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Research Article

Molecular Characterization and Antibiogram Study of Bacteria Isolated from Dental Plaque Samples from Dental Carries Patients in Northern Bangladesh

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Abstract

The attachment of salivary bacteria initiates the formation of dental plaque to the acquired pellicle covering the tooth surface. This Research aimed to investigate the prevalence and antibiogram study of isolated bacteria from Tasmia dental care Saidpur, Bangladesh. In this study, we selected 200 patients (aged 20-60 years) to isolate desired isolates. *16S rRNA* gene sequencing techniques were applied for molecular confirmation of isolated bacteria. 55.83% of patients exhibited molar caries, 27.5% between molar and premolar, 12.5% at the incisor, and 4.17% at the cervical border. Dental caries was ($P < 0.001$) prevalent in age groups 31-40 (82.86%), 41-50 (72.22%), and above 50 (34.29%), respectively. In contrast to patients from high- and middle-income households, those from low-income families had a considerably higher prevalence of dental caries (79.78%) ($P < 0.001$). Dental caries was observed to be substantially ($P < 0.001$) more prevalent among the illiterate (84.0%) than among the educated (25.0%) or the very worried (8.33%). *Staphylococcus* spp. (33.33%), *E. coli* (25.0%), *Streptococcus* spp. (20.0%), *Acromobacter xylosoxidans* (16.67%) and *Pseudomonas* spp. (5.0%) were the most often isolated bacteria. *Acromobacter_ xylosoxidans*_strain_LMG_1863 was identified with 144bp in this Research. *A. xylosoxidans*, and *E. coli* were resistant to over six antibiotics. At the same time, all *Pseudomonas* spp. were resistant except for Ciprofloxacin and colistin. *Staphylococcus* spp. and *Streptococcus* spp. were also resistant to more than five antibiotic discs. Triclosan and fluoride-containing antibiotic-free toothpaste can be the best preventive methods for cavities.

Keywords: Antibiotics; Bacteria; Bangladesh; Dental Caries; Resistant

Abbreviations

EMB: Eosin Methylene Blue Agar; MSA: Mannitol Salt Agar; MR: Methyl Red; VP: Voges-Proskauer; TSI: Triple Sugar Iron; MIU: Motility Indole Urease; PCR: Polymerase Chain Reaction

Introduction

Dental caries is one of the world's most prevalent chronic infectious disorders [1]. It is a chronic sickness that can affect people of any age. Caries (tooth decay) is the most frequent chronic oral disease in the world, affecting more than 90% of the population. Nonetheless, the severity of the illness varies considerably amongst individuals [2]. Sixty to ninety percent of children and adults suffer tooth decay [3]. Dental caries is a disorder caused by the interaction of specific bacteria with the dietary components of a biofilm known as "dental plaque". Indigenous mouth flora, the primary cause of dental caries, is detected in bacterial plaques collected over time

on tooth surfaces [4]. *Streptococcus mutans* causes dental caries. It coexists with 500 other bacteria as tooth biofilm [5]. Bacterial host infections are caused by local bacteria, most notably aerobic gram-positive cocci, anaerobic gram-negative rods, and gram-negative rods [6]. Dental caries is a slow-moving, long-term disease that causes permanent damage to some teeth.

For a long time, the microbial flora of dental caries has been linked to the disease. This includes bacteria that can live with or without oxygen, *Lactobacillus acidophilus*, and *Lactobacillus casei* [7]. Previous Research reported that *Streptococcus sanguinis* is linked to oral health, and *S. mutans*, *Veillonella spp.*, *Lactobacillus fermentum*, *Actinomyces spp*., and *Bifidobacterium* spp. are linked to caries [8]. Acid-producing bacteria induce tooth decay in fermentable carbohydrates like sucrose, fructose, and glucose [9]. Even though dental caries is easy to spot, other diagnostic tools, such as x-rays, are used to find less obvious decay and figure out how bad the damage is [10]. Caries-related bacteria have traditionally been found using culture-based methods, eliminating uncultured species. Molecular approaches are utilized to identify and count microorganisms connected to tooth caries, even if some are uncultivable. Munson., *et al.* used cultural and genetic techniques to identify tooth cleft species [11]. Using molecular techniques, Chhor., *et al.* evaluated bacterial diversity in adult tooth decay [12]. People who don't brush and floss frequently get caries. Wei and Hyman said most children don't brush long enough or eliminate plaque legally [13]. Antibiotics are the most common caries treatment. Most antibiotics are useless against oral infections due to overuse, accessible availability, and cost-efficiency. Dental caries problems require proper diagnosis. For more effective medications, pathogens and antibiotic sensitivity must be recognized. Therefore, this Research aims to determine which bacteria are the most dangerous and responsible for dental caries in northern Bangladesh, as well as to define the antibiogram profile of those bacteria.

Research Methodology

Study design and settling

Dental plaque samples were collected from patients of different age's patients and transferred to the Department of Microbiology (HSTU) for microbiological tests. In this Research, 200 patients were selected from other locations in the Dinajpur district of Bangladesh. For total bacterial count, samples were serially diluted in a PCA medium (Hi media, India). The principal medium for isolating bacteria from pus samples of dental caries patients is nutrient agar. Then, specific bacteria were isolated using selective media such as MacConkey agar, Eosin methylene blue agar, Cetrimide agar, Mannitol salt agar, and Blood agar. Using gram staining methods, bacterial morphology and gram properties were discovered [14]. Then, according to the protocol [15], traditional biochemical assays were conducted to detect bacteria that cause tooth caries. We purchased all bacterial cultural media from Hi Media, India.

DNA sequencing and phylogenic tree analysis

In this Research for bacterial genomic DNA extraction, a pure bacterial colony was selected and followed the previous laboratory protocol. DNA was extracted from bacterial cells using a DNA removal test kit, followed by lab protocol [16]. The bacterial cells were centrifuged for 10 minutes at 2400g in a multi-speed IEC CL31R centrifuge (Thermo Scientific). With moderate agitation, the pellets were suspended 400l Tris EDTA buffer containing 4001 0.01M Tris –HCL, pH 7.4, and 0.001 M EDTA. The suspension was then treated with 10% SDS (Fisher Scientific) and proteinase K. (Ambion). At 65°C for 60 minutes, the rest was incubated in a hybridization oven (Biometra OV2, Menachem, U.K.). 100 l 5M NaCl and then 100 l CTAB/NaCl (warm at 70°C) were added to the solutions during incubation. After 20 minutes of incubation at 65°C, the solution was cooled for 5 minutes at room temperature. Using a 24:1 (Sigma-Aldrich) ratio of chloroform to isoamyl alcohol, the solution was centrifuged at 1300 g for 15 minutes. The supernatants were incubated at 37°C for 30 minutes with 5l RNase A (5mg/ml in 0.5 M NaCl, 0.01 M EDTA). PCR protocol was followed by the previous [17]. Applied Biosystems Genetic Analyzer 3130 was used to sequence the genome of *A. xylosoxidans* at the National Institute of Biotechnology, Savar. Molecular evolutionary genetics was used to examine changed DNA sequences [18]. Using the neighbor-joining method, a phylogenetic tree with 1,000 bootstrap replications was constructed [19]. The nucleotide sequence is 468 (dental plaque sample/adult/2018). A PCR reaction amplified a 144-bp region with 98% similarity to *Achromobacter insolitus* strain LMG 6003 to confirm *Achromobacter xylosoxidans* strain LMG 1863 using forward Primer-(5'GACCTCGGTTTAGTTCACAGA 3') and reverse Primer-(5'CACACGCTGACGCTGACCA 3').

Antibiogram study

Using Kirby-Bauer disk diffusion techniques, mueller-Hinton agar plates and commercial antibiotic discs were used to determine antibiotic sensitivity. For the antibiotic sensitivity test, first prepared the bacterial solution and then poured it into a Mueller-Hinton agar (Oxoid, TM, U.K.) plate. Then add antibiotic disc (Hi media, India) with different concentrations. A total of 15 commercial antibiotics such as Gentamicin (10µg), Chloramphenicol (30µg), Neomycin (30µg), Penicillin(10µg), Cloxacillin(1µg), Ciprofloxacin (10µg), Amikacin (30µg), Vancomycin (2µg), Ampicillin (10µg), Kanamycin (30µg), Amoxicillin(30µg), Norfloxacin(10µg), Levofloxacin(5µg), Colistin(10µg), Erythromycin(15µg) were used for antibiotic sensitivity test. Finally, all dishes were incubated at 37°C for 24 hours. After 24 hours, keep the record of the zone of inhibition. According to Clinical and Laboratory Standards Institution [20] recommendations, the bacterial zone of inhibition was estimated on a millimetre scale and classified as sensitive, resistant, or intermediate.

Data analysis

The raw data were entered into an XL table sheet and analyzed using a statistical package for social science for Windows version 20 (SPSS) (Chicago, IL, USA). The level of statistical significance was fixed at ≤ 0.05.

Results and Discussion

Isolation and identification of bacterial isolates

In our Research, 60 bacterial isolates were isolated, including gram-positive and gram-negative bacteria. Of 60 isolates, 32 (53.33%) were gram-positive, and 28 (46.67%) were gram-negative. Figure 1 shows the frequency of isolates. The highest number of isolates was *Staphylococcus* spp. 20 (33.33%), followed by *E. coli* 15 (25%), *Streptococcus* spp. 12(20%), *A. xylosoxidans* 10 (16.67%) and *Pseudomonas* spp. 3(5.0%) respectively.

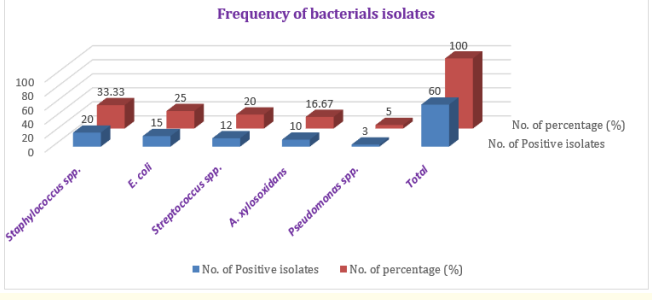

Figure 1: Frequency of Bacterial isolates.

According to the site of infection, the prevalence of isolates were mentioned in figure 2. In this study, out of 200 suspected patients, 120 (60%) positive patients were identified with 60 isolates. All samples were collected from Tasmia dental care at Saidpur, Dinajpur, Bangladesh. The most infections were found in a cavity at motor 67 (55.83%), whereas the lowest was found in the cavity in central margin 5 (4.17%). Dental caries was significantly (P < 0.001) highest at the molar teeth and lowest at the cervical margin.

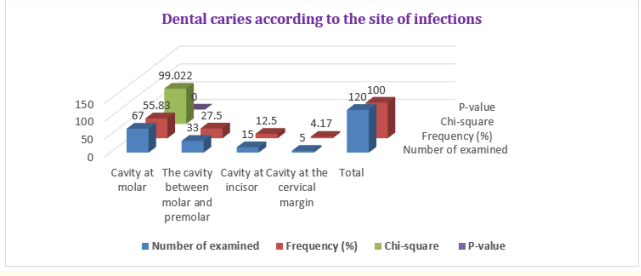

Figure 2: Prevalence of dental carries based on site of infection.

According to the patient's age group, the frequency of dental caries is shown in figure 3. Age affects human dental caries significantly. The prevalence of dental caries was highest (P < 0.001) in the 31-40 age group (82.86%), followed by 41-50 (72.22%), above 50 (34.29%), and 20-30 (26.83%).

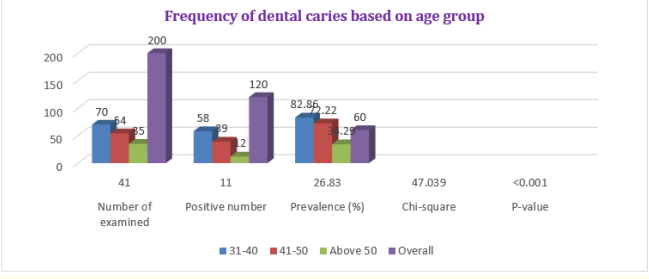

Figure 3: Dental caries frequency based on age group.

In the current study, which is provided in table 1, there was no significant (P > 0.05) effect of gender on the frequency of dental caries. These results indicate those female patients had a greater prevalence than male patients.

As shown in table 2, the frequency was significantly (P < 0.001) highest in low-income families (79.78%) and lowest in medium households (34.38%), while it was 57.45% in patients from good families.

Sex	Number of examined	Positive number	Prevalence (%)	Chi-square	P-value
Male Female	76 124	41 79	53.95 63.71	1.871	0.171 (N.S.)
Overall	200	120	60.00		

Table 1: Prevalence results in men based on sex-wise.

Family status	Number of examined	Positive number	Prevalence (%)	Chi-square	P-value
Poor Medium Good	89 64 47	71 22 27	79.78 34.38 57.45	22.135	<0.001
Overall	200	120	60.00		

Table 2: Prevalence of dental caries based on family status.

In the current Research, table 3 shows that illiterate people (84.0%) had a considerably (P < 0.001) higher rate of dental caries compared to graduates (25.00%) and highly concerned people (8.33%), whereas the rates for below class 5 (76.09%), class 5 to below SSC (67.19%), and SSC to HSC (43.24%).

Educational status	Number of samples	Number of positive patients	Prevalence (%)	Chi-square	P-value
Illiterate Below class 5 Class 5 to below SSC SSC to HSC Graduation Highly concern people	25 46 64 37 16 12	21 35 43 16 4 1	84.00 76.09 67.19 43.24 25.00 8.33	38.034	<0.001
Overall	200	120	60.00		

Table 3: Frequency of dental carries based on educational status.

Figure 4 shows that persons living in small towns (72.04%) had a higher prevalence rate than those living in villages (48.28%) and cities (51.02%).

Antibiotic sensitivity test of gram-negative and gram-positive bacteria

A. xylosoxidans, *E. coli*, *Pseudomonas* spp., *Staphylococcus* spp., and *Streptococcus* spp. were treated with commercially available

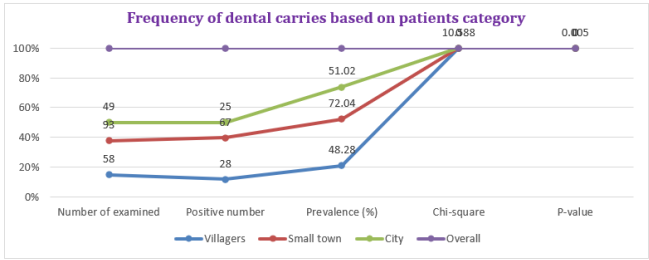

Figure 4: Frequency of dental carries based on patients category.

antibiotics, which are listed in table 4. All *A. xylosoxidans* isolates were sensitive to Gentamicin and Neomycin (90%), followed by Chloramphenicol and Colistin (80%), but resistant to Penicillin and Amoxicillin whereas *E. coli* were 100% sensitive to Gentamycin, Chloramphenicol, Levofloxacin, and Erythromycin, 86.67% susceptible to Ciprofloxacin, and 100% resistant to Cloxacillin, Amoxicillin, and Ampicillin. *Pseudomonas* spp. were susceptible to Colistin (100%), Ciprofloxacin (66.67%), and Amikacin (66.67%).

Antibiotics with disc concentration (µg)	*A. xylosoxidans* (10)		*E. coli* (15)		*Pseudomonas* spp. (3)	
	%R	%S	%R	%S	%R	%S
Ciprofloxacin(5)	3(30)	7 (70)	2(13.33)	13(86.67)	1(33.33)	2(66.67)
Chloramphenicol (30)	2(20)	8 (80)	0(0)	15(100)	3(100)	0(0)
Penicillin(10)	10(100)	0(0)	NT	NT	3(100)	0(0)
Cloxacillin(1)	NT	NT	15(100)	0(0)	NT	NT
Kanamycin(30)	7(70)	3 (30)	3(20)	12 (80)	3(100)	0(0)
Gentamycin(10)	1(10)	9(90)	0(0)	15(100)	3(100)	0(0)
Vancomycin(30)	8(80)	2(20)	14(93.33)	1(6.67)	3(100)	0(0)
Neomycin(30)	1(10)	9(90)	5(33.33)	10(66.67)	3(100)	0(0)
Amoxicillin(30)	10(100)	0(0)	15(100)	0(0)	3(100)	0(0)
Ampicillin(25)	10(100)	0(0)	15(100)	0(0)	3(100)	0(0)
Amikacin(30)	7(70)	3(30)	14(93.33)	1(6.67)	2(66.67)	1(33.33)
Norfloxacin(10)	NT	NT	6(40)	9(60)	NT	NT
Levofloxacin(5)	NT	NT	0(0)	15(100)	NT	NT
Colistin(10)	2(10)	8(80)	NT	NT	0(0)	3(100)
Erythromycin(15)	NT	NT	0(0)	15(100)	NT	NT

Table 4: Antibiotic sensitivity tests of gram-negative bacteria.

Legends: S = Sensitive, R = Resistance, % = Percentage and NT = Not Tested.

Figure 5 (A, B) demonstrate the antibiotic sensitivity pattern of gram-positive bacteria. The antibiotic discs were used for a total of 20 *Staphylococcus* spp. and 12 *Streptococcus* spp. *Staphylococcus* spp. were highly sensitive to Ciprofloxacin and chloramphenicol (100%), followed by novobiocin and levofloxacin (95%), whereas highly resistant shown in amoxicillin, amikacin and cloxacillin. Moreover, *Streptococcus* spp. were found to be extremely sensitive to ofloxacin (100%) and kanamycin (83.33%) and extremely resistant to penicillin, amoxicillin, ampicillin, and vancomycin (100%), chloramphenicol, and amikacin (91.6%), respectively.

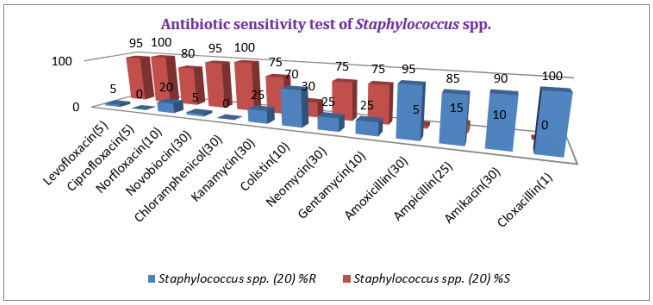

Figure 5(A): Antibiotic sensitivity test of *Staphylococcus* spp.

Gene sequencing and phylogenic tree analysis

About 40% of samples tested positive for *A. xylosoxidans*. This bacteria is first identified from dental caries sample. The PCR band was an amplifier with 98% homogeneity, and *A. xylosoxidans* strain

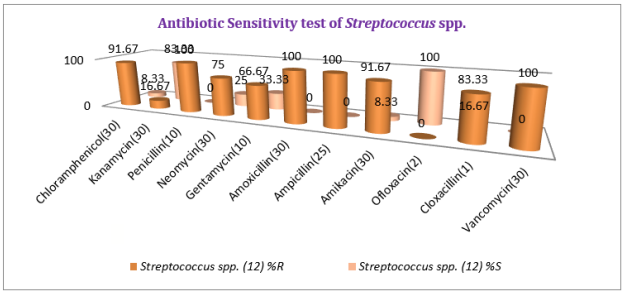

Figure 5(B): Antibiotic sensitivity test of *Streptococcus* spp.

LMG 1863 was determined using the universal Forward primer and Reverse primer 144 bp. The National Institute of Biotechnology sequenced the PCR amplification which was shown in figure 6. Figure 7 shows the phylogenic tree analysis.

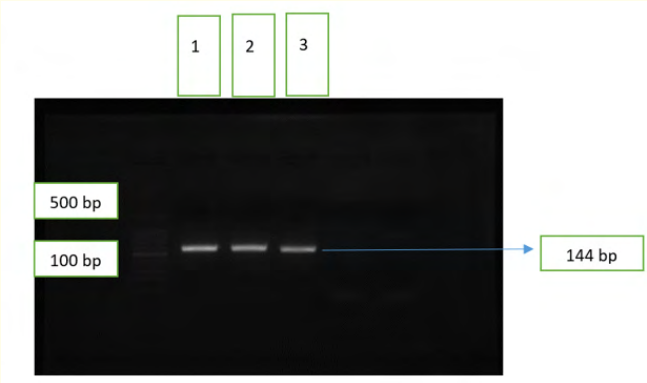

Figure 6: PCR amplification of *A. xylosoxidans* (144bp) with 500 bp marker.

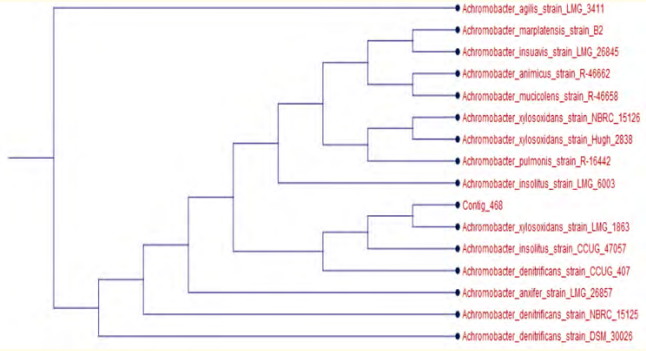

Figure 7: Phylogenic tree of *A. xylosoxidans* strain LMG 1863.

A. xylosoxidands is a prominent emerging pathogen among immunocompromised individuals globally, and it is present in most soil and streams [21,22]. In this investigation, we found *A. xylosoxidians* in dental plaque from people of varying ages for the first time. This study's results corroborate those of [23], which found that the prevalence of dental caries was highest among those aged 31 to 40. Similar to the current study [24], revealed that the maximum prevalence (32.60%) of infection was established in the 26-35 age group, while the lowest prevalence (10.18%) was identified in the under-15 age group. According to sex-based Research, our findings are almost identical to those of [25], who reported that females had a higher caries frequency than males [23,25]. It discovered that a more significant proportion of females (80%) were affected by dental caries, which is consistent with the current study's findings. The bacterial frequency based on isolates was more or less similar to the study of [13,24], which identified 41.75% *Staphylococcus aureus* and 17.58% *Streptococcus* spp. *Streptococcus* spp. (39%), *Staphylococcus* spp. (21%), and *E. coli* (6.4%) were found in dental plaque biofilms, according to a report [26]. PCR technique identified *A. xylosoxidans* with a 144 bp band, comparable to previous publications. The drug resistance profile of each isolate was determined using the disc-diffusion approach and a variety of commercially available antibiotics. The antibiotic sensitivity test results were generally consistent with those of [9,27]. Usually, *A. xylosoxidans* is detected in lung disease, UTI, pneumonia, and immunocompromised patients, especially chronic ones. *A. xylosoxidans* was detected in the environment and in clinical samples, which is an important emerging pathogen worldwide. This study indicates that appropriate antibiotics and dental care can prevent tooth decay. Comparing environmental and clinical isolates and elucidating the causes and treatments for dental carries will be the focus of future studies. Future Research is required to discover antibiotics that are effective against *A. xylosoxidans* and other infectious bacteria.

Conclusion

Decay, the most widespread health issue affecting people, can affect any tooth surface. Dental caries were more typically identified in molar teeth in 31-40-year-old female patients, and we could locate several bacteria in the dental plaque area. Low-income, rural, and illiterate people experienced more dental decay. Almost all of the bacteria in this study are pathogenic, which can

cause tooth decay. We noticed that most antibiotics were highly resistant, that's why it is challenging to control dental decay. The present study may contribute to developing effective treatment and prevention strategies for tooth decay. Our study suggested that without a proper prescription, antibiotics should be avoided.

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Conflict of Interest

The authors have no conflict of interest.

Author's Contribution

Md. Aoulad Hosen and Nazmi Ara Rumi contributed as the first authors, designed the experiment, lab work, collected data, and wrote the manuscript. Tauhidur Islam helps to sample collection and prepared the tables. Md. Shajedur Rahaman gave his guidelines during manuscript draft and also designed the figures. Md. Khaled Hossain and Md. Fakhruzzaman critically evaluated the manuscript. Md. Aoulad Hosen checked plagiarism and corrected grammatical errors. Md. Aoulad Hosen also analyzed data. All authors participated in exhaustive research and revised the work before its final publication.

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