ORIGINAL ARTICLE

Metronidazole Susceptibility and Resistance Pattern among Anaerobes causing Periodontitis in tertiary care unit

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ABSTRACT

Background: Periodontitis is a global health problem affecting a large number of individuals. The disease is generally caused by anaerobes. It is well managed by proper oral hygiene. However, systemic antibiotics are also prescribed to avoid any complication. Metronidazole is a frequently prescribed antibiotic for the treatment of periodontitis.
Aim: To evaluate the susceptibility to metronidazole by anaerobic microbes isolated from individuals suffering from periodontitis.
Methodology: observational study was carried out in period from January to June 2016 dental clinic of Jinnah Postgraduate Medical Centre, Karachi, Pakistan. For this purpose, 100 samples were collected from the periodontal pockets of the patients. The samples were processed for identification of anaerobic bacteria through conventional culture and biochemical tests. Metronidazole susceptibility testing was performed by DDM.
Results: Out of 100 samples, anaerobes were isolated from 41 samples. Of them 41 samples *Prevotella denticola* (14.63%), *Prevotella loecsheii* (4.89%), *Prevotella melaninogenica* (12.19%), *Prevotella oris* (17.07%), *Prevotella oralis* (4.89%), *Fusobacterium nucleatum* (9.76%), *Tannerella forsythensis* (9.76%), *Peptostreptococcus anaerobicus* (14.63%), *Peptostreptococcus micros* (2.43%) and *Veillonella spp*. (9.76%) were identified or isolated. About 56.13% of anaerobic isolates were found to be resistant to Metronidazole.
Conclusion: Resistance to Metronidazole will ultimately lead to therapeutic failures. Measures such as controlled use of antibiotic should be implemented to prevent the emerging resistance of the drug in the society.
Key words: Anaerobes, metronidazole, periodontitis, resistance, susceptibility

INTRODUCTION

Periodontitis is an inflammatory disease of the gums. It is a public health problem and if not treated could lead to tooth loss as well as systemic consequences[1]. The incidence of periodontitis increases with age. It affects around 50 % of the world adult population[2]. The sixth most occurring human disease is severe periodontitis with a prevalence rate of 11.2%[2]. Periodontitis is caused by mixed organisms predominantly anaerobes. It is reported that *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are the two most common species causing periodontal disease. Apart from them, *Fusobacterium sp., Treponema sp., Prevotella sp., Eikenella sp., Campylobacter sp., Selenomonas sp. and Peptostreptococcus sp.* are also found responsible for causing periodontitis[4].

Periodontitis is generally managed with proper oral hygiene measures along with mechanical and surgical treatment.[5]However, despite of diligent therapy patients may suffer from periodontal tissue damage, aggressive periodontitis and even loss of tooth. These complications are managed by using systemic antibiotics[6]. Antibiotics generally recommended for periodontitis are Amoxicillin, Tetracycline, Doxycycline, Minocycline, Metronidazole, Azithromycin and Clindamycin. These drugs can be used either alone or in combinations depending upon the condition of the patient and sensitivity of bacterial pathogens[7].

Systemic antibiotics have been used for the past 35-40 years in treating periodontitis.[8,9] However, it has been reported that affected patients in the United States are found to be resistant to the antibiotics commonly prescribed to treat periodontitis[10.11].

The wide antibiotic-resistance among periodontitis pathogens is an alarming issue. The aim of the present study is to isolate anaerobes from patients suffering from periodontitis and investigate the susceptibility of metronidazole in the isolated anaerobes.

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MATERIALS AND METHODS

Sample Collection: Present study was carried out in outpatient dental clinic of Jinnah Postgraduate Medical Centre, Karachi, Pakistan after obtaining approval from the ethical committee BMSI, JPMC (IRB#F.1-2/2016/BMSI-E.COMT/049/JPMC). Patients clinically diagnosed with periodontitis were enrolled in the study during the period of January 2016–June 2016 using convenience sampling. Patients were excluded if they had dentures or receiving any antibiotic therapy. Informed patient consent was

obtained from all patients prior to the sample collection and confidentiality of each case was observed. A total of 100 patients were included in the study. Samples were collected from periodontal pocket of enrolled patients with the help of sterile syringe. Modified enriched thioglycolate broth was used as transportation medium to transport samples to the Clinical Laboratory (Microbiology section of Citilab, Karachi).

Sample incubation: The samples were processed within 24 hours for the isolation of periodontal bacteria. Aerobic incubation was performed by culturing each sample on Blood agar and MacConkey agar for recovery of aerobes and facultative anaerobes. They were incubated at 37ºC for 24 hours.

For the recovery of micro-aerophillic anaerobes, carbon dioxide enriched incubation was carried out. Each sample was cultured on Chocolate agar and was incubated in candle jar at 37ºC.

Anaerobes and Facultative anaerobes were recovered by culturing each sample on freshly prepared Blood agar supplemented with vitamin K and Thioglycolate broth. Incubation was carried out in anaerobic jar at 37ºC for 48 hours. Anaerobic atmosphere was produced using Anaerocult A (Kit manufactured by Merck).

Aerotolerant testing of individual colonies obtained from anaerobic and microaerophilic incubation was also performed. Anaerobic isolates were processed for further identification.

Antibiotics discs of Kanamycin 1 mg, Vancomycin 5 mcg and Colistin 10 mcg were used for identification of each colony type subcultured on the Blood Agar. Sodium polyanethol sulfonate (SPS) disk was added when the Gram stained smear showed the presence of Gram positive cocci. Plates were incubated anaerobically at 37ºC for 48 hours. Isolates showing gram negative bacilli were incubated separately anaerobically at 37ºC for 7 days to rule out the presence of black pigment producing anaerobes. Identification of the organisms isolated were carried out using conventional and rapid biochemical tests such as nitrate reduction test, indole test, esculin hydrolysis test and gelatin liquefaction test, 20% bile tolerance test, rapid urease test, rapid carbohydrate fermentation test and oxidase test[12].

Metronidazole susceptibility testing: Metronidazole 16 mcg disc was used to perform the susceptibility testing of the anaerobic isolates. Antibiotic susceptibility testing of anaerobe positive isolates was performed by disc diffusion method described by Wilkins et al[13,14].

It was performed by adding 1.5 ml of inoculum cultured for 18 to 24 hours at maximum turbidity in thiogylycolate medium to 10 ml of Brain Heart Infusion agar which had been previously melted and cooled at 50ºC. After mixing the contents twice by inversion they were poured in 90 mm petri dishes and were solidified at room temperature. Discs containing 16 mcg of Metronidazole were applied on the solidified agar plates. The plates were incubated anaerobically at 37º C for 24 hours in an anaerobic jar using Anaerocult A kit. Zones of inhibition were measured against a black background with a ruler. Sensitivity was defined as zone diameter greater than or equal to 17 mm whereas zone diameter less than or equal to 15 mm was considered resistance to metronidazole.

The data was analyzed by using SPSS ver. 19. The categorical data variables such as numbers of isolates, antibiotic resistance and sensitivity were represented by frequencies and percentages. While mean and SD were used for description of zone of inhibition (mm).

RESULTS

Samples were obtained from 100 periodontitis patients. Out of 100 samples, 3 failed to show the presence of any organisms. Facultative anaerobes were found in the remaining 97 samples while anaerobes were present and isolated from 41 samples. Only 2 samples showed the presence of aerobes. Table 1 shows the different anaerobes isolated from the 41 samples.

Table 1: Anaerobes isolated from Periodontitis Patients

Anaerobic Organisms	**No. of Isolates%**
Gram Negative Bacilli (n=41)	
Fusobacterium nucleatum	4(9.76)
Prevotella denticola	6(14.63)
Prevotella loescheii	2(4.89)
Prevotella melaninogenica	5(12.19)
Prevotella oris	7(17.07)
Prevotella oralis	2(4.89)
Tannerella forsythensis	4(9.76)
Gram Positive Cocci	
Peptostreptococcus anaerobicus	6(14.63)
Peptostreptococcus micros	1(2.43)
Gram Negative Cocci	
Veillonella spp.	4(9.76)

Table 2(a): Metronidazole Sensitivity Results

Anaerobic Organisms	**Sensitive %** (n=18)	**Resistant%** (n=23)
Fusobacterium nucleatum	2 (4.89)	2 (4.89)
Prevotella denticola	2 (4.89)	4 (9.76)
Prevotella loescheii	1 (2.43)	1 (2.43)
Prevotella melaninogenica	3 (7.32)	2 (4.89)
Prevotella oris	3 (7.32)	4 (9.76)
Prevotella oralis	2 (4.89)	0 (0)
Tannerella forsythensis	0 (0)	4 (9.76)
Peptostreptococcus anaerobicus	3 (7.32)	3 (7.32)
Peptostreptococcus micros	1 (2.43)	0 (0)
Veillonella spp.	1 (2.43)	3 (7.32)

Table 2(b): Comparison of zone of inhibition as assessed by disc diffusion assay among susceptible and resistance strains of isolated anaerobes

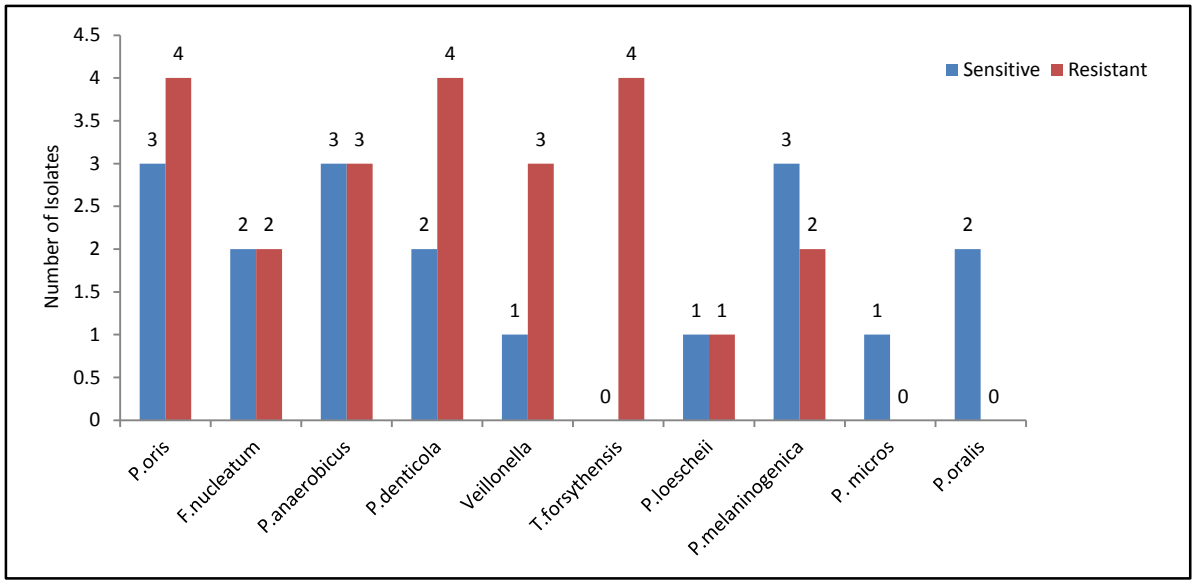
Zone of Inhibition	**Sensitive Mean ± SD**	**Resistant Mean ± SD**	**P-value**
Fusobacterium nucleatum	24.5 ± 0.1	12.0 ± 1.41	<0.001
Prevotella denticola	24.52 ± 1.44	11.7 ± 0.68	<0.001
Prevotella melaninogenica	23.98 ± 0.524	12.27 ± 1.8	<0.001
Prevotella oris	22.46 ± 1.02	11.9 ± 1.15	<0.001
Peptostreptococcus anaerobicus	23.82 ± 0.5	10.92 ± 0.6	<0.001

The different anaerobic organisms were subjected to metronidazole susceptibility testing using diffusion disc method. On the basis of zone of inhibition, resistance and sensitivity to 16mcg metronidazole disc was identified. Table 2 (a) and Figure 1 summarize the number of

anaerobic organisms that were found to be resistant and sensitive to metronidazole. Table 2(b) described the comparison of zone of inhibition among susceptible and resistance strains of anaerobes.

Out of total 41 about 23 isolates (56.13%) showed resistance to metronidazole while only 18 isolates (42.5%) were found to be sensitive to metronidazole As depicted in table 2(a). There were statistically highly significant differences among the zones of inhibition of metronidazole susceptible and resistance strains of anaerobes. As illustrated in table 2(b).

Figure 1: Metronidazole susceptible and resistance strains of Anaerobes



DISCUSSION

Metronidazole, a nitroimidazole derivative has been used for more than 50 years for clinical treatment of a number of diseases. The use of metronidazole against anaerobes (Bacteroides necrophorus) was first reported in 1964. The drug is highly active against gram positive and gram negative anaerobes[15].

Metronidazole acts by interacting with DNA thereby inhibiting nucleic acid synthesis. Resistance to metronidazole occurs due to increased activity of enzymes responsible for DNA repair or decreased activation of the drug or increased efflux of the drug or enhanced oxygen scavenging capabilities[16].

Metronidazole is preferred in periodontitis because of high efficacy and fewer side effects. However, resistance to metronidazole is now being developed by the anaerobic bacteria[17.] This developing trend seems to be under reported because sensitivity analysis is not routinely being performed by most of microbiology laboratories.

However, it has been studied globally and reported over the past few years. Maestre et.al reported less than 6% of metronidazole resistance among 261 isolates of anaerobes[18]. Comparatively, in 2013, Junlin et al cited resistance to metronidazole in 24.5% isolates of anaerobes[19]. Similarly, in 2016, Padnekar et al also reported resistance to metronidazole in 8% of anaerobic isolates[17].

The current study was conducted to identify the anaerobes in periodontitis patients and their susceptibility to metronidazole by using conventional method. Out of 100 samples, anaereobes were isolated from 41 samples. Prevotella, Fusobacterium, Tanerella, Peptostrptocoocus and Vellionella were isolated among anaerobes. When these anaerobes were subjected to metronidazole susceptibility testing, it was about 56.3% that more than half of the total isolates were resistant to metronidazole which was consistence with study can conducted by Rugarabamu (2017). This was cross sectional study in which they collected 70 different samples from patients with various forms of orofacial infections. In about 83% of isolated samples anaerobes were isolated and out of which40% of samples shows resistance to metronidazole.

CONCLUSION

Increasing resistance to metronidazole by anaerobes would be a great challenge to combat these infections. In anaerobic infections, therapeutic failures secondary to metronidazole resistance could only be documented by performing sensitivity testing of anaerobic isolates routinely. Using conventional method is cost effective and yields reliable results. This study highlights the needs of investigating the susceptibility of anaerobes to antibiotics as routine practice and further studies to clarify the mechanism of metronidazole resistance. Adoption of measures such as proper dose, route of administration, prohibition of over the counter dispensing of the drug and awareness programs may assist in the prevention of resistance and improved management of anaerobic infections.

Ethical Considerations: This study complies with the conditions set out as per the principles of declaration of Helsinki.

Conflict of Interest: The authors declare they have no conflict of interest

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