni* eggs were detected in the mice faeces at 8 weeks post-infection. The detected egg was large round ovoid, non-operculated egg, containing fully mature miracidium with lateral spine **(**Supplementary figure. a**)**. On contrary, the infection was detected by touchdown PCR at 2 weeks post-infection (Ct values

≤ 29 are strong positive, Ct values 30-39 are moderate positive and Ct values ≥ 40 are weak reaction) **(**Supplementary figure. b, c and d**)**.

logical changes in liver with the antioxidant enzymes and oxidative stress markers.

The results of ALT and AST activities were in



Supplementary figure. Faecal examination and touch- down PCR for confirmation the infection by S. mansoni in mice. (a) Faecal examination. (b), (c), and (d) Touchdown PCR.bile ducts besides congestion of hepatic blood vessels and inflammatory cells (arrow). P.I.; post infection.

DISCUSSION

Schistosomiasis is a debilitating disease with high economic impact, affects many people all over the world leading to high morbidity and mortality (Curtis and Minchella, 2000). Animal models are used as tools for understanding the host-parasite relationships. Mice have been shown to be permissive to *S. mansoni* and they have been widely used to answer fundamental questions on the dynamics of *Schistosoma* infections, including diagnosis (Cheever et al., 2002 and Wang et al., 2004). Because of problems in collecting sufficient numbers of well-defined samples from human patients (from recently acquired infections), this study was conducted on *S. mansoni* infected mouse model and correlated the histopathoagreement with previous studies (Gharib et al., 1999 and EL-Sokkary et al., 2004). Such increase in transaminase enzymes activities after 4, 6 and 8 weeks P.I. might be attributed to presence of immature and mature *Schistosoma* worms in portal vein and infiltration of this area with inflammatory cells, congestion in the portal hepatic vessels and severe hydropic degeneration in the hepatocytes and hepatocytes replacement by focal areas of coagulative necrosis. All of these changes leading to decrease hepatocytes population, and increased cell membrane permeability, subsequently; transaminase enzymes were released into the circulation. Additionally, this complete destruction of hepatocytes, which are responsible for albumin synthesis might explain the decrease

Table 3. Correlation between liver histopathology score and serum ALT, AST activities, total proteins and albumin levels

Bivariate correlation	Kendall's tau-b	Spearman's rho correlation
Histopathology score * ALT	0.473 **	0.655 **
Histopathology score * AST	0.514**	0.676 **
Histopathology score * T.P	0.775**	0.901 *
Histopathology score * albumin	-0.741**	-0.851 **
Histopathology score * globulin	0.864**	0.949**

***P<0.05 a strong significant correlation between liver histopathology score and serum biochemical variable*

Table 4. Correlation between liver histopathology score and liver tissue antioxidant enzymes and oxidative stress marker

Bivariate correlation	Kendall's tau-b	Spearman's rho correlation
Histopathology score * Catalase	-0.823**	-0.927 **
Histopathology score * GSH	0.844 **	0.938**
Histopathology score * MDA	0.926**	0.982 **

***P* < 0.05 a strong significant correlation between liver histopathology score and liver tissue antioxidant enzymes

in serum albumin level 6 weeks P.I. The decrease in albumin level was in accordance with previous studies (Gharib et al., 1999 and EL-Sokkary et al., 2004).

Liver plays an important role in protein metabolism; thereafter the hepatocytes damage will be reflected on the total protein levels (Mbuh et al., 2005). Therefore, the replacement of hepatic cells by fibrous tissue might explain the significance increase ($P< 0.05$) in T.P. levels 4, 6 and 8 weeks P.I. The increase in serum T.P. levels was in agreement with previous study (Page et al., 1972). In addition, the increase in GSH level might be interprets the increase in T.P. level, due to the critical role of GSH in proteins synthesis (Sen, 1997 and Gul et al., 2000). Moreover, the increase in globulin fraction as apart of body immunity response to the parasitic infestation (Harfoush et al., 2003) might be another theory explained the increase in serum T.P. level.

It was previously reported that in parasitic diseases there is a complex and a dynamic physiological relationship between the parasite and the antioxidant defense components of the host (Coutinho et al., 2007). Catalase enzyme has the ability to protect the cell from the accumulated H2O2 produced from dismutation of superoxide anion (Nare et al., 1990). The decrease in liver tissue catalase enzyme activity 4 weeks P.I. was in accordance with (Dessein et al., 1999 and EL-Sokkary et al., 2004). The depletion in the catalase enzyme at 4 weeks P.I. might be attributed to the rapid destruction that observed in hepatic tissue, which by its role consume the enzyme by high amount.

The results of tissue-reduced glutathione (GSH) were in accordance with Hirota et al., 1989 and Song et al., 2000. GSH is an important intracellular antioxidant and play a major role in protecting cells against reactive oxygen species (ROS) and free radicals pro-

duced even in normal metabolism (Sen, 1997 and Gul et al., 2000). Hepatic tissue represents the major GSH reservoir for extra-hepatic levels (Lew et al. 1995). Both hepatic and extra-hepatic GSH are released into the circulation by the help of stressors through an alpha –receptor mechanism (Lew et al., 1985 and Song et al., 2000). The increase in GSH level 4 weeks post-infection might be attributed to the replacement of the hepatic cells with coagulative necrosis and fibrous tissue, which stimulate the extra-hepatic reservoir to secrete high amount of GSH to overcome the shortage resulted from this damage.

Lipid peroxides were elevated by *S. mansoni* throughout the infection (Shaheen et al., 1996 and Pascal et al., 2000). Lipid peroxidation resulted in oxidative destruction of cellular membrane. Subsequently, the toxic free radicals were secreted and MDA is one of the most important free radicals (Cheeseman et al., 1993 and Paradis et al., 1997). Therefore, the elevation of MDA level was observed at 2 weeks post-infection. This finding was in accordance with previous reports (Shaheen et al., 1996 and Pascal et al., 2000).

This study aims to contribute in unveiling the correlation of different biochemical parameters either in serum or in liver tissue with hepatic tissue changes. In conclusion, liver tissue antioxidants enzymes and the oxidative stress marker are sensitive biochemical parameters to the stage of *S. mansoni* infection, and they may be useful to expect the host response to treatment in clinical case. The more increase in the level of AST, ALT, T.P. and globulin in serum or liver tissue MDA and GSH indicating severs histopathological changed into the affected liver and hopeless prognosis is expected. On contrary, the increase in albumin level in serum or catalase level in liver tissue of affected patient/animal demonstrating mild liver histopathological changes. Subsequently, good prognosis and response to anti-schistosomal treatment will be anticipated. However, we applied this preliminary correlation in mice, more studies are required to correlate the serum levels of other biochemical variables that originated from the liver as acute phase proteins with the histopathological changes in liver and other affected organs in schistosomiasis. Furthermore, other studies are required to include chronic stage of the disease on more prolonged period of the infection in mice.

CONFLICT OF INTEREST

The authors of this paper have declared that no competing interests exist.

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Research article
Ερευνητικό άρθρο

Determination of Metal Contamination in Seafood from the Black, Marmara, Aegean and Mediterranean Sea Metal Contamination in Seafood

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ABSTRACT. Seafood is one of the most important components of a healthy diet due to its composition. With the Black, Marmara, Aegean and Mediterranean Sea, Turkey has substantial sources of seafood. Seas are highly impacted by environmental pollution. Among these, heavy metal pollution has long been recognized as a serious problem for seafood. As heavy metals cannot be degraded, they are deposited, assimilated or incorporated in water, sediment and aquatic animals. By these properties, they can be transferred to humans through the food chain especially by the consumption of fish and shellfish. The aim of this study is to determine the concentrations of Cd, As, Pb and Hg levels in selected fish species and marine animals from all of the 4 seas of Turkey by using the ICP-MS technique, and to compare the results with the legislations safe limits. For this purpose, 13 different fish species, mussels and shrimps have been obtained from the Black, Marmara, Aegean and Mediterranean Seas. According to the results, metal concentrations decrease in the order As>Pb>Hg>Cd. In all the seas, the same order was found. Statistically significant differences were observed in the metal levels between fish species and the shellfish in all regions. Except for the two samples, all the results was found compatible with the Turkish Food Codex and European Commission Regulation limits. Arsenic levels were detected between 0,076-4,230 mg/kg within the samples. Cadmium levels were detected as higher than the limits in two samples obtained from the Mediterranean Sea, *Scophthalmus maximus* and *Mullus barbatus* species as 0,076 mg/kg and 0,064 mg/kg, respectively. The highest and the lowest levels of mercury and lead were measured as 0,005-0,405 and 0,015-0,405 mg/kg, respectively. The results obtained from this study revealed that, except for a few cases, the selected heavy metal concentrations in most samples were below the limits. Also, besides the mussels and the shrimps, there was no single type of fish that was consistently high in all metals. The examined seas and the seafood were found to be safe for human consumption.

Keywords: Fish Species, Shellfish, ICP-MS, Heavy metal, Contamination

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INTRODUCTION

Seafood is one of the most important components of a healthy diet due to its proteins of high biological value, vitamin D, vitamin E, iodine, and the long chain omega-3 polyunsaturated fatty acids (Sioen et al., 2007). With the Black, Marmara, Aegean and Mediterranean Sea, Turkey has substantial sources of seafood. According to the last data's, seafood production in Turkey is 537,345 tons, and 392,972 tons of it is obtained from the seas by fishing. For many coastal cities in Turkey fishing is a major economic activity and a means of subsistence. Because of its geographical location and unique habitat, Turkey's seas are characterized by a rich marine biodiversity (Anon, 2016a).

Seas are highly impacted by environmental pollution. Among these, heavy metal pollution has long been recognized as a serious problem for seafood (Caçador et al., 2012). Although adverse health effects of heavy metals have been known for a long time, exposure to them continues. The sources of heavy metals can be anthropogenic or natural. Draining of sewage, dumping of wastes, recreational, mining and other industrial activities are generally the causes of the anthropogenic contamination. Naturally heavy metals may enter into aquatic system by ore-bearing rocks, windblown dust, forest fires and vegetation in small amounts (Fernandez-Leborans and Herrero, 2000; Jarup, 2003). To avoid the pollution of the marine waters, countries taking measures. For example in the EU the main goal of the Marine Directive is to achieve Good Environmental Status (GES) of EU marine waters by 2020. GES was defined by the directive as the environmental status of marine waters where these provide ecologically diverse and dynamic oceans and seas which are clean, healthy and productive (Anon., 2016c). Cadmium, mercury, zinc, lead, arsenic and manganese are some of the heavy metals that can be toxic, persistent and bioaccumulative in aquatic environments. As heavy metals cannot be degraded, they are deposited, assimilated or incorporated in water, sediment and aquatic animals. With these properties, heavy metals are transferred to humans through the food chain especially by the seafood (Malik et al., 2010).

Besides the ecological damage, heavy metals cause carcinogenic and other adverse effects on human health. Some of the heavy metals are essential for the body but some are toxic even in small amounts. Also, essential metals can have toxic effects when they are consumed in excessive amounts (Ubillus et al., 2000). Cadmium (Cd) was classified as human carcinogen by Environmental Protection Agency (EPA). Cd causes kidney failure and fragile bones in human. Lead (Pb) affects the nervous system. Mercury (Hg) causes lung damage and brain function loss. High levels of arsenic (As) can be fatal while small amounts affect the cardiovascular system. Chronic effects after long exposure to heavy metals are more important but the acute cases have also been witnessed in Minimata and Itai-itai diseases (Martin and Griswold, 2009).

Heavy metal pollution in the aquatic environment is identified by measuring its concentration in water, sediment and living organisms (Boran, 2010). Especially marine animals such as fish, accumulate metals higher than present in water and sediment so it is crucial to determine the level of heavy metals in fish and the other sea products to evaluate the possible risk to human health (Bat, 2012). The metal accumulation, changes according to the location, distribution, habitat, feeding habits, age and size of the organism (Velusamy, 2014).

There are different methods like Inductively Coupled Plasma Atomic Emission Spectrometric Method (ICP-MS), Flame Atomic Absorption Spectrometric (FAAS), Atomic Absorption Spectrometric with Graphite Furnace (GFAAS), Electro-Thermal Evaporation Inductively Coupled Plasma Mass Spectrometry (ETV-ID-ICP-MS), Inductively Coupled Plasma Optical Spectrometry (ICP-OES), Inductively Coupled Plasma Spectrometry Having Isotope (ID-ICP-MS), Inductively Coupled Plasma Flame Emission Spectrometry (ICP-AES) to detect metal levels in fish and the other marine animals (Aygun and Abanoz, 2011, Caçador et al., 2012, Hussein and Khaled, 2014, Uysal et al., 2008).

Within these methods, ICP-MS has become more common in food analysis. Compared to the other techniques ICP-MS has some advantages like simultaneous multielement measurement capability, coupled with very low detection limits. Also this tech-

nique offers a wider linear dynamic range and provides simpler spectral interpretation and isotopic information (Nardi et al., 2009).

The aim of this study to determine the concentrations of Cd, As, Pb and Hg levels in selected fish species and marine animals from all of the 4 seas of Turkey by using the ICP-MS technique, and compare the results with the Turkish Food Codex according to the safety limits.

MATERIALS AND METHODS

Sample collection

For this study different fish species, mussels and shrimps were obtained from the Black, Marmara, Aegean and Mediterranean seacoast. Among the Black Sea mussels (*Mytilus galloprovincialis*) and 13 fish species were selected; mullet (*Mugil soiuy*), mackarel (*Trachurus trachurus*), seabass (*Dicentrarchus labrax*), shad (*Alosa fallax*), red mullet (*Mullus barbatus*), turbot (*Scophthalmus maximus*), anchovy (*Engraliusencrasicolus*), whitting (*Merlangius euxmus*), blue fish (*Pomatomus saltatrix*), bonito (*Sarda sarda*), grey mullet (*Mugil cephalus*), garfish (*Belone belone*), hake (*Merluccius merluccius*) . From the Marmara Sea mullet (*Mugil soiuy, Mugil cephalus*), mackarel (*Trachurus trachurus*), seabass (*Dicentrarchus labrax*), shad (*Alosa fallax*), red mullet (*Mullus barbatus*), turbot (*Scophthalmus maximus*), anchovy (*Engralius encrasicolus*), whitting (*Merlangius euxmus*), blue fish (*Pomatomus saltatrix*), bonito (*Sarda sarda*), grey mullet (*Mugil cephalus*), garfish (*Belone belone*), hake (*Merluccius merluccius*), seabream (*Sparus auratus*), 14 fish species were obtained along with mussel (*Mytilus galloprovincialis*) and shrimp (*Penaeus indicus).* Mussel (*Mytilus galloprovincialis*), shrimp (*Penaeus indicus)* and selected 9 species selected from the Aegean and Mediterranean Sea are; mullet (*Mugil cephalus*), shad (*Alosafallax*), hake (*Merluccius merluccius*), whitting (*Merlangius euxmus*), seabass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*), red mullet (*Mullus barbatus*), blue fish (*Pomatomus saltatrix*), seabream (*Sparus auratus*).

One and a half kg from each fish species, shrimp and mussel, were purchased from 3 different fishermen or fish markets of each seacoast in 3 fishing seasons. Samples were washed in clean water and 1 kg of muscles with skin from the each party fish, mussel and shrimp were homogenized. All the prepared samples were stored in the freezer at -20°C until the analysis.

Standards and Reagents

All reagents were of analytical reagent grade. Ultrapure water was used for all dilutions. Nitric acid was not less than 65 %, with a density of approximately 1,4 g/mL. The element standard solutions from Merck Millipore were used for the calibrations and prepared by diluting stock solutions of mg/L.

Standard Preparation

The concentrations of the stock solutions were 20 mg/L for As, 10 mg/L for Cd, 10 mg/L for Pb. Mercury stock solution was prepared by diluting 1 mL of Hg and 1 mL of nitric acid in a 100 ml volumetric flask.

Extraction and Clean-up

Heavy metal analysis was done according to the method of the Nordic Committee on Food Analyses No.186 2007 (Anon, 2007). All the glassware and plastics were held overnight in 10% (v/v) nitric acid. Before its use, it was rinsed with distilled water and deionized water and dried. Boneless muscle tissues (with skin for fish samples) were removed using stainless steel knife and were digested to a strong acid digestion. From each individual sample, 2 aliquots of 1 g homogenized specimen were taken for extraction. Microwave system was used for the extraction of the samples. A digestion solution was prepared with 5 ml of 65 % nitric acid and 5 ml ultra-pure water, with this solution samples were digested under 600 W power, 10 min. Ramp time, 450 Psi pressure at 180 °C in 10 minutes. Then the content was decanted to the falcon tubes and ultra-pure water was added up to 50 ml for the quantification in ICP-MS (Agilent 7500c ICP-MS). For the calibration 3 different concentrations of the metal solutions were used. 1 µg/L, 5 µg/L and 20 µg/L were used for As, while 0,5 µg/L, 2,5 µg/L and 10 µg/L for the Cd, Hg and Pb. During the test of mercury, gold was added in order to stabilize the Hg. Analytical blanks were run in the same way.

Table 1. The settings for the ICP-MS

Parameter	Setting
RF-Power (W)	1500
Carrier gas flow l min -1	1,2
Plasma gas flow l min –1	15
Auxiliary gas flow l min –1	1,0
Nebuliser	Babington
Spray chamber	Water cooled double pass
Spray chamber temperature °C	2
Lens voltage	4,5
Mass resolution	0,8
Integration time points/ms	3
Points per peak	3
Replicates	3

Table 2. Instrumental detection limits (LOD) and limits of quantification (LOQ)

Metal	LOD (µg/g)	LOQ (mg/kg)
As	0,5	0,023
Pb	0,3	0,0013
Hg	1	0,047
Cd	0,5	0,033

Icp-Ms

Mass spectrometer with inductively coupled argon plasma was operated in a mass range from 5 – 240 AMU. The settings of the ICP-MS are illustrated in Table 1. The recovery of the calibration was ranged within 10% . Instrumental detection limits (LOD) and limits of quantification (LOQ) was listed in Table 2. The calculation of the detection limit is based on the 3 times of SD of the blank solution. After calibration of the instrument, the test solutions were analyzed. The samples obtained by pressure digestion were diluted before measurement in order to avoid interference by high concentrations of matrix elements. Within suitable short intervals (after 10 samples) the blank solution and one calibration solution were checked regularly. Calculation of the concentration was done automatically by the software of the ICP-MS instrument.

Statistical analyses

Statistical analyses was conducted with one-way ANOVA test for the difference between seas and Student-t test for the difference between fish and shellfish, $p<0,05$ applied as the minimum level of significance. Statistical software SPSS 14.01 was used for the analyses.

RESULTS

Mean concentrations of all analyzed metals are shown in Table 3. The metal concentrations decrease in the order As>Pb>Hg>Cd, and are the same in all seas. The results of mussels and shrimps were found higher than the other fish species for every metal, in every region. Statistically significant differences were found in the metal levels between fish species and the shellfish in all regions. Between the fish species, *Merluccius merluccius* has the highest concentrations of arsenic in the Marmara, Aegean and Mediterranean Sea; also this species has the second highest concentration of arsenic in the Black Sea. In Turkish Food Codex, there are no specific limits for the As in foods.

For Cd, from all the regions, highest levels were detected in mussels. *Engralius encrasicolus* in the Black and Marmara Sea; *Scophthalmus maximus* in the Aegean and Mediterranean Sea has the highest levels within the fish species. Cadmium levels were

Table 3. Mean concentrations of all analyzed metals.

Region	Sample	Arsenic (mg/kg)	Cadmium (mg/kg)	Mercury (mg/kg)	Lead (mg/kg)
Black	*Mugil soiuy*	0,374	0,034	0,068	0,159
	Trachurus trachurus	0,915	0,024	0,035	0,182
	Dicentrarchus labrax	0,168	0,012	0,023	0,113
	Alosa fallax	1,56	0,021	0,043	0,096
	Mullus barbatus	2,44	0,016	0,032	0,165
	Scophthalmus maximus	0,637	0,027	0,065	0,191
	Engralius encrasicolus	0,896	0,063	0,007	0,071
	Mytilus galloprovincialis	4,23	0,097	0,405	0,375
	Merlangius euxmus	0,463	0,013	0,081	0,099
	Pomatomus saltatrix	0,484	0,016	0,046	0,085
	Sarda sarda	0,257	0,008	0,064	0,072
	Mugil cephalus	0,269	0,011	0,008	0,045
	Belone belone	0,209	0,018	0,023	0,086
	Merluccius merluccius	2,43	0,014	0,049	0,128
Marmara	*Mugil cephalus*	0,335	0,015	0,036	0,055
	Alosa fallax	0,689	0,017	0,037	0,087
	Merluccius merluccius	2,09	0,009	0,062	0,115
	Engralius encrasicolus	0,565	0,032	0,005	0,044
	Dicentrarchus labrax	0,173	0,006	0,026	0,143
	Scophthalmus maximus	0,482	0,014	0,039	0,152
	Belone belone	0,303	0,007	0,013	0,069
	Mullus barbatus	0,942	0,009	0,016	0,098
	Merlangius euxmus	0,324	0,011	0,043	0,054
	Sarda sarda	0,197	0,005	0,028	0,061
	Mytilus galloprovincialis	2,85	0,087	0,341	0,267
	Sparus auratus	0,264	0,012	0,025	0,078
	Trachurus trachurus	0,707	0,016	0,022	0,136
	Penaeus indicus	6,93	0,037	0,061	0,173
	Mugil soiuy	0,279	0,026	0,032	0,093
	Pomatomus saltatrix	0,345	0,006	0,018	0,047

Aegean	*Mugil soiuy*	0.175	0,037	0,014	0,078
	Alosa fallax	0,403	0,015	0,028	0,046
	Merluccius merluccius	1,15	0,025	0,034	0,075
	Merlangius euxmus	0,118	0,009	0,063	0,059
	Dicentrarchus labrax	0,093	0,005	0,008	0,04
	Scophthalmus maximus	0.613	0,108	0,045	0,139
	Mytilus galloprovincialis	1,96	0,143	0,079	0,405
	Mullus barbatus	0,551	0,028	0,016	0,099
	Pomatomus saltatrix	0,182	0,011	0,025	0,049
	Penaeus indicus	2,76	0,051	0,094	0,015
	Sparus auratus	0,118	0,027	0,019	0,077
Mediterranean	*Pomatomus saltatrix*	0,076	0,008	0,012	0,027
	Merlangius euxmus	0,272	0,012	0,048	0,071
	Sparus auratus	0,193	0,035	0,086	0,101
	Alosa fallax	0,298	0,044	0,013	0,086
	Merluccius merluccius	0,843	0,038	0,035	0,108
	Mugil soiuy	0,316	0,012	0,008	0,051
	Mytilus galloprovincialis	1,38	0,112	0,203	0,366
	Mullus barbatus	0,773	0,064	0,032	0,104
	Penaeus indicus	1,82	0,091	0,014	0,068
	Dicentrarchus labrax	0,116	0,015	0,006	0,032
	Scophthalmus maximus	0,443	0,076	0,018	0,045

detected as higher than the limits in two samples obtained from the Mediterranean Sea, *Scophthalmus maximus* and *Mullus barbatus* species as 0,076 mg/kg and 0,064 mg/kg, respectively.

Results of Hg showed that mussels are the most contaminated samples in the Black, Marmara and Mediterranean Sea, while the shrimp was in the Aegean Sea. Within the fish species *Merlangius euxmus* in the Aegean and Black Sea, *Merluccius merluccius* and *Sparus auratus* in the Marmara and Mediterranean Sea has the highest levels of Hg, respectively. It was found that, none of the samples have higher levels than the Turkish Food Codex and European Commission Regulation limits.

According to the lead data, mussels were found to have the highest contamination levels in all regions. Highest levels of lead were detected in *Scophthalmus maximus* species from the Black, Marmara and Aegean Sea. *Merluccius merluccius* was found the most contaminated fish species in the Mediterranean Sea. None of the samples showed higher levels than the Turkish Food Codex and European Commission Regulation limits.

DISCUSSION

Studying the concentration of heavy metals in marine organisms, provides an opportunity to observe the mechanism of bioaccumulation, envi-

ronmental pollution and to assess the effects on public health. It is known that, muscles of the marine organisms are not the active parts of accumulation. But because they are edible parts and showed exceeded levels in the studies, we preferred muscle for the analysis. In our study toxic elements like As, Cd, Pb and Hg was detected in some fish species, mussels and shrimps obtained from 3 seas of Turkey.

The metal concentrations was found in the same order in all seas as, As>Pb>Hg>Cd. In compliance with the other studies, Bat et al. (2012), Damino et al. (2011), Monikh et al. (2013) and Turkmen et al. (2009). Cd was reported as the lowest contaminant.

Statistically significant differences were found in the metal levels between fish species and the shellfish in all regions, according to the student t test ($p<0,05$). It is known that mollusks are filter feeders, they adsorb high doses of contaminants from the environment they lived in. Also, especially mussels were lived near to the coastal so they are affected much more by coastal contamination such as boat sewage and draining of sewerage. Similarly, Culha et al. (2007) detected a significant difference in the metal levels between mussels, sea snails and the fish species. Also in Copat et al. (2013) study Cd, Cr, Pb, Mn, Ni and V levels found higher in *Donax trunculus* then the other fish species. Hussein and Khaled (2014) compared the levels of metals of two mussels (*Pinctada radiate* and *Paphia textile*) with the other fish species they detected. They reported the levels of all metals they analyzed higher in bivalves than the fish species. Their study also revealed that the large bivalves recorded higher values for Fe, Zn, Mn, Cu, and Cd; whereas the small ones recorded highest levels for Cr, Ni and Pb.

Arsenic is a naturally occurring ubiquitous, highly mobilized element and mainly cycled by water in the environment. Because of this character fish and the other marine organisms are possible contamination routes for food chain. According the results of our study, As was the highest contaminated metal in all samples, in all regions. It is well known that, factors such as seasonal cycle of absorption, temperature and salinity might enhance the large bioaccumulation of As. Also, previous findings suggest that non-toxic As was accumulated more in marine organisms then the toxic form coming from anthropogenic activities. Because of the complexity in differantiating the toxic and non-toxic forms, no specific limits can be determined for As. There is still not a limit for seafood, in Turkish Food Codex and European Commission Regulations (Anon 2016b). In Bilandzic et al. (2011) study, four fish species from the Adriatic Sea, detected for arsenic and the concentrations ranged from 0.01 to 70.9 mg/kg. Tuzen (2009) reported the arsenic level in muscles of fishes in the Black Sea, ranged from 0.11 to 0.32 mg/kg. In Spain, lower levels of arsenic were reported in the following ranges (μg/g): 15.39–17.77 in red mullet, 3.93–5.42 in anchovy and 1.73–7.47 in mackerel (Falcó et al., 2006). Gorur et al. (2012) revealed the highest and lowest mean arsenic concentrations of *T. mediterranus* and *M. merlangus* as 4.40 μg/g and 1.32 μg/gdw.

One of the most important properties of Cd is to be biomagnified in the environment. From the upper parts of the food chain, the level of Cd accumulation getting higher. More developed species accommodate more Cd in their systems (Velusamy et al., 2014). Trace metal content of nine fish species harvested from the Black and Aegean Sea were determined in Uluozlu et al. (2007) study, the lowest cadmium content was found 0.45 μg/g in *M. cephalus* and *M. barbatus*, while the highest cadmium content was found 0.90 μg/g in *S. sarda*. Cd concentrations have been reported as 0.02–0.24 mg/kg for muscles of fish from the Black Sea coasts (Topcuoglu et al., 2002). Damino et al. (2011) reported that, the average concentrations did not exceed 0.30 μg/g in fishes from the Mediterranean and Atlantic area. In our study levels were detected as higher than the limits in two samples, *Scophthalmus maximus* and *Mullus barbatus* species obtained from the Mediterranean Sea. The Mediterranean Sea is surrounded by some of the most populated and industrialized countries in the world, and it is almost a closed basin. This cause a regular and constant pollution of the sea by toxic compounds. These may be the reasons that the Mediterranean fishes tend to exhibit high levels of heavy metal than those of populations inhabiting other areas (Storelli et al., 2005). Also in Mendil et al. (2010) study, Cd levels was found higher than the recommended levels in Atlantic bonito red mullet, mackerel and whiting. Tuzen et al. (2009) also have compatible results with our study, they reported high levels of

Cd than the legal limits, from the different fish species caught from the Black Sea. In Turkmen et al. (2008) study, Cd levels of twelve fish species from the Mediterranean, Marmara, Aegean Sea's was also found higher than the permissible limits.

In our study in the Black and Marmara Sea, *Engraluis encrasicolus* has the highest Cd levels inside the fish species. This is considerable cause, in Turkey this species has the big percentage of the fishing with 179 615 tons (Anon, 2016a), and being cheap and widespread makes this species the most preferred one. Similarly in Nisbet et al. (2010) study, *Engraluis encrasicolus* was found to have the higher level for Cd.

Mercury is one of the most toxic heavy metal in the environment and generally human exposure to mercury through the consumption of seafood that may contain methyl-mercury in their tissues. We found that, none of the samples detected in this study, have higher Hg concentrations than the Turkish Food Codex and European Commission Regulation limits. Differently from our study, Nisbet et al. (2010) reported that, they were not detected Hg from the fishes they analyzed from the Black Sea. But in Tuzen et al. (2009) study, mercury levels were found between 0.025-0.084 µg/kg in the fish species obtained from the Black Sea. Harmelin-Vivien et al. (2009) indicated the level of mercury in red mullets from the Mediterranean and Black Sea's as 0.16 µg/kg. These differences can be the result of the methods that used for the analyses, as we mentioned above there were different methods which have different detection limits and sensitivity.

Lead is a toxic element causes carcinogenic effects in marine organisms and humans. Uluozlu et al. (2007) reported the levels of Pb between 0.33-0.93 mg/kg for the fishes they analyzed from the Black and Aegean Sea. In another study levels was found to be in a range of 0.22-0.85 mg/kg for muscles of fish from the Black Sea (Tuzen et al., 2003). From a study conducted in Portugal 0.01-0.15 mg/kg Pb determined in the muscles of fish (Cid et al. 2001). In contrast with these results, in Mendil et al. (2010) and Tuzen et al. (2009) studies, lead levels were reported above the legal limits. In our study, *Merluccius merluccius* was found the most contaminated fish species with Pb, in the Mediterranean Sea. Likewise, in Findik and Cicek (2011) study, it was determined that *Merluccius merluccius* has a higher mean of metal concentration when compared with the other species. But in the study authors indicated that, the levels of Pb was at least 6 times higher than the Turkish Food Codex for *M. barbarous* and 34 times for *M. merluccius*. Copat et al. (2012) was reported that the amount of Pb found in *E. encrasicolus* from the Mediterranean Sea exceeded the limits set by the EC Regulation.

CONCLUSION

The results obtained from this study revealed that, except in a few cases, the selected heavy metal concentrations in most samples were below the limits. Also, instead of the mussels and the shrimps, there was no single type of fish that was consistently high from all metals. The examined seas and the seafood were found to be safe for human consumption.

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Research article
Ερευνητικό άρθρο

An overview of sheep farming features and management practices in the region of south western Peloponnese and how they reflect on milk microbial load

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ABSTRACT. In this study the demographic features, various farm characteristics and milk quality parameters were investigated in sheep farms (n =128) from the North West Peloponnese region, using a prescreen questionnaire. The interviewed sheep farmers were under the control of the regional milk control laboratory of the Hellenic Milk Organization ELGO «Dimitra», which provided us with the corresponding milk quality data for the first quarter of 2014. Our findings regarding the demographic data demonstrated that 23% of farmers were 31-40 years old, 64% of them were high school graduates and 28% had received relevant farming general training; namely 17% of them has designated as "young farmers" in the frame of Third Axis of the Operational Program «Rural Development-Regeneration of the Countryside 2000-2006» and 11% have attended briefings by veterinarians and agriculturists. In terms of farm characteristics, it has been revealed that the mean flock size was 148 sheep and regarding the farming system, the majority of the farms (89.15%) applied a mixed extensive / indoors system. Additionally, regarding building infrastructures and the way of milking, there was limited technological penetration, since the buildings were old-style (77%) and the milking procedure was performed by hands (83%). Concerning the milk composition, the measurements showed mean values of (%) content of fat, protein, lactose and non-fat dry matter (NFDM) at 6.54±0.88, 5.56±0.36, 4.59±0.23 and 10.95±0.42, respectively. Furthermore, the mean value of the total bacterial count (TBC) was found 5.38±0.55 log cfu/ml and the pH value at 6.71±0.11. In general, sheep farmers had a

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good perception of the integrated management and their prospect for sheep farming was optimistic. The impact of this study is that farmers increase their trust to the educational institutions and are prompted to become more receptive to new adaptive trends.

Keywords: sheep; milk; farmers; management; perception; training

ΠΕΡΙΛΗΨΗ. Στην έρευνα αυτή εξετάσθηκαν δημογραφικά στοιχεία, ζωοτεχνικά χαρακτηριστικά και παράμετροι της ποιότητας του γάλακτος σε εκτροφές γαλακτοπαραγωγικών προβάτων, στην περιοχή της βορειοδυτικής Πελοποννήσου. Η έρευνα περιέλαβε 128 εκτροφείς προβάτων και διεξήχθη με χρήση προσχεδιασμένου ερωτηματολογίου. Οι παραγωγοί στους οποίους έγινε η έρευνα, ήταν υπό τον έλεγχο του ΕΛΓΟ «Δήμητρα» και παρέδιδαν το γάλα σε μη αυτοελεγχόμενες τυροκομικές μονάδες. Από τα στοιχεία της έρευνας προέκυψαν τα πιο κάτω δεδομένα. Το 23.26% των κτηνοτρόφων ήταν από 31 έως40 ετών. Το 64% αυτών ήταν απόφοιτοι δευτεροβάθμιας εκπαίδευσης και είχαν παρακολουθήσει κάποια ενημερωτικά σεμινάρια γεωργικού και κτηνοτροφικού περιεχομένου ως «Νέοι αγρότες» (17%) ή από ενημερώσεις κτηνιάτρων της κτηνιατρικής υπηρεσίας της περιοχής τους (11%). Το μέσο μέγεθος της εκτροφής ήταν 148 πρόβατα. Στο 77% των ερωτηθέντων οι υποδομές ήταν παραδοσιακού τύπου. Πολύ περιορισμένη ήταν η τεχνολογική διείσδυση στο θέμα της άμελξης αφού στο 83% διεξαγόταν με παραδοσιακό τρόπο. Σε ότι αφορά την ποιότητα του γάλακτος, από τα δεδομένα των μετρήσεων που μας παραχωρήθηκαν από το εργαστήριο ποιοτικού ελέγχου του ΕΛΓΟ «Δήμητρα», υπολογίστηκαν οι μέσες τιμές της (%) περιεκτικότητας σε λίπος, πρωτεΐνη, λακτόζη και στο στερεό υπόλοιπο άνευ λίπους, στα 6.54 ± 0.88, 5. 56 ± 0.36, 4.59 ± 0.23 και 10.95 ± 0.42 αντίστοιχα. Επιπλέον, η μέση τιμή του συνολικού μικροβιακού φορτίου βρέθηκε στο 5.38 ± 0.55 log cfu/ml και η μέση τιμή του pH των εξετασθέντων δειγμάτων γάλακτος ήταν στο 6.71 ± 0.11. Από την επεξεργασία των απαντήσεων στις ερωτήσεις που απευθύνθηκαν στους κτηνοτρόφους, προέκυψε ότι σε γενικές γραμμές, οι εκτροφείς των προβάτων είχαν καλή αντίληψη σε θέματα που αφορούσαν την ολοκληρωμένη διαχείριση και η προοπτική για την εκτροφή προβάτων στο μέλλον φάνηκε να είναι αισιόδοξη. Ως αντίκτυπος της μελέτης αυτής φάνηκε ότι ενισχύθηκε η εμπιστοσύνη των κτηνοτρόφων στα εκπαιδευτικά ιδρύματα και από τη συζήτηση μαζί τους προέκυψε ότι είναι δεκτικοί σε αλλαγές και επιζητούν την ενημέρωση και τη γενικότερη υποστήριξη.

Λέξεις κλειδιά: Πρόβατο, γάλα, κτηνοτρόφοι, διαχείριση, αντίληψη, εκπαίδευση

1. INTRODUCTION

Sheep and goat farming in Greece is one of the most important sectors of animal production; it has been practiced for thousands of years by exploiting mountainous and semi-arid regions, which are not suitable for crop cultivation (Degen, 2007). Despite the existing difficulties and constraints, the sector remains of high economic and social significance for the country as a whole. It is thus considered that any decline will greatly affect large areas of the country, leading, among others, to the loss of a culture that has survived almost unchanged for centuries (Zygoyiannis, 2006).

The sheep and goat livestock in Greece extents to 9.5 million sheep and 4.5 million goats, compared to just 154.000 dairy cows. As a result, sheep and goat milk production amounts almost up to 60% of the total milk production while the remaining 40% stands for cow milk. Nearly 80% of sheep and goat milk derives from small and family farms, which are highly dependent on family labour, with almost 115.000 families engaged in farming and over 300.000 people working part or full time in dairy sheep farming sector. The small ruminant farming is more laborious considered mainly semi-intensive rather than intensive. Additionally, the milk processing sector comprises 53 big dairy companies processing > 5.000 tons of milk per year and 671 small and medium-sized enterprises (SMEs) or family dairy units processing < 5.000 tons of milk per year. A total of 730.589 tons of cow milk, 735.669 of sheep milk and

Table 1. List of Farms' characteristics

	Replies	(%) Percentage of replies
Demographic data		
Farmers' age		
	25-30	8.53
	31-40	23.26
	41-50	36.43
	51-60	22.48
	61-70	9.30
Farmers' educational level		
	Elementary	30.23
	Secondary	64.34
	Higher	5.43
Farmer' training		
	Yes	27.91
General Characteristics		
Farms' location		
	Hills	58.14
	Mountains	36.43
	Lowland	5.43
Flock size		
	<100	45.00
	100-300	49.00
	>300	6.00
Farming system		
	Mixed	89.15
	Indoor	7.75
	Transhumance	3.10
Breed		
	Local sheep	61.71
	Lacaune	6.20
Milk yield (kg)		
	<1,5	42.63
	1,5-2,0	52.64
Milking		
	Traditional	82.95
	Mechanical	17.05
Infrastructures		
	Traditional	77.34
	Organized	9.40
	Semiorganized	13.26

350.871 tons of goat milk is processing to a big variety of products, with the major ones being pasteurized milk, yogurt and cheese (Parpouna, 2016).

In Greece, the farms have a small herd size and are highly dependent on family labour. Usually lack mechanised milking systems, despite the attempts of restructuring and modernization in recent years. At the same time, it should be stressed that 19 out of the 21 Greek Protected Designation of Origin (PDO) cheeses are produced from sheep or goat milk or mixtures of them; EC Regulation 510 (2006), thus reinforcing the need to produce milk of good quality. However, there is still a need for intensifying reform efforts at both the level of animal farming and the milk processing. Proper training and life-long learning of farmers and cheese makers should be the basis of these efforts. The training programs should cover issues ranging from animal feeding and hygiene to the quality of the final products, as all these determine the value of the products and thus the economic cost-benefit ratio of the small enterprises.

Taking the above into account, the objective of this study was to provide an overview of sheep farming features and management practices in North Western Peloponnese and to evaluate milk and dairy products quality therein.

2. MATERIAL AND METHODS

2.1 Study area and participants

According to Hellenic Agricultural Organization - DIMITER (HAO-DIMITER) published data for 2013, the Greek sheep breeders for milk production, for 2011, amount to 40.000. However, the study area of the present work represents North Western Peloponnese and in particular the regions of Korinthos, Achaia and Eleia



Fig 1. The study area

(Figure1), which according to retrieved data from Hellenic Statistic Authority (HSA), for the year 2010 accommodate 4.000 sheep farms consisting the 10% of the total exclusive sheep farmers of Greece. Among them only the 176 exclusive sheep farmers are routinely controlled by the local quality control laboratory of HAO-DIMITER representing the 4.4% of the area sheep farmers. Out of them 128 were surveyed. The farmers' participation was voluntary and the participants hold the right to withdraw consent at any time, without excuse.

2.2 Data Collection

Research was carried out via personal interviews with the sheep farmers. For the purpose of this survey, a questionnaire of 36 questions was drawn up to obtain information about demographic and general farm data (10 questions) and estimate farmers' perception about welfare and health management aspects (15 questions), the animal feeding and milk yield issues (3 questions) the milk and dairy products' quality aspects (6 questions) and finally two questions concerned the perspective of farming conditions improvement and possible modification of flock size.

In order to assess the produced milk microbial load of the 128 surveyed sheep farms, data regarding 1318 milk samples were provided by HAO-DIMITER (Table 3), during the first semester of 2014 including two monthly measurements in average. After excluding outliners and log10 transformation in total bacteria counts data of milk properties were summarized by descriptive statistics.

2.3 Data analysis

Data collected from the farmers' replies were analyzed by Stat graphics Centurion Software (version XVII). Presentation of answers as (%) percentage is shown in Tables 1 and 2. Data were tested for Normality by the *Kolmogorov-Smirnov* test. For the milk microbial load evaluation, the mean values ± standard deviation of fat, protein, lactose, non-fat dry matter content, temperature, pH and bacteria total

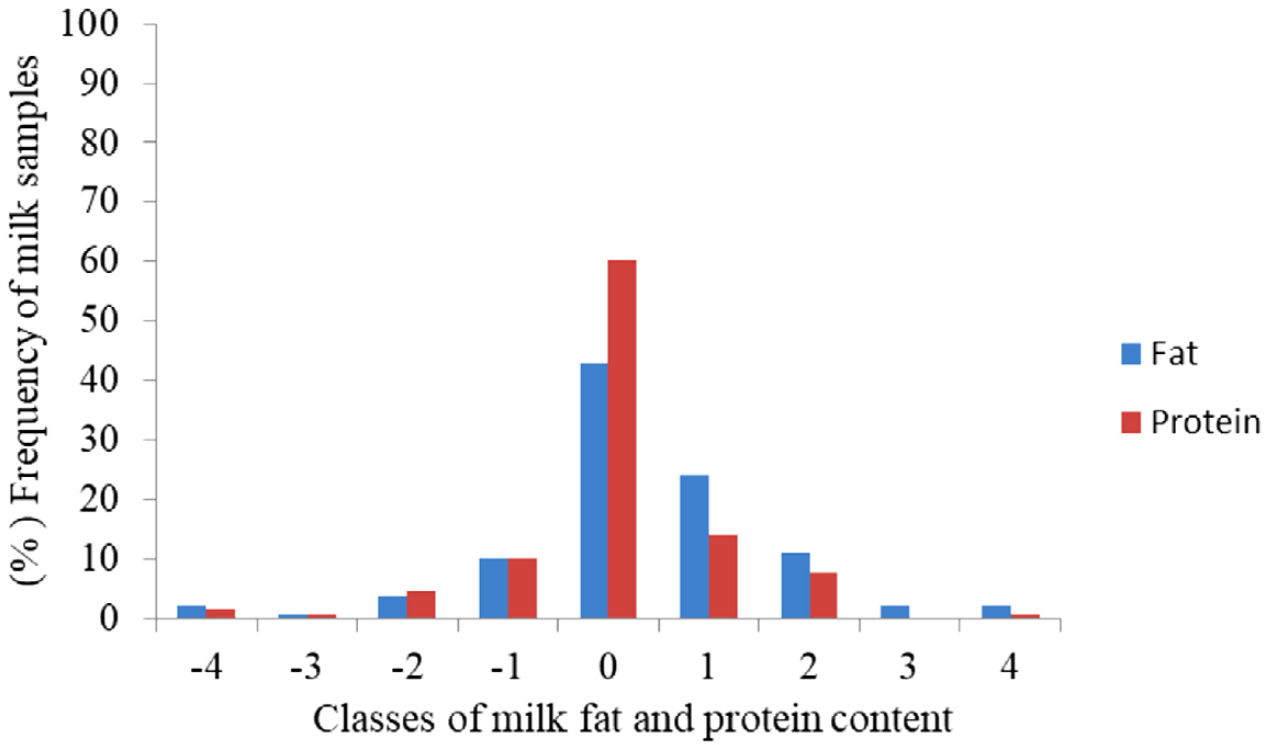


Fig 2. Classification of milk samples - Fat and Protein classes

counts' measurements were calculated. Particularly, data of fat content were classified in nine classes; one basic (0) ranging from 6.09 to 6.69, four awarding with increasing rate value of 0.3 coded as (1) 6.69-6.99, (2) 6.99-7.29, (3) 7.29-7.59 and (4) >7.59) and four penalizing classes with increasing rate value of 0.3 coded as (-1) 5.79-6.09, (-2) 5.49-5.79, (-3) 5.19-5.49 and (-4) <5.19). Regarding the protein content the rating was: one basic class coded as (0) from 5.33 to 5.73 four awarding classes with increasing rate value 0.2 coded as follows: (1) 5.73-5.93, (2) 5.93-6.13, (3) 6.13-6.33 and (4) >6.3 and four penalizing with increasing rate value of 0,2 characterized as (-1) 5.13-5.33, (-2) 4.93-5.13, (-3) 4.73-4.93 and (-4) <4.73. The milk microbial load expressed as colony forming units per ml (cfu/ml) was classified according to the evaluation of milk hygienic quality published by Pirisi et al. (2007), into four classes, namely class AA (≤ 200.000 cfu/ml), class A (200.000-500.000 cfu/ml, class B (500.000-1.500.000 cfu/ml) and class C(≥ 1.500.000 cfu/ml). From the above classification concerning fat and protein content and total bacteria count, emerged an estimation of the milk quality produced in the 128 farms as shown in (Figures 2 and 3).

3. RESULTS

3.1 Sheep farmers' demographic data and farm characteristics

The replies of the 128 farmers on the 36 questions are presented in Tables 1 and 2. All farms were conventional, family owned and operated. The majority of the farmers (68%) were between 25 to 50 years old while the rest (32%) over 50 years. Concerning educational and training level, even though 64 % of the farmers were high school graduates only 28 % has been trained on relevant sheep farming key topics, such as breed selection, pregnancy, lambing, grazing management, milk production, and/or sheep health. Regarding farm location the 58.14% of 128 farms was located on hills while the 36.43% and 5.43% were located on mountains and lowlands respectively. The total amount of sheep in all farms was 18.985,

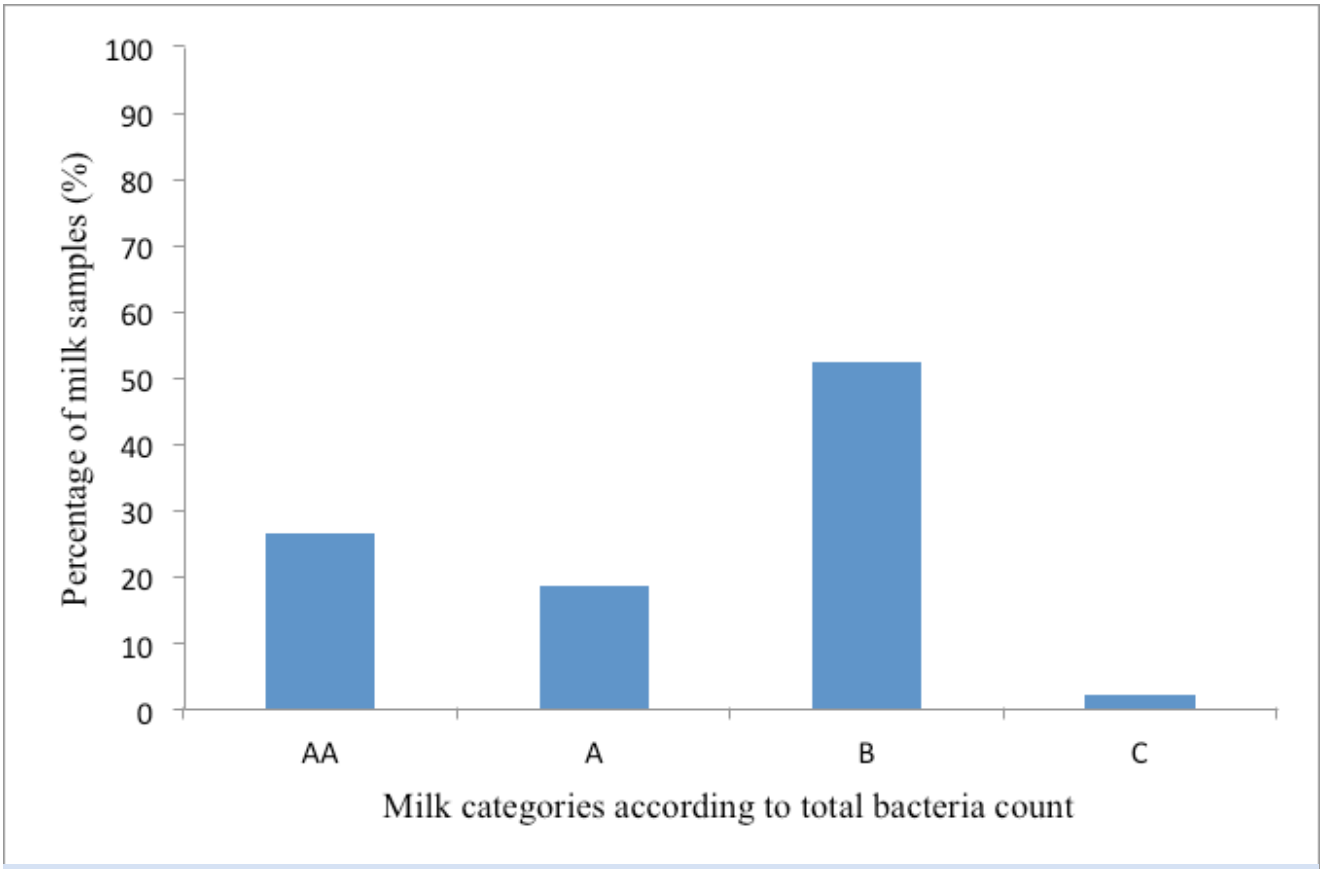


Fig 3. Classification of milk samples - Total Bacteria Count classes

averaged at 148 ± 11.29 with reliability 95% and confidence limit ±22.21. The flock size varied considerably among flocks, ranging from < 100 to over 300 sheep. Precisely, 84.49% of the farmers owned up to 200 sheep and only 15.51% between 200 and 1000 sheep. On the basis of the animal feed origin (farming system), the majority of the farms (89.15%) applied a mixed extensive / indoors system, 7.75% an intensive one, and only 3.1% was characterized as pastoral, as farmers move within the region and use the rights to graze on common pastures. The local breed was found in 61.71% of the farms. The daily milk yield was 1.5 kg at 52.64% (Table.2) during a milking period of 160-180 days. In most cases, milking was performed traditionally (83%) and only 17% of the farmers used milking machines. The bedding in the majority of the farms (77.34%) was wheat straw and there was no distinct parlour of milking.

Almost all farmers (99.2%) knew about the existence of differences among sheep and perceived the significance of this issue in sheep farming. The same was valid regarding the impact of the weather conditions (99%) and the farmer's behavior (100%) on sheep welfare, while 73.64% believed that pasture grazing favors best sheep living when compared to indoors feeding (10%) or combination 15.5%.

Farmers in their vast majority (99%) had understood the advantage of prevention vs. therapy and also the importance of udder anatomy in the sheep health (95%) and milk microbial load (89%) and thus they used it as an animal selection criterion for their flocks**.**

Regarding risk effects deriving from grazing, 70% of the farmers believed that these mainly affect the animal health while the rest 30% the milk microbial load. The one half of the farmers believes that these risks are related with bacteria and pests and the other with chemical substances.

As regards clinical or subclinical mastitis the 75% of them receive occasionally advice by a vet but only for the 35.94% of farmers the vet hold the health records; however, all farmers administered antibiotics to diseased animals even applying old prescrip-

Table 2. List of questions in the survey for sheep farmers' practices and perceptions

Questions	(%) percentage of positive replies
Do you believe that among sheep there are differences	99.20
Do you believe that farmer's behavior effect sheep welfare	98.45
Do you believe that weather affects the sheep welfare	100.00
Do you believe that pasture grazing is better for sheep welfare	73.64
Do you believe that indoors living system is good for sheep welfare	10.08
Do you believe that a combined living system is good for sheep welfare	15.50
Do you believe prevention is better than therapy for sheep health	98.45
Do you consider the udder shape of primary importance for the animals' selection	95.35
Do you consider the udder anatomy an important factor in milk quality	89.15
Do you consider as grazing risks the bacteria and pests or the chemical substances	50.00
The mentioned grazing risks affect the animal health or the milk quality	70.00
Is the diagnosis of mastitis performed by veterinarians	74.40
Do you apply antibiotics	99.22
Do you reject the milk of diseased and treated with antibiotics sheep	100.00
Who keeps the sheep health records	
The farmer	64.06
The veterinarian	35.94
For increased milk yield do you consider as more important,	
The food energy content	70.00
The food quantity	30.00
Does the feeding modification during lactation affect,	
The milk yield	20.00
The milk composition	40.00
Inadequate food consumption is estimated by	
The physical body condition	70.00
The milk yield	30.00
Do you consume raw milk	7.75
Do you consider raw milk as hazard for human health	90.00
Do you know that the bulk tank temperature is a risk factor for milk quality	98.40
Do you believe that raw milk quality ensures the safety and quality of dairy products	99.22
Does the manufacturing process affect the product quality	72.09
Do you know that the milk fat content is affected by the roughages' consumption	89.84
Can you improve,	
Feeding	38.75
Hygienic factors	27.90
Both of them	50.38
Do you plan to continue sheep farming	49.63

Table 3. Bulk tank milk samples characteristics

Milk parameters	Number of measurements (n)	Milk Parameter (Mean value ± s.d.)
Temperature (°C)	1308	4.37±0.65
Freezing Point (°C)	1170	0.56±0.01
pH	1318	6.71±0.11
Fat (%)	1238	6.54±0.88
Protein (%)	1240	5.56±0.37
Lactose (%)	1240	4.59±0.24
NFDM (%)	1239	10.95±0.43
Log (cfu/ml)	1223	5.38±0.56

*NFDM=Not Fat Dry Matter

tions. All were convinced that the milk of diseased sheep must be rejected in accordance to the instructions of the recommended therapy.

The nutrition was a primary concern for all farmers but the feeding system is not part of this article, which is only dealing with the farmers' opinions only on specific feeding aspects as shown in Table 2. The majority of the farmers (70%) considered the food energy content as the main factor for a high milk yield in contrast to the rest that considered the food quantity. However, 40% believed that the feeding system modifications during lactation affect milk quantity and composition. Moreover, 70% of the farmers trusted the physical condition as a good criterion for feeding assessment while the rest 30% considered the milk yield.

In order to determine farmers' perceptions on issues related to consumers' safety aspects, the 92.25% that means the 118 farmers comprehended that the raw milk consumption is hazardous for human health. The 125 (98%) considered the bulk tank temperature as the main factor for milk deterioration. All of them were convinced that the raw milk microbial load vastly ensures the quality and the safety of the dairy products while the 70% of them assessed the importance of the manufacturing process in the milk quality aspects. Regarding milk composition the 115 farmers (90%) knew about the effect of roughages' consumption in the milk fat content (Table 2).

When farmers were asked about factors that could improve the effectiveness of feeding and the hygienic conditions of their sheep farms, interestingly enough, almost 33% of the farmers replied that there is no space for improvement in the management of flocks and milk. Others believed that they could improve both feeding and hygienic conditions (50%) or simply hygienic conditions (28%).

Half of the farmers intended to continue the sheep farming either with the same flock size or they planned to increase it (Table 2).

3.2 Milk quality

All chemical and microbiological data were fitted to the Normal distribution when tested by the *Kolmogorov-Smirnov* test P-values, 0.477 and 0.29 respectively. The evaluation of milk samples' composition is shown in Figure 2. Regarding fat and

protein content 43 and 60% were fitted in the basic quality class respectively, which included the mean values for fat (6.54±0.8813) and then protein content (5.56±0.3665). The penalizing classes for both fat and protein content were found at the same level (17.18%), while the awarding ones were at 40 and 23% respectively. The microbial milk microbial load is shown in Figure 3. Thus, 26.56% of the samples were classified in class AA (excellent quality) and 18.75% in class A (very good quality). This means that 45.31% of the samples had a bacterial count load less than 500.000 cfu/ml, which it accepted as a critical threshold for the processing of raw milk without thermal treatment. On the contrary, the majority of the samples (52.34%) belonged to class B, which is considered as accepted by EC regulation 1662/2006 for cheese manufacturing after milk thermal treatment.

4. DISCUSSION

According to the results on the demographic characteristics, sheep farming is mainly practiced by men (87%) and in a lesser degree by women (13%). These results agree with a previous study on the region of Thessaly, Greece (Lioutas et al., 2010) and are higher than 76% with those reported by Giannenas et al. (2008) concerning Eordea Kozanis area in Northern Greece. Data regarding the age follow normal distribution with a maximum in the category of 41-50 and this is in accordance with the corresponding data of the above mentioned studies.

The 64.34% were high school graduates. In general, training is needed not only for knowing but also for behaving differently and managing their farms better. Even though farmers wish to attend training seminars, the majority of them (84%) replied that this is practically not feasible, since a) training seminars are usually conducted far away from their villages, b) according to their opinion, the seminar's content they do not meet their needs, and c) their opinion that their participation in an educational process will make them feel uncomfortable. Nevertheless, 27.91% have participated in training programs and it is high enough in relation with 3.4% reported by Lioutas et al. (2010).

Regarding the flock size, it was shown that most sheep farms were of low-intensity with a few animals, nevertheless flocks were bigger than those reported by Zervas et al. (1996) for the same area and smaller than the corresponding ones in North-Eastern Greece (Alexopoulos et al., 2011).

In regard to the farming system, differences were observed when compared to data reported for North-Eastern Greece by Alexopoulos (2011). Our findings revealed transhumance, indoor and mixed systems for 3.1%, 7.75% and 89.15% of the farms, respectively, while in North-Eastern Greece the respective numbers were 5%, 43% and 52%. The indoor farming system was very limited and this is in accordance to Zervas et al. (1996), who concluded that farming in Feneos, a location included in our study area, is characterized by an extensive livestock production system. As farmers admitted, the farming system depended "on their economic status and the weather conditions". According to their statement "the sheep farming is extensive when the weather is good and intensive when finances are prosperous".

Genetic material of various types and origins from different geographic areas has contributed to the foundation of Greek sheep breeds which date back to the very remote past, Hatziminaoglou et al., 1990. Since many foreign breeds have been imported into the country and are used in breed crossing with local ones, a large breed biodiversity appeared in sheep farms. This was confirmed by the current situation of the surveyed farms; where about 60% of the sheep consisted of a "specific local breed" according the farmers, while the rest of the animals were crossbred with either the Greek breeds Karagouniko and the sheep of Chios (34.00%) or the foreign breeds Lacaunae (6.20%). According Kominakis et al. (2001), the absence of integrated rational approach of breed improvement has led to reduction or even extinction of some rare indigenous breeds.

The mean daily milk yield was around the 1.5 kg at 95.27% of flocks and only for 4.73% the yield was around 2.5 kg. The increased milk yield may be attributed to the foreign breeds, like Lacaunae, joined in flocks (6.20%). In the study area the mechanical milking does not seem to be an extended practice, in contrast to the North-Eastern Greece where it is applied at 91%. It is known that although mechanical milking for small ruminants was intro-

duced in Greece in the early 1980s it has not been widely applied, probably due to either the insufficient diffusion of its advantages, or to the high investment cost. Maybe the size of farms and the limited financial sources were the reasons that in the majority of farms there was one main room divided in two places where milking system was available. This data are similar with the findings of Giannenas et al. (2008).

Regarding sheep welfare, farmers were aware of the fact that different behaviour among sheep could designate sheep diversity. The weather and the farmer's behaviour were considered as stressful conditions, when these were improper. According to welfare guidelines for sheep farmers the weather is a key factor (Guideline, 2003). This fits with the majority of the farmers who agree with the above and moreover believe that sheep live better, when these are free in grasslands.

Concerning health management, for 95% of the farmers the udder anatomy is a major criterion in animal selection, as it influences animal health, facilitates milking and affects the milk microbial load. The majority of the farmers (74%) collaborates with veterinarians rather fragmentary and applies treatment with antibiotics (99%) thus the 25% of farmers self-activate without preceding veterinary consulting. However, all farmers verified that milk from sheep treated with antibiotics must be rejected. Some farmers believed that the veterinarians were not qualified enough to properly deal with health management aspects, as previously reported by Kristensen and Enevoldsen (2008). The lack of systematic subsequent support from the side of veterinarians is pointed out by the low percentage (36%) of farmers, who replied that the health-treatment records were kept by the veterinarians.

All farms aim at increased milk yield of high quality for good milk price and manufacturing of quality products. Given that there are no official and global regulations on fat and protein content except the minimum requirements, in the frame of this study the bulk tank milk evaluation has been done only by fat and protein content and total bacteria count, according to Pirisi et al. (2007). This has been revealed that in the 83% of examined samples the (%) fat and protein content was equal or bigger to the threshold values of class (0) and at the 17% of the samples these were found lower. Regarding the evaluation of microbial quality bulk tank, the 98% of the samples appeared acceptable count. In addition, the microbial count of 45.31% of samples was less than 500.000 cfu/ml, the limit that permits to the milk to be treated without any previous thermal treatment. These findings were very interesting to evaluate production processes and cleaning given the great bacteria diversity found in the sheep farms, the lack of milking machines and maybe the inadequate operation of cold tanks according to D'Amico et al 2010. Freezing point estimation has also been used to identify adulteration due to water addition and the pH value as an estimator of milk microbial load (Morgan et al., 2001). The mean values were in accordance with values stated at the Codex Alimentarius (2003) (data not shown).

As small ruminants' milk is mostly transformed into cheese, its quality is mainly comprised in fat and protein content (Zervas, thus Tsiplakou 2011) thus the feeding is the major factor affecting sheep milk chemical quality and consequently the yield in cheese. According to Kitsopanidis (2000) from a productivity analysis of the farm resources used in sheep farming, the need for better use of the available pasture and more quantities of silage instead of concentrates, have been pointed out. Animal feeding affects animal energy balance, milk composition, and nutrition value especially in early lactation stage (Nudda et al., 2014) as well as the "terroir" profile of milk products (Martin et al., 2005). In this study, 70% of the farmers were aware of this, while 30% believed that the feed quantity has an impact mainly on milk yield and not on the nutritional value of milk.

The study highlights that the majority of farmers were aware of the risk factors associated with milk and dairy products quality; 90% of them knew that their health was in risk by consuming raw milk but, it was not clear to the farmers whether grazing risks could also influence milk microbial load. The farmers' perception that the herd size and the farm management practices influence bacteria counts in bulk tank milk is in accordance with Kelly et al., (2009) which studied the associations between herd management factors and bulk tank total bacterial count in grass-based dairy herds as well as the study of Alexopoulos et al., 2011, which also explored the role of different factors on

the quality of raw ovine milk. **T**he statement of 39% of farmers, that they should improve nutrition, could be correlated with farmers who supply milk of low fat and protein content, or those who follow unsatisfactory feeding. Concerning the microbial quality of milk, even though the high percentage of samples with high bacterial load, only a small percentage of farmers believed that they should improve the hygienic conditions of their farm. This is indicative of farmers' insufficient training on issues concerning the hygienic production.

Overall, despite these shortcomings, it is very optimistic that about 50% of farmers plan to continue the job of sheep farming and increase their flock size. The mean herd size consisted of 100-200 sheep while the herd had low genetic potential and therefore low productivity. The mean profile of the sheep farmer that emerged from this survey was a man of less than 50 years old, with high school educational level. He considered sheep farming as primary occupation and deals with this job for many years. He had not attended any training program even he very much wanted to do so. He had no feeding strategy. The limited farmers' finances resulted in insufficient veterinary support. The farming management was almost empirical. His knowledge was well established especially on milk hygienic quality issues and thus the microbiological milk microbial load was greatly within the limits of EC regulations. Even though in general he was not satisfied, he wanted to keep on farming but needs state support, technical assistance and training to improve the overall management and increase the consequent income as well.

5. CONCLUSIONS

The present study depicted the sheep herds' of NW Peloponnese current status and investigated the sheep farmers' level of knowledge with regards aspects of integrated production management of milk and dairy products. The survey highlighted positive outcomes regarding the age, the educational level, the good perception of many farming aspects and the willingness to continue and improve the sheep farming. On the contrary, as negative findings emerged the limited knowledge input through agricultural education/ training programs, the small flock size and the traditional farm infrastructures. Regarding the milk quality evaluation, the survey represented that objective of farmers must be to increase the milk quantity, to stabilize the composition in protein and fat and their ratio since protein ensures the cheese quantity and fat the cheese quality. Additionally, must be mentioned the farmers' belief that the establishment of a farmer-friendly environment is necessary and could contribute to the improvement of the sector growth, the advancing of the productivity, the labor conditions and their incomes. Considering the limited of actual farm data from Greek sheep flocks, the development of a technical manuscript addressing useful issues for the farm management system should be a useful future approach.

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Research article
Ερευνητικό άρθρο

Comparative analysis of changes in the lungs of experimental animals' induced conventional and lung protective ventilation

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ABSTRACT. Mechanical ventilation has long been the leader in the treatment of critically ill and injured patients in an intensive care unit. The aim of this study was to examine the impact of the application of positive end-expiratory pressure on histopathological findings and on the parameters of ventilation, oxygenation and acid-base status. The experimental study included 42 animals (piglets), which were divided into of tree groups, each containing 14. The animals of the control group (conventional ventilation) were ventilated with the tidal volume of 10-15 mL/kg. Tidal volume of 6 mL/kg was applied in the low tidal ventilation group, whereas the ventilation strategy in the lung protective ventilation group meant the application of a tidal volume of 6 mL/kg and the 7 mbar of positive end-expiratory pressure. Mechanical ventilation in each animal lasted for 4 hours. After conducting mechanical ventilation, samples were taken from the lung tissue, which were sent for histopathological examination. The parameters of ventilation, oxygenation and acid-base status were measured after each hour's duration of mechanical ventilation. Application of positive end-expiratory pressure 5-10 mbar during mechanical ventilation is a safe and useful method which is not followed by the occurrence of significant abnormalities in the structure of the ventilated lung. However, a low tidal volume without positive end-expiratory pressure causes significant changes in the histological structure of healthy lungs. Positive end-expiratory pressure keeps the alveoli open throughout the respiratory cycle which allows the lungs to maintain homeostasis in terms of adequate ventilation, oxygenation and acid-base status.

Keywords: Low tidal ventilation, positive end-expiratory pressure, Animal Experimentation

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INTRODUCTION

Mechanical ventilation of the lungs in the past few decades was a basic life support, for support of critically ill patients. It is a widely applied therapeutic measure in intensive care units and is an integral part of the therapeutic treatment of patients with Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS) (Hiil JD et al., 1965). It has found wide use in a number of operational procedures that are performed under general anesthesia, when the application of various medications and procedures terminated spontaneously breathing patients, and this vital function is enabled using the ventilator incorporated in modern equipment for anesthesia. Although essential for the successful treatment of respiratory failure, mechanical ventilation can worsen or indirectly damage lungs (Ashbaugh DG et al., 1967; Dreyfuss D et al., 2001). Too low end-expiratory lung volume may be related inter aliato cyclic opening and collapse of unstable lung units, which is promoted by ventilation with zero or inadequate PEEP (Positive End-Expiratory Pressure). The repeated opening and collapse of the alveoli and bronchial tree end segments generate forces tangent to alveolar basement membranes (sometimes not aptly referred to as "cutting"). The entire phenomenon of multidirectional changes in stress is called atelectrauma. In this context, the detrimental effects of ventilation may be alleviated by the application of PEEP to prevent the cyclic derecruitment of pulmonary alveoli but not high enough to lead to their excessive inflation (De Prost N et al., 2011; Chiumello D et al. 2008; Caironi P et al., 2010). When a given group of alveoli collapses, the traction force exerted on their walls by the adjacent relaxed units multiplies due to the so-called parenchymal (interstitial) interdependence, which results primarily from the route of connective tissue fibres in the lung structure (connective tissue syncytium) (Whitehead T et al., 2002; Gattinoni L et al., 2011; Marini JJ, 2001). Although these forces favour re-aeration of atelectatic units, they may reach values that cause substantial local stress at the link between the collapsed and relaxed pulmonary zones. A small amount of applied PEEP is used in most mechanically ventilated patients to mitigate end-expiratory alveolar collapse. A higher level of applied PEEP is sometimes used to improve hypoxemia or reduce ventilator-associated lung injury in patients with acute lung injury, acute respiratory distress syndrome, or other types of hypoxemic respiratory failure. Objectives: In this paper, we present some types (strategies) of mechanical lung ventilation applied in experimental conditions, their impact on the occurrence of certain histopathological changes, as well as the repercussions of these changes on lung function in maintaining the homeostasis of gas exchange and acid-base status.

MATERIAL AND METHODS

This study was conducted as a prospective and randomised experimental study at the Institute of Experimental Medicine in Kosovska Mitrovica. The research is to begin after obtaining approval from the ethics committee of the Medical Faculty, number 2777/2013, with the obligation to respect the provisions of the Animal Health Protection (Official Gazette of RS No.37 / 91, 50/92, 33 / 93,52 / 93,53 / 95, 52/96 and 25/2000), the Law on environmental Protection (Official Gazette of RS No.66 / 91, 83/92 and 53/95), Directive 86/609 / EEC (1986) Council of Ministers of member states and European Convention for the protection of Vertebrate Animals used for experimental and other scientific purposes (1990). The experimental study included 42 animals (piglets), which were divided into three groups (control group CV - Conventional Ventilation, study group LVtV – Low-Tidal-Volume Ventilation and study group LPV- Lung Protective Ventilation), each containing 14. As anesthetics, we used ketamine hydrochloride (Calypsol Silkroad Online Pharmacy) and midazolam (Dormicum – Roche). We administered 20 mg/kg of body weight of ketamine hydrochloride and 0.5 mg/kg of body weight for midazolam. Application of the drug was carried out by intramuscular injection in the cervical muscles. Anesthesia was performed over a period of 3 to 5 minutes after the administration of the anesthetic. Immediately we placed the peripheral intravenous cannula in the vein of the left or right ears. After the establishment of the peripheral venous line, we applied the continuous intravenous infusion (15 mL/kg/h of body weight) of 0.9 % NaCl. After the induction of anesthesia, which was characterised by preservation of spontaneous breathing, the experi-

Table 1. Anesthetic/Analgesic/ Muscle relaxant agents commonly used in ventilation of experimental animals

Anesthetic/Analgesic/ Muscle relaxant	Dose
ketamine hydrochloride (Calypsol – Silkroad Online Pharmacy)	Loading dose 20 mg/kg b.w., intramuscular
midazolam (Dormicum – Roche)	Loading dose 0,5 mg/kg b.w., intramuscular
propofol (Diprivan – AstraZeneca)	0.06–0.7 mg/kg/min b.w., TIVA
fentanyl (Fentanyl – Janssen)	Loading dose: 1–7 μg/kg/h, b.w. TIVA
pancuronium bromide (Pavulon- Organon)	0,15 mg/kg b.w., intravenous

 # TIVA - the method of total intravenous anesthesia

Table 2. Initial ventilator settings for pigs with normal pulmonary function

Ventilator Parameter	Control group - CV	Study group - LVtV	Study group - LPV
Model of mechanical ventilation	Intermittent Positive-Pressure Ventilation (IPPV)	Intermittent Positive-Pressure Ventilation (IPPV)	Intermittent Positive-Pressure Ventilation (IPPV)
Duration of mechanical ventilation	4 h	4 h	4 h
Fraction of inspired oxygen (FiO_2)	40 % (0,4)	40 % (0,4)	40 % (0,4)
Tidal volume (Vt)	15 mL/kg	6 mL/kg	6 mL/kg
PEEP	0 mbar	0 mbar	7 mbar
Respiratory rate	12 breaths per minute	12 breaths per minute	12 breaths per minute
I:E ratio	1:2	1:2	1:2
Inspiratory time	~1,7 s	~1,7 s	~1,7 s
Expiratory time	~3,3 s	~3,3 s	~3,3 s

 # CV – Conventional Ventilation; LVtV – Low Tidal Volume Ventilation; LPV – Lung Protective Ventilation; PEEP – Positive End-Expiratory Pressure; I:E ratio – the ratio of the duration of inspiration to the duration of expiration.

mental animal was placed on the operating table on dorsal recumbency (supine position) with the aim of establishing an operational airway (tracheotomy). Tracheotomy allowed successful establishment of airway in all experimental animals, avoiding complications related to endotracheal intubation (prolonged, difficult or impossible intubation, laryngospasm, bronchospasm). Upon the establishment of the airway and initiation of mechanical ventilation of lungs, maintenance of anesthesia was achieved using the method of Total Intra-Venous Anesthesia (TIVA) (Table 1). Continuous intravenous administration of analgesics and anesthetics was done using Braun FM perfusor. The ventilator was started with mechanical ventilation by a previously the well-established mode of ventilation. The control group (conventional ventilation) consisted of the experimental animals, which were ventilated with a tidal volume of 15 mL/kg of body weight, a respiratory rate of 12 breaths per minute, an inspiratory oxygen fraction of 40 % (FiO2 0.4) and a PEEP equal to 0. A mechanical ventilation with the low tidal volume of 6 mL/kg per IPPV (Intermittent Positive Pressure Ventilation) was applied in the test group, without the application of PEEP. In experimental animals of the study group, LPV was applied with a tidal volume of 6 mL/kg of body weight, a respiratory rate of 12 breaths per minute, an inspiratory oxygen fraction of 40 % and a PEEP of 7 mbar (Table 2). The parameters of ventilation, oxygenation and acid-base status were determined at the end of the first (T1), second (T2), third (T3) and fourth (T4) hour of implementation of

the mechanical ventilation. To perform the mechanical ventilation of lungs of experimental animals the ventilator Dräger Savina®300 was used. Assessment of the pulmonary function of experimental animals was performed by monitoring parameters of ventilation, oxygenation and acid-base status. Monitoring of ventilation included the following parameters: tidal volume (Vt), minute volume ventilation, peak pressure (Ppeak), plato pressure (Pplato) and mean airway pressure (Paw.mean), partial pressure of carbon dioxide in the arterial blood of experimental animals (PaCO2). Monitoring of oxygenation involved saturation of hemoglobin in arterial blood (SaO2) and partial pressure of oxygen in arterial blood (PaO2). Evaluation of acid-base status was performed on the basis of the values of arterial blood pH. Values of the intrapulmonary shunt (Qs/Qt) were also monitored during the implementation of the mechanical ventilation of lungs of experimental animals. Monitoring of ventilation, oxygenation and acid-base status were made at specified time intervals (T) during the experiment. Determining the value of the monitored parameters was performed at the end of every hour duration of mechanical ventilation of the lungs (T1-4). For the monitoring of experimental animals during the implementation of mechanical ventilation of lungs we used: Monitor Infinity Gamma XL - Dräger, gas analyzer GEM Premier 3000 Instrumentation Laboratory and monitor incorporated in the ventilator type Dräger Savina®300. Immediately after the establishment of the airway and initiation of mechanical ventilation to the lungs of experimental animals, we started the preparing of the femoral artery. The most common are the prepared right femoral artery. The blood sample for gas analysis was taken by connecting separate, vacuum packed syringes with heparin to previously marketed intra-arterial cannula whose apex is in the lumen of the femoral artery immediately after the completion of the surgical preparation of the same. Arterial blood gas analyses of experimental animals were made at the end of each time of the research phase.

At the end of a four-hour ventilation, while the experimental animal was under general anesthesia, on mechanical ventilation of the lungs, the median sternotomy was done, the chest was opened, coming to the lungs and then surgical resection of certain parts of the lungs (tops, bases, ventral and dorsal) take clips of tissue for histopathological examination. From each lung was taken five tissue sections (a total of 10 for one experimental animal). Clips of lung tissue were placed in special plastic containers filled with formalin solution, hermetically closed, identified and sent to the Institute of Pathology for making a histopathological preparation and their analysis and description by a pathologist. Analysis of *pathohistological* samples was performed by a pathologist who was blinded to the experimental research protocol. The tissue was fixed 24 h in 4 % neutral buffered formalin, processed with a standard sequence of water-alcohol-xylene-paraffin, paraffin cast in molds, cut on a rotary microtome LEICA RM 2235 and routinely stained with hemalum and eosin. Gradation degree of the histopathological changes in the lungs of piglets was based on the following divisions: (4) expressed - histopathological changes were present in the 6 to 10 of the preparation taken from both lungs of experimental animals; (3) moderately expressed - changes were present in 3 to 5 of a total of 10 preparations; (2) minimally present (expressed) - present in 1 to 2 histological preparations of the 10 examined and (1) histopathological changes not present. Histological samples were observed using Axiovert 200M Inverted Microscopes - Carl Zeiss at an increment of x100.

Table 3. Body weight of experimental animals and statistical analysis (t- test and Anova test. The result is significant at p<0,01)

Group	Control group (CV)	LVtV group	LPV group
Body weight (kg±SD)	24,64 ± 2,46	24,71±2,39	24,92 ± 2,52
t- test (p value)	CV/LVtV 0,938715	CV/LPV 0, 764525	LVtV/LPV 0,819788
Anova test (p value)		0,950404	

 # CV – Conventional Ventilation; LVtV – Low Tidal Volume Ventilation; LPV – Lung Protective Ventilation; SD - Standard Deviation

STATISTICAL ANALYSIS

The analysis of obtained data was performed using the SPSS 15.0 software as well as Microsoft Excel 2010. Descriptive statistics was used to determine the relative numbers and measures of the central tendency: the arithmetic mean (X), a measure of variability (standard deviation - SD), and the relative proportions (percentages). Student's t-test of independent samples was used to test the statistical significance of the differences among the mean values of the observed parameters in different groups. The one-way Anova test was also used in the statistical processing of the obtained results. The one way, or one-factor, Anova test for independent measures is designed to compare the means of three or more independent samples (treatments) simultaneously. A p-value<0.01 was considered statically significant.

RESULTS

Table 3 provides the values of average body weight

Table 4. Comparison of the presence and severity of histopathological changes in the lung parenchyma experimental animals (pigs) control and study groups (1- no expressed, 2 - minimum expressed, 3 - moderately expressed, 4 - expressed).

Histopathological changes	The presence and severity			t – test (p value)			ANOVA p - value
	CV	LVtV	LPV	CV/LVtV	CV/LPV	LVtV/LV	
Perivascular edema	1,71±0,61	2,28±0,61	1,14±0,36	0,020236	0,006672*	<0,00001*	<0,000011*
Interstital edema	2,28±0,46	3,36±0,74	1,85±0,53	0,000157*	0,032901	<0,00001*	<0,00001*
Alveolar edema	1,35±0,49	2,79±0,69	1	<0,00001*	0,018635	<0,00001*	<0,00001*
Bleeding in the lung parenchyma	1,71±0,46	2,07±0,73	1,35±0,49	0,137665	0,061403	0,006029*	0,008798*
Distension of the alveoli	2,78±0,59	1,86±0,66	3,21±0,57	0,00055*	0,060969	<0,00001*	<0,00001*
Rupture of alveoli	1,85±0,36	1,78±0,69	2,21±0,42	0,738092	0,024702	0,063274	0,060372
The collapse of the alveoli	1,35±0,49	3,35±0,63	1,28±0,47	<0,00001*	0,698937	<0,00001*	<0,00001*
Microatelectasis	1,5±0,52	3,21±0,69	1,28±0,47	<0,00001*	0,26211	<0,00001*	<0,00001*
Cellular infiltration of perivascular space	1,78±0,42	2,78±0,58	1	<0,00001*	<0,00001*	<0,00001*	<0,00001*
Cellular infiltration of interstitial space	1,71±0,49	2,78±0,42	1	<0,00001*	<0,00001*	<0,00001*	<0,00001*
Cellular infiltration of intraalveolar space	1,21±0,42	1,71±0,46	1	<0,00001*	0,082276	<0,00001*	0,000025*
Small airways- obstruction	1,43±0,51	3,14±0,77	1,14±0,36	<0,00001*	0,102449	<0,00001*	<0,00001*
Small airways - dilatatio	1,5±0,52	1,07±0,26	3,07±0,47	<0,00001*	<0,00001*	<0,00001*	<0,00001*
The rifts in the lung parenchyma	1,43±0,51	1,14±0,36	2,14±0,36	0,102449	0,000293*	<0,00001*	<0,00001*

*# CV – Conventional Ventilation; LVtV – Low Tidal volume Ventilation; LPV – Lung Protective Ventilation * p<0,01 – the results ist significant*

and its standard deviation. The comparative analysis of these values did not give a statistical significance ($p<0,01$) between the control and study groups of experimental animals.

The histopathological report on lung preparations after ventilation with the tidal volume of 15 mL/kg without the application of PEEP (conventional ventilation) reveals a weak presence of a perivascular and interstitial edema with minimal cellular infiltration of the interstitial and perivascular space. Some preparations (lower parts of the lungs which rest against the spine and the rear wall of the rib cage) also show parts of the lung parenchyme with the presence of bleeding, and the collapse of the surrounding alveoli which creates microatelectasis. In the other parts of the lungs (tips and the upper parts of the left and the right lungs), there are moderately distended alveoli, with a weak presence of alveolar wall rupture (Figure 1).

Ventilation with a lower tidal volume (Vt 6 mL/kg, PEEP 0 mbar; Group LVtV) revealed a completely different pathohistological picture when compared with the conventional lung protective ventilation. There is a vivid presence of the alveolar collapse with the creation of microatelectasis. The collapse of small airways, a moderate cellular infiltration of the perivascular and the interstitial space, with low infiltration of alveolar spaces were also detected. Some preparations also reveal parts of the lung parenchyma with the presence of minor bleeding (Figure 2). In some lung regions, there are also moderately distended alveoli with a rare presence of the alveolar wall rupture.

Lung protective ventilation (Group LPV) is characterised by different degrees of alveolar distension with a low level of alveolar wall rupture. In certain parts of the lungs, there is a presence of tears, i.e. holes in the lung parenchyma. They do not occur frequently and are small. Small airways are dilated, although there are parts of the lungs where certain contents can be noticed in the lumen. Bleeding is insignificant and present subpleurally. No microatelectasis. Cellular infiltration of the perivascular, interstitial or alveolar space is not noticed (Figure 3). By testing the differences of histopathological changes in the lungs of experimental animals in the control and study groups LVtV and LPV, using t-test and Anova test, we come to the conclusion that there is a statistically significant difference ($p<0,01$) in the changes that have occurred in the lungs of experimental animals of the study group LVtV and LPV compared to the experimental animals in the control group (Table 4).

Monitoring the values of Ppeak, Pplato i Paw.mean during intervals of time (T1-T4), a gradual rise has been noticed during mechanical lung ventilation. The lowest values are noted in the LVtV study group

Table 5. Review of mean values P peak, P plato, and P aw.mean by time stages of research and testing the significance of differences between control and study groups using t-test and Anova test.

Parameters	P_{peak} (mbar ± standard deviation)				P_{plato} (mbar ± standard deviation)				$P_{aw.\ mean}$ (mbar ± standard deviation)			
Groups	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
CV	20,06±1,81	21,14±1,40	21,28±1,26	22±0,87	18,14±2,07	18,92±1,38	19,64±1,08	20,07±1,07	5,62±0,62	6,07±0,61	6,54±0,74	6,91±0,73
LVtV	11,71±0,72	12,5±0,65	12,64±0,49	13,71±0,46	10,28±0,82	11,21±0,57	11,35±0,49	12,07±0,47	3,64±0,63	3,85±0,36	4,28±0,46	4,64±0,49
LPV	19,07±0,73	20,21±0,97	20,5±1,28	21,92±0,91	17,07±0,61	18,35±0,93	18,71±1,32	20±0,87	11,07±0,61	11,5±0,65	11,85±0,86	12,14±0,66
Statistical analysis (t - test and Anova test. The result is significant at p<0,01*)												
t - test CV/LVtV	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*
CV/LPV	0,07697	0,05397	0,11544	0,83481	0,08282	0,21266	0,05310	0,84851	<0,00001*	<0,00001*	<0,00001*	<0,00001*
LVtV/LPV	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*
Anova	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*

 # CV – Conventional Ventilation; LVtV – Low Tidal Volume Ventilation; LPV – Lung Protective Ventilation; Ppeak – Peak pressure; Pplato – Plato pressure; Paw.mean – Mean airway pressure

Table 6. Review of mean values PaCO2, pH arterial blood and minute volume of ventilation by time stages of research and testing the significance of differences between control and study groups using t-test and Anova test.

Parameters	$PaCO_2$ (mmHg ± standard deviation)				pH arterial blood				MVV (L/min)
Groups	T1	T2	T3	T4	T1	T2	T3	T4	
CV	36,7±2,64	33±1,79	28,4±2,09	26±0,67	7,427	7,47	7,52	7,549	3,43
LVtV	41,6±1,86	46,4±1,74	59,6±3,36	73,7±4,85	7,401	7,322	7,221	7,118	1,79
LPV	37,8±2,65	43,8±1,79	51,5±2,27	54,6±2,09	7,419	7,38	7,35	7,333	1,82
Statistical analysis (t-test and Anova test. The result is significant at p<0,01*)									
t-test CV/LVtV	<0,00001*	<0,00001*	<0,00001*	<0,00001*	0,010239	<0,00001*	<0,00001*	<0,00001*	<0,00001*
CV/LPV	0,264244	<0,00001*	<0,00001*	<0,00001*	0,290688	<0,00001*	<0,00001*	<0,00001*	<0,00001*
LVtV/LPV	0,000221*	0,000689*	<0,00001*	<0,00001*	0,046285	<0,00001*	<0,00001*	<0,00001*	0,739,429
Anova	<0,00001*	<0,00001*	<0,00001*	<0,00001*	0,955433	0,046901	0,052097	0,031131	<0,00001*

 # PaCO2 – The partial pressure of carbon dioxide in arterial blood; CV – Conventional Ventilation; LVtV – Low Tidal Volume Ventilation; LPV – Lung Protective Ventilation; MVV – Minute Volume of Ventilation

and the highest in the study group LPV. Due to the application of PEEP in the LPV group, Paw.mean in this group has the highest values and there is a statistically significant difference (p <0,00001) in relation to the control and LVtV groups. Table 5 represents a review of mean values Ppeak, Pplato and Paw.mean by time stages of research and testing the significance of differences between control and study groups using t-test and Anova test.

Conventional lung ventilation (control group) in the duration of 4 h led to a gradual decrease of the PaCO2 value from 36,7 mmHg to 26 mmHg and the increase of arterial blood pH from 7,427 to 7,549 (moderate respiratory alkalosis). Low tidal volume ventilation (LVtV group) caused significant hypercapnia (PaCO2: 41,6–73,7 mmHg) with the decrease of artery blood pH (7,401–7,118) and the creation of a heavy respiratory acidosis. Table 6 shows the review of mean values of PaCO2 and arterial pH by time stages of research and test of the significance of differences between control and study groups using t-test and Anova test.

All the three applied models of mechanical ventilation (Conventional Ventilation, Ventilation Low Ttidal Vvolume and Lung Protective Ventilation) in the duration of 4 hours were characterised by the PaO2 and SaO2 values within the normal range. Values of intrapulmonary shunt recorded a significant increase in experimental animals from during the mechanical ventilation of the lungs with low tidal volume. The conventional and the ventilation of low tidal volume and PEEP is being followed by a minimal increase of Qs/Qt in experimental animals from the control and LPV groups. Table 7 shows the review of mean values PaCO2 and arterial pH by time stages of research and test of the significance of differences between control and study groups using t-test and Anova test.

DISCUSSION

Today it is considered (there is ample evidence in recent experimental and clinical studies) that lung damage can occur during mechanical ventilation with low tidal volume. On the histopathological preparations from the lungs of experimental animals in the LVtV group, there are marked changes in the form of existence of microatelectasis, alveolar collapse, perivascular, interstitial and alveolar edema, cellular infiltration, collapse of small airways, etc.

However, it is observed that not all parts of the lungs are affected the same by pathological changes. Previously described changes are present almost in all regions of the left and right lungs. However, at certain parts of the lungs (upper and perihilar region), there is the presence of moderate distension alveoli with minimal rupturam of alveolar walls and the absence of edema. This type of change was represented in the upper parts of the lungs (bearing in mind that during mechanical ventilation experimental animals were in a supine position). This suggests that the applied airway pressure may be ideal for opening and ventilation of some lung units, insufficient to open the largest part of the atelectatic zone and cause excessive distension in areas with satisfactory compliance (Gattinoni L et al., 1993; Rimensberger PC et al., 1999; Silva PL et al., 2015). The positive end-expiratory pressure (PEEP) applied during mechanical ventilation in LPV group prevented the formation of significant edema of perivascular, interstitial and alveolar space, which is accompanied by low expressed cellular presence in them. At the same time, PEEP was holding open the largest number of alveoli and small airways. It seems that ventilation with low lung volume can be a cause of lung damage. Several mechanisms may explain the occurrence of lung injury induced by low tidal volume ventilation. Cyclic opening and closing of small airway/lung units may lead to increased local stress and the occurrence of atelectrauma. PEEP effectively holds open distal airways, which makes possible the ventilatory cycle. This was fully defined in animal models, but the significance in humans has not been established. Several studies suggest that the adverse effects of mechanical ventilation can be reduced by the application of PEEP (Webb HH et al. 1974; Argiras EP et al. 1987; Dreyfuss D et al. 1988; Sandhar BK et al. 1988; Yardimci Ç et al., 2001). Ventilation washed isolated rat lung airways with low volume (5-6 mL/kg) and low PEEP or PEEP equal to zero, causing lung injury that can be reduced by high levels of PEEP (Muscedere JG et al., 1994). The foregoing experimental studies that are treating VILI (Ventilator-Induced Lung Injury) showed that the application of PEEP has a protective effect on the lungs during the implementation of mechanical ventilation. However, the application of PEEP of 10 mbar during the mechanical ventilation with tidal volume of 20 mL/kg is being accompanied by severe histopathological changes in the lungs of experimental animals and higher mortality. At the same time, these authors in this group of experimental animals recorded increased Ppeak above 30 cm H2O. The authors believe that increase of Ppeak value above 30 cm H2O during the mechanical ventilation of lungs is being followed by the loss of protective effect PEEP (Vilar et al., 2009). Then a PEEP flow further contributes to lung damage. Proof of this is the appearance of perivascular edema, inflammatory infiltrates and foci of small bleeding in the lung parenchyma of these experimental animals.

Mechanical ventilation of the lungs with low tidal volume (LVtV group) and constant breathing frequency, characterised by low minute volume ventilation, has resulted in a significant increase in the partial pressure of carbon dioxide (PaCO2) and a decrease of pH value of arterial blood. The main reason for the occurrence of severe hypercapnia, which is accompanied by respiratory acidosis is a hypoventilation of lungs that was present throughout the duration of this type of mechanical ventilation. The effect of low tidal volumes and low insuflation pressure leads to an inability recruit a large number of alveoli for gas exchange. As a result, there is an occurrence of microatelectasis. A significant reduction in the surface of the alveolar-capillary membrane is available for gas exchange, leading to significant distortions of ventilation-perfusion relationships, increased intrapulmonary shunt and alveolar dead space. With a further duration of the mechanical ventilation, the intensity of the existing histopathological changes increases and created new, which basically leads to worsening hypercapnia and respiratory acidosis in experimental animals. The low values of PaCO2 and intrapulmonary shunt (Qs/Qt), as well as high values of pH in LPV study grup, are a direct result of effective pulmonary ventilation. The use of PEEP enabled more effective lung ventilation holding a large number of alveoli that were opened during the respiratory cycle. This leads to a reduction of the intrapulmonary shunt, alveolar dead space and maintenance of ventilation-perfusion relationships in different lung regions in approximately the physiological range. It is clear that the partial pressure of

Table 7. Review of mean values PaO2, SaO2, and Os/Qt by time stages of research and testing the significance of differences between control and study groups using t-test and Anova test.

Parameters	PaO_2 (mmHg ± standard deviation)				SaO_2 (% ± standard deviation)				Qs/Qt (% ± standard deviation)			
Groups	T1	T2	T3	T4	T3	T4	T3	T4	T3	T4	T3	T4
CV	176±5,86	166±2,01	173±3,49	175±3,27	99,8±0,42	99,07±0,47	98,9 ±0,61	99,6 ±0,51	8,57±2,41	9,43±2,21	9,93±2,2	10,42 ±2,53
LVtV	150±6,94	146±5,48	142±7,41	140±6,5	98,7±0,61	98,35±0,63	98,2 ±0,58	97,4 ±0,94	14,21±1,36	17,57±1,15	21,5±1,16	24,85 ±1,03
LPV	159±5,67	161±4,79	156±4,43	158±4,16	99,35±0,61	99,42±0,97	99,1 ±0,61	99,2±0,53	8,71±1,89	10,97±1,9	11,14±1,56	12,21±0,97
Statistical analysis (t-test and Anova test. The result is significant at p<0,01*)												
t test CV/LVtV	<0,00001*	<0,00001*	<0,00001*	<0,00001*	0,046901	0,067167	0,544649	0,039905	<0,00001*	<0,00001*	<0,00001*	<0,00001*
CV/LPV	<0,00001*	0,00055*	<0,00001*	<0,00001*	<0,00001*	0,002485*	0,003966*	<0,00001*	0,863068	0,41673	0,105471	0,024991
LVtV/LPV	0,001827*	<0,00001*	<0,00001*	<0,00001*	0,011149	<0,00001*	0,000801*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*
Anova	<0,00001*	<0,00001*	<0,00001*	<0,00001*	0,000054*	0,000025*	0,001142*	<0,00001*	<0,00001*	<0,00001*	<0,00001*	<0,00001*

 # CV – Conventional Ventilation; LVtV – Low Tidal Volume Ventilation; LPV – Lung Protective Ventilation; SaO2 – Saturation of hemoglobin in arterial blood; PaO2 – the partial pressure of arterial oxygen; Qs/Qt – Intrapulmonary shunt

carbon dioxide in the arterial blood and arterial pH can be kept within normal limits by adjusting the respiratory rate (increasing or decreasing) and minute ventilation during the implementation of mechanical ventilation of the lungs. The pressure of carbon dioxide in arterial blood reflects the minute ventilation. The protective ventilatory strategy with low tidal volumes in Aacute Rrespiratory Ddistress Ssyndrome (ARDS) can lead to an increase in PaCO2 (permissive hypercapnia) and mild respiratory acidosis. Some research gives the suggestion that hypercapnia has a protective effect on the lungs in terms of injury, although there is no evidence from clinical research (Laffey JG et al., 2001; Masterson C et al., 2015; Ni Chonghaile M et al., 2005; Marhong J et al., 2014; Contreras M et al., 2015; Masterson C et al., 2015 Marhong J et al., 2014). The values of the monitored parameters of oxygenation (SaO2, PaO2) are largely dependent on the inspiratory oxygen concentration in the inhaled air (FiO2) and to a lesser extent on recorded minute ventilation. For these reasons, the duration of ventilation with low tidal volume, records less variation of SaO2 and PaO2. In the LPV group where PEEP is applied, there has been an increase in the value of PaO2 and SaO2 with the duration of mechanical ventilation. The beneficial effect of PEEP on oxygenation and saturation has been proven in many clinical studies (Tugrul S et al., 2005; Toth I et al., 2007; Badet M et al., 2009; Sánchez Casado M et al., 2012; Şentürk M et al., 2015). Nowadays, protective lung ventilation has become standard procedure in the treatment of patients with ARDS. Secondary analysis of the strategies of mechanical ventilation applied in ARDS has shown that a reduction in tidal volume from 12 to 6 mL/kg is being accompanied by certain benefits regardless of the value of the pressure plateau. Multicentric, randomissed trials and meta-analyses did not demonstrate that the values of PEEP over 12 mbar improve the condition of patients with ARDS (Briel M et al., 2010; Meade MO et al., 2008; Brower RG et al., 2004; Mercat A et al., 2008). Over the last few decades, there is a tendency of decrease in tidal volume in clinical practice (intraoperatively and in intensive care units) (Esteban A et al., 2013). The application of PEEP and low tidal volume (6 mL/kg) during cardiac surgery may lead to improvements in lung mechanics and gas exchange, with simultaneous prevention of postoperative intrapulmonary shunt compared with standard ventilation tidal volume of 12 mL/kg and PEEP of 5 mbar (Chaney et al., 2000). Other authors, have demonstrated in their research that received Vt of 6 mL/kg for large operations in abdominal surgery did not reduce the deterioration of lung function in the postoperative period compared to conventional ventilation with tidal volume of 12 mL/kg and a PEEP of 5 mbar (Treschan et al., 2012). However, anhother group of authors, in their study showed

that, compared with conventional ventilation (Vt 9 mL/kg, without the use of PEEP), the use of protective ventilation (Vt 7 mL/kg, PEEP 10 mbar) during intra-abdominal surgery lasting more than 2 hours leads to improved lung function tests in the next 5 postoperative days, with a reduction of the modified Clinical Pulmonary Infection Scores (mCPIS). At the same time, it reduces the incidence of pulmonary complications and improves oxygenation (Severgnini et al., 2013). Furthermore, it was concluded that the protective use of PEEP during surgery leads to better postoperative oxygenation and reduced formation of atelectatic fields (Imberger et al., 2010). Group the auothors, in their meta-analysis, which includes the most recent test, came to the conclusion that protective lung ventilation with low tidal volume, with or without PEEP, in critically ill patients without previous lung damage, is being accompanied by favourable clinical outcome in terms of reducing the incidence of ARDS and lung infection. However, there has not been a decline in mortality, reduction in the incidence of atelectasis or a shorter hospital stay. Respiratory monitoring is of great help adjusting optimal ventilation in order to prevent injury due to mechanical ventilation and to timely detect postoperative pulmonary complications in the perioperative period (Yuda Sutherasan et al., 2014). It remains an open question what PEEP values should be used. Today, that is the subject of many experimental and clinical studies. Available evidence indicates that high levels of PEEP, as compared with low levels, did not reduce mortality before hospital discharge. The data also shows that high levels of PEEP produced no significant difference in the risk of barotrauma, but rather improved participants' oxygenation to the first, third, and seventh days (Santa Cruz R et al., 2013). Regardless of the results, adjusting the value of PEEP should be compared to individual assessment for each patient (Gattinoni L et al., 2012).

CONCLUSION

Application of the moderate values of PEEP (7 mbar) during the implementation of the strategy of mechanical ventilation of the lungs with low tidal volume is being accompanied by minimal histological abnormalities in the structure of the ventilated lung. However, a low tidal volume without PEEP causes significant changes in the histological structure of healthy lungs. PEEP keeps the alveoli open throughout the respiratory cycle which allows the lungs to maintain homeostasis in terms of adequate ventilation, oxygenation and acid-base status.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have not competing interests.

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Research article
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Identification of new avian Infectious Bronchitis virus variants in Iranian poultry flocks by High Resolution Melting curve analysis

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ABSTRACT. Avian Infectious bronchitis (IB) is a common coronavirus infection of chickens and responsible for performance reduction and increasing mortality due to subsequent respiratory, renal and/or reproductive disorders. Classification of causative agent is necessary to plan successful vaccination strategies to prevent the infection due to poor inter-strains cross-reaction. To identify dominant circulating strains in Iran, a Real-time PCR combined with 3' Un-Translated Region (3' UTR) High Resolution Melting (HRM) analysis designed as a rapid and reliable method for IB Virus (IBV) detection and differentiation. Samples collected from 20-suspected flocks and after PCR products, HRM curves of samples as well as 6 commercial IB live vaccines with 2 standard strains, were analyzed as references. IBV genomes detected in 11 samples while according to HRM analysis and calculating Genotype Confidence Percentage (GCP), 6 positive specimens identified as 793/B field strains and the left 5 found as new IBV variant strains. Then obtained PCR products sent for nucleotide sequencing to determine genotype relativity. All five infectious agents, related to QX-like type and indicating circulation of new variants in Iran as a probable cause of vaccination failures and consequent economical losses.

Key words: High Resolution Melting analysis, Identification, Infectious Bronchitis Virus, Iranian, New variant, Poultry

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1. INTRODUCTION

Avian Infectious Bronchitis Virus (IBV) considered as a causative agent of a worldwide, acute, highly contagious disease of chicken called (IB). IBV belongs to group 3 coronavirus genus in Coronaviridae family. Its genome consists of 27,000 bp nucleotides coding four structural proteins including Spike (S), Membrane (M), Nucleocapsid (N) and envelope (E) (Cavanagh and Gelb, 2008). In susceptible chicks, IBV causes respiratory disease followed by reduced weight gain, increased feed conversion ratio (FCR) and mortality, especially by concurrent bacterial infections. In commercial layers and breeders, IBV replication in oviduct causes decrease in egg production and quality. IBV is highly transmissible and the only way to control it is by prevention, because there is no treatment for IB. Although strict biosecurity measures and vaccination schedules are implemented widely, the success may not be achieved, since there is low cross-immunity between multiple serotypes of IBV and there is risk of vaccine reversion to more virulent strains. Therefore, identification of dominant IBV strains in the field is so important to avoid vaccination failures and control consequent economic losses (Cavanagh and Gelb, 2008; Cook, 2012).

Although there are several methods to differentiate and classify IBV strains, the most effective way is the nucleotide sequencing of IBV's genome, especially S1 gene. However, due to high propensity of this gene to mutation or recombination, it cannot solely be reliable (Hewson, 2009; Hewson, 2010). Moreover, S1 gene sequencing is not rapid enough to plan an appropriate vaccination protocol in order to control IBV outbreaks (Hewson, 2009; Hewson, 2010). Due to presence of hyper-variable region in the 3' UTR of IBV with conserved flanking regions, sequence analyzing of 3' UTR can be used to differentiate IBV strains (Williams, 1993)1993. It has been recognized that a deletion in the 3'UTR of the Australian subtype B vaccines that not observed in the same serotype of field isolates (Hewson, 2009). In another study suggested that the 3'UTR could be used to differentiate vaccine and field isolates by using real-time polymerase chain reaction (RT-PCR), which is more rapid than sequence analysis (Hewson, 2010).

High Resolution Melt (HRM) analysis method was reported to accurately identify single base changes in nucleotide sequences of up to 400 base pair (bp) in length (Lin, 2008).

Recently, despite vaccination, there are several reports of IB outbreaks in Iranian broiler and layer flocks. The aim of this study was to evaluate IBV strains status and determinate new variants in Iranian poultry flocks by using HRM curve analysis of IBV-3' UTR region as a rapid and reliable method for differentiation and characterization of IBV strains.

MATERIALS AND METHODS

Field sampling

Twenty tracheal samples were collected from suspected broiler and layer flocks as well as one Iranian IBV field isolate registered as IR/773/2001 (793/B) (Razi institute; Iran), one standard M41 strain (Veterinary Laboratories Agency Central Veterinary Laboratory, Weybridge; UK) and seven current IB commercial live vaccines, as reference strains (Table1).

Table 1: IBV strains

References	Specification	Sources
Vac.1	Nobilis IB 4/91(793/B)	Merck Animal Health-Netherland
Vac.2	Nobilis Ma5	Merck Animal Health- Netherland
Vac.3	Bronhikal SPF (H120)	Veterina- Croatia
Vac.4	H120	Razi- Iran
Vac.5	Cevac Vitabron L (H120+PHY.LMV.42)	Ceva- Hungary
Vac.6	Bioral H120	Merial- France
Ref.1	M41	Veterinary Laboratories Agency, Weybridge-UK
Ref.2	IR/773/2001 (793/B)	Razi-Iran

VIRAL RNA EXTRACTION AND CDNA SYNTHESIS

Easy-spin® RNA extraction kit (iNtron Biotechnology inc; South Korea) was applied to extract viral RNA. The tracheal scrapings were homogenized in PBS and all vaccines and other reference strains were diluted to 2 ml distilled water before RNA extraction. To start RNA extraction, 100 µl of diluted vaccine or PBS containing field tracheal scrapings, lysed in 900 µl of Lysis buffer and continued according to the manufacturer's instruction. The extracted RNA was added to a reaction micro-tube contains 5 µl final solution including 20 units Ribolock TM (RNase inhibitor) enzyme, 10 mM dNTPs, 200 units RevertAid TM (murine Moloney leukemia virus reverse transcriptase), 100 pmol Randome Hexamer, 12.5 µl DEPC- treated water (Fermentas- Termo Fisher Scientific; USA). This was incubated at 25 degree centigrade (ºC) for 10 minutes, after which it was heated at 42 ºC for 60 minutes and then 70 ºC for 70 minutes (Hewson, 2009; Hewson, 2010).

Real-time RT-PCR and HRM analysis

For Real-time RT-PCR, all of 25 µl RT reactions contained 2.5 µl 10× Buffer, 5 µM Syto9® (Invitrogen), 50 mM MgCl2, 1.2 µM dNTP, 10 mM forward primer and 10 mM reverse primer, 2 µl cDNA as template. The sequence of the forward primer (All 1-F) was CAGCGCCAAAACAACAGCG and reverse primer (Del 1-R) was CATTTCCCTGGCGATAGAC (Cinagen; Iran) (Hewson, 2009; Hewson, 2010). The 3' UTR nucleotide sequences for vaccine strains and references were unavailable in GenBank.

RT-PCR was performed using a Rotorgene 6000 (Corbett Life Science; Germany) with an initial denaturation step of 94 ºC for 1 minute, followed by 40 cycles of 94 ºC for 20 seconds, 57 ºC for 20 seconds, 72 ºC for 30 seconds, and a final extension of 72 ºC for 2 minutes (Hewson, 2009; Hewson, 2010).

HRM curve analysis was carried out on the 3' UTR PCR products immediately after PCR using a Rotorgene 6000 and Rotor-Gene Q Series 2.0.2.4 version (Corbett Life Science). The 3' UTR PCR prod-

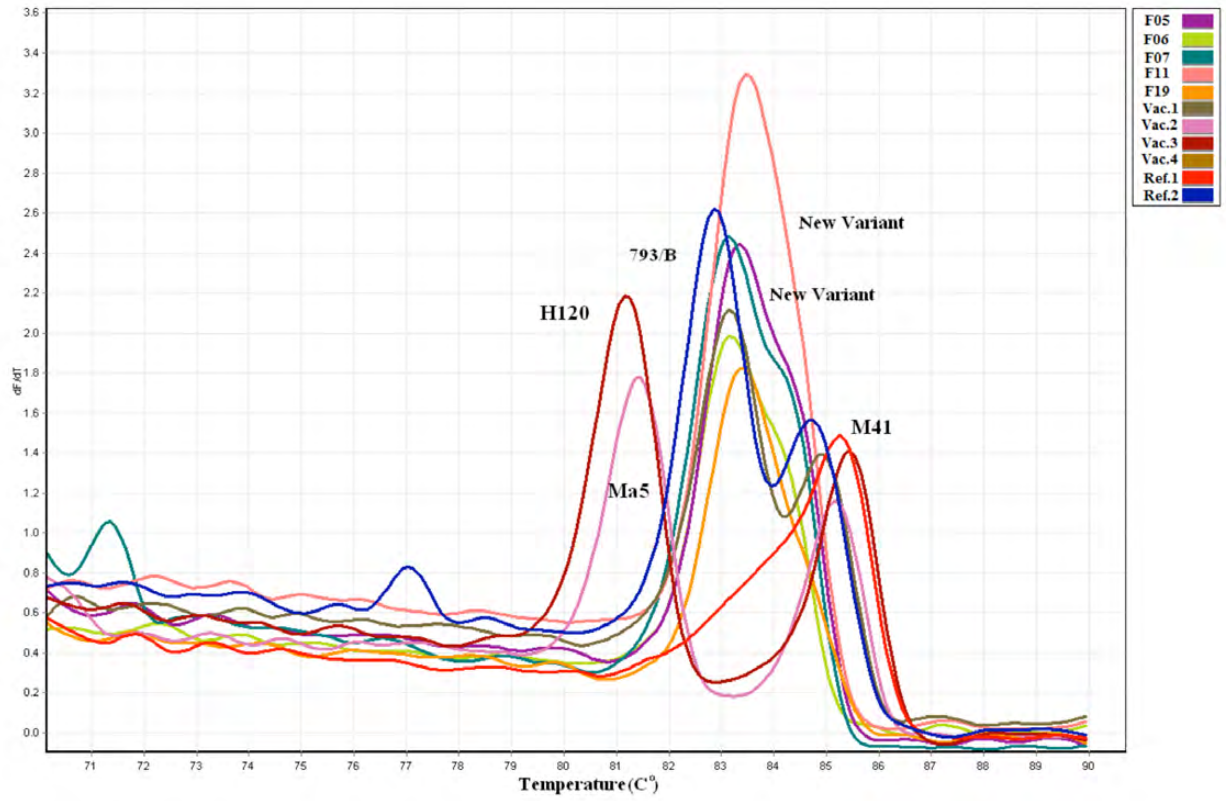


Fig 1: Conventional melt curves produced by the amplicons from 3' UTR of F.05, F06, F.07, F. 11 and F.19 field samples, determined as Varaint' QX-like type and compated to refrence strains and vaccines.

ucts were subjected to temperature increasing ramps of 0.3 ºC between 70 ºC and 90 ºC (Hewson, 2009; Hewson, 2010).

Then each strain was set as a 'genotype' comparing to known reference strains and the HRM Genotype Confidence Percentages (GCPs), valued attributed to each strain being compared to each genotype. Strains with GCP equivalent or more than 95% indicating the same genotype.

After HRM analysis of positive field samples, amplicons related to the new variants sent to Kavoosh Fanavar Kosar Company to be sequenced. Then initial identification all PCR products were carried out online by BLAST software (http://blast.ncbi.nlm.nih.gov). Moreover, in order to evaluate 3'UTR sequence of the vaccine and field IB strains for planning a differentiation tool, another commercial 793/B IB vaccine (Gallivac IB88; Merial) as well as Vac.1 (Nobilis 4/91; MSD) and one field strain (IR/773/2001-Razi) were subjected to find probable differences.

Table 2: Genotype Confidence Percentage of 3' UTR amplicons obtained from 11 positive samples, refrence strains and vaccines at a ramp of 0.3 Cº

	Vac.1	Vac.2	Vac.3	Vac.4	Vac.5	Vac.6	Ref.1	Ref.2	F.01	F.02	F.03	F.04	F.05	F.06	F.07	F.11	F.12	F.19	F.20
V ac .1	10 0	0. 18	0. 09	0. 08	0. 07	0. 07	0. 0 5	9 0. 5 8	8 9. 9 7	8 9. 9 0	8 8. 6 5	8 9. 0 2	4 5. 2 0	3 8. 1 7	4 9. 1 8	5 2. 2 0	8 7. 4 9	4 1. 6 4	9 0. 8 9
V ac .2		10 0	80 .8 7	82 .4 3	87 .4 0	83 .3 4	0. 0 0	0. 4 8	0. 4 4	0. 1 5	0. 3 8	0. 0 7	0. 0 3	0. 0 5	0. 0 5	0. 0 2	0. 7 8	0. 0 3	0 0 4
V ac .3			10 0	90 .0 0	89 .2 6	95 .7 7	0. 0 1	0. 2 0	0. 1 5	0. 0 4	0. 1 7	0. 0 3	0. 0 1	0. 0 1	0. 0 1	0. 2 3	0. 0 1	0. 0 3	0 0 9
V ac .4				10 0	97 .3 7	88 .5 0	0. 0 0	0. 2 3	0. 1 9	0. 0 6	0. 1 8	0. 0 3	0. 0 1	0. 0 2	0. 0 2	0. 0 1	0. 3 6	0. 0 1	0 0 2
V ac .5					10 0	92 .0 5	0. 0 0	0. 2 0	0. 1 5	0. 0 5	0. 1 5	0. 0 2	0. 0 1	0. 0 1	0. 0 1	0. 0 1	0. 2 8	0. 0 1	0 0 2
V ac .6						10 0	0. 0 1	0. 1 5	0. 0 9	0. 0 3	0. 0 3	0. 0 2	0. 0 1	0. 0 1	0. 0 1	0. 0 0	0. 1 6	0. 0 1	0 0 3
R ef. 1							1 0	0. 1 0	0. 2 9	0. 0 0	0. 9 7	0. 8 3	0. 4 1	0. 0 5	0. 1 0	0. 3 3	0. 0 3	0. 3 7	0. 3 1

R	1	9	9	9	9	4	5	5	4	9	5	9
ef.	0	6.	5.	0.	1.	9.	0.	9.	0.	0.	0.	2.
2	0	8	8	4	3	5	0	7	4	0	0	9
		3	0	1	7	7	5	9	9	8	6	6
F.		1	9	9	8	5	7	7	4	8	8	9
01		0	5.	0.	9.	7.	0.	7.	9.	8.	9.	1.
		0	6	7	1	9	1	2	4	0	1	3
			9	7	4	7	9	2	5	3	7	6
F.			1	9	9	4	4	5	5	8	8	9
02			0	1.	0.	1.	7.	9.	3.	8.	9.	0.
			0	6	7	2	5	7	6	6	1	4
				1	9	5	5	2	9	9	6	7
F.				1	9	6	5	5	6	9	5	9
03				0	6.	1.	9.	8.	0.	0.	0.	0.
				0	4	2	7	9	0	3	0	0
					3	0	9	5	5	8	7	9
F.					1	5	3	4	5	8	3	9
04					0	5.	6.	9.	3.	9.	0.	1.
					0	7	9	1	2	5	0	3
						7	8	0	8	2	7	3
F.						1	9	9	9	2	9	2
05						0	5.	6.	0.	4.	1.	0.
						0	2	0	9	9	6	1
							6	2	5	1	4	6
F.							1	9	9	4	8	5.
06							0	7.	1.	9.	9.	5
							0	1	2	0	9	1
								9	8	0	7	
F.								1	9	4	8	8.
07								0	0.	9.	9.	2
								0	4	3	3	7
									2	8	5	
F.									1	2	9	1
11									0	0.	7.	9.
									0	1	0	6
										1	6	2
F.										1	2	7
12										0	5.	2.
										0	3	1
											3	0
F.											1	2
19											0	0.
											0	3
												8
F.												1
20												0
												0

RESULTS

Real time RT-PCR showed 11 IBV field samples were positive. Melt curves for references have been categorized in three types including H120 and Ma5 vaccines, 793/B vaccine (Nobilis 4/91) and Razi filed isolate, M41 strain and field samples. Massachusetts strain vaccines melt curves showed two peaks in the rage of 79 to 87º C, which the first peak was taller than the second (Figure). IBV 793/B types (Vac.1 and field), had two distinct but similar melt patterns which the melt curves ranged from 81 to 86.2º C with two followed peaks that the first was taller than the second one with shorter interval as compared with Massachusetts types (Figure). Moreover, 793/B vaccinal strain (Vac.1-4/91) showed first peak at higher temperature compared to Razi field isolate (Ref.2) (Figure). Ref.1 sample (M41) generated a different melt pattern in the range of 81to 86.8 º C having just one peak (Figure). None of field samples melt curves was similar to those vaccines and reference strains. After HRM curves analysis, GCP calculated by the mentioned software and the results showed a correlation with the melt patterns of specimens (Table 2).

According to visual examination of HRM graph's patterns and comparison of GCPs, positive samples classified in six groups (Table3). Group 1, 2, 5 and 6 had similar melt curve pattern to 793/B types but just differed from Razi field isolate. Two left groups had different melt curves pattern from references and 793/B type, therefore determined as new variant types. Group 3's (F.05, F.06 and F.07) melt curves had a sharp spike-like peak at 83.5º C and group 4 (F.11 and F.19) showed a high peak with a mild bulging step in descending curve side and melt occurred between 81 to 85.2º C (Figure).

According to BLAST results, all new variants (5 filed samples) showed maximum similarity of 95% to three QX-like strains including CK/CH/LZJ/111113 (JX195175.1), CK/SWE/0658946/10 (JQ088078.1) and ITA/90254/2005(FN430414.1).

After comparing 3' UTR fragment sequence of 793/B vaccine and field strains, an 18 bp deletion found in the initial part of vaccine's amplicon that not seen in the Razi's field isolate.

Finally based on HRM curve analysis and GCP, five specimens were found as new IBV variants (Table 3).

Table 3: Classification of positive field samples based on visual evaluation of melt curves and GCP (> 95%).

Field specimen's HRM analysis group	Sample Code	Genotype
1	F.01- F.02	793/B
2	F.03- F.04	793/B
3	F.05- F06- F.07	New Variant
4	F.11- F.19	New Variant
5	F.12	793/B
6	F.20	793/B

DISCUSSION

Since the first detection of IBV in Iran (Aghakhan et al., 1994), several studies were done to evaluate the situation of IB outbreaks. Recent studies reported that 793/B is the dominant strain in Iranian poultry flocks (Seyfi Abad Shapouri., 2004; Shoushtari., 2008). In order to establish a successful vaccination schedule, as a most important part of prevention strategies, differentiation of IBV is so critical and currently performs according to S1 gene sequencing. The main problem of S1 gene sequencing as a golden standard method for IBV differentiation is a high propensity of this gene to mutation even in multiple passages and field circulation or be a probable site of recombination (Capua., 1999; Li., 2010) (4). Moreover other limitations such as lack of reliable pair of primers for amplification of S1 gene for all IBV strains and large size of its PCR products, which may lead to false negative results, have been reported for this method (Hewson., 2010). To set a reliable method for differentiating IBV, other part of IBV's genome such as 3' UTR have been suggested and some pairs of primers have been introduced for this fragment (Adzhar., 1996). The IBV 3' UTR hyper-variable region was used for HRM analysis, as it appears less prone to spontaneous mutation than S1 gene and because of its small size of 3'UTR amplicons (Hewson., 2010). Moreover, a strong inter-strain correlation between the S1 gene and the hyper-variable region in the 3'UTR assists in validating the use of the 3'UTR for IBV strain differentiation (Hewson., 2010). It has been reported that IBV vaccine strains can be differentiated from field infections due to occurrence of deletion in 3'UTR sequenc-

es detected by Restriction Fragment Length Polymorphism (RFLP) and HRM analysis (Hewson., 2009; Hewson., 2010; Majdani., 2012; Mardani., 2006).

Following recent outbreaks of IB infection in Iranian poultry farms despite vaccination and due to presence of some restrictions in current diagnostic techniques for IBV identification and differentiation, such as time consuming and the high cost of operation, this study conducted to evaluate ability of HRM analysis in differentiation of current Iranian's IBV strains. Previous studies about application of HRM technique mentioned that this method is much faster than conventional DNA sequencing and suggested 3' UTR HRM curves analysis as a useful method to detect and differentiate IBV strains (Hewson., 2009; Hewson., 2010).

In current study, detection and identification of IBV strain by HRM analysis done for the first time in Iran. HRM analysis in conjunction with GCPs can differentiate current live IB vaccine in Iran from field isolates and a useful curve melt pattern demonstrated for those mentioned references. Moreover, a 18 bp deletion found in the first part of 3'UTR PCR products of 793/B vaccine strains, which not happened in field reference of 793/B strain that led to different melt curve pattern by which these vaccine strains can be differentiated from the same field serotypes, that was experienced previously in a Australian study which reported that a 58 bp deletion found in current Australian vaccine comparing by field isolates (Hewson., 2009; Mardani., 2006).

Finding of 5 specimens by above 95% similarity to confirmed QX-like strains was indicating presence of new variant in Iranian poultry farm. This result can be a probable cause of vaccination failure in the premises. Before this finding, many poultry veterinarians reported similar clinical and post mortem lesion of QX-like strains in flocks (reports not published) which seen despite of extreme use of Massachusetts vaccines in farms. One study mentioned that due to consistent use of Massachusetts vaccines from 1997 in Sweden, genotyping variation occurred in circulating IB strains, which leaded to vaccination failure (Farsang., 2002) that may refer to the same phenomenon in Iran. Moreover there are many reports of QX-like in the European country which indicates wide spread of this new variant around the world (Beato., 2005; Bochkov., 2006; Worthington., 2008).

This study concluded the molecular presence of QX-like variants in Iranian poultry flock by using HRM curve analysis, as a new reliable, fast and cost benefit molecular technique and it is suggested that in order to complete genotyping of these new strains, other procedure such as viral isolation and S1 sequencing for phylogenic studies would be preferable to confirm statement.

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Research article
Ερευνητικό άρθρο

Molecular Diversity Analysis of Jattal and Dera Din Panah Goat Breeds of Pakistan using Microsatellite Markers

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ABSTRACT. This paper evaluates the genetic diversity of goat breeds in Pakistan, a country blessed with a wide range of goats spread throughout with distinct features contributing to a rich Animal Genetic Resource. The genetic diversity of two goat breeds (Jattal and Dera Din Panah) was assessed with 25 animals representing the two breeds using 16 microsatellite markers. The mean observed and expected heterozygosity of both goat breed populations were observed as 0.83+0.21. The average number of observed alleles was 3.6+1.6 for all loci. The mean polymorphic information content for a goat breed was 0.45, indicating the usefulness of markers panel. Highest Nei's standard genetic distance (Ds) value of 0.0612 was observed between Jattal and Dera Din Panah goats, and the mean Fst value was 0.013. The measures of genetic variation revealed a good scope for effective improvement, conservation, and designing national breeding policies, in future, for Pakistan goat breeds.

Key Words: Microsatellite markers, genetic diversity, goat breeds, Pakistan

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INTRODUCTION

Pakistan, which is the third largest goat producing and the tenth largest goat producing country in the world, has 30 goat breeds (*Capra hircus*) that play a major role in the economy of poor farmers (Khan et al. 2008). For the effective utilization of this rich Animal Genetic Resource of Pakistan through suitable breeding and conservation strategies, characterization and evaluation of genetic variations among these breeds are necessary. The first step in conservation of biodiversity identifies the current genetic variability and then build a pool of genetic information for conservation. The current study was designed to genetically characterize the selected two goat breeds of Pakistan using microsatellite markers. Microsatellite loci are currently used, due to its high degree of polymorphism, and it is a most powerful tool for inferring genetic diversity (Bruford and Wayne 1993; Marikar and Mustafa 2013). Microsatellites, also called as short tandem repeats (STR), are among the preferred methods of genetic assessment because of their abundance, extremely high polymorphism, low mutation rate, ease of amplification through PCR, and small product size (Jouquand, Priat et al. 2000; Teneva, Todorouska et al. 2005).

This paper reports the first molecular investigation of two endemic breeds of goats in Pakistan region in which Jattal and Dera Din Panah goat breeds were selected for characterization. Dera Din Panah goat breed is an inhabitant of Muzaffargarh and Multan districts of Punjab, while Jattal goat locates in Azad Jammu and Kashmir (1985). These two breeds are very difficult to get in to the research because both are living in the Kashmir region and Punjab and less attention was given. Therefore the main objective of this study was to characterize and observe genetic variation in goat breeds through microsatellite markers for making effective strategies for the conservation and proper management of the biological resources of Pakistan, mainly because they are endemic and endangered species.

MATERIALS AND METHODS

Animal selection and DNA Extraction

Ten (10) ml of blood samples were collected aseptically from the jugular vein of 25 animals, each from Jattal and Dera Din Panah goat breeds, into 15 ml falcon tubes containing 200 µL ethylenediamine tetraacetic acid (EDTA). Selected animals were unrelated as they were collected from breeding areas of Jattal breed in Mirpur and Kotli districts in Azad Jammu and Kashmir region close to boarder area while Dera Din Panah samples were collected from Government Livestock Farm at Rukh Khery Wala District Layyah in Punjab province.. Genomic DNA was extracted using an inorganic method of extraction in the molecular laboratory (Sambrook and Russell 2001). The final concentration of extracted DNA was made to 50 ng/uL through spectrophotometry and 0.8% agarose gel electrophoresis.

Markers Selection and Genotyping

Sixteen (16) microsatellite markers (FAO recommended) were selected. Table 1 presents details of all microsatellite loci. All microsatellites were optimized for PCR amplification through BioRad thermo cycler using reaction mixture of 25 uL containing 100 ng DNA, 50 mM KCl, 10 mM Tris-HCl, 2 mM dNTPs, 1.5 mM $MgCl_2$, 1.25 pmol/ uL of forward and reverse

Table 1. I.Microsatellite markers description; marker, product size and chromosome number

Marker	ASR	Chromosome
MAF70	127-188	4
OarFCB11	130-161	2
OarAE101	100-134	6
MAF33	113-147	9
OarFCB128	106-136	2
OarFCB304	123-201	19
OarHH47	138-177	18
OarVH72	122-150	25
BM0757	138-189	9
INRA32	150-194	11
BM1818	259-313	32
ILSTS011	300-382	24
MM12	93-131	9
ETH152	181-236	5
INRA032	194-230	11
OarFCB48	104-187	17

primers, and 0.15 uL of 5U Taq polymerase (Fermentas, USA). Touch-down PCR was used for amplification. The initial denaturing at 950C for 4 minutes was followed by 35 cycles each for 30 seconds at 940C for denaturation, 45 seconds at 640-540 C for annealing, and 45 seconds at 720 C for extension. This was followed by 10 minutes at 720C for the final extension. The products were electrophoresed on 12% non-denaturing polyacrylamide gel in 1X TAE buffer at 120 volts for 7 hours.

Statistical Analysis

The results of polyacrylamide gel electrophoresis were analyzed by the relative flow method. Statistical analysis was performed for each microsatellite marker to calculate the genetic variability measures such as the number of alleles, expected and observed heterozygosity, homozygosity, and Polymorphic Information Content (PIC). Genetic distances between the breeds were determined according to Nei (Nei 1973). The unweighted pair-group method (UPGM) helped to make the genetic distance dendrogram, while POPGENE 1.31 and POWER STAT software were used for calculations (Yeh and Yong 1999).

RESULTS

A total of 59 alleles were found in the 16 loci in all two breeds as a whole. The number of alleles per locus ranged from 2 (OarAE101, MAF33, OarFCB128, OarHH47, BM1818, and INRA032) to 6 (MAF70, INRA32, ETH152, and OarFCB48) and the observed number of alleles (na) for all populations per loci was 3.6+1.6 (Table 2). The observed heterozygosities in all studied breeds ranged from 0.32 (OarFCB11) to 1 (MAF70, OarFCB304, OarVH72, ILSTS011, MM12, ETH152, and OarFCB48) with an average of 0.83+0.21, while the expected heterozygosities throughout all the breeds ranked between 0.48 (OarFCB128) and 0.0.79 (OarFCB48); and the average was 0.61+0.12. The average heterozygosity in Jattal and D.D.P. was observed as 0.543. The average number of alleles per breed was observed from between 2.8 (Jattal and Dera Din Panah) (Table 3), and the average PIC value for both goat populations was 0.45. Goat populations revealed a significant genetic distance from each other (Figure 1) (Nei 1978).

Table 2. Summary F-Statistics and Gene Flow for All Loci in Jattal and Dera Din Panah goat breeds

Marker	Fis	Fit	Fst	Nm
MAF70	-0.5133	-0.5078	0.0036	68.8333
OarFCB11	0.5077	0.5077	0	****
OarAE101	-0.5651	-0.5422	0.0146	16.8611
MAF33	-1	-1	0	****
OarFCB128	-0.5612	-0.5606	0.0004	624.5
OarFCB304	-0.3998	-0.3877	0.0086	28.8065
OarHH47	-0.7707	-0.7241	0.0263	9.2656
OarVH72	-1	-1	0	****
BM0757	-0.5978	-0.5595	0.024	10.1825
INRA32	****	****	0	****
BM1818	-0.7707	-0.7241	0.0263	9.2656
ILSTS011	-0.4351	-0.4314	0.0026	96.7778
MM12	-0.5567	-0.5333	0.015	16.3878
ETH152	-0.3617	-0.2652	0.0709	3.2786
INRA032	-0.7214	-0.7207	0.0004	624.5
OarFCB48	-0.3691	-0.3691	0	****
Mean	-0.5158	-0.4949	0.0138	17.8172
St. Dev				

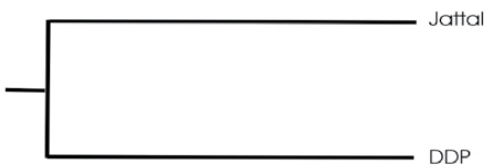


Figure. 1. Dendrogram Based Nei's (1972) Genetic distance: Method = UPGMA, Modified from NEIGHBOR procedure of PHYLIP Version 3.5

The genetic identity of Jattal and Dera Din Panah goat was 0.940 (Table 4) (Nei 1978), and the genetic distance between two goat breeds was 0.0612 with more similarity between Jattal and D.D.P goat breeds. The dendrogram based on Nei's (1978) genetic distance using UPGMA method indicated the close relationship between Jattal and D.D.P. goat breeds; however, these goat breeds were distinct (Figure 1).

Table 3. Summary of Genetic Variation Statistics and Heterozygosity Statistics for All Loci

Marker	Na	Ne	I	Obs_Hom	Obs_Het	Exp_Hom*	Exp_Het*	Nei**	Ave_Het_Het
MAF70	4	2.9691	1.2316	0	1	0.3301	0.6699	0.6632	0.6608
OarFCB11	3	2.3191	0.9308	0.72	0.28	0.4255	0.5745	0.5688	0.5688
OarAE101	2	1.9716	0.6859	0.24	0.76	0.5022	0.4978	0.4928	0.4856
MAF33	2	2	0.6931	0	1	0.4949	0.5051	0.5	0.5
OarFCB128	2	1.9992	0.6929	0.22	0.78	0.4952	0.5048	0.4998	0.4996
OarFCB304	4	3.5791	1.3223	0	1	0.2721	0.7279	0.7206	0.7144
OarHH47	2	1.9501	0.6803	0.16	0.84	0.5079	0.4921	0.4872	0.4744
OarVH72	2	2	0.6931	0	1	0.4949	0.5051	0.5	0.5
BM0757	3	2.1088	0.8475	0.18	0.82	0.4689	0.5311	0.5258	0.5132
INRA32	1	1	0	1	0	1	0	0	0
BM1818	2	1.9501	0.6803	0.16	0.84	0.5079	0.4921	0.4872	0.4744
ILSTS011	4	3.3179	1.2721	0	1	0.2943	0.7057	0.6986	0.6968
MM12	4	2.8752	1.1754	0	1	0.3412	0.6588	0.6522	0.6424
ETH152	6	4.771	1.6164	0	1	0.2016	0.7984	0.7904	0.7344
INRA032	2	1.9992	0.6929	0.14	0.86	0.4952	0.5048	0.4998	0.4996
OarFCB48	4	3.7092	1.3466	0	1	0.2622	0.7378	0.7304	0.7304
Mean	2.9375	2.5325	0.9101	0.1762	0.8237	0.4434	0.5566	0.5511	0.5434
St. Dev	1.2894	0.9352	0.3951	0.2864	0.2864	0.1825	0.1825	0.1806	0.1761

Table: 4. Nei's Unbiased Measures of Genetic Identity and Genetic distance (Nei1978). Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Population ID	Jattal	D.D.P
Jattal	****	0.9784
D.D.P	0.0218	****

DISCUSSION

Studies on genetic characterization of goat breeds are extremely limited in Pakistan. Most of the selected microsatellite markers showed polymorphism, indicating their usefulness. The average heterozygosity of Pakistani goat was 0.543, which is lower than Chinese goat populations 0.677 (Wang, Yang et al. 2006) and 0.77 (Yang, Zhao et al. 1999), and higher than Indian goat populations 0.426 (Fatimaa, Bhonga et al. 2008; Rout, Joshi et al. 2008).

The observed heterozygosity in Jattal and Dera Din Panah goat (0.822, 0.825) breeds was higher than expected heterozygosity values of goats (0.559, 0.549), indicating no overall loss of heterozygosity (allele fixation) in the studied breeds. Mean values of Fis (heterozygote deficit) were -0.515 for goat breeds are low, representing lower inbreeding coefficient between breeds, which is evident from the home

track distance of the studied breeds in contrast to the positive Fis values (high level of inbreeding) in Italian goat breeds (Bozzi, Degl'Innocenti et al. 2009).

Polymorphism information content (PIC) is another good indicator of marker efficiency for genetic studies. In this study, the average PIC value of 16 loci was 0.45 in Jattal and Dera Din Panah goat breeds, while 0.53, which was lower than Indian domestic goats (0.60) (Pandey, Sharma et al. 2010), Chinese (0.62), and Saanen (0.57) goat breeds, but greater than Korean goats (0.35) (Kim, Yeo et al. 2002). The high average PIC values of the panel of microsatellites used for all breeds supported the appropriateness of markers for genetic diversity analysis in Pakistan goat breeds.

Genetic distance between the studied goat breeds showed genetic differentiation among them and reflected the morphological characters as well e.g. Jattal breed is meat type small breed with medium size ears, hair present on chin, spiraled horns in males but smooth in females, small udder and teats while Dera Din Panah is a large black color dairy goat breed having long hair, long hanging and twisted ears and long spiral horns along with well-developed udder and teats. Further, the dendrogram explains the close relationship between Jattal and Dera Din Panah goats, revealing a considerable genetic relatedness.

This study concludes about a high genetic diversity in Pakistani goat breeds and confirms that genotyping through microsatellites is an effective tool for genetic evaluation of different breeds. This microsatellite panel can be used on other domestic and wild goat breeds of Pakistan, and the variation in the values of this study with other reports may be due to the difference of markers, breeding plans, laboratory techniques, and sample size.

CONCLUSION

Future researchers can use these results as a basic guide for better understanding of the genetic relationship and breed differences in goat breeds for making prospective breeding policies and conservation plans to protect any loss in allelic variation in goat breeds in the country.

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Research article
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Serum C-Reactive Protein, Erythrocyte Sedimentation Rate, and White Blood Cell Count in Romanov Sheep with Infectious Keratoconjunctivitis

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ABSTRACT. This study aimed to evaluate the use of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and white blood cell count (WBC) as markers of the severity of inflammation in the eyes of the Romanov breed sheep with infectious keratoconjunctivitis. A total of 10 Romanov breed sheep between the 1.5 and 2 years of age , including the ones carrying infectious keratoconjunctivitis (G1, n = 5) and healthy ones (G2, n = 5), which were housed under the same care and nutrition conditions were examined, in a sheep breeding enterprise within the boundaries of Siirt province. Staphylococcus aureus sp., Clostridium spp., and Penicillium spp. were detected based on the microscopic morphology of the colonies in swabs collected from the eyes of sick animals. Biochemical tests were performed relevant to the suspected agents while no growth was detected in the swabs of the control group. There was a statistically significant difference in serum CRP and WBC levels between the G1 and G2 groups ($p<0.05$). No statistically significant difference was found between the values of the other parameters tested.

Higher levels of CRP and WBC were determined in sheep having infectious keratoconjunctivitis, are compared to those in healthy animals.

Key words: Infectious Keratoconjunctivitis, Albumin, Erythrocyte Sedimentation Rate, C Reactive Protein, White Blood Cell Count

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INTRODUCTION

Infectious keratoconjunctivitis, also known as 'pink eye', in sheep is seen in many parts of the world and is an economically important and contagious disease of small ruminants (Egwu, 1991; Van Halderen et al., 1994). Clinical findings may begin unilaterally, however, bilateral insult has been observed in many cases (Van Halderen et al., 1994). The first indication of the disease is conjunctival hyperemia, serous lacrimation, increased blinking, photophobia and blepharospasm (Akerstedt et al., 2004). Subsequently, the conjunctival veins dilate and proceed over the cornea. On the surface of the cornea, especially in the peripheral parts, blackish and grayish formations are observed. Within 2-5 days, the serous discharge becomes purulent, due to bacterial infection. In the latter stages of the disease, keratitis and corneal ulcers may develop, leading to permanent loss of vision (Van Halderen et al., 1994, Greig, 1989).

The disease usually occurs during the winter, when the animals are kept in enclosed stalls, in some regions, and in dry and dusty environments during the summer. The main source of inection is infected ocular discharge. The infection spreads through susceptible animals in a short time via direct contact or via vectors (Egwu et al., 1989). Although the disease is mostly seen in 5-10 day old lambs, it can be detected in all age groups (Greig, 1989; Naglit et al., 1999).

Although many different microorganisms are incriminated in the etiology of the disease, the causes and predisposing factors of the disease are still being investigated. *Branhamella ovis* (*B. ovis*), *Chlamydia psittaci* (*C. psittaci*) and *Mycoplasma conjunctiva* (M. *conjunctiva*) are reported as the major known pathogens of the disease (Greig, 1989; Giacometti, et al., 1999).

Acute phase response is a non-genuine process that occurs against infection, inflammation and trauma (Long et al., 2008; Gabay et al., 1999). The changes in distribution of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and shape of blood elements are part of the acute phase response and are frequently used as indirect diagnostic methods for infectious diseases (Sãez-Llorens et al., 1993). In practice, ESR and CRP and leukocyte count (WBC) tests are used to assess the severity of the inflammation. These tests result in higher values in infectious diseases (especially bacterial ones), malign diseases, traumas, infarcts, inflammatory arthritis and vasculitis (Hatipoğlu et al., 2011).

The purpose of this study was to determine the usability of ESR, CRP and WBC as markers of the severity of inflammation in the eye in sheep with infectious keratoconjunctivitis by comparing their values in affected sheep with those in healthy sheep of the same breed.

MATERIAL AND METHOD

Animal selection and inclusion criteria

The study population was constituted of a total of 10 Romanov sheep between the ages of 1.5 and 2 years, including the ones with infectious keratoconjunctivitis (G1, n = 5) and healthy ones (G2, n = 5), which were housed under the same care and nutrition conditions, in a sheep breeding enterprise within the boundaries of Siirt province. Clinical examination of all animals (body temperature, pulsation, number of respirations, lymph nodes, tracheal palpation, lung auscultation and percussion) was carried out in detail. Animals with conjunctival hyperemia, blepharospasm, photophobia, purulent lacrimation, and corneal opacity at various grades were evaluated as having infectious keratoconjunctivitis and were allocated in group G1. The animals that did not lose appetite and performance, and had normal clinical and ophthalmological examination results were healthy and were allocated in group G2.

Laboratory tests

Blood samples were transferred from the vena jugularis to sterile gel-containing biochemical tubes (Becton Dickinson and Company, Franklin Lakes, NJ), and serum samples were derived in 30 minutes by centrifuging for 15 min at 3000 rpm. CRP, total protein (TP) and albumin (ALB) concentrations (advia 1800 chemistry system) were measured. Hematocrit (HCT) and WBC values (MINDRAY BC-2800 VET) were measured in whole blood samples collected in EDTA coated tubes. ESR values were determined by the Westergren method.

Microbiological analyses

Ocular specimens were collected from the right and left eyes of the control group and from the animals with the disease with a moistened brush with sterile saline and applied to surfaces of conjunctiva. Samples were taken into tubes containing Cary-Blair transport medium (Lab M, UK) and were sent to Uludag University Veterinary Faculty Microbiology Department with cold chain in Cary Blair transport medium. Swabs were inoculated into blood agar (Merck, Germany), Mac Conkey agar (Merck, Germany), Eosine Methylene Blue agar (Merck, Germany), pathogenic fungi (Merck, Germany) and *Mycoplasma* agar (Merck, Germany) and incubated at 37 °C for 24-96 hours in both aerobic and microaerophilic conditions. After examination of the microscopic morphology of the colonies, biochem-

ical tests were performed relevant to the suspected agents.

Statistical analyses

Statistical analyses were performed using the IBM SPSS Statistics 20 program pack and the Mann-Whitney U test was used in nonparametric tests. Level of significance was set at $p<0.05$.

RESULTS

In group G1, serum CRP level was found to be higher than in group G2 ($p<0.05$). Although the WBC count was within the reference range, it was found to be higher in G1 group than this in the G2 group, and this difference was statistically significant ($p<0.05$). ALB concentration was below the lower reference value in both G1 and G2. No statistically significant difference was found between the groups. There was no statistically significant difference between ESR, HCT and TP values ($p>0.05$) (Table 1).

DISCUSSION

Many of the acute phase proteins (APP) have been extensively studied in the field of human medicine and are routinely used today in the diagnosis and prognosis of various diseases. Recent studies have also pointed out that APPs also have important areas of use in veterinary medicine (Eckersall et al., 2004).

As there are many APPs known today, their concentration in blood and their diagnostic importance vary in different animal species. CRP is not considered to be an acute phase protein for ruminants, however, its concentration increases during inflammation and infections. Studies have reported that CRP levels are associated with body condition score, lactation status and animal health, and it rapidly increases in t case of infection. For this reason, it is reported that CRP may be a marker for herd health evaluation (When-Chuan et al., 2003). In practice, ESR and CRP tests are used to evaluate the acute phase response (Barati et al., 2008). Polycythemia, erythrocyte deformities, elevation in bile salts, non-steroidal anti-inflammatory drug use, and cardiac failure may reduce ESR (Celik, 1996). In the presented study, the difference in the CRP concentration of the G1 group compared to the G2 group was found to be statistically significant.

The measurement of APPs is in particular important in terms of differentiate bacterial from viral involvement in the infection and administer the appropriate treatment. However, APPs can't form a reagent between bacterial and viral diseases in CRP production due to the wide of individual varieties (Petersen et al., 2004). When used for this purpose, it strengthens the diagnosis and provides more accurate information on the prognosis of infected animals (Gruys et al., 2005; Gruys et al., 1994). Although the increase in CRP and WBC values in this study suggests infectious keratoconjunctivitis, microbiological analyses have been performed for definite diagnosis, and *Staphylococcus aureus* sp., *Clostridium* spp., and *Penicillium* spp. were isolated based on the results of Gram and Lactophenol Cotton Blue staining and biochemical tests of colonies that grew from the samples collected from the eyes of sick animals while no growth was detected in the swabs of the control group.

The acute phase response emerges as a complex reaction characterized by local and systemic changes initiated by inflammatory mediators in the area of tissue destruction (Gruys et al., 1994). Local reactions during acute phase response include: increased capillary permeability, leukocyte passage in the inflammatory zone, and release of various chemical mediators (Ganheim, 2007). The increase in the WBC level in the blood of the animals in G1 group of the study supports this situation. The resulting inflammatory response lasts for 1-2 days and then gradually disap-

Table 1: Median (Min-Max) values in infectious keratoconjunctivitis group (G1) and in control group (G2)

Parameter	G1 Median(Min-Max)	G2 Median(Min-Max)	p
CRP	6,60 (5,50 - 7,60)^{A}	4,50 (4,20 - 4,70)^{B}	0,008
ESR	2,00 (2,00 - 3,00)	2,00 (2,00 - 3,00)	0,690
WBC	9,33 (6,76 - 12,86)^{A}	6,01 (5,97 - 7,76)^{B}	0,016
HCT	27,70 (20,60 - 32,00)	26,30 (25,30 - 40,40)	0,841
ALB	3,10 (2,30 - 3,80)	3,40 (3,40 – 3,90)	0,421
TP	7,50 (7,10 - 8,50)	7,20 (7,00 – 7,90)	0,421

A, B: There is statistical significance between values expressed in different letters on the same line ($p<0.05$)
(CRP, C Reactive proteins; ESR, Erythrocyte Sedimentation Rate; WBC, White Blood Cell; HCT, Hematocrit; ALB, Albumin; TP, Total protein)

pears. However, the presence of the stimulant causing the acute phase or the status' becoming chronic also causes this process to be prolonged (Petersen et al., 2002). The finding of keratitis and corneal ulcers in the ophthalmologic examination of sheep included in the study indicates that the disease is chronic, and increased serum CRP levels indicate that the acute phase response is prolonged.

ESR, which always occurs within certain limits in humans and animals, shows differences in different species. ESR is also commonly linked to the speed of rouleaux formation (Zhao et al., 1993). It is indicated that ESR increases when some active plasma proteins increase in blood. It has been reported that the most effective of the acute phase proteins is fibrinogen (O'donnell, J., et al., 1997), and that the ESR is also increased when fibrinogen concentration is increased. And it is suggested that ALB, which is one of the plasma proteins, reduces the ESR (O'donnell, J., et al., 1997). It is stated that the amount of plasma ALB in humans, sheep, goats and dogs is higher than that of globulins (O'donnell, J., et al., 1997). As is known, in inflammatory diseases, the WBC counts in blood increase and ESR also increases due to the increased level of fibrinogen (Alexy et al., 2015). This suggests that the number of WBCs along with ESR and both of them provide evidence for inflammatory diseases. For this reason, the WBC number was also taken into account in our study; however, no relationship with ESR was found. It is noteworthy that the HCT value, which is among the factors that might affect ESR in sheep, was positively correlated with ESR, even if it was not statistically significant.

CONCLUSION

In conclusion, determining high levels of CRP and WBC in sheep having infectious keratoconjunctivitis, when inflammatory parameters such as ESR, CRP and WBC are compared with healthy animals, supports the fact that these parameters can be used to evaluate the severity of inflammation in the eyes of sheep with infectious keratoconjunctivitis.

Animal Rights Statement

This study was approved by the Siirt University Animal Research Local Ethics Committee

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Research article
Ερευνητικό άρθρο

Different estrus induction protocols and fixed time artificial insemination during the anoestrous period in non-lactating Kivircik ewes

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ABSTRACT. The efficiency of medroxyprogesterone acetate (MAP) sponges or norgestomet ear implants (half or entire) for synchronizing and inducing the estrous cycle in non-lactating Kivircik ewes was investigated during the natural non-breeding period. Ewes were treated for 11 days either with 60 mg MAP sponges (group 1, *n*=27) or with 1.5 mg norgestomet (group 2, *n*=25) or with 3 mg norgestomet (group 3, *n*=27) ear implants. In addition, each ewe received an intramuscular injection of 500 IU of equine chorionic gonadotropin (eCG) and 125 μg cloprostenol (PGF2α), 48 h prior to progestagen removal. Double Cervical Artificial Insemination (AI) with diluted fresh semen was performed at a fixed time (36 and 48 h) following progestagen withdrawal. Mean values for estrous detection rates at the first 12 ± 6 h and within 72 h, the time from progestagen removal to the onset of estrous, the duration of the induced estrous and pregnancy rate were found to be 46.8%, 86.1%, 26.1 ± 7.3 h, 27.0 ± 10.7 h and 27.8%, respectively. There were significant differences between groups 2 and 3 in the time of induced estrous onset ($P<0.05$). These results indicate that, each of the three protocols was equally efficient in inducing and synchronizing estrus in non-lactating Kivircik ewes during the natural non-breeding period.

Keywords: anestrous, cloprostenol, eCG, ewe, norgestomet, MAP

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INTRODUCTION

The majority of sheep breeds perform different reproduction activities depending on season changes, latitude/longitude, the length of the photoperiod, the nutrition, the male effect and other factors (Gordon, 1999; Dogan and Nur, 2006). Therefore, estrous synchronization and induction together with Al in ewes is important for the improvement of reproductive efficiency and management processes (Gordon, 1999; Gokcen et al., 2000; Wildeus, 2000; Menchaca and Rubianes, 2004). Controlled breeding of sheep involves artificial control of estrus and ovulation with exogenous hormone treatments (Keisler and Buckrell, 1997; Menchaca and Rubianes, 2004). Intravaginal sponges impregnated with progesterone or its synthetic analogues, namely medroxyprogesterone acetate (MAP) and fluorogestone acetate (FGA) are usually inserted over periods of 6 to 14 day in conjunction with eCG, especially during natural anestrous period. Sometimes prostaglandin F2α injected at sponge removal or 48 h prior to sponge removal (Gordon, 1999; Wildeus, 2000; Ungerfeld and Rubianes, 2002; Dogan and Nur, 2006; Ustuner et al., 2007). Moreover, norgestomet ear implants can easily be used in sheep for estrous synchronization (Cardwell et al., 1998; Awel et al., 2009; Ataman et al., 2009; Garoussi et al., 2012; Uslu et al., 2012; Blaschi et al., 2014). Gonadotrophins, such as equine chorionic gonadotrophin (eCG), have been shown to stimulate follicular growth, increase ovulation rate and fertility and induce a tighter synchrony of ovulation in both anestrous (Maurel et al., 2003; Dogan and Nur, 2006) and cycling sheep (Ustuner et al., 2007). Prostaglandin F2α and its synthetic analogs are luteolytic factors that can be used, particularly in breeding period (Keisler and Buckrell, 1997; Wildeus, 2000; Menchaca and Rubianes, 2004; Dogan and Nur, 2006). Estrous synchronization or induction in sheep has been accomplished using several protocols with varying degrees of success (Scaramuzzi and Martin, 1984; Menchaca and Rubianes, 2004). Furthermore, the effects of these protocols on fertility rates are variable (Wildeus, 2000). Hormone cost is one of the biggest factors limiting estrus induction and synchronization in sheep. Thus, many farmers are reluctant to use this technique. Lowering the cost can make both AI and estrus synchronization more common. The purpose of this study was, therefore, to evaluate the effectiveness of half or entire doses of norgestomet implants in combination with eCG and cloprestenol: a) on estrous induction and synchronization rates and b) on the fertility rates obtained after AI, compared to that of the MAP sponges (in combination with eCG and cloprestenol) in non-lactating Kivircik ewes, during the natural non-breeding period.

MATERIAL AND METHODS

The study was carried out at village Bursa (latitude 40° 13`E , longitude 29° 00` N, altitude 100 m), located in Inegol, in western Turkey, during March (the natural non-breeding period) under natural lighting. A total of 79 non-lactating Kivircik ewes, 2 to 4 years old, weighing 35 to 58 kg and with body condition scores ranging from 2.5 to 3.5 [evaluate on a scale of 0 to 5, according to Morand-Fehr et al. (1989)], were used in this study. In addition, 7 Kivircik rams of proven fertility and 5 teaser rams were used in the present study. The sheep were allowed to graze on natural pasture from 07:30 to 11:30 h and from 12:30 to 17:30 h and kept in pen overnight. Water and a mineral salt lick were provided *ad libitum*. In addition, the ewes received 0.5 kg concentrate per ewe per day during the entire period of this study. The management of the ewes did not change throughout the entire experimental period.

These females were equally assigned, according to age, body weight and body condition scores, to one of three 11 day progestagen treatments. Group 1 received a 60 mg MAP (Esponjavet, Hipra, Spain) vaginal sponge (group 1; n=27). The rest ewes received either a half (group 2; n= 25) or an entire (group 3; n=27) ear implant (impregnated with 3 mg of norgestomet, Crestar, Intervet, Netherlands). The half (0.2 cm in diameter and 1.5 cm in length) and the entire (0.2 cm in diameter and 3 cm in length) implants were inserted subcutaneously into the upper side of the ear using the implanting device provided by the manufacturer. All ewes received an intramuscular injections of 500 IU eCG (Chrono-Gest, Intervet, Netherlands) and 125 μg cloprostenol (Dalmz-

in, Fatro, Italy) 48 h prior to progestagen removal. Estrous was monitored with the aid of 5 teaser rams, every 6 h from 12 to 72 h following progestagen sponge and implant withdrawal. The ewes were considered in estrus when they were mounted by the teaser rams. Estrous onset was defined as the time elapsed between sponge/implant removal and the first accepted mount. Estrous duration was defined as the time between the first and last accepted mount, within the same estrous period.

One ejaculate from each ram was collected by electroejaculation. During collection and examination, the semen was protected from temperature shock. Each ejaculate was immediately evaluated for volume and wave motility (Mylne et al., 1997). Only ejaculates with a volume higher than 0.5 ml and good wave motility (≥3) were used. The volume was determined in the collection tube, which was graduated in divisions of 0.1 ml and the motility was assessed by depositing a drop of semen on a slide and was examined under the phase contrast microscopy (x40; Nikon, Japan) equipped with a warm stage (35°C). The semen sample was scored using a scale ranging from 0 (no wave movement) to 5 (extreme wave movement). Only ejaculates with scores of 3 and higher were used. The density was determined with the aid of a haemocytometer. The semen was diluted (one step dilution) at 30°C to a sperm concentration of 800 x 106 motile cells/ml. The diluent consisted of sterilized cow skim milk, 1000 IU/ml sodium G penicillin and 1000 μg/ml dihydrostreptomycine sulfate. Thereafter, diluted semen samples were pooled in the same test tube. The diluted semen was then cooled to 4°C over a 90 min period and kept at this temperature until insemination. All ewes were inseminated intracervically twice at a fixed time 36 and 48 h following sponge or implant withdrawal with a 0.25 ml straw containing 200 x 10^6 spermatozoa. All ewes were restrained in a standing position and the external opening of the cervix was located with the aid of a speculum and a head lamp. The Al gun was carefully inserted as far as possible into the cervical canal without force, where the semen was slowly deposited. All ewes were inseminated by the same inseminator. Seventy-five days post AI ewes were screened for pregnancy diagnosis using a real-time ultrasound equipped with a 3.5- MHz linear array transabdominal transducer (Honda, HS-1500, Japan). The fetal heartbeat and the fetal image were checked for the

Table 1. The mean estrous detection rate, time from progesterone removal to estrous onset and, estrous duration (± S.E.), and pregnancy rate in Kivircik ewes following different estrous synchronization treatments and AI at a fixed time

Treatment group	n	Estrous detection rate (%)		Time from progesterone removal to onset of estrous (h)	Estrous duration (h)	Pregnancy rate (%)
		12 ± 6 h	within 72 h			
Group 1	27	(17/10) 37.0[a]	(6/21) 77.8[a]	26.3±6.1[ab]	26.6±9.4[a]	(19/8) 29.7[a]
Group 2	25	(14/11) 44.0[a]	(1/24) 96.0[a]	28.3±8.6[a]	24.5±10.2[a]	(19/9) 36.0[a]
Group 3	27	(11/16) 59.3[a]	(4/23) 85.2[a]	24.0±6.3[b]	30.0±12.0[a]	(22/5) 18.5[a]
Total	79	(42/37) 46.8	(11/68) 86.1	26.1±7.3	27.0±10.7	(57/22) 27.8

a,b means in the same column with different superscripts indicate a significant difference (P<0.05)
Group 1: MAP impregnated sponge plus eCG and PGF2α, Group 2: half norgestomet ear implant plus eCG and PGF2α, Group 3: entire norgestomet ear implant plus eCG and PGF2α.

diagnosis of pregnancy.

The time from progesterone removal to onset of estrus and the duration of induced estrous periods were subjected to analyses of variance (one-way ANOVA). The differences between means were tested for significance with the Fisher's PLSD. Estrous detection and pregnancy rates were analyzed using the *chi-square test* and *Fisher's exact test*, respectively; the 95% significance level was noted. SPSS 10.0 software was used for statistical analyses (Instat, 1990-1993).

RESULTS

The mean values of estrous rates for the first 12.0 ± 6.0 h and within 72.0 h, the time from progesterone withdrawal to the onset of estrus, the duration of the induced estrous and the pregnancy rates are presented in Table 1. Figure 1 illustrates the percentage of ewes in different groups that detected in estrus at different intervals after sponge or implant removal. Six ewes from Group 1, one ewe from Group 2 and four ewes from Group 3 did not show any overt signs of estrus during the observation period. Estrous onset for the rest ewes occurred between 12 and 72 h after the end of treatment. Only the time from progesterone withdrawal to the onset of estrus was significantly different between groups 2 and 3. All the other parameters were not significantly different among the 3 treatment groups. Thus, the data were pooled and the overall (for the three groups) estrous detection rate for the first 12 ± 6 h period and within 72 h was 46.8% and 86.1%, respectively. The overall (in the 3 groups)

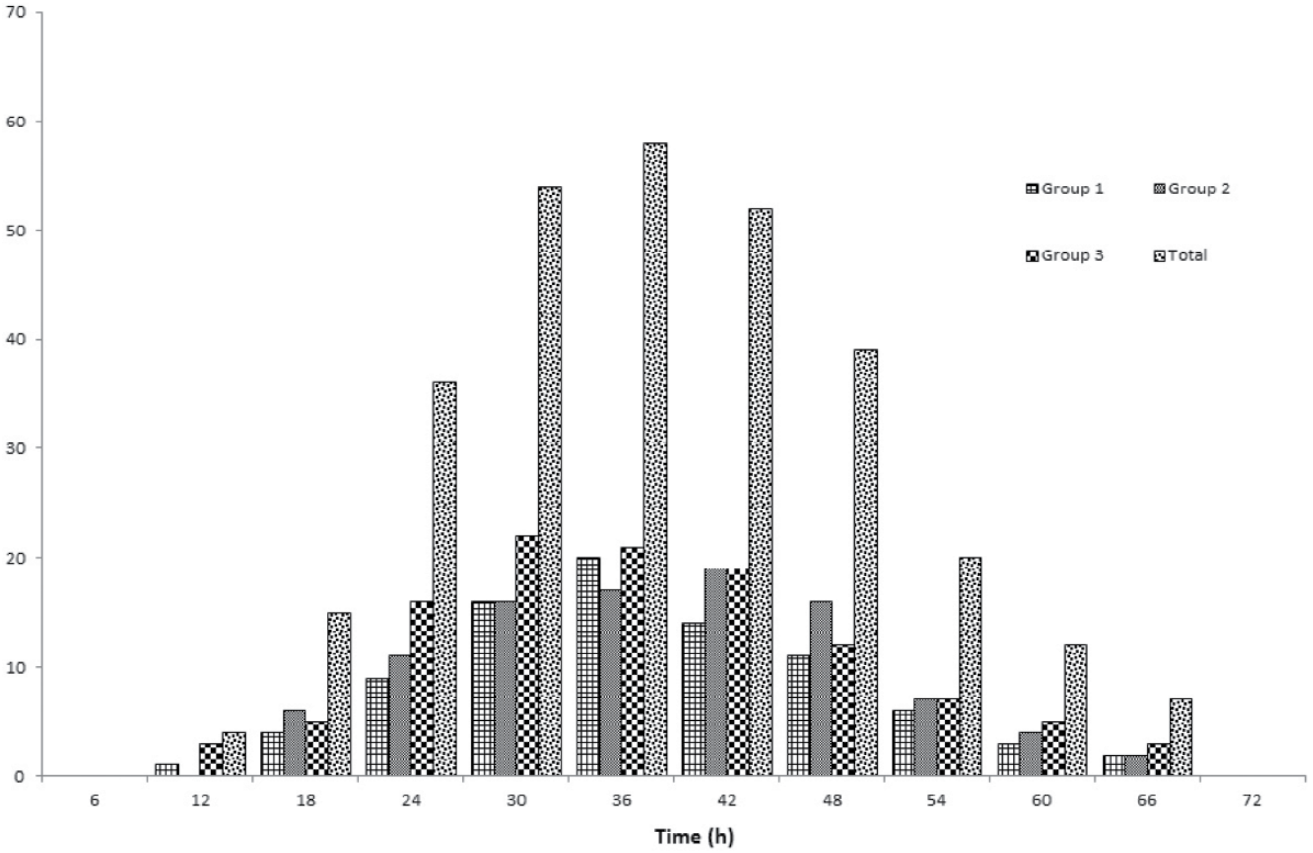


Figure 1. Percentages of ewes at different intervals between sponge or implant removal and the onset of estrous in Kivircik ewes after different progestogen treatments.
Group 1: MAP impregnated sponge plus eCG and PGF2α, Group 2: half norgestomet ear implant plus eCG and PGF2α, Group 3: entire norgestomet ear implant plus eCG and PGF2α.

mean time from progesterone withdrawal to onset and the duration of estrous were 26.1 ± 7.3 h and 27.0 ± 10.7 h, respectively. The overall mean pregnancy rate 75 days after AI was 27.8%. Three of the 11 ewes did not show any estrous signs, but diagnosed pregnant at day 75 after AI.

DISCUSSION

The three treatments used in this study were found to be efficient for estrous induction and synchronization of ewes during the non-breeding period. Although there was no significant difference among the three groups, the estrous detection rate obtained in Group 3 (59.3%) was the highest at the first 12.0 ± 6.0 h. Furthermore, the estrous detection rate at the first 12.0 ± 6.0 h period was higher in ewes treated with half (44.0%) or entire (59.3%) norgestomet implant in comparison with those treated with MAP (37.0%) sponges. Taking in mind these results, it could be concluded that ewes receiving norgestomet showed estrus earlier and closer synchrony compared to those received MAP sponges. This could be explained by the easier absorption of ear implant containing norgestomet compared with sponges. Similarly, Ataman et al. (2009) reported earlier estrous detection rate in Akkaraman cross bred ewes treated with ear implant containing 3 mg norgestomet, compared to ewes treated with intravaginal sponge containing 30 or 40 mg FGA at the transition from non-breeding to breeding period. Considering our overall estrous detection rate for the first 12.0 ± 6.0 h period (46.8%), our result was higher than that reported by Ungerfeld and Rubianes (1999), Das et al. (2000), Simonetti et al. (2000), Dogan and Nur (2006), Hashemi et al. (2006), Ustuner et al. (2007) and Blaschi et al. (2014). There were no significant differences among the 3 groups in terms of estrous detection rate within 72 h after progesterone removal. The estrous detection rate recorded during the 72 h observation period following the cessation of treatment (overall mean rate 86.1%) is within the range of 42.0-100% quoted in treatment with progesterone (Das et al., 2000) or FGA or MAP intravaginal sponges alone (Ungerfeld and Rubianes, 1999; Simonetti et al., 2000; Ataman et al., 2009) or in combination with eCG (Zarkawi et al., 1999; Gokcen et al., 2000; Ungerfeld and Rubianes, 2002; Zeleke et al., 2005; Hashemi et al., 2006; Ustuner et al., 2007) and PGF2α (Dogan and Nur, 2006) or norgestomet ear implants alone (Ataman et al., 2009) or in combination with eCG (Cardwell et al., 1998; Awel et al., 2009; Garoussi et al., 2012; Uslu et al., 2012) and PGF2α (Blaschi et al., 2014), in different breeds of ewes under different environmental conditions.

It has been reported that the onset of estrous occured within 18-144 h following withdrawal of progesterone impregnated sponges (Das et al., 2000), MAP or FGA impregnated sponges (Ungerfeld and Rubianes, 1999; Simonetti et al., 2000; Gokcen et al., 2000; Dogan and Nur, 2006; Hashemi et al., 2006; Ustuner et al., 2007) or norgestomet ear implants (Cardwell et al., 1998; Ataman et al., 2009; Blaschi et al., 2014). In the present study, ewes detected in estrous between 12 and 72 h after sponge and implant removal; the highest incidence of estrous onset occured between 30 and 42 hours (Fig. 1). The distribution of estrus in our groups was similar to that reported by Zarkawi et al. (1999), Simonetti et al. (2000), Dogan and Nur, (2006), Hashemi et al. (2006), Ataman et al. (2009) and Blaschi et al. (2014). Nevertheless, these results are not in agreement with Ungerfeld and Rubianes (1999) who found the highest incidence of estrous onset occurring between 72 and 96 h after MAP sponge withdrawal. This deference could be due to the different rate of absorption and metabolization of each progestagen or progesterone. In the present study, the mean overall interval to the onset of estrus following progestagen removal was 26.1 ± 7.3 h and it was significantly longer in the half implant group, compared to the entire implant group ($P<0.05$) (Table 1). The mean overall interval obtained in this trial is in agreement with the findings of Hashemi et al., (2006), who reported a 29.6 h interval to the onset of estrus when using 60 mg MAP sponges and 500 IU eCG. As concern the onset of estrous, on the other hand, longer periods have been reported by Ungerfeld and Rubianes (1999), Das et al. (2000), Simonetti et al. (2000), Ungerfeld and Rubianes (2002), Zeleke et al. (2005), Dogan and Nur (2006) and Ustuner et al. (2007), where ewes synchronized with progesterone

or MAP or FGA impregnated sponges. In a previous study, Cardwell et al. (1998) used norgestomet alone or in combination with eCG and reported an estrous onset interval of 46.0 and 32.6 h, respectively. Furthermore, Usta et al. (2012) reported an interval of 45.6 h for the onset of estrus, after using 1.5 mg norgestomet ear implant treatment for 10 days with 500 IU eCG, in lactating Morkaraman ewes during non-breeding period. Similarly, Blaschi et al. (2014) reported a 44.0, 41.9 and 34.2 h interval to estrus onset in Santa Inês x Texel cross bred ewes, by using 1.5 mg norgestomet for 5, 9, and 14 days, respectively, in combination with 400 IU eCG and 22.5 μg D-cloprostenol at the time of implant removal. The reason for these discrepancies is indefinite; it may be attributed to differences in breed, nutrition, season, hormone-based protocols (Hashemi et al., 2006), use of gonadotrophins and presence of the male after sponge removal (Ungerfeld and Rubianes, 1999). All these factors are known to influence this parameter (Gordon, 1999; Das et al., 2000; Wildeus, 2000; Maurel et al., 2003; Zeleke et al., 2005, Blaschi et al., 2014). Having in mind all these result, our protocols lead to earlier estrus onset.

The mean overall duration of the induced estrous period (27.0±10.7 h) recorded in this study is similar to that reported by Dogan and Nur (2006) and Blaschi et al. (2014), longer than that reported by Das et al. (2000), Zeleke et al. (2005) and Hashemi et al. (2006) and shorter than that reported by Ustuner et al (2007). The shortest mean estrus duration was recorded in the half-implant treatment group (24.5±10.2 h), but it was not significantly different compared to the other 2 treatment groups. Maurel et al. (2003) reported high blood oestrogen levels after induced luteolysis and stimulation of follicular growth due to FSH or exogenous eCG. Thus, blood oestrogen concentration could be related to the contradictory results among our finding and those of previous studies. Further research is needed to confirm the reasons for these differences.

None of the treatment protocols showed any significant advantage over the other as concern the conception rate. These results are in agreement with Ataman et al. (2009), who did not record any differences between FGA sponges and norgestomet ear implants. The overall post-treatment conception rate obtained after AI with fresh diluted semen in this study was 27.8%. The pregnancy rate obtained in this study is not within the range of 33.3 to 100% reported for ewes synchronized with intravaginal progestagen sponges during the breeding (Gokcen et al., 2000; Simonetti et al., 2000; Ustuner et al., 2007), non-breeding (Dogan and Nur, 2006) and transition period (Zeleke et al., 2005) after AI with fresh diluted semen. Furthermore, the present results were lower than those of Blaschi et al. (2014), who recorded a pregnancy rate of 47.8, 60.9 and 83.3 % in short (5 days), medium (9 days) and long-term (14 days) norgestomet implant treated ewes after fixed-time AI with fresh diluted semen, during the natural breeding period. Nonetheless, the current pregnancy rates are not in agreement with none of those reported in the literature. However, pregnancy rates depend on many parameters, such as the breed, the time of AI, the synchronization protocols, the lifespan of spermatozoon and oocyte, and the overall managerial conditions (Gordon, 1999; Wildeus, 2000; Menchaca and Rubianes, 2004; Zeleke et al., 2005). Further research is needed to determine the exact reasons.

Three of 11 ewes did not show any overt signs of estrus, but were diagnosed pregnant at day 75 after AI. This finding is in agreement with a previous report in Anatolian black goats (Dogan et al., 2005) and Kivircik ewes (Dogan and Nur, 2006), synchronized with MAP or FGA sponges. Allison and Robinson (1970) suggested that these silent ovulations may be related to inadequate endogenous progesterone levels with consequent inability of the ewe to respond to endogenous oestrogen. Besides, absence of estrus and ovulation may be due to insufficient gonadotrophic hormone released by the pituitary, to a poor ovary response to the exogenous eCG or to the variation in responsiveness of animals to eCG (Maurel et al., 2003; Menchaca and Rubianes, 2004; Bartlewski et al., 2011).

CONCLUSIONS

In conclusion, it can be said that estrous detection rate and pregnancy rate were not significant-

ly different among MAP impregnated sponges, half and entire norgestomet ear implants groups. However, the time from progesterone removal to estrus onset and the duration of induced estrus were significantly different between half and entire norgestoment groups. Therefore, protocols used in the present study are equally efficient in synchronizing and inducting estrus in non lactating Kivircik ewes during the natural anoestrous period. Half implant could be used to reduce the cost of the treatment.

CONFLICT OF INTEREST

There is no conflict of interest to declare.

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Research article
Ερευνητικό άρθρο

Welfare parameters in dairy cows reared in tie-stall and open-stall farming systems: pilot study

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ABSTRACT. Animals can experience pain or distress as a result of widely accepted management practices. In dairy cattle, housing system can affect animal welfare. The assessment of animal welfare requires the use of multiple indicators in order to analyse the heterogeneity of the aspects involved. The aim of this study was to compare the welfare of dairy cows reared in a tie-stall (TS) and open-stall (OS) system by metabolic, immunological and stress related parameters.

The study involved 80 pluriparous lactating cows belonging to eight dairy farms located in the area of Mugello (Florence, Italy) reared in TS and OS systems. Ten blood samples were collected at morning time to measure alanine-aminotransferase (ALT), aspartate-aminotransferase (AST), blood urea nitrogen (BUN), β-hydroxybutyrate (BHBA), creatinine (Creat), non-esterified fatty acids (NEFA), total proteins (TP), calcium (Ca), phosphorus (P), and potassium (K), serum lisozyme (SL), bactericidal activity (SBA), haptoglobin (Hp), oxygen free radicals (OFR), and hair cortisol levels. At the same time a body condition score (BCS) was recorded. Statistical analysis was performed by ANOVA. The experiment was carried out in accordance with European Commission regulations (Directive 2010/63/EC and Directive 98/58/EC).

The results showed that the housing system affected various parameters such as ALT, BHBA, OFR, cortisol ($P \leq 0.01$) and AST, BUN ($P \leq 0.05$). Most of these parameters showed mean values within the range of reference without revealing any signs of suffering. An interesting outcome regarded the OFR levels, which was higher in the OS system (68.2±34.51 U.Carr. vs 36.1±21.39 U.Carr.), probably as a consequence of the high productive effort. In conclusion, it is possible to state that TS did not show a comparable overall situation with serious signs of welfare impairment.

Key words: dairy cattle, welfare, farming system, metabolic parameters, immunological parameters

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INTRODUCTION

Concern regarding animal welfare is widespread. It regards not only the absence of illness or injury, but also focuses on the pain or distress that the animals might experience as a result of managerial practices (Fraser, 2008), that in dairy industry mainly consist in animals reared in tie-stall (TS) and open-stall (OS) housing system (EFSA, 2015).

In terms of animal welfare, the TS housing system of dairy cows is controversial. According to some authors, this system, restricting voluntary movement and the social behaviour of cows, cannot be considered suitable (Popescu et al., 2013). Regarding the milk performance, health, fertility and behaviour (Zdziarski et al., 2002), some authors have not reported large differences between the two types of housing system, while others (Kara et al., 2015) report a direct effect on milk yield and animal health. Despite the criticism, TS housing systems are still widely used for dairy cows in many parts of the world (Popescu et al., 2013). In Italy, the TS system is mainly adopted on small sized farms where structural and economic constraints limit the possibility of reorganisation (Corazzin et al., 2010).

Animal welfare is a multidimensional concept (Fraser, 1995), its assessment can be performed by several approaches that can rely on farm resources and management, or on animal based indicators (EFSA, 2009; De Vries, 2015). Among the last, the health status of animals represents an important aspect that have to be taken into account. Biochemical investigation is used to check metabolic disorders (Radkowska and Herbut, 2014). Minerals have an important role for productive and reproductive performances (Galindo et al., 2014). The immune status of the animals can indicate a predisposition to developing diseases conditioned by stressful events. Some authors indicate that serum lysozyme, bactericidal activity, and haptoglobin are broader indicators of bovine non-specific immune reactivity in different breeding conditions (Bonizzi et al., 2003). Oxidative stress highlights the possible imbalance between reactive oxygen metabolite production and the neutralizing capacity of antioxidant mechanisms (Bernabucci et al., 2005), such oxidative stress may be involved in several pathological conditions, including those related to production and to the general welfare of the cows (Lykkesfeldt and Svendsen, 2007).

An evaluation of the pituitary-adrenal axis activity is very important because it regulates many biological processes such as energy balance, reproduction and immune responses (Comin et al., 2013). The measurement of glucocorticoids together with other indices of stress such as immune function, metabolism, and nitrogen balance reveal how animals perceive and adapt to their environment.

Furthermore, the body condition score (BCS) can indicate an effective nutritional management (Roche et al., 2009; 2013).

This study compared the animal welfare of dairy cows reared in TS and OS systems by means of metabolic, immunological, stress related parameters and BCS.

MATERIALS AND METHODS

The experiment was performed in accordance with European Commission regulations, and the animal handling followed the recommendations of Directive 98/58/EC concerning the protection of animals kept for farming purposes.

The study was carried out between April and May 2014 and involved 80 pluriparous, 3.5-6 years old, lactating cows belonging to eight dairy farms located in the area of Mugello (Florence, Italy): three farms reared animals in the TS system and the other five farms reared the animals in the OS system. The TS farms were smaller than the OS farms in terms of number of animals (mean 22 ± 10.2 *vs* 110 ± 35.8 heads) and farm size (mean 33 ± 12.1 *vs* 458±709.8 ha). Feeding management based on the use of unifeed was followed in the OS farms, and hay and meals in the TS farms.

No animal enrolled experienced a change in social group or had been affected by any diseases in the period before the study.

In each farm, ten blood samples were collected in the morning from the jugular vein using vacutainer tubes. The blood samples were kept in iceboxes and immediately sent to the laboratory of Rome, in the Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana (IZSLT). The following parameters were measured by an automated biochemical analyser (Olympus AU 400) using a commercial enzymatic test kits (Beckman-Coulter) and according to the manufacturer's instructions: alanine-aminotransfer-

ase (ALT), aspartate-aminotransferase (AST), blood urea nitrogen (BUN), creatinine (Creat), total proteins (TP), calcium (Ca), phosphorus (P), and potassium (K). Furthemore, non-esterified fatty acids (NEFA) and β-hidroxibutirrate (BHBA) were analysed by two different commercial enzymatic test kits, Randox and Catachem respectively, according to the manufacturer's instructions. Haptoglobin (Hp) was determined by an ELISA commercial method (Tridelta), and oxygen free radicals' levels (OFR) was monitored by a commercial colorimetric method (DIACRON) both according to the manufacturer's instructions. Finally, the serum lisozyme (SL) and bactericidal activity (SBA) determinations were performed according to validated procedures by bacteriological assay (Osserman and Lawlor 1966, Bonizzi et al. 1989, Ponti et al. 1989, Amadori at al. 2002).

Cortisol was analysed in a tail hair matrix following Accorsi et al. (2008) method. Hair samples were carefully cut from the tail switch using clippers and were frozen to -20°C to prevent lice, which are often found in this body area.

Blood and hair samples were collected during the daily routine in order not to disturb the animals and in compliance with the current legislation on animal welfare.

At the same time, the body condition score (BCS) was recorded by the same observer using the 1-5 scale according to Ferguson et al. (1994) along with an increasing level of fattening.

An ANOVA test was performed by JMP (The Statistical Discovery Software, SAS Institute, 2002). The model included the type of housing system and the farm nested in the type of housing system as variability factors. A 5% and 1% ($P \leq 0.05$; $P \leq 0.01$) significance levels were used.

RESULTS

Some parameters displayed significant differences related to the housing system: ALT, AST, BUN, BHBA, OFR and hair cortisol. Most of the investigated parameters showed values within the reference range. Table 1 summarizes the results obtained in the study.

Table 1. Metabolic, immunological and stress parameters related to the two housing systems.

	Housing system						
	TS (n = 30)		OS (n = 50)				
	mean	SE	mean	SE	P	Normal range*	UM
ALT	42.4 ↑	12.42	35.4	7.59	0.0002	14-38	U/L
AST	87.4	19.16	97. 8	29.24	0.0513	60-118	U/L
BUN	14.6 ↓	6.18	13.3 ↓	3.96	0.0395	20-30	mg/dl
BHBA	6.4	1.30	8.3	2.09	0.0001	<10.5	mg/dl
Creat	1.03	0.138	1.02	0.142	0.6212	1-2.7	mg/dl
NEFA	73.2 ↓	16.14	68.5 ↓	68.89	0.7051	89-618	mmol/L
TP	7.5	0.39	7.6	0.52	0.1228	5.7-8.1	g/dl
Ca	9.6	0.33	9.5	0.39	0.4956	8-10.5	mg/dl
P	5.3	1.20	5.3	0.77	0.8627	4-7	mg/dl
K	4.7	0.48	4.6	0.49	0.0989	3.9-5.8	mmol/L
SL	1.0	0.87	0.8 ↓	0.65	0.0591	1-3	ug/ml
SBA	81.2 ↓	16.31	81.5 ↓	11.35	0.8991	>90	%
Hp	0.1	0.44	0.1	0.24	0.9794	0.0-0.5	mg/ml
OFR	36.1	21.39	68.2	34.51	0.0001		U.Carr.**
Cortisol	2.8	1.43	1.6	1.05	0.0001		pg/mg
BCS	3.14	0.090	3.04	0.070	0.3996	1-5	

*Reference values were provided by the laboratory of IZSLT

**U.Carr. is an arbitrary unit; 1 U.Carr. is equivalent to 0.08 mg of H 202/100 mL.

↑ Values over the threshold of the normal range; ↓ Values under the threshold of the normal range.

Cows reared in the TS showed a significantly lower (P≤0.05) serum AST activity (87.4±19.16 U/L) than those reared in the OS system (97.8±29.24 U/L). Radkowska and Herbut (2014) observed a similar trend in cows reared in stalls with access to an outdoor area compared with those reared without it. On the contrary, ALT values resulted higher in TS group (42.4±12.42 U/L), contrasting the observations of the previous authors.

BUN also showed mean values under the normal range.

Creatinine resulted within the range of normality, revealing a proper kidney function.

NEFA values were under the normal range in both groups, highlighting that this parameter was not influenced by the housing system.

TP, Ca, P, K values fell within the normal range.

In our study SL, SBA and Hp did not vary within the groups; however, SL was close to significance. Mean SBA values were under the normal threshold in the two groups, while the SL mean value was low only in the OS group.

Hp was not affected by the housing system and the values were within the normal range.

OFR was significantly higher (P≤0.01) in the OS group (68.2±34.51 U.Carr), data regarding the normal range are not available.

Hair cortisol values showed a significant higher (P≤0.01) mean values in TS (2.8±1.43 pg/mg and 1.6±1.05 pg/mg).

The cows reared in the two housing systems did not differ in terms of BCS, which were 3.1 and 3.0 respectively for both systems.

DISCUSSION

ALT values in the TS group slightly exceeded the normal range. Moreover, as AST, BHBA and Creat were within normal range, animal health and then welfare did not seem to be impaired.

Usually the plasma NEFA concentration increases in response to increased energy needs accompanied by inadequate feed intake (Overton and Waldron, 2004), while low NEFA concentrations are not to be considered biologically important (Oetzel, 2004).

Since NEFA and BHBA are indicators of negative energy balance, the picture described in this study did not reflect such situation (Adewuyi, 2005).

The low level of BUN could be explained by a deficient protein intake (Lee et al., 1978) although in this study TP showed values within the normal range and did not differ between the two groups.

The interpretation of blood mineral concentrations varied according to each specific mineral: Ca is generally an ineffective means of assessing calcium intake, probably because of the sensitive homeostatic mechanism, while P and K blood concentrations are good measures of nutritional supply (Herdt et al., 2000).

SL, SBA and Hp represent a nonspecific cellular immune response. The alteration in these parameters may indicate inadequate hygienic and sanitary conditions of the herd or an inappropriate feed and management approach (Bonizzi et al., 2003). As SL is involved in the immune system, it is one of the most predictive parameters of disease. Variations in its levels have been found in response to inflammation or metabolic stress-related conditions in early lactation (Trevisi et al., 2012). Our results indicated a slightly altered immune response. Some authors have reported a decrease in SL in cows during the transition period (Bonizzi et al., 2003). On the other hand, others indicated SBA values of around 90% as being a significant alteration of the physiological conditions, thus indicating the predisposition to developing diseases conditioned by stressful events (Amadori et al., 2002).

The high level of OFR in the OS group is interesting given its influence on oxidative stress, which can lead to the modification of important physiological and metabolic functions. OFR values resulted unexpected in the OS farming system. Since failure in the adequate control of free radicals within metabolically active tissues results in oxidative stress and possibly increased health disorders in high-producing dairy cattle (Sordillo et al., 2009), our results would highlight the importance of antioxidants supplementation in the diet of dairy cows, particularly in those reared in OS.

It is likely that animals reared in OS are selected more for productive purposes than other animals, thus health problems could have a higher incidence together with animal welfare impairment.

In the present study the mean milk production of cows reared in OS reached a significantly higher level of milk production (P≤0.001) than those reared in TS (respectively 9558.68±384.803 l and 5996.76±496.779 l).

Cortisol in the tail hair matrix normally shows a higher concentration compared to other parts of the body. The hairs also grow back in very short time in the tail, suggesting that this is the most suitable location to collect hair samples (Moya et al., 2013). The recorded values of hair cortisol were lower than found in the literature, but comparable with those reported by Rizzo et al. (2007) in pregnant cows (45.14±2.08 and 49.16±2.08 U.Carr). Del Rosario et al. (2011) reported hair cortisol concentrations equal to 12.1 ±1.85 pg/mg in 2-year-old cows, and Burnett et al. (2014) found a cortisol level equal to 11.0±1.2 pg/mg in lactating dairy cows. These observations led us to consider that the obtained values of hair cortisol did not indicate a situation of chronic stress in the cows reared in TS.

Typically, psychological stress is associated with fear, such as that experienced during commingling or social mixing, exposure to new environments, loud and unusual noises, and restraint (Carroll and Forsberg, 2007). These conditions scarcely affected the animals reared in TS. The limitations of TS, such as the lack of areas for movement and the boredom, may be compensated by the comforting and reassuring environment.

These moderate values, together with low plasma levels of OFR, NEFA and BHBA could indicate a reduced risk of metabolic disorders (Bernabucci, 2005).

The living conditions of cows reared in TS did not have a negative impact on the examined parameters, which in some cases were more suitable than those reared in OS.

CONCLUSIONS

In conclusion, the study evidenced that some parameters significantly varied in the two farming systems but, in consequence of the fact that most of them are within the normal range, the welfare of the animals seem not to be impaired. Finally, animals reared in the TS system did not show alterations ascribable to evident signs of suffering.

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Isolation, identification and molecular characterization of *Mycoplasma bovis* in mastitic dairy cattle by PCR and culture methods

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ABSTRACT. *Mycoplasma bovis* is well known as one of the major causative agents of mastitis in dairy cattle herds. The aim of this study was the identification of *Mycoplasma bovis* strains by PCR and traditional culture methods from a total number of 328 milk samples collected from cows with clinical mastitis symptoms from all over Iran. First step cultures in PPLO broth and agar showed 58 samples (17.69%) as positive. Out of 328 samples, 97 samples (29.57%) were positive for *Mycoplasma* genus according to the amplification of the *16SrRNA* gene performed by PCR and from them, 31 (31.97%) samples were positive by PCR on the *P48* gene. The purified *P48* positive PCR products were sequenced and results were compared to *M. bovis* reference strain *PG45* (CP002188). A phylogenic tree was created using Neighbor-joining method in MEGA6 software. The studied strain IB220 showed 100% identity with the reference strain of *M. bovis* and followed the same phylogenetic roots while studied strain IB216 showed 99.7% homology with the reference strain. Twelve selected geographical isolated strains were subjected to Gene Bank under accession numbers of KX772789 to KX772800. This is the first study of the molecular characterization of *Mycoplasma bovis* in dairy cattle with clinical mastitis from Iran.

Keywords: *Mycoplasma bovis*, cattle, isolation, PCR, mastitis

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1. INTRODUCTION

Mycoplasma bovis is well known as one of the major causative agents of mastitis in dairy cattle herds. The bacteria infection can also cause abortion, infertility, arthritis, kerato-conjunctivitis, otitis media, pneumonia, and subcutaneous abscesses which lead to huge economic losses worldwide (Giacometti et al., 1999; Nicholas and Ayling, 2003). There are at least 11 other *Mycoplasma* species associated with mastitis outbreaks in cattle, but the infection induced by *M. bovis* is characterized by the increased number of severe clinical mastitis that are mostly non-responsive to treatment. *Mycoplasma* are small prokaryotes with simple encoding gene treasure which cause numerous diseases in cattle of dairy farms such as arthritis and mastitis which lead to huge economic losses worldwide (Hotzel et al., 2003). The organism is also known as the main causative agent of therapy-resistant mastitis on large dairy farms. Traditional methods of detection and isolation of *Mycoplasma* strains such as serological and culture methods are time consuming, so the more sensitive biological methods such as PCR, are widely used for the detection and molecular analysis of various bacteria such as *M. bovis* strains in every microbial laboratories worldwide (Kirk and Lauerman, 1994). *P48* is a membranous lipoprotein of slightly lower than 48kDa that is homologous to the family of the Macrophage Activator Lipoproteins (MALPs) and it is coded via a conserved sequences specific to the *M. bovis* species (Wawegama et al., 2014), so the sequence of *M. bovis* strain *PG45* under accession number of CP002188 was selected (Wise et al., 2011) to compare with the strains detected in this study. The encoding gene sequence of lipoprotein *P48* is recorded as accession numbers of DQ020481 and DQ020482 at Gene Bank (Lysnyansky et al., 2008) and it is known as one of the virulence factors of *M. bovis* species (Li et al., 2011). As the *P48* gene is well conserved in all *M. bovis* strains, primers with high sensitivity and specificity were designed (Fu et al., 2014), and used for the amplification of *P48* gene. The immunogenicity of *P48* is also well known as one of the identified immunodominant membrane protein of *M. bovis* surface antigenic variation (Behrens et al., 1994). Robino also studied the conserved surface lipoprotein *P48* as a marker for the detection of *M. bovis* strains (Robino et al., 2005). One of the housekeeping genes which are known as conserved sequences in organisms, is *uvrC* encoding gene considered as *M. bovis* marker's too (Thomas et al., 2004). The *uvrC* encodes deoxyribodipyrimidine photolyase which removes damaged DNA and play the role in the repairing system in *Mycoplasma* species (Hotzel et al., 2003). The study of the sequences of the *uvrC* encoding gene sequence may lead to understanding of the phylogenic relations of different strains of *M. bovis* species. Rossetti used a novel designed pair of primers for the amplification of the *uvrC* gene by Real-time-PCR as the bio marker of *M. bovis* strains (Rossetti et al., 2010). Because of the important role of *M. bovis* in clinical mastitis in dairy herds, this study was designed to isolate and characterize *M. bovis* strains originated from different clinical mastitis cases from all over Iran, using two traditional culture-biochemical methods in comparison to highly sensitive molecular tools such as the PCR method.

2. MATERIALS AND METHODS

Sampling was done from a huge survey population of dairy cow husbandries from all over Iran, from April 2015 to May 2016. Raw milk samples were collected from 328 cows suffering from clinical signs of mastitis. The samples were immediately placed in test tubes with transport *Mycoplasma* medium and then transferred on ice to the *Mycoplasma* reference laboratory of Razi Vaccine and Serum Research Institute, Karaj, Iran, in 24 hours and incubated at 37°C for 12-18 hours with CO_2. After the primary concentration of *Mycoplasma* bacteria in the PPLO broth, 700µl of each cultured sample was added to 5ml new sterile PPLO broth using specific antibacterial filter (PVDF) which is permeable to organisms smaller than 450nm in size such as *Mycoplasma* and viruses. Main cultures were incubated at 37° under CO_2 gas treatment for two weeks. As *M. bovis* considered a non-glucose fermentative species, the red color of the PPLO cultures was notified as a positive biochemical sign of the specific growth of *M. bovis* species. 700µl of each sample were stored at -70°C for DNA extraction. 200µl of *M. bovis* PPLO culture were used on PPLO agar and incubated at 37° under CO_2 condition. The detection of *Mycoplasma* specific colonies after 5-7 days, was considered as a positive sign of the isolation of *M. bovis* bacteria.

Table 1. Primer pairs for amplification three encoding genes of 16SrRNA, P48 and uvrC.

Kojima et al., 1997	163bp	GCTGCGGTGAATACGTTCT	16Sr RNA	M1F	Genus specific primers
		TCCCCACGTTCTCGTAGGG		M3R	
Fu et al., 2014	1341bp	GCTTCATGTGGTGATAAATACTTTA	P48	IMB-F	Species specific primers (1)
		CTATTTTTGTGTTTCTTTAGCCAAT		IMB-R	
Bashiruddin et al., 2005	1626bp	TTACGCAAGAGAATGCTTCA	uvrC	MbouvrC-L	Species specific primers (2)
		TAGGAAAGCACCCTATTGAT		MbouvrC-R	

2.1. DNA Extraction and primers design

DNA was extracted from identified biochemically *Mycoplasma* colonies using Phenol-Chloroform extraction method (Pourbakhsh, 2010). A pair of *Mycoplasma* genus specific primers was designed for the first step of screening and the discrimination from other probable bacteria. *16SrRNA* gene was selected as the target gene for detecting the genus, as described by Kojima (Kojima et al., 1997). Then two pairs of *M. bovis* species specific primers on *P48* and *uvrC* genes were designed as described by Fu and Sabramaniam, respectively (Fu et al., 2014; Sabramaniam et al., 1998) (Table 1).

2.2. PCR of *16SrRNA* gene for detection of Genus *Mycoplasma*

10ng of extracted DNA subjected as template in 100 microliter total reaction volume of 2µl of each primers (20µM, Cinnagen, Iran), 2µl of each dATP, dTTP, dGTP and dCTP (200µM Fermentase), 10µl of 10X PCR buffer, 2.5U Taq DNA polymerase enzyme (2500U - 5U/µl - Cat. No. DP1603) (Cinnagen, Iran), 1.5 mM $MgCl_2$ in an automated Thermo cycler (*FALC*, Germany) under the following programs:

a) for the *16SrRNA* encoding gene (Kojima et al., 1997), 94 °C (7.5min) of initial denaturation and then 30 cycles of denaturation at 94 °C (30s), annealing at 56 °C (30s), elongation at 72 °C (60s) and final extension in 72 °C (5min).
b) for the amplification of *P48* encoding gene (Fu et al., 2014), 94 °C (4min) of initial denaturation and then 35 cycles of denaturation at 94 °C (60s), annealing at 58 °C (60s), elongation at 72 °C (100s) and final extension in 72 °C (10min).
c) for the amplification of *uvrC* encoding gene (Bashiruddin et al., 2005), 94 °C (2min) of initial denaturation and then 35 cycles of denaturation at 94 °C (30s), annealing at 52 °C (30s), elongation at 72 °C (60s) and final extension in 72 °C (7min).

PCR products were subjected to electrophoresis on 1.5% agarose gel in TBE buffer (1X) at 100V and were visualized under UV light by *Erythro-gel* staining (*Biotium*, USA). All the positive detected *P48* PCR products presenting the specific 1341bp length band, were purified by PCR Product Purification Kit following the instructions of the manufacturer (*MBST*, Iran). The purified PCR products of the *P48* amplified target gene were sent for bidirectional sequencing (*Bioneer*, Korea). Sequencing results were compared with other *P48* sequences recorded in Gene Bank using the Bio-Edit software. Phylogenic tree were designed by the Bootstrap1000 and Neighbor-Joining Tree analysis. The statistical analysis for significance of difference between age, rate of production and herd size with the prevalence, was done using Chi-Square test.

3. RESULTS

Culture results on *PPLO a*gar growth showed fried egg appearance in 58 out of total 328 (17.69%) raw milk samples under light microscopy. Cultures showed that 270 milk samples (82.31%) were negative. We also analyzed with PCR the negative cultures of the samples. *16SrRNA* gene PCR results showed 97 out of 328 (29.57%) positive samples, including the 58 positive culture results, which indicates that the 39 samples with negative culture results were positive by PCR indicating the higher sensitivity of the method. 231 samples (70.43%) were negative according to the PCR of the *16SrRNA* gene. All of the culture positive samples were showed as positive by the PCR results, as shown in Figure 1.

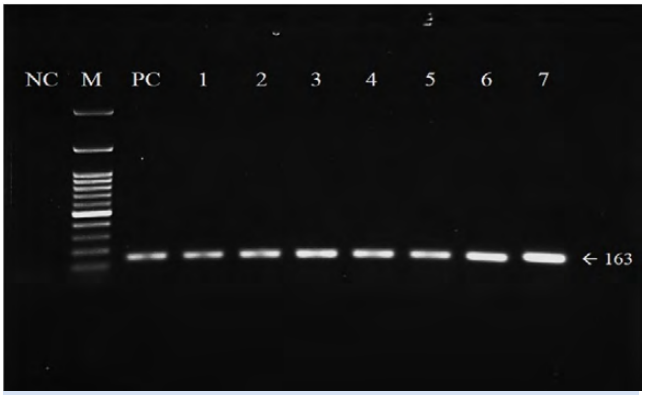


Fig 1. PCR results of the amplification of the *16SrRNA* gene.
M: Marker 100bp, PC: Positive Control (*M. bovis* ATCC 25523/ PG45), NC: Negative Control, 1-7: *Mycoplasma* detected samples.

Species specific PCR on *P48* gene was performed on samples which were proved as *Mycoplasma* by PCR of the *16SrRNA* gene. Results showed 31 out of 97 strains as *M. bovis* species and 66 samples were negative, belonging to other *Mycoplasma* species, Figure 2. The prevalence of clinical mastitis caused by *M. bovis* in this study was calculated as 31.97%.

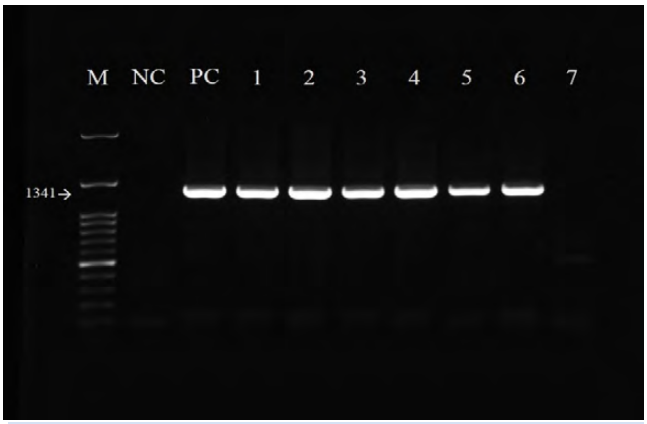


Fig 2. PCR results using specific Primers *P48*.
M: Marker 100bp, NC: Negative Control (*M. agalactiae* NCTC 10123), PC: Positive Control (*M. bovis* ATCC 25523/ PG45), 1-6: 1341 bp length PCR products of M. bovis species.

Another PCR with species specific designed primers on the *uvrC* gene, was performed on randomly selected samples which proved as *M. bovis* by *P48* specific primers as mentioned above. Results showed a 1626 bp PCR product as expected, Figure 3.

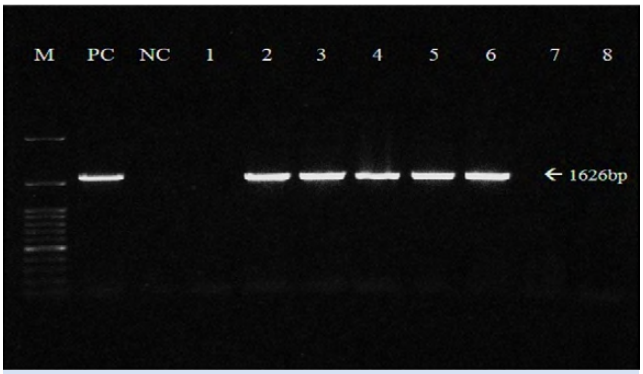


Fig 3. PCR results using specific Primers uvrC.
M: Marker 100bp, PC: Positive Control (*M. bovis* ATCC 25523/ PG45), NC: Negative Control, 2-6: Suspected samples, 1, 7, 8 are negative samples.

12 sequenced strains of this study as shown in the table 2 were aligned and analyzed by Bio-Edit software and were compared to *Mycoplasma bovis PG45* MU clone A2 complete genome, recorded at Gene Bank under accession number CP002188. PCR products of *PG45* amplification test were selected for sequencing and results were compared using online multi nucleotide sequence BLAST software. Twelve strains isolated from different geographical areas were compared. Results showed the maximum and minimum identity of 100% and 99% among these geographical obtained strains as shown in table 2. From the 12 selected strains only two strains (Query_160084 and 160085) had 99% identity.

Strain IB220 showed the maximum identity with the reference *M. bovis* sequence and the other strains showed a minimum identity of 99% (Table 3).

Finally, the above twelve strains were further analyzed for phylogenic tree (Figure 4). The phylogenic tree that was based on the molecular analysis and BLAST comparison results also confirmed the 100% identity of the strain IB220 with the reference strain of *M. bovis* in Gene Bank following the same phylogenetic roots, while the studied strain of IB216 has the identity of 99.7% with the reference strain. Alignment results showed 99.7% similarity between IB220 and IB216 samples and 99.8% similarity to other strains of the study as well. All the 12 geographical isolated strains from different parts of the country that were sequenced in this study were subjected to Gene Bank under accession numbers of KX772789 to KX772800.

Table 2. Results of antimicrobial susceptibility testing in Salmonella isolates.

	Max score	Total score	Query cover	E value	Identity	Accession
IB252	2091	2091	100%	0.0	100%	Query_160091
IB240	2091	2091	100%	0.0	100%	Query_160089
IB239	2091	2091	100%	0.0	100%	Query_160088
IB230	2091	2091	100%	0.0	100%	Query_160087
IB228	2091	2091	100%	0.0	100%	Query_160086
IB215	2091	2091	100%	0.0	100%	Query_160083
IB40	2091	2091	100%	0.0	100%	Query_160082
IB20	2091	2091	100%	0.0	100%	Query_160081
IB13	2091	2091	100%	0.0	100%	Query_160080
IB216	2085	2085	100%	0.0	99%	Query_160084
IB241	1890	1890	90%	0.0	100%	Query_160090
IB220	1801	1801	86%	0.0	99%	Query_160085

Table 3. BLAST comparison analysis of 12 selected strains with the reference strain of PG45 ATCC 25523. IB220 showed 100% identity with the reference strain.

	Max score	Total score	Query cover	E value	Identity	Accession
IB20	2252	2849	0%	0.0	99%	Query_41471
IB228	2248	2846	0%	0.0	99%	Query_41476
IB252	2242	2840	0%	0.0	99%	Query_41481
IB230	2242	2840	0%	0.0	99%	Query_41477
IB240	2231	2829	0%	0.0	99%	Query_41479
IB239	2231	2829	0%	0.0	99%	Query_41478
IB216	2217	2809	0%	0.0	99%	Query_41474
IB40	2213	2810	0%	0.0	99%	Query_41472
IB215	2207	2805	0%	0.0	99%	Query_41473
IB13	2085	2683	0%	0.0	99%	Query_41470
IB241	1884	2473	0%	0.0	99%	Query_41480
IB220	1807	2367	0%	0.0	100%	Query_41475

In order to study the relation between the age and the prevalence of the infection, samples were classified in five different groups including 65 samples (2 positive, 63 negative) at age 24-36 months, 56 samples at age 36-48 months (4 positive, 52 negative), 76 samples at age 48-60 months (8 positive, 68 negative), 42 samples (12 positive, 30 negative) at age 60-72 months and 89 samples at age more than 72 months (5 positive and 84 negative). In our study, there was statistical significance in the incidence of *M. bovis* mastitis among the different age groups ($P<0.05$).

Study analysis showed the prevalence of 38.71% infection in high rate of production cows, 38.71% in cows with moderate rate of production and 22.58 % in cows with low rate of milk production. This study did not show statistically significant between the prevalence and rate of production ($P>0.05$). The majority of positive samples were in the herds with the average size of over than 800 cows. There was significant relation between herd size with the incidence of infection ($P<0.05$). Results also showed that 142 out of total 328 milk samples were taken from

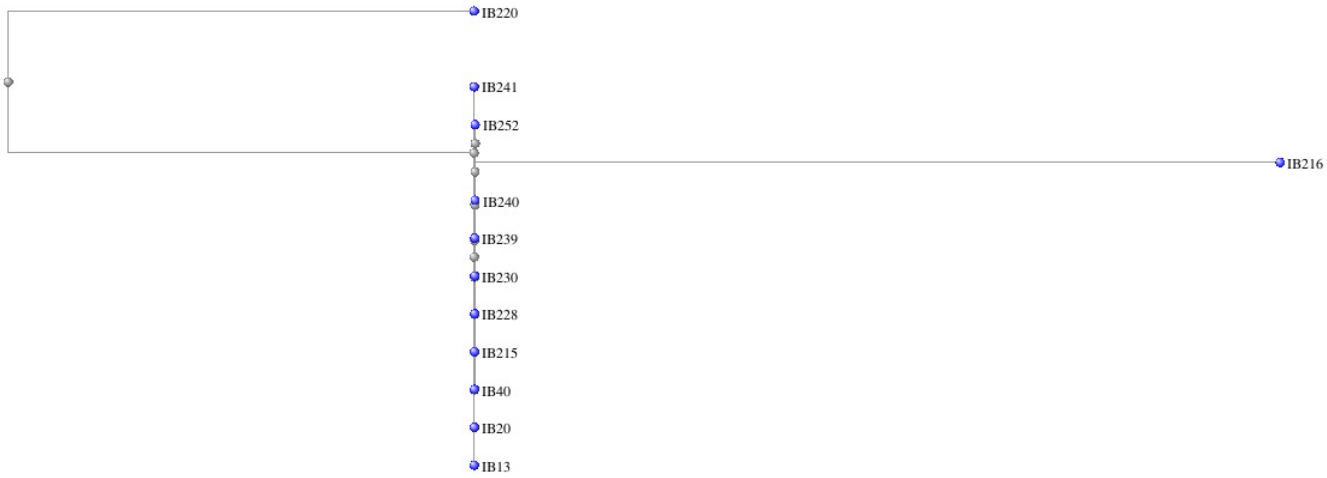


Fig. 4. The Phylogenic Tree drawn by Tree was drawn using Neighbor-joining method in MEGA6 software.

cows with the clinical recorded history of 1 to 10 times acute mastitis.

4. DISCUSSION

Mycoplasma bovis is widely known to be the most important etiological agent of various bovine diseases leading to huge economic losses (Pfützner and Sachse, 1996). In current study *Mycoplasma bovis* from mastitic milk of cattle farms in Iran were isolated and identified using PCR and culture methods. As clinical and pathological signs are not characteristic for *M. bovis* infection, laboratory diagnosis is necessary. Traditional methods for detection and isolation of *Mycoplasma* strains are time consuming, so more sensitive methods such as PCR, are widely used in every microbial labs. In this study culture results of milk samples showed 58 out of total 328 (17.69%) *M. bovis* bacteria with fried egg appearance under light microscopy. In a conducted study, comparing obtained isolates with existing isolates in Gene Bank based on the gene sequence *16SrRNA* proved 100 percent similarity between isolates (Cai et al., 2005).

Except for the 58 samples identified as positive by cultures, 39 samples from the negative cultures were found positive in the *16SrRNA* encoding gene PCR. All of the positive samples detected by culture were led to positive PCR results. The comparison of the two methods of PCR and the cultures for the detection of mycoplasma species in milk samples, showed that sensitivity and specifity of PCR are 96.2% and 99.1%, respectively. Also, the sensitivity and specificity of PCR on bulk tank milk compared with cultures of the same samples are 100% and 99.8%, respectively (Baird et al., 1999). In the study by Pinnow and colleagues, the sensitivity of Nested-PCR for the detection of *M. bovis* in milk kept for 2 years, was 100%, while the sensitivity of culture was 27%; so this approach was introduced as an appropriate method to identify and isolate *M. bovis* in clinical samples (Pinnow et al., 2001).

It was indicated that the identification of *M. bovis* strains in cattle herds and the evaluation of their pathogenicity and antigenic variation is important (Behrens et al., 1994). *P48* is a membranous lipoprotein of 48kDa weight that is homologous to the family of the Macrophage Activator Lipoproteins (MALPs) and is coded via a conserved sequence specific to the *M. bovis* species (Wawegama et al., 2014). As the *P48* gene is a conserved sequence in all *M. bovis* strains, designed primers by Fu (Fu et al., 2014), were used for amplification the *P48* gene in this study. Robino also studied the conservative surface lipoprotein *P48* as a marker for the detection of *M. bovis* strains (Robino et al., 2005). Study of *P48* sequence may lead to understand the phylogenic relations of different strains of *M. bovis* species. Specific PCR of *P48* gene was done on samples identified as *Mycoplasma* by PCR on *16SrRNA* gene. Results revealed that 31 out of 97 *Mycoplasma* detected samples were belonged to *M. bovis* species (31.97%) and 66 samples (68.04%) were belonged to

other *Mycoplasma* species. The results of the study demonstrated that the cattle farms from Iran were infected with *M. bovis*. The pollution is very different compare to the rest of the world as the presence of bacteria in milk samples in this study was lower than its presence in Italy (Radaelli et al., 2011) and higher than other countries such as France (Arcangioli et al., 2011). Sequenced strains of this study were aligned and analyzed by Bio-Edit software and were compared to other *P48* sequences of *M. bovis* recorded at Gene Bank (NCBI). The results of alignment analysis indicated that the majority of the *M. bovis* strains, which was isolated from Iranian cows, had 99-100% homology to the reference strain of *M. bovis*. In previous studies, other gene was used for evaluating phylogenetic relationship among the isolated *M. bovis* strains. So far, there was no study based on *P48* gene examining the phylogenetic relation between *M. bovis* strains, but other studies have been conducted on the basis of other genes. In Egypt, assigning of *Vsp* genes sequence for molecular analysis of the isolated *M. bovis* strains showed that some isolates have homology with reference strain PG45 and some other were divided into different groups (Eissa et al., 2012). In Germany, the phenotypic changes of *M. bovis* isolated from culture methods and specific PCR was shown that the isolated strains are different from *PG45* and there is dissimilarity between them; 5 isolates had identical DNA profiles but three other isolates performed different patterns. The results showed that protein patterns within species *M. bovis* strains can be very useful for the comparison (Hala and Hotzel, 2013).

According to studies, cows in all ages may be affected by this type of mastitis but fresh cows mostly show severe symptoms (Radostits et al., 2000). In this study, most of positive cases were in 60-72 months age group with 38.8% and 48-60 months with 25.8%, respectively. The results of this study are inconsistent with the other studies. Fu showed that the highest rate of the prevalence of infection were seen in cows in age more than 1 year old with the prevalence of 10.5% infection following by a rate of infection up to 9.61% in cows at age 3 with no reliable relations between the age of prevalence and rate of pregnancy in cattle (Fu et al., 2014). The most positive cases were related to cattle with herd size 800-1500 (45.16%) and cattle with 1500 herd size and higher (32.25%), respectively. This is consistent with findings in other studies. Research in United States showed that an increase of mycoplasma mastitis is associated with an increase in herd size. It had been shown that risk of *M. bovis* mastitis and possibility of a positive sample in a big herd with more than 350 cattle, is 15 times higher than herds with fewer than 350 cattle and the same it is in their milk tanks (Park, 2014). In other study in dairy herds in Northern Italy that was conducted on a sample of clinical mastitis in dairy cows, the results showed that the occurrence of mastitis is common in larger herds (Radaelli et al., 2011).

5. CONCLUSIONS

In this study results showed the highest prevalence of positive samples in cows with average and high production rates. The highest prevalence belongs to 4-6 years old group, high production cows with the average size of over than 800 cows. This is the first study on the isolation, identification and molecular characterization of *Mycoplasma bovis* infection in dairy cattle with clinical mastitis from Iran. Results suggested *M. bovis* as one of the main causative agents of dairy cow mastitis in Iran.

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Research article
Ερευνητικό άρθρο

Efficacy of recombinant VAXXITEK HVT-IBDv vaccine against very virulent Infectious bursal disease virus (vvIBDv) challenge in layer chicks: A pilot study

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ABSTRACT. The infectious bursal disease virus (IBDv) is widespread in poultry flocks all around the world. Various biotypes have emerged and because of that, adequate management practices and vaccination of chicks are of paramount importance for the protection against field strains. One day old Lohmann Brown chicks were vaccinated with intermediate vaccines and the recombinant VAXXITEK HVT-IBDv vaccine formulation, and challenged at 48 days of life with the very virulent IBDv (vvIBDv) strain CH/99. The best protection (100%) was achieved with the recombinant vaccine administered by the subcutaneous or intramuscular route at a day old, while intermediate and intermediate plus vaccines protected 80% of birds from clinical symptoms. The highest bursa body ratio (5.33, 3.50 and 4.12) was accomplished in non- vaccinated and non-challenged birds and birds vaccinated with the VAXXITEK HVT-IBDv vaccine. The recombinant VAXXITEK HVT-IBDv vaccine has provided protection for commercial chicks against challenge with the vvIBDv strain in this experiment. Under field conditions, additional vaccination is possibly needed with supplementary application of live attenuated vaccines. However, the recombinant vector vaccines are providing significant aid against clinical signs and immunosupression caused by the vvIBDv.

Keywords: poultry, IBDv, intermediate vaccines, recombinant vaccines, challenge

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INTRODUCTION

The infectious bursal disease virus (IBDv) causes health problems in poultry flocks all around the world. This virus is capable of spreading across a long distance and survives in a poultry farm environment, even if the farms were thoroughly cleaned and disinfected between production cycles. Once the farm is contaminated with the IBDv it is almost impossible to prevent the infection of chicks. Vaccination of chicks against IBDv is often inefficient due to the interference of maternally derived antibodies with vaccine viruses (Chettle et al., 1989). Studying molecular genetics of the virus and various vaccination approaches substantially contributed to understanding the biology of the IBDv and has improved control strategies (Müller et al., 2012).

The IBDv is a double stranded RNA virus with a bisegmented genome and it belongs to the genus *Avibirnavirus* of the family *Birnaviridae* (Müller et al., 1979). The larger segment of the virus encodes the polyprotein, which is autocatalytically cleaved to VP2, VP3 and VP4 proteins, while a small overlapping open reading frame fragment encodes the protein VP5, which has a role in cell lysis and the apoptosis process. The smaller segment encodes an RNA dependent RNA polymerase (Mundt et al., 1995). The most studied is the capsid protein VP2, since it is exposed at the surface of the virus and possesses conformational epitopes with different amino acid arrangements in various biotypes (Bayliss et al., 1990). Therefore, the VP2 protein has become an important target for genetically engineered vaccines, some of which are based on the recombinant technology (Darteil et al., 1995).

The turkey herpes virus (HVT) is an avirulent, cell associated virus and has been used for decades for the vaccination of chicks against Marek's disease (MD). As the Marek's disease virus is ubiquitous, all commercial chicks have to be vaccinated in hatcheries subcutaneously at the first day of life or *in ovo* (Gimeno, 2008). Because of the cell associated nature of the HVT, it has become an attractive target for the development of vector vaccines. The basic concept for the vaccination of chicks with rHVT-VP2 depends on the expression of the VP2 at the surface of the cell which in turn induces immune responses. The vector virus (VAXXITEK HVT-IBDv) was found in the feathers follicles, which means that the pathogenesis of the vaccine strains was well established in the recombinant formulation (Gelb et al., 2016). The partial aid, when it comes to the protection from MD, is also possible with some of the HVT recombinant vaccines (Aly et al., 2012). However, the results from research studies are different and the success of field vaccination depends on multiple factors such as management practices, and virulence and antigenicity of the field strains.

Outbreaks of vvIBDv still occur in broiler and layer chicks in Serbia. Most commonly the vaccination with the intermediate plus vaccines takes place at approximately 10 days of age and second vaccine is given to chicks 7 to 10 days apart. In cases when for a longer period of time new outbreaks are not recorded, the intermediate vaccines are continuously used. Such control has shown some benefits but in farms with poor management practices it is still highly risky to replace vaccination with the intermediate vaccines only. In such circumstances the option to vaccinate chicks as early as at day one or *in ovo* with the recombinant rHVT-VP2 seems to be promising. Hence, a challenge experiment was done to evaluate several vaccination protocols in order to gain experience with the rHVT-VP2 in Serbia. The goal was to perform an experimental infection of chicks vaccinated with the intermediate or intermediate plus vaccines *per os* and with the rHVT-VP2 vaccine subcutaneously or intramuscularly, in order to determine the level of protection against challenge with the vvIBDv strain.

MATERIAL AND METHODS

General description

The following work was conducted at the Faculty of Veterinary Medicine in Belgrade. The facilities where the experiments were carried out were separated by concrete walls. The walls and concrete floors were easy to disinfect. The commercial feed produced by technology for Lohmann brown provenience was prepared in a local feed factory according to HACCP quality assurance system and ISO 22000 standards implemented. Water was provided ad libitum. The facility is the only establishment certified

for experiments involving poultry and as such it has permit issued by the Ministry of Agriculture of the Republic of Serbia, Veterinary Directorate (permit number 323-07-02263/2014-05/2). The chicks from all the experimental groups were vaccinated in the hatchery against MD, infectious bronchitis (IB) and Newcastle disease (NDV). The type of MD vaccines used in this study is presented in experimental infection. During the experiment, the complete vaccination program against poultry diseases commonly applied in Serbia was also performed. The vaccines included those against Newcastle disease virus and infectious bronchitis virus i.e. Nobilis Ma5-Clone30, Nobilis ND clone30, IB Bioral H120 (MSD Animal Health, The Netherlands).

Vaccines against IBDv

Three types of commercial live IBD vaccines were used in the study: the intermediate (D78) and intermediate plus vaccines (228E), (MSD Animal Health, The Netherlands) and the recombinant vector vaccine VAXXITEK HVT-IBDv (Merial-Sanofy, France).

Challenge virus

For the challenge experiment, the local field vvIBDv strain CH/99 was used. The CH/99 IBDv is the standard challenge strain used in Serbia. It was not titrated on chickens but it causes mortality of 50% layer chicks which are free of maternal antibodies. According to the amino acid sequences of the hypervariable domain of the VP2, this virus belongs to the very virulent biotype (GeneBank accession number KF439863), (Dobrosavljević et al., 2014).

Experimental infection

Sixty one-day old commercial Lohmann Brown chicks were held in isolation units and provided with feed and water ad libitum. Each vaccinated group and two control groups consisted of 10 chicks. Groups G1 and G2 had received the VAXXITEK HVT-IBDv vaccine subcutaneously or by intra muscular route, respectively. They were also vaccinated with the CVI988 vaccine. Chickens in group G3, G4 and G5 were vaccinated at one-day of age, with the Cryomarex (CVI 988-Rispens+HVT) vaccine (Merial-Sanofi, France). No interference between rHVT and CVI988 has been established so far (Hein et al., 2011). G3 group of chicks received the intermediate vaccine at 28 days of age, while G4 group received the intermediate plus vaccine at 26 days of age using the per/os method (water mixed with skim milk). The timing for the vaccination against IBD was estimated using Deventer formula based on an ELISA antibody titer of day old chicks, in order to avoid the interference with maternally derived antibodies (MDA). After seven days, groups G3 and G4 were vaccinated one more time against IBDv with the intermediate vaccines. Groups G5A and G5B were not vaccinated against IBDv. Group G5A was a challenge control, while group G5B was not vaccinated nor challenged during the experiment. Challenge with the CH/99 virus (10 birds from each group except G5B) was done at 48 days of age by oculo/nasal administration of 50µl of the crude bursal homogenate which was prepared as described previously (Dobrosavljević et al., 2014). Clinical symptoms, mortality (Le Nouen et al., 2012) and bursa/body weight ratios (Sharma et al., 1989) were used to evaluate the success of the vaccinations. Bursas were collected from six chicks from each group sacrificed at 59 days of age. The number of samples for statistical analysis was determined according to the following formula, for the minimal size of the sample in population.

$$n = \left[\frac{z \cdot \sigma}{G}\right]^2$$

z- Confidence level

σ - Basic standard deviation

G – The maximum permissible error

Chicks were sacrificed according to the EU Directive 2010/63 of the European Parliament and the Council on the protection of animals used for scientific purposes (Directive 2010/63/EU). The Committee of animal welfare of the Republic of Serbia has provided the permit (permit number 323-07-07812/2014/05/1) for the challenge experiment.

STATISTICAL ANALYSIS

The statistical analysis was done by using descriptive statistical parameters (analysis of variance-ANOVA and Tukey test). The established statistical sig-

nificance was at the level of 5 and 1% and was further elaborated using software GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California USA, www.graphpadHYPERLINK "http://www.graphpad.com/".com and MS Excel programs.

RESULTS AND DISCUSSION

During the experiment the health status of the birds was recorded on a daily basis until the termination of the experiment. The symptoms of the acute IBDv (such as depression, ruffled feathers and watery diarrhoea) were recorded 48 hours after challenge in all birds from the non-vaccinated challenged group of chicks (group G5/A) and the mortality was 50%. In the groups of chicks vaccinated with the intermediate and intermediate plus vaccines (groups G3 and G4 respectively) the symptoms of acute IBDv were noticed in two chicks from each group, within two days from the challenge control chicks and those birds had succumbed to the infection. In necropsy discrete bleedings on pectoral muscle were seen and the bursa was swollen in gelatinous edema. The IBDv was confirmed in the bursa applying immunodiffusion test. At the time of challenge, residual MDA in four chicks from groups G3 and G4 respectively, may have still been high at 26 and 28 days of age and active immune responses may have been delayed or even absent at the time of challenge. Hence, the second vaccination with the intermediate vaccine did not provide sufficient protection for the chicks which was also observed in the field (Aliyu et al., 2016) and experimental conditions (Massi et al., 2008). However, in chicks that received the recombinant vector vaccine formulation (G1, G2) and in the control group unvaccinated and unchallenged (G5B), no clinical symptoms or mortality were recorded.

Table 1

The mean bursa/body weight ratio was significantly higher ($p<0.01$) in the control non-challenged group and chicks that were vaccinated with the recombinant vector vaccine, compared to non-vaccinated challenged birds and birds that received the intermediate and intermediate plus vaccines. Also, the mean bursa/body weight ratio (5.33+1.02) was significantly higher ($p<0.05$) in the control non-challenged group (G5/B) compared with that in the chicks vaccinated

Table 1: Clinical symptoms, mortality rate and bursa/body index in vaccinated and challenged chickens at 59 days (11 days post challenge)

Group	Vaccination against IBDV	Clinical symptoms*	Mortality**	Mean bursa weight/g	Bursa/body index
G.1	vHVT13 s/c.	0/10	0/10	2.3	3.50
G 2	vHVT13 i/m.	0/10	0/10	2.85	4.12
G.3	"Intermediate"	2/10	2/10	1.0	1.55
G.4	"Intermediate plus"	2/10	2/10	0.75	1.22
G5/A	Non-vaccinated/ infected	10/10	5/10	0.45	0.69
G5/B	Non-vaccinated/ non-infected	0/10	0/10	3.73	5.33

**number of chickens with clinical symptoms/total number of chickens in experimental group,*
***Number of chickens that succumb infection/total number of chickens in experimental group*

with the recombinant vector vaccine in group G2 (mean B/B weight ratio of 4.12+0.55), and was significantly higher ($p<0.01$) compared with that in the chicks vaccinated with the recombinant vector vaccine in group G1 (mean B/B weight ratio of 3.50+0.20), while there were no significant differences between groups G2 and G1 ($p>0.05$). The mean bursa/body weight ratio in group G1 (3.50+0.20) and G2 (4.12+0.55) was significantly higher ($p<0.01$) in comparison with those in groups G3, G4 and G5/A.

Table 2

The results of the challenge experiment are in agreement with the results obtained by Massi et al., 2008. In their experiment**,** 100% protection was established after the subcutaneous application of vHVT13 in chicks that had been challenged with the vvIBDv. Our experiments are also in agreement with Darteil et al., (1995), who accomplished 100% protection against IBDv with 105 plaque forming units (pfu) of vaccine dose and 60% with 104 pfu vaccine dose per bird at one day of age, with the vHVT002 recombinant vector vaccine. In this vaccine, the open reading frame of the VP2 was inserted at the deleted locus of the glycoprotein gene *gl* under the control of the human cytomegalovirus immediate early promoter. It was established that the efficacy of the vector vaccines depends on the potency of the promoter (Tsukamoto et al., 2002) as well as the proper target site used for insertion of the foreign gene (Darteil et al., 1995).

In the field situation (or in experiments with the commercial chicks), the titers of the maternal antibodies are high at the first day of age and the interference with live-attenuated IBDv vaccine strains is plausible but not with the recombinant IBDv-HVT vaccines. The bursa of experimental birds that were examined 11 days post challenge showed clear differences between the groups of chicks in our study. The size of the bursa and spleen was larger in the non-challenged and the birds vaccinated with the recombinant vaccine, compared to challenged control and groups vaccinated with the intermediate vaccines.

Figure 1

Vaccination with live IBD vaccines is controversial since the occurrence of immunity depends on many attributes even in controlled experiments. In commer-

Table 2: Data on descriptive statistic analysis of the B/B weight ratio at 59 days of age (11 days post challenge)

Groups of chickens	N	$\bar{x}$	SD	Sy	CV (%)	X max	X min
G1	6	$3,50^{dhij}$	0,20	0,0982	5,61	3,75	3,27
G2	6	$4,12^{Aefg}$	0,55	0,2714	13,17	4,59	3,65
G3	6	$1,55^{ceh}$	0,16	0,0780	10,05	1,75	1,37
G4	6	$1,22^{bfi}$	0,09	0,0460	7,54	1,35	1,15
G5/A	6	$0,69^{agj}$	0,30	0,1489	43,12	1,04	0,35
G5/B	6	$5,33^{abcdA}$	1,02	0,5094	19,11	6,74	4,36

Different superscript letters indicates statistical significance between experimental groups of chickens: a, b, c, d, e, f, g, h, i, j, p<0,01; A, p<0.05, N, total number of chickens per group; $\bar{x}$ *the arithmetic average, SD, standard deviation; Sy, standard error; CV, coefficient of variation; X max, maximal value of the bursa/body weight ratio; X min, minimal value of the bursa/body weight ration.*

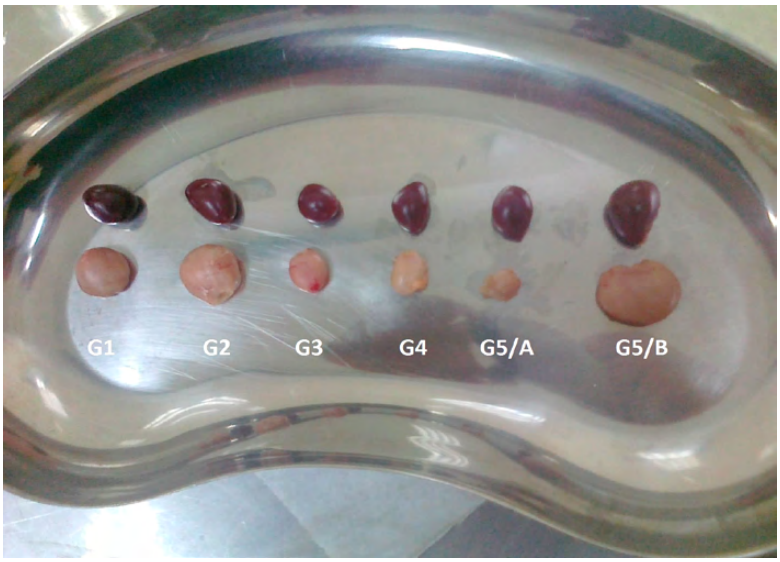


Fig 1: Bursa and spleen of chicks which have been sacrificed at 11 days post infection with vvIBDv (CH/99 challenge strain)

cial broilers the vaccine viruses could cause transient destruction of the bursa and it was postulated that a delay in bursa recovery influences the decrease of the number of target cells in the bursa which are then less available for the pathogenic virus (Rautenschlein et al., 2005). However, in experiments presented here, 2 out of 10 birds in groups G3 and G4 had symptoms of acute IBD, and had succumbed to the infection

It has been established recently that even in the presence of high titers of maternal antibodies, the recombinant vaccine (vHVT13) was efficiently protecting chicks against classical, very virulent and variant IBDv (Bublot et al., 2007, Perozo et al., 2009, Prandini et al., 2016). A few experiments with the rHVT-IBDv have been done in commercial broilers and a good antibody response to IBDv was obtained in the research work conducted in Italy (Le Gros et al., 2009), Slovenia (Zorman-Rojs et al., 2011) and Jhenaidha (Rashid et al., 2013) after a subcutaneous application and when the *in ovo* vaccine delivery system was used (Roh et al., 2016). It was also shown that the recombinant VAXXITEK HVT-IBDv vaccine provided a high maternal antibody titer in progeny from parents vaccinated with a single recombinant vaccine or if the rHVT-IBDv vaccine was combined with the inactivated vaccine, compared to a single inactivated vaccine. The protection of broiler chicks originating from parents vaccinated with the recombinant VAXXITEK HVT-IBDv which have been vaccinated with rHVT-IBDv *in ovo* was superior comparing to chicks originating from parents vaccinated with a single inactivated vaccine, even in the face of high levels of MDA (Lemiere et al., 2013). Authors concluded that the clinical protection of broilers under field conditions could be achieved after vaccination of parent flocks and their progeny with the rHVT-IBDv vaccines. Gelb et al., (2016) has shown that the recombinant VAXXITEK HVT-IBDv vaccines offer clinical protection of broiler chicks with MDA against challenge with homologous and heterologous IBDv strains. These birds were protected based on the incidence of microscopic lesions in the bursa even if interference with the active immune response was observed. Active protection in specific pathogen free chicks was achieved at 18 DPV, onwards in their experiments. However, it is still not known whether the application of the recombinant HVT-IBDv vaccine alone at one day of age, in the circumstances where vvIBDv persist on poultry farms, is sufficient for the clinical protection of birds**.**

In situations when broilers are not routinely vaccinated against MDV, the HVT type of vector vaccine may provide some convenience for the protection against both diseases or to minimize the immunosuppression caused by both viruses (Aly et al., 2012). Therefore, the successes of the application of recombinant vaccines under field conditions and the experience gained from various epidemiological situations will determine the application of genetically engineered vaccines for the present and future in Serbia.

In conclusion, the protective ability of the recombinant VAXXITEK HVT-IBDv vaccine against the challenge with vvIBDv (strain CH/99) was established in this experiment. However, in the future, a more detailed investigation should be performed using virus titration, immunohistochemistry detection of the IBDv antigen in lymphoid organs of chicks, serology testing and PCR in order to gain more experience with the recombinant HVT-IBDv vaccines. Good management practice on poultry farms has to

become imperative as much as vaccination, especially in developing countries, where vvIBDv still causes significant economical losses.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

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Research article
Ερευνητικό άρθρο

The effect of aminophylline on trans-diaphragmatic pressure in isoflurane anaesthetised dogs undergoing castration

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ABSTRACT. The trans-diaphragmatic pressure (P_{di})is the main indicator of diaphragmatic contractility and the influence of many different drugs on P_{di} has already been studied. The main aim of this study was to investigate the effect of aminophylline on P_{di} in anaesthetised dogs. Eighteen, healthy, client-owned, male dogs undergoing castration were recruited in the study. All the animals were premedicated with dexmedetomidine and morphine. Anesthesia was induced with propofol to effect and maintained with isoflurane in oxygen. Animals were randomly allocated into two groups. In the animals of the aminophylline group (group A), aminophylline was administered as an intravenous bolus at 4mg/kg after induction, whereas in the animals of the control group (group C), no aminophylline was given. When the surgical plane of anesthesia was achieved, two balloon catheters, one in the stomach and one in the mid-third of the oesophagus were inserted for P_{di} measurement. The two groups differed non-significantly with regards to P_{di} (p=0.182). The results of the study could be viewed as an indication that the aminophylline might increase diaphragmatic contractility if also proven in a larger population of animals'.

Keywords: diaphragm, trans-diaphragmatic pressure, oesophageal balloon catheters, dog

ΠΕΡΙΛΗΨΗ. Η δια-διαφραγματική πίεση (P_{di}) είναι ο κύριος δείκτης της συσπαστικότητας του διαφράγματος και η επίδραση αρκετών διαφορετικών φαρμάκων σε αυτήν έχει μελετηθεί. Ο κύριος στόχος αυτής της μελέτης είναι να μελετηθεί η επίδραση της αμινοφυλλίνης στη P_{di} σε σκύλους υπό αναισθησία. Σε αυτή την μελέτη συμπεριλήφθηκαν δεκαοχτώ, υγιείς, ιδιόκτητοι, αρσενικοί σκύλοι οι οποίοι υποβλήθηκαν σε ορχεκτομή. Η προαναισθητική αγωγή σε όλα

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τα ζώα ήταν η δεξμεδετομιδίνη και η μορφίνη. Η εγκατάσταση της αναισθησίας έγινε με προποφόλη και η διατήρηση με ισοφλουράνιο σε οξυγόνο. Τα ζώα κατανεμήθηκαν τυχαία σε δύο ομάδες. Στην ομάδα της αμινοφυλλίνης (ομάδα Α), η χορήγηση της αμινοφυλλίνης γινόταν ενδοφλέβια ως μια δόση στα 4 mg/kg αμέσως μετά την εγκατάσταση της αναισθησίας, ενώ στην ομάδα control (ομάδα C) δεν χορηγούνταν αμινοφυλλίνη. Όταν η χειρουργική αναισθησία επιτυγχανόταν, εισάγονταν δύο καθετήρες με μπαλόνι, ο ένας στο στόμαχο και ο άλλος στο μέσο τρίτο του οισοφάγου, για τη μέτρηση της P_{di}. Οι δύο ομάδες δε διέφεραν στατιστικά σημαντικά όσον αφορά τη P_{di} (p=0,182). Τα αποτελέσματα της μελέτης θα μπορούσαν να αποτελούν μια ένδειξη ότι η αμινοφυλλίνη ίσως αυξάνει την συσπαστικότητα του διαφράγματος εάν αποδειχθεί σε μεγαλύτερο πληθυσμό ζώων.

Λέξεις-κλειδιά: διάφραγμα, δια-διαφραγματική πίεση, οισοφαγικοί καθετήρες, σκύλος

INTRODUCTION

The diaphragm is the main inspiratory muscle and its normal function is of great importance for the anesthesiologist. Diaphragmatic insufficiency, especially diaphragmatic fatigue, has been associated with many respiratory diseases in humans and animals. Moreover, several pharmacological agents, such as anesthetic drugs (propofol, halothane, isoflurane) have been found to depress diaphragmatic contractility in experimental studies (Jagers et al.2009; Kochi et al. 1992; Nishina et al.2003; Zhang et al.2009). In veterinary clinical practice, the effect of four anesthetic drugs (isoflurane, fentanyl, propofol, ketamine) on diaphragmatic contractility in dogs has already been studied (Pavlidou et al. 2013).

Methylxanthines have several pharmacological actions of therapeutic interest. They relax smooth muscles, especially the bronchial muscles, and they stimulate the central nervous system (Jagers et al. 2009). They are also positive chronotropes and inotropes, as well as mild diuretics. In human medicine, theophylline is used to increase contractility of fatigued diaphragm in healthy patients and in patients with chronic obstructive pulmonary disease (Aubier 1985; Jagers et al. 2009; Sigrist et al. 1982). In small animal clinical practice, theophylline and aminophylline are used as bronchodilators and they have also been used experimentally to improve diaphragmatic contractility in both normal and fatigued diaphragm in dogs (Aubier et al. 1983b; Gayan-Ramirez et al. 1994; Sigrist et al.1982).

Aminophylline is a methylxanthine, which has been widely studied in human and veterinary clinical practice. In one study it has been shown that aminophylline may increase trans-diaphragmatic pressure (P_{di}) in a fatigued human diaphragm (Aubier et al. 1981), while another study has failed to support this finding (Levy et al. 1990). Moreover, theophylline seems to improve exercise capability in patients with chronic obstructive pulmonary disease (Murciano et al.1984). *In vivo* studies in anaesthetised laboratory animals and *in vitro* studies in skeletal muscle fibers support the significant positive effect of methylxanthines on diaphragmatic contractility (Aubier 1985; Jagers et al.2009; Levy et al.1990; Wanke et al.1994). It has been shown that in anesthetized dogs, inspiratory muscle contractility was improved in a dose-dependent manner after the administration of aminophylline in a paralyzed diaphragm (Sigrist et al.1982).

Trans-diaphragmatic pressure (P_{di}) is a very good indicator of diaphragmatic contractility in clinical practice (Laporta and Grassino 1985). The measurement of P_{di} during maximum inspiratory effort as an indicator of diaphragmatic strength helps in the assessment of patients with respiratory muscle weakness (Hillman et al. 1990; Zakynthinos and Roussos 2005). Trans-diaphragmatic pressure is the difference between intra-abdominal (P_{abd}) and intra-pleural (P_{pl}) pressure (Hubmayr et al.1990). However, the measurement of Pa_{bd} and P_{pl} is difficult under clinical conditions and therefore, various methods have been proposed for the measurement of P_{di}. Thus, Pa_{bd} and P_{pl} are measured in the stomach (Pg_{ast}) and in the oesophagus (Po_{es}), respectively (Benditt 2005). In an attempt to obtain the maximum deflections of

P_{gast}, P_{oes}, and P_{di} during a respiratory cycle, patients are allowed to breath while the upper airway is fully occluded in a clinical setting. As a result, during inspiration the maximum increase in Pgast, and the maximum decrease in P_{pl} (detected as maximum decrease in P_{oes}) will be observed, and the maximum deflection of P_{di} could be calculated. Maximum deflections of P_{gast}, P_{oes}, and P_{di} during a respiratory cycle are obtained with a special technique, which is called the Mueller's maneuver. In human medicine, the Mueller's maneuver is an attempt of inspiration with closed mouth and nose (or the glottis). A modified technique for the application of Mueller's maneuver has already been described in dogs under anesthesia in clinical conditions (Pavlidou et al. 2014).

To our knowledge, there is no published study in dogs on the effect of methylxanthines on a non-fatigued diaphragm. The aim of this study was to investigate the effect of aminophylline on P_{di} in isoflurane anesthetized male dogs. Our hypothesis was that the administration of aminophylline during anaesthesia might increase diaphragmatic contractility in dogs.

MATERIALS AND METHODS

For this prospective, randomized, non-blinded clinical study, approval from the Ethics Committee of Aristotle University of Thessaloniki was obtained. Each owner was informed in detail about the study protocol and a signed written consent was taken. The study population was animals admitted to the Companion Animal Clinic of Aristotle University of Thessaloniki for castration. Exclusion criteria were status ASA 3 or higher, any active respiratory disease, a history of a chronic respiratory problem, and anticipated necessity to apply intermittent positive pressure ventilation (IPPV) during surgery.

All the animals underwent only castration without any other abdominal/thoracic surgical procedure so as to avoid any effects of the atmospheric pressure on the open cavities and of the surgical manipulations on the P_{oes} and P_{gast}. Castration was performed in left or right lateral recumbency, in an attempt to minimize the effect of body position on the P_{oes} and P_{gast}. Obese animals were excluded from the study since it has been shown that obesity may decrease diaphragmatic contractility in humans (Ora et al. 2011). The evaluation of the obesity was based on the body condition scoring system (1-5). Obese animals were scored as 4 or 5. Furthermore, it has been shown that in very small or large breed dogs, the introduction and proper placement of the balloon catheter into the stomach is difficult and the P_{gast} measurement is inaccurate (Pavlidou et al. 2014). Therefore, medium size dogs were used in this study.

The animals were hospitalized in the Clinic for at least one day before surgery. Pre-anesthetic evaluation included physical examination, complete blood count, serum biochemistry (determinations of albumin, urea, creatinine, potassium and glucose concentrations, alkaline phosphatase/ALP and alanine transaminase/ALT activities) and thoracic /abdominal radiographs. The animals were fasted for eight hours with dry food and they had free access to water for up to two hours before premedication.

Animal preparation

Eighteen, healthy, male, client-owned dogs, 1-10 (1.7±1.3) years (mean ± standard deviation) old and 5-30 (15.5 ± 6.5) kg body weight were enrolled. All animals were premedicated with dexmedetomidine (Dexdomitor, Pfizer Hellas, Athens, Greece) at 175μg/m2 and morphine (Morphine sulfate, Famar SA, Athens, Greece) at 0.1 mg/kg intramuscularly. Twenty minutes later the cephalic vein was catheterized and the administration of Lactated Ringer's solution (LR's, Vioser, Trikala, Greece) at 10 ml/kg/h intravenously (IV) commenced. Anesthesia was induced with propofol (Propofol MCT/LCT, Fresenius, Fresenius Kabi Greece) to effect. In particular, an initial dose of 2 mg/kg was given IV and then incremental doses of 1 mg/kg were injected until endotracheal intubation could easily be performed. Anesthesia was maintained with isoflurane (Isoflurane, Bayer, Leverkusen, Germany) in oxygen. All the animals were breathing spontaneously. Fresh gas flow was delivered at 1.5 L/min through a semiclosed circle rebreathing system. Carprofen (Rimadyl, Pfizer Hellas, Athens, Greece) was administered just after intubation at 2 mg/kg IV. The animals were randomly allocated into two groups. In the animals of group A, aminophylline (Aminophylline,

Demo, Athens, Greece) was administered as an intravenous bolus at 4 mg/kg just after induction, whereas in the animals of group C, no aminophylline was given.

Heart rate (HR), respiratory rate (RR), mean arterial blood pressure (MAP) (measured non-invasively using oscillometry with the cuff placed around the forelimb), end-tidal carbon dioxide partial pressure ($PE'CO_2$) and end-tidal isoflurane fraction (FE'iso) were constantly monitored (Datex-Ohmeda S/5, GE Healthcare, Helsinki, Finland) and recorded every 5 minutes. In case of a $PE'CO_2$ higher than 8.5 kPa, artificial ventilation was applied (McDonell and Kerr 2007) and the measurement of P_{di} was cancelled. During surgery, all the animals were placed in lateral recumbency, left or right.

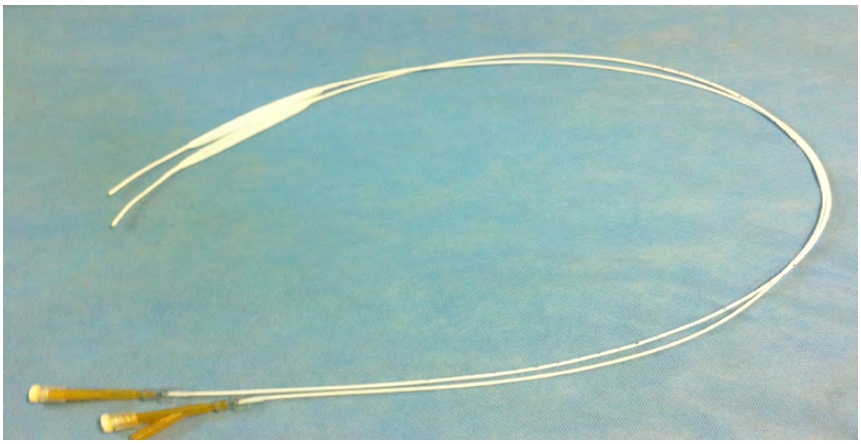
Clinical signs along with electronic monitoring readings were used in order to assess the adequacy of anesthetic depth. Such signs were lack of reflexes, presence of adequate muscle relaxation, and a lack of physiological response to surgical stimulation characterized by less than 10 % change in HR, RR and MAP during surgical stimulation.

Trans-diaphragmatic pressure measurement

When a surgical plane of anesthesia was achieved, two 90 cm long oesophageal balloon catheters (Esophageal Balloon Catheter Set, CooperSurgical Company, Trumbull, USA) (Figure 1) with guide wires were used for the measurements of P_{oes} and P_{gast}. The balloon of the first catheter was introduced into the stomach for the measurement of P_{gast} and the distal end of the second catheter was positioned in the mid-third of the oesophagus for the measurement of P_{oes}. The correct positioning of the two catheters was confirmed by the observation of the respective pressure tracings on the computer screen: a positive deflection (increase in pressure) during each inspiration was an indication of the correct intra-gastric position of the balloon of the catheter, whereas a negative deflection (decrease in pressure) during each inspiration, confirmed placement of the balloon of the second catheter into the oesophagus. The proximal end of each catheter was connected to a pressure-transducer and then to a recording device (Pressure Monitoring system Buzzer-II, Michael Roehrich, Austria). The catheters were secured in place by fixing them with an adhesive tape to the endotracheal tube. Following the removal of the guide wires, the balloons were inflated with 0.5-1 ml of air. All the pressure measurements were saved with a sampling rate of 10 Hz.

For the measurement of P_{di}, it was necessary to obtain the maximum deflections of Pgast (ΔP_{gast}) and P_{oes} (ΔP_{oes}) during a respiratory cycle. To achieve this, the endotracheal tube was disconnected from the anesthetic circuit and tightly closed with a thumb after the end of expiration, so that the animal was forced to breathe against a completely closed airway (Laporta and Grassino 1985; Pavlidou et al. 2014) (modified Mueller's maneuver). P_{gast} and P_{oes} were assessed 3 times during a period of 60 minutes independently of the duration of the surgery. In order to calculate P_{di}, the Mueller's maneuver of a single obstruction was performed three separate times on each animal, with a 30-minute interval between each measurement (one just after the anesthetic induction, one just after the start of the surgery and one at the end of the 60 minutes period). The balloon catheters were removed after the end of the 60-minute period independently of the duration of surgery.

Fig 1. Oesophageal balloon catheter



All the data from the recording device were saved in a spread sheet. Then, they were analyzed with a signal analysis software (Qtiplot, MicroCal, Northampton,

Massachusetts, USA). From the data for each pressure (gastric or oesophageal), a positive curve for gastric pressure and a negative curve for oesophageal pressure respectively, were drawn. The baseline of gastric and oesophageal curves was zeroed. In every set of measurements, the P_{di} value was calculated (difference between P_{gast} and P_{oes}). The area under the curve (AUC) for each variable was calculated, using the trapezoid method (Matthews et al. 1990). The AUCs were then standardized by the duration of measurements (60 min).

Statistical analysis was performed with a statistical software (SPSS 19, IBM company, Illinois, USA). AUCst from all variables were evaluated for normality using the Shapiro-Wilk test, and for difference of means with the t-test. $p< 0.05$ was considered to be statistically significant.

RESULTS

The two groups were homogenous regarding the age (p=0.450) and the weight (p=0.864) of the animals. The mean values of P_{di} as well as of all measured hemodynamic and respiratory parameters are shown in Table 1.

The mean±standard deviation of AUC of P_{di} was 14.42±4.87 mmHg (1.87±0.63 kPa) in group A and 11.62±3.54 mmHg (1.51±0.46 kPa) in group C, with the difference being statistically non-significant (p=0.182). Moreover, the mean±standard deviation of AUC of $PE'CO_2$ was higher in group A (6.8 ± 0.8 kPa) than in group C (6.5±0.3 kPa) without a statistically significant difference (p=0.316). Regarding AUC of FE'iso, it was not significantly (p=0.181) different in group A (1.4±0.2 %) than in group C (1.6±0.2 %).

DISCUSSION

The present clinical study was designed to investigate the effect of aminophylline on diaphragmatic contractility in dogs under anesthesia. The evaluation of diaphragmatic contractility was based on the modified technique for P_{di} measurement with balloon catheters (Pavlidou et al. 2014). P_{di} value in control group was 11.62±3.54 mmHg and this finding is in accordance with the P_{di} reference published values in our previous study (Pavlidou et al. 2013). Regarding the anesthetic protocol, it was the same with the protocol (premedication, induction, maintenance) that has been used in the other two published clinical studies about P_{di} (Pavlidou et al. 2013, Pavlidou et al. 2014). This anesthetic protocol seems to have a weak effect on diaphragmatic contractility.

In both human and veterinary medicine, the main indication for use of methylxanthines is their bronchodilator action, especially in patients with respiratory and cardiovascular problems (Aubier et al. 1981; Plumb 2002). Aminophylline enhances diaphragmatic contractility and increases P_{di} in experimental studies (Aubier 1981; Aubier et al. 1983b; Aubier

Table 1. Mean ± standard deviation (SD) and p values of P_{di}, haemodynamic and respiratory variables in the two groups (group A, aminophylline-group C, control).

Variable	Statistic	Group	
		A	C
P_{di} (mmHg) (p=0.182)	N	9	9
	Mean	14.4	11.6
	SD	4.8	3.5
HR (min^{-1}) (p=0.719)	N	9	9
	Mean	82.0	83.6
	SD	8	10.5
RR (min^{-1}) (p=0.956)	N	9	9
	Mean	9.7	9.6
	SD	5.2	5.3
MAP (mmHg) (p=0.124)	N	9	9
	Mean	88.6	75.7
	SD	15.3	9.8
$PE'CO_2$ (kPa) (p=0.316)	N	9	9
	Mean	6.8	6.5
	SD	0.8	0.3
FE'iso (%) (p=0.181)	N	7	8
	Mean	1.4	1.6
	SD	0.2	0.2

1985; Jagers et al. 2009). The exact mechanism by which aminophylline improves diaphragmatic contractility is not yet known. It has been suggested that methylxanthines (theophylline and aminophylline) induce their therapeutic effects by inhibiting phosphodiesterase, which catalyzes the hydrolysis of cyclic AMP (Mazza 1982). However, this hypothesis was questioned and other theories, like prostaglandin inhibition and alteration of calcium movement across the cell membrane, have been proposed. The transfer of intracellular calcium may be responsible for the direct action of aminophylline on skeletal muscles (Aubier et al. 1981; Aubier et al. 1983b; Aubier 1985). Another possible mechanism of action is the elongation of the diaphragmatic fibers. Methylxanthines have been shown to strengthen and elongate the fibers of skeletal muscles (especially of the diaphragm) after the electrical stimulation of these muscles (Aubier et al. 1983b).

In an experimental study, various ventilatory parameters, P_{di} and electromyographic activity in awake canines were measured. It was found that aminophylline, in the usual dose rate for humans (a loading dose of 7 mg/kg followed by a continuous rate infusion at 1.5 mg/kg/h), increases ventilation and contractility of the respiratory muscles (Jagers et al. 2009).

In humans, methylxanthines have been used in many clinical trials and mainly in patients with respiratory disease (Aubier et al. 1981; Eldridge et al. 1983; Levy et al. 1990; Moxham et al. 1985; Murciano et al. 1984; Murciano et al. 1989; Sigrist et al. 1982). Most of the trials support the inotropic effect of aminophylline on respiratory muscles and especially on diaphragm (Aubier et al. 1981). Additionally, different doses of aminophylline have also been used in humans in order to investigate its effect on diaphragmatic contractility and P_{di} (Levy et al. 1990).

In dogs, the therapeutic dose of aminophylline is 6-11 mg/kg per os, intramuscularly, or intravenously (Plumb 2002). Methylxanthines have a low therapeutic index and therefore, the dosage should be calculated carefully. The main side effects of methylxanthines in dogs and cats are vomiting, nausea, insomnia, diarrhea, polyphagia, polyuria and polydipsia. Moreover, aminophylline can affect the cardiovascular system causing tachycardia, hypotension, arrhythmias and acute respiratory failure, especially when it is injected intravenously and in non-anesthetized patients (Plumb 2002). A dose of 4 mg/kg as an intravenous bolus of aminophylline was used in this clinical study. This is a low dose but it was chosen so that any adverse effects of aminophylline would be eliminated, as this was a clinical investigation. Moreover, the clinical study was not fully blinded so as the anesthetist was aware of the chosen group in order to recognize and treat any side effects of aminophylline.

In this study, P_{di} was higher in the aminophylline group(14.4 mmHg) than in the control group(11.6 mmHg), although the difference was statistically non-significant ($p=0.182$, achieved power, i.e. 1-β error probability = 0.378). If there is no true effect of aminophylline in P_{di}, our results suggest that there is an 18.2% probability of this difference not being a random effect. Although there is a high probability of error if we accept a true effect of aminophylline, this may be of clinical importance, especially in compromised animals. Moreover, there was also a statistically non-significant difference between the two groups with regards to $PE'CO_2$($p=0.316$), although $PE'CO_2$ was higher in group A than in group C. In animal studies, it has been shown that minute ventilation increased with increasing the concentration of aminophylline in plasma in conscious dogs. This phenomenon has been explained by the direct effect of the drug on the respiratory centers or by increasing the sensitivity of these centers to the carbon dioxide or hypoxia (Aubier et al. 1983a).

Aminophylline can cause tachycardia and hypotension via positive inotropic and vasodilatory actions (Gayan-Ramirez et al. 1994; Rutherford et al. 1981). However, in the present clinical study, the mean arterial pressure was higher in the aminophylline group (A) than in the control group (C), and the heart rate was higher in group C than in group A, although both differences were statistically non-significant. Hypotension, as an adverse effect of aminophylline administration may not have been caused in this clinical study, because of the low dose administered (4 mg/kg), as hypotension was observed when ami-

nophylline was administered at higher doses (over 7 mg/kg) (Mazza 1982; Pearl et al. 1984).

CONCLUSIONS

Although our sample size and the used dose rate of aminophylline were small, there seems to be a tendency of aminophylline to increase diaphragmatic contractility, in dogs under general anesthesia. The clinical importance of this finding has to be evaluated further in a larger number of clinical cases. Moreover, it would be of interest to investigate the effect of aminophylline administration on diminishing or even cancelling the depressant effects of other anesthetic or analgesic drugs on P_{di} intra-operatively especially in patients with respiratory disease.

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Research article
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Analysis of the *PRNP* gene polymorphisms in healthy Greek sheep during 2012 - 2016

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ABSTRACT. Scrapie is a slowly progressive infectious disease of sheep and goats that causes degeneration of the central nervous system. Scrapie is one of several transmissible spongiform encephalopathies (TSEs), like the bovine spongiform encephalopathy (BSE). In sheep, polymorphisms at codons 136, 154 and 171 of the host gene PRPN that encodes the PrP protein, are known to be closely linked to susceptibility or resistance to natural and experimental classical scrapie. In many countries, but not in Greece, breeding programs have been implemented to increase genetic resistance. This study was supported mainly by the private initiatives of farmers willing to improve their flocks by increasing the resistance to scrapie. Thus, the PrP genotypes (of the three mentioned codons) from 5815 blood samples of clinically healthy rams from 160 healthy flocks during the period 2012 – 2016 were determined. Additionally, 1399 blood samples were genotyped only for the 171 codon. Samples were analyzed by Real Time PCR (TaqMan probes) with specific labeled probes. Our results showed an increased percentage of the two genotypes, ARR/ARR and ARR/ARQ linked with resistance to the disease (27.29% and 34.6%, respectively) and relatively reduced percentage of the genotype ARQ/ARQ (24.23%) which is associated with susceptibility to disease and is the most common genotype in the Greek flocks. This joined effort has resulted in the establishment of an important number of farms with an increased population of genetically resistant rams to classical scrapie.

Keywords: classical scrapie resistance/susceptibility, PrP genotypes, breeding programme

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ΠΕΡΙΛΗΨΗ. Η τρομώδης νόσος (scrapie) αποτελεί μια βραδέως εξελισσόμενη λοιμώδης νόσος των προβάτων και των αιγών, η οποία οδηγεί σε εκφυλισμό του κεντρικού νευρικού συστήματος. Η scrapie είναι μια μορφή των μεταδιδόμενων σπογγωδών εγκεφαλοπαθειών (TSEs) όπως και η σπογγώδη εγκεφαλοπάθεια των βοειδών (BSE). Στα πρόβατα είναι γνωστό ότι οι πολυμορφισμοί στα κωδικόνια 136, 154 και 171 του γονιδίου PRPN που κωδικοποιεί τη πρωτεΐνη PrP σχετίζονται άμεσα με την ευαισθησία/ανθεκτικότητα της φυσικής και πειραματικής κλασικής scrapie. Σε πολλές χώρες, αλλά όχι στην Ελλάδα έχουν εφαρμοστεί προγράμματα με στόχο την αύξηση της ανθεκτικότητας στη νόσο. Η παρούσα εργασία στηρίχτηκε κυρίως στην ιδιωτική πρωτοβουλία παραγωγών που ήταν πρόθυμοι να βελτιώσουν την εκτροφή τους αυξάνοντας την ανθεκτικότητα στην τρομώδη νόσο. Έτσι μελετήθηκαν οι γονότυποι του γονιδίου PrP (των τριών προαναφερθέντων κωδικονίων) σε 5815 δείγματα αίματος από υγιή κριάρια προερχόμενα από 160 υγιείς εκτροφές, κατά τη χρονική περίοδο 2012-2016. Επιπρόσθετα σε 1399 δείγματα αίματος αναλύθηκαν μόνο οι πολυμορφισμοί του κωδικονίου 171. Οι γονοτυπικές αναλύσεις πραγματοποιήθηκαν με τη χρήση της Real Time PCR (TaqMan ανιχνευτές) χρησιμοποιώντας ειδικά σημασμένους ανιχνευτές. Τα αποτελέσματά μας παρουσιάζουν αυξημένα ποσοστά των δύο γονοτύπων ARR/ARR και ARR/ARQ, οι οποίοι συνδέονται με ανθεκτικότητα έναντι της νόσου (27.29% και 34.6%, αντίστοιχα) και σχετικά μειωμένο το ποσοστό του γονότυπου ARQ/ARQ (24.23%) που συνδέεται με ευαισθησία έναντι της νόσου και ο οποίος είναι ο πιο κοινός γονότυπος στις Ελληνικές εκτροφές.
Αυτή η κοινή προσπάθεια οδήγησε στην καθιέρωση ενός σημαντικού αριθμού εκτροφών που διαθέτουν αυξημένο πληθυσμό κριαριών γενετικά ανθεκτικών στην κλασική scrapie.

ΛΕΞΕΙΣ ΕΥΡΕΤΗΡΙΑΣΗΣ: ευαισθησία/ανθεκτικότητα scrapie, PrP γονότυποι, πρόγραμμα γενετικής βελτίωσης

INTRODUCTION

Scrapie is a slowly progressive infectious disease of sheep and goats that causes degeneration of the central nervous system. The main constituent of the infectious agent is an aberrant isoform (PrP^{Sc}) of the normal cellular (PrPC) prion protein (PrP), which is a cell–surface glycoprotein [19]. It has been showed that the PrP abnormal prion protein form consists of an approximately 40% of beta sheet folding that transforms it to protease resistant and infectious (Prusiner, 1991). The disease is fatal after a long incubation period and continues to exist within a herd by spread between herdmates or by transmission from ewe to lamb (Goldmann et al., 1990). The transmission as well as the incubation period of the disease depends on the exposure to the infectious agent, the scrapie strain and the genetic background of the host (O'Rourke et al., 1997). Prion protein of sheep is a protein of 256 amino acids. More than 15 polymorphisms of the PRPN gene that encodes PrP of sheep have been reported (DeSilva et al., 2003) but only the polymorphisms at codons 136, 154 and 171 are known to be closely linked to susceptibility to natural and experimental classical scrapie (Bossers et al., 1996, Hunter et al., 1996, Dawson et al., 1998, Elsen et al., 1999, Thorgeirsdottir et al., 1999, Tranulis et al., 1999). The polymorphisms of the codon 136 (Alanine/Valine/Threonine; A/V/T), codon 154 (Arginine/Histidine; R/H) and codon 171 (Arginine/Glutamine/Histidine/Lysine; R/Q/H/K) have been analyzed in many studies (Goldmann et al., 1994, Hunter et al., 1994, Glouscard et al., 1995). It has been shown that the VRQ haplotype is strictly associated with susceptibility in homozygosis and heterozygosis (Belt et al., 1995, Hunter et al., 1996), while the ARR/ARR genotype is correlated with resistance to scrapie (Goldmann et al., 1994, Hunter et al., 1994, Baylis et al., 2002). This information is used in European countries for the implementation of national breeding programmes to reduce the existed susceptibility to scrapie (Arnold et al., 2002). The haplotype AHQ may be associated with increased resistance and incubation time in some breeds (Hunter et al., 1996, Dawson et al., 1998, Elsen et al., 1999, Thorgeirsdottir et al., 1999, O' Doherty et al., 2002), while it is associated with high susceptibility in purebred and crossbred German Merinoland sheep (Lunken et al., 2004) as well

as in a Romanov flock (Diaz-Avalos et al., 2005). In Greece, Histidine in codon 154 has been found at a significantly high frequency in the Chios crossbred scrapie-affected sheep, suggesting that probably: (a) this allele is associated with increased susceptibility, at least in Chios breed, (b) there is a local scrapie strain strongly correlated with Histidine in codon 154 or (c) there is a combination of the allele susceptibility and the scrapie strain tropism (Ekateriniadou et al., 2007a). The ARQ haplotype's susceptibility varies between sheep breeds, while the ARH and TRQ haplotypes seem to be rather neutral (Dawson et al., 1998, Billinis et al., 2004). Little is known about haplotype ARK and it has not been associated with scrapie resistance or susceptibility (Acutis et al., 2004, Alexander et al., 2005).

In Greece, where the sheep population stands at about 9.5 million, not much is known about PrP alleles' distribution in healthy crossbred sheep. Scrapie was firstly diagnosed in 1986 (Leontides et al., 2000), moreover Billinis et al., (2004) have found some polymorphisms in healthy and scrapie-affected sheep. Ekateriniadou et al., (2007b) have described the alleles and genotype frequencies of healthy sheep from 13 rare breeds as well as from healthy and scrapie-affected sheep in the period 2003-2005 where the ARQ/ARQ genotype was predominated in all three sheep groups tested (Ekateriniadou et al., 2007a). Greece is the EU-country with the second highest incidence of positive scrapie cases after Cyprus (EU- Health and Food Safety 2014). In many countries as mentioned above, but not in Greece, breeding programs have been officially implemented to increase genetic resistance. The purpose of the current study was to determine the PrP genotypes (codons 136, 154 and 171) from blood samples of clinically healthy rams from 160 healthy flocks during the period 2012-2016. The study was based on the private initiatives of farmers and both freelancers and state veterinarians for the establishment of an important number of farms with an increased population of resistant rams.

MATERIALS AND METHODS

Samples

In total 7214 sheep have been analyzed. The samples originated from 160 Greek healthy flocks of scrapie positive and negative areas. The majority of these flocks (105) originated from Northern Greece, 45 from Central and Western Greece and 10 from Southern Greece. The most farmers sent for genotyping all their males. A lot of these flocks were sending samples each year for genotyping their newborns.

The 5815 samples were fully analyzed by identifying the genotypes of the three amino acids (aa) 171, 136 and 154, while in the 1399 samples only the 171aa was genotyped. These 1399 samples were the newborns from some of these flocks (40) mentioned above that had sent samples more than once and adopted breeding schemes having as a target to increase the genetic resistance to scrapie by keeping only the most resistant to scrapie animals.

Genomic DNA extraction

Genomic DNA was extracted from the blood samples using the PureLink Genomic DNA kit (Life Science) according to the protocol for blood lysate.

Table 1. Taqman probes used for the detection of SNPs at codons 136, 154 and 171 by Real-Time PCR.

Target aa	Sequence (5′-3′)	5′-Label	3′-Label
A136	CTCATGGCACTTCCCA	6FAM	BHQ1
V136	CTGCTCATGACACTTCCCAG	HEX	BHQ1
R154	CCGTTACTATCGTGAAAACATGTAC	ROX	BHQ2
H154	CCGTTACTATCATGAAAACATGTACC	Cy5	BHQ2
R171	CCAGTGGATCGGTATAGTAACCA	6FAM	BHQ1
H171	AGACCAGTGGATCATTATAGTAACCA	HEX	BHQ1
Q171	CCAGTGGATCAGTATAGTAACCAGA	ROX	BHQ2
K171	ACCAGTGGATAAGTATAGTAACCAGA	Cy5	BHQ2

PrP genotype analysis

A Real Time PCR method was used to detect the eight polymorphisms: 171Q/R/H/K, 136A/V and 154R/H. The analyses were performed by two tetraplex Real-Time PCR reactions. The first one was for the detection of the four polymorphisms of the 171aa and the second one for the polymorphisms of the 136aa and 154aa. In both tetraplex reactions a DNA fragment of 180bp was amplified using the primers: ScF-5'-GCC TTG GTG GCT ACA TG-3' and ScR-5'- CTG TGA TGT TGA CAC AGT CAT-3'. The sequences of the labelled probes used for the detection are shown at Table 1. Amplification reaction mixtures were prepared at a final volume of 15 μl containing 1X KAPA PROBE FAST qPCR kit Master Mix Universal, 400nM of each primer and probes and 40-50 ng of sample's DNA. qPCRs were performed in a Chromo4™ Real-Time Detector. The cycling conditions for all the reactions consisted of the initial denaturation at 95oC for 3min, and 45 cycles of denaturation at 95oC for 3sec and annealing/extension at 62oC for 30sec. The method has been validated by applying it in samples that the PrP gene has been sequenced and also in control samples sent by the European Reference Lab (AHVLA). In case of double heterozygosis, the genotypes order e.g. ARR/AHQ and not AHR/ARQ, has been determined following the *PRPN* genotypes' order that has been recorded through the relevant literature.

Statistical analysis

The results were analysed statistically using the Chi square test to compare the frequencies of the Types 1, 2 and 3 of the EU Scrapie Plan during 2012-2016.

RESULTS

PrP allelic variants

The allelic variants in codons 136 (A/V) and 154 (R/H) were genotyped in 5815 healthy rams. In codon 136, Alanine was detected with a frequency of 99.61% and Valine with a frequency of 0.39%, having the A/A homozygotes predominated (99.3%) over the V/V homozygotes (0.03%), appeared in only 2 samples. As far as codon 154 is concerned, Arginine was the predominant amino acid (96.1%) with H/R heterozygotes found with a frequency of 7.2%.

Table 2. Frequency of the PrP alleles in codons 136, 154 (n=5815) and 171 (*n= 7214) detected in healthy crossbred sheep

PrP Codon	Allelic variant (aa)	Frequency(%)
136	A	99.61
	V	0.39
154	R	96.1
	H	3.9
171*	Q	50
	R	47.26
	H	2
	K	0.74

The tetramorphism Q/R/H/K in codon 171 was genotyped in 7214 healthy males and Glutamine was the predominant amino acid detected at the 50% of the samples. Arginine was detected in a high frequency (47.26%) not only in heterozygotes but also in homozygosis (26.84%), while Histidine and Lysine were detected in the very low frequencies of 2 and 0.74%, respectively. The frequency distribution of the alleles is shown in Table 2.

PrP haplotype variants

In the 5815 analysed samples, 9 known haplotypes have been appeared. The most frequent haplotype was ARR with a frequency of 47.78%. The ARQ haplotype has been found in a very high frequency (44.93%), while the AHQ and ARH haplotypes were detected with a frequency of 3.86% and 2.24%, respectively.

Table 3. Frequency distribution of the PrP haplotype variants detected in n=5815 healthy crossbred sheep.

Prp haplotypes	Frequency (%)
ARR	47.78
ARQ	44.93
AHQ	3.86
ARH	2.24
ARK	0.75
AHR	0.02
AHH	0.03
VRQ	0.36
VRH	0.03

The 5 rest haplotypes (ARK, AHR, AHH, VRQ and VRH) were the less frequent detected in very low frequencies varying from 0.75 to 0.02%. The frequency distribution of the PrP haplotypes is shown in Table 3.

PrP genotypes

The 9 different haplotypes resulted in 23 genotypes of the 5815 analysed samples. These genotypes were categorized into 5 Types according to the level of resistance/susceptibility to scrapie as defined by the EU Scrapie Plan. Type 1 is comprised only by the ARR/ARR genotype and is the most resistant to scrapie. Type 2 is comprised by the genotypes ARR/ARQ, ARR/AHQ, ARR/ARH, ARR/ARK and AHR/AHQ presenting high level of resistance to scrapie, while Type 3 is represented by the ARQ/ARQ, ARQ/AHQ, ARQ/ARH, ARQ/ARK, AHQ/ AHQ, ARH/AHQ, ARK/ARK, ARH/ARK, ARH/ARH, AHQ/ARH and AHQ/ARK genotypes presenting no resistance to scrapie. Finally, Type 4 is represented by the genotypes ARR/VRQ and AHQ/VRQ and Type 5 by the ARQ/VRQ, ARQ/VRH, ARK/VRQ and VRQ/VRQ genotypes which are the most susceptible to scrapie and completely unsuitable for breeding schemes.

Table 4. Classification and frequency the 23 PrP genotypes detected in 5815 health crossbred sheep based on the EU Scrapie plan.

Type	Degree of resistance	Genotype	Frequency (%)
1	The most resistant	ARR/ARR	27.29
2	High level of resistance to scrapie	ARR/ARQ	34.6
2		ARR/AHQ	3.37
2		ARR/ARH	2.05
2		ARR/ARK	0.69
2		AHR/AHQ	0.03
3	Susceptible / Little resistance to scrapie	ARQ/ARQ	24.23
3		ARQ/AHQ	3.59
3		ARQ/ARH	2.15
3		ARQ/ARK	0.67
3		AHQ/ AHQ	0.29
3		ARH/AHQ	0.05
3		ARK/ARK	0.02
3		ARH/ARK	0.05
3		ARH/ARH	0.09
3		AHQ/ARH	0.07
3		AHQ/ARK	0.03
4	Susceptible to scrapie not to be used for breeding	ARR/VRQ	0.29
4		AHQ/VRQ	0.03
5	Highly susceptible to scrapie, not to be used for breeding	ARQ/VRH	0.05
5		ARQ/VRQ	0.31
5		ARK/VRQ	0.02
5		VRQ/VRQ	0.03

The most frequent genotype was ARR/ARQ (34.6%) which is related with a high level of resistance to scrapie. The ARR/ARR genotype that is considered as the most resistant to scrapie was present in a frequency of 27.29%. The ARR/- genotypes associated with resistance to the disease had a total frequency of 68.29% while the susceptible to scrapie ARR/VRQ, ARQ/VRQ and VRQ/VRQ genotypes were found in a frequency of 0.29%, 0.31% and 0.03%, respectively. ARQ/-AHQ/- and ARH/- genotypes as well as VRQ/- genotypes (the three mentioned above genotypes plus the ARK/VRQ and AHQ/VRQ genotypes) that are associated with high susceptibility to scrapie, were found in a frequency of 31.05%, 7.41%, 4.41% and 0.68%, respectively. Table 4 lists the genotypes and their frequencies.

The analysis performed showed a significant increase ($p<0.01$) in Type 1 (from 14.53% to 22.67%) and Type 2 (from 26.5% to 41.28%) genotypes along with a significant decrease ($p<0.01$) in Type 3 (from 58% to 35.11%) genotypes during the years 2012-2016. The Type 4 and Type 5 genotypes' frequencies were too low constantly with no specific trend (Figure 1).

In the genotype analysis of the 171 codon in the 1399 samples, a significant increase ($p<0.01$) of Arginine frequency was also observed both in homozygosis and heterozygosis from 14.88 to 33.77% and from 28.84 to 47.49%, respectively, while a significant decrease ($p<0.01$) was observed in the frequency of Glutamine both in homozygosis and heterozygosis from 53.95 to 17.28% and from 2.33 to 1.46%.

DISCUSSION

Scrapie of small ruminants remains a problem in Greece since no national breeding programme has been implemented. The aim of the present study was

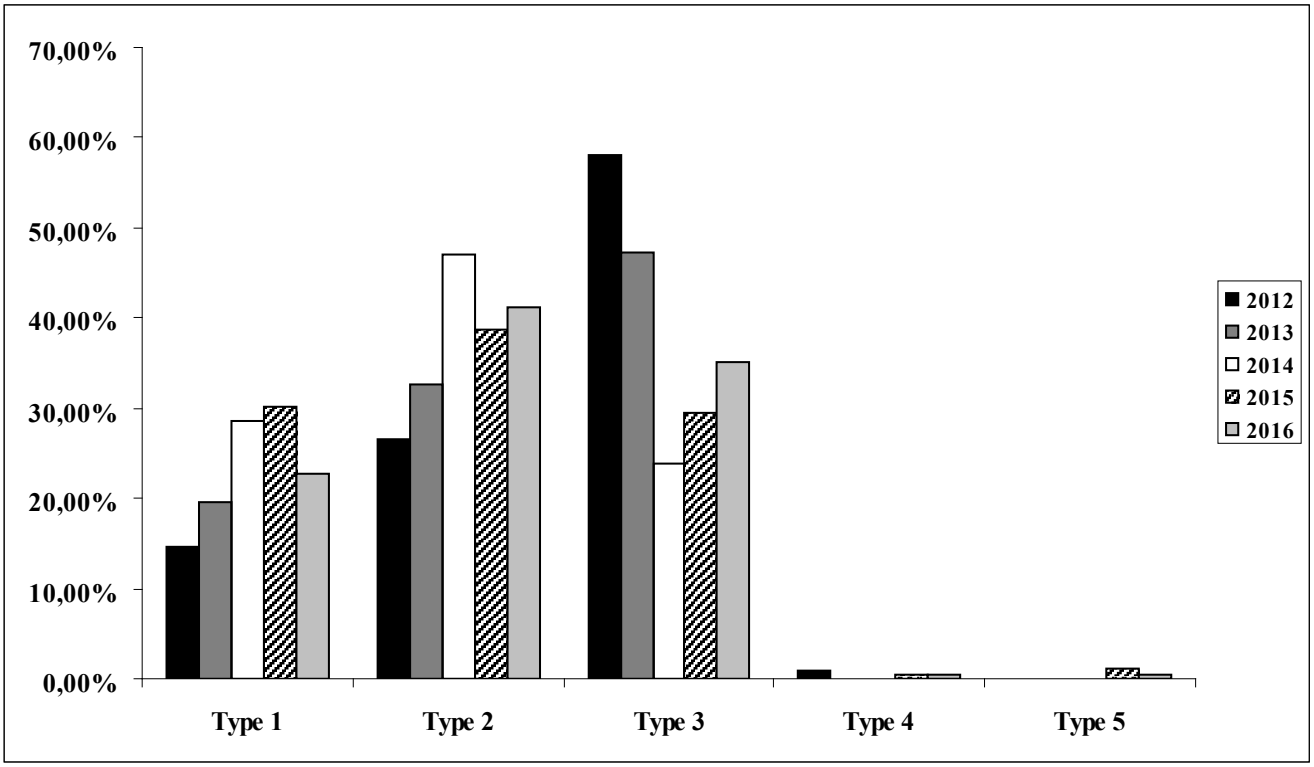


Figure 1. Distribution of scrapie resistant/susceptible Types based on the EU Scrapie Plan classification, detected during the time period 2012-2016

to determine the genotypes of the three codons of the PrP protein linked to resistance/susceptibility to scrapie in healthy Greek sheep flocks.

The results showed the frequencies of the alleles, the haplotypes, as well as those of the genotypes, for the PrP codons 136 and 154 in 5815 samples and for the codon 171 in 7521 samples. The analyses showed not only the existence of the most frequent haplotypes ARR, ARQ, AHQ, ARH, VRQ (Elsen et al., 1999, Thorgeirsdottir et al., 1999, O'Doherty et al., 2002, Billinis et al., 2004) but also the ARK haplotype, a rare haplotype that has been detected in Italian Bielese breed with a frequency of 2.5%; (Acutis et al., 2004), Mongolian Khallkh and Sartuul with a frequency 0.6%; (Gombojav et al., 2003), in one Spanish animal (Acin et al., 2004), in Oklahoma at 0.35%, in Kivircik sheep breed in Turkey at 0.35% (Oner et al., 2011) as well as in Greek purebred and crossbred animals at 1.6% (Billinis et al., 2004). Two other rare haplotypes, AHH and VRH were detected both in the very low frequency of 0.03%. In the 9 haplotypes found in the samples, ARR was the predominant one in all flocks (47.78%) being followed by haplotype ARQ (44.93%). The AHQ haplotype was found in low frequency (3.86%), lower than the frequency that has been detected in a previous study (Ekateriniadou et al., 2007b) in rare Greek breeds (6.31%). The rare ARK haplotype was also detected in a low frequency of 0.75%. The VRQ haplotype associated with susceptibility to scrapie was also detected at a very low frequency (0.36%), whereas the neutral haplotype ARH was found in a frequency of 2.24%.

The analysis of the 5815 samples revealed 23 different genotypes. The ARR/ARQ genotype (Type 2) was the predominant one with a frequency of 34.6%. The ARR/ARQ genotype is also appears as the most frequent genotype in other countries/breeds that have begun an effort to increase the resistance to scrapie, such as the Sicilian sheep with a frequency of 39.9% (Reale et al., 2015) and the Polish Pomeranian Landrace sheep with a frequency of 35.9% (Proskura et al., 2013). The second most frequent genotype was the resistant genotype to scrapie (Type 1) ARR/ARR (27.29%) while the most frequent Greek genotype ARQ/ARQ (Type 3), was the third most frequent genotype (24.23%).

The analyses during the period 2012-2016 showed a significant increase ($p<0.01$) of the scrapie resistant genotypes (Types 1 and 2) with a parallel decrease in the scrapie susceptible (Type 3) genotypes (Figure 1). The increasing trend of Type 1 and Type 2 genotypes disrupted presenting a small decrease in 2016 and in 2015, respectively, accompanied with a slight increase of Type 3 genotypes during 2015-2016 (Figure 1). These results could be attributed to the analyses of the newborns samples from 40 flocks that had sent samples more than once and adopted breeding schemes, focusing in the analysis of the 171aa. The analyses of these samples as mentioned above, presented a significant increase ($p<0.01$) in the frequencies of Arginine and a significant decrease ($p<0.01$) in the frequencies of Glutamine in both homozygosis and heterozygosis. A decrease of the allelic variants RR and R/- was observed in 2014 due to the small number of samples analyzed only for the 171aa since the newborns genotyped in 2012 and 2013 needed at least a year for introducing them to the reproducing procedure.

Finally, the most susceptible to scrapie Types 4 and 5 genotypes were detected during this time period at very low frequencies.

The farmers along with the freelancers and state veterinarians successfully followed the suggestions for selection breeding against scrapie based on the *PRPN* analyses performed during 2012-2016. The conversion time of a flock to scrapie resistant is depending on the number of the scrapie resistant rams and ewes and on the level of scrapie resistance that the breeding scheme was begun. Arnold's model (Arnold et al., 2002) suggests that it will take at least 20 years to ensure that all slaughter lambs carry at least one ARR allele.

CONCLUSION

The initiative of the farmers and private and state veterinarians to genotype their future breeders began a significant effort for the transformation of their flocks from scrapie susceptible to scrapie resistant.

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Research article
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Polymorphism of ompH gene of *Pasteurella multocida* serotype A strains isolated in Iran

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ABSTRACT. One of the most frequent causes of respiratory infection and death in sheep and goats is *Pasteurella multocida*. In humans, it has been associated with diseases of the respiratory tracts, arthritis, osteomyelitis and meningitis. Outer membrane protein H (OmpH) has a role in immunogenicity and pathogenicity of *P. multocida.* The aim of this study was to characterize the genetic diversity of ompH gene of a panel of *P. multocida* serotype A strains isolated in sheep. Forty *P. multocida* serotype A strains isolated in previous study were selected and analyzed by restriction fragment length polymorphism (RFLP) of a species-specific PCR assay. RFLP amplified fragment produced five different cleavage patterns. On the basis of combinations resulting from ompH gene digestion, the 40 *P. multocida* isolates were classified in six RFLP type. It seems that isolates with variants genetic profile represent different pathogenecity. New vaccine formulation should consider multivariants of *P. multocida* in order to confer a wider protection.

Keywords: *Pasteurella multocida*; RFLP-PCR; ompH gene.

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INTRODUCTION

Pasteurella multocida, a gram-negative facultative bacterium, is one of the notorious animal pathogen causing widespread infections in various domestic animals; snuffles in rabbits, pneumonia and haemorrhagic septicaemia in cattles, sheep and goats, fowl cholera in chickens and atrophic rhinitis in pigs (Lee et al., 2007). Sheep pasteurellosis is one of the most common infectious and economically important bacterial diseases which occur in temperate and subtropical areas (Prabhakar et al., 2010; Sahragard 2016). *P. multocida* is an endemic disease in Iran such as West Azarbaijan, Mazandaran, Gilan, Khozestan and Fars Provinces (Tabatabaei *et al* 2002, Shayegh *et al* 2009, Mirghafari 2016). In Iran, as in many other countries, strains of *P. multocida* have frequently been isolated from sheep and goats, and they represent a significant cause of outbreaks of respiratory infections (Danesh 2013).

Virulence factors of *P. multocida* are defined by various cell-surface expressed components. Among these, the most important are polysaccharide capsule and typical constituents of the outer membrane of the cell wall such as lipopolysaccharides (LPS), a limited number of major proteins, and several minor proteins presented in very high copy numbers (Miguel et al., 2014). On the other hand, as antigenic determinants, they stimulate antibody production of the adaptive immune system (Sellyei et al., 2012). Studies utilizing outer membrane proteins (OMPs) of Gram negative bacteria indicated OMPs as protective immunogens that could play an important role in bacterial adherence and invasion (Singh et al., 2011). Protein H, or porin H, is the major outer membrane protein in the envelope of *P. multocida* (Luo et al., 1997). These proteins are at the interface between pathogen and host and are subject to various selective pressures depending on their function (Davies et al., 2003). Consequently, OMPs exhibit varying degrees of inter-strain heterogeneity and this can be used to assess intra-species diversity and determine epidemiological relationships (Chang et al., 2012). Outer membrane protein H (OmpH) gene encoded the major outer membrane protein that has a role in immunogenicity and pathogenicity of *P. multocida* isolates in mice, (Tan et al., 2010). ompH is one such major protein in the envelope of *P. multocida* that has been purified and characterized as a porin (Singh et al., 2011). In fact it is structurally and functionally related to the super family of porins of Gram negative bacteria (Luo et al., 1997). The sequence analysis of the ompH gene demonstrated the increased diversity of the porin protein and revealed major variations in two discrete regions encoding large external loops that presumably interact with the host immune system (Sellyei et al., 2012). OmpH as well as ompA proteins show considerable heterogeneity, and at least among avian *P. multocida* strains, a number of different variants appear to be associated with certain capsular serotypes (B, D, or F) (Davies et al., 2003; Williams et al., 1990).

The infectious serogroups of *P. multocida* associated with outbreaks of pneumonic pasteurellosis in

Table 1. Primer and Sequence used in the present study.

Primer	Gene	Name	Sequence	Amplicon size (bp)
A l lPASS	KMT1	KMT1T7	ATCCGCGATTTACCCAGTGG	460
		KMTSP6	GCTGTAAACGAACTCGCCAC	
Outer membrane protein	OMPH	ompH -FWD	ACTATGAAAAAGACAATGGTAG	1200
		ompH -RWD	GATCCATTCCTTGCAACTTATT	

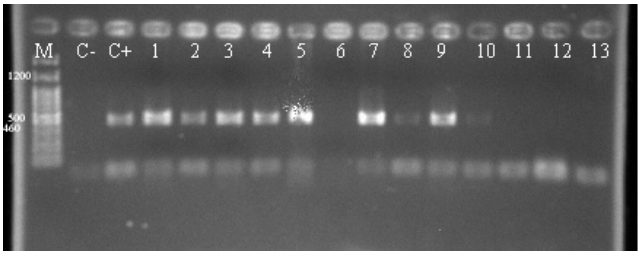
sheep and goats (A and D) have been incriminated as both primary and secondary agents of pneumonia (Zamrisaad et al., 1996; Sahragard et al., 2011; Danesh 2013). Our findings support the need for the development of a multivariant vaccine using the prevalent *P. multocida* serotype circulating in Iran as well as strategic deworming, and improved housing conditions for sheep and goats (Tahamtan 2014). But before that, classification and ranking of many *P. multocida* isolated is necessary to identify different variants.

There are several classifications of *P. multocida* based on the pathogenesis, mortality and genetic diversity in the animal models. But because of complex pathogenesis of *P. multocida* and interaction between host and bacterium, the past classification did not provide more information about that (Harper et al., 2006; Tahamtan and Mirghafari, 2016). Recent molecular techniques, especially restriction fragment length polymorphism (PCR –RFLP), are the most effective methods for identification and classification of genetic variation in bacterial animal isolates (Tshikhudo, 2013; La et al., 2006). PCR-RFLP is widely applied for analyzing polymorphism within a gene segment (Sellyei et al., 2012). Therefore, the aim of this study was to apply PCR-RFLP analysis to the ompH gene to characterize the genetic variation of local *Pasteurella* isolates from sheep.

MATERIALS AND METHODS

Sample: Fourty *P. multocida* serotype A strains collected in previous study were isolated from sheep with respiratory diseases, in different parts of Fars province, Iran (Tahamtan and Mirghafari, 2016).

Fig 1. PCR products of 460 bp resulting after amplification of all pass primers. M: molecular size marker; C-: negative control; C+: positive control; numbers (1-5 & 7-10): some positive samples; numbers (6, 11, 12 & 13): some negative samples.



PCR

DNA extraction and PCRs

DNA extraction was carried out according to previous study (Tahamtan et al., 2016; Jabbari et al., 2005). Briefly, bacterial cells were lysed by EDTA (0.5 M), SDS (0.5%) and proteinase K (20 ng/ml) followed by phenol-choloroform- isoamyl alcohol extraction, chloroform/ isoamyl alcohol (24:24:1) mixture. Genomic DNA was precipitated by addition of sodium acetate and absolute ethanol. Then ethanol 70% was added and after dried at room temperature, re-suspended in TE buffer (pH 8) (Jabbari et al., 2005). PCR was performed in a thermal cycler (Master Gradient Eppendorf, Germany). PCR mixture contained 3 µL of 1× PCR buffer, 3 µL of deoxyribonucleotide triphosphate (dNTP) blend (2.5 mM each dNTP), 2 µL of primers (10 picomole each) (Table 1). One µL of template DNA, and 0.2 µL of high fidelity Taq DNA polymerase were added with distilled water to reach 25 µL. One kb DNA marker (Fermantase) was used. The PCR conditions were: 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 30 sec, 56°C for 30 sec, and 68°C for 6 min, and 1 cycle of 25°C for 1 min (Tsai et al, 2011). The isolates from sheep were identified as ShI 1 - ShI 40.

PCR-RFLP

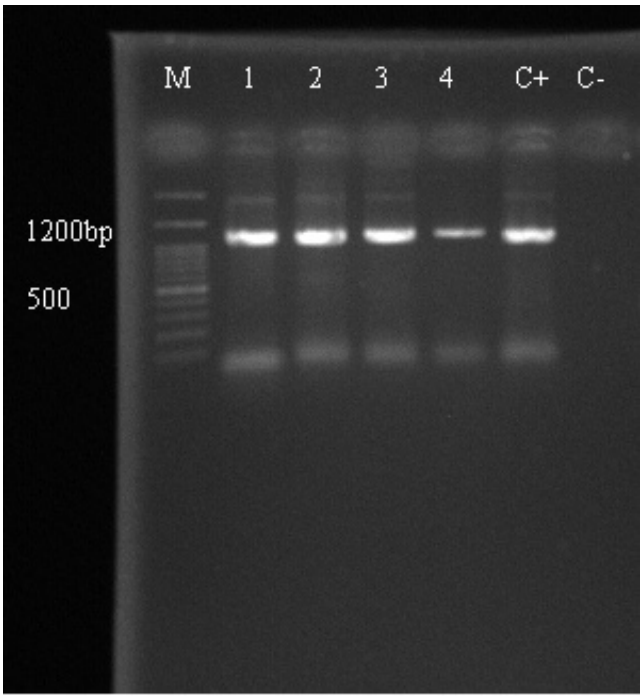
The RFLP procedure was carried out by digesting the amplified PCR products with *EcoRI* and *Hind III* endonucleasese enzymes in the supplied buffer according to the manufacturer's protocols. The PCR product (4 µl) was digested by the addition of 4 µl of water, 1 µl of 10X buffer, and 1 µl (10 U) of restriction enzyme, and incubated at 37°C for 2 hrs. The RFLP products were run on 2.0% agarose gels at 5 V/cm for 45 min, stained with ethidium bromide, and then visualized under ultraviolet illumination.

RESULTS

PCR amplification of the isolates using Pasteurella multocida species specific primers produced a single 460 bp band (figure 1). PCR product of ompH gene is shown in figure 2. PCR amplification of ompH- primers results in a 1.2 kb fragment.

PCR-RFLP typing of P. multocida isolates is presented in table 2. Twenty seven and 13 cloned EcoRI fragments indicate A and B patterns respectively. Twenty

Fig 2. PCR product of 1200 bp obtained with ompH primers. M: molecular size marker; C-: negative control; C+: positive control; numbers 1-4: some positive samples.



one, 14 and 5 cloned fragments representing C, D and E patterns to a different single restriction fragment in a genomic Hind III digestion. Figure 3 shows P. multocida isolates with 2 cleavage pattern when digested with EcoRI and 3 cleavage pattern when using Hind III restriction enzymes.

EcoRI and Hind III digestion resulted in fragments of 700-500, 900-300 and 1200 bp.

Combination of different restriction patterns grouped the 40 P. multocida strains into six RFLP types (table 3). On the basis of combinations, two to three bands in each digests are produced among the 40 isolates and identify six RFLP types.

DISCUSSION

Variations in the molecular mass of ompH among different P. multocida strains have also been reported (Hussaini et al., 2013). The role of ompH as a protective antigen has been identified against homologous infection by Ghanizadeh et al (2015). They have successfully used PCR analysis based on RFLP in ompH gene to investigate genetic heterogeneity and to classify avian P. multocida isolates. There was also considerable variation in the gene content of ompH (Prasannavadhana et al., 2014), but the genetic variation among P. multocida isolates is not well established in Iran. The present paper represents the first report of PCR-RFLP characterization based on ompH gene of P. multocida ovine strains isolated in Iran.

Although several studies have investigated the phylogeny of P. multocida species on the basis of 16S rDNA gene sequences (Michael and Abbott, 2007; Dousse et al., 2008; Bhimani et al., 2014) only few studies have used the ompH gene analysis (Tan et al., 2010).

In current research 40 P. multocida isolates were identified by genomic methods and divided into six different RFLP type. Similarly to what has been reported by Sellyei et al. (2012) our results demonstrated that a PCR-RFLP approach targeting the ompH gene using EcoR I and Hind III restriction endonucleases has been useful for characterization of P. multocida. The differentiation obtained by PCR-RFLP analysis enables a clear separation of the P. multocida group. ompH gene is more suitable for genetic differentiation of closely related species within the same cluster compared to 16S rRNA sequences because of its higher rate of nucleotide

Table 2. PCR-RFLP typing of *P. multocida* isolates with different fragment size.

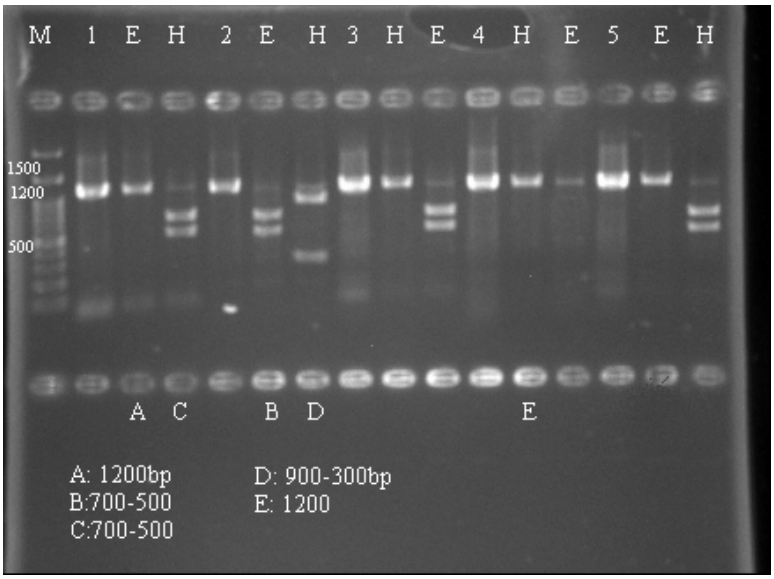
Endonuclease enzyme	Profile pattern	Size of fragments (bp)	Number of isolates
EcoR I	A	1200	27
	B	700-500	13
Hind III	C	700-500	21
	D	900-300	14
	E	1200	5

Table 3. Combination of different cleavage patterns of *P. multocida* isolated from sheep following EcoRI and Hind III restriction enzyme digestion.

Pattern	Restriction enzymes		Bacteria
RFLP type	EcoR I	Hind III	*P. multocida* Isolates
I	A(1200bp)	C(500-700)	ShI*1, ShI4,ShI6, ShI8, ShI10, ShI18
II	B(500-700)	D(900-300)	ShI2, ShI3, ShI5, ShI17, ShI20, ShI21
III	A(1200)	E(1200)	ShI7, ShI9, ShI11, ShI12, ShI14, ShI19, ShI22, ShI26, ShI27, ShI30, ShI33
IV	A(1200)	D(900-300)	ShI13, ShI15, ShI16, ShI23
V	B(500-700)	C(500-700)	ShI24, ShI25, ShI28, ShI29, ShI31, ShI32, ShI39, ShI40
VI	B(500-700)	E(1200)	ShI34, ShI35, ShI36, ShI37, ShI38

**ShI: sheep isolates*

Fig 3. The DNA patterns results of isolates after digestion with EcoR I and Hind III enzyme (The pattern A-E).Number1-5 is PCR products, E: EcoRI ,H:Hind III digestion.



polymorphism (Jabbari et al., 2005). The phenotypic heterogenecity of P. multocida isolates showed more genetic diversity among ovine isolates. We found that the sheep isolates were formed diverse RFLP type with various groups. This genetic diversity reflected the phenotype characteristic such as diseases in sheep, because they isolated from sheep with history of pasteurelosis. In current study, although the genetic diversity among the isolated strains was observed, the exact relationship between RFLP type and strain characteristics such as pathogenecity and immunogenecity remain to be determined.

CONCLUSION

In conclusion, in the present study, we applied two efficient, economic, and alternative molecular methods (single multiplex PCR and RFLP PCR) to characterize and to demonstrate genetic diversity of ovine *P. multocida* species. Both proposed methods accurately detected and distinguished six different types of heterogenic *P. multocida* in Iran. The possible effect of the presence of multiple serotypes on the PCR-RFLP profile needs further examinations. This genetic diversity among the isolates reflected the diseases in sheep, because they represented the history of pasteurelosis.

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Research article
Ερευνητικό άρθρο

Effects of yeast based toxin binder supplementation on growth performance and intestinal microarchitecture in male buffalo calves (*Bubalus bubalis*) exposed to different concentrations of Aflatoxin B1.

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ABSTRACT. The purpose of study was to analyze the effects of yeast based toxin binder (YTB) on growth performance and intestinal microarchitecture of buffalo calves exposed to different concentrations of aflatoxin B1 (AFB1). Male buffalo calves (n=72) having no disease exposure in the last four months, aged 10 ± 1 months and weighing 150 ± 12.22 kg (Mean ± standard deviation)were purchased from a buffalo calf fattening farm and divided in 8 equal groups (n=9 animals in each group). Animals that were fed common basal diet and basal diet along with yeast cell wall based toxin binder (YTB) and had no exposure to AFB1 were served as controls. Different concentrations of AFB1 (6mg, 8mg and 10mg per kg of feed) were given to 27 animals, similarly 2mg of YTB (per kg of contaminated feed) was added to concentrate with different AFB1 concentrations and fed to another 27 animals. Average daily gain of each animal was recorded. Animals were slaughtered after 27 days and intestinal segments from duodenum, jejunum, ileum and colon were collected which were stained with hematoxylin/eosin or combined alcian blue and periodic acid schiff stain.

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Average daily gain and intestinal morphometric parameters (villus height, villus width, crypt depth and surface area) in all the selected intestinal segments decreased (P<0.05) whereas, intraepithelial lymphocytes, goblet cells (having acidic and neutral mucins) and proliferative cells increased (P<0.05) with AFB1 exposure. It was also observed that YTB neutralized (P<0.05) the harmful effects of AFB1 on intestinal morphology, boosted activity of goblet cells and decreased (P<0.05) number of proliferative cells. It was concluded that YTB supplementation is advantageous in eliminating the negative effects of AFB1 on gut of buffalo calves.

Keywords: Buffaloes, aflatoxin B1, yeast based toxin binder supplementation, growth performance, intestinal histology.

INTRODUCTION

Aflatoxins (AF), a cluster of intoxicating mycotoxins are secondary metabolites from certain strains of filamentous fungi specially *Aspergillus Flavus* and *Aspergillus Parasiticus* (Rashid et al., 2012). These fungi colonize on feed and food products and produce AF whose presence may lead to economic losses and raise public health concerns which at times can prove to be fatal (Mendoza et al., 2011). Harmful outcomes of AF which include teratogenic, mutagenic, growth inhibitory and carcinogenic effects have been widely studied (Oguz et al., 2000, Sur and Celik, 2003). Aflatoxins suppress the immune system and cause both macro and micro pathological alterations in the normal structure of liver, kidney and spleen making animal more susceptible to infectious agents (Magnoli et al., 2011).

To decrease the toxic effects of AF toxin binders (TB) are being used (Schatzmayr et al., 2003). Inclusion of TB in feed contaminated with AF has been the most promising approach for reducing the detrimental effects of AF (Galvano et al., 2001). In-vivo and in-vitro trails have been conducted over the last decade with TB for estimating their efficacy against toxins but results from both the trials do not correlate very well (Doll et al., 2004; Diaz et al., 2004). Many complex indigestible carbohydrates (polysaccharides in yeast cell wall and cellulose) and bacteria (glucomannans and peptidoglycans) have been reported to absorb AF among these yeast cell wall based toxin binder (YTB) is being preferred for its better nutritional properties and superior binding activity than other commercially available toxin binders (Huwig et al., 2001).

Among several types of AF aflatoxin B1 (AFB1) is most commonly encountered and is considered most potent than any other type (Yunus et al., 2011), causing stern health concerns for animal populations (Sirajudeen et al., 2009). Negative effects of AF have been an active area of research (Yunus et al., 2011), and studies have revealed that the primary site for absorption of AF is small intestine (predominantly duodenum) (Mendoza et al., 2011). To the best of our knowledge influence of YTB supplementation on the quantification and differentiation of goblet cells (GC), role of intraepithelial lymphocytes (IEL) as an indicator of mucosal damage and immunohistochemistry of proliferative cells in intestine of buffalo calves exposed to different concentrations of AFB1 has not been assed. This study will be pivotal in recognizing the structural alterations in buffalo gut associated with AFB1 and YBT supplementation as it aims to evaluate the effects of different concentrations of AFB1 on intestinal microarchitecture (morphometric variables, IEL count, GC quantification and differentiation and immunohistochemistry of proliferative cells) in gut of buffalo calves and efficacy of YTB to counter toxic effects of AFB1 on intestinal morphology.

MATERIALS AND METHODS

Experimental design and grouping of animals

A total of 72 having no disease exposure in the last four months, aged 10 ± 1 months and weighing 150 ± 12.22 kg (Mean ± standard deviation) were purchased from a buffalo calf fattening farm and divided in 8 equal groups (n=9 animals in each group). These animals were then acclimatized for 15 days in an environmentally control shed having an optimum temperature of 22 ± 2 ºC and a relative humidity of 55 ± 10 % before the start of trial during which they were given ad-libitum corn silage (Table 1). Silage was tested for the presence of AFB1 on weekly basis by high performance liquid chromatog-

raphy. Animals were dewormed and vaccinated for foot and mouth disease and hemorrhagic septicemia (UVAS-FMD+HS-VAC, Pakistan). At the start of trail all animals were individually housed in separate pens made of stainless steel and were offered corn silage (free of AFB1, 15 kg per animal) and concentrate (1 kg per animal) (Table 1) per day and there refusal were weighed daily. Animals were divided into eight groups with each group having (n = 9) animals. Basal diet (BD) and BD plus YTB (BD-YTB) were not exposed to AFB1. For investigating the harmful effects of AFB1 different concentrations of AFB1 were given to animals of 3 groups which included AFB1 6mg per kg of concentrate (AFB1 0.6), AFB1 8mg per kg of concentrate (AFB1 0.8) and AFB1 10mg per kg of concentrate (AFB1 1.0) after being formulated in the concentrate (Table 1) for 27 days. Furthermore for analysis the effects of YTB during aflatoxicosis in buffalo calves, 2mg per kg of YTB (Fixar viva dry yeast based, Bentoli Agrinutrition Asia Pte. Ltd, USA) was mixed in feed having different concentrations of AFB1 and given to another 3 groups of buffalo calves (AFB1 0.6-YTB, AFB1 0.8-YTB, and AFB1 1.0-YTB). Throughout the trail animals had open access to water. This study was conducted according to the guidelines of Animal Care and Use Committee, University of Veterinary and Animal Sciences (UVAS), Lahore.

Production of AFB1,preparation of diet and safety measures

Aflatoxin B1 being used in the trial was produced in Microbiology laboratory UVAS using toxic strains of *Aspergillus Flavus* IMI-90, obtained from International Mycology Institute, London as described by Tessari et al., (2006). Briefly, chloroform (30 ml chloroform per 10 grams of culture) was used to extract coconut culture after shaking for 30 minutes. Contents post filtration via filter paper (Whatman#1) was evaporated to dryness. Densitometry was applied for quantification and water bath (WNE 14 water bath, Memmert, USA) was used for evaporation of chloroform solution having AFB1 at 60 ºC. Contents were resuspended in fish oil that had been proven negative for AF presence. This fish oil with different concentrations

Table 1. Ingredients (as fed basis) and nutrient composition (% dry matter basis) of concentrate and corn silage fed to male buffalo calves.

Ingredients (Concentrate)	**% unless indicated**
Corn Meal	22.8
Sugarcane molasses	2
Wheat midds	65.8
Soyabean meal	3.3
Limestone	3.6
Fish oil	1
Salt	1
Premix*	0.4
Chemical composition (Concentrate)	
Dry matter	86
Crude protein	16.8
Crude fat	4.2
Crude fiber	12.3
Neutral detergent fiber	38.6
Metabolizable energy	2.6 M cal/kg
Chemical composition (Corn Silage)	
Dry matter	35.7
Crude protein	7.3
Crude fat	1.3
Crude fiber	26.4
Neutral detergent fiber	66.2
Metabolizable energy	2.2 M cal/kg

M cal, Mega calories.
** Each kg of premix contained; vitamin A, 495 IU; vitamin E, 0.33 IU; vitamin D3, 165 IU; zinc, 16mg; copper, 4mg; selenium, 0.1mg; iodine, 0.6mg; and cobalt, 0.6mg.*

(6mg, 8mg and 10mg) of AFB1 was used in the concentrate formulation (Table 1). Final concentrations of AFB1 in concentrate to be offered were confirmed using procedure used by Shephard et al., (1990).

Aflatoxin B1 is a toxic substance therefore it was manipulated as solutions to avoid formation of aerosol and dust. Aprons, masks and nitrile gloves were used at every step of manufacturing and manipulation (Corcuera et al., 2011).

Growth performance, sampling and histological morphometry

Animals were weighed on weekly basis and after completion of trail were slaughtered by Halal slaughter method as described by Gregory et al., (2008), and 3cm intestinal segments at midpoints of duodenum, jejunum, ileum and colon were collected. Samples were washed with physiological saline solution, opened longitudinally and immediately fixed in 10% buffered formaldehyde solution for 48 hours. Intestinal segments were then rinsed with water and after dehydration with graded series of absolute alcohol (50%, 60%, 70%, 80%, 90% and 100%) were cleared twice with benzene. Sections were embedded with paraffin and 4 µm thin sections were obtained which were stained with hematoxylin/eosin (Wang et al., 2009). Slides were observed under light microscope (Labomed CXL, New York Microscope Co, USA) fitted with camera (Moticam CMOS Digital Camera, New York Microscope Co, USA) and measurements were made with commercial morphometric program (Pixel Pro, Labomed, USA). Double blind analysis was done in triplicate on 5 well oriented villi that were selected on the basis of intact lamina propria. Variables that were measured included villus height (from tip of villus to villus crypt junction), villus width, crypt depth (from base to the transition region between villus and crypt), surface area (2 × villus width/2 ×villus length) (Solis de los Santos et al., 2007), and IEL (rounded cell that had central or slightly eccentric nucleus with scant cytoplasm) number.

Histo-chemistry of goblet cells

Slides obtained from paraffin embedding technique were subjected to alcian blue and periodic acid Schiff staining methods (Bancroft and Stevens, 2007), to evaluate goblet cells containing acidic and neutral mucin types respectively. Acidic mucins were stained blue by AB whereas neutral mucins were stained magenta by PAS (Leknes, 2010).

Immunohistochemistry of proliferative cells

Proliferative cells were counted in all the groups with antibody MIB-1 (Sigma-Aldrich, St. Louis, MO., USA) directed against proliferation marker Ki-67. Regarding antigen accessibility sections were incubated in boiling 0.01 M citrate buffer having pH 6 and washing was carried out in phosphate buffered saline (PBS) with 0.05% Tween (PBS-T). For blocking the endogenous peroxidase activity slides were treated with 1% hydrogen peroxide and to avoid binding of unspecific antibody slides were treated with 10% goat serum (Life technologies, Scientific supplies Ltd. Pakistan). At 4 ºC binding of MIB-1 (1:50 in PBS) was done overnight. Sections were then incubated with polyclonal goat anti-mouse antibody which had been labeled with horse reddish peroxidase. Visualization was done with 3,3′ diaminobenzidine solution (KPL Inc., Gaithersburg, Maryland USA). Quantification of proliferative cells positive to MIB-1 antibody was done as described by Masanatz et al., (2010) in the five consecutive crypts of selected intestinal segments and the average of the results were reported.

STATISTICAL ANALYSIS

Statistical differences were analyzed by using Statistical Package for Social Sciences (SPSS 13.3, SPSS, Chicago, USA). For estimating the normal distribution of data Shapiro- Wilk test was applied, data was found to be normally distributed. Data for comparison among groups were subjected to one way ANOVA keeping BD, different concentrations of AFB1, treatment of AFB1 contaminated groups with YTB and inclusion of YTB to BD as constant factors. Statistical differences among means were considered significant at $P < 0.05$ and calculated using Duncan's multiple range test.

RESULTS

Effects of feeding different levels of AFB1 on average daily gain and morphometric variables (villus

height, villus width, crypt depth, villus: crypt ratio and surface area) of selected intestinal segments are shown in Table 2. Exposure of buffalo calves to AFB1 resulted in lower (P<0.05) average daily gain and decreased (P<0.05) morphometric variables except for crypt depth which increased (P<0.05) with AFB1 for all the selected intestinal segments. Villus height, villus width, villus: crypt ratio and surface area decreased (P<0.05) whereas crypt depth increased (P<0.05) as AFB1 concentrations were increased. It was observed that by addition of YTB harmful effects of AFB1 on intestinal microarchitecture were neutralized as no difference was observed for morphometric variables in AFB1 0.6-YTB, AFB1 0.8-YTB and AFB1 1.0-YTB compared to BD. Moreover inclusion of YTB in BD not contaminated with AFB1 increased (P<0.05) villus height, villus width, and surface area.

A higher (P<0.05) IEL count was observed in all selected intestinal segments of male buffalo calves exposed to different concentrations of AFB1 compared to BD animals. Inclusion of YTB to contaminated diet decreased (P<0.05) IEL count of animals compared to those exposed to AFB1. Number of GC, GC having acidic mucin and neutral mucin increased (P<0.05) with exposure to AFB1 compared to BD and gradually increased (P<0.05) with increasing AFB1 concentrations in feed. Supplementation of YTB caused an increase (P<0.05) in GC having acidic mucin and neutral mucin in selected intestinal segments of animals exposed to AFB1 and BD-YTB animals.

For all the selected intestinal segments number of proliferative cells increased (P<0.05) with increas-

Fig 1. Acidic type of goblet cells in in the duodenum of calves fed AFB1 0.8 -YTB.

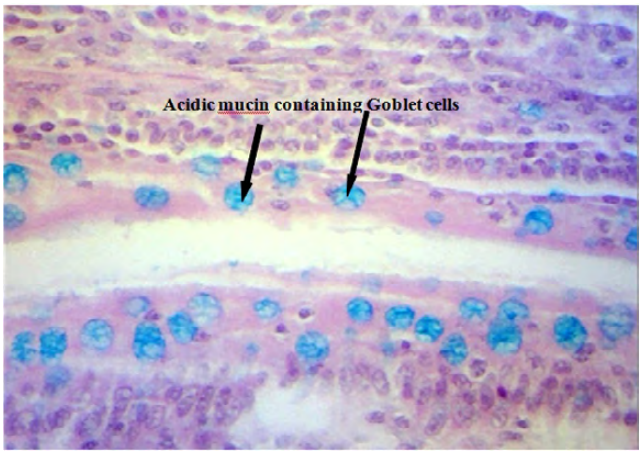


Fig 2. Mixed type of goblet cells in in the duodenum of calves fed AFB1 0.8 -YTB.

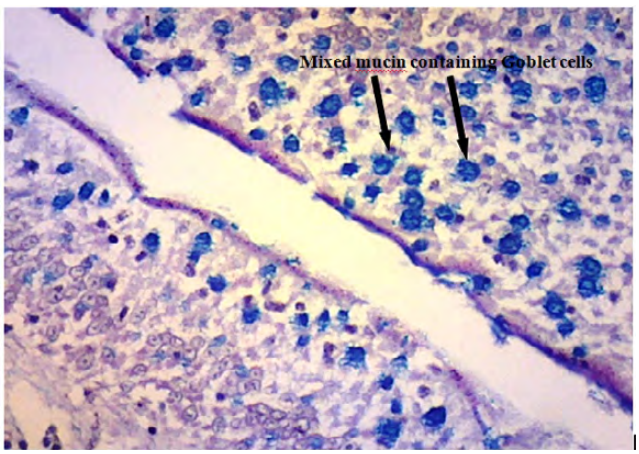


Fig 3. Acidic and mixed mucin having types of goblet cells in in the duodenum of calves fed AFB1 -0.8.

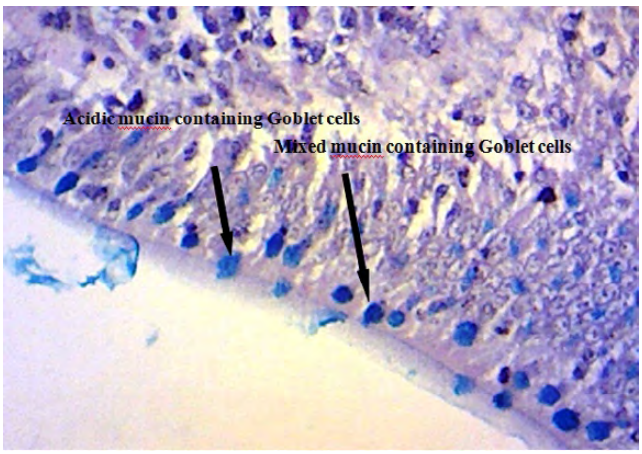


Fig 4. Intra epithelial lymphocytes in the Duodenum of calves fed AFB1- 0.8.

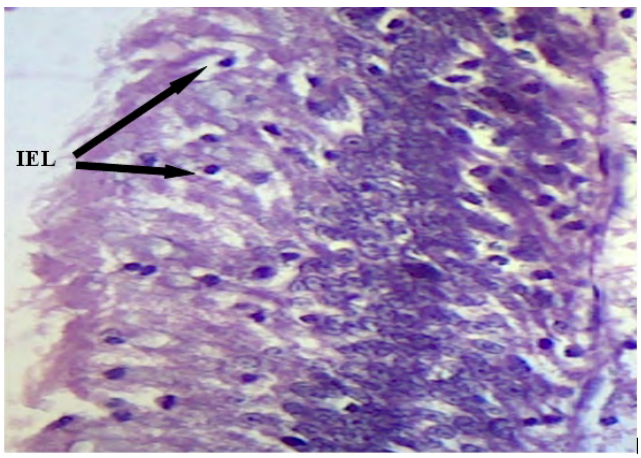


Table 2. Comparison of intestinal morphometric parameters and intraepithelial lymphocyte count in intestinal segments of male buffalo calves.

Intestinal segments	Variables	BD	Contaminated			Treated			BD-YTB	SEM	*P*-Value
			AFB_1 0.6	AFB_1 0.8	AFB_1 1.0	AFB_1 0.6-YTB	AFB_1 0.8-YTB	AFB_1 1.0-YTB			
Duodenum	Average daily gain (grams)	502^{d}	411^{c}	372^{b}	310^{a}	491^{d}	505^{d}	511^{d}	557^{e}	31.75	< 0.01
	Villus height (mm)	0.73^{d}	0.51^{c}	0.45^{b}	0.39^{a}	0.72^{d}	0.71^{d}	0.68^{d}	0.78^{e}	0.04	< 0.01
	Villus width (mm)	0.15^{d}	0.11^{c}	0.09^{b}	0.06^{a}	0.12^{d}	0.11^{d}	0.12^{d}	0.18^{e}	0.01	<0.01
	Crypt depth (mm)	0.23^{a}	0.35^{e}	0.41^{d}	0.50^{e}	0.24^{a}	0.21^{a}	0.25^{a}	0.27^{ab}	0.04	< 0.01
	Villus: crypt ratio	3.17^{e}	1.45^{e}	1.09^{b}	0.78^{a}	3.01^{e}	3.38^{f}	2.72^{d}	2.88^{de}	0.39	< 0.01
	Surface area (mm^2)	0.37^{e}	0.18^{c}	0.15^{b}	0.08^{a}	0.27^{d}	0.25^{d}	0.26^{d}	0.45^{e}	0.04	< 0.01
	Intraepithelial lymphocytes	24.61^{a}	38.14^{d}	42.73^{e}	49.55^{f}	27.32^{ab}	30.24^{b}	33.17^{b}	24.18^{a}	3.38	< 0.01
Jejunum	Villus height (mm)	0.69^{d}	0.44^{c}	0.36^{b}	0.25^{a}	0.68^{d}	0.71^{d}	0.73^{d}	0.79^{e}	0.07	< 0.01
	Villus width (mm)	0.14^{c}	0.08^{b}	0.07^{b}	0.05^{a}	0.13^{c}	0.15^{c}	0.15^{c}	0.19^{d}	0.01	< 0.01
	Crypt depth (mm)	0.26^{a}	0.38^{e}	0.44^{d}	0.48^{e}	0.25^{a}	0.27^{a}	0.29^{a}	0.31^{ab}	0.03	< 0.01
	Villus: crypt ratio	2.65^{d}	1.15^{e}	0.81^{b}	0.52^{a}	2.72^{d}	2.62^{d}	2.51^{d}	2.54^{d}	0.35	< 0.01
	Surface area (mm^2)	0.32^{d}	0.17^{c}	0.08^{b}	0.04^{a}	0.28^{d}	0.31^{d}	0.34^{d}	0.45^{e}	0.05	< 0.01
	Intraepithelial lymphocytes	22.61^{a}	31.25^{d}	35.43^{e}	42.72^{f}	23.69^{ab}	25.57^{b}	24.91^{ab}	21.89^{a}	2.64	< 0.01
Ileum	Villus height (mm)	0.64^{e}	0.41^{c}	0.35^{b}	0.33^{a}	0.61^{de}	0.58^{d}	0.53^{d}	0.71^{f}	0.05	< 0.01
	Villus width (mm)	0.13^{d}	0.09^{c}	0.06^{b}	0.04^{a}	0.11^{d}	0.12^{d}	0.11^{d}	0.15^{e}	0.01	< 0.01
	Crypt depth (mm)	0.33^{a}	0.39^{c}	0.47^{d}	0.53^{e}	0.29^{a}	0.31^{a}	0.34^{a}	0.36^{ab}	0.03	< 0.01
	Villus: crypt ratio	1.93^{ef}	1.05^{c}	0.74^{b}	0.62^{a}	2.10^{e}	1.87^{e}	1.55^{d}	1.97^{ef}	0.22	< 0.01
	Surface area (mm^2)	0.28^{e}	0.12^{c}	0.07^{b}	0.04^{a}	0.21^{d}	0.22^{d}	0.19^{d}	0.34^{f}	0.03	< 0.01
	Intraepithelial lymphocytes	27.19^{a}	37.27^{d}	44.31^{e}	46.22^{f}	29.64^{b}	30.43^{b}	28.88^{ab}	26.91^{a}	2.81	< 0.01
Colon	Villus height (mm)	0.59^{d}	0.45^{c}	0.39^{b}	0.32^{a}	0.57^{d}	0.55^{d}	0.58^{d}	0.67^{e}	0.04	< 0.01
	Villus width (mm)	0.11^{d}	0.07^{c}	0.05^{b}	0.04^{a}	0.11^{d}	0.10^{d}	0.12^{d}	0.16^{e}	0.01	< 0.01
	Crypt depth (mm)	0.37^{a}	0.43^{c}	0.49^{d}	0.57^{e}	0.36^{a}	0.39^{a}	0.41^{a}	0.43^{ab}	0.02	< 0.01
	Villus: crypt ratio	1.59^{e}	1.04^{c}	0.79^{b}	0.56^{a}	1.58^{c}	1.41^{d}	1.42^{d}	1.55^{e}	0.15	< 0.01
	Surface area (mm^2)	0.22^{d}	0.09^{c}	0.06^{b}	0.04^{a}	0.19^{d}	0.17^{d}	0.21^{d}	0.28^{e}	0.03	< 0.01
	Intraepithelial lymphocytes	31.11^{a}	47.02^{e}	54.55^{d}	59.88^{c}	35.62^{a}	34.47^{a}	35.23^{a}	31.97^{a}	3.92	< 0.01

Results are demonstrated as mean ± s.e.m.
Difference of superscripts (a-f) within a row indicates significance difference P < 0.05.
BD, basal diet (negative for AFB1); AFB1 0.6, aflatoxin B1 6mg per kg of concentrate; AFB1 0.8, aflatoxin B1 8mg per kg of concentrate; AFB1 1.0, aflatoxin B1 10 mg per kg of concentrate; AFB1 0.6- YTB, aflatoxin B1 6mg per kg of concentrate plus 2mg of yeast based toxin binder per kg of feed; AFB1 0.8- YTB, aflatoxin B1 8mg per kg of concentrate plus 2mg of yeast based toxin binder per kg of feed; AFB1 1.0- YTB, aflatoxin B1 10mg per kg of concentrate plus 2mg of yeast based toxin binder per kg of feed; BD-YTB, basal diet plus 2mg per kg of yeast based toxin binder; SEM, standard error of mean.

Table 3. Goblet cells, histochemistry of goblet cells and immunohistochemistry of proliferative cells in intestinal segments of male buffalo calves.

Variables	Intestinal segments	BD	Contaminated			Treated			BD-YTB	SEM	*P*-Value
			AFB_1 0.6	AFB_1 0.8	AFB_1 1.0	AFB_1 0.6-YTB	AFB_1 0.8-YTB	AFB_1 1.0-YTB			
Goblet cells (per villus)	Duodenum	49.21^{a}	59.24^{b}	62.34^{bc}	65.37^{c}	67.52^{d}	69.98^{d}	73.29^{e}	99.86^{f}	5.56	< 0.01
	Jejunum	55.82^{a}	68.13^{b}	71.14^{b}	75.02^{bc}	78.09^{d}	81.27^{de}	85.12^{e}	116.71^{f}	6.69	< 0.01
	Ileum	71.65^{a}	79.84^{b}	80.23^{b}	83.26^{b}	89.17^{c}	93.45^{c}	96.15^{c}	141.34^{d}	8.12	< 0.01
	Colon	88.91^{a}	93.23^{b}	95.77^{b}	98.65^{c}	101.24^{d}	105.36^{d}	108.91^{d}	168.79^{e}	9.65	< 0.01
Goblet cells having acidic mucin (per villus)	Duodenum	18.14^{a}	31.86^{b}	34.25^{c}	36.97^{c}	41.69^{d}	44.26^{d}	49.87^{d}	51.27^{e}	3.59	< 0.01
	Jejunum	26.61^{a}	30.74^{b}	33.37^{b}	35.42^{c}	39.19^{d}	43.84^{e}	44.36^{e}	58.17^{f}	3.43	< 0.01
	Ileum	29.22^{a}	36.11^{b}	40.42^{bc}	41.24^{c}	45.13^{d}	48.27^{d}	51.86^{d}	62.88^{e}	3.87	< 0.01
	Colon	33.15^{a}	38.21^{b}	40.07^{b}	41.14^{c}	49.11^{d}	51.24^{d}	53.62^{d}	74.16^{e}	4.84	< 0.01
Goblet cells having neutral mucin (per villus)	Duodenum	15.26^{a}	22.17^{b}	23.79^{b}	25.32^{c}	21.35^{d}	22.13^{d}	23.03^{d}	48.13^{e}	3.71	< 0.01
	Jejunum	24.27^{a}	27.21^{b}	28.53^{b}	30.79^{b}	32.84^{c}	34.21^{c}	36.18^{c}	49.27^{d}	2.97	< 0.01
	Ileum	25.04^{a}	30.18^{b}	33.29^{c}	34.96^{c}	38.21^{d}	40.39^{d}	42.58^{d}	57.83^{e}	3.72	< 0.01
	Colon	29.04^{a}	34.55^{b}	36.11^{b}	37.25^{c}	42.24^{d}	44.95^{d}	47.14^{e}	65.39^{f}	4.19	< 0.01
Proliferative cells (per mm of crypt)	Duodenum	38.41^{b}	42.91^{c}	43.74^{c}	46.19^{d}	36.29^{b}	39.57^{b}	39.86^{b}	34.79^{a}	1.45	< 0.01
	Jejunum	32.13^{b}	35.62^{c}	37.89^{cd}	39.27^{d}	30.61^{b}	31.46^{b}	32.87^{b}	29.28^{a}	1.36	<0.01
	Ileum	27.57^{b}	31.73^{c}	33.54^{d}	37.28^{e}	26.12^{b}	28.99^{b}	29.15^{b}	24.85^{a}	1.54	< 0.01
	Colon	25.38^{b}	28.25^{d}	30.42^{d}	34.67^{e}	24.11^{bc}	26.43^{bc}	27.49^{c}	21.09^{a}	1.53	< 0.01

Results are demonstrated as mean ± s.e.m.
Difference of superscripts (a-f) within a row indicates significance difference P < 0.05.
BD, basal diet (negative for AFB1); AFB1 0.6, aflatoxin B1 6mg per kg of concentrate; AFB1 0.8, aflatoxin B1 8mg per kg of concentrate; AFB1 1.0, aflatoxin B1 10 mg per kg of concentrate; AFB1 0.6- YTB, aflatoxin B1 6mg per kg of concentrate plus 2mg of yeast based toxin binder per kg of feed; AFB1 0.8- YTB, aflatoxin B1 8mg per kg of concentrate plus 2mg of yeast based toxin binder per kg of feed; AFB1 1.0- YTB, aflatoxin B1 10mg per kg of concentrate plus 2mg of yeast based toxin binder per kg of feed; BD-YTB, basal diet plus 2mg per kg of yeast based toxin binder; SEM, standard error of mean.

ing AFB1 concentrations but YTB supplementation decreased (P<0.05) there number and bought it back to normal levels as no difference for proliferative cells was observed between animals of AFB1 0.6-YTB, AFB1 0.8-YTB, AFB1 1.0-YTB and BD groups. However, BD-YTB animals had the least number (P<0.05) of proliferative cells in the selected intestinal segments compared to animals of other groups.

DISCUSSION

Outcomes of YTB supplementation in BD and feed contaminated with different concentrations of AFB1 on intestinal morphology were studied in male buffalo calves. Impairments in the intestinal mucosa caused by AFB1 decreases its nutrient absorbing ability (Liu et al., 2011). Villus surface area depends upon the dimensions of villus (Hou et al., 2012), and higher growth rates are associated with greater villus

surface area (Awad et al., 2009), and increased villus: crypt ratio (Wu et al., 2004), which is in agreement with our results. Aflatoxin B1 induces alterations in intestinal microarchitecture (Yang et al., 2012), such as short villi, deep crypts, reduced villus: crypt ratio and surface area (Wan et al., 2013). This study confirms previous reports by Dogi et al., (2011), and Motawe et al., (2014), that YTB supplementation nullifies the harmful effects of AFB1 on intestinal morphology. Yeast cell wall (YCW) binds with AFB1, reduces its absorption in intestine and thus is useful in protecting ruminants from detrimental effects of AFB1 (Firmin et al., 2011). Yeast improves intestinal microarchitecture by increasing concentration of useful microbes present in the intestine and suppressing the concentration of pathogenic bacteria thus increasing growth performance of animal (Gao et al., 2008). It was also observed that YTB supplementation improved intestinal microarchitecture in BD-YTB animals compared to BD, similar results were seen in duodenum and jejunum of broilers by Gao et al., (2008), and in duodenum of pigs by Shen et al., (2009), who reported that YCW supplementation increases villus height and villus: crypt ratio.

The IELs play an important role in the regulations of immune response as they form specialized lymphoid compartments and are the first cells to encounter antigens if intestinal lumen (Finamore et al., 2008). Increased number of IEL in all the selected intestinal segments are results of intestinal inflammatory response (Quinteiro-Filho et al., 2010), towards AFB1. This increase in IEL count may also be associated with lesions (O,Handley et al., 2001), caused by AFB1. Beta-glucans (ß-glucans) are carbohydrates having linked glucose molecules which are major components of yeast cell wall (Volman et al., 2008). These components have the ability to enhance mucosal immunity of intestine (Battilana et al., 2001), as after oral administration ß-glucans regulate immune cells of Peyer's patches and IELs (Suzuki et al., 1990;Tsukada et al., 2003). Supplementation of YTB decreased number of IEL in intestinal mucosa compared to AFB1. Intestinal motility is due to stimulation of parasympathetic nerves, since lymphocytes carry cholinergic receptors it can be assumed that stimulation of parasympathetic nerves then activate the mucosal immune system in the intestine leading to an increase in IEL number.

Highly viscous mucus layer that covers intestinal mucosa, helps in lubrication of ingested food and

Fig 5. Intra epithelial lymphocytes in the Duodenum of calves fed AFB1 0.8- YTB.

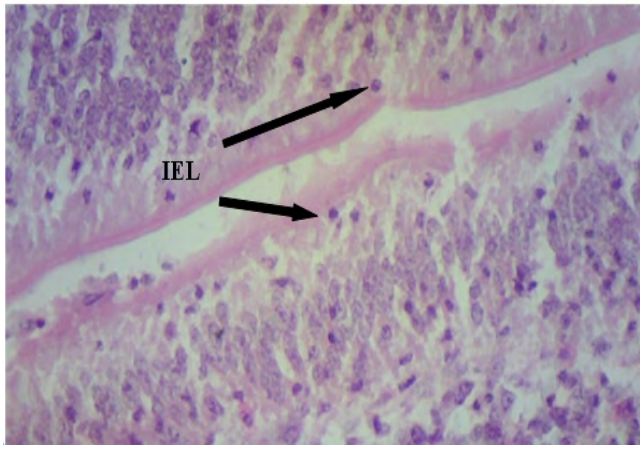


Fig 6. Histological section of duodenum showing villus height of calves fed AFB1 0.8- YTB.

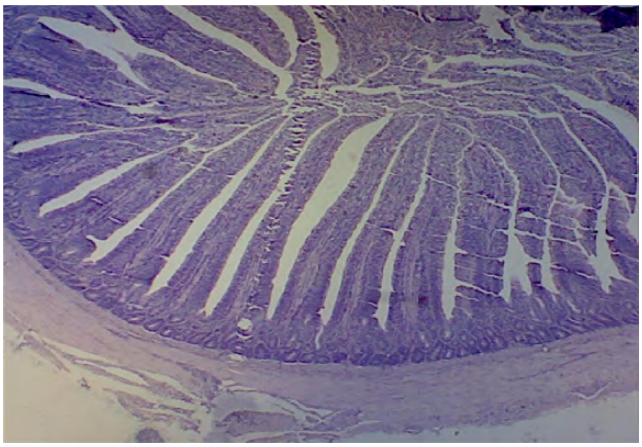
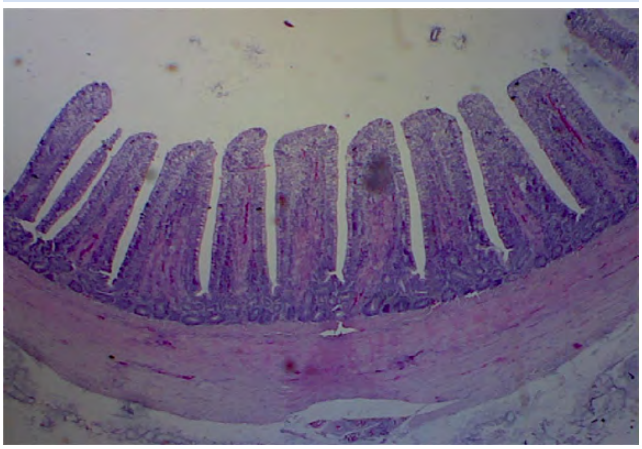


Fig 6. Histological section of duodenum showing villus height of calves fed AFB1 0.8.



protects intestinal epithelium by acting as a physical barrier to pathogens is secreted by GC (Kim and Ho, 2010). Goblet cells are responsible for secretion of mucin which after hydration becomes mucus (Cone, 2009). Number of GC in intestinal epithelium may vary with challenging substances and diet (Machado-Neto et al., 2013). Number of intestinal GC on exposure to AFB1 increased in our study, similar results were observed by Kenawy et al., (2009), who observed an increased GC number in intestine after exposure to AFB1 contaminated diet. Moreover, addition of YCW increases GC number in intestine (Chee et al., 2010; Morales-Lopez et al., 2010; Muthusamy et al., 2012), but there is no consensus on whether increase in GC number is considered an improvement in animal health or not (Lea et al., 2013).

To our knowledge no data is present regarding histochemistry of GC and immunohistochemistry of proliferative cells in male buffalo calves exposed to AFB1 or supplemented with YTB. Mucins secreated by goblet cells are either acidic, neutral or mixed in nature. In fish GC having acidic mucins protect intestinal epithelium against chemical agents while GC having neutral mucins provide protection against chemical agents (Cruz et al., 2014). Factors that lead to an increase in GC having acidic and neutral mucin under the influence of AFB1 or YTB supplementation are yet to be investigated. However increased number of GC having acidic or neutral mucin in response to YTB supplementation indicates greater intestinal protection against pathogens in male buffalo calves.

Amplified villus length is associated with an increase in number of proliferative cells as longer villi indicate faster proliferation in intestinal crypts (Wu et al., 2013). Decline in proliferative cells number reduces the amount of energy required for maintaining microarchitecture of gut (Masanetz et al., 2010). Yet the exact mechanisms that caused a diminution in proliferative cells number after YTB supplementation are needed to be explored.

CONCLUSIONS

Collectively, results of the current study confirm that feeding aflatoxin B1 (AFB1) contaminated diet exerts deleterious effects on intestinal microarchitecture. Supplementation of 2mg/kg yeast cell wall based toxin binder (YTB) not only alleviates harmful effects on intestinal microarchitecture induced by AFB1 but also supports modulations in defense system of male buffalo calves. Therefore, use of YTB in AFB1 contaminated feed is beneficial and recommended.

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Research article
Ερευνητικό άρθρο

Prevalence and antimicrobial resistance of *Salmonella* isolated from bovine and ovine samples in slaughterhouses of Algiers, Algeria

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ABSTRACT. The aims of our work are to estimate the prevalence of Salmonella isolated from carcasses and feces of cattle and sheep in the two biggest slaughterhouses in Algiers, Algeria, and to characterize the obtained strains by serotyping and antimicrobial resistance testing. The detection of *Salmonella* was performed by the conventional culture method and isolates were confirmed by PCR. Susceptibility to antibiotics was carried out by agar disc diffusion method. The results showed that 10.17% of samples were *Salmonella* positive. Carcass samples were more contaminated than fecal samples. Serotyping of the 84 *Salmonella* isolates has enabled to identify 10 different serovars; the most predominant was *S.* Muenster. The invA gene was detected in 96.43% of isolates whereas all *S.* Typhimurium strains were positive for spy gene. Sixty-eight (80.95%) isolates were resistant to at least one of the 28 antibiotics tested and exhibited 17 different antimicrobial resistance patterns. The most frequently observed resistance was to streptomycin (69.05%). While 22.62 % of the isolates were MDR, two *S.* Typhimurium showed an"ACSSuT" pentaresistance pattern. Considering the importance of this group of bacteria for public health, *Salmonella* control is necessary at several steps of food production to ensure safe products for consumers.

Keywords: *Salmonella*, slaughterhouse, prevalence, serovars, antimicrobial resistance.

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INTRODUCTION

The genus *Salmonella* is a Gram negative rod-shaped bacteria belonged to the family of *Enterobacteriaceae*. It includes more than 2500 serovars that inhabit the gastrointestinal tracts of various domestic and wild animal species (Bahnass et al., 2015). It is estimated that salmonellosis represents 93.8 (16%) million cases of human gastroenteritis among an estimated 582 million cases of 22 different food borne enteric diseases, and it is responsible for 155 000 deaths worldwide each year (Elgroud et al., 2015; Manoj et al., 2015). *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) is the causative agent of 46% of outbreaks (Ahmed and Shimamoto, 2014). The most common contaminated foods associated with human salmonellosis are poultry, beef, eggs, seafood, and dairy products (Abbassi-Ghozzi et al., 2012). At the production level, inadequate sanitation in slaughterhouses, improper handling of meat, and cross- contamination through processing equipment are some of the most frequent causes of the spread of *Salmonella* (Singh and Mustapha, 2014).

Conventional bacterial culture methods are still used most often to detect and identify *Salmonella* and require at least 3-11 days including selective enrichment and plating followed by biochemical tests (Karmi, 2013). Recently, PCR-based techniques are used effectively for rapid detection of *Salmonella* serovars (Can et al., 2014).

The purposes of this study are to monitor the prevalence of *Salmonella* in bovine and ovine carcasses and feces in Algiers, by using conventional culture method and PCR assay, and to determine the antimicrobial resistance profiles of the isolates. Bovine and ovine carcasses were used as test items because they are widely consumed in Algeria. Additionally, a few national studies have been conducted on the prevalence of *Salmonella* in red meats.

MATERIAL AND METHODS

Samples collection

Cattle and sheep were brought to El-Harrach and Hussein Dey slaughterhouses from different regions of the country. During two periods, from February to June 2013, and from December 2013 to May 2014, 826 ovine and bovine samples were collected among which 190 from bovine carcasses, 251 from ovine carcasses, 160 bovine feces and 225 from ovine feces. Samples were obtained immediately after evisceration. Carcass samples were obtained using the wet and dry swabbing method with 2 sponges for each of the four sites chosen in accordance with Annex A of the ISO standard 176048. The four wet-dry swabs pairs from each carcass were pooled, and processed as one sample. Fresh fecal samples were collected directly from rectum at the time of slaughtering and packed into separate sterile polyethylene bags. Then, all samples were transported on ice to the laboratory for immediate processing and analysis.

Isolation and identification of *Salmonella* spp. by conventional method

Isolation of *Salmonella* spp. from all samples was performed according to the ISO norm 6579:2002 (Annex D010705). Briefly, fecal samples were diluted at 10-1 with buffered peptone water (Institut Pasteur d'Algérie [IPA], Algiers, Algeria). Carcass swabs were put into 100 mL BPW.

After incubation, 1 and 0.1mL of pre-enriched broth were, respectively, transferred to Müller Kauffmann Tetrathionate- novobiocin broth (IPA, Algiers, Algeria) and to Rappaport-Vassiliadis with soya (IPA, Algiers, Algeria), then incubated for 24 h at 37 °C and 42°C, respectively. A loopful from each selective enrichment broth was streaked onto selective xylose-lysine-deoxycholate and Hektoen agar plates (IPA, Algiers, Algeria), and incubated at 37°C for 24 h. Presumptive *Salmonella* colonies were characterized using classical biochemical tests with Triple Sugar Iron (TSI; IPA) agar slant, indole urea reagent (IPA), Lysine Decarboxylase (LDC; IPA) reagent and *ortho*-NitroPhenyl-β-galactoside (ONPG; IPA) , as well as Api 20E gallery (Biomérieux, SA, France).

Serotyping was carried out using slide agglutination with commercial poly O (OMA, OMB), and poly H antigen specific antisera (Difco, Sparks, MD. USA). Once the antigenic formulae were obtained, the Kauffmann-White scheme was used to name the serovars.

Molecular confirmation

Molecular confirmation of isolated *Salmonella*

strains was carried out using the PCR technique. *S.* Typhimurium ATCC 14028 was used as positive control in this study.

DNA extraction

The genomic DNA was extracted from a culture incubated overnight. One or two colonies were suspended in 500 µL of molecular biology water (AccuGENE®, Lonza Group Ltd., Basel, Switzerland), and boiled 10 minutes at 95°C. After adding 100 µL of 5 M NaCl, and centrifuging, the supernatant was removed to a new tube, and 500 µL of cold 100% ethanol were added. A second centrifugation was performed and the supernatant was poured and the DNA pellet was washed in 500 µL of 70% ethanol, and centrifuged, then dried for 30 min at 37°C. Finally, the DNA was re-suspended in 100 µL of DEPC water (Sigma-Aldrich, St. Louis, MO, USA), and stored at - 20°C until use.

Primers sets and PCR amplification

Specific primers for *Salmonella* spp. and *S.* Typhimurium have been published previously (Rahn et al., 1992; Olsen et al., 1995).

Individual PCR assays were performed according to the original published protocols (Rahn et al., 1992; Can et al., 2014). The PCR mixture was consisted of 25 µL final volume containing 1X PCR buffer (Sigma- Aldrich, St-Louis, USA), 200µM of each dNTPs (Dr. Zeydanlı Life Sciences Ltd., Ankara, Turkey), 3mM of MgCl2 (Sigma- Aldrich, St-Louis, USA), 1U *Taq* polymerase (Sigma- Aldrich, St-Louis, USA), 0.4 µM of each primer, and 2 µL template DNA.

The following amplification conditions were used: an initial denaturation step for 3 min at 94°C. Then, 30 cycles, each one consisting of denaturation at 94°C for 30 seconds, 58°C as annealing temperature for 45 seconds, and elongation at 72°C for 60 seconds. Finally, a terminal elongation step of 5 min at 72°C was performed.

For *Salmonella* serovar Typhimurium, PCR protocol was the same, except the annealing temperature, which was established at 55°C (Can et al., 2014).

Electrophoresis of PCR products

The PCR amplified products were electrophoresed in 1.5% agarose gel (AXYGEN Bioscience), stained with 3 µL/g of Ethidium Bromide (Dr. Zeydanlı Life Sciences Ltd., Ankara, Turkey). A 100 pb ladder (AXYGEN Bioscience) was served as a molecular weight marker. In each PCR run, a non-template control (negative control) was included to detect possible external DNA contamination. DNA bands were visualized under UV transillumination (UVP, Upland, USA) and photographed.

Antimicrobial susceptibility test

Antimicrobial susceptibility patterns of *Salmonella* isolates were determined by the agar disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) using Mueller- Hinton agar (IPA, Algiers, Algeria). The plates were incubated at 35°C for 24h. The following antibiotic discs (Oxoid, Hampshire, United Kingdom) were used: ampicillin (AMP, 10 µg), ticarcillin (TIC, 75 µg), piperacillin (PRL, 100 µg), amoxicillin (AML, 25 µg), mecillinam (MEL,10 µg), cefazolin (KZ, 30 µg), cefoxitin (FOX, 30 µg), ceftazidine (CAZ, 30 µg), ceftriaxone (CRO, 30 µg), cefepime (FEP, 30 µg), amoxicillin/clavulanate (AMC, 20 µg/10 µg), aztreonam (ATM, 30 µg), imipenem (IPM, 10 µg), kanamycin (K, 30 µg), gentamicin (GM, 10 µg), netilmicin (NET, 30 µg), streptomycin (S, 10 µg), nalidixic acid (NA, 30 µg), norfloxacin (NOR, 10 µg), ciprofloxacin (CIP, 5 µg), sulphonamides (SSS, 300 µg), trimethoprim (W, 5 µg), trimethoprim/sulfamethoxazole (SXT, 1.25 µg/23.75 µg), furans (F, 300 µg), chloramphenicol (C, 30 µg), tetracycline (Te, 30 µg), colistin (CT, 10 µg), and fosfomycin (FOS, 50 µg). The results were recorded by measuring the inhibition zones and scored as sensitive, intermediate, and resistant according to the Clinical Laboratory Standards Institute (CLSI, 2008) criteria.

RESULTS

***Salmonella* prevalence**

Out of 826 samples, 84 (10.17%) were *Salmonella* positive. The relative prevalence within the slaughterhouses was 14.7% (56/381) for El-Harrach and 6.29% (28/ 445) for Hussein Dey. *Salmonella* was detected in all types of samples with different frequencies, 20.52% (39/190) in bovine carcasses, 12.74% (32/251) in ovine carcasses, 6.87% (11/160)

in bovine feces, and 0.89% (2/225) in ovine feces. Bovine samples were more contaminated (14.28%) than ovine samples (7.14%). The frequency of isolation was higher from carcasses (16.10%) than from the feces ones (3.37%) (Table 1).

(14.28%) and *S.* Anatum (13.09%). The highest proportions of *S.* Muenster were recovered from bovine and ovine carcasses, with 48.48% and 33.33%, respectively, compared to bovine and ovine feces (15.15% and 3.03% respectively) (Table 2).

Table 1. Prevalence of *Salmonella* in bovine and sheep carcass and fecal samples from the two slaughterhouses

Animal species	Slaughterhouses								**Total**	
	El-Harrach				Hussein Dey					
	Carcasses		Feces		Carcasses		Feces			
	n	n Positive (%)	n	n Positive (%)	n	n Positive (%)	n	n Positive (%)	**n**	**n Positive (%)**
Bovine	85	29 (34.12%)	78	9 (11.54%)	105	10 (9.52%)	82	2 (2.44%)	**350**	**50 (14.28%)**
Ovine	112	18 (16.1%)	106	00 (0.0%)	139	14 (10.1%)	119	2 (3.4%)	**476**	**34 (7.14%)**
Total	**197**	**47 (23.86%)**	**184**	**9 (4.89%)**	**244**	**24 (9.84%)**	**201**	**4 (1.99%)**	**826**	**84 (10.17%)**

n: number of samples.

Table 2. Distribution of *Salmonella* serovars.

Serovar	**Slaughterhouses**												**Total (%)**
	El-Harrach						**Hussein Dey**						
	Bv C	Ov C	**Total**	Bv F	Ov F	**Total**	Bv C	Ov C	**Total**	Bv F	Ov F	**Total**	
S. Muenster	14	7	**21**	5	0	**5**	2	4	**6**	0	1	**1**	**33 (39.28)**
S. Kentucky	3	2	**5**	0	0	**0**	6	2	**8**	0	0	**0**	**13 (15.47)**
S. Infantis	4	6	**10**	2	0	**2**	0	0	**0**	0	0	**0**	**12 (14.28)**
S. Anatum	1	0	**1**	0	0	**0**	1	7	**8**	1	1	**2**	**11 (13.09)**
S. Richmond	4	0	**4**	0	0	**0**	0	0	**0**	0	0	**0**	**4 (4.76)**
S. Havana	2	0	**2**	0	0	**0**	1	0	**1**	0	0	**0**	**3 (3.57)**
S. Typhimurium	0	2	**2**	0	0	**0**	0	1	**1**	0	0	**0**	**3 (3.57)**
S. Montevideo	1	0	**1**	2	0	**2**	0	0	**0**	0	0	**0**	**3 (3.57)**
S. Virginia	0	1	**1**	0	0	**0**	0	0	**0**	0	0	**0**	**1 (1.19)**
S.Braenderup	0	0	**0**	0	0	0	0	0	**0**	1	0	**1**	**1 (1.19)**
Total (%)	**29**	**18**	**47 (55.95)**	**9**	**0**	**9 (10.71)**	**10**	**14**	**24 (28.57)**	**2**	**2**	**4 (4.76)**	**84**

Bv C: bovine carcasses, Ov C: ovine carcasses, Bv F: bovine feces, Ov F: ovine feces.

Distribution of *Salmonella* serovars

A position ten (10) different serovars were identified. The most common was *S.* Muenster (39.28%) followed by *S.* Kentucky (15.47%), *S.* Infantis

Molecular confirmation

B position in PCR assay, using S139 and S141 primers belonging to *inv*A gene that amplifies a 284 bp sequence of the *inv*A gene, 96.43% (81/84) of pos-

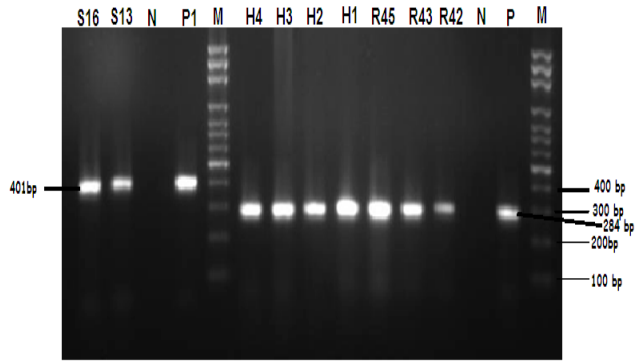


Fig. 1 Specific PCR of *Salmonella* isolates using primer sets *inv*A and *Spy*.
M: 100bp Marker (AXYGEN Bioscience), P: Positive Control (*Salmonella* spp.), N: Negative control (PCR mixture without DNA), R42 R43 R45 H1 H2 H3 and H4: Analyzed isolates showing positive 284 bp DNA fragment of *inv*A gene specific for *Salmonella* spp., P1: Positive control (*Salmonella* Typhimurium), S13and S16: Analyzed isolates showing positive 410bp DNA of *Spy* gene specific for *Salmonella* Typhimurium.

itive samples in conventional culture method including *arizona* generated a single 284 bp amplified DNA fragment on agarose gel (Fig. 1).

Serotyping revealed 3 *S.* Typhimurium isolates of ovine carcass origin and PCR assay shown the presence of specific amplified product 410bp obtained with S. Typhimurium primers chosen from the *Spy* gene (Fig. 1)

Antimicrobial resistance of isolates

From a total of 84 *Salmonella* isolates evaluated for resistance against a panel of 28 selected antimicrobial agents, 68 (80.95%) were resistant to at least one antimicrobial.

All *Salmonella* isolates were susceptible to cefoxitin, ceftazidine, ceftriaxone, cefepime, aztreonam, imipenem, kanamicin, netilmicin, colistin, and fosfomycin. Resistance to the remaining 18 antimicrobials varied between 1.19% and 69.05%. The highest resistance rate observed was to streptomycin (69.05%), followed by sulphonamides (32.14%), then ampicillin, ticarcillin, piperacillin, amoxicillin, nalidixic acid and tetracycline (each 17.86%) (Table 3).

Out of the 68 resistant *Salmonella* isolates, 19 (22.62%) were multidrug resistant (MDR) among which 15 isolates were resistant to more than 5 antimicrobials. Twelve isolates of *S.* Kentucky isolated displayed resistance to at least 10 antimicrobials including fluoroquinolones. The three *S.* Typhimurium strains expressed resistance to more than 9 antimicrobials, including the ‘‘ACSSuT’’ pentaresistance pattern showed by two strains. Among the 10 serovars identified, resistance was found in 9 of them. Only the 3 isolates of *S.* Havana did not show any resistance to all antimicrobials tested. Table 4 displayed 17 different resistance patterns including 14 MDR patterns.

DISCUSSION

Salmonella prevalence

Table 3. Antimicrobial resistance of *Salmonella* isolates from bovine and ovine samples

Serovars	n	Antibiotics*																			Recapitulatory		
		AMP	TIC	PRL	AML	AMC	MEL	KZ	GM	S	SSS	W	SXT	NA	NOR	CIP	C	TE	F	0	1	2-5	+5
S. Muenster	33	0	0	0	0	0	0	0	0	19	8	0	0	0	0	0	0	0	6	27	0	0
S. Anatum	11	0	0	0	0	0	0	0	0	3	2	0	0	0	0	0	0	1	0	7	2	2	0
S. Infantis	12	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	12	0	0
S. Kentucky	13	12	12	12	12	6	4	10	9	13	13	1	1	12	11	12	0	11	0	0	0	1	12
S.Havana	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0
S. Richmond	4	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	4	0	0
S. Typhimurium	3	3	3	3	3	2	0	0	0	3	3	1	1	2	0	0	2	3	1	0	0	0	3
S. Montevideo	3	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	3	0	0
S. Virginia	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
S. Braenderup	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0
Total	84	15	15	15	15	8	4	10	9	58	27	2	2	15	11	12	2	15	1	16	49	4	15
(%)	100	17.86	17.86	17.86	17.86	9.52	4.76	11.90	10.71	69.05	32.14	2.38	2.38	17.86	13.09	14.28	2.38	17.86	1.19	19.05	58.33	4.76	17.86

*n: number of isolates, AMP: ampicillin, TIC: ticarcillin, PRL: piperacillin, AML: amoxicillin, AMC: amoxicillin/clavulanate, MEL: mecillinam, KZ: cefazolin, GM: gentamicin, S: streptomycin, SSS: sulphonamides, W: trimethoprim, SXT: trimethoprim/sulfamethoxazole, NA: nalidixic acid, NOR: norfloxacin, CIP: ciprofloxacin, C: chloramphenicol, Te: tetracycline, F: furans.*All Salmonella isolates were susceptible to cefoxitin, ceftazidine, ceftriaxone, cefepime, aztreonam, imipenem, kanamicin, netilmicin, colistin, and fosfomycin.*

Table 4. Resistance pattern profiles of isolated *Salmonella* strains.

Salmonella serovars (n/N)	Resistance patterns	n
S. Muenster (27/33)	S	19
	SSS	8
S. Anatum (4/11)	S	2
	S,SSS	1
	SSS,TE	1
S. Infantis (12/12)	S	12
S. Kentucky (13/13)	S,SSS,W,SXT	1
	AMP,TIC,PRL,AML,KZ,S,SSS,NA,CIP,TE	1
	AMP,TIC,PRL,AML,KZ,S,SSS,NA,NOR,CIP	1
	AMP,TIC,PRL,AML,KZ,S,SSS,NA,NOR,CIP,TE	1
	AMP,TIC,PRL,AML,GM,S,SSS,NA,NOR,CIP,TE	1
	AMP,TIC,PRL,AML,KZ,GM,S,SSS,NA,NOR,CIP,TE	1
	AMP,TIC,PRL,AML,MEL,GM,S,SSS,NA,NOR,CIP,TE	1
	AMP,TIC,PRL,AML,AMC,KZ,GM,S,SSS,NA,NOR,CIP,TE	3
	AMP,TIC,PRL,AML,AMC,MEL,KZ,GM,S,SSS,NA,NOR,CIP,TE	3
S. Richmond (4/4)	S	4
S. Typhimurium (3/3)	AMP,TIC,PRL,AML,S,SSS,W,SXT,TE	1
	AMP,TIC,PRL,AML,AMC,S,SSS,NA,C,TE	1
	AMP,TIC,PRL,AML,AMC,S,SSS,NA,C,TE,FU	1
S. Montevideo (3/3)	S	3
S. Virginia (1/1)	NA	1
S. Braenderup (1/1)	S,SSS	1

N: total of isolates, n: number of resistant isolates, AMP: ampicillin, TIC: ticarcillin, PRL: piperacillin, AML: amoxicillin, AMC: amoxicillin/clavulanate, MEL: mecillinam, KZ: cefazolin, GM: gentamicin, S: streptomycin, SSS: sulphonamides, W: trimethoprim, SXT: trimethoprim/sulfamethoxazole, NA: nalidixic acid, NOR: norfloxacin, CIP: ciprofloxacin, C: chloramphenicol, Te: tetracycline, F: furans.

In the current study, out of 826 tested samples, 84 (10.17%) were positive for *Salmonella* reflecting the failure of hygiene practices during the slaughtering. Furthermore, it is suggested that the presence of even small numbers of *Salmonella* species in carcasses may lead to heavy contamination of the finished retail product (Dabassa and Bacha, 2012; Ateba and Mochaiwa, 2014). According to the results shown in table 1, bovine samples were more contaminated (14.28%) than ovine samples (7.14%). This result in agreement with previous Algerian data (Nouichi and Hamdi, 2009; Mezali and Hamdi, 2012), could be due to the particular susceptibility of bovine species to *Salmonella* infection (Nouichi and Hamdi, 2009).

Bovine carcasses are most contaminated by *Salmonella* (20.52%). Previously, we have reported a lower rate of contamination (10%) in the same type of samples (Nouichi and Hamdi, 2009). Worldwide, previous reports indicated a variable prevalence of *Salmonella* in beef meat and carcasses ranged from 1.4% to 13.3% (Dabassa and Bacha, 2012; Tafida et al., 2013; Ahmed and Shimamoto, 2014; Ateba and Mochaiwa, 2014; Dong et al., 2014).

In ovine carcasses, the prevalence was 12.74%. While Teklu and Negussie (2011) registered a similar result (14.1%), other studies reported lower rates of contamination: 1.11% (Nouichi and Hamdi, 2009), and 3.3% (Dabassa and Bacha, 2012).

The prevalence of *Salmonella* in bovine feces was 6.87%. Compared to other studies that evaluated *Salmonella* in cattle fecal samples, our results corroborate relatively the reports of Addis et al. (2011), and Bahnass et al. (2015), who found 7.69% and 8.5%, respectively. Yet, it is more than 7 times lower than the 52% found by Kagambèga et al. (2013). On the other hand, Bordonaro et al. (2015) reported a very low rate (1.7%).

The prevalence of *Salmonella* in ovine fecal samples remains fairly low (0.89%) compared to that

recorded in feces of cattle (7.5%), and that reported in previous studies: 3.3% (Dabassa and Bacha, 2012), and 6.4% (Bahnass et al., 2015).

The present study showed a considerably higher prevalence of *Salmonella* in carcass samples (16.10%) than feces (3.38%), which is consistent with the findings from previous reports of Teklu and Negussie (2011)), Dabassa and Bacha (2012) on sheep and cattle samples, respectively. However, our results contrast with the study's finding of Dong et al. (2014), which indicated higher *Salmonella* prevalence in feces than in carcasses. The relationship between fecal shedding and carcass contamination seems weak. It may be related to the fact that healthy carrier animals especially bovines excrete only a few number of *Salmonella*, unless they undergo some kind of stress (Teklu and Negussie, 2011), and the high level of *Salmonella* on carcasses might be explained by contamination from other sources such as animal skins, operators' hands and equipment, considering the non-respect of slaughtering hygienic rules observed during our study. Nevertheless, the presence of even a carrier animal can be a potential source of contamination of carcasses, environment, material and personnel. Furthermore, fecal samples tend to be less clean than carcasses and other food products samples, and therefore, it is more difficult to grow and detect *Salmonella* in these samples because of other organisms and species of competitive bacteria (Bordonaro et al., 2015).

Distribution of *Salmonella* serovars

Ten serovars were identified in the current study. Based on the results obtained, there seemed to be a difference in the types of *Salmonella* serovars from the different sources: *S.* Muenster and *S.* Anatum were isolated from the four categories of samples. This would likely reflect cross-contamination from multiple sources and poor hygiene conditions in the slaughterhouses. While certain serovars were exclusively recovered from only one source, such as, *S.* Richmond and *S.* Typhimurium, which were isolated from cattle and sheep carcasses, respectively. The identification of *S.* Kentucky only in carcass samples could be explained that the origin of the contamination wasn't feces.

S. Muenster found predominant in this study (39.28%), was also the most prevalent serovar in each category of samples. According to Van Cauteren et al. (2009), this serovar is rarely identified from humans, foods or animals.

The other serovars recovered, including *S.* Kentucky, *S.* Infantis, *S.* Anatum, *S.* Typhimurium and *S.* Montevideo are, without respecting the ranked order, among the top 10 serovars encountered in Africa (Hendriksen et al., 2011), in Europe (EFSA, 2014), and in USA (CDC, 2014).

S. Kentucky was isolated from 15.47 % of samples. According to our results during this study and the international studies pre-established in Algeria (Bouzidi et al., 2012; Elgroud et al., 2015), Morocco (El Allaoui et al., 2014), Tunisia (Abbassi-Ghozzi et al., 2012), and Nigeria (Tafida et al., 2013), this result is alarming because the sudden emergence and worrying of *S.* Kentucky has shown an increasingly insensitive to almost all families of antibiotics.

Previous national studies demonstrated that *S.* Anatum was found to be the most prevalent serovar isolated from bovine and ovine carcasses (Nouichi and Hamdi, 2009) and from red meat and their products (Mezali and Hamdi, 2012) in Algiers. Otherwise, *S.* Typhimurium, *S.* Heidelberg, *S.* Enteritidis and *S.* Hadar were mainly isolated from broilers by Bounar-Kechih et al. (2012), Bouzidi et al. (2012), Elgroud et al. (2015), respectively. These findings suggest that several serovars of *Salmonella* that may cause significant animal and human illnesses occur in Algeria.

At the international level, *S.* Enteritidis and *S.* Typhimurium were reported to be the most frequently isolated (Hendriksen et al., 2011; CDC, 2014; EFSA, 2015).

These differences in the prevalence of *Salmonella* and the distribution of serovars between studies may depend on the isolation methodology, the geographic area, and the housing and husbandry conditions.

Molecular confirmation

The present study supports the ability of the S139 and S141 primers targeting the *inv*A gene specific of *Salmonella* spp. to confirm the isolated colonies.

Out of the 84 *Salmonella* isolates tested, 81 yield-

ed desired amplified products of approximately 284 bp similar to that of reference strain of *Salmonella* using the primer pairs for *inv*A. The efficiency was 96.43%. Our report corroborates many recent studies in Egypt (Maysa and Abd-Elall, 2015) and Nigeria (Smith et al., 2015) conducted on *Salmonella* isolated from human, animals, food and water samples in which *inv*A gene (284 bp) was prevalent at 96%. Karmi (2013) reported that all *Salmonella* isolates positive for the presence of *inv*A gene, have the capacity to invade and survive in macrophages.

In contrary, Karmi (2013), Tafida et al. (2013), and Dong et al. (2014) have detected and reported the *inv*A gene in all *Salmonella* isolates tested.

In this study, the DNA of three *Salmonella* isolates confirmed biochemically and serotyped (one *S.* Muenster, one *S.* Anatum, and one *S.* Infantis) was not amplified by PCR. Although, Rahn et al. (1992) could not detect S. Litchfield and S. Senftenberg by using S139 and S141 primers. Malorny et al. (2003) managed to do it with the same primers after modification in the thermal cycling conditions and using hot start PCR.

The failure to amplify *inv*A homologues sequences was most likely due to the absence of the *inv*A gene in these *Salmonella* strains (Rahn et al., 1992). Ginocchio et al. (1997) determined that the invasion-associated pathogenicity island which has previously been shown to be linked to the *inv* locus remains unstable in certain *Salmonella* serovars. It would also suggest that these organisms would not be invasive or, alternatively, that they may possess other pathways of invasion independent of *inv*A gene (Rahn et al., 1992; Ginocchio et al., 1997). In addition some *Salmonella* species also could not be detected by other PCR methods (Van Kessel et al., 2003).

Interestingly, the strains that were shown to lack of the *inv*A sequences in the two studies of Rahn et al. (1992) and Ginocchio et al. (1997) had been recovered from environmental samples and were not specifically associated with disease. Even whether the three isolates found negative for *inv*A gene during our study were isolated from carcasses; it is possible that all of them were originated from environment.

After identifying the *inv*A gene as specific to *Salmonella*, PCR has also been used for distinguishing of *Salmonella* serovars. As for *S.* Typhimurium, the *Salmonella* plasmid virulence s*pv* genes were used (Olsen et al., 2014). These genes are particularly required for systemic infection (Nickerson and Curtiss, 1997). In our study, all isolates serotyped *S.* Typhimurium were found positive for *spv* gene. The sensibility of the primer used in this study has also been also well demonstrated using simplex or multiplex PCR assays (Ahmed and Shimamoto, 2014; Can et al., 2014; Manoj et al., 2015).

Antimicrobial resistance of isolates strains

In the current study, the rate of resistance to at least one antimicrobial is high (80.95%) and could be explained by the widespread and indiscriminate use of the drugs for therapeutic and prophylactic purposes both in veterinary and human health sectors. This is in accordance with previous results recorded in Algeria (80% [Elgroud et al., 2009]; 68.42% [Bouzidi et al., 2012]; 90.32% [Mezali and Hamdi, 2012]), in Egypt (100% [Sallam et al., 2014]) and in Morocco (93.5% [El Allaoui et al., 2014]). Resistance to streptomycin was quite common (69.05%, n=58) and corroborates the finding of Elgroud et al. (2009; 58%) and Aouf et al. (2011; 68.75%), while 17.86% (n=15) of isolates were found resistant to tetracyclines, which is lower than that noted by Bouzidi et al. (2012; 36.9%) and Aouf et al. (2011; 100%). As for sulphonamides, our result (32.14%) was higher than that reported in Algeria by Bounar-Kechih et al. (2012) and Mezali and Hamdi (2012) who recorded 13% and 16.13%, respectively. Streptomycin, sulphonamides and tetracycline are old first-intention molecules and have been widely used in animal husbandry. In addition to streptomycin, resistance to aminoglycosides involved also gentamicin, (10.71%); all of the isolates resistant to this drug are belonging to *S.* Kentucky serovar. A similar result has been registered by Bouzidi et al. (2012) and Le Hello et al. (2013).

Only 2 isolates displayed resistance to the association trimethoprim/sulfamethoxazole, which is lower than the result of Aouf et al. (2011). In the present study, 15 (17.86%) isolates were resistant to nalidixic acid; 18.75% were recorded by Aouf et al. (2011). Increasing resistance to this antimicrobial has been

also reported by the national studies (Elgroud et al., 2009; Bounar-Kechih et al., 2012; Mezali and Hamdi, 2012).

As for furans and chloramphenicol 1.19% and 2.38% were recorded respectively. These findings corroborate those of Mezali and Hamdi (2012) and could be explained by the moderate use of these drugs because of their removing from the Algerian nomenclature.

Moreover, all of the *S.* Kentucky isolates were found to be resistant to fluoroquinolones (norfloxacin and/or ciprofloxacin). To our knowledge, this is the first national paper showing resistance to these drugs in *Salmonella* strains isolated from red meats since the other previous studies in Algeria (Elgroud et al., 2009; Bouzidi et al., 2012) were done on poultry.

This finding is more worrying as fluoroquinolones should be reserved for the treatment of serious gastrointestinal infections in adults. This may be linked to a non-prudent use of these molecules, although expensive in animal husbandries in Algeria (Elgroud et al., 2009).

Full resistance to quinolones is achieved when two cumulative mutations in genes that encode the targets of these drugs are present concurrently (Le Hello et al., 2013; El Allaoui et al., 2014).

It appears that isolates tested in this study underwent this kind of mutation only because of which they were resistant to nalidixic acid and to other quinolones molecules such as norfloxacin and ciprofloxacin. Resistance to β-lactams involved only penicillins (ampicillin, ticarcillin, piperacillin, amoxicillin, mecillinam and the combination amoxicillin/clavulanic acid) and first generation cephalosporins (cefazolin). Conversely, the absence of resistance to third generation cephalosporins was an important finding since they are clinically essential in the treatment of invasive salmonellosis in humans. Multiple drug resistance in *Salmonella* may result from random chromosomal mutations and transfer of resistance genes (Abbassi-Ghozzi et al., 2012). Multidrug resistant *Salmonella* serovars have been proposed to be more virulent than non-multidrug resistant ones (Sallam et al., 2014).

In our study, 14 different MDR patterns were found. The two serovars commonly involved in food-borne outbreaks, *S.* Kentucky and *S.* Typhimurium presented the greatest number of multi-resistance phenotypes. Transmission of multi-resistant *Salmonella* to humans through food chain may involve a high risk for public health by compromising the effectiveness of medical treatment and increasing the number of invasive infections. Two *S.* Typhimurium isolates displayed an ‘‘ACSSuT’’ pentaresistance pattern. This is another worrying antimicrobial pattern evidenced during this study was also found by Mezali and Hamdi in 2012. In Europe, resistance to ‘‘ACSSuT’’ was the most common multidrug-resistant pattern recorded among the multidrug-resistant *Salmonella* Typhimurium isolated from human and food sources (EFSA, 2015). All the strains displaying MDR were isolated from both ovine and bovine carcasses. By contrast, all *Salmonella* isolated from feces were resistant only to streptomycin or sulphonamides. This finding confirms that the high number of *Salmonella* isolated from carcasses during this study is not mainly associated with fecal carriage.

CONCLUSIONS

Overall, our findings showed that the bovine and ovine carcasses are considered as an important source of multidrug-resistant *Salmonella* serovars and can pose a high risk for the consumer; subsequently, hygienic measures should be undertaken to reduce contamination of meat with virulent strains of *Salmonella*, and strict guidelines for the use of antibiotics should be necessary to prevent the dissemination and acquisition of antimicrobial resistance. This study also demonstrated that 96.43% of *Salmonella* isolates were positive for the presence of virulence gene (*inv*A) that responsible for cell invasion. Furthermore, it is important to emphasize that PCR method based on *inv*A gene should be used for rapid identification of *Salmonella* serovars and could replace the conventional bacteriological and biochemical methods.

CONFLICT OF INTEREST STATEMENT

The authors report no conflict of interest.

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Short communication
Σύντομη δημοσίευση

Aflatoxin M1 in Nili-ravi buffaloes and its detoxification using organic and inorganic toxin binders

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ABSTRACT. The present study had two objectives: first, to determine the carry over or excretion percentage of aflatoxin B1 (AFBI) in milk in form of aflatoxin M1 (AFM1) and second, to assess the reduction in excretion of AFM1 in milk using different organic and inorganic toxin binders available in Pakistani market. Lactating Nili-Ravi buffaloes (n=16) were randomly selected and were divided into four treatment groups designated as A, B, C and D. In each treatment 500 µg/Kg of aflatoxin B1 (AFB1) was fed along with no sequestering agent added (control); and three toxin binders: Fixar Viva in group B, Mycosorb in group C and T5X in group D. These toxin binders were added at concentration of 0.25% of dry matter intake of animal. It resulted in 2.13% carryover in milk as AFM1. A significant reduction ($P<0.05$) in dry matter intake, milk production, milk fat and protein percentage was also observed by feeding AFB1. Addition of three toxin binders Mycosorb, Fixar Viva, and T5X at a concentration of 0.25% in ration resulted in 47%, 39%, and 35% reduction in AFM1 secretion respectively. The present study also indicated that percentage carryover of AFM1 in buffaloes is higher than that reported in lactating cows as well as in goats and Mycosorb is capable of reducing the excretion of AFM1 into milk by improving the dry matter intake, milk production and protein contents. These findings may be applicable in field to reduce AFM1 release in milk of Nili-Ravi buffaloes.

Keywords: Aflatoxins, Buffaloes, Carry-over percentage, Milk, Toxin binders

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INTRODUCTION

Aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. The most abundant aflatoxin in naturally contaminated dairy rations is aflatoxin B1 (Soufiani et al., 2016). The occurrence of aflatoxin in feed and feed commodities is more common in countries like Pakistan where temperature and humidity are higher due to optimum growing conditions for molds. Aflatoxins cause liver damage, decrease growth rate and milk production by deteriorating its quality. After ingestion in lactating animals aflatoxin B1 (AFB1) is metabolically bio-transformed by cytochrome P enzyme into hydroxylated form "Aflatoxin M1" that is excreted in milk (Chohan et al., 2016). Contamination of aflatoxin M1 in milk is a matter of serious concern due to its carcinogenic activity as reported by International Agency for Research on Cancer (IARC). The level of AFM1 is also not reduced during pasteurization due to its heat stability; therefore it remains in UHT milk (Diaz et al., 1995) and milk products. Approximately 0.3-6.2% of AFB1 in animal feed is transformed to AFM1 in milk but this carry over may be affected by different factors such as animal species involved. As almost all the previous studies involve cows (Iqbal and Asi, 2013), sheep or goat as experimental animals but not a single study had been conducted on buffaloes that is contributing to 75% of Pakistan's milk yield. Therefore we need to investigate the carry-over percentage of AFM1 in Nili-ravi buffaloes that are the main source of milk in our country.

Moreover, due to high potential risks to human health and economic losses of dairy industry associated with aflatoxins, World Health Organization (WHO) recommends their detoxification to minimum level. Use of toxin binders is reported to be a good practicable method for aflatoxin decontamination. Currently a number of toxin binders for aflatoxins are commercially available in Pakistan. However, there is need to evaluate their effectiveness on carry-over reduction of AFB1 to AFM1 in milk.

Keeping in view the above facts the present study was designed with two objectives; first to determine the excretion percentage of AFM1 in lactating buffaloes and second to uncover the effect of organic and inorganic toxin binders (commercially available in Pakistan) on detoxification of aflatoxin B1 in these animals. The conclusion and recommendations drawn from this study will be helpful for policy makers to implement a strict regulation on AFB1 in food and feed to reduce or avoid the contamination of AFM1 in milk and dairy products.

MATERIALS AND METHODS

Production of AFB1

To harvest AFB1, *Aspergillus flavus* strain NRRL 2999 culture was used as inoculant according to method described by Shotwell et al. (1966). Briefly, 30g of polished rice and 10 mL of distilled water was added to a 300 mL Erlenmeyer flask, autoclaved at 15 psi for 15 min and cooled down to room temperature, and inoculated with culture for fermentation. Further, flasks were placed on an orbital shaker with 130 revolution/min for 10-14 days at 25°C. This process was repeated again and again until required amount of aflatoxin was obtained. Rice was harvested when its color turned brown.

Quantification of AFB1

Brown color fermented rice were collected and mixed in 60 mL of chloroform for 30g of fermented rice. Extraction was made by refluxing for 4hrs and then extract was filtered through cheesecloth. Refluxing was conducted three times to recovered maximum quantity of aflatoxin B1. Then filtrate was dried over thin layer chromatography plate and was transferred in chromatography tank. Methanol (3mL) was added in 97 mL of chloroform and was transferred in tank along with chromatography plate (Gallo et al., 2010). Plates were inspected for aflatoxin B1 in a chromate-viewer to find the components by fluorescence. Quantification of aflatoxin B1 was determined by visual comparison of fluorescence zone with the known quantity of zone formed by the standards of aflatoxin. Aflatoxin B1 was separated by scratching the chromatogram. This was stored in freezer at-20°C to prevent its breakdown.

Experimental design

The experiment was carried out in Livestock Experiment Station, Haroonabad, Pakistan. Lactating Nili-Ravi buffaloes (n=16) were randomly selected and were divided into four treatment groups designated as A, B, C and D. In each treatment 500 µg/Kg of aflatoxin B1 (AFB1) was fed along with no seques-

tering agent added (control); and with three toxin binders: Fixar Viva in group B, Mycosorb in group C and T5X in group D. These toxin binders were added at concentration of 0.25% of dry matter intake of animal. Experiment was divided in two phases. First phase was pre-experimental (6 days) and second was experimental phase (28 days). During the pre-experimental phase buffaloes were fed with ration containing no AFB1 in each treatment. This was done to assure the complete removal of residual aflatoxin M1 in the milk as previous studies show the removal of AFM1 in 3-4 days after withdrawing AFB1 feeding (Kangethe and Langa, 2009). During the experimental phase all lactating buffaloes were offered AFB1 contaminated ration containing Fixar Viva, Mycosorb and T5X at 0.25% of dry matter in treatment B, C and D respectively. Group A was positive control and no toxin binder was added in it. Daily dry matter intake and milk production were recorded. A composite sample was collected from morning and evening milk collection after every 5th day of experiment. A total of 112 samples were collected and were stored in freezer until analyzed for AFM1, milk protein%, milk fat%, lactose% and solid not fat (SNF%). AFM1 was analyzed by competitive ELISA method using Helica Inc. kit.

Milk sample preparation

Half of milk sample was defatted by removing the fat layer after centrifuging samples at 4000 rpm. These defatted samples were analyzed for AFM1 using Enzyme Linked Immunosorbent Assay (ELISA) using microtitre plates (Helica Inc. Pakistan). A total of 112 samples were analyzed for AFM1. Plates were read by using ELISA reader ELX800. Half of the milk was used for analysis of Milk fat%, Protein%, Lactose% and solid non fat% (SNF%) by proximate analysis as described in AOAC 16th edition.

Statistical analysis

Treatment differences were identified by one-way analysis of variance (ANOVA) by the general linear model procedure of SPSS. Statistical significance was considered for $P < 0.05$.

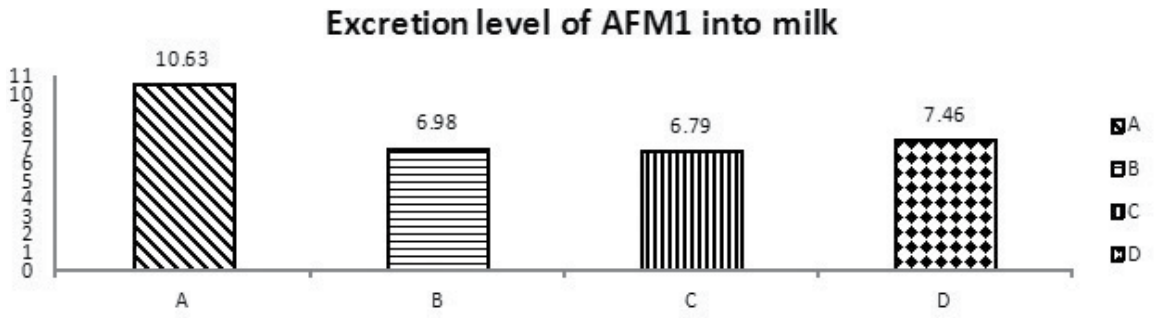
RESULTS AND DISCUSSION

Carry-over percentage of Aflatoxin M1

In pre-experimental phase there was a non significant difference in excretion of AFM1 in all groups. On the other hand at 5th day of experimental phase there was increased excretion of AFM1 in all groups. The highest excretion of AFM1 was observed in control group A (10.031 ±0.29), while in remaining groups AFM1 excretion was lower than control group and minimum AFM1 was observed in group C containing Mycosorb (6.621 ±0.22). On 10th day of experimental phase the level of AFM1 excretion further increased in all groups except in group C where the level of AFM1 did not increase significantly (6.828 ±0.39). While on 15th day of experimental phase a significant decrease in AFM1 excretion was observed in group C (6.73±0.39). This decrease continued on day 25th and 28th of experimental phase with significant values in group C as compared to other groups. Percentage reduction of AFM1 in group B, C and D was 39%, 47% and 35% respectively. However results indicated that this decrease in AFM1 in group B and D was not much significant (Figure 1).

Our results also indicated that a commercially available toxin binder called Mycosorb that pertain glucomannan resulted in increased percentage

Figure 1



reduction of AFM1. Glucomannan is a cell wall derivative of *Sacchromyces cerevisiae* that acts as organic toxin binder in minimizing the adverse effects of aflatoxins. This is in accordance with Murthy and Devegowda (2004) who reported that modified glucomannan readily adsorb several mycotoxins better than inorganic binders in broilers. Similarly a 36 % reduction in carry over from AFB1 in feed to AFM1 in milk was reported in an *in vivo* study by Galvano et al. (1996). Akhtar et al. (2014) also reported its better efficacy in terms of improved hematological and biochemical parameters.

However, our results contradict the findings of Mojtahedi et al. (2013) who reported that inclusion of Mycosorb up to 36g/d (3 times more than recommended dosage) was not effective in reducing AFM1 concentrations in lactating Holstein cows. Similar results were published by Kissell et al. (2013) who described non efficacy of Mycosorb in reducing AFM1 milk concentrations in dairy cows by feeding 10gm Mycosorb per cow per day. This may explain the differential action of organic compounds (glucomannan) in cows and buffaloes.

Our results indicated 2.13% carry-over of aflatoxin M (AFM1) in milk when 500 µg/Kg of aflatoxin B1 was given. This conversion of AFB1 into AFM1 is in accordance with VanEgmond, (1989) who reported the carryover ratio in a range of 1- 4% in cows. However our values were less than that reported by Britzi et al. (2013) in high producing Israeli-Holstein dairy cows. They described a carryover percentage of 5.8% and 2.5%. This may be due to the amount of aflatoxin given as they gave feed containing ~86 µg AFB1 for 7 days. Similarly a number of other factors including species, production level, season as well as feeding and milking routines of animals may affect the carryover percentage of AFM1 (Veldman et al., 1992; Coppock and Christian, 2007; Hussain et al., 2010). Moreover, the differences in ruminal fluid composition of cows and buffaloes may be responsible for its differential carryover in both species. As Upadhaya et al. (2010) reported that aflatoxin B1 degradation in rumen fluid was influenced by the species of animal and types of forage fed to the animals. This may be due to differential biotransformation of aflatoxin in rumen of cattle and buffaloes.

Effect of AFM1 on dry matter intake and milk production

Highest dry matter intake (DMI) and milk production was observed in group C (given Mycosorb) as compared to control and other treatment groups (Table 1). This may be due to organic nature of Mycosob in comparison to Fixer Viva (inorganic) and T5X (mixture of both organic and inorganic). This may be increased efficacy of Mycosorb (containing glucomannan) that improves digestibility and intake in lactating animals (Wohlt et al., 1991).

Effect of AFM1 on milk components

The results of present study indicated that use of Fixar Viva and T5X did not improve protein and fat contents of milk in group B and D. While on other hand, the feeding of Mycosorb significantly increased milk protein and fat contents in group C. This is not in line with the studies of Diaz-Llano and Smith (2006) and Korosteleva et al. (2007) that described no effect of glucomannan mycotoxin absorbent (GMA) on milk composition in dairy cows and lactating sows. This shows a major role of species involved for the effects of toxin binders. In addition, there was not

Table 1. Milk Production and Carry-Over Percentage of Aflatoxin M1 and Its Reduction by Using Toxin Binders in Experimental Groups

Treatments	Toxin Binder (gms) Gm/buffalo/day	Aflatoxin B1 (µg/buffalo/day)	Aflatoxin M1 (µg/kg)	Milk production (kg/day)	Aflatoxin M1 secretion	Carryover %age	Percentage reduction
Group A	0gms	500	1.76	6.04	10.64[a]	2.13%	
Group B	30gms	500	1.07	6.47±0.75	6.98[c]	1.39%	39%
Group C	30gms	500	0.93	7.25±1.33	6.79[b]	1.35%	47%
Group D	30gms	500	1.13	6.70±0.81	7.46[c]	1.49%	35%

Table 2. Percentages of Various Milk Components in Experimental and Control Groups

Parameters	Group A	Group B	Group C	Group D
Dry matter Intake	12.04 ±0.71[a]	12.30 ±0.30[b]	12.83 ±0.28[c]	12.42 ±1.05[b]
Milk Production	6.04 ±0.95[a]	6.47±0.75[a]	7.25± 1.33[b]	6.70 ± 0.81[a]
Protein % in milk	3.61±0.34[a]	3.66±0.63[ab]	3.94±0.35[b]	3.72±0.29[ab]
Fat % in milk	5.37±0.72[a]	5.31±0.76[ab]	5.56±.75[b]	5.56± 0.45[ab]
Lactose % in milk	5.20±0.54[a]	4.96±0.84[a]	5.12±0.35[a]	5.13±0.27[a]

a significant difference in lactose percentage of all groups (Table 2).

CONCLUSIONS

The findings of present study indicate that percentage carryover of AFM1 in buffaloes is higher than that reported in lactating cows as well as in goats and Mycosorb is capable of reducing the excretion of AFM1 into milk by improving the dry matter intake, milk production and protein contents. These findings may be applicable in field to reduce AFM1 release in milk of Nili-Ravi buffaloes.

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Case report
Κλινικό περιστατικό

A case of osteofibroma on the symphysis mandible in a cow

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ABSTRACT. Osteofibroma or ossifying fibroma both in human and animals is a rarely seen benign neoplasia. It usually locates on the mandible. Although it is commonly seen in young horses no case reports were described in cattle. In this case report, an osteofibroma located at the symphysis mandible in a cow was described. The tumor mass was reported to grow over in a two-month period to the size of soccer ball. The mass was totally extirpated and histopathological examination was performed. The mass was diagnosed as osteofibroma in microscopic examination.

Keywords: Osteofibroma, symphysis mandible, cow

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INTRODUCTION

Ossifying fibromas are fibro-osseous neoplasms characterized by the replacement of normal bone by a fibrous cellular stroma containing foci of mineralized bone trabeculae and cementum-like material (Barnes et al., 2005).

Ossifying fibroma most frequently affects the mandible of human and animals causing distortion of the normal bone contour, displacement or loss of teeth, and difficulty with swallowing or eating (Morse et al., 1988; Whitten et al., 2006). It can also cause difficulty in breathing when present on the nasal cavity causing obstruction. Although osssifying fibromas are rare in animals, they are more frequently reported in young horses, and categorized as "equine juvenile ossifying fibroma"(Morse et al., 1988). While these neoplasias have also been reported in cats (Turrel and Pool 1982; Quigley and Leedale 1983), dogs (Miller et al., 2008), lama (McCauley et al., 2007), sheep (Rogers and Gould 1998), and goats (Pritchard 1984) we have not come across any data on cows.

Although it constitutes a significant group of fibro-osseous lesions combining characteristic clinical and microscopic features, the etiology of ossifying fibroma is not entirely understood. However, some authors associate it with neoplastic and metabolic imbalance (Bahl et al., 2012).

Here, we report a case of osteofibroma located in the mandibular symphysis of a 5-year-old pregnant Simmental cow.

CASE HISTORY

A 5-year-old Simmental cow in the sixth month of pregnancy was brought to Kafkas University, Faculty of Veterinary Medicine Clinics. The anamnesis revealed that a walnut-sized mass was identified in the mandibular symphysis, and it grew to the size of a soccer ball in 2 months (Figure 1, Figure 2).

The mass significantly interfered with the animal's feeding ability. On clinical examination, the mass was firm in consistency and firmly attached to the mandible, making difficult for the animal to open its mouth. Incisor teeth were pushed towards the inside of the mouth. We decided to perform a surgical operation to relieve the animal's feeding difficulty.

After the routine preparations for the surgery, ring-shaped local infiltration anesthesia (40 ml, 2% lidocaine HCl, Adokain®, Sanovel) was performed on the root of the mass with sedation (0.2 mg/kg intravenous, xylazine HCl, Rompun®, Bayer), and mental nerve block (7 ml, 2% lidocaine HCl, Adokain®, Sanovel) was performed. Then the mass was totally extirpated (Figure 3, Figure 4). After the mass was removed, one incisor tooth spontaneously fell out. The bleeding caused in the cavity formed after the removal of the mass was controlled using electrocautery. The cavity was then filled with an absorbable hemostatic agent (SURGICEL® Original Absorbable Hemostat, Ethicon), and the incision line was closed with simple separate stitches using polyglactin 910 sutures (No 2, Vicryl®, Ethicon).

In addition to postoperative use of an antiseptic mouthwash (Ülkem® Glycerin Iodine, Ülkem İlaç), wound care and parenteral antibiotic therapy (20,000 IU procaine benzylpenicillin, 20 mg dihydrostreptomycin sulfate/ kg/live weight, Reptopen S, CEVA-DIF) were administered for one week.

In the postoperative period following the removal



Fig 1: Preoperative view of the case



Fig 2: View of the mass



Fig 3: Postoperative view

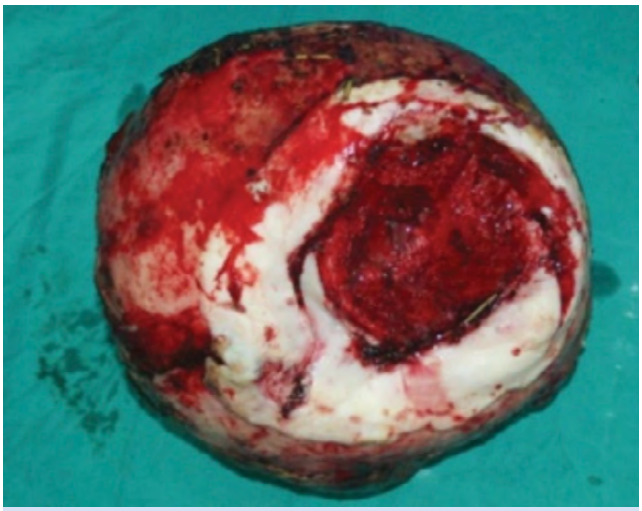


Fig 4: Extirpated mass



Fig 5: The view of area in postoperative 2-month



Fig 6: 2th month postoperative view

of the mass, a significant relief was observed in jaw movements, and the animal was able to eat and drink water more comfortably. The stitches in the surgical wound were removed on the 10th postoperative day, and no complication was seen in the region where the mass was located or in the general condition or pregnancy of the animal during the 2-month follow-up period (Figure 5, Figure 6).

The samples obtained from the extirpated mass were fixed in a 10% buffered formaldehyde solution for histopathological examination. Cross-sections prepared following routine procedures were stained with hematoxylin-eosine, and examined under a light microscopy.

RESULTS AND DISCUSSION

Ossifying fibroma, which is particularly specific to the mandible and described as benign progressive proliferation of the fibro-osseous tissue, is most frequently reported in very young horses or various domestic animal species such as cats, dogs, lama, sheep and goats (McCauley et al., 2007; Miller et al., 2008; Morse et al., 1988; Quigley and Leedale 1983; Pritchard 1984; Rogers and Gould 1998; Turrel and Pool 1982; Whitten et al., 2006). However, we have not come across any previous case report on its incidence in cows. In this case presentation, histopathologic features and the surgical manipulations and the follow ups of the mass located in the mandibular symphysis of a 5-year-old Simmental cow, which was 6 month-pregnant, was described. The mass was reported to grow significantly over a 2-month period and negatively affected the animal's jaw movements and feeding ability. After the histopathological examinatios the mass was named as osteofibroma. Due to the absence of previous reports on osteofibroma in cattle, the current case was considered to worth presenting.

Histologically, osteofibromas are characterized by dense fibroblastic stroma resembling disordered granulation tissue with isomorphic fibroblasts transforming into osteoblasts. Relatively regular spicules and trabeculae of bone rimmed by osteoblasts may be formed in a moderately vascularized fibro-osseous stroma (Carvalho et al., 2012; Morse et al., 1988). Osteofibroma can be histopathologically distinguished from fibrous dysplasia, osteoma and osteosarcoma (Kodaira et al., 2010; Morse et al., 1988). Generally, it is more like fibrous dysplasia. Fibrous dysplasia is the proliferation of bone trabeculae in benign dense fibrous stroma. However, bone trabeculae are not rimmed by osteoblasts in fibrous dysplasia as they are in ossifying fibroma. The connective tissue cells of ossifying fibroma lack pleomorphism and do not have the high mitotic index of osteosarcoma (Kodaira et al., 2010). In the present case, the tissue in the center of the mass was a

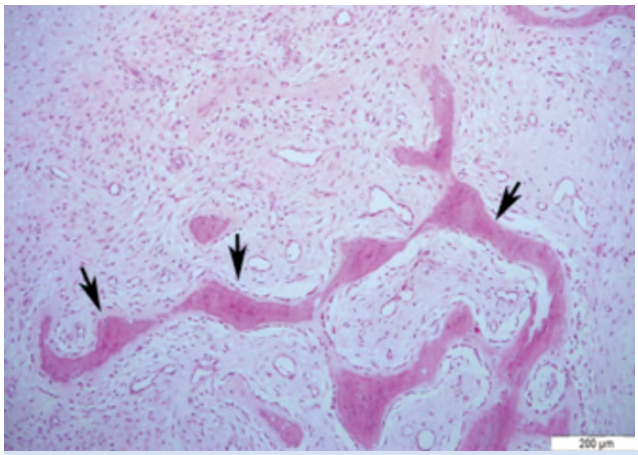


Fig 7: Thin bone trabeculae (arrows) and well vascularized loose connective tissue among them, hematoxylin eosin

loose connective tissue composed of newly formed thin bone trabeculae, and well-vascularized fusiform cells and fibrils between these trabeculae. The peripheral regions of the mass were fibrotic with no trabeculae. The skin covering the mass was hyperplastic. The diagnosis of osteofibroma was made based on the histopathological findings (Figure 7). In our case no mitotic figures were seen and the microscopic morphology was consistent with osteofibroma; therefore it is presented as a case of osteomafibroma.

Successful treatment of ossifying fibroma requires curettage of the affected tissue or complete removal of the tumor. It was reported that if these surgical manipulations were performed well relapses do not occur (Bertrand et al., 1993; Richardson et al., 1991; Suarez-Soto et al., 2013). However, a relapse rate of 35% has been reported for humans since removal of the affected bone with surgical operations such as mandibulectomy or hemimaxillectomy is not always possible (Richardson et al., 1991; Suarez-Soto et al., 2013). In the present case, we did not perform mandibulectomy for total extirpation of the mass, but used electrocautery to control the bleeding during the removal of the mass, and to cauterize the tumor base. No relapse or complication was seen during the postoperative 2-month routine control period, and none was reported on follow-up telephone calls for one year.

While osteofibroma is mostly reported in the mandible, maxilla, or skull of humans or animals such as lamas or rabbit (McCauley et al., 2007; Miller et al., 2008; Morse et al., 1988; Quigley and Leedale 1983; Pritchard 1984; Rogers and Gould 1998; Turrel and Pool 1982; Whitten et al., 2006), it is reported to be located in the rostral mandible in a majority of the cases of osteofibroma in horses (Richardson et al., 1991). Current case resembles to the clinical cases of horses with osteofibromas because of the rostral localization of the mass.

In conclusion, osteofibroma is rare in cows and of vital importance due to its location. Our case may be viewed as a contribution to the literature and veterinary practice.

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Case Report
Κλινικό Περιστατικό

Effective hands-on treatment of a corneal dermoid in a calf

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ABSTRACT. In the present report, an effective hands-on treatment for a corneal dermoid that extended to the third eyelid, in the left eye of a Japanese Black calf is described. Xylazine sedation and local infiltration anesthesia with 2% lidocaine on the upper and lower eyelids as well as the third eyelid, in combination with topical anesthesia was performed. A Weitlaner retractor allowed immobilizing the patient's eyeball during surgery, and superficial lamellar keratectomy was performed to surgically excise the mass. A temporary tarsorrhaphy was placed in order to allow the surgical corneal wound to heal as well as to treat keratitis. After an uneventful postoperative recovery, complete cure 70 days post-operatively was achived. This hands-on method represents a practical and effective treatment for ocular dermoids in calves.

Keywords: Calf, hands-on treatment, ocular dermoid, temporary tarsorrhaphy

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CASE HISTORY

A 1-month-old male Japanese Black calf (weight, 77 kg) was referred to the Iwate University Veterinary Teaching Hospital with a history of palpebral enlargement and epiphora on the left eye. Upon ophthalmic examination, a bunch of hairs were protruding from the left eyeball, oriented from the lower eyelid (Figure 1A). The hairs originated from a mass attached to the cornea, bulbar conjunctiva and bulbar aspect of the third eyelid (Figure 1B). Superficial corneal ulceration and corneal opacity (keratitis) as a result of trichiasis were also noticed.

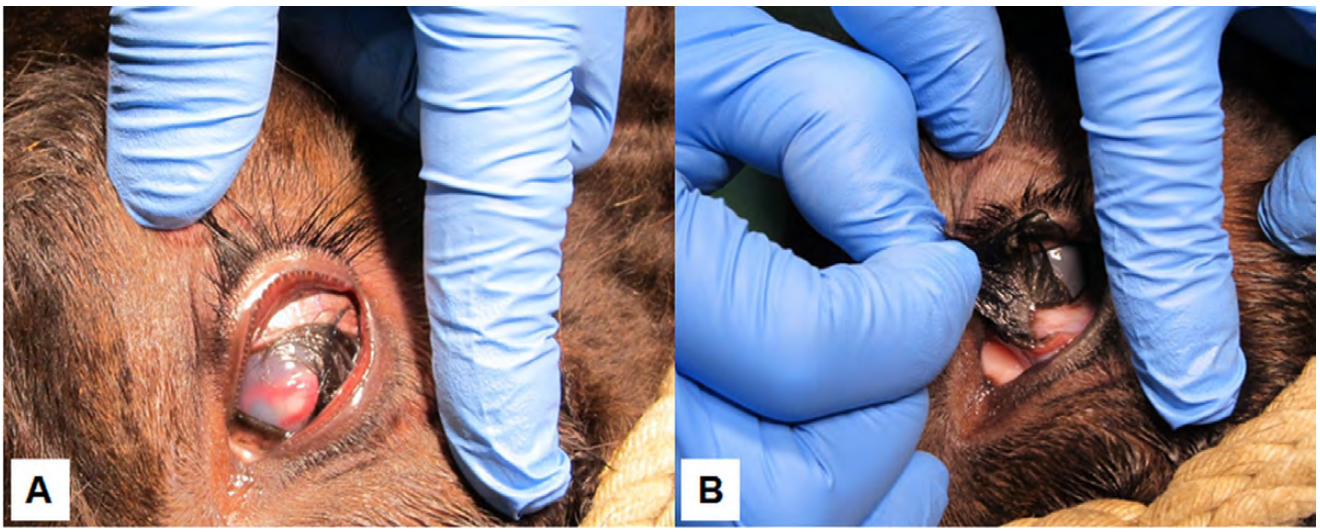
The calf was sedated with 0.1 mg/kg body weight (BW) xylazine intravenously while procaine penicillin G 20,000 unit/kg BW was also administered intramuscularly. The animal was positioned in the right lateral recumbency, with the left side of the face upward. The skin around the left eye was prepared aseptically. Local infiltration anesthesia was performed by injection with a 21 gauge needle of 5-mL of 2% lidocaine solution, along the upper and lower eyelids (Figure 2A). Additionally, 1-mL lidocaine solution was instilled on the ocular surface.

A Weitlaner retractor was used to keep the eye open and the third eyelid was gently clamped by the assistant to suppress eye movement. In order to perform superficial lamellar keratectomy, the mass was retracted with a pair of tissue forceps, and peeled off from the third eyelid and the cornea using a surgical scalpel with a no. 11 blade (Feather Safety Razor, Osaka, Japan) (Figure 2B and C). After hemostasis and irrigation, an erythromycin-containing ophthalmic ointment (Elicosin ophthalmic ointment; Santen Pharmaceutical, Osaka, Japan) was applied inside of the conjunctival sac. A temporary tarsorrhaphy was performed (Figure 2D) with one horizontal synthetic absorbable mattress suture (Coated VICRYL 2-0, J333H; Ethicon, Somerville, NJ, USA) and infusion tubing. The surgery was completed in 30 min after xylazine administration, whereas the calf was given intravenous (0.01 mg/kg BW) atipamezole to reverse the sedation.

After complete recovery from sedation the calf returned to the farm. Procaine penicillin G (20,000 unit/kg BW, intramuscularly, SID) was suggested for 3 days postoperatively. The temporary tarsorrhaphy was removed 14 days post-op; Formation of granulation tissue on the cornea and the third eyelid were noticed whereas the corneal opacity was improved (Figure 3A). No granulation tissue was seen 70 days post-operatively (Figure 3B).

The histopathological examination confirmed that the hairy mass (Figure 4A) was an ocular dermoid accompanied by keratinized squamous epithelium with deposition of melanin and keratohyaline granules (Figure 4B). The dermic layer had hair follicles and the sebaceous and sweat glands were associated with lymphocytic infiltration (Figure 4C).

Fig 1. Pre-surgical photographs of the ocular dermoid in a Japanese Black calf. (A) Corneal opacity (keratitis) and hairs originating from the ventral eyelid. (B) The mass located in the cornea and the third eyelid.



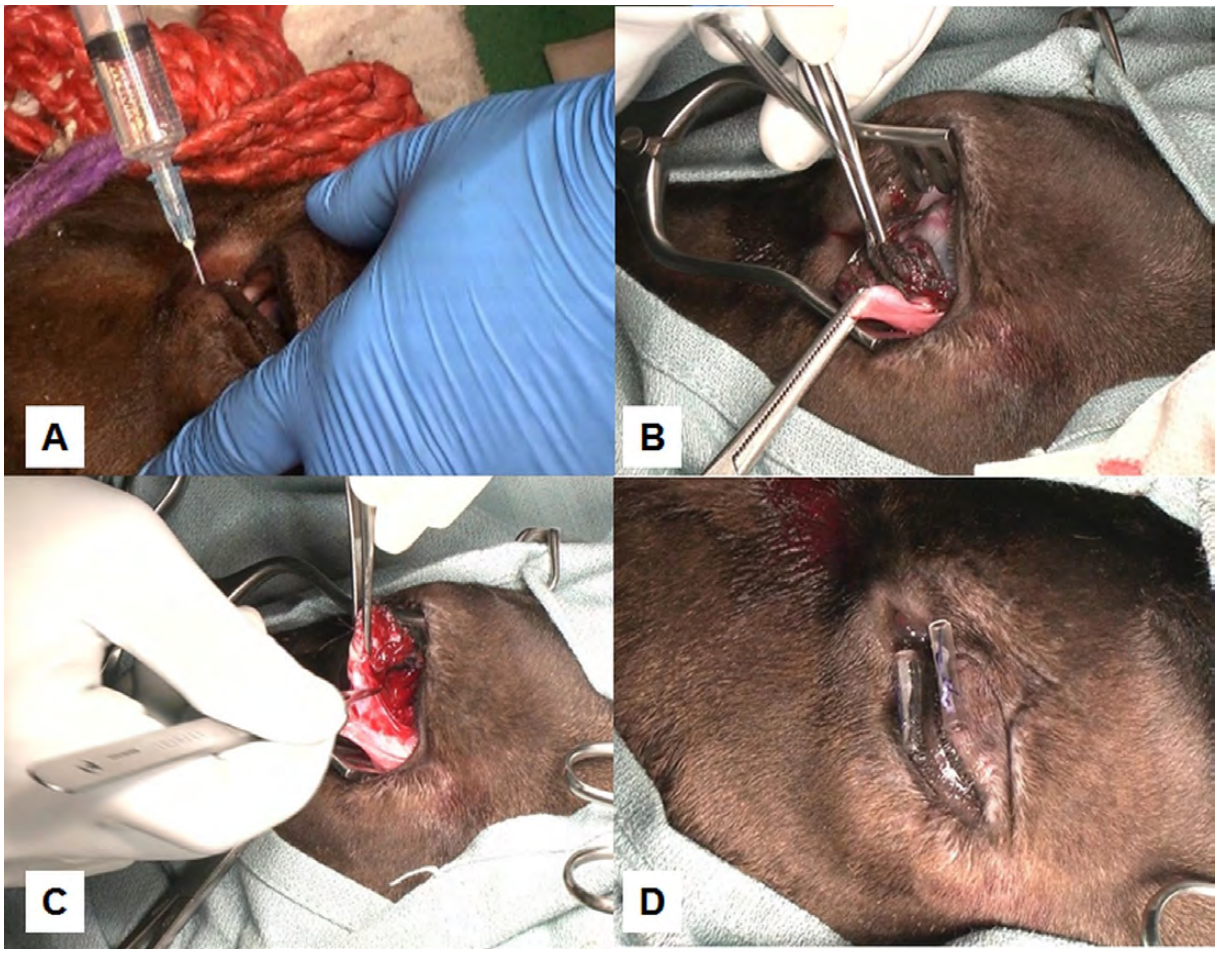


Fig 2. Photographs of surgical excision of an ocular dermoid in a Japanese Black calf. (A) Inserting the needle to achieve local infiltration anesthesia with the angle aimed at the third eyelid. (B) Use of a Weitlaner retractor and appropriate positioning of the third eyelid by the assistant. (C) The mass peeled off by applying a superficial lamellar keratectomy. (D) Temporary tarsorrhaphy placed on the left eyelid.

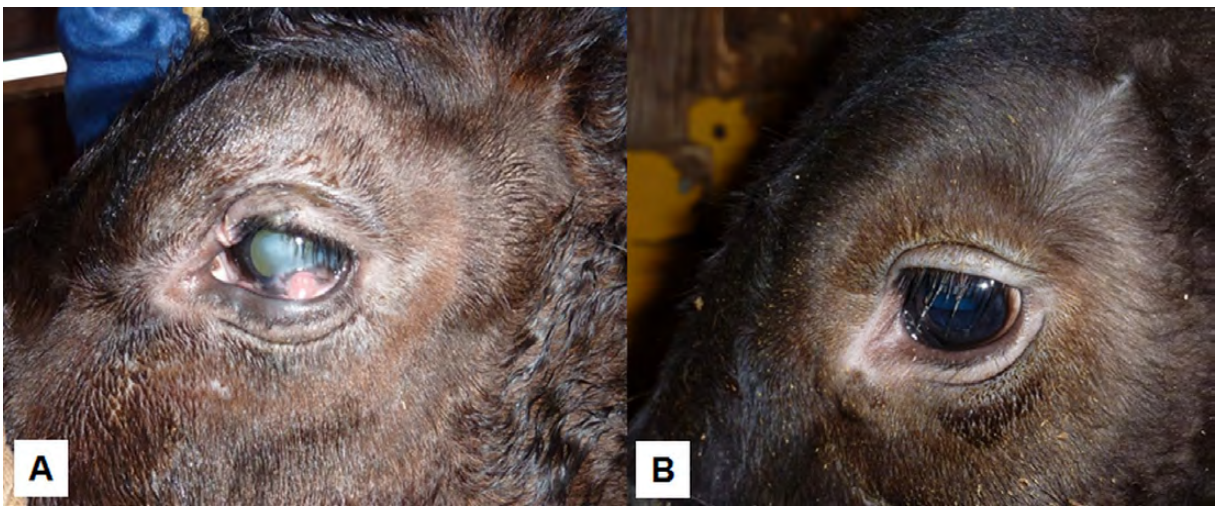
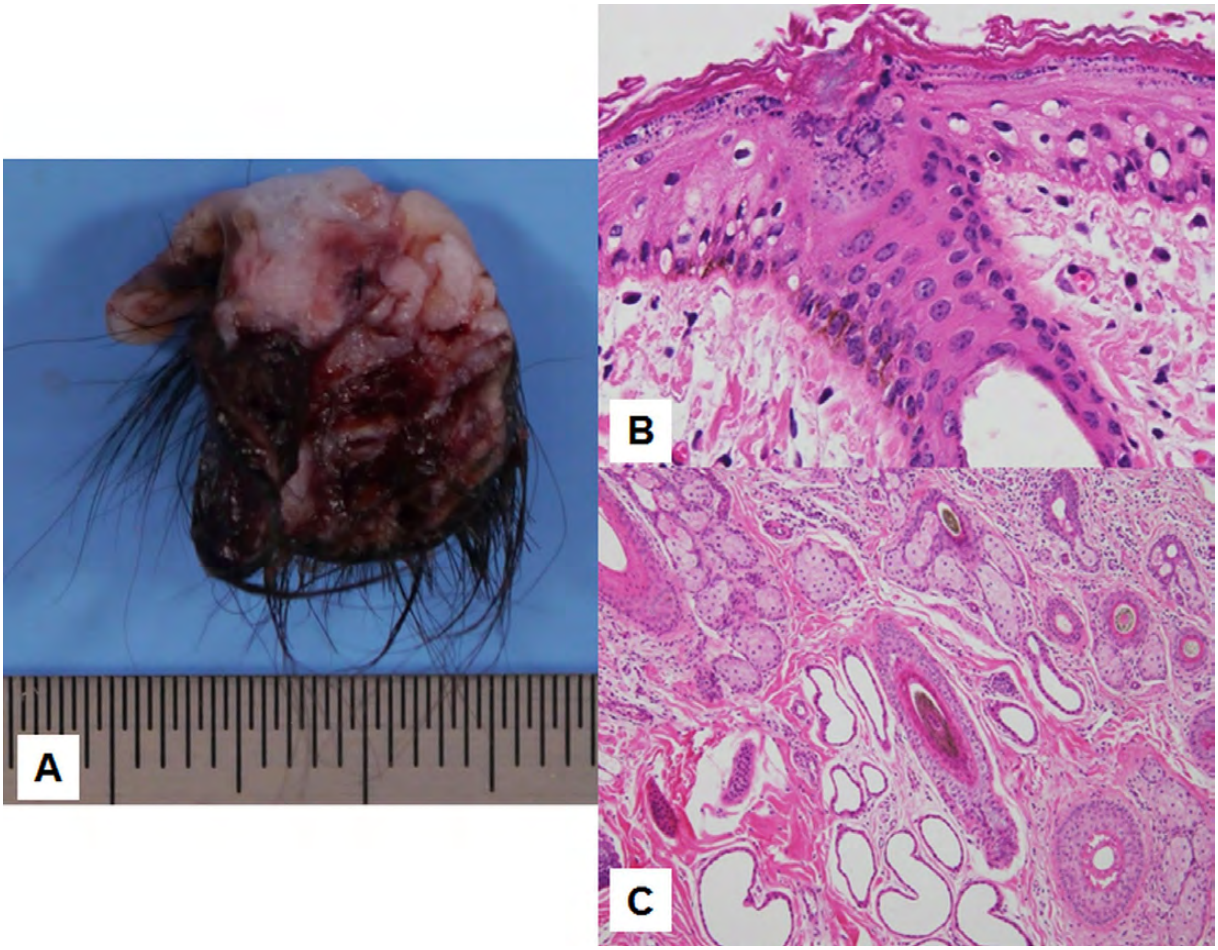


Fig 3. Post-surgical photographs of an ocular dermoid in a Japanese Black calf. (A) Corneal opacity had improved 14 days after surgery, associated with the formation of granulation tissue on the cornea and third eyelid. (B) The granulation tissue had disappeared completely 70 days after surgery.

Fig 4. Gross (A) and histopathological (B and C) findings of the ocular dermoid resected from a Japanese Black calf. (A) Macroscopic appearance of the resected tissue with hairs. (B) Keratinized squamous epithelium with melanin and keratohyaline granules; hematoxylin-eosin staining, ×240. (C) Hair follicles and the sebaceous and sweat glands in the dermal layer; hematoxylin-eosin staining, ×100.



DISCUSSION

An ocular dermoid, which is a congenital ophthalmologic disease of calves, is a choristoma that may be found in the cornea, conjunctiva, limbus, or eyelid (Jena et al., 2015). Dermoid cysts originate as ectopic deposition of epidermal tissues during closure of the neural tube (Alam and Rahman, 2012). The cyst includes accessory organs of the skin, such as the epidermis, hair, hair follicles, and glandular epithelium (Kiliç et al., 2012). This hairy mass irritates the surface of the eye and causes epiphora and ocular inflammation (Jena et al., 2015). The eyelids, cornea, conjunctiva, and sclera are the major sites for ocular dermoids (Alam and Rahman, 2012; Yeruham et al., 2012). Surgical removal of these masses is recommended, and superficial lamellar keratectomy is the most common surgical procedure (Fubini and Ducharme, 2004). Compared with the previous reports (Simon et al., 2010; Alam and Rahman, 2012; Kiliç et al., 2012; Mudasir et al., 2012; Yeruham et al., 2012; Jena et al., 2015; Nagar et al., 2015), the present report precisely describes our experience in surgically treating an ocular dermoid in a calf with an uneventful prognosis.

The calf in this report was placed in the lateral recumbency under xylazine sedation, which is the most common method to immobilize cattle. A retrobulbar nerve block was used in previous reports to surgically resect corneal dermoids in cattle under sedation (Simon et al., 2010; Yeruham et al., 2012; Jena et al, 2015; Nagar et al., 2015). The advantage of this nerve block is immobilization of the eyeball during surgery (Riebold et al., 1995; Tranquilli et al., 2007). However, this technique requires more surgical skill to insert

the needle accurately into the retrobulbar space, which may induce several complications, such as an optic nerve injury, retrobulbar hemorrhage, or increased intraocular pressure (Tranquilli et al., 2007). Auriculopalpebral nerve block for ocular surgery in bovines has also been reported but this kind of anesthesia can be used only in combination with local anesthesia, since it does not provide analgesic effect (Simon et al., 2010; Mudasir et al., 2012; Nagar et al., 2015). In our case, local infiltration anesthesia was easily performed into the upper and lower eyelids, as well as the third eyelid in combination with topical anesthesia on the ocular surface. Although local anesthesia did not restrain eyeball movement, appropriate retention of the third eyelid with the use of a Weitraner retractor allowed a successful surgical procedure.

Corneal ulceration and keratitis are often observed in ocular dermoid cases, related to the ocular irritation caused by hairs on the mass (Kiliç et al., 2012; Yeruham et al., 2012; Jena et al, 2015; Nagar et al., 2015). In the present case, a temporary tarsorrhaphy was placed in order to allow the surgical corneal wound to heal as well as to treat keratitis, as shown previously by others (Simon et al., 2010; Nagar et al., 2015). The corneal opacity in our case healed 14 days after surgery, as the temporary tarsorrhaphy promoted repair of the corneal epithelium allowing an effective recovery (Birchard and Sherding, 2000).

The prevalence of ocular dermoids is lower in cattle compared to that in other species (Yeruham et al., 2012). Epiphora, blepharospasm, irritation and consequent inflammation of the ocular surface as well as visual impairment could be severe, so surgical removal is recommended. (Fubini and Ducharme, 2004). Our calf underwent a successful surgery after xylazine sedation in combination with local infiltration anesthesia with less anesthetic risk of complications. Even the globe motion was not eliminated; it was controlled adequately by the surgical assistant, allowing the successful removal of the dermoid. According to the authors' opinion, this effective surgical procedure represents a feasible and hands-on treatment for ocular dermoids in calves.

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1. ΟΝΟΜΑΣΙΑ ΤΟΥ ΚΤΗΝΙΑΤΡΙΚΟΥ ΦΑΡΜΑΚΕΥΤΙΚΟΥ ΠΡΟΪΟΝΤΟΣ LETIFEND, λυοφιλοποιημένο υλικό και διαλύτης για ενέσιμο διάλυμα για σκύλους **2. ΠΟΙΟΤΙΚΗ ΚΑΙ ΠΟΣΟΤΙΚΗ ΣΥΝΘΕΣΗ** Κάθε δόση 0,5 ml του εμβολίου περιέχει: Λυοφιλοποιημένο υλικό Δραστικό συστατικό: Ανασυνδυασμένη πρωτεΐνη Q από *Leishmania infantum* MON-1 ≥ 36,7 μονάδες ELISA (EE)* * Το περιεχόμενο αντιγόνου καθορίζεται σε ELISA έναντι εσωτερικού προτύπου. Βλ. τον πλήρη κατάλογο εκδόχων στο κεφάλαιο 6.1. **3. ΦΑΡΜΑΚΟΤΕΧΝΙΚΗ ΜΟΡΦΗ** Λυοφιλοποιημένο υλικό και διαλύτης για ενέσιμο διάλυμα. Λευκό λυοφιλοποιημένο υλικό. **4. ΚΛΙΝΙΚΑ ΣΤΟΙΧΕΙΑ 4.1 Είδη ζώων** Σκύλοι. **4.2 Θεραπευτικές ενδείξεις προσδιορίζοντας τα είδη ζώων** Για τον ενεργό εμβολιασμό μη μολυσμένων σκύλων από την ηλικία των 6 μηνών για τη μείωση του κινδύνου ανάπτυξης ενεργού λοίμωξης ή/και κλινικής νόσου μετά την έκθεση σε *Leishmania infantum*. Η αποτελεσματικότητα του εμβολίου έχει καταδειχθεί σε επιτόπια μελέτη, στις οποίες οι σκύλοι εκτέθηκαν φυσικά σε *Leishmania infantum* σε ζώνες με υψηλή πίεση μόλυνσης για χρονικό διάστημα δύο ετών. Σε εργαστηριακές μελέτες που περιελάμβαναν πειραματική πρόκληση με Leishmania infantum, το εμβόλιο μείωσε τη βαρύτητα της νόσου, συμπεριλαμβανομένων των κλινικών σημείων και του φορτίου παρασίτων στον σπλήνα και τους λεμφαδένες. Εγκατάσταση ανοσίας: 4 εβδομάδες μετά τον εμβολιασμό. Διάρκεια ανοσίας: 1 έτος μετά τον εμβολιασμό. **4.3 Αντενδείξεις** Καμία. **4.4 Ειδικές προειδοποιήσεις για κάθε είδος ζώου** Το εμβόλιο είναι ασφαλές για μολυσμένους σκύλους. Ο επαναληπτικός εμβολιασμός μολυσμένων σκύλων δεν επιδείνωσε την πορεία της νόσου (στη διάρκεια της δίμηνης περιόδου παρακολούθησης). Δεν έχει καταδειχθεί αποτελεσματικότητα σε αυτά τα ζώα. Πριν από τον εμβολιασμό συνιστάται δοκιμή για την ανίχνευση της Λεϊσμανίασης. Η επίδραση του εμβολίου στη δημόσια υγεία και τον έλεγχο της λοίμωξης ανθρώπων δεν μπορεί να εκτιμηθεί από τα διαθέσιμα δεδομένα. **4.5 Ιδιαίτερες προφυλάξεις κατά τη χρήση** Ιδιαίτερες προφυλάξεις κατά τη χρήση σε ζώα Εμβολιάζετε μόνο υγιή και μη μολυσμένα ζώα. Ξυνιστάται η αποπαρασίτωση των μολυσμένων σκύλων πριν από τον εμβολιασμό. Είναι απαραίτητο να λαμβάνονται μέτρα για τη μείωση της έκθεσης σε σκνίπες σε εμβολιασμένα ζώα. Ιδιαίτερες προφυλάξεις που πρέπει να λαμβάνονται από το άτομο που χορηγεί το φαρμακευτικό προϊόν σε ζώα Δεν απαιτούνται. **4.6 Ανεπιθύμητες ενέργειες (συχνότητα και σοβαρότητα)** Μετά τον εμβολιασμό στους σκύλους έχει παρατηρηθεί πολύ συχνά κνησμός στη θέση της ένεσης. Έχει παρατηρηθεί η αυτόματη υποχώρηση αυτής της αντίδρασης εντός 4 ωρών. Η συχνότητα των ανεπιθύμητων ενεργειών καθορίζεται ως ακολούθως: - πολύ συχνή (περισσότερο από 1 στα 10 ζώα παρουσιάζουν ανεπιθύμητες ενέργειες) - συχνή (περισσότερο από 1 αλλά λιγότερο από 10 ζώα στα 100 υπό θεραπεία ζώα) - μη συνηθισμένη (περισσότερο από 1 αλλά λιγότερο από 10 ζώα στα 1.000 υπό θεραπεία ζώα) - σπάνια (περισσότερο από 1 αλλά λιγότερο από 10 ζώα στα 10.000 υπό θεραπεία ζώα) - πολύ σπάνια (λιγότερο από 1 στα 10.000 υπό θεραπεία ζώα, συμπεριλαμβανομένων μεμονωμένων αναφορών) **4.7 Χρήση κατά την κύηση, τη γαλουχία ή την ωοτοκία** Η ασφάλεια του κτηνιατρικού φαρμακευτικού προϊόντος δεν έχει αποδειχθεί κατά τη διάρκεια της κύησης ή της γαλουχίας. Συνεπώς, δεν συνιστάται η χρήση κατά τη διάρκεια της κύησης και της γαλουχίας. **4.8 Αλληλεπιδράσεις με άλλα φαρμακευτικά προϊόντα και άλλες μορφές αλληλεπίδρασης** Δεν υπάρχουν πληροφορίες σχετικά με την ασφάλεια και την αποτελεσματικότητα από την χρήση του εν λόγω εμβολίου με οποιοδήποτε άλλο κτηνιατρικό φάρμακο. Η απόφαση για τη χορήγηση του εν λόγω εμβολίου πριν ή μετά από τη χορήγηση οποιουδήποτε άλλου κτηνιατρικού φαρμάκου, θα πρέπει να λαμβάνεται κατά περίπτωση. **4.9 Δοσολογία και τρόπος χορήγησης** Υποδόρια χρήση. Δοσολογικό σχήμα πρωτεύοντος εμβολιασμού: Μία εφάπαξ δόση εμβολίου (0,5 ml) για χορήγηση σε σκύλους από την ηλικία των 6 μηνών. Δοσολογικό σχήμα επαναληπτικού εμβολιασμού: Μία εφάπαξ δόση εμβολίου (0,5 ml) για χορήγηση ετησίως στα εξής. Μέθοδος χορήγησης: Ανασυστήστε ένα φιαλίδιο του λευκού λυοφιλοποιημένου υλικού χρησιμοποιώντας 0,5 ml διαλύτη. Ανακινήστε απαλά για να παράξετε διαφανές διάλυμα και χορηγήστε αμέσως όλο το περιεχόμενο (0,5 ml) του ανασυσταθέντος προϊόντος. **4.10 Υπερδοσολογία (συμπτώματα, μέτρα αντιμετώπισης, αντίδοτα), εάν είναι απαραίτητα** Μετά τη χορήγηση διπλής δόσης, οι αντιδράσεις είναι παρόμοιες φύσης με αυτές που παρατηρούνται μετά τη χορήγηση εφάπαξ δόσης (βλ. παράγραφο 4.6). **4.11 Χρόνος(οι) αναμονής** Δεν απαιτείται. **5. ΑΝΟΣΟΛΟΓΙΚΕΣ ΙΔΙΟΤΗΤΕΣ** Φαρμακοθεραπευτική ομάδα: Ανοσολογικά φαρμακευτικά προϊόντα για κυνίδες – σκύλος – ανενεργά παρασιτικά εμβόλια – λεϊσμανίαση. Κωδικός ATCvet: QI07AO01 Για την ενεργοποίηση ενεργητικής ανοσίας κατά της νόσου που προκαλείται από παράσιτα *Leishmania infantum*. Τα διαγνωστικά εργαλεία για την ανίχνευση των αντισωμάτων *Leishmania* (SLA ή IFAT ή ταχείες διαγνωστικές δοκιμές rk-39) θα πρέπει να είναι καταλληλα ώστε να επιτρέτουν τη διάκριση μεταξύ σκύλων που έχουν εμβολιαστεί με αυτό το εμβόλιο και σκύλων που έχουν μολυνθεί από *Leishmania infantum*. Η αποτελεσματικότητα του εμβολίου έχει καταδειχθεί σε επιτόπια μελέτη στην οποία ορισμένοι σκύλοι διαφόρων φυλών εκτέθηκαν φυσικά σε *Leishmania infantum* σε ζώνες με υψηλή πίεση μόλυνσης για χρονικό διάστημα δύο ετών. Τα δεδομένα κατέδειξαν ότι ένας εμβολιασμένος σκύλος διατρέχει 9,8 φορές μικρότερο κίνδυνο να εμφανίσει κλινικά σημεία, 3,5 φορές μικρότερο κίνδυνο να φέρει ανιχνεύσιμα παράσιτα και 5 φορές μικρότερο κίνδυνο να αναπτύξει κλινική νόσο από ό,τι ένας μη εμβολιασμένος σκύλος. **6. ΦΑΡΜΑΚΕΥΤΙΚΑ ΣΤΟΙΧΕΙΑ 6.1 Κατάλογος εκδόχων** Λυοφιλοποιημένο υλικό: Χλωριούχο νάτριο Υδροχλωρική αργινίνη Βορικό οξύ. Διαλύτης: Ενέσιμο ύδωρ. **6.2 Κύριες ασυμβατότητες** Να μην αναμιγνύεται με άλλο κτηνιατρικό φαρμακευτικό προϊόν, εκτός του διαλύτη ο οποίος διατίθεται για χρήση με αυτό το κτηνιατρικό φαρμακευτικό προϊόν. **6.3 Διάρκεια ζωής** Λυοφιλοποιημένο υλικό: Διάρκεια ζωής του κτηνιατρικού φαρμακευτικού προϊόντος σύμφωνα με τη συσκευασία πώλησης: 3 έτη. Διαλύτης: Διάρκεια ζωής του διαλύτης: 4 έτη. Διάρκεια ζωής μετά την ανασύσταση σύμφωνα με τις οδηγίες: άμεση χρήση. **6.4. Ιδιαίτερες προφυλάξεις κατά τη φύλαξη του προϊόντος** Φυλάσσετε σε ψυγείο (2 °C - 8 °C). Μην καταψύχετε. **6.5 Φύση και σύνθεση της στοιχειώδους συσκευασίας** Φιαλίδιο λυοφιλοποιημένου υλικού Τύπου Ι γυάλινα φιαλίδια που περιέχουν 1 δόση του εμβολίου Φιαλίδιο διαλύτη Τύπου Ι γυάλινα φιαλίδια που περιέχουν 0,8 ml διαλύτη. Τα φιαλίδια κλείνονται αμφότερα με πώμα εισχώρησης βρωμοβουτυλίου και πώμα αλουμινίου. Μενεξέδη συσκευασίας: Πλαστικό κουτί που περιέχει 1 φιαλίδιο 1 δόσης λυοφιλοποιημένου υλικού και 1 φιαλίδιο 0,8 ml διαλύτη. Πλαστικό κουτί που περιέχει 4 φιαλίδια 1 δόσης λυοφιλοποιημένου υλικού και 4 φιαλίδια 0,8 ml διαλύτη. Πλαστικό κουτί που περιέχει 5 φιαλίδια 1 δόσης λυοφιλοποιημένου υλικού και 5 φιαλίδια 0,8 ml διαλύτη. Πλαστικό κουτί που περιέχει 10 φιαλίδια 1 δόσης λυοφιλοποιημένου υλικού και 10 φιαλίδια 0,8 ml διαλύτη. Πλαστικό κουτί που περιέχει 20 φιαλίδια 1 δόσης λυοφιλοποιημένου υλικού και 20 φιαλίδια 0,8 ml διαλύτη. Πλαστικό κουτί που περιέχει 25 φιαλίδια 1 δόσης λυοφιλοποιημένου υλικού και 25 φιαλίδια 0,8 ml διαλύτη. Πλαστικό κουτί που περιέχει 50 φιαλίδια 1 δόσης λυοφιλοποιημένου υλικού και 50 φιαλίδια 0,8 ml διαλύτη. Πλαστικό κουτί που περιέχει 100 φιαλίδια 1 δόσης λυοφιλοποιημένου υλικού και 100 φιαλίδια 0,8 ml διαλύτη. Μπορεί να μην κυκλοφορούν όλες οι συσκευασίες. **6.6 Ειδικές προφυλάξεις απόρριψης ενός χρησιμοποιηθέντος κτηνιατρικού φαρμακευτικού προϊόντος ή άλλων υλικών που προέρχονται από τη χρήση του προϊόντος, αν υπάρχουν** Τα μη χρησιμοποιηθέντα κτηνιατρικά φαρμακευτικά προϊόντα ή μη χρησιμοποιηθέντα υπολείμματά τους πρέπει να απορρίπτονται σύμφωνα με τις αγχίουσες εθνικές απαιτήσεις. **7. ΚΑΤΟΧΟΣ ΑΔΕΙΑΣ ΚΥΚΛΟΦΟΡΙΑΣ** Laboratorios LETI, S.L., unipersonal C/ Del Sol 5, Polígono Industrial Norte Tres Cantos 28760 Madrid ΙΣΠΑΝΙΑ +34 91 771 17 90 **8. ΑΡΙΘΜΟΣ(ΟΙ) ΑΔΕΙΑΣ ΚΥΚΛΟΦΟΡΙΑΣ** EU/2/16/195/001-008 **9. 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