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Original Article

CULTURE-DEPENDENT APPROACHES AMONG THE CARIES PATHOGENS ISOLATED FROM THE CARIOUS DENTINE AND THEIR EMERGING DRUG RESISTANCE

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ABSTRACT

Objective: To evaluate the commonest caries pathogens from different type of caries andto screen for the emergence of drug resistance among the caries causing pathogenic bacteria.

Methods: A cross-sectional study was conducted in the department of Microbiology. Sample Size was 44 and samples from carious dentine which was immediately transported to the microbiology laboratory. Samples were processed microbiologically to isolate the caries pathogens. Identification of strains were done by standard biochemical characterization studies. The isolates were subjected to antimicrobial sensitivity test. The results were recorded and analyzed for drug resistance.

Results: Out of 44 patients, 40 aerobic bacteria, 2 anaerobic bacteria and 2 fungi were isolated. Out of the 40 aerobic bacteria, the most common isolated was *Klebsiella pneumoniae* followed by *Pseudomonas spp. and Streptococcus viridians*. Ciprofloxacin, Gentamycin and Linezolid showed excellent activity against Gram-positive Bacteria. The most frequently involved teeth of dental caries were mandibular 1st molar (54.54%) followed by Mandibular 2nd molar (13.63%).

Conclusion: In our study we found that *Klebsiella pneumoniae, Pseudomonas sp., Streptococcus viridians* were the most frequent organisms encountered. The study implicates the need for time to time antimicrobial susceptibility examination of the dental caries pathogens that will help to prevent the emergence of resistance property among the dentinal pathogenic organisms.

Keywords: Dental caries, Drug resistance, Pathogens, Antimicrobial susceptibility, Microbial cultute

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INTRODUCTION

Dental caries, which is also referred to as tooth decay or cavities, is one of the most common widespread, persistent diseases today and is also one of the most preventable. When you eat certain foods, the bacteria on your teeth breaks them down and produces acids that have the ability to seriously damage the hard tissues of your tooth. The result is the formation of dental caries. The cavities may be a number of different colors, from yellow to black. Symptoms may include pain and difficulty with eating. Complications may include inflammation of the tissue around the tooth, tooth loss, and infection or abscess formation. Dental caries, being the most common unmet public health problem, indicates its need to urge dentists to overcome this problem globally. Caries exhibit in different types and is found to be associated with the co-aggregation property of microbial flora with other oral hygienic factors. In spite of the surgical removals, excavations and administration of antimicrobials for carious dentine, there seems to be repeated infection and chronic prevalence of caries. A complete understanding of microbial etiology and prevention of emerging drug-resistant strains will aid in the eradication of this chronic dentine problem condition from the oral cavity.

Dental caries continues to be a significant public health problem in many parts of the world. Although the bacteria responsible for caries initiation and early caries progression have been studied extensively, the microbiology of dentine caries has been reported to show considerable diversity and has not yet been fully characterized. Dissolution by acid of the surface enamel, particularly the lactic acid produced by *Streptococcus mutans* is considered to be the primary event in caries development [Van Ruyven FJ *et al., 2000*] [1]. This exposes the underlying avascular mineralized connective tissue matrix of dentine, which is prone to invasion. This occurs by the migration of bacteria into the network of tubules occupied by the processes of the pulpal odontoblasts [Love RM *et al.*, 2002] [2].

Microorganisms causing dental caries

Reports suggest that the early stage of invasion involves *Lactobacillus sp*., *Actinomyces sp*., and *Streptococcus mutans* [Llena-Puy MC *et al.,* 2000] [3]. This phase is followed by an invasion of more diverse group of organisms, including Gram-negative anaerobes [Hoshino E. *et al.,* 1985] [4]. There is evidence that interspecies cooperation enhances the migration of the mixed bacterial flora through the dental tubules [Nagaoka S. *et al.,* 1995] [5]. *Streptococcus mutans* is more pathogenic than others and they also vary in their potential to produce dental caries. The precise role of *Lactobacilli sp*., in the initiation and progression of dental caries cannot be defined clearly but studies suggest that it plays a vital role in the initiation stages to the progression and deep lesion stages [Martin FE *et al.,* 2002] [6]. *Actinomyces sp.,* is a commonly found species on dental plaques and many species of *Actinomyces* play significant roles in different form of caries [Brailsford SR *et al.*, 1999] [7]. Yeasts have also been reported to aid the caries progression in the affected cases. *Candida albicans*, a Gram-positive budding yeast, has been reported from primary endodontic infections and is very much possible to isolate them from caries [Ferrari PH *et al.,* 2005] [8].

Drug resistance among dental caries

From past so many years, surgical treatment has been followed in many dental offices to remove the decayed tooth structure. The flaw in this approach is that the causative organisms are not removed completely and to overcome these problems, antimicrobials like penicillin erythromycin was used. But with time, resistance has developed among the various caries pathogens against these antimicrobial drugs. This results in compelling evidence to progress with clinical assessments of antibiotic susceptibility and various microbiological efforts to understand the limits of this everlasting problem [Girija AS Smiline *et al.,* 2010][9].

Objective

With this background, this study was carried out to evaluate the commonest caries pathogens from different type of caries and to screen for the emergence of drug resistance among the caries causing pathogenic bacteria.

MATERIALS AND METHODS

Study design and setting

A cross-sectional study was carried out in the Department of Microbiology, MGIMS, Sewagram, during the period of May-June 2019 after due approval from the Institutional Review Board.

Study participants

Clinically suspected patients of dental caries who visited the Dental OPD MGIMS, Sewagram, Wardha (M. S.) during the study period were screened for routine examination and investigations.

Sample size

44 samples are included in the Study.

Inclusion criteria

- All patients visiting the dental OPD for dental caries treatment
- Giving consent

Exclusion criteria

- The patients who used antimicrobial agents within the preceding 30 d of their visit
- Not giving consent

Collection and processing of clinical samples

In patients who attended dental OPD for the treatment of dental caries. The source of material for the isolation of the caries pathogens were carious dentine removed from the carious tooth during endodontic restorative procedures. The specimens were received after obtaining the informed consent from the patients. A proforma was recorded for each study case to analyze the age, sex, occupation, marital status, risk factors such as drinking, smoking, tobacco chewing, tattooing and intravenous drug (IV) users, food habits viz., vegetarian/non-vegetarian, economical status and detailed clinical examination. After the removal of superficial plaque and debris overlying the lesion, the carious zone of decalcified and partially decalcified dentine was washed with sterile saline. The carious dentine was then excavated with sterile dental explorers and was transferred into vials of sterile thioglycollate broth (TGB) and brain heart infusion broth (BHI) to a concentration of approximately 10 mg (wet weight) of dentine per ml prior to processing [10]. The samples were brought to the microbiology laboratory and were processed within 3 h of collection.

Sample were dispersed in the transport medium by using a Vortex mixer and the broth was incubated at 37 °C/10% CO2 for 2 h. After incubation the broth was inoculated onto sterile brain heart infusion blood agar (BHIBA), thioglycollate agar (TGA) and Sabouraud's dextrose agar (SDA). Incubation was performed at 37 °C/10% CO2 for 48 h. SDA was incubated at 37 °C/24 h aerobically. After incubation, the isolates were identified by colony morphology, gram's staining and were characterized biochemically [11]. The isolation rate was analyzed statistically using appropriate statistical test. The isolates were later subjected for the antimicrobial susceptibility test. Preliminary screening of the emergence of drug resistance among the isolated caries pathogens were studied by conventional Kirby–Bauer method [12].

Briefly, a minimum of four colonies of the test organisms were touched with a sterile loop and transferred into sterile Mueller Hinton broth under aseptic conditions and was incubated for two hours at 37 °C/2 h. After incubation, the density of each microbial suspension was adjusted equal to that of 10^6 CFU/ml (standardized to that of 0.5 McFarland standard) and was used as inoculum for performing the disc diffusion test. One hundred microliter of the inoculum of each test organism was spread as lawn cultures onto sterile Mueller Hinton agar plates in such a way to achieve a confluent growth. Similarly, for the caries bacterial pathogens, chlorhexidine (0.2%) discs were used. Standard ATCC organisms were included as controls. All the plates were incubated at 37 °C/18 h and the zone of inhibition will be measured using antibiotic sensitivity scale [Hi-media] and recorded. Absence of the inhibition zone indicated the resistance property of the caries pathogens. For anaerobic pathogens isolation, samples from TGB were dispersed and plated onto the BHI agar plates supplemented with 5% defibrinated sheep blood, 0.6% yeast extract, 5 mg/l hemin, and 1 mg/l menadione using spread culture technique. Next, plates of each sample were immediately transferred to anaerobic jars, which were evacuated and filled three times with an anaerobic atmosphere (90% N2, 5% H2, and 5% CO2). The anaerobic jars were incubated in a traditional incubator for up to 7 d at 37 °C. For the unidentified bacterial, fungal pathogens and facultative anaerobic bacteria, identification and antimicrobial susceptibility test was performed by VITEK 2 GN, GP, YST and ANC card identification system, respectively.

OBSERVATION AND RESULTS

During the period of study, 44 patients with dental caries entered into the study. Dental caries were predominantly seen in males (54.54%) with a male-to-female ration of 1.2:1. The age of patients ranged from 15–60 y with mean age occurrence being 31.84 y. The most commonly affected age group was 21–30 y followed by 41-50 y [fig. 1].

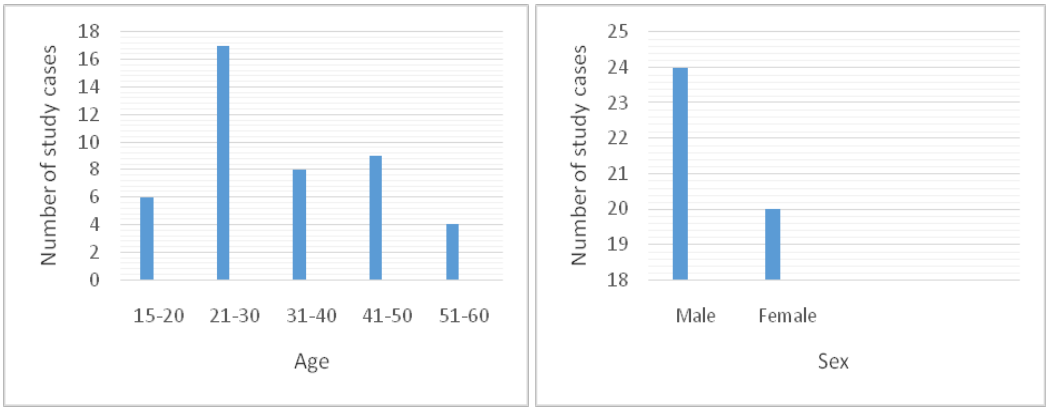

Fig. 1: Proportion of dental caries in relation to certain demographic variables

On microbiological analysis of carious dentine, 88.88% were showing growth of aerobic organism; 3.70% were showing no growth aerobically; 3.70% were anaerobic and 3.70% were fungi (table 1).

Table 1: Result of microbiological analysis

Result	No.	%
No growth aerobically	2	3.70
Aerobic organisms	48	88.88
Anaerobic organisms	2	3.70
Fungus	2	3.70
Total isolated	54	

Of all the pathogens, in the aerobic group, *Klebsiella pneumoniae* was the most common organism isolated, (26.92%). The other organisms were *Streptococcus viridans* (19.23%); *Pseudomonas spp.* (17.30%); *NonA Non B beta hemolytic Streptococcus* (7.69%); *Group A Streptococcus* (5.76%); *Staphylococcus schleiferi, Staphylococcus hemolyticus, Citrobacter koseri and E. coli* (3.84%). Two anaerobic organisms were isolated in our study. The growth and isolation of anaerobic organism was 3.84 %. The growth and isolation of fungus was 3.84%. The fungus so isolated was *Candida albicans* [table 2].

Table 2: Number of organism isolated

Organisms	No.	%
Staphylococcus schleiferi	2	3.84
Staphylococcus hemolyticus	2	3.84
Streptococcus viridans	10	19.23
Group A Streptococcus	3	5.76
Non A Non B beta hemolytic Streptococcus	4	7.69
E. coli	2	3.84
Klebsiella pneumoniae	16	26.92
Pseudomonas spp.	10	17.30
Citrobacter koseri	2	3.84
Lactobacillus	2	3.84
Candida albicans	2	3.84
Total	54	

As seen in table 3, Ciprofloxacin, Gentamycin and Linezolid showed excellent activity against Gram-positive Bacteria. Ampicillin(83.3%) *and* Cefuroxime (11.11%) showed poor activity against *Enterobacteriaceae*. All the antimicrobials had excellent activity against *Pseudomonas spp.*

Table 3: Antibiotic resistance: gram-positive bacteria

Antibiotics	% Resistance to gram positive bacteria N=21
Penicillin	19.04
Erythromycin	19.04
Ciprofloxacin	0
Co-Trimoxazole	28.57
Oxacillin	47.6
Gentamycin	0
Linezolid	0

The most frequently involved teeth were mandibular 1st molar (54.54%) followed by Mandibular 2nd molar (13.63%) (table 4).

Table 4: Distribution of patients according to the frequency of the tooth-causing dental caries (N = 44)

S. No.	Tooth	Frequency	Percentage
1.	Lower 3rd molar	2	4.54
2.	Lower 2nd molar	6	13.63
3.	Lower 1st molar	24	54.54
4.	Lower 1st premolar	0	0
5.	Lower 2nd premolar	2	4.54
6.	Lower canine	0	0
7.	Lower lateral incisor	0	0
8.	Lower central incisor	0	0
9.	Upper 3rd molar	0	0
10.	Upper 2nd molar	2	4.54
11.	Upper 1st molar	2	4.54
12.	Upper 1st premolar	2	4.54
13.	Upper 2nd premolar	2	4.54
14.	Upper canine	0	0
15.	Upper lateral incisor	0	0
16.	Upper central incisor	0	0

DISCUSSION

Dental caries is the most common chronic disease and is the biggest unmet health care need among human population. Prevalence of dental caries in our community results due to poor oral hygiene. Socioeconomic disparities in both rates of disease and treatment are a major public health issue [13]. To date, effective biological interventions to prevent caries have not been developed. Surgical excavation being the major treatment procedure in caries, the use of antimicrobials like chlorhexidine, povidone iodine, fluorides,

penicillin or other antifungal agents are also in use to kill a broad spectrum of organisms. They may be semi selective, in that medicaments can be prescribed that preferentially affect Gram-positive, Gram-Negative, anaerobic or aerobic organisms, but they still kill an array of like organisms. Thus it is concluded that broad spectrum antibiotics are not effective long-term unless their application is periodically repeated. This repeated suppression can be effective as long as resistant strains of the bacterial pathogens do not develop and also due to the suppression of the normal flora [14].

Out of the 44 enrolled in this study, 24 were males and 20 were females. Male preponderance evident in this study was in accordance with the other studies in the literature [15-19].

The age of the patients ranged from 15-56 with the mean age of 31.84 y.

In this study, the dental samples have showed a high incidence rate of *Klebsiella pneumoniae*, *Streptococcus viridans* and *Pseudomonas spp*. Consistent with the other studies, the aerobic species outnumbered the anaerobic species [19, 20]. Further comparison shows there is an absence of anaerobic organisms in contrast to other studies [21-23] which may be because of technical factors which could have decreased the number of fastidious anaerobe isolated in our study.

Similar to study by Singh *et al.* [22], the mandibular first molars were involved in maximum number of cases in this study population (n = 44), unlike the other studies that have reported the most frequent involvement of mandibular third molars [24, 25]. This might be because it is the first permanent tooth to erupt in the oral cavity and is apparently most susceptible to caries.

Dental caries has been treated by removal of diseased tooth structure or faulty restorations prior to placement of materials to restore form and function. The underlying thought for this approach must be that surgical removal of the nidus of infection will inhibit the disease processes. However, the flaw in this method is that the removal of the demineralized/diseased tooth structure does not result in complete elimination of the causative infection because if foci of infection persist it will result in recurrence, so the next step is to treat the remaining infection with effective antimicrobial agents [26]. It has been reported that the introduction of antimicrobials in the prophylactic treatment has reduced the infection, but the long term use of these antibiotics could be compromised by emergence of resistant strains [26].

The utilization of antimicrobials in treating dental caries has gained much attention worldwide. But the widespread concern is emerging drug resistance among the pathogenic population has worsened the treatment regimens in dentistry. The developing resistance of bacteria to these common antimicrobial agents is a cause of concern for the dentist [26]. The resistance developed to antimicrobial agents can arise from the selection of resistant strains among naturally susceptible species or from the ingress of newer strains of naturally resistant species. Greater use of a single antimicrobial agent in a given environment directly dictates the rate of resistance among the different microbial populations [27].

Overuse and abuse of antibiotics are the main factors responsible for the development of resistance against antibiotics.

19.04% of gram positive bacteria were found to be resistant to both penicillin and erythromycin. These values were significantly less when compared to a previous studies done by Uwaezuoke *etal*., and Dwivedi *et al.*, in which they found 95.8% and 48% samples resistant to penicillin respectively [27,28].47.6% of Gram positive bacteria shows resistance against Oxacillin. *Pseudomonas*, most of its samples were found to be sensitive to all antimicrobials that were tested, which is in contrast with a previous study done by Navneet Kaur*et al.*, [29]. Selection of an antibiotic regimen should always be based on knowledge of the efficacy of an antibiotic for the bacteria. It should also be remembered that dental caries represent an ecosystem of bacteria in which by-product of one species of bacteria may be nutrient of other bacteria. Thus, an antibiotic that is effective against a few species of bacteria in polymicrobial infection, may indirectly affect all or some of these bacteria in that ecosystem. Therefore, the study results depict that the growing resistance among pathogenic bacteria against the commonly prescribed antibiotics will be eliminated by the usage of newer antibiotics [30].

CONCLUSION

In our study we found that *Klebsiella pneumoniae, Pseudomonas sp., Streptococcus viridians* were the most frequent organisms encountered. The antimicrobial susceptibility examination results don't give an exact conclusion. This may be due to a small sample size and hence a more extensive study should be done. This implicates the need for time to time antimicrobial susceptibility examination of the dental caries pathogens that will help to prevent the emergence of resistance property among the dentinal pathogenic organisms.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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