IJMPR 2018, 2(4), 68-73

International Journal of Modern Pharmaceutical Research
www.ijmpronline.com

ISSN: 2319-5878
IJMPR
Research Article

SJIF Impact Factor: 3.498

ISOLATION AND IDENTIFICATION OF ORAL MICROBIOTA FROM CLINICAL SPECIMENS

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Received on: 10/05/2018
Revised on: 31/05/2018
Accepted on: 21//06/2018

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ABSTRACT

Over 700 bacterial species have been identified from studies of the oral cavity with many studies focusing on the diversity in the oral cavity during disease. Some pathogenic bacteria are known to have formed symbiotic relationships in order to interact and co-inhabit niches within the mouth The study was aimed to isolated and identified the oral microbiota from 1638 oral specimens were collected from the Department of Microbiology, R.V.S Dental College and hospital, Coimbatore. This study isolated and identified several oral bacterial strains which belonged to the species Streptococcus, lactobeccilus, actinomyces, Fusobacterium spp, Bifidobacteriumdentium, Peptostreptococcus spp and Enterococcus with varying antibiotic resistance patterns.

KEYWORDS: Microbiota; Streptococcus; Enterococcus; antibiotic resistance.

INTRODUCTION

Over 700 bacterial species have been identified from studies of the oral cavity with many studies focusing on the diversity in the oral cavity during disease.[1] Studies that have examined microbial diversity in several sites of the oral cavity have identified many bacterial taxa from six phyla as being components of healthy oral cavities. These comprise of Firmicutes, including Streptococcus, Gemella, Eubacterium, Selenomonas and Veillonella species, Actinobacteria, including Actinomyces, Atopobium and Rothia species, Proteobacteria, including Neisseria, Eikenella and Campylobacter species ,Bacteroidetes, including Porphyromonas, Prevotella and Capnocytophaga species Fusobacteria, including Fusobacterium and Leptotrichia species, and members of the TM7 phylum,[2] This phylum has no culturable members, but bacteria belonging to this phylum are seen in both healthy oral cavities and in oral diseases such as halitosis (bad breath) and mild periodontitis (wastage of the gums).[3]

Some pathogenic bacteria are known to have formed symbiotic relationships in order to interact and co-inhabit niches within the mouth. This is certainly the case for Porphyromonas gingivalis, one of the major causes of periodontal disease, which is known to coexist with several pathogenic species.[4] It has also been shown that P. gingivalis can use the metabolic by-products of its cohabitants in order to promote growth and that in turn, its own metabolic byproducts can be utilised by the species it co-habits with, resulting in a symbiotic existence. These interactions may also enhance overall pathogenic growth rate and virulence of disease.[5]

MATERIALS AND METHODS

This study was conducted in the Department of Microbiology, R.V.S Dental College and hospital, Coimbatore. A total of 1638 oral specimens were collected from a period of 2years from August 2014 to july 2016 processed according to standard recommended procedures, for bacterial isolates of as predominant/pure growth. The samples were examined microscopically for pus cells and bacteria.

Sample Collection and processing: Clinical specimens: The various clinical samples obtained from oral cavity were mouth swabs, plaque scrapings, subgingival crevices, mouth rinses, Saliva, Suppragingival plaques.

Inclusion Criteria: Only the samples with predominant bacterial growth were included in the study.

Exclusion Criteria: The samples with scanty bacterial growth will be excluded from the study.

Aerobic Sample Collection: Unstimulated Saliva sample was collected from the dental caries patients attending the OPD Conservative Dentistry Department in to a sterile container.

Anaerobic Sample Collection: Saliva sample was collected from dental caries patients in directly to the Robertsons Cooked Meat Medium. All the samples were properly labeled and transported to the Microbiology Laboratory, R.V.S dental college and hospital for analysis as soon as possible to prevent overgrowth of

contamination at microorganism and death of potential pathogens.

Aerobic Isolation: The saliva sample was streaked on to Nutrient Agar, Blood Agar, MacConkey Agar plates for aerobic isolation. All the plates incubate 37ºc for 24 hours. After overnight incubation Nutrient Agar, Blood Agar, MacConkey Agar plate colonies was examined.

Anaerobic Isolation: The saliva sample was directly collected to Robertson's Cooked Meat Medium and incubates all the RCM tubes for 37ºc for 48 hours. After 48 hours incubation colonies picked from the RC Medium and streaked on to Blood Agar, Trypticase Soy Agar, Brain Heart Infusion Agar plates and inoculate Thioglycolate broth for anaerobic isolation. All the plates incubate 37ºc for 48hours at anaerobic condition. Anaerobic jar with anaerobic Gas pack used to create an oxygen free environment for the growth of anaerobic microorganism. After the 48 hours incubation all the plates were examined. Smear was made from the colonies for Gram staining method to determine the morphology and further biochemical processed as per standard methods. Isolated colonies was first identified depending on their Gram staining for microscopic examination

Identification: The isolates were identified up to the genus and species level by Gram's stain, motility testing, colonial morphology andhemolysis in blood agar plate (BAP), growth in bile aesculin agar, oxidase, catalase test. bacitracin resistance and arginine hydrolysis, and esculin hydrolysis. fermentation of adonitol, starch, arabinose, cellobiose, dextrin, dextrose, dulcitol, galactose, glucose, inulin, lactose, maltose, mannose, manitol. And using standard microbiological techniques, Species level identification was done by MALDI-TOF.

Antimicrobial susceptibility testing: The Aeroobic bacterial isolates were checked for the Susceptibility to antibiotics. It was determined using the disc diffusion assay on Muller Hinton agar plates supplemented with 5% defibrinated sheep blood, using the following antibiotics (diffusible amount): Amoxicillin (25 µg), Ampicillin (10 µg), Amoxicillin/ Clavulanic acid (20/10 µg), TIC: Ticarcillin (75 µg), Cefalotin (30 µg), Ceftazidime (30 µg), Amikacin (30 µg), Gentamicin (500 µg), Kanamycin (1000 µg), Tobramycin (10 µg), Streptomycin (500 µg), Erythromycin (15 UI), Lincomycin (10 µg), Bacitracin (10 UI), Colistin (10 µg), Trimethoprim-Sulfamethoxazole (1.25/23.75 µg), Nalidixic acid (30 µg), Ciprofloxacin (5 µg), Ofloxacin (5 µg), Nitroxolin (20 µg) and Vancomycin (30 µg). After 18 h of incubation at 37°C, inhibition zone diameters around each disc were measured and the strains were categorized as resistant, intermediate resistant, or susceptible to the antimicrobial agents based on the inhibition zone size.

RESULTS AND DISCUSSION

A totalof 1896oral isolates were obtained from 1638 clinical samples from oral cavity. They consisted of 1256 aerobic isolates and 640 anaerobic isolates. Bacterial isolates were identified upto species level by using MALDI-TOF and the isolates were listed in table 1. Common isolates.

Antimicrobial susceptibility testing: The antibiotic susceptibility of the Aerobic isolated oral bacteria showed the presence of multiresistant strains (Table 2). Resistance profiles of Enterococci to the antimicrobial agents were as follows: penicillin, ticarcillin, Ceftazidime, Amikacin, Tobramycin and Streptomycin, 100%; Colistin, 91%, Trimethoprim-Sulfamethoxazole, 71%, Ampicillin, 33%, Amoxicillin, 29%, Amoxicillin/ Clavulanic acid, Gentamicin and Kanamycin, 24%. In our study1638 patients oral samples there was more isolation of Gram positive bacteria than Gram negative bacteria in oral samples. Gram positive (80%) more frequently were isolated than Gram negative (15%). And also more frequently isolation of Gram positive bacteria than gram negative bacteria in normal controls subjects. Enterococcus faecalis in saliva (45.5%) and subgingival biofilm samples (47.8%) from periodontitis patients compared to periodontally healthy controls. However very few studies have evaluated the correlation between the prevalence of Enterococcus faecalis and dental caries disease Rams et al., (1992) detected that Enterococcus faecalis in 1% of early onset dental caries and 5.1% of dental caries patients using culture methods, whereas Souto and Colombo, (2008) found a much higher prevalence of this species (80%) in a large number of subgingival biofilm sample from dental caries patients. In addition, these authors observed this bacterium was much more prevalent in healthy sites from dental caries patients as compared to sites in dental caries healthy individuals. Streptococcus mutans levels correlate with caries incidence at the population level, but not necessarily at the individual level. Streptococcus mutans counts in saliva and plaque are not linearly associated with caries incidence in an individual patient, despite evidence for a linear caries progression over time.8 Streptococcus mutans negative individuals with coronal or root surface caries can be found, albeit at low rates (typically 2 percent). S. mutans was found at low frequency even in infants with caries, but was isolated more often from those infants with caries compared to those who were caries-free (29.7 vs 9.8%),however differences in the isolation frequencies of S. sobrinus (2.7 vs 1.3%) were not significant. In our study (43.3%) isolated from 30 dental cariespatients saliva sample. The micro flora of dental caries is characterized by a high proportion of facultative anaerobic Gram-negative bacteria (30%). In our study also isolated Klebsiella pneumoniae (13.3%), Pseudomonas aeruginosa (6.6%), and Escherichia coli (20%) isolated from saliva sample in 30 dental caries patients. The genus Fusobacterium is frequently reported in infections of the dental caries with reports indicating that Fusobacterium species can be detected in up to 52% of specimens[6]. In our study

(26.6%) was isolated from the saliva sample in dental caries patients. (Uematsu et al., 1993)[7] isolated and identified 422 strains from the seven patients with dental caries. They showed that 42% of these isolates were asaccharolytic Eubacterium species or closely related strains. (Hoshino et al., 1992)[8] reported that a saccharolytic Eubacterium species are the predominant bacteria of infectious lesions in smooth surface decay and of the infected layers of dentin, suggesting that these bacterial species are involved in the progression of dental caries. In our study also showed prevalence of Eubacterium spp (16.6%) isolated in saliva sample in 30 dental caries patients A correlation also exists between Lactobacillus rates in dental plaque and in saliva. If bacteria from the genus Lactobacillus represent 0.1% of the total salivary flora, a critical concentration of 105 CFU/ml of saliva is necessary for the detection of lactobacilli on the surface of enamel. Lactobacilli absent from the oral cavity of newborns appear during the first year of the life. (Mc Carthy et al., 1965)[9] observed the presence of this species in 50% of newborns during their first year with a rate from 200 to 30000 bacteria. In children without caries, the rate of salivary Lactobacilli varied among the different studies. (Carlsson et al., 1975).[10] considered that Lactobacilli became regularly present in 50% of children and only since the age of 2. Later, (Kohler and Bjarnason, 1987),[11] indicated that 40% of a population of 3-year old children harboured Lactobacilli in rates varying from 2.103 to 4.104 CFU/ml of saliva. For older children (from 6 to 16 years old), this rate is slightly bigger (54.6%). On the other hand, other authors reported the presence of Lactobacilli in 100% of sampled children. One factor that could influence the rate of salivary Lactobacilli during childhood is the carbohydrate intake. In our study is also isolated more frequently (36.6%) Lactobacillus spp isolated in saliva sample at 30 dental caries patients. Gram positive anaerobic bacteria, especially Peptostreptococcus spp were isolated with high rates in dental caries patients[12]. In also our study Peptostreptococcus spp isolated in high rates. It was isolated (40%) in saliva sample at 30 dental caries patients. Actinomycetes spp are abundant in the human mouth and induce root surface caries in hamsters and gnotobiotic rats. They are also carbohydrate users, but are not powerfully acidogenic or acid tolerant. Actinobacillus suis is not easy to routinely diagnose,[13] as it can be isolated along with other bacteria, and may be present in chronic cases.[14] In our study Actinobacillus spp isolated for conventional method and also in this study showed Actinobacillus spp was isolated more prevalence (33.3%) from saliva sample in 30 dental cariespatients.[15] The microflora of severe, moderate and minimal lesions in young adults with rapidly progressing dental caries, and have observed microbial complexes associated with severe and moderate lesions, while in small lesions species Actinomyces, Capnocytophaga ochracea, Haemophilus segnis and Veillonella parvula were identified. Veillonella species Fusobacterium and P.gingivalis have all been associated with dental caries infection. (Daniluk et al., 2006).[16] In our study Peptostreptococcus spp (40%), Actinobacillus spp (33.3%), Fusobacterium spp (26.6%), and Porphyromonas spp (16.6%) was isolated in saliva sample at 30 dental caries patients.[17] In this study, we concluded the aerobic and anaerobic microflora from patients with dental caries and without dental caries. Our data demonstrated that these species showed a trend to be more frequently detected in association with tooth surface and inner surface compared to controls. Further molecular studies are required for a better understanding of this association.

Table 1: Identification of oral Isolates based on MALDI-TOF.

Genus and species	Number of strains
Streptococcus salivarius	388
Streptococcus mutans	336
Streptococcussanguinis	124
Streptococcusmitis	128
Streptococcussobrinus	20
Streptococcus milleri	5
Streptococcusinfantis	2
Streptococcus pneumoniae	8
Streptococcusgordonil	3
Streptococcus intermedius	2
Streptococcusvestibularis	1
Neisseria catarrhalis	21
Staphylococcus aureus	30
Enterococcus feacalis	76
Enterococcus feacium	91
Lactobacillus gasseri	41
Lactobacillusfermentum	26
Lactobacilluscasei	35
Lactobacilluscrispatus	25
Lactobacillussalivarius	22

Lactobacillusvaginalis	21
Lactobacillus mucosae	17
Lactobacillusoris	16
Lactobacillusultunensis	19
Actinomycessp.	76
Actinomycesodontolyticus	36
Veillonelladispar	18
Veillonella atypical	25
Bifidobacteriumdentium	31
Peptostreptococcus spp	82
Actinobacillus spp	46
Fusobacterium spp	32
Eubacterium spp	14
Porphyromonas spp	13

Table 2: Micro-organisms and their sensitivity pattern towards all the antibiotics.

Drugs organism		Amikacin		Amoxicillin-Clavulanic acid.		Ampicillin/ Amoxicillin		Cefotaxime/ Ceftriaxone		Cefuroxime		Cotrimoxazole		Gentamicin		Netilmicin		Norfloxacin	
	No. of strains	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Streptococcus salivarius	388		67		74		48		56		32		29		35		31		16
Streptococcus mutans	336		62		72		49		40		35		30		25		13		10
Streptococcussanguinis	124		11		20		19		23		21		9		5		7		9
Streptococcusmitis	128		17		10		13		21		19		12		11		14		11
Streptococcussobrinus	20		6		2		2		1		3		3		1		1		1
Streptococcus milleri	5		1		1		1		1		0		0		0		1		0
Streptococcusinfantis	2		1		0		0		0		1		0		0		0		0
Streptococcus pneumoniae	8		2		1		1		1		1		0		0		1		1
Streptococcusgordonil	3		0		0		1		1		1		0		0		0		0
Streptococcus intermedius	2		0		0		1		0		0		0		1		0		0
Streptococcusvestibularis	1		0		0		1		0		0		0		0		0		0
Neisseria catarrhalis	21		3		1		2		5		2		3		4		0		1
Staphylococcus aureus	30		4		8		6		5		2		2		2		0		1
Enterococcus feacalis	76		14		12		14		5		12		11		4		4		0
Enterococcus feacium	91		18		12		16		19		12		5		5		2		2
Total	1256		206		213		164		178		141		104		93		74		52

CONCLUSION

In the present study, we were able to isolate and identify several oral bacterial strains which belonged to the species Streptococcus and Enterococcus with varying antibiotic resistance patterns in the collected clinical sample.

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