INTERNATIONAL JOURNAL OF
PHARMACEUTICAL RESEARCH AND BIO-SCIENCE

EVALUATION OF COINFECTION OF ANAEROBES AMONG PATIENTS WITH PERIODONTAL INFECTIONS AT UNIVERSITY OF PORT HARCOURT TEACHING HOSPITAL

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Accepted Date: 21/05/2014; Published Date: 27/10/2014

Abstract: BACKGROUND: Anaerobes are the common bacteria found in the human oral cavity and are often isolated and identified from periodontal infections and other infections involving other body sites. **OBJECTIVES:** To isolate and characterize anaerobes from patients with periodontal infections attending UPTH, Port Harcourt in Nigeria and to determine their level of occurrence and co-infection. **METHODS**: Ninety-seven supragingival and subgingival specimen were collected from patients with different periodontal infections and were studied by culture, PCR and biochemical analysis, Susceptibility testing to Amoxicillin and Metronidazole was determined by agar dilution method. **RESULTS**: Sixty-one (62.8%) anaerobic isolates were obtained from the 97 patients.43 of the isolates were fully characterized and identified as *F. nucleatum P. Gingivalis* and *Prevotalla intermedia* species. **CONCLUSION**: The isolation of anaerobes from these periodontal infections shows they can be found in human and also known to cause infections. The use of PCR has been shown to be rapid and sensitive in the identification of anaerobes. It is important for other anaerobic species to be studied and fully identified in order to achieve and administer accurate treatment of other oral infections.

Keywords: Anaerobes, Periodontal infections, Co-infection, PCR (Polymerase chain reaction)

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PAPER-QR CODE

Access Online On:

www.ijprbs.com

How to Cite This Article:

Anyiam IV, Ibe SN, Abu GO, Braimoh OB, Nwaokorie FO; IJPRBS, 2014; Volume 3(5): 1-15

INTRODUCTION

The oral cavity harbors about 300-700 different bacterial species (1, 2). *A. actinomycetemcomitans, Treponema denticola, P. gingivalis, Bacteroides forsythus*, *Peptostreptococcus, Tannerella forsythia Prevotella* spp, and *Fusobacterium* species have been defined as the periodontal pathogens accounting for infections in the oral cavity (3). These organisms are acquired from early stages of life from food, kisses and people at close proximity with children. However, the acquisition and stability of these periodontal pathogens remains undefined (3). Some of these oral pathogens are commonly co-isolated in sub gingival biofilm samples (3). This consistent coexistence suggests that a strong ecological relationship may exist among these microbial species. Acute periodontitis, Chronic periodontitis, Dental alveolar abscess, Dental caries, Epical periodontitis, Gingivitis, Lateral periodontal abscess, are the most common oral bacterial disease of man which result from an interaction between a susceptible host, commensal microbiota and the environment. Although some specific microorganisms have been implicated in the pathogenesis of these conditions, resulting to complex diseases due to breakdown in the homeostasis between the human host and microbiota. Periodontitis is a chronic immuno inflammatory infectious disease of the gingiva tissue, alveolar bone that leads to the destruction of periodontal ligament and adjacent supportive alveolar bone resulting to tooth loss and mouth odour induced by biofilms containing numerous periodontal pathogens (4, 5, 6). In Nigeria, the prevalence of periodontal disease with deep pocketing among patients aged 15 years and above range between 15% and 58% (7, 8).Periodontal infections seems to be the source of microorganisms involved in infections in other extra oral sites (9). This infection, occur worldwide (10, 11, 12, 13) and is the major cause of dental loss in the adult population and is considered a serious health problem globally. These oral diseases are seen frequently in Nigerian hospitals among different age population. However, there are good number of reports to substantiate the understanding and knowledge of predominant pathogens, though most sources acknowledge anaerobic bacteria as the causative agent in chronic oral infections (14, 15). Consequently, It was seen to be responsible for 24.8% of oro-facial infections seen in a naval base hospital in Lagos, Nigeria (16) and a major predisposing factor for dentalveolar abscess (17).The specific identification of these anaerobic strains and their prevalence in specific oral infections remains a problem in Nigeria because of our environmental factors which tend to limit their growth and the difficulties involved in the conventional identification of anaerobes unlike the aerobic species, culturing of anaerobes requires defined media and the time-consuming during incubation; an important factor to consider when the clinical condition of a patient is involved and the need for fast laboratory results. Based on continuous discovery of identification techniques most of them can presently be studied. The `introduction of rapid methods of identification for anaerobes using commercial kits, is well recognized (18), fatty acid methyl esters (FAME) analysis (19), DNA–DNA

hybridization (20). There is also the use of molecular methods which is a recent improvement in the accurate detection of anaerobes from clinical specimens such as polymerase chain reaction (PCR) and genetic sequencing (21, 22). However, these techniques are associated with challenges such as the high cost of obtaining the equipment, tedious nature of the laboratory procedures and inconsistent results. This study details molecular identification of anaerobes; and shows the level of occurrence and co infection of these anaerobes among patients with periodontal infections.

MATERIALS AND METHODS

Study Population: All consenting consecutive patients from both sexes and from all age groups with pocket formation attending oral diagnostic unit of University of Port Harcourt Teaching Hospital were eligible to participate in the study.

Criteria

Patients who are not on any antibiotics three months before the specimen collection and with periodontal pockets greater than or equals to 5mm. Patients with malignant tumors and congenital defects were excluded from this study.

A total of ninety-seven (97) supragingival and sub gingival specimen, were collected from patients who had symptoms and signs of anaerobic infection on clinical examination were enrolled for the study. Inclusion criteria included informed consent, and patients who are not on any antibiotics within the three months before the study and with periodontal pockets greater than or equals to 5mm. Exclusion criteria; patients with malignant tumors and congenital defects.

Specimen Collection: Specimen were collected into pre-reduced dental transport media (oxoid) (pre- reduced thioglycolate broth) using a sterile paper point inserted into the periodontal pockets with depth > 5 mm for I min. Two paper points were used on each patient. One was placed into the sterile pre-reduced transport medium in a transportation vial for culture and the other into a 1.5 ml eppendorf tube containing thioglycolate medium for DNA extraction. The specimen collected was processed within two (2) hours of collection,

Bacterial Isolation and Identification:

Specimens were streaked on Bacteroides Bile Esculin (BBE) agar and Fastidious anaerobic agar (FAA)(Lab M) supplemented with 5µg/ml hemin,1µg/ml vitamin K, and 5% of sheep blood and incubated at 37^{o}C for 7 days to allow for pigmentation to appear in an anaerobic jar (Merck KGaA, Germany)containing 90% N_2 and 10% CO_2 generated by sachets of gas generating kit (Merck, Germany).The isolates were gram stained and subcultured on FAA. The growth on the plate was examined macroscopically gram stained and subculture onto fastidious anaerobic agar (Lab M). Antibiotic disc susceptibility tests for Amoxicillin and Metronidazole was carried

out using brucella blood agar plate supplemented with 5 µg/ml hemin, 1µg/ml vitamin K, and 5% of sheep blood incubated at 37^0C for7 days in an anaerobic jar (Merck KGaA, Germany) and checked for the presence or absence of zones of inhibition.

Biochemical Tests: Biochemical tests were performed using API 20 A (bio Mérieux SA, Mercy-l'Etoile, France) according to the manufacturer's instructions. Indole reaction was performed onIndole reagent (Anaerobe systems). The identified isolates were stored in 10% skimmed milk at -80^0C.

Antimicrobial Suscetibility testing

Antibiotics: The potency powder of the following antibiotics was used for the Minimum Inhibitory Concentration (MIC), Metronidazole and Amoxicillin.

The agar dilution procedure recommended by Clinical Laboratory Standard Institute was used (23). Brucella agar with sterile horse blood was prepared as the basal medium. An inoculum was grown in Brain Heart Infusion broth supplemented with hemin and menadione for 24hourrs and the turbidity was adjusted to 0.5 McFarland standards. Double dilution of each antimicrobial agent (ranging from 0.06-32µg/ml) was incorporated into the sterile molten Brucella agar which was supplemented with hemin (5µg/ml), vitamin K (1µg/ml) on the day of the test. A 200ul vol of the overnight broth were inoculated on the plates and incubated anaerobically for 2days at 37^0C. Control plates without Metronidazole and Amoxicillin were inoculated and incubated in the same way. Reference strain *Bacteroides fragilis* ATCC 25285 was included as control. The MICs were recorded as the lowest concentration of the antibiotic in the medium that inhibited bacterial growth, gave a faint haze of growth or with no more than one discrete bacterial colony. The MICs were interpreted as being resistant or sensitive by applying the breakpoints that were proposed by CLSI (23).

Bacterial Identification by Polymerase

Chain Reaction: Conventional PCR assay was conducted to confirm phenotypic identification of the isolates as *P. gingivalis, F. nucleatum* and *P. intermedia.* Molecular identification of the isolates to species level was performed by simple and rapid PCR based method using a primer previously published by Avila-Campos *et al* (24).

DNA Extraction: The DNA extraction was done directly on the clinical specimens stored in -80^0C, using the boiling method. A 1.5ml of the clinical specimen in broth was centrifuged for 5minutes at 10,000rpm and the supernatant discarded. About 500µl of sterile water was added to the pellet and vortexed to homogenize with the sterile water using a vortex mixer. In an Eppendorf thermo mixer comfort (22331 Hamburg, Germany), the specimen was boiled in a heating block (Fisher Scientific) for 10 minutes and vortexed, and then centrifuged at 10,000rpm for 5 minutes. About 500µl of the DNA solution was transferred into 1.5ml

eppendorf tubes. The Nanodrop spectrophotometer (ND-1000, UV/VIS spectrophotometer USA) was used to estimate the concentration and purity of the extracted DNA using a blank solution containing sterile water as the standard.

DNA Amplification- This was performed using species-specific primers. The amplification was carried out using the primer set: *Prevotella interm*-Forward (FCGT GGA CCA AAG ATT CAT CGG TGG A) and *Prevotella interm*-Reverse (CCG CTT TAC TCC CCA ACA AA) for *Prevotella intermedia*, FN5059S-F (ATTGTGGCTAAAAATTATAGTT) and FN5059S-R for *Fusobacteria*spp, Porphyromonas gin-F (AGGCAGCTTGCCATACTGCG) and Porphyromonas gin-R(ACTGTTAGCAACTACCGATGT) for *Porphyromonas gingivalis*, and Bacteroides frag-F (TGATTCCGCATGGTTTCATT) and Bacteroides frag-R (CGACCCATAGAGCCTTCATC) for *Bacteroides fragilis*. The amplification reaction was carried out in an Eppendorf Master Cycler gradient. The cycling parameter consisted of an initial denaturation step at 94^0C for 3 minutes; followed by 35 consecutive cycles of denaturation at 95^0C for 1 minute, the primer-specific annealing temperature at 54^0C for 1 minute, an extension step at 72^0C for 2 minutes. This was followed by a final extension step at 72^0C for 5 minutes (25).All PCR products were separated using a 1.5% agarose gel electrophoresis performed in an electrophoresis tank at 100volts for 2.5hours (Sigma chemical company), stained with 0.5µg/ml ethidium bromide and photographed under UV trans illuminator using a digital camera (Optima UVT 260D.S/N-268002). A DNA ladder digest of 100bp ladder was used as molecular weight marker.

Statistical Analysis: Data obtained from this study are presented as frequency distribution and percentage of oral infections. Chi-sqaure and Correlations were used to test for significant difference at 95% confidence limit.

RESULTS

Of the clinical dental conditions, the number of patients with periodontal infections were eighty (82.5%) Fifty-four (55.7%) of the 97 patients, fifty-four were females (55.7%) and forty-three were males (44.3%) The highest number of patients infected was within the age 20-29 years. Female patients within the age bracket 20-29 years and the male patients within 30-39 years were the most infected.

Table 1 shows the distribution of oral infections by age group. The highest incidence was seen in the age bracket 20-29years while the lowest in the age bracket 70-79 years.

Table 2 shows the distribution of oral infections by sex. Chronic periododntitis was the infection with the highest number of occurrence seen in both male 18(18.6%) and female 25(25.8%) while the lowest was Acute periodontitis 4(4.1%) for female and Epical periodontitis 5(5.2%) for male.

The highest number of isolated organism was *Prevotella intermedia* 19(23.8%) while the least was *Peptostreptococcus* spp 2(2.5%) by culture as shown in Table 3.

Co-infection was mostly seen in patients with caries and the anaerobes involved were *Prevotella intermedia* and *Fusobacterium nucleatum* as shown in Table 4.

Fifty-two (85%) of the 61 isolates were identified by API 20A (bioMérieuxSA, Mercyl'Etoile, France). 30 (49.2%) isolates produced indole on indole reagent (Anaerobe Systems, USA). 43 isolates were correctly identified and confirmed as *F. nucleatumP. Gingivalis* and *Prevotalla intermedia* species by (Figure 1, Figure 2 and Figure 3) using species-specific primers.

The antibiotic sensitivity test shows Metronidazole was the most sensitive to the anaerobes (Table 5).

Table 1: Age distribution of oral infections among patients attending the UPTH dental clinic

Age Group (years)	Male	Female	Total infected	Total number
0-9	1	2	3	2
10-19	5	7	12	9
20-29	12	23	35	30
30-39	14	8	22	17
40-49	4	7	11	10
50-59	5	0	5	4
60-69	1	6	7	7
70-79	1	0	1	0
80-89	0	1	1	1
Total	43	54	97	80

Table 2: Distribution of oral infections by sex among patients attending the UPTH dental clinic

Oral infection	Number (%) Male	Female	Total
Acute periodontitis	7(7.2)	4(4.1)	11(11.3)
Epical periodontitis	5(5.2)	8(8.2)	13(13.4)
Lateral periodontal	6(6.2)	7(7.2)	13(13.4)
abscess	18(18.6)	25(25.8)	43(44.3)
Chronic periodontitis	36(37.1)	44(45.4)	80(82.5)

Table 3: Frequency occurrence of isolated organisms by culture among patients at UPTH dental clinic

Isolated organism	Number of times	(%)
Bacteriodesfragilis	9	11.3
Fusobacterium nucleatum	11	13.8
Micromonas spp	7	8.8
Peptostreptococcus spp	2	2.5
Porphyromonas gingivalis	13	16.3
Prevotella intermedia	19	23.8
Total	61	76.5

Table 4: Co-infection of isolated organisms in patients with oral infections attending the UPTH

Isolated organisms	Number of co-infection	Type of infection	(%)
Bacteriodes fragilis	1	Caries	1.3
Fusobacterium nucleatum			
Fusobacterium nucleatum	1	Caries	1.3
Porphyromonas gingivalis			
Micromonas spp	1	Caries	1.3
Fusobacterium nucleatum			
Micromonas spp	1	Gingivitis	1.3
Fusobacterium nucleatum			
Micromonas spp	1	Periodontitis	1.3
Fusobacterium nucleatum			
Prevotella intermedia	2	Caries	2.5
Fusobacterium nucleatum			

Table 5: Antibiotic Susceptibility of oral isolates from patients attending UPTH to Amoxicillin and Metronidazole

Isolates	Amoxicillin		Metronidazole	
	Range tested (ug/ml) Breaking point (ug/ml)	MIC Obtained	Range tested (ug/ml) Breaking point (ug/ml)	MIC Obtained
1	≤ 0.5-32	0.25	≤ 0.5-32	2
2	≤ 0.5-32	0.25	≤ 0.5-32	0.5
3	≤ 0.5-32	1	≤ 0.5-32	0.5
4	≤ 0.5-32	0.25	≤ 0.5-32	2
5	≤ 0.5-32	0.5	≤ 0.5-32	0.25
6	≤ 0.5-32	32	≤ 0.5-32	0.125
7	≤ 0.5-32	0.125	≤ 0.5-32	0.5
8	≤ 0.5-32	16	≤ 0.5-32	32
9	≤ 0.5-32	0.5	≤ 0.5-32	0.25
10	≤ 0.5-32	0.5	≤ 0.5-32	0.5
11	≤ 0.5-32	0.25	≤ 0.5-32	32
12	≤ 0.5-32	16	≤ 0.5-32	0.125
13	≤ 0.5-32	16	≤ 0.5-32	16
14	≤ 0.5-32	0.125	≤ 0.5-32	16
15	≤ 0.5-32	4	≤ 0.5-32	0.25
16	≤ 0.5-32	0.5	≤ 0.5-32	1
17	≤ 0.5-32	0.5	≤ 0.5-32	0.5
18	≤ 0.5-32	0.25	≤ 0.5-32	0.5
19	≤ 0.5-32	0.25	≤ 0.5-32	0.05
20	≤ 0.5-32	0.125	≤ 0.5-32	1

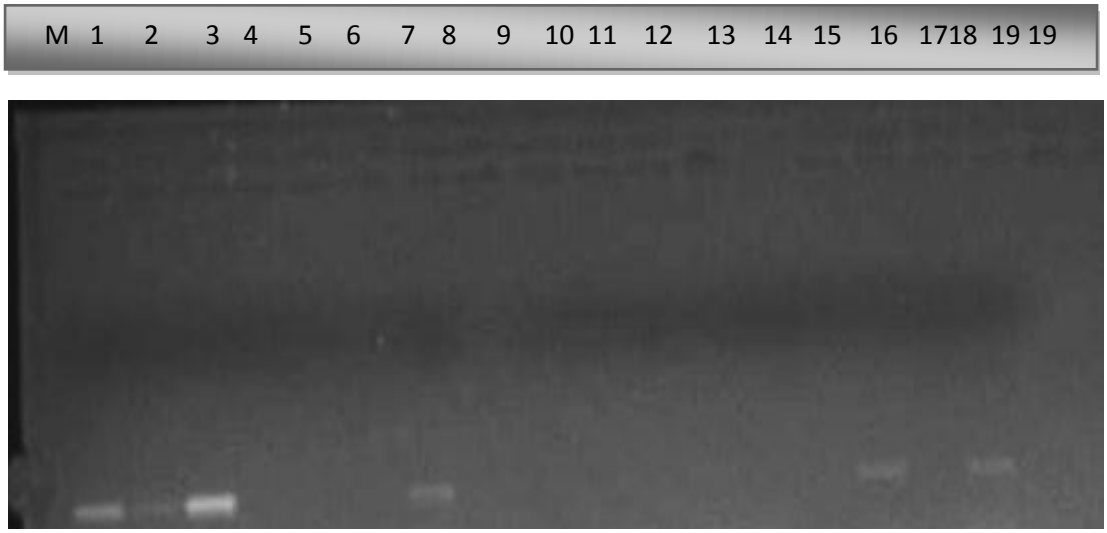

Figure 1: Lane M, 1Kb DNA ladder, Lanes 1 negative control, lanes 2,4-19 shows no amplication bands, lanes 3showsamplification bands of *Fusobacterium nucleatum*, in gel electrophoresis of using species specific primer

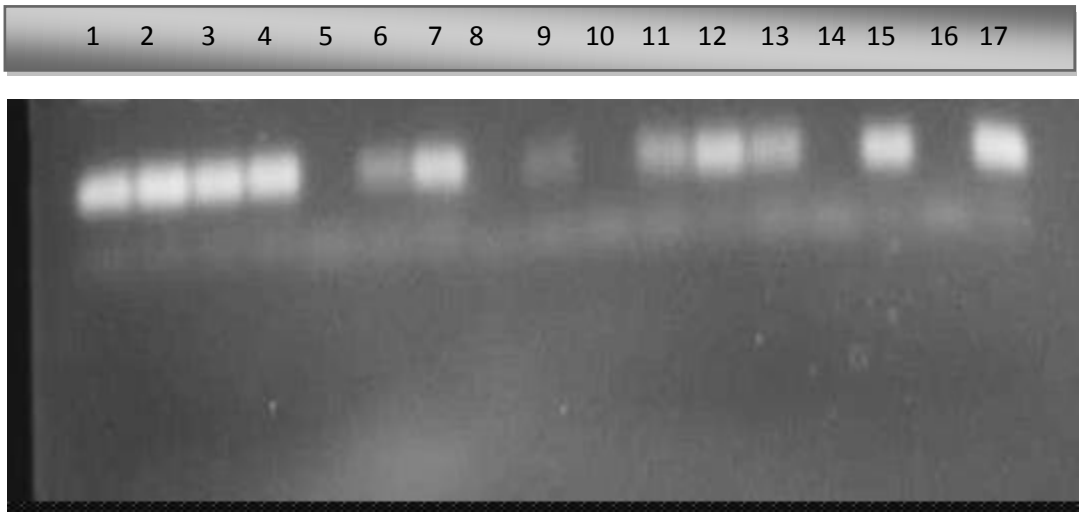

Figure 2:Lanes 1-4, 7, 12, 15 and 17 shows amplification bands, lanes 5,6 ,8-11,13, 14 and 16 shows no amplification bands in gel electrophoresis of *Porphyromonas gingivalis*, using species specific primer

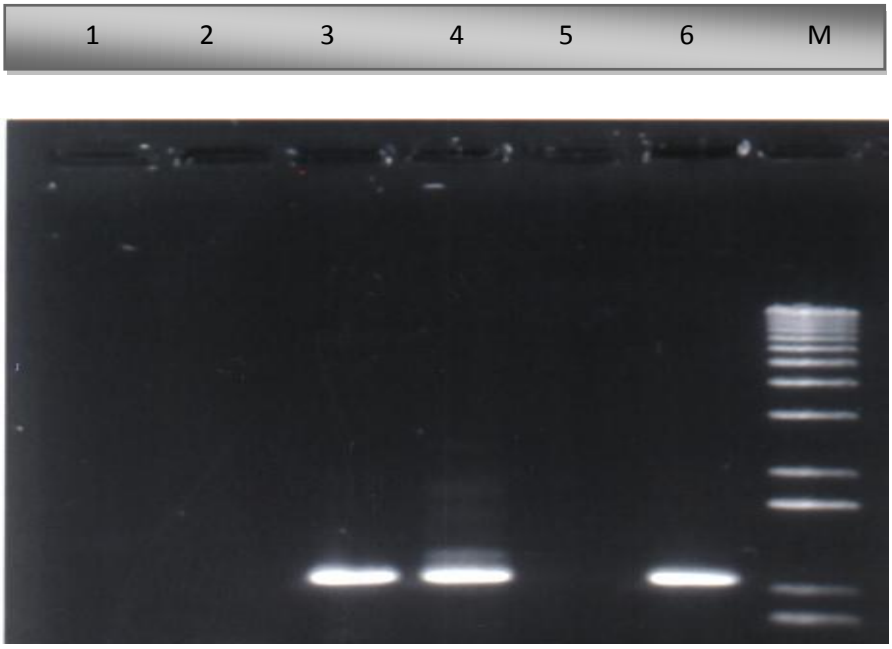

Figure 3: Lane M, 1Kb DNA ladder, Lane 1 negative control, lanes 2, 5 shows noamplification bands, lanes 3,4, 6 shows amplification in gel electrophoresis of *Prevotella intermedia* using species specific primer.

DISCUSSION

The involvement of anaerobes in human infections has shown to be a concern recently. However, there are now literatures on the study of these bacterial species in the low income countries. The significance of anaerobes in the development of oro-facial infections as well as infection in other organs has drawn attention due to its potential pathogenicity, frequency in periodontal lesions, production of tissue irritants, and their ability to aggregate with other periodontal pathogens in oro-facial infections of microbial etiology (26).

The presence of *Bacteroides fragilis, Fusobacterium nucleatum, Micromonas* spp*, Peptostreptococcus* spp*, P. gingivalis,* and *Prevotella intermedia* in Nigerian population suggests their role in the etiology and pathogenesis of oral infections. Dental caries is an oral infection caused by the inflammation of bone usually associated with pus formation. Chronic periodontitis involves overgrowth of oral bacteria in dental biofilm and whose interaction with the host's immune response leads to inflammation and loss of the tooth attachment (26).

The culture technique from this study has identified *Bacteroides fragilis, Fusobacterium nucleatum, Micromonas* spp*, Peptostreptococcus* spp*, P. gingivalis,* and *Prevotella* species based on their physiological and morphological and biochemical characteristics. Fifty- two (85%) of the 61 isolates were identified by API 20A (bioMérieuxSA, Mercyl'Etoile, France). 30 (49.2%) isolates produced indole on indole reagent (Anaerobe Systems, USA). The Nigerian

reports on prevalence of periodontal infections varies; tests are of limited value in its routine diagnosis. Metronidazole was shown to be the most effective antibiotic for these isolates. This agrees with a previous study (27). Metronidazole is the effective treatment for anaerobes.

Majority of the isolates were obtained from patients with chronic periodontitis. This may be due to the purulent nature of this infection which is a major characteristic of anaerobic infection. When the specific pathogens responsible for these infections are defined, it is possible to prevent the infections before they occur or on the other hand treat infected persons without much problem.

Female patients were the most predominant group as seen in this study; however this is not a significant difference and seem to agree with previous findings suggesting that both sexes are predisposed and equally liable to oro-facial infections (28). The prevalent oral infection was chronic periodontitis. The age group mostly infected was between 20-29years, this could be as a result of their adventure into diets and poor oral hygiene practices.

Using PCR method, it was possible to identify and confirm 43 isolates as *Fusobacterium nucleatum, P. gingivalis,* and *Prevotella* species correctly by using species-specific primers because they have been found to be the most predominant periodontal pathogens. There is a possibility that the other species identified by culture but not by PCR may have been other oral species of these anaerobes. Previous study reported the PCR method can detect specific microorganisms, and has been shown to be rapid, reliable, time effective than traditional methods of culturing and identifying anaerobes especially those associated with periodontal disease (29)

The identification of anaerobic species in Nigeria has been improved despite the high cost of materials involved and the tedious nature of its isolation and characterization. A report in Nigeria has shown with PCR technique, identification time is reduced especially when using direct clinical specimen and may alleviate the difficulties encountered during anaerobic cultures and characterization. The specificity of this technique may assist in defining the species and strains common in our environment (26). The use of this molecular method in this study has satisfactorily shown it could be used to identifying anaerobic species in low income countries especially when using direct clinical specimen and this may help to reduce the challenges encountered during anaerobic cultures.

CONCLUSION

The presence of anaerobes from the different periodontal infections shows they can be cause infections in human and this could be attributed to poor oral hygiene practices. Co-infection was mostly seen among the population above 25years. *Prevotella intermedia* and *Fusobacterium nucleatum* were the predominant species of anaerobes found to co infect these young adults. Metronidazole was very effective against the species of anaerobes identified in

this study. The identification of anaerobic species is not practiced in Nigeria because of lack of skills. PCR has been shown to be rapid and sensitive in the identification of anaerobes, it was able characterize the anaerobes at molecular level. It is important for other anaerobic species to be studied and fully identified in order to achieve and administer accurate treatment of oral infections.

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