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AP-PCR and Antimicrobial Susceptibility Patterns of *Fusobacterium nucleatum* Associated with Chronic Periodontitis among Patients at Lagos University Teaching Hospital

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Authors' contributions

This work was carried out in collaboration between all authors. FON designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. AOC and FTO supervised the work. MJA-C managed the analyses of the study. G-JEJr. performed the practical laboratory activities and literature searches. POA, KAU and KOS recruited and examined the patients. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To isolate, identify and evaluate the genetic diversity and antimicrobial susceptibility of *F. nucleatum* recovered from Nigerian patients with chronic periodontitis.
Study Design: Cross-sectional design.

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Place and Duration of Study: Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos, between January 2007 and July 2008.
Methodology: We analyzed *F. nucleatum* species recovered from Nigerian patients with chronic periodontitis. Bacterial identification was done using colonial morphology; Grams stain reaction, conventional biochemical tests, API 20-A and Polymerase chain reaction (PCR). The minimum inhibitory concentration (MIC) of 6 antibiotics was determined by agar dilution method on Brucella blood agar while the bacterial genetic diversity was studied using the Arbitrarily Primed-PCR (AP-PCR) method with the arbitrary primer OPA-05. The inter-relationship and genetic similarity matrix among the isolates was determined and by Numerical taxonomy and multivariate analysis system (NTSYS-pc) statistical package.
Results: We obtained 48 isolates of *F. nucleatum* from 50 Nigerian patients (28 males and 22 females) with chronic periodontitis. They were susceptible to metronidazole, clindamycin, cefoxitin, tetracycline, amoxicillin and clavulanate. One was resistant to amoxicillin (MIC >32 µg/ml) and produced β-lactamase. The isolates were further placed into five groups (A, B, C, D and E) based on their AP-PCR profile.
Conclusion: The AP-PCR analysis showed heterogeneity among strains. By using AP-PCR, we observed a single β-lactamase producing clone resistant to amoxicillin which eventually formed a distinct group showing that such genetic difference may have contributed to the formation of a separate clone.

Keywords: Arbitrarily primed-PCR; anaerobic bacteria; fusobacterium nucleatum; antimicrobial susceptibility; chronic periodontitis.

1. INTRODUCTION

Fusobacterium nucleatum is a gram-negative anaerobic bacterium which is a member of the resident oral microbiota and other mucous membranes of humans and animals. The presence of this microorganism in the oral cavity contributes to the initiation and progression of infections particularly in patients with poor oral hygiene. Although commonly considered to belong to the oral microbiota commensals, *F. nucleatum* species are involved in chronic periodontitis, intrauterine infection and are common isolates in other human disease conditions (Signat et al., 2011, Takenaka et al., 2012, Castellarin et al., 2012).

Periodontitis is a bacterially induced chronic inflammatory disease that destroys the connective tissue and bone of supporting teeth (Chaushu et al., 2012). In Nigeria, the prevalence of periodontal disease with deep pocketing among patients aged 15 years and above range between 15 and 58% (Akpata, 2004; Okeigbemen, 2011). In addition, the involvement of *Fusobacterium* species in oral infections including *cancrum oris* (NOMA); a severe infection frequently found in immune compromised or malnourished children with poor oral hygiene is well documented (Adiola and Obiadazie, 2009; Ogbureke and Ogbureke, 2010). Species of *F. nucleatum* are heterogeneous with known differences in their virulence, binding specifications, nutritional requirements and antibiotic susceptibility pattern and these are key factors in their colonization, pathogenesis and response to antimicrobials (Koeth et al., 2004). The ability to associate these species with a definite periodontal condition may provide a better understanding of their involvements in periodontitis and also assist in epidemiological typing of *F. nucleatum* during infections especially those involving other body sites.

Species of *F. nucleatum* are susceptible to most antibiotics but resistance exists among different strains (Nyfors et al., 2003; Takenaka, et al., 2012). Like other anaerobes, when *Fusobacterium* species are involved in infections, treatments are usually empirical. However, the global concern over emergence of resistance among anaerobic species has increased the demand for susceptibility testing of implicated strains (Ardila et al., 2010; Goldstein et al., 2011). Moreover, variations in the genetic composition of different strains may be responsible for reduced susceptibility or indeed resistance to some antibiotics used in therapy. This is believed to be common in multicultural environments where self-medication is a problem.

Although studies have recognized the presence of *F. nucleatum* in certain disease conditions in Nigerian population, the genetic profiles of strains involved and their susceptibility to antimicrobials used in therapy are yet to be established. It is therefore necessary, to define the different genotypes of these anaerobes in relation to their susceptibility patterns in order to establish epidemiological relationship during infections as well as guidelines towards empirical therapy. Thus, this study was carried out to determine the genetic profile and antimicrobial susceptibility pattern of oral *F. nucleatum* isolated from Nigerian patients with chronic periodontitis attending Lagos University Teaching Hospital.

2. MATERIALS AND METHODS

2.1 Study Design/Patients

This is a cross sectional study. A total of 174 patients were enrolled from the dental clinic of the Lagos University Teaching Hospital (LUTH) out of which 50 patients aged between 17 and 74 years (mean age 44 years ± SD), who had symptoms and signs of chronic periodontitis with probing depth ≥ 5mm on clinical examination, were recruited for the study. The 50 patients selected for microbial sampling were 28 males and 22 females. The patients had not received professional cleaning nor used antibiotic therapy within the three months preceding the study. Those showing odontogenic anomalies such as macrodontia and microdontia, root fractures, systemic diseases especially endocarditis, arthritis, septicemia and arteriosclerosis, or severe acute apical periodontitis were excluded. Patients were initially screened using the Community Periodontal Index of Treatment Needs (CPITN) (Ainamo et al., 1982); a rapid and less invasive index for determining periodontal status of patients. Patients with sextants scoring codes 3 and 4) were initially selected. The Oral hygiene Index was used to assess the oral hygiene status of the patients based on the amount of debris and calculus on 6 index teeth (Greene and Vermillion, 1964). Sites were selected following initial screening and determined by the presence of bleeding on probing (1) and no bleeding on probing (0). An index tooth was probed, using the probe as a “sensing” instrument to determine pocket depth and to detect sub gingival calculus and bleeding response. The sensing force used was no more than 20 grams. The probe tip was inserted gently into the gingival pocket and the depth of insertion read against the colour coding of the probe. The total extent of the pocket was explored. At least 6 points on each tooth were examined: mesio-buccal, mid-buccal, disto-buccal and the corresponding lingual or palatal sites. The Williams probe was then used to determine the specific pocket depth on the tooth sites. Patients having at least two teeth with interproximal attachment loss of 6 millimeters or more and at least one tooth with 5 millimeters or more of pocket depth at interproximal sites were diagnosed with severe chronic periodontitis and used for the microbiological evaluation. All patients were given written informed consent to be recruited for this study, which was approved by the Research and Ethics Committee of the College of Medicine, University of Lagos, Proc. No. CM/COM/8/VOL.XIX and Lagos University

Teaching Hospital (LUTH) Idi-Araba Proc. No. ADM/DCST/221/VOL.10. The remaining 124 patients were used for another set of study.

2.1.1 Collection of samples

Samples were aseptically collected from two non-contiguous periodontal pockets with depth ≥ 5 mm by inserting two sterile paper points (No. 30, UnoDent, England) for 60 s. Each paper point was carefully placed into a separate Dental transport medium (Anaerobe systems, USA) immediately after collection. Approximately 2-5 ml of pus was aseptically aspirated with needle and syringe directly from apical and dentoalveolar abscesses and this was aseptically injected into transport medium. The samples were transported to the laboratory and processed within 2 h of sampling.

2.1.2 Bacterial isolation and identification

The sub gingival samples were vigorously vortexed (30s) and 0.1 ml was streaked on fastidious anaerobe agar (Lab M) and *Fusobacterium* selective agar (Anaerobe Systems, USA). Both media were originally supplemented with 5 µg/ml hemin, 1 µg/ml vitamin K, and 5% of sheep blood. Plates were incubated in anaerobic jars (Merck, Germany) with 90% N_2 plus 10% CO_2, at 37°C for 7 days. Characteristic grayish white granular colonies were sub-cultured on fastidious anaerobe agar to obtain pure cultures. One strain per patient was selected and inoculated in duplicate into 5 ml of brain heart infusion (BHI) incubated for 48 h at 37°Cin anaerobiosis and used for the study. Other isolates obtained were placed in 10% skimmed milk (Oxoid, UK) at -80°C in ultra low freezer for further studies. Bacterial identification was done by conventional biochemical tests (Summanen et al., 1993) and API 20-A system (bioMérieux SA, Mercy-l'Etoile, France) as recommended by the manufacturer. The isolates were maintained in 10% skimmed milk at - 80°C.

2.3 Detection of *F. nucleatum* by PCR

The species were finally confirmed by PCR using species-specific oligonucleotide primer pair FN 5059S (F: 5'- ATT GTG GCT AAA AAT TAT AGTT -3') and FN 5059S (R: 5'- ACC CTC ACT TTG AGG ATT ATA G -3') as described by Avila-Campos et al. (1999).

2.4 Antimicrobial Susceptibility Testing

In this study, strains from patients were subjected to antimicrobial susceptibility testing according to the method recommended by Clinical Laboratory Standard Institute (CLSI, 2007). Antimicrobial agents used were as follows: cefoxitin (Merck Inc., West Point, PA, USA); clindamycin and metronidazole (RodhiaFarma Ltd, Sao Paulo, SP, Brazil); tetracycline (Forchemicals Ltd., Sao Paulo, SP, Brazil); amoxicillin and clavulanate (Glaxo SmithKline, Philadelphia, PA, USA). Reference strains *Bacteroides fragilis* ATCC 25285 was included as control. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the antibiotic that yielded no bacterial growth.

2.5 Beta-lactamase Production

Amoxicillin-resistant strains were evaluated to verify β-lactamase production using Beta-lactamase Identification Sticks (Oxoid Ltd., Basingstoke, Hampshire, England). A β-lactamase producing strain; *Bacteroides fragilis* ATCC 43858 was used as control.

2.6 Genetic Diversity Using AP-PCR Method

Amplifications of the extracted DNA were carried out using final volumes of 25 µl, containing 1X PCR buffer, 50 mM $MgCl_2$, 0.2 mMdNTP, 0.4 µM OPA-05 primers, 0.5 U Platinum *Taq* DNA polymerase (Invitrogen Ltd, Sao Paulo, SP, Brazil) and 5 ng DNA. The 10-base random oligonucleotide primer OPA-05 (5'-AGG GGT CTTG-3') (Operon Technologies, Inc., Alameda, CA) was used as described by Hsuch et al. (2002).

2.7 Data Collection and Statistical Analysis

Each DNA fragment generated was treated as a separate character and scored as a discrete variable using 1 to indicate presence and 0 for absence. A rectangular binary data matrix was obtained and statistical analysis was performed using the Numerical taxonomy and multivariate analysis system NTSYS-pc statistical package (Rohlf, 1993). A pair wise similarity matrix was generated by means of simple matching coefficient and unweighted pair-group method using arithmetic average. Cluster analysis was performed to develop a dendogram and a batch mode of NYSYS-pc was used to show the inter-relationship and genetic similarity matrix among the strains. An index of discrimination (D) based on Simpson's index of diversity was used to determine the discriminating power of AP-PCR in sub typing local isolates of *F. nucleatum* according to the formula described by Hunter and Gaston in 1988.

3. RESULTS AND DISCUSSION

High prevalence of pathogenic gram negative anaerobes especially *Porphyromonas gingivalis*, *Prevotella intermedia*, *F. nucleatum* and *Bacteroides* species are observed in patients with periodontal infections than in those with healthy periodontium (Mane et al., 2009; Signat et al., 2011). Only the presence of *F. nucleatum* strains was determined, because it is the most important fusobacteria in colonization and development of periodontal disease (Mane et al., 2009). Any effort to control their proliferation may likely limit the rate and intensity of periodontal infections (Akpata, 2004). A total of 174 patients were enrolled for the study, out of which, 50 patients had symptoms and clinical signs of chronic anaerobic infection, on clinical examination and thus were selected. Of the 50 patients evaluated for microbial studies, 48(96%) isolates of *F. nucleatum* were isolated one from each patient and identified on the basis of their unique spindle shape with tapered ends, indole reaction, susceptibility to kanamycin and colistin and resistance to vancomycin. Two samples did not yield any growth of *F. nucleatum* both in fastidious anaerobe agar and *Fusobacterium* selective agar. According to this microbial evaluation, most patients with chronic periodontitis harbored *F. nucleatum*.

The genetic variation among the isolates obtained was determined by random amplified polymorphic DNA (RAPD) patterns generated by AP-PCR. Fig. 1a and 1b showed the different bands produced by the isolates using AP-PCR. Some of the isolates produced none or very few bands. Out of the 48 isolates studied, only 13 (27.1%) were typeable with OPA-5 primer while the remaining 35 (72.9%) were non typeable. The primer produced amplification of 0.5 kb and 6.280 kb bands (Figs. 1a & b).

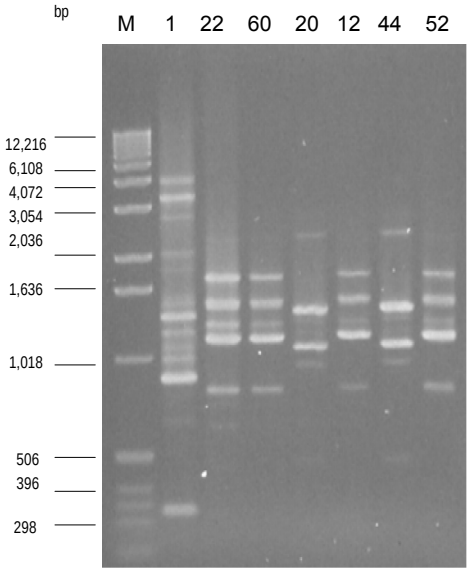

Fig. 1a. AP-PCR profile of *F. nucleatum* species using oligonucleotide primer OPA-05. Lane M; DNA marker; lane 1; *F. nucleatum* ATCC 10953. Lanes 22, 60, 20, 12, 44 are isolates of *F. nucleatum*.

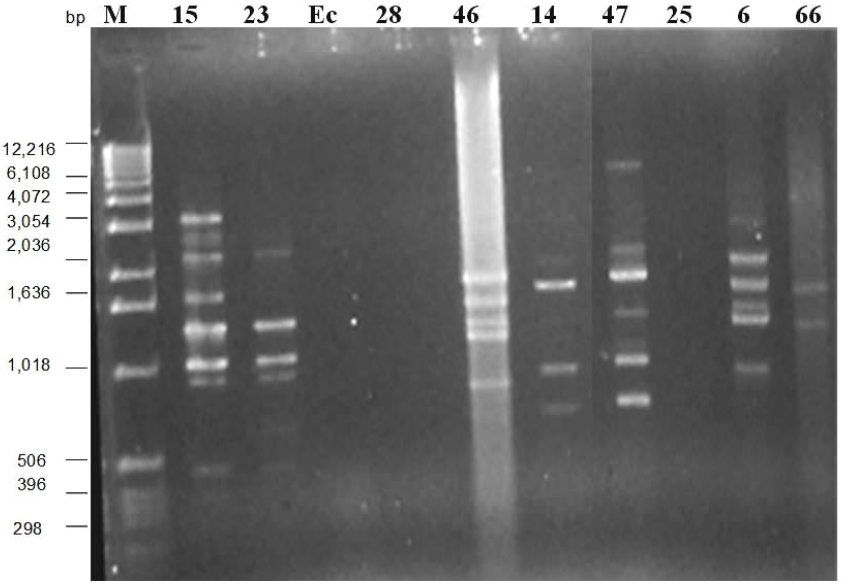

Fig.1b. Lane M; DNA marker, Lane Ec; *Escherichia coli* J53 pACYC 184 (ECOLI), lanes 15, 23, 28, 46, 14, 47, 25, 6, 66 are *F. nucleatum* isolates.

The distance between the different bands produced were analyzed using numerical taxonomy and multivariate system software (NTSYS). The numerical index of discrimination of this method was high (D = 0.977) but degree of typeabilty was low (27%). All amplified

and typeable isolates had 80% similarities. By using the OPA-05 primer five groups A (I), B (II), C (III), D (IV) and E (VI) were found (Fig. 2). Group A had two subgroups with 90% similarities. Subgroup 1 showed four identical isolates representing one clone, while subgroup 2 harbored only one isolate. Group B and C had only one isolate each which was obtained from a female patient of 70 years and a male patient of 25 years respectively. Group D was divided into subgroups 1 and 2 with 90% similarities. Subgroup 1 showed 98% similarities and the isolates were genotyped into three separate clones, while subgroup 2 showed two similar isolates which belong to the same clone. Group E had one isolate (FN 15) which was obtained from a 57 years old female patient, and this was the only amoxicillin-resistant isolate.

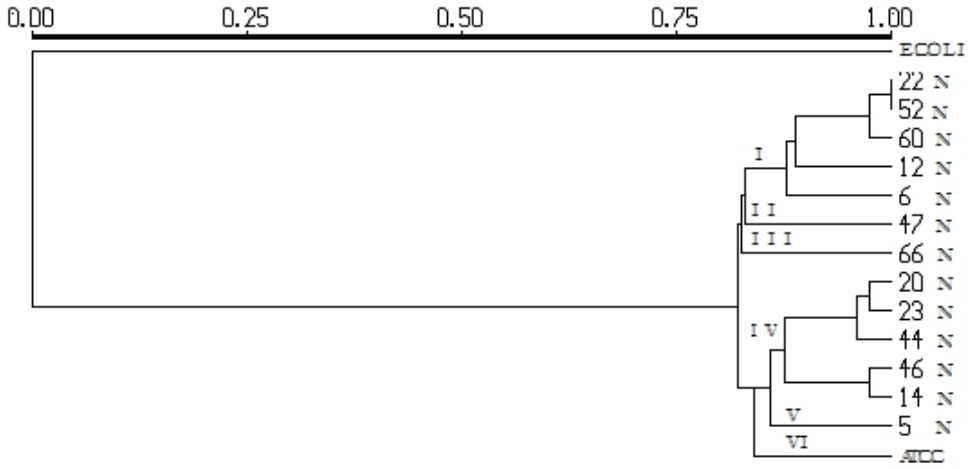

Fig. 2. Genotypic analysis of the *F. nucleatum* isolates obtained from Nigerian patients (N) with chronic periodontitis. *E. coli* J53 pACYC 184 (ECOLI); *F. nucleatum* ATCC 10953 (ATCC). The isolates were genotyped into 5 groups A, B, C, D and E

Bacterial clone vary considerably not only on type of infection or specimen collected, but also on the isolates, species, strains and sites studied. Different strains of *F. nucleatum* can be found in healthy periodontium and the number tends to increase in diseased conditions especially in patients with chronic periodontitis (Signat et al., 2011). In this study, a standard stain *F. nucleatum* ATCC 10953 and one isolate from each patient isolated from two non-contiguous periodontal sites and fully identified as *F. nucleatum* were selected based on diversity of epidemiologic source and studied. This is because in this study, if more isolates from same patient were analyzed, genetically identical microorganisms may have been tested, producing non-reliable results. Further studies following this pilot analysis will look at typing strains from other sources in Nigerian population.

The use of molecular methods especially PCR have greatly improved the identification of anaerobic species of clinical importance. In this study, Gram negative reaction, unique spindle shaped morphology with pointed ends characteristic of *F. nucleatum* spp. was used for the presumptive identification of the isolates in addition to indole reaction. However, the identity of the isolates was confirmed by PCR using a set of primer specific to *F. nucleatum* species prior to genotyping. Typing of bacteria cells is useful in differentiating heterogeneous strains from isolates mainly to evaluate epidemiological relationships. In this study, the AP-PCR was able to differentiate the isolates into five distinct groups (A, B, C, D, E) confirming the broad genetic difference that exits among the different clones of *F. nucleatum.*

Retrospective studies have correlated clinical therapeutic failures with antibiotic resistance (Brook, 2006). Patterns of antimicrobial resistance in fusobacteria are quite variable around the world. This phenomenon may partly be related to the genetic diversity within the species. In respect to this, our study evaluated the genetic variations of isolates obtained in relation to their susceptibility pattern to antibiotics administered to patients who present with periodontal infections. All the isolates were susceptible to cefoxitin, clavulanic acid, clindamycin, metronidazole and tetracycline. This agreed with the findings of some authors (Mosca et al., 2007; Jacinto et al., 2008) but different from others (Serrano et al., 2009; Gomes et al., 2011; Takenaka et al., 2012). However, one isolate (FN 15) was resistant to amoxicillin (MIC > 32 µg/ml) with beta lactamase production. According to our analysis this was the only isolate in Group E. Showing that genetic variations should not be overlooked when considering the antibiotic susceptibilitily pattern of *F. nucleatum* species.

This method showed a great polymorphism among the *F. nucleatum* species from which five genetic groups were established. Although some of the isolates were genotypically identical, clonal differences among species were observed and it may be explained by the fact that the patients were not epidemiologically linked. However, one can suggest that these clones may be associated with chronic periodontitis. There is a need for further verification. Different clonal types have been defined among *F. nucleatum* species. Moraes et al. (2003) reported four clonal types obtained from patients with root canal infections. Similarly, Haraldsson et al. (2004) detected 7 AP-PCR types in a single salivary sample. Two non-contiguous periodontal sites were evaluated (Wara-aswapati et al., 2007; Mane et al., 2009). Positive cultures of *F. nucleatum* were obtained from majority of the patients. Although it appears that our selection of a single isolate from each patient did not affect the degree of isolation since almost all the samples gave a positive culture and five distinct genetic groups, it may have limited the number of groups formed. Therefore, there is a future need to carry out a comparative study that would look at the genetic profile of periodontal strains from several periodontal sites and probing depths.

Furthermore, different commercial OPA primers have been used to study bacterial cells but not every arbitrary primer produced useful banding pattern following amplification (Bidet et al., 2001). We decided to use OPA-05 based on high discriminating capacity observed in a previous study (Hsuch et al., 2002) and within the limit of our available resources. Based on the Simpson's index of diversity (Hunter et al., 1988) this primer was quite rapid and discriminatory but showed low level of type-ability because visible bands were observed in only 27.1% (13/48) of isolates studied. The unamplified ones showing no visible bands were not typeable by OPA-05 primers. We achieved good discrimination with just one primer but irrespective of our proper standardization of the PCR conditions, we were faced with lack of reproducibility described as the major challenge of AP-PCR technique (Bidet et al., 2001). This may have greatly increased the percentage of non type-able strains observed supporting the need to evaluate the strains with other AP-PCR primers with high type-ability (Moraes et al., 2003). In addition, other typing methods such as PCR-Ribotyping, Pulsed-Field Gel Electrophoresis, Restriction enzyme analysis among others may be used to study these species and determine the most convenient and alternative typing technique.

4. CONCLUSION

The AP-PCR analysis showed heterogeneity among strains. By using AP-PCR, we observed a single β-lactamase producing clone resistant to amoxicillin which eventually formed a distinct group showing that such genetic difference may have contributed to the formation of

a separate clone. To the best of our knowledge, this is the first report on the genetic profile in oral *F. nucleatum* isolated from Nigerian patients.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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