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## *ORIGINAL ARTICLE*

# Isolation and Molecular Identification of *Fusobacterium nucleatum* from Nigerian Patients with Oro-facial Infections

## *L'isolement et l'identification moléculaire de Fusobacterium nucleatum de patients nigérians présentant une infection bucco-faciale*

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**ABSTRACT**

BACKGROUND*: Fusobacterium nucleatum* is one of the most common anaerobic bacteria present in the oral cavity and is often isolated from infections involving other body sites.

OBJECTIVE: To characterise *F. nucleatum* strains from patients attending a teaching hospital in Nigeria in order to provide information on the methods for accurate identification of anaerobes in clinical specimen.

METHODS: *Fusobacterium nucleatum* specie from 50 patients presenting with oro-facial infections were studied by culture on *Fusobacterium* selective agar and fastidious anaerobe agar. The isolates were characterised based on colonial morphology, microscopy, lipase production, susceptibility to kanamycin and colistin and resistance to vancomycin. Biochemical tests were performed using a commercial test kit. The identity of the isolates was confirmed based on molecular characterization performed using polymerase chain reaction (PCR) analysis.

RESULTS: **Forty-eight (96%)** *F. nucleatum* isolates were obtained from the 50 patients by culture and all the isolates were identified by colonial appearance and microscopy based on their unique spindle shape with tapered ends. Only 26 (54.2%) of the 48 isolates were identified by commercial API 20A test kit while PCR confirmed the identity of all the isolates.

CONCLUSION: **Anaerobes are involved in human infections and their study is quite cumbersome due to tedious nature and high cost of the techniques involved. Cultural method is reliable in the isolation and identification of *F. nucleatum* species. PCR is a rapid and simple method that can complement the phenotypic identification of anaerobes and would assist in their full identification. WAJM 2011; 30(2): 125–129.**

Keywords: **Anaerobes,** *Fusobacterium nucleatum,* **Oro-facial infections, Polymerase chain reaction.**

*RÉSUMÉ*

**CONTEXTE:** Fusobacterium nucleatum est l'une des bactéries les plus courantes des anaérobies présentes dans la cavité buccale et est souvent isolé des infections de sites d'autres corps.

**OBJECTIF:** Caractériser les souches de F. nucleatum chez de patients fréquentant un hôpital d'enseignement au Nigéria afin de fournir des informations sur les méthodes d'identification précise des bactéries anaérobies dans un échantillon clinique.

**METHODES:** Fusobacterium nucleatum de 50 patients présentant des infections oro-faciales a été étudié par la culture sur agar sélectif et Fusobacterium anaérobie fastidieux. Les isolats ont été caractérisés en fonction de la morphologie coloniale, la microscopie, la production de lipase, la sensibilité à la kanamycine et colistine et la résistance à la vancomycine. Les tests biochimiques ont été réalisés en utilisant une trousse commerciale. L'identité des isolats a été confirmée sur la base caractérisation moléculaire effectuée en utilisant la réaction en chaîne par polymérase (PCR).

**RÉSULTATS:** Quarante-huit (96%) isolats de F. nucleatum ont été obtenus à partir des 50 patients par la culture et tous les isolats ont été identifiés par l'aspect colonial et la microscopie en fonction de leur forme en fuseau unique aux extrémités effilées. Seulement 26 (54,2%) des 48 isolats ont été identifiés par le test du kit commercial API 20A. La confirmation de l'identité a été faite par PCR pour tous les isolats.

**CONCLUSION:** Les anaérobies sont impliqués dans les infections humaines et leur étude est assez difficile en raison de la nature pénible et du coût élevé des techniques concernées. La méthode de culture est fiable dans l'isolement et l'identification des espèces F. nucleatum. La PCR est une méthode simple et rapide qui peut compléter l'identification phénotypique des bactéries anaérobies et aiderait dans leur identification complète. **WAJM 2011; 30(2): 125–129.**

*Mots-clés:* Anaérobies, Fusobacterium nucleatum, Infections oro-faciales, la réaction en chaîne par polymérase.

## INTRODUCTION

The genus *Fusobacterium* is a heterogenous group of anaerobic bacteria implicated in several human and animal diseases and *F. nucleatum* is considered to be one of the potential pathogens.[1] This dominant species of the 500 or more organisms that co-exist in the oral cavity[2–5] are among the first anaerobic species to colonize the mouths of infants and considered a key species in the building and development of complex dental biofilms.[6] The unusual length (5 to 10μm long), adhesive nature and other cell surface properties possessed by these species make it possible for them to coaggregate and promote the survival of periodontal disease-causing bacteria such as *Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter actinomycetomcomitans, Treponema denticola* and *Streptococcus* species.[7] They are numerously present in most patients with poor oral hygiene which supports their involvement in chronic oro-facial infections.

Oral infections, especially periodontitis, occurs worldwide.[8–11] It is the major cause of dental loss in the adult population and is considered a serious health problem globally. In Nigeria, *Fusobacterium* species have been recovered from patients with noma (Canrum oris),[12] oro-facial infections,[13] dentoaveolar abscess[14] and from oral cavities of malnourished children.[15] Activity of *F. nucleatum* in infections of extra-oral sites is well documented, having been isolated from patients with systemic infections such as bacteraemia, brain and liver abscesses, urinary tract infection, and preterm delivery with low birth weight.[16–18]

Unlike the aerobic species, cultivation of anaerobes requires defined media incubated in the absence of oxygen for seven to 14 days which is a long time when considering the clinical condition of a patient and the need for fast laboratory results. The introduction of rapid methods of identification for anaerobes using commercial kits, is well recognized.[19] Other identification methods such as fatty acid methyl analysis,[20] DNA–DNA hybridization,[21] composition of the peptidoglycan layer[22] and glutamate dehydrogenase electrophoretic pattern[23] are available. However, these techniques are associated with set backs such as the high cost of obtaining the equipment, tedious nature of the laboratory procedures and inconsistent results. Introduction of molecular methods and genetic sequencing has contributed to the recent improvement in the identification of anaerobes especially direct from clinical specimens.[24]

Irrespective of the established procedures and results obtained from other continents, there is little information on the isolation and characterisation of *F. nucleatum* in Africa because it is difficult and time-consuming to cultivate, coupled with high cost of materials. This report details microbiological and molecular identification of *F. nucleatum* from oro-facial infections.

## SUBJECTS, MATERIALS, AND METHODS

**Study Site and Patients:** A total of 174 patients were enrolled from dental clinic of Lagos University Teaching Hospital (LUTH) out of which 50 patients with ages between 17–74 years (mean age 44 years), who had symptoms and signs suggestive of anaerobic infection, on clinical examination were recruited for the study. Inclusion criteria included willingness to give a written informed consent, and the patient had not received professional cleaning nor used an antibiotic within the three months preceding the study.

Specimens were collected from patients showing radiographic evidence of bone loss, clinical characteristics of periodontal inflammation (bleeding on probing) or chronic discharge. The clinical conditions considered for the study included acute periodontitis, chronic periodontitis, juvenile periodontitis, chronic gingivitis, chronic pulpitis, dental abscess, dental caries, periapical abscesses and root canal infections.

**Sample Collection:** Sub-gingival biofilm was collected aseptically by inserting two sterile paper points (No. 30, UnoDent, England), into the periodontal pockets with depth $\geq 5$ mm for 60s and placed into Dental transport medium (Anaerobe systems, USA). Approximately 2–5 ml of pus was aseptically aspirated with a needle and syringe directly from apical and dentoalveolar abscesses. All specimens were transported in transport media and processed within 2 h of sampling.

**Bacterial Isolation and Identification:** Samples were vortexed for 30s and streaked on *Fusobacterium* selective agar (FSA) (Anaerobe Systems, USA) 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°C for 7 days in an anaerobic jar (Merck KGaA, Germany) containing 90% $N_2$ and 10% $CO_2$ generated by sachets of gas generating kit (Merck, Germany) and checked visually in accordance with manufacturers. The isolates were gram stained and sub-cultured on FAA. The growth on the plate was examined macroscopically and each white or gray speckled colony that were dry, irregular, crumb-like circular with an entire edge measuring 0.5–2 mm in diameter, were Gram stained and sub-cultured onto fastidious anaerobic agar (Lab M). Gram-negative, spindle shaped isolates with pointed ends were presumed to be *F. nucleatum*. Antibiotic disc susceptibility tests for colistin (10μg), kanamycin (1000μg) and vancomycin (5μg) 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°C for 7 days in an anaerobic jar (Merck KGaA, Germany) and checked visually for presence or absence of inhibition zones.

**Biochemical Tests:** Biochemical tests were performed using API 20A (bioMérieux SA, Mercy-l'Etoile, France) according to the manufacturer's instructions. Indole reaction was performed on both API 20A and Indole/nitrate reagent (Anaerobe systems). Lipase production and lecitinase activities were studied using egg yolk agar (Anaerobe Systems, USA). The identified isolates were stored in 10% skimmed milk at –80°C.

**Bacterial Identification by Polymerase Chain Reaction:** Conventional PCR assay was conducted to confirm phenotypic identification of the isolates as *F. nucleatum*. 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.* [25]

**DNA Extraction:** Chromosomal DNA was extracted from 48 h growth of *F. nucleatum* in Brain Heath infusion (BHI) broth (5ml) harvested by centrifugation at 14, 000 x g for 10 min. The cells were washed three times in one ml of ultra pure water by centrifuging at 12,000 x g for 5 min before DNA extraction and purified using QIAamp DNA mini kit (Qiagen, Hilden, Germany). The resulting filtrate (DNA) was used as a template for PCR assay.

**DNA Amplification**: Amplification of DNA was performed using species-specific primer pairs: FN 5059S (5′ ATT GTG GCT AAA AAT TAT AGTT 3′) and FN 5059S (5′ ACC CTC ACT TTG AGG ATT ATA G 3′)[25] carried out using a final volume of 25μl, with the reaction mix containing sterile water, 1 X PCR buffer, 2.5 mM MgCl$_2$ 0.2 mMol dNTP, each 0.4 μM of primer, 0.5 U *Taq* DNA polymerase and 5 ng of DNA. Amplification was programmed to run following cycling conditions of initial denaturing at 94°C for 5 min; 35 cycles of denaturing at 94°C for 1 min, annealing at 40°C for 2 min, extension at 72°C for 2 min, followed by final extension at 72°C for 10 min and the reactions were carried out in a Thermocycler Gene Amp PCR system 9700. A reference strain *F. nucleatum* ATCC 10953 was used as positive control and PCR mix without a DNA template was included as a negative control.

Amplification products were analyzed by 1% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 2 mM EDTA [Ph 8.3]) performed at 70 V for 2.5 h. Gels were stained with 0.5μg/ml of ethidium bromide for 45 min, examined under ultra-violet (UV) transilluminator and photographed using a Digital Kodak Science 120 system. A DNA ladder of 100 bp (Invitrogen, São Paulo, SP, Brazil) was used as molecular weight marker.

**Statistical Analysis**

Statistical analyses were performed using statistical package for social sciences SPSS software version 13.0 to compare the percentage efficacy of identification by culture, rapid method and PCR using a bar chart. Demographic data are presented as frequency distribution and percentage of respondents and orofacial conditions.

**RESULTS**

A total of 50 patients were studied with an age range of 17–74 years. The patients were 28(56%) males and 22 (44%) females (mean age 44 years). Nine clinical conditions were diagnosed, viz acute periodontitis 5 (10.0%) chronic periodontitis 30 (60.0%), juvenile periodontitis 1 (2.0%), chronic gingivitis 2 (4.0%), chronic pulpitis 1 (2.0%) dental abscess 3 (6.0%) dental caries 3(6.0%), periapical abscesses 2 (4.0%) and root canal infection 3 (2.0%) as shown in Table 1. Forty-eight (96%) *F. nucleatum* isolates were obtained from the 50 patients by culture and all the isolates (100%) were identified as *F. nucleatum* species by microscopy based on their unique spindle shape with tapered ends.

The isolates were lipase negative, susceptibile to kanamycin and colistin but resistant to vancomycin, Only 26 (54.2%) of the 48 isolates were identified by API 20A (bioMérieux SA, Mercy-l'Etoile, France) mostly by positive indole reaction. In addition 25 isolates (52.08%) produced indole on indole/nitrate reagent (Anaerobe Systems, USA). All 48 isolates were correctly identified and confirmed as *F. nucleatum* species with the presence of a single band of approximately 502 bp by PCR (Figure 2) using species-specific primers.

**DISCUSSION**

The involvement of anaerobes in human infections has generated global interest in recent times. However, there is the absence in literature on the study of these bacterial species in Africa. The significance of *F. nucleatum* 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 its ability to aggregate with other periodontal pathogens in oro-facial infections of microbial etiology. The presence of *F. nucleatum* in Nigerian population suggests its role in the aetiology of orofacial infections. Chronic periodontitis is an oral infection caused by the 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. 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. This study demonstrated that male patients were predominant though this was not statistically significant. However, a similar number of male and female patients had chronic periodontitis which was the most prevalent clinical condition recoded. This was similar to previous findings,[13] suggesting that both sexes are equally liable to oro-facial infections.

The Nigerian reports on prevalence of dental caries vary considerably;

**Table 1: Distribution of Oro-facial Infections by Sex**

| Clinical Condition | Number (%) | | |
| --- | --- | --- | --- |
| | **Male** | **Female** | **All** |
| Acute periodontitis | 3(6) | 2(4) | 5(10.0) |
| Chronic periodontitis | 15(30) | 15(30) | 30(60.0) |
| Chronic gingivitis | 0 | 2(4) | 2(4.0) |
| Chronic pulpitis | 0 | 1(2) | 1(2.0) |
| Dental abscess | 2(4) | 1(2) | 3(6.0) |
| Dental caries | 2(4) | 1(2) | 3(6.0) |
| Juvenile periodontitis | 1(2) | 0 | 1(2.0) |
| Peri-apical abscess | 2(4) | 0 | 2(4.0) |
| Root canal infection | 3(6) | 0 | 3(6.0) |
| **Total** | **28(56.0)** | **22(44.0)** | **50(100.0)** |

# PRINTER, SEE FIG. 1

**Fig. 1: Agarose gel containing representative amplicons of *Fusobacterium nucleatum* specific primers. Lane M, molecular size marker (100bp DNA ladder); Lane –ve, negative control lane; lane +ve, *F. nucleatum* subsp. *polymorphum* ATCC 10953; Lanes 1–4, test strains of *Fusobacterium nucleatum* isolates.**
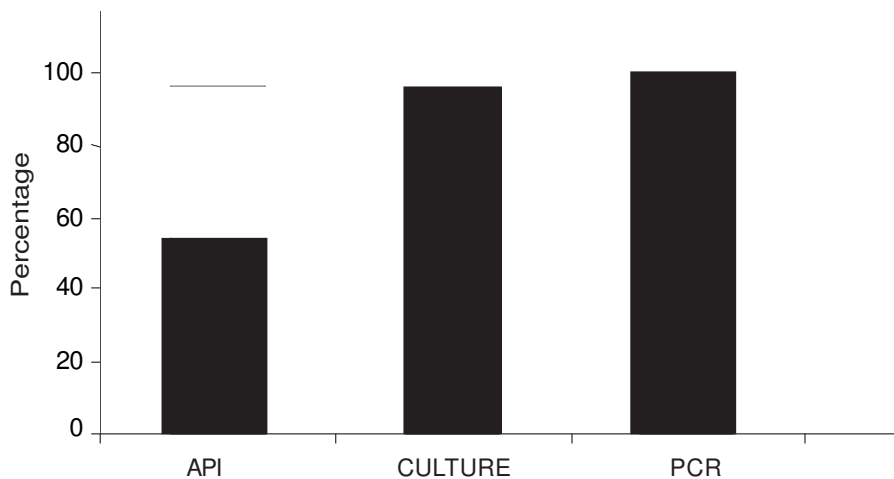


**Fig. 2: Isolation and identification of *F. nucleatum* by conventional, rapid and molecular methods.** API, Analytical Profile Index; PCR, Polymerase Chain Reaction.

however, most results show higher prevalence in children aged between 12–15 years.[26] In this study, a low prevalence of dental caries was observed and this was not unexpected because specimens were collected from patients aged 17 years and above with focus on anaerobic pathogens.

*Fusobacterium* is a member of the resident microbiota which are very fastidious. We isolated 48 (96.0%) *F. nucleatum* species from 50 patients using cultural methods at an average of one isolate from each patient. The use of fastidious anaerobe agar (FAA) greatly improves the recovery of *Fusobacterium* species from clinical specimens, which was also observed by Falkler *et al.* (2000) who used FAA and *Fusobacterium*

selective agar (FSA).[15] The characteristic spindle shape of *F. nucleatum* on gram stain was a reliable feature in the identification of *F. nucleatum* though this is not the case for all *Fusobacterium* spp, some of which (like *F. necrophorum)* are pleomorphic without an obvious spindle shape.[27]

A transport medium with a low redox potential that provide the anaerobic environment with the purpose of maintaining the viability of the isolates is essential to the isolation of anaerobes.[28] We used the dental transport medium which supported the survival of *F. nucleatum* for up to three days before culture.[28]

*Fusobacterium nucleatum* is weakly fermentative and biochemical

tests are of limited value in its routine identification. This was highlighted by the problems of identification encountered with the use of the API 20A which identified only 54.2% of the isolates. A similar finding was previously reported by Murray and co-workers.[29] Indole reaction is the major significant biochemical tests for *F. nucleatum.* In place of using API kit, the indole/nitrate medium which also detects indole production and is more economical may be used.

By using PCR method, it was possible to identify all the isolates. As previously reported, PCR can detect specific microorganisms, and has been shown to be rapid, reliable, sensitive and less cumbersome than traditional methods of culturing and identifying anaerobes especially those associated with periodontal disease.[30] Although this method was specific in identifying the isolates it can be used directly on clinical specimens.

The identification of *F. nucleatum* and other anaerobic species is relatively difficult in Nigeria due to the high cost of materials and the tedious nature of its isolation and characterization. All the isolates obtained through culture were correctly identified as *F. nucleatum* by PCR. With the PCR technique, identification time is reduced especially when using direct clinical specimen and this may alleviate the difficulties encountered during anaerobic cultures and characterization. In addition, the specificity of this technique will assist in defining the species and strains common in our environment.

Oro-facial infections are polymicrobial in nature and are seen very frequently in Nigerian hospitals.[12–14] Both aerobes and anaerobes are involved. Unfortunately, during clinical diagnosis, laboratory studies are focused on the aerobes because the techniques involved in their identifications are simple, easy and faster. The inability to study anaerobes leads to misdiagnosis, antibiotic resistance and treatment failures. This study has highlighted the phenotypic and molecular method for the identification of anaerobes in our environment. It is important therefore, that other anaerobic species are fully identified in order to foster accurate treatment of infections.

## Conclusion

The isolation of *F. nucleatum* from patients with oro-facial infections suggests their involvement in these infections. Cultural method is reliable in the isolation and identification of *F. nucleatum* species. However, the introduction of molecular methods complements the phenotypic identification of anaerobes and would assist in their full identification during infections in order to provide information for epidemiological purposes and management of anaerobic infections.

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