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Biochemical and molecular analysis of bacteria isolated from dental caries

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ABSTRACT

Dental caries, next to common cold, is one of the most common disorders. It is a bacterial process which damage hard tooth structures (enamel, dentine and cementum). In order to study the cause of dental caries, this study was carried out to isolate the predominant cariogenic organisms from caries samples. A total of 10 caries samples were collected and from the samples 405 microbials isolates were obtained. Among this, 28 morphologically different colonies were identified and from them, 10 predominant were selected for identification. The biochemical and molecular analysis reveals that two organisms belongs to the genus Enterobacter and two belongs to the genus Bacillus. The organisms such as Enterobacter hormaechei, Enterobacter sp., Micrococcus luteus, Klebsiella pneumoniae, Exiguobacterium sp., Staphylococcus sciuri, Acinetobacter radioresistens, Weissella confusa, Bacillus cereus and Bacillus subtilis were identified as predominant organisms in oral cavity.

Keywords: Dental caries, cariogenic organisms, isolation, biochemical characterization, 16S rRNA sequencing, phylogenesis

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INTRODUCTION

Dental caries is a polymicrobial infectious disease, which cause biofilm accumulation on dental surface and leads to frequent consumption of fermentable sugars [1]. It is considered that the accumulation and metabolism of bacteria on teeth and implants surfaces are the primary cause of caries, gingivitis, peridontities, periimplantitis and breathe [2]. More than 500 bacterial species have been identified from the oral cavity [3,4] and a large proportion of the microbes present in the mouth have not yet been cultured [5]. Now a days, advanced molecular methods such as polymerase chain reaction (PCR) and 16S rRNA gene sequencing analysis were used to identify the microbes and it have been revealed that the bacterial involvement in the development of dental caries is more complex than previously believed [6].

In order to evaluate the status of dental caries as a public health problem, there must be a research on each fact of the disease process. The first step of the study may be finding the organisms associated with caries. So the aim of the study is to isolate and identify the bacteria from dental caries affected teeth.

EXPERIMENTAL SECTION

Collection of dental caries samples

The caries samples were collected from 10 patients of age group 7-67yrs from Rose Dental Clinic, Nagercoil, K.K. Dist., Tamil Nadu, under aseptic condition. Prior to the collection of sample, the patients were made to rinse the tooth with 3% hydrogen peroxide and then cleaned with decontaminating solution (2.5% sodium hypochlorite) [7]. The caries samples were collected from the patients using the sterile swab by a dentist and were introduced into 2ml of normal saline in sterile screw cap tubes. The samples were transported to the laboratory for further study and stored at 4ºC.

Isolation and screening of bacteria

For isolation of bacteria, the method of Chandrabhan *et al*. [8] was followed with minor modifications. Each sample in tubes was inoculated separately into 25 ml of nutrient broth (Himedia) and incubated at 35°C ± 1°C for 48 hours. One ml of freshly grown culture from each dental plaque was serially diluted up to 10^{-10} with distilled water. 100 µl serially diluted samples were spread over nutrient agar plates. The inoculated plates were incubated at 37°C for 3 days under aerobic conditions. The predominant and morphologically different colonies were then cultured using standard streak plate technique for the isolation of the microorganisms in the nutrient agar media. The isolated colonies were subsequently streaked on nutrient agar slant for pure culture preservation.

Morphological and biochemical identification of isolates

The bacteria were gram stained and observed under light microscope (40×). Motility was checked using hanging drop method as referred by Aneja [9]. The presence or absence of spore was checked using malachite green [9]. Biochemical properties of the isolates were tested according to Bergey's Manual of Systematic Bacteriology [10]. The following properties were determined: IMViC test series, nitrate reduction, urease test, starch hydrolysis, gelatin liquefaction, oxidase, catalse, hydrogen sulfide production and carbohydrate utilization test.

Identification of the isolates by 16S rRNA sequencing

DNA isolation reagent (SoluteReady® Genomic DNA purification kit), PCR Master Mix, Agarose gel electrophoresis consumables and primers were purchased from HELINI Biomolecules, Chennai, India. The DNA extraction was performed by following the manufacturer's instructions. The forward and reverse DNA sequencing reaction of PCR amplicon was carried out with the following 16S rRNA Forward primer: AGA GTT TGA TCC TGG CTC AG and 16S rRNA Reverse primer: ACG GCT ACC TTG TTA CGA CTT using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The size and amount of amplicons were examined by electrophoresis on 1% agarose gel with 10µl of 100bp DNA ladder.

Phylogenetic analysis and provisional taxonomy

The 16S rDNA gene sequences were used to carry out BLAST with the nrdatabase of NCBI genbank database and were provisionally identified. Based on maximum identity score first two sequences from each isolates were selected and aligned using multiple alignment software program Clustal W. The evolutionary history was inferred using the Maximum Parsimony (MP) method. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm [11] with search level 2 in which the initial trees were obtained by the random addition of sequences (10 replicates). Evolutionary analyses were conducted in MEGA6 [12].

RESULTS

Isolation and Screening of bacteria from dental caries

During the study period, a total of ten samples (S1 to S10) were collected from patients affected with dental caries. The age of the patients was from 7 to 67. The total viable count (TVC) of S1 to S10 at 10^{-5} dilution revealed that the total numbers of colonies were 405 among all the age group. The sample S3 (age 21) showed a maximum of 72 colonies, whereas sample S10 (age 67) showed a minimum of 8 colonies. Among the total 405 colonies, twenty eight morphologically different colonies were found (Table 1), and among which ten predominant colonies were selected for further study. Accordingly the isolates were named as A1-A10 (Plate 1).

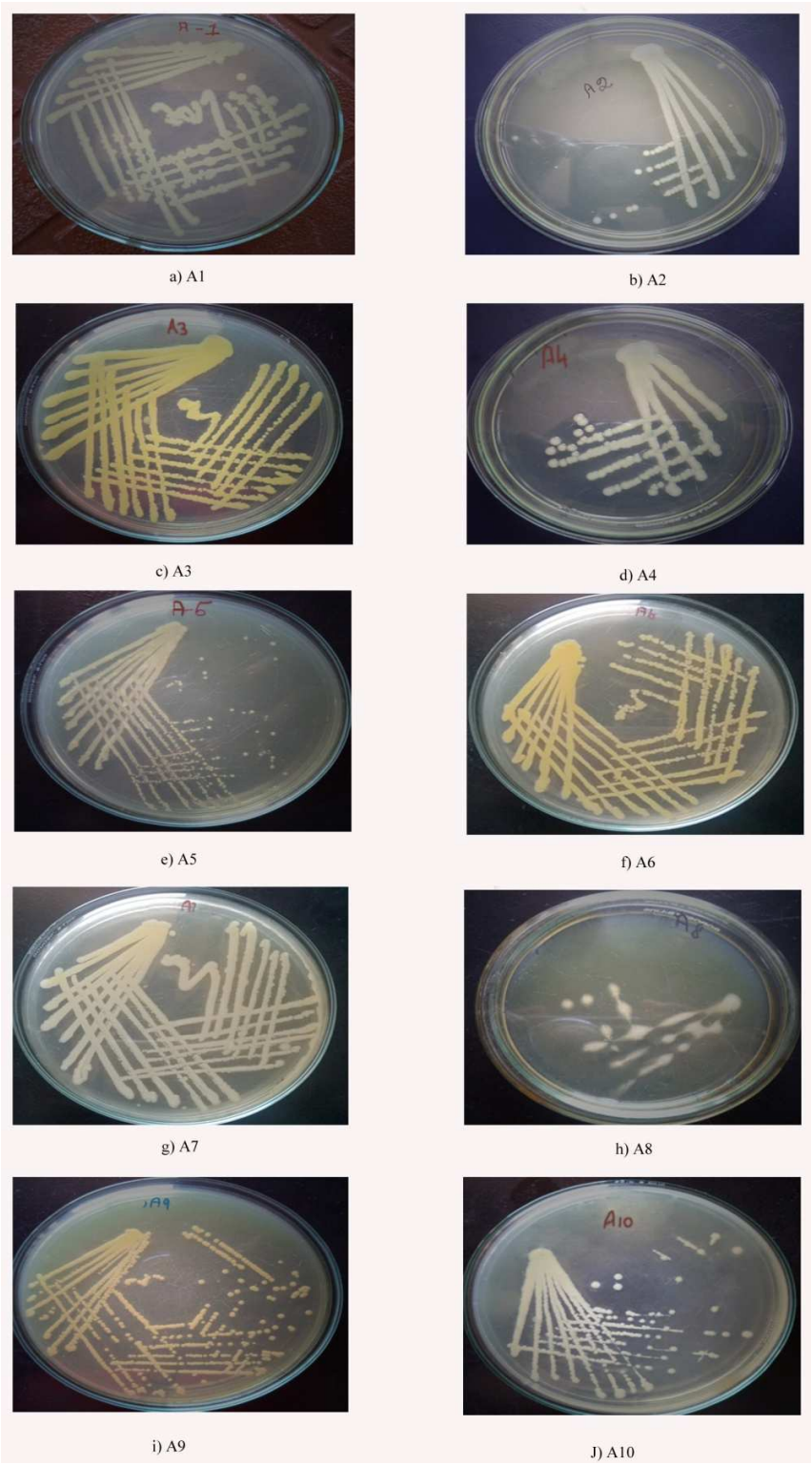

Plate 1 Predominant colonies isolated from dental caries infected teeth

Table 1 Distribution of bacteria in patients affected with dental caries

Sl.No.	Sample name and age	TVC CFU/ml (10^{-5})	No. of morphologically different colonies
1	S1(7)	10	28
2	S2(17)	23	
3	S3(21)	72	
4	S4(27)	69	
5	S5(30)	65	
6	S6(35)	60	
7	S7(38)	62	
8	S8(47)	18	
9	S9(58)	18	
10	S10(67)	8	
Total number of isolates		405	

Morphological and biochemical identification of isolates

The results of morphological and biochemical tests indicated that, five isolates were rods, three isolates were short rods and two were cocci. The gram staining results showed the isolates A1, A2, A4, and A7 were gram negative and the other isolates were gram positive. The isolates such as A1, A2, A3, A5, A9 and A10 were motile and A4, A6, A7 and A8 were non motile. Spore forming organisms such as A3, A9, and A10 were also isolated. In IMViC test series, isolates A8 and A10 were positive and all the other isolates were negative for indole production test. Three isolates such as A3, A5 and A8 were positive to methyl red. Voges Proskauer results showed that five isolates were positive. Six isolates utilizes citrate and four isolates failed to utilize citrate, as carbon source. The isolates A1, A2, A4, A6, A9 and A10 were found to be positive for nitrate reduction. Majority of the isolates displayed negative result to urease test and only A4 and A5 showed positive result. In starch and gelatin hydrolyzing ability, isolates A3, A5 and A10 showed positive to both hydrolysis. A2 hydrolyse neither starch nor gelatin. Three isolates (A1, A6 and A8) does not hydrolyse starch but hydrolyse gelatin. Isolates such as A4, A7 and A9 hydrolyse starch and failed to hydrolyse gelatin. A3, A6, and A10 were positive to both oxidase and catalase, whereas A2 and A8 were negative. All the other isolates such as A1, A4, A5, A7 and A9 displayed oxidase negative and catalase positive. Three isolates (A6, A7 and A9) produces hydrogen sulphide. When the isolates are checked for the utilization of carbohydrates variations were noted. The results were recorded in Table 2.

Table 2 Morphological and Biochemical tests of 10 selected organisms

Sl.No	Test name		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
1.	Morphology		Rods	Short rods	Cocci	Rods	Rods	Cocci	Rods	Short rods	Rods	Short rods
2.	Gram staining		-	-	+	-	+	+	-	+	+	+
3.	Motility		+	+	+	-	+	-	-	-	+	+
4.	Spore		-	-	+	-	-	-	-	-	+	+
5.	Indole		-	-	-	-	-	-	-	+	-	+
6.	Methyl Red		-	-	+	-	+	-	-	+	-	-
7.	Voges Proskauer		+	+	-	+	-	-	-	-	+	+
8.	Citrate utilization		+	+	+	+	-	-	+	-	-	+
9.	Nitrate reduction		+	+	-	+	-	+	-	-	+	+
10.	Urease		-	-	-	+	+	-	-	-	-	-
11.	Starch hydrolysis		-	-	+	+	+	-	+	-	+	+
12.	Gelatin liquefaction		+	-	+	-	+	+	-	+	-	+
13.	Oxidase		-	-	+	-	-	+	-	-	-	+
14.	Catalase		+	-	+	+	+	+	+	-	+	+
15.	Hydrogen Sulphide production		-	-	-	-	-	+	+	-	+	-
16.	Carbohydrate fermentation test	Glucose	+	+	+	+	+	+	-	+	+	+
17.		Sucrose	-	+	-	+	+	+	+	+	+	+
18.		Lactose	-	+	-	+	-	+	-	-	+	-
19.		Maltose	+	+	+	+	+	-	+	+	+	+
20.		Mannitol	+	+	-	+	+	+	-	-	-	+
21.		Fructose	-	-	+	+	+	+	-	+	+	+
22.		Arabinose	+	+	-	+	+	-	-	-	+	+
23.		Mannose	+	+	-	+	-	+	-	-	+	+

+ positive, - negative

Identification of the isolates by 16S rRNA sequencing and phylogenetic analysis

Fragment of 16S rDNA gene were amplified by PCR. A single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose. After that the PCR amplicon was purified to remove contaminants. The forward and reverse DNA sequencing reaction of PCR amplicon were carried out using BDT v3.1 Cycle sequencing kit on ABI 3730xl genetic analyzer.

The phylogenetic tree was constructed using Maximum Parsimony method. The consensus tree inferred from 10 most parsimonious trees were shown in Figure 1. Branches corresponding to partitions reproduced in less than 50% trees are collapsed. The consistency index was 0.778473 (0.738938), the retention index was 0.883629 (0.883629), and the composite index was 0.687882 (0.652947) for all sites and parsimony-informative sites (in parentheses). The analysis involved 30 nucleotide sequences. Codon positions included were 1st. There were a total of 521 positions in the final dataset.

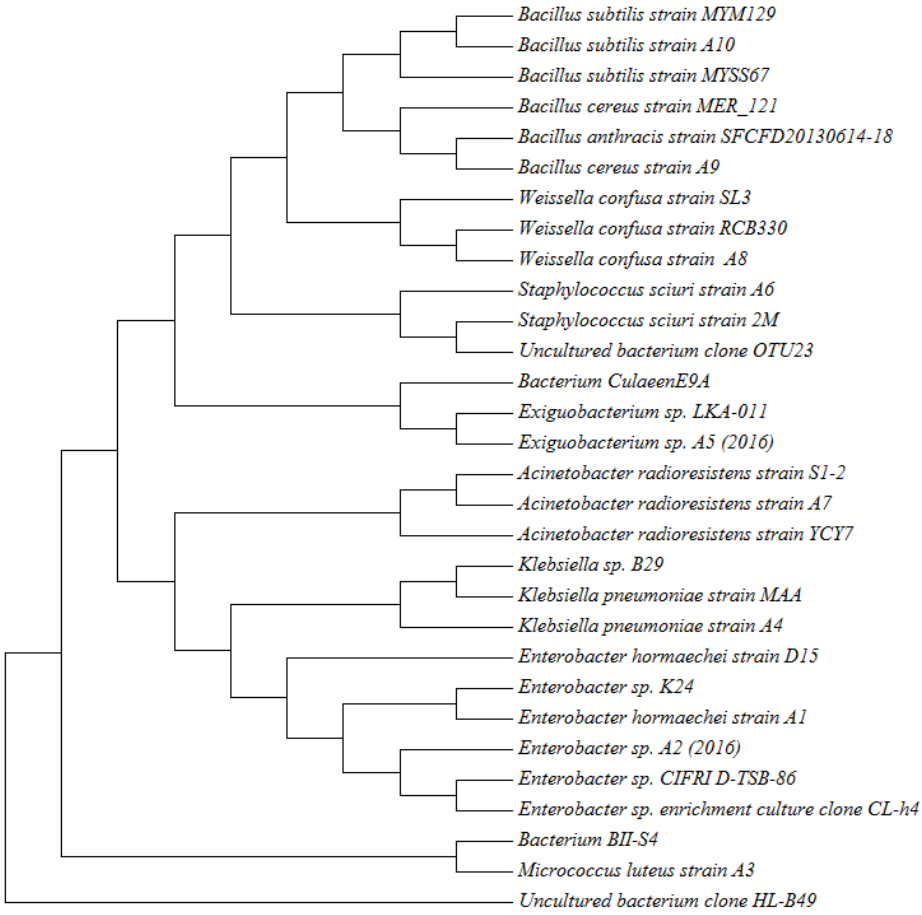

Figure 1 Phylogenetic analysis of bacterial taxa found in clinical samples of caries infected teeth

Based on the nucleotide homology and phylogenetic analysis, the bacterial strains A1 and A2 were identified and assigned as *Enterobacter hormaechei* strain A1 and *Enterobacter* sp. A2 (2016) (GenBank Accession Numbers: KT598356.1 and KT598355.1). The nearest homolog species of these strains were found to be *Enterobacter* sp. K24 and *Enterobacter* sp. CIFRI D-TSB-86 (GenBank Accession Number: KM377654.1 and KP117096.1). The isolates A1 and A2 showed 99% similarity with their respective homolog species. The nearest homolog species of the strain A3 was found to be Bacterium BII-S4 (GenBank Accession Number: HG800059.1) and the strain A3 was identified and assigned as *Micrococcus luteus* strain A3 (GenBank Accession Number: KT598358.1). The similarity was 99%. The bacterial strain A4 was identified and assigned as *Klebsiella pneumoniae* strain A4 (GenBank Accession Number: KT598354). The strain has 100% similarity with its nearest homolog species, and the nearest homolog species of this strain was found to be *Klebsiella* sp. B29 (GenBank Accession Number: KT316422.1). The strain A5

was identified and assigned as *Exiguobacterium* sp. A5 (2016) (GenBank Accession Number: KT440887.1) and its nearest homolog species was found to be *Exiguobacterium* sp. LKA-011 (GenBank Accession Number: JN626953.1) and the similarity was 99%. The closest relative of the strain A6 was *Staphylococci sciuri* strain 2M (GenBank Accession Number: KT339323.1) with 98% similarity and hence the strain A6 was identified and assigned as *Staphylococci sciuri* A6 and the GenBank accession number was KT440886.1. The strain *Acinetobacter radioresistens* strain A7 (GenBank Accession Number: KT440885.1) which was previously assigned as strain A7 has 99% similarity with its nearest homolog species *Acinetobacter radioresistens* strain YCY7 (GenBank Accession Number: JF775420.1). Based on 16S rRNA sequencing, the strain A8 was identified as *Weissella confusa* strain A8 and deposited to GenBank with Accession Number: KT440884.1. Its nearest homolog species was *Weissella confusa* strain RCB330 (GenBank Accession Number: KT260542.1) and the percentage of similarity was 99. Based on morphological and biochemical analysis, it was identified that the strains A9 and A10 belongs to the genus Bacillus. By 16S rRNA sequencing, their species level were identified and assigned as *Bacillus cereus* and *Bacillus subtilis* with GenBank accession numbers KT598357.1 and KT440888.1 respectively. Their nearest homolog species were found to be *Bacillus cereus* strain MER_121 (GenBank accession number KT719696.1) and *Bacillus subtilis* strain MYM129 (GenBank accession number KR827431.1). The similarity was found to be 100 and 99 percentage respectively (Table 3).

Table 3 Identification of bacterial strains isolated from dental caries with partial sequence of 16S rRNA genes referenced to accession number in GenBank

Sl.No.	Isolates	Name of the organism with accession number	Closest relative (obtained from BLAST search)	Similarity (%)
1	A1	*Enterobacter hormaechei* strain A1(KT598356.1)	*Enterobacter* sp. K24 (KM377654.1)	99
2	A2	*Enterobacter* sp.A2(2016) (KT598355.1)	*Enterobacter* sp. CIFRI D-TSB-86 (KP117096.1)	99
3	A3	*Micrococcus luteus* strain A3(KT598358.1)	*Bacterium* BII-S4 (HG800059.1)	99
4	A4	*Klebsiella pneumoniae* strain A4 (KT598354.1)	*Klebsiella* sp. B29 (KT316422.1)	100
5	A5	*Exiguobacterium* sp.A5(2016) (KT440887.1)	*Exiguobacterium* sp. LKA-011 (JN626953.1)	99
6	A6	*Staphylococcus sciuri* strain A6 (KT440886.1)	*Staphylococcus sciuri* strain 2M (KT339323.1)	98
7	A7	*Acinetobacter radioresistens* strain A7 (KT440885.1)	*Acinetobacter radioresistens* strain YCY7 (JF775420.1)	99
8	A8	*Weissella confusa* strain A8 (KT440884.1)	*Weissella confusa* strain RCB330 (KT260542.1)	99
9	A9	*Bacillus cereus* strain A9 (KT598357.1)	*Bacillus cereus* strain MER_121 (KT719696.1)	100
10	A10	*Bacillus subtilis* strain A10 (KT440888.1)	*Bacillus subtilis* strain MYM129 (KR827431.1)	99

DISCUSSION

Dental disease is a progressive bacterial damage to teeth exposed to oral environment. It is one of the most common diseases found in all age groups because of its relatively rapid progress and it is the main cause of tooth loss in younger people. The ultimate effect of caries is to break down enamel and dentine to open a path for bacteria to reach the underlying tissue [13]. Devi *et al*. [14] recovered 320 isolates from 150 samples. Mohapatra *et al*. [13] processed 100 caries samples on different isolation media like blood agar, Lactobacilli isolation agar, actinomycetes isolation agar and sabouraud dextrose agar and isolated 218 microbial colonies and Chandrabhan *et al*. [8] isolated 39 bacteria from 50 dental plaque samples. In the present investigation, from 10 caries samples a total of 405 colonies were observed at 10^{-5} dilution.

Dental caries is placed into the category of a bacterially dependent problem. Actually the cause of caries is not due to a single organism, but a series of microorganisms cooperatively responsible for this [15]. In the present findings, organisms such as *E. hormaechei*, *Enterobacter* sp, *M. luteus*, *A. radioresistens*, *Exiguobacterium* sp, *W. confusa* (basonym- *Lactobacillus confuses*), *S. sciuri* and *K. pneumoniae*, *B. cereus and B. subtilis* were identified.

Among the selected isolates, two gram negative, oxidase negative, fermentative and nonpigmented rods with the general characteristics of the family Enterobacteriaceae and of the genus Enterobacter were noted. The isolates were further identified as *E. hormaechei* and *Enterobacter* sp by 16S rRNA sequencing. Kim *et al.* [16] and O'Hara *et al.* [17] found the dominance of *E. hormaechei* by sequence analysis from the supragingival plaque. Back-Brito *et al.* [18] suggested that the microorganisms of the Enterobacteriaceae family present in the oral cavity can serve as a reservoir, and can severely compromise the lives of immunocompromised individuals. Mahopatra *et al.* [13] worked on samples of dental caries from different hospitals and clinics of Bhubaneswar and have reported the presence of *Staphylococcus* sp and *Klebsiella* sp. In the present study, *S. sciuri* and *K. pneumoniae* were isolated from dental caries. *S. albus* and *K. pneumoniae* were also reported by Olorunjuwon *et al.* [19] in the infected teeth.

In the present study, a gram positive short rod was identified as *W. confusa.* The organism was previously reported by Olano *et al.* [20] who states that *W. confusa* can be identified and differentiated from other species such as *Enterococcus* spp; *Sreptococcus* spp; *Lactobacillus* spp; and *Leuconostoc* spp. by its biochemical and physiological properties. Lee *et al.* [21] studied on ten patients with bacteraemia caused by *W. confusa* which are rarely reported.
Among the cultures isolated, the routine identification of *A. radioresistens* and *Exigoubacterium* sp. were not possible by a phenotypic approach, due to their absence in the databases of all commercial biochemical kits. Thus only identification by using a molecular approach was done to evaluate the accurate identification. Dortet *et al.* [22] also used the molecular approach to identify two organisms namely, *A. ursingii* and *A. schindleri.* Similarly, Anderson *et al.* [23] found *E. aurantiacum* among eight identified taxa isolated from secondary root canal infections for the first time and had not been detected in root-filled teeth before.

The isolate A5 is a gram positive, cocci and motile bacteria which is identified as *M. luteus*. It also has been identified from oral flora by Raju and Anitha [24]. They also reported that *M. luteus* and *B. subtilis* were the most predominant bacteria among all the isolates in dental caries.

Bacillus sp. was discovered by Cohn and Koch in the 19th century in which this group contains gram positive endospore forming rods. In the present investigation, the isolates A9 and A10 are gram positive, endospore forming and motile bacteria. Isolate A9 is a rod shaped bacterium whereas A10 is a short rod. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the bacteria belongs to the genus Bacillus and were identified as *B. subtilis* (A9) and *B. cereus* (A10). Jain, [25] also isolated and identified *B. subtilis* from oral samples. *B. subtilis* is the one of the predominant bacteria as mentioned earlier by Raju and Anitha, [24] and they too isolated *B. cereus* from oral samples.

In summary, the present study showed that 16S rRNA sequence analysis is a fruitful approach to determine the phylogenetic relationships of cariogenic bacteria.

CONCLUSION

The results obtained from the study shows that, the reliability of phenotypic tests for the identification of cariogenic organisms is inadequate especially for *Acinetobacter radioresistens* and *Exiguobacterium* sp. Indeed, phenotypic analysis was combined with molecular identification method ie, 16S rRNA sequencing. Even if the prevalence of the organisms remained unclear, this study highlights the importance of *Enterobacter* sp. *Micrococcus luteus, Klebsiella pneumoniae, Exiguobacterium* sp., *Staphylococcus sciuri, Acinetobacter radioresistens, Weissella confusa, Bacillus cereus* and *Bacillus subtilis* among opportunistic pathogens. Additional studies were needed for species level identification of the organisms *Exiguobacterium* sp. A5 (2016) and *Enterobacter* sp. A2 (2016) and may be achieved by other molecular methods. Finally, more isolates will be needed to increase the knowledge about cariogenic organisms.

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