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Research Article

Phenotypic Detection of Adhesive Activity and Biofilm Formation among Prevotella spp. Isolated from Patients Suffering from Acute Inflammation in Root Canal Teeth

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Abstract

In this study, 150 patients were visited to dental clinic in Al - Hilla city suffering from acute inflammation in root canal teeth, the samples were cultivated anaerobically culture at (37°C) for (48) hrs. Identification of Prevotella spp. was depended on the colonial morphology, microscopically, and biochemical tests as initial identification, after that, these isolates were identified by specific negative cards of Vitek 2 system, out of 150 specimens only 17(11.4%) isolates were belonged to Prevotella, while 133(88.6%) related to other types of microorganisms. Identification of Prevotella spp. by Vitek 2 system were classify these bacteria in to three species, the results showed that, out of 17 Prevotella isolate, 12(70.6%) were related to Prevotella intermedia, 3(17.7%) isolates were related to Prevotella nigresceens and 2(11.7%) isolates were related to Prevotella tannerae. In order to identify which Prevotella bacteria are present in the subject, the researchers extracted DNA from the numerous Prevotella strains that had previously been identified with the Vitek2 system. They then used the extracted DNA to conduct PCR with specific primers for the 16S RNA gene to amplify it. Gel electrophoresis revealed that all of the Prevotella intermedia samples (100%) generated a 660 bp DNA fragment, which was unique to the strain. Isolates that previously tested positive for Prevotella nigresceens using the Vitek2 method were identified with molecular testing for 16srRNA gene. The findings revealed that every one of the 3 test subjects (100%) was positive. Bands showing a higher yield as compared to the genomic sequence appear while looking for more than 800 bp of sequence. As well, 16srRNA gene detection was performed on 5 Prevotella tannerae isolates, and it was determined that the 2 (100%) isolates tested positive for this gene. The findings showed an increase in (1110 bp) band size compared to the normal allelic ladder. Epithelial cell adhesion is thought to be a vital component in bacterial infection. All the examined species of Prevotella (100% of the group) were positive for the findings. Additional studies to measure quantitatively the quantity of biofilm production were done in a microtiter with Trypticase Soy Broth plus (1 percent) glucose. To improve the accuracy of this test, it was performed three times. The study's findings were translated into three categories of biofilm formers: non-biopersistent, moderate, and chronic bacteria biofilm formers based on the level of OD value, which can be read as (OD<0.120, OD=0.120-0.240, and OD>0.240). The findings concluded that all Prevotella isolates produced biofilms (100 percent) and Prevotella intermedia biofilms were present in a majority of specimens (10 percent), the majority of Prevotella nigrescens also displayed biofilms (3 out of 3, 100 percent), and the minority of Prevotella tannerae expressed moderate biofilms (1 out of 2, 50 percent) while only the minority of Prevotella intermedia specimens showed any trace of biofilm (2 out of 12, percent) (50 percent). To test for in vitro antibiotic resistance in root tooth-related acute inflammation, Prevotella spp. isolates from all Prevotella isolates were put through the modified Kirby-Bauer disc diffusion technique. Clindamycin, Amoxillin, Ceftazidime, Tetracycline, Chloramphenicol, Aztreonam, and metronidazole are all antibiotics that have been shown to be effective against Prevotella spp. isolates. The results compare according to Clinical Laboratory Standard Institute guidelines (CLSI, 2019) as resistant. Highest rate of resistant is seen to almost antibiotics used in present study, 16(94.1%) isolates were resistant to Clindamycin, 15(88.2%) isolates were resistant to Amoxillin, 13(76.47%) isolates were resistant to Chloramphenicol, 11(64.7%) isolates were resistant to Tetracycline, 9(52.9%) isolates were resistant to Aztreonam and 3(17.6%) isolates were resistant to metronidazole.

Keywords

Prevotella spp., acute inflammation in root teeth, adhesive factor, biofilm formation, antibiotic resistance, 16srRNA.

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Introduction

Hundreds of microbe species reside in the oral cavity, with each person having between 100–200 of them, all with their own ecosystem in your mouth (Krishnan et al., 2017). Once the canal is contaminated with bacteria, infection may advance into the periapical tissues, leading to apical periodontitis (Sabharwal et al., 2019). When you're ill, you may have a dental medicine infection. They have a polymicrobial character, and in initial infections, a majority of the microbiota consists of obligate anaerobic bacteria (Rajaram et al., 2016). Perhaps it is time to revisit the origins of periodontitis, where the attack of oral bacteria makes a big difference (Kugaji et al., 2019). Members of the oral, vaginal, and gut microbiota like Prevotella spp. are often seen in illnesses with anaerobic tracts (Tett et al., 2021). Prevotella bacteria are a kind of gram-negative, anaerobic rod with saccharolytic activity, sensitive to bile, and ready to populate the world with bacteria (Azzawi et al., 2018). These bacteria are a component of the human oral, intestinal, and system floras, and they may be involved in infections and illnesses affecting anything from the mouth and gut to the brain and urogenital tract (Hurst, 2018). Respiratory diseases that may follow aspiration include aspiration pneumonia, organ abscess, empyema, and chronic ear irritation (Komiya & Kadota, 2020). In addition to isolation from infections, residents must be separated from sores in the oral area such as abscesses and burns. Other items on the list include bite wounds, paronychia, infection from a wound in the foot, osteomyelitis, brain abscesses, and infections in the upper tracts (Toprak et al., 2020). In periodontitis and dental abscesses, Prevotella spp. are the most common among the Gram-negative anaerobes. Periodontal diseases are defined as lesions with inflammation, and they include acute necrotizing lesion gingivitis, periodontitis, and adult periodontitis. Prevotella intermedia and Prevotella nigrescens, when found in such lesions, are considered to be periodontal illnesses (Dahlen et al., 2019). The black-pigmenting anaerobes are known as Porphyromonas gingivalis. Each one needs haemin for their development because of the lack of iron in their diet (Zambon & Haraszthy, 2021). These species were shown to bind free lactoferrin which was discovered in inflammatory and disease-related neutrophils (Lu et al., 2020). The Prevotella species, however, remain unaffected by lactoferrin's inhibitory effects on P. gingivalis growth. P. intermedia does not support inorganic iron or iron-binding macromolecules such siderophilin and lactoferrin, but it grows in the presence of hemin–iron-containing complexes which include hemin, human hemoglobin, bovine hemoglobin, and bovine enzyme (Rosa et al., 2021). It has been shown that P. intermedia has hemoglobin-binding protein on its cell surface (Naito et al., 2021). Invasion of animal tissue cells is a very important step within the pathological process of the many infections, the flexibility to survive intracellularly permits bacterium to evade the system and probably to pass around (Rey et al., 2020). The flexibility to persist at intervals the host cell has been incontestable to be vital for the virulence of those pathogens (Ghazaei, 2018).

Materials and Methods

Patients and collection of samples

The project ran from November 2020 to July, spanning a time of (9) months (2021). Around 150 people suffering from severe inflammation in their root canal teeth went to the Al-Hilla dental clinic. For the 30-second collection period, the researchers took samples from infected root canals using a disposable paper tip, as per normal practice. They were gathered with the utmost care to prevent contamination. The specimen was transferred to the Department of Microbiology for further investigations, it was inoculated into Blood agar and PYG liquid medium agar medium, then was incubated at (37°C) for (48) hours anaerobic culture. Bacterial isolated were diagnosed by Gram stain, colony morphology, biochemical test and Vitek 2 system and 16srRNA for identification of Prevotella spp. (Hessan & Jassam, 2021).

Ethical Approval

In order to be included in the research, each patient had to provide their written permission.

Identification of bacterial isolates by gram stain, biochemical tests

Identification tests for each strain were conducted, including cultural, morphological, and

biochemical features (Baron et al., 1994, McFadden, 2000).

Identification of Prevotella spp. isolates with Compact VITEK-2 System:

All Prevotella spp. isolates were identified using the Compact VITEK-2 System, which was also used to screen isolates (BioMerieux). The isolates are identified using biochemical reactions in this phenotypic identification type. Vitek-2 has 64 wells for fluorescence biochemical tests in each card. In addition to the carbohydrate assimilation tests, phosphatase, urea, and nitrate assays were used on 20 samples. In addition to these processes, the Vitek-2 device also automated card handling by the machine, including card filling, sealing, and moving to the incubator. Output reports are decoded using a specified algorithm. The obtained findings were ID-GN (identification of Gram-negative bacteria) identified according to the ID-GN databank. The findings from these systems are delivered with the help of the appropriate supporting software, which generates the ID data. "Repeat results" were utilized if the first tests showed "low discrimination" or "no ID," and the results were run again if there was "low discrimination" or "no ID" as the results. All strains have been inoculated on anaerobic medium and subsequently incubated at 37°C throughout the night. A single colony was utilized for identification using the manufacturer's technique on the VITEK-2 Systems, which is as described by the manufacturer (BioMerieux).

DNA extraction form bacterial culture

To carry out DNA extraction and isolate specimens, following manufacturer's instructions (Geneaid, USA). To see them, they were illuminated using UV lights.

Identification of Prevotella spp. by 16SrRNA gene

The sequence of primer and PCR settings that used in study are listed in Table (1).

Table (1):
16SrRNA genes primers sequences with their amplicon size Base pair (bp) and their condition.

16Sr RNA Genes	Primer sequence (5'-3')	Size (bp)	PCR condition	Reference
Prevotella intermedia	F: TT GCG TGC ACT CAA GTC CGC C R: AAG GAG GTG ATC CAG CCG CA	660	Stage 1: 2 min., 95°C Stage 2: 30 sec., 95°C, Stage 3: 30 sec., 62.4°C Stage 4: 70.0 sec., 72°C, Stage 5: 29 extra periods Replication stage 2-4 Stage 6: 5 min., 72°C Step 7: Holding, 4°C	Mahdi et al., (2020)
Prevotella nigresceens	F: CCAGCAGCCGCGGTAATACG R: TACCAGGGTATCTAATCC	800	Stage 1: 2 min., 95°C, Stage 2: 30 sec., 95°C, Stage 3: 57.6°C reduction 0.5°C each sequence for 30 sec. Stage 4: 80.0 sec., 72°C, Stage 5: Recurrence stages 2-4 for 14 extra periods Stage 6: 30 sec., 95°C, Stage 7: 30 sec., 50.6°C, Stage 8: 80.0 sec., 72°C	Hadi et al., (2020)

Prevotella tannerae	F: CGG CCT AAT ACC TCA TGG CA R: AGA TCG TGT TTG CAC AAA AT	1110	Stage 9: Recurrence stages 6-8 19 extra periods Stage 10: 5 min., 72°C, Stage 11: holding 4°C, Stage 1: 2 min., 95°C, Stage 2: 30 sec., 95°C, Stage 3: 57.6°C reduction 0.5°C each sequence for 30 sec. Stage 4: 80.0 sec., 72°C, Stage 5: Recurrence stages 2-4 for 14 extra periods Stage 6: 30 sec., 95°C, Stage 7: 30 sec., 50.6°C, Stage 8: 80.0 sec., 72°C Stage 9: Recurrence stages 6-8 19 extra periods Stage 10: 5 min., 72°C, Stage 11: holding 4°C,	De Lima et al., (2018)

Adherence Activity

One of the key virulence characteristics of Prevotella is its capacity to stick to intestinal cells, and it is discovered using the methods of (Lagha et al., 2017).

Biofilm Production

In the study done by Avdić et al. (2019), the tissue culture plate technique (TCP) assay was the method that was often used to determine if a microorganism had biofilm forming abilities Table (2).

Table (2)
Classification of bacteria biofilm formation by TCB method

Mean of O.D. value at 630 nm	Biofilm formation
<0.120	weak
0.120-0.240	Moderate
>0.240	strong

Antibiotics Susceptibility Test by Disk Diffusion Test (DDT)

A previously discovered bacterial organism was used to carry out the operation using a culture. This test used bacterial suspension of moderate turbidity created by incubating moderate turbidity from ready-made (0.5) McFarland tube standard in nutrient broth after it was inoculated with growth taken from (5) colonies established on blood agar plates. To acquire a sample from the standardized culture, a sterile swab was utilized, and that sample was then spread onto a Muller–Hinton agar plate (CLSI, 2019).

Results

In this research, 150 patients were sent to the dental clinic in Al-Hillah, Iraq, to treat their infected

root canal teeth. After 48 hours, samples were cultured at 37°C for the allotted time. Prevotella spp. were first identified using colony morphology, microscopy, and biochemical testing. These isolates were then identified using a Vitek 2 system by running particular negative cards of this system, which returned a matching response for 17 (11.4%) of the 150 isolates tested (3).

Table (3):
Identification of Prevotella spp. depended on the colonial morphology, microscopically, and biochemical tests and specific positive cards of Vitek 2 system

Total No. of samples	Initial identification of Prevotella spp.	Other microorganisms	Identification of Prevotella spp. by Vitek 2 system
150	17(11.4%)	133(88.6%)	17(11.4%)

Identification of Prevotella spp. by Vitek 2 system were classify these bacteria in to three species, the results showed that, out of 17 Prevotella isolate, 12(70.6%) were related to Prevotella intermedia, 3(17.7%) isolates were related to Prevotella nigresceens and 2(11.7%) isolates were related to Prevotella tannerae as shown in Table (4).

Table (4):
Identification of Prevotella spp. depended positive cards of Vitek 2 system

Bacteria	Name of Species	No. of isolate	Percentage
Prevotella spp.	Prevotella intermedia	12	70.6%
	Prevotella nigresceens	3	17.7%
	Prevotella tannerae	2	11.7%
	Total	17	100%

The DNA was isolated from all previously known Prevotella isolates using the Vitek2 method, and particular 16srRNA gene sequences were amplified using this DNA for the purposes of sequencing, by employing primers for three of the Prevotella kinds (as shown in Table 1). (1). Gel electrophoresis revealed that all of the Prevotella intermedia samples (100%) generated a 660 bp DNA fragment, which was unique to the strain. Isolates that previously tested positive for Prevotella nigresceens using the Vitek2 method were identified with molecular testing for 16srRNA gene. The findings revealed that every one of the 3 test subjects (100%) was positive. Bands showing a higher yield as compared to the genomic sequence appear while looking for more than 800 bp of sequence. As well, 16srRNA gene detection was performed on 5 Prevotella tannerae isolates, and it was determined that the 2 (100%) isolates tested positive for this gene. the band that looked like the allelic ladder revealed that there were some good outcomes in the experiment, as shown in the figure (1).

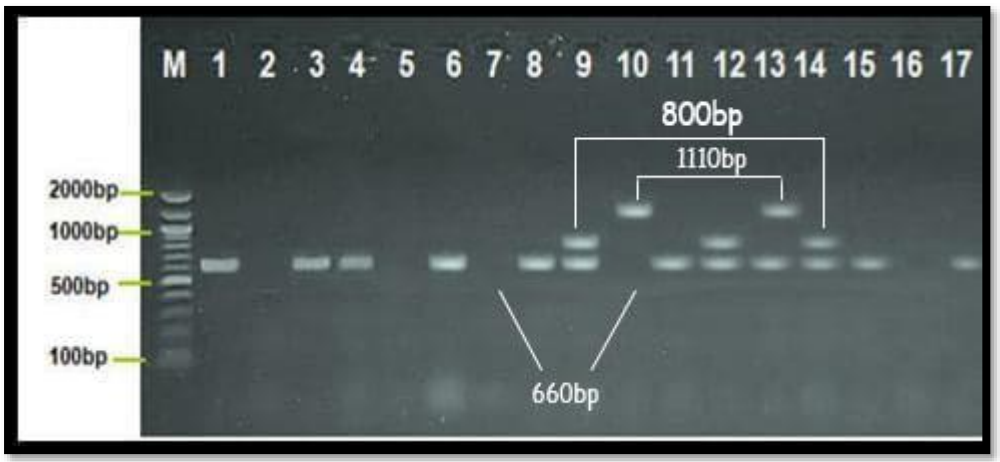

Figure (1): Agarose gel electrophoresis image of PCR product analysis for 16srRNA gene in Prevotella

isolates. M (Marker ladder 2000-100bp). Lanes at (660 bp) product size related to 16srRNA gene in Prevotella intermedia isolates, Lanes at (800 bp) product size related to 16srRNA gene in Prevotella nigresceens isolates and Lanes at (1110 bp) product size related to 16srRNA gene in Prevotella tannerae isolates Epithelial cell adhesion is thought to be a vital component in bacterial infection. All species of Prevotella (100 percent) were shown to attach to epithelial cells, as the table indicates (5).

Table (5):
Adhesion of Prevotella spp. on epithelial cells

No	Bacterial species	Adhesion assays	
		no. of isolates (17)	Epithelial cells
1	Prevotella intermedia	12	12(100%)
2	Prevotella nigresceens	3	3(100%)
3	Prevotella tannerae	2	2(100%)

As well, a microtiter (biofilm test) was used using glucose-containing (1%) Trypticase SoyBroth. To improve the accuracy of this test, it was performed three times. The study's findings were translated into three categories of biofilm formers: non-biopersistent, moderate, and chronic bacteria biofilm formers based on the level of OD value, which can be read as (OD<0.120, OD=0.120-0.240, and OD>0.240). The findings concluded that all Prevotella isolates produced biofilms (100 percent) and Prevotella intermedia biofilms were present in a majority of specimens (10 percent), the majority of Prevotella nigrescens also displayed biofilms (3 out of 3, 100 percent), and the minority of Prevotella tannerae expressed moderate biofilms (1 out of 2, 50 percent) while only the minority of Prevotella intermedia specimens showed any trace of biofilm (2 out of 12, percent) (50 percent). these findings were shown in Table (6).

Table (6)
Production of biofilm in Prevotella spp.

Bacterial isolate	No. of isolates	Biofilm Strong	Moderate	Weak	% of biofilm Formation
Prevotella intermedia	12	10(%)	2(%)	0(0%)	100
Prevotella nigresceens	3	3(100%)	0(0%)	0(0%)	100
Prevotella tannerae	2	1(50%)	1(50%)	0(0%)	100

All the identified of Prevotella spp. isolates from acute inflammation of root teeth were subjected to in vitro antibiotic resistant test by modified Kirby - Bauer disc diffusion method. Selective antibiotics are used to show their effected on Prevotella spp. isolates such as Clindamycin, Amoxillin, Ceftazidime, Tetracycline, Chloramphenicol, Aztreonam and metronidazole. The results compare according to Clinical Laboratory Standard Institute guidelines (CLSI, 2019) as resistant. Highest rate of resistant is seen to almost antibiotics used in present study, 16(94.1%) isolates were resistant to Clindamycin, 15(88.2%) isolates were resistant to Amoxillin, 13(76.47%) isolates were resistant to Chloramphenicol, 11(64.7%) isolates were resistant to Tetracycline, 9(52.9%) isolates were resistant to Aztreonam and 3(17.6%) isolates were resistant to metronidazole. The results were shown in Figure (2).

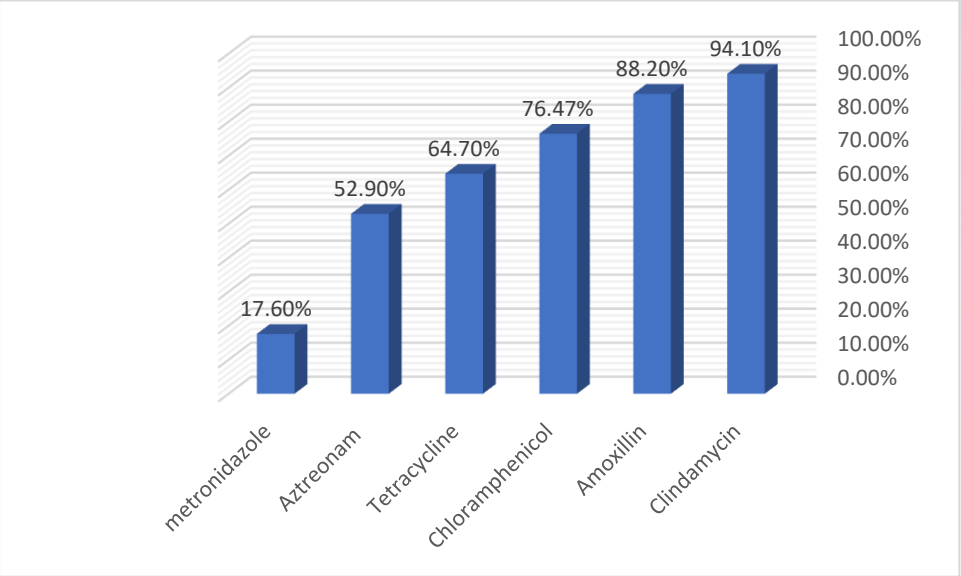

Figure (2) Antimicrobial resistant for of tested Prevotella spp.

Discussion

Prevotella species detected in acute inflammation of root teeth embrace Prevotella intermedia, Prevotella nigresceens and Prevotella tannerae almost like the results of Viscount Nelson et al., (2018). Prevotella is a non-pigmented, Gram-negative, anaerobic bacterium which has been shown to be connected to many severe oral and general infections. This was shown by Kotrashetti et al. (2018). Many clinical specimens with Prevotella have had culture and organic chemistry tests performed, which are usually inaccurate (Agarwal & Lewis, 2021). PCR primers for Prevotella were discovered via comparison of neighboring species' 16SrRNA genes and selecting PCR primers that were unique to Prevotella (Fehlner-Peach et al., 2019). Of all samples, 36.2% (17) of them had Prevotella DNA, (Prevotella DNA was present in all patients.) according to Harjunmaa (2018). Prevotella in clinical specimens may be differentiated using a sensitive, specific, and accurate PCR test (Price et al., 2020). A considerable amount of the Prevotella genus is made up of black-pigmented anaerobic species, and the various members contribute to oral disease (Tett et al., 2021). An individual's risk of developing necrotizing gingivitis and acute gum disease (often referred to as trench mouth) has been heavily linked to Prevotella intermedia. Prevotella intermedia and its closely related species, Prevotella nigrescens, both inhabit the cracks between teeth (Zhang et al., 2017). Besides being found in oral cavity infections, Prevotella intermedia, P. nigresecens, and Prevotella tannerae have also been recognized (De Peru et al., 2018). PCR, which is becoming a new mainstay of identifying methods, may possibly replace old microbiological culture methods in usage (Idelevich et al., 2018). But thus far, we've seen no ways to identify Prevotella using clinical specimens (Kostrzewa et al., 2019). Microorganisms may be solubilized from their cell surfaces in adherence activity. When bacteria attach to an appropriate tissue matrix, they may cause colonization. The gift research showed that P. intermedia could cling to tissue well, which allows them to cause colonization. Pathogens' ability to adhere to oral surfaces is seen as an important part of the infectious illness process (de Oliveira Marre et al., 2020). Studies have demonstrated that in nutrient-laden fluid, P. intermedia may adhere to animal tissue cells and bacteria can form biofilms on any surface. Microorganisms, a solid surface, and a fluid medium are all essential to biofilm development (Frasca et al., 2018). The biofilm development process goes through three phases. First, molecules that are both organic and inorganic are absorbed into the surface, where they combine to create a learning layer. Stage 2: Bacterial Adhesion to the Conditioned Layer: pH, temperature, surface energy of the substrate, organic process availability, and duration of contact with microbe are some of the variables that influence the microorganism adhesion (Berne et al., 2018). There are three stages of bacterial substrate interaction: Fimbriae, pili, flagella, and extracellular polysaccharides are methods microorganisms use to move themselves and other microorganisms to the surface of a substrate, thereby contributing to colonization of the surfaces of substrates (glycocalyx). First non-specific adhesion of microbes happens because of their static attraction, the ability to bind to substrates through valence and atomic number 1 bonding, and the dipole and hydrophobic interactions. Step 3:

Microbial substrate-specific adhesion phase. This step involves the organism's molecules being stuck to receptor molecules on the substrate. During the third stage, biofilm and biofilm development takes place. In the second stage, the first microbial layer attracts more colonizers and secondary microcolonies, which all contribute to the creation of the whole biofilm structure (Gutmann & Manjarrés, 2018). Dentistry microbe biofilms are all distinguished by their presence and location: as intractable biofilms inside a canal, of the tooth, and found outside the tooth or in the surrounding bone and soft tissue (Pavithra, 2020). AMR develops after the development of many bacteria and other microorganisms that adapt to being susceptible to medical treatment. While much of the discussion is on the misuse of antibiotics, it's obvious that there's a bigger problem with pharmaceutical quality (Graham et al., 2019). Medicines with a lower dose of the active ingredient will cause resistance. Ways aimed toward addressing antimicrobial resistance embrace making certain broad access to reasonable medicines, correct situation of existing antimicrobial treatments, investments within the development of latest treatments. Most isolates were susceptible to antibiotic and metronidazole, with solely 3 isolates immune to metronidazole. Singh et al., (2021) found that, Antibiotics are utilized extensively to treat oral infection, and a number of other and several other studies have examined the result of persistent antibiotic use on microorganism resistance. All Prevotella isolates as liable to antibiotic (Lamoureux et al., 2021), while antibiotic resistance is rare, and antibiotic drug resistance amongst Prevotella has nevertheless to be detected (Heirali et al., 2017). Furthermore, antiprotozoal resistance was solely detected during a tiny number of isolates. Lee et al., (2021) found, metronidazole resistance (21%) was highest within the oral isolate and exceeded the amount rumored by others (Odo, 2018; Boekhoud et al., 2020). Antibiotic condition in Prevotella spp. was demonstrates that, meropenem, piperacillin/tazobactam, chloramphenicol and metronidazole are probably to be the foremost effective antibiotics if treatment is indicated (Forbes et al., 2021). Antimicrobial resistance among anaerobes has systematically enhanced within the past three decades, and also the condition of anaerobic microorganism to antimicrobial agents has subsided predictable. Antibiotic drug, a biological process agent, is active against most anaerobic bacteria however never used (Ishak et al., 2020). Resistance to the current drug is rare, though it's been rumored for a few Prevotella spp. (Maraki et al., 2020). One should bear in mind that antibiotic resistance of antibiotic drug typically cluster round the susceptibility breakpoint (McDermott & Davis, 2021). Chloramphenicol was regarded in the past because the drug of alternative for treatment of significant anaerobic infections once the character and condition of the infecting organisms (Brook, 2017).

Conclusion

Prevotella spp. is the one of the causative agent of acute inflammation of root canal teeth, most species of clinical isolates of Prevotella were presence of adhesive factor, and it can produce biofilm by quantitative method, and it was highly antibiotic resistance mainly Clindamycin and Amoxillin.

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