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Original Article

The Complex Microbiome of Caries-Active and Caries-Free Supragingival Plaques in Permanent Dentition

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ABSTRACT

Background and Aim: Dental caries is one of the most common diseases seen in the oral cavity in all periods of deciduous, mixed, and permanent dentition. A comprehensive study of the oral microbiome is required to understand its polymicrobial etiology. The aim of this study was to reveal the plaque microbiome of caries-active and caries-free adults. **Materials and Methods:** A total of 52 samples were collected from 26 caries-active patients and 26 caries-free controls. Dental supragingival plaque samples were collected from each subject and the bacterial 16S rDNA, expanded V3–V4 region, was amplified using next generation sequencing. **Results:** The core microbiome was defined with 235 shared bacteria in genus level, and among all microbiome 14.8% of all bacteria showed significant difference ($P < 0.05$). The bacteria responsible of caries may be listed as *Anaeroglobus*, *Atopobium*, *Bifidobacterium*, *Centipeda*, *Cryptobacterium*, *Desulfobulbus*, *Filifactor*, *Howardella*, *Lactobacillus*, *Leptotrichiaceae* (unclassified), *Megasphaera*, *Mycoplasma*, *Olsenella*, *Phocaeicola*, *Propionibacterium*, *Pseudoramibacter*, *Scardovia*, *Schwartzia*, *Treponema*, *and Veillonellaceae* (unclassified). **Conclusion:** The present study provides comprehensive knowledge of the microbiological etiology of caries in permanent dentition.

KEYWORDS: *Caries, microbiome, next generation sequencing, permanent dentition*

INTRODUCTION

Biofilm is a layer of prokaryotic and eukaryotic cells that are firmly attached to a living or non-living surface and embedded in an organic matrix of biological origin.[1] The oral cavity of an adult person, which contains approximately 50–100 billion bacteria, is an extremely complex structure.[2] There are approximately 700 species of prokaryotes in the human oral cavity and these bacteria live in balance within the framework of certain connections and relationships.[3-5]

Dental caries is one of the most common diseases seen in the oral cavity in all periods of deciduous, mixed, and permanent dentition.[6] Caries formation is the result of multifactorial events such as plaque microflora, host immune system, dietary habits, and oral hygiene of an individual.[7]

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The detection of oral microorganisms by culture method, besides being time consuming, remains inadequate in most cases due to the unculturable nature of oral bacteria.[3,8] Molecular methods provide a great advantage in the diagnosis of bacteria that cannot be cultured and/or difficult to culture.[9,10]

Next generation sequencing (NGS) method can analyze a large number of sequences with high accuracy and ultra-fast speed.[11] In addition, it allows the genome to be sequenced without any host cell and provides rich and original information.[12,13]

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Therefore, the aim of our study is to define the bacteria responsible for carious and non-carious plaque in permanent dentition with the NGS technology and reveal the bacterial nature of carious lesions.

Materials and Methods

A total of 52 patients aged between 18 and 50 years were divided into two main groups: caries-active (n = 26) and caries-free (n = 26). All subjects were systemically healthy, having no hereditary, morphologically and drug-related structural changes (amelogenesis imperfecta, dentinogenesis imperfecta, fluorosis, molar-incisal hypomineralization, etc.) in their teeth, not received any antibiotic treatment in the last 6 weeks, and having oral hygiene habits were similar (brushing their teeth with toothpaste at least once a day) were included. Caries classification was made on the basis of ICDAS II criteria.[14]

All patients had signed the informed consent form, and before sample collection, they were instructed to avoid any dietary and oral hygiene practices after 20:00 pm. Plaque samples (12 h) were collected with a sharp periodontal curette between 08:00 and 09:00 am transferred to Eppendorf tubes with DNA/RNA shield solution. (Ethical approval of a study was obtained from the Marmara University Faculty of Dentistry Ethics Committee (06.06.2017; #2017-100).

DNA isolation was performed according to the manufacturer's instructions on the isolation kit.

Samples were processed and analyzed by ZymoBIOMICS™ Service – Targeted Metagenomic Sequencing (ZymoResearch, Irvine, CA, USA). 16S library preparation: Bacterial 16S ribosomal RNA gene-targeted sequencing was performed using the Quick-16S™ NGS Library Prep Kit (Zymo Research, Irvine, CA, USA). The bacterial 16S primers used expanded the V3–V4 region of the 16S rRNA gene. These primers were specially designed by Zymo Research to provide the best coverage of the 16S gene with high sensitivity. They potentiate the V3–V4 region of the 16S rRNA gene. The sequencing library was prepared using an innovative library preparation process where PCR reactions were performed on real t-PCR machines to control the loops and thus prevent PCR chimera formation. The final PCR products were pooled based on equal molarity as measured by qPCR fluorescence readings. Finally, the common library was filtered with Select-a-Size DNA Clean & Concentrator™ (Zymo Research, Irvine, CA, USA), then measured with TapeStation® and Qubit®.

Sequencing: The final library was sequenced on Illumina® MiSeq™ with a V3 reagent kit (600 cycles). Sequencing was performed with >10% PhiX. Bioinformatics analysis of raw data Bioinformatics analysis was carried out by researchers (ZCC & AC) using NGS Mothur software on usegalaxy.org open-source address. The raw data of our study provided by ZymoResearch by following the Standard Operating Procedure presented by the Schloss laboratory within Galaxy, the creator of the Mothur software package.[15] These analyzes took place under the following headings and subheadings:

- Obtaining and preparing data
- Understanding the input data
- Transfer data to Galaxy program
- Quality control
- Sequencing and reduction of sequencing and PCR errors
- Evaluation of error rates according to sample population
- OTU analysis
- Calculating the diversity of species
- Visualization
  - Phinch
  - Krona-Pie-Chart
- Additional operations
- Determining the statistical significance of clusters

Data were analyzed descriptively using the software Stata v. 15.1 (StataCorp, College Station, TX, USA); with the level of significance set at $P < 0.05$ (excluding Bonferroni corrections). Pearson Chi-square and Fisher's exact tests were used to evaluate the relationships between categorical variables. Histogram, Kolmogorov–Smirnov, Q–Q Plot tests, Shapiro-Wilk, Skewness-Kurtosis tests were applied to evaluate the distribution in terms of normality. Since the data distributions were not parametric, Kruskal Wallis tests were used for the evaluation of more than two groups and Mann–Whitney U tests were used for intergroup post-hoc analysis. The P significance level for the Kruskal Wallis test was re-determined as 0.05/4 = 0.0125 according to the number of groups, and statistical significance was examined. Mann–Whitney U tests were used for comparisons between the two groups and $P < 0.05$ was considered statistically significant.

Results

Demographic information, tooth-brushing habits, caries information were shown in Table 1.

The caries status of the patients was diagnosed and recorded according to ICDAS II criteria shown in Table 2.

While 85.2% of the bacteria isolated from carious and non-carious plaque samples did not differ, 14.8% (n = 35) were found to be statistically significant. Descriptive data (median = p50) were listed in Table 3.

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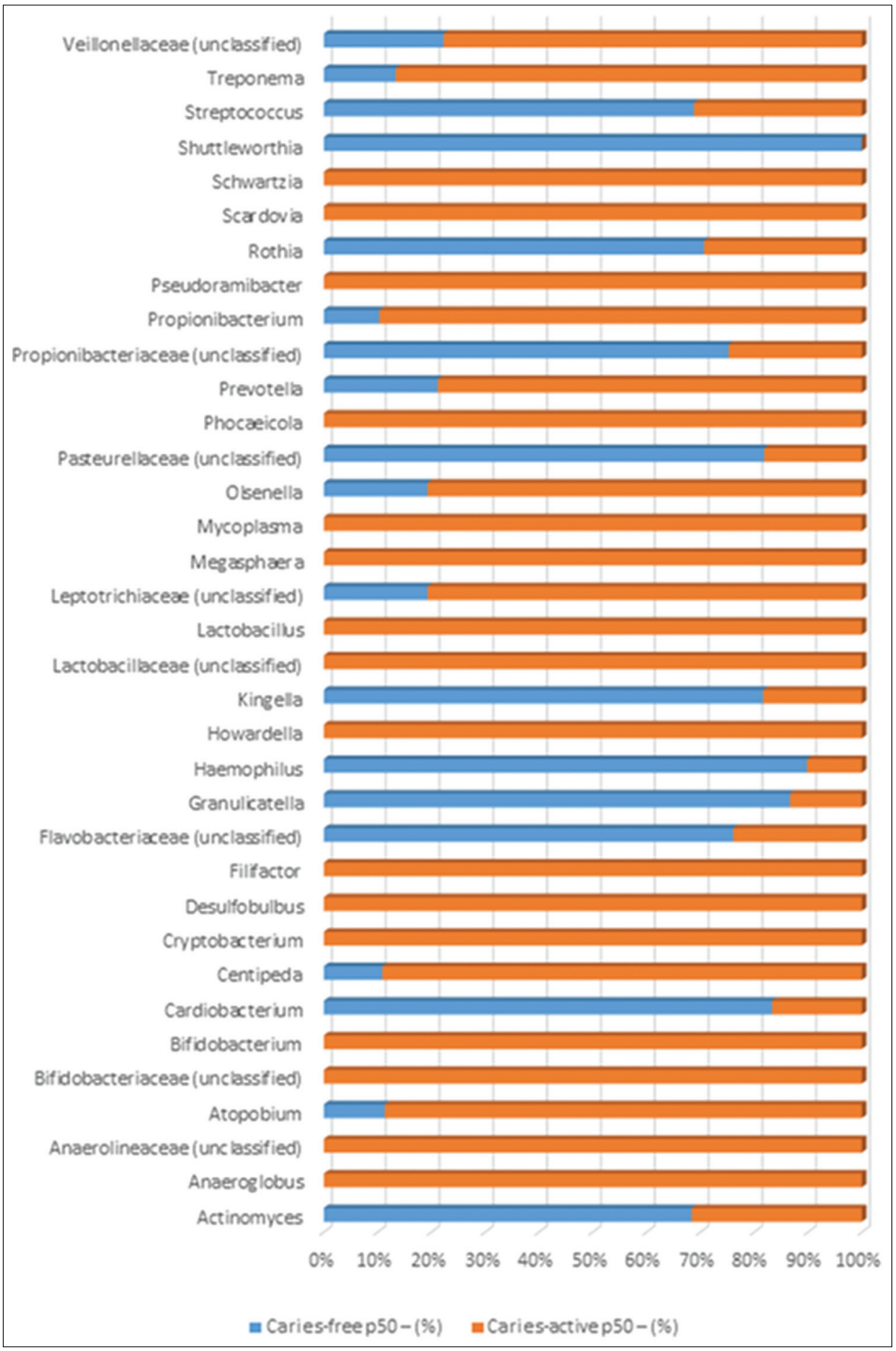


Figure 1: Distribution of oral bacteria in caries-free and caries-active plaques

Distribution of all bacteria in caries-free and caries-active plaques samples was shown in Figure 1.

Discussion

Contemporary concepts of dental caries etiology and pathogenesis emphasize a "mixed bacterial-ecological approach" as being responsible for lesion initiation and progression.[16] Rather than being considered an "infectious disease" caused by a specific organism, dental caries are now understood to be a biofilm-mediated disease.[17] Dental caries can thus be considered to be anendogenous infection, which may occur when members of resident flora obtain a selective ecological advantage over other species, disrupting the homeostatic balance of the biofilm, and thereby initiating the disease process.[18]

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Table 1: Demographic information, tooth-brushing habits, caries information of caries-acrive, and caries-free groups

	Caries-active			Caries-free		
	Female	Male	Total	Female	Male	Total
Age						
Mean±SD	26,2±6,9	30,8.4±7,8	28,7±7,1	24,9±5,8	29,1±8,2	26.7±8.3
Gender						
n (%)	12 (46)	14 (54)	26 (100)	15 (58)	11 (42)	26 (100)
Tooth-brushing habit						
n/day (%)						
None/rare	3 (25)	5 (36)	8 (30)	0 (0)	0 (0)	0 (0)
1	7 (58)	6 (43)	13 (50)	5 (33)	3 (27)	8 (31)
2	2 (17)	3 (21)	5 (20)	7 (47)	6 (55)	13 (50)
2+	0 (0)	0 (0)	0 (0)	3 (20)	2 (18)	5 (19)
Average caries						
n_{median} ICDAS II	8	11	10	0	0	0

Table 2: Caries status of caries-active and caries-free groups

	ICDAS 0	ICDAS 1	ICDAS 2	ICDAS 3	ICDAS 4	ICDAS 5	ICDAS 6	Total
Caries-active *n* (%)	53 (9)	23 (4)	35 (6)	187 (32)	129 (22)	88 (15)	70 (12)	585 (100)
Caries-free *n* (%)	366 (67)	115 (21)	65 (12)	0 (0)	0 (0)	0 (0)	0 (0)	546 (100)

In this study, microbial diversity of caries-free and caries-active plaques in permanent dentition were investigated by using NGS.

In a study performed by Cephas *et al*.,[19] high bacterial diversity was noted in saliva of adults and infants. *Haemophilus*, *Neisseria*, *Veillonella*, *Fusobacterium*, *Oribacterium*, *Rothia*, *Treponema*, and *Actinomyces* were predominant in adults. Another study using the ICDAS II classification in the diagnosis of caries showed that Shuttleworthia was significantly higher in caries-free compared to caries-affected children ($P = 0.031$).[20] In addition, microbiota the black discolored plaque, which was found to be associated with caries-free status, were listed as listed *Actinomyces*, *Cardiobacterium*, *Haemophilus*, *Corynebacterium*, *Tannerella*, and *Treponema*.[10,21] Consistent with these findings, in our study, *Actinomyces*, *Shuttleworthia*, *Rothia*, *Haemophilus*, and *Granulicatella* were found to be statistically higher in caries-free plaques.

On the other hand, it has been suggested that *Streptococcus mutans* play a role in the main etiology of dental caries in the history of dentistry.[22,23] However, consistent with our study, some other studies contradict and suggest other bacterial species such as *Lactobacillus*, *Actinomyces* or *Bifidobacterium*, *Veillonella* may be dominant in the carious plaque.[6,24,25] Recent molecular studies support these findings, expanding the list of potential cariogenic bacteria to other bacterial species such as *Veillonella*, *Propionibacterium*, *Selenomonas*, *Neisseria*, *Eikenella*, *Fusobacterium*, *Leptotrichia*, *Enterococcus*, and *Atopobium*.[26-29] Additionally, *Scardovia wiggsiae* was found to be significantly associated with severe-early caries in the presence and absence of *S. mutans*.[30] In our study, consistent with these findings, *Atopobium*, *Bifidobacterium*, *Lactobacillus*, *Propionibacterium*, *Scardovia*, *Veillonellaceae* bacteria were found to be statistically higher in carious plaque samples ($P < 0.05$). In addition to these bacteria, *Anaeroglobus*, *Bifidobacteriaceae* (unclassified), *Centipeda*, *Cryptobacterium*, *Desulfobulbus*, *Filifactor*, *Howardella*, *Lactobacillaceae* (unclassified), *Leptotrichiaceae* (unclassified), *Megasphaera*, *Mycoplasma*, *Olsenella*, *Phocaeicola*, *Pseudoramibacter*, *Schwartzia*, and *Treponema* were also found to be related to caries formation.

The NGS method makes it possible to detect previously undetected species, but it also brings with a more detailed microbial analysis. The NGS method allows the analysis of a vast number of bacteria, quickly and precisely.[13] In this method, the 16S rRNA gene used to detect bacterial diversity has several properties that make it suitable for our purpose. It increases the reliability of the method by being present in all prokaryotes, having highly protected and variable regions, and being the most widely used gene for the identification of bacteria. Using the NGS technology, the present study aids to understand complex microbial diversity in caries-free and caries-active plaques in permanent dentition.

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Table 3: Microbial differences of caries-free and caries-active plaque samples

Bacteria	Caries-free p50 -(%)	Caries-active p50 -(%)	*P*
Actinomyces	3,45	1,56	0.0019
Anaeroglobus	0	0,032	0.0108
Anaerolineaceae (unclassified)	0	0,0022	0.0011
Atopobium	0,0036	0,028	0.0002
Bifidobacteriaceae (unclassified)	0	0,016	0.0000
Bifidobacterium	0	0,023	0.0000
Cardiobacterium	1,2	0,24	0.0001
Centipeda	0,0012	0,0098	0.0236
Cryptobacterium	0	0,002	0.0004
Desulfobulbus	0	0,002	0.0132
Filifactor	0	0,26	0.0077
Flavobacteriaceae (unclassified)	0,58	0,182	0.0083
Granulicatella	0,13	0,02	0.0071
Haemophilus	0,23	0,026	0.0061
Howardella	0	0,0024	0.0303
Kingella	0,74	0,216	0.0032
Lactobacillaceae (unclassified)	0	0,0035	0.0011
Lactobacillus	0	0,036	0.0000
Leptotrichiaceae (unclassified)*)*	0,0072	0,0301	0.0061
Megasphaera	0	0,042	0.0041
Mycoplasma	0	0,0114	0.0037
Olsenella	0,055	0,23	0.0071
Pasteurellaceae (unclassified)	10,4	2,32	0.0023
Phocaeicola	0	0,004	0.0004
Prevotella	3,2	12,4	0.0071
Propionibacteriaceae (unclassified)	0,14	0,046	0.0005
Propionibacterium	0,0037	0,032	0.0060
Pseudoramibacter	0	0,0116	0.0068
Rothia	0,64	0,258	0.0483
Scardovia	0	0,008	0.0090
Schwartzia	0	0,0035	0.0269
Shuttleworthia	0,0093	0	0.0105
Streptococcus	5,6	2,54	0.0225
Treponema	0,26	1,68	0.0483
Veillonellaceae (unclassified)	0,38	1,28	0.0378

P50: median

Conflicts of interest

There are no conflicts of interest.

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Table of Contents | Author Institution Mapping | Reviewer Institution Mapping | Citations | Access Statistics | View as eBook

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	Issue 3 (March) Page Nos. 215-378 Accessed - 22455	Issue 2 (February) Page Nos. 123-214 Accessed - 21515	Issue 1 (January) Page Nos. 1-122 Accessed - 25262	
Vol.24 2021	Issue 12 (Dec) Page Nos. 1759-1869 Accessed - 25754	Issue 11 (November) Page Nos. 1573-1758 Accessed - 36361	Issue 10 (October) Page Nos. 1415-1571 Accessed - 26683	Issue 9 (September) Page Nos. 1259-1413 Accessed - 21677
	Issue 8 (August) Page Nos. 1109-1258 Accessed - 21090	Issue 7 (July) Page Nos. 965-1107 Accessed - 23527	Issue 6 (June) Page Nos. 789-963 Accessed - 43543	Issue 5 (May) Page Nos. 633-788 Accessed - 17125
	Issue 4 (April) Page Nos. 457-631 Accessed - 36122	Issue 3 (March) Page Nos. 299-455 Accessed - 71118	Issue 2 (February) Page Nos. 143-298 Accessed - 28453	Issue 1 (January) Page Nos. 1-141 Accessed - 32750
Vol.23 2020	Issue 12 (Dec) Page Nos. 1629-1779 Accessed - 51268	Issue 11 (November) Page Nos. 1487-1627 Accessed - 48517	Issue 10 (October) Page Nos. 1333-1486 Accessed - 54184	Issue 9 (September) Page Nos. 1183-1331 Accessed - 64638
	Issue 8 (August) Page Nos. 1033-1182 Accessed - 38454	Issue 7 (July) Page Nos. 889-1032 Accessed - 77286	Issue 6 (June) Page Nos. 751-888 Accessed - 59620	Issue 5 (May) Page Nos. 589-749 Accessed - 56678
	Issue 4 (April) Page Nos. 437-588 Accessed - 51049	Issue 3 (March) Page Nos. 275-436 Accessed - 50291	Issue 2 (February) Page Nos. 129-274 Accessed - 43051	Issue 1 (January) Page Nos. 1-127 Accessed - 55890
Vol.22 2019	Issue 12 (Dec) Page Nos. 1629-1789 Accessed - 111585	Issue 11 (November) Page Nos. 1463-1628 Accessed - 87825	Issue 10 (October) Page Nos. 1313-1462 Accessed - 97330	Issue 9 (September) Page Nos. 1175-1311 Accessed - 96763
	Issue 8 (August) Page Nos. 1033-1174 Accessed - 78793	Issue 7 (July) Page Nos. 881-1031 Accessed - 101168	Issue 6 (June) Page Nos. 745-880 Accessed - 92580	Issue 5 (May) Page Nos. 591-744 Accessed - 63241
	Issue 4 (April) Page Nos. 445-590 Accessed - 117050	Issue 3 (March) Page Nos. 289-444 Accessed - 117182	Issue 2 (February) Page Nos. 149-288 Accessed - 65158	Issue 1 (January) Page Nos. 1-147 Accessed - 75655
Vol.21 2018	Issue 12 (December) Page Nos. 1549-1684 Accessed - 99888	Issue 11 (November) Page Nos. 1391-1547 Accessed - 85240	Issue 10 (October) Page Nos. 1243-1390 Accessed - 76097	Issue 9 (September) Page Nos. 1087-1241 Accessed - 103434
	Issue 8 (August) Page Nos. 949-1085 Accessed - 127124	Issue 7 (July) Page Nos. 821-948 Accessed - 81283	Issue 6 (June) Page Nos. 687-819 Accessed - 108001	Issue 5 (May) Page Nos. 539-686 Accessed - 166368

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	Issue 4 (April) Page Nos. 401-537 Accessed - 104187	Issue 3 (March) Page Nos. 257-400 Accessed - 85995	Issue 2 (February) Page Nos. 125-255 Accessed - 93548	Issue 1 (January) Page Nos. 1-118 Accessed - 50560
Vol.20 2017	Issue 12 (December) Page Nos. 1517-1658 Accessed - 97578	Issue 11 (November) Page Nos. 1355-1515 Accessed - 105546	Issue 10 (October) Page Nos. 1221-1354 Accessed - 88176	Issue 9 (September) Page Nos. 1053-1220 Accessed - 117134
	Issue 8 (August) Page Nos. 919-1051 Accessed - 105103	Issue 7 (July) Page Nos. 783-918 Accessed - 87615	Issue 6 (June) Page Nos. 647-782 Accessed - 116699	Issue 5 (May) Page Nos. 507-646 Accessed - 111282
	Issue 4 (April) Page Nos. 399-506 Accessed - 81784	Issue 3 (March) Page Nos. 267-398 Accessed - 106420	Issue 2 (February) Page Nos. 131-265 Accessed - 145197	Issue 1 (January) Page Nos. 1-125 Accessed - 111032
Vol.19 2016	Issue 6 (Nov-Dec) Page Nos. 695-839 Accessed - 121359	Issue 5 (September-October) Page Nos. 573-694 Accessed - 107059	Issue 4 (July-August) Page Nos. 431-572 Accessed - 127780	Issue 3 (May-June) Page Nos. 303-430 Accessed - 109246
	Issue 2 (March-April) Page Nos. 161-302 Accessed - 132897	Issue 1 (January-February) Page Nos. 1-155 Accessed - 118645		
Vol.18 2015	Suppl 1 (December) Page Nos. s1-s78 Accessed - 142072	Issue 6 (November-December) Page Nos. 703-832 Accessed - 144173	Issue 5 (September-October) Page Nos. 577-701 Accessed - 102870	Issue 4 (July-August) Page Nos. 437-576 Accessed - 276408
	Issue 3 (May-June) Page Nos. 307-435 Accessed - 117025	Issue 2 (March-April) Page Nos. 163-306 Accessed - 186633	Issue 1 (January-February) Page Nos. 1-148 Accessed - 103542	
Vol.17 2014	Issue 6 (November-December) Page Nos. 667-818 Accessed - 184205	Issue 5 (September-October) Page Nos. 543-665 Accessed - 131169	Issue 4 (July-August) Page Nos. 397-542 Accessed - 168096	Issue 3 (May-June) Page Nos. 267-396 Accessed - 161495
	Issue 2 (March-April) Page Nos. 127-266 Accessed - 164954	Issue 1 (January-February) Page Nos. 1-124 Accessed - 198823		
Vol.16 2013	Issue 4 (October-December) Page Nos. 407-558 Accessed - 178254	Issue 3 (July-September) Page Nos. 269-405 Accessed - 248747	Issue 2 (April-June) Page Nos. 129-268 Accessed - 229803	Issue 1 (January-March) Page Nos. 1-126 Accessed - 172724
Vol.15 2012	Issue 4 (October-December) Page Nos. 373-499 Accessed - 152334	Issue 3 (July-September) Page Nos. 247-372 Accessed - 192038	Issue 2 (April-June) Page Nos. 125-246 Accessed - 156853	Issue 1 (January-March) Page Nos. 1-115 Accessed - 178099
Vol.14 2011	Issue 4 (October-December) Page Nos. 383-509 Accessed - 213780	Issue 3 (July-September) Page Nos. 253-382 Accessed - 210146	Issue 2 (April-June) Page Nos. 129-252 Accessed - 220009	Issue 1 (January-March) Page Nos. 1-122 Accessed - 200556
Vol.13 2010	Issue 4 (October-December) Page Nos. 360-479 Accessed - 147131	Issue 3 (July-September) Page Nos. 240-359 Accessed - 52670	Issue 2 (April-June) Page Nos. 120-239 Accessed - 54908	Issue 1 (January-March) Page Nos. 1-119 Accessed - 37748
Vol.12 2009	Issue 4 (December) Page Nos. 345-464 Accessed - 17729	Issue 3 (September) Page Nos. 229-3377 Accessed - 17225	Issue 2 (June) Page Nos. 113-228 Accessed - 17518	Issue 1 (March) Page Nos. 1-112 Accessed - 16006
Vol.11 2008	Issue 4 (December) Page Nos. 291-401 Accessed - 18307	Issue 3 (September) Page Nos. 176-289 Accessed - 15067	Issue 2 (June) Page Nos. 89-176 Accessed - 12604	Issue 1 (March) Page Nos. 1-87 Accessed - 13717
Vol.10 2007	Issue 4 (December) Page Nos. 272-356	Issue 3 (September) Page Nos. 184-270	Issue 2 (June) Page Nos. 95-183	Issue 1 (March) Page Nos. 1-94

	Accessed - 11822	Accessed - 13961	Accessed - 11652	Accessed - 11626
Vol.9 2006	Issue 2 (December) Page Nos. 96-182 Accessed - 10637		Issue 1 (June) Page Nos. 1-93 Accessed - 14130	
Vol.8 2005	Issue 2 (December) Page Nos. 65-137 Accessed - 11966		Issue 1 (June) Page Nos. 1-64 Accessed - 9350	

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