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Evaluation of *Fusobacterium nucleatum* in Amniotic Fluid of Women with Preterm Labour

Fusobacterium nucleatum in Amniotic Fluid of Women with Preterm Labour

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Abstract

Objective: The effect of *Fusobacterium nucleatum*, an oral commensal, on pregnancy outcomes is of global concern. This study screened for *F. nucleatum* and the *fadA* gene in amniotic fluid obtained from women presenting with preterm labour.

Methods: This is a cross-sectional pilot study. Amniotic fluid was collected from women with preterm labour at Lagos University Teaching Hospital and analysed using conventional polymerase chain reaction (PCR). 16SrRNA species-specific primers were used to assess the presence of *F. nucleatum* species and *fadA* genes for virulence potentials. Questionnaire-based data obtained was analysed using SPSS version 20.0. and amplicon analysis was measured on base pairs.

Results: Forty participants had preterm deliveries between April and August 2018. Of these 34 (85%) were enrolled, out of which 25 amniotic fluid samples (AF) were collected. The mean age of the participants was 30.5 years. Among them, 10 (29.4%) were primigravida, and 24 (70.6%) were multigravida. Six (1.8%) of the women had a history of gestational diabetes, and four (1.2%) had hypertension. Only 4 (1.2%) were aware that bacteria could cause preterm labour. 11 (32%) visited the dentist during the period of their pregnancy. There was no amplification of 16SrRNA or the *fadA* genes in the tested samples.

Conclusions: This study showed that preterm delivery was more common in multigravida pregnant women aged 31-35 years. Although some of the samples were cloudy and purulent, *Fusobacterium nucleatum* species-specific and virulent *fadA* genes were not detected in amniotic fluid obtained from the population of women with preterm labour studied.

Keywords: Anaerobes, *Fusobacterium nucleatum*, Preterm labour, Primigravida, Multigravida, Amniotic fluid, Polymerase chain reaction

Plain English Summary

Microbiological analysis reveals the presence of infectious agents in preterm deliveries. This study used a molecular-based method to analyse the presence of an anaerobe *F. nucleatum*, known for causing preterm delivery in women, and its virulence gene potential. The study did not detect the species of interest but created awareness about the involvement of microbes in preterm delivery. It also highlighted that preterm delivery was seen more in multigravida pregnant women aged 31-35 years.

Background

Preterm delivery is defined as birth before 37 weeks of gestation (1). Studies have shown that

risk factors range from immunological to genetic disorders, endocrine disorders and pre-eclampsia (1, 2, 3). Others are lifestyle and

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behavioural factors, uterine leiomyoma, family history, the interval between pregnancy, and medical, gynaecological and obstetric risk factors (3). According to WHO's key fact (4), an estimated 15 million babies are born preterm annually. Neonatal mortality contributes to under-five mortality, including the contribution of preterm neonatal mortality. The burden exerted by Nigeria as a nation with the second-highest number of neonatal deaths worldwide cannot be overlooked.

Globally, the prevalence of preterm birth ranges from 9.0–12.0%, with Europe, India, China, Nigeria, Bangladesh, and Indonesia accounting for a greater percentage of the burden in that order (5). The prevalence rate associated with preterm delivery in Enugu, Nigeria, is 16.9% (6), 11.8% in Ilorin (7) and 8% in Lagos (8). None of these studies reported microbiological analysis or laboratory-specific diagnostic findings on the contributions of infectious agents to preterm delivery.

Maternal infections (16.6%) account for the second highest risk factor of preterm delivery next to maternal malnutrition (17.5%) in low- and middle-income countries (9). Reported infections are mainly those of HIV, malaria, syphilis, chlamydia, asymptomatic bacteriuria, periodontal infection, bacterial vaginosis and Group B streptococcal colonization. Periodontal diseases are worse during pregnancy due to the presence of hormonal imbalances. A good percentage of women report excessive gum bleeding during pregnancy due to pregnancy-associated gingivitis, and plaque-induced inflammation of the gingiva (10). In Ibadan, Opeodu *et al.* (11) demonstrated women's periodontal condition and treatment needs during pregnancy. The fact that 89.3% of the women had never visited a dentist in their lives and observed conditions showed the need for preventive dental visits. The periodontal pathogens responsible for this undergo haematological translocation from the oral cavity to cause extra-oral infections (12). *Fusobacterium nucleatum* is an oral commensal associated with gingivitis, periodontitis, and oral cancer (13, 14). It is implicated in metastatic infections involving the heart valves (15), liver (16), brain, joints (17), and septic thrombophlebitis in extra-pharyngeal locations (2), and the uterus (14). It is also isolated commonly in intrauterine infections (14).

Periodontitis is caused by bacteria that produce significant amounts of proinflammatory mediators, mainly IL-1, IL-6, PGE2, and TNF- α (18). *F. nucleatum* has the characteristic ability to locate, adhere and rapidly colonize (19, 20) to elude the host's defence mechanisms and to produce substances that directly contribute to

the destruction of tissues (21). They can translocate to placenta sites, or the by-products they produce may reach the placenta and enter the amniotic fluid and fetal circulation, serving to activate inflammatory signalling pathways. This may promote labour activation through the production of placental and chorion–amion prostaglandin E2 (PGE2) (22). Women with preterm birth (PTB) demonstrated significantly increased gingival crevicular fluid (GCF) levels of IL-6 and PGE2 compared with those who had full-term births (23).

Specifically, *F. nucleatum* stimulates arachidonic acid metabolism, a rate-limiting step for prostaglandin synthesis, in the human uterine endometrium. These metabolites are important mediators of inflammation. In addition, *F. nucleatum* harbours the conserved *Fusobacterium Adhesin A (fadA)* that encodes the host colonisation factor. Additionally, it produces phospholipases that play a role in remodelling the stability of lipid membranes, therefore affecting placental and foetal development in pregnancy (24). The *FadA* and N-Utilisation Substances (*nusG*) virulence genes peculiar to pathogenic strains of *Fusobacterium* species have been studied and linked as having major virulent potential (15). Recent investigations have demonstrated that *Fusobacterium nucleatum* colonizes the placenta by binding its Fap2 lectin to the placentally displayed Gal-GalNAc (25).

Several risk factors have been associated with preterm and low-weight births, and these include genitourinary and periodontal infections (26). Another important risk factor is the distinct bacteria communities that are associated with the placental tissues of patients with severe chorioamnionitis. *Fusobacterium* species have been detected in the placenta tissues of women who had severe chorioamnionitis (27, 28, 29). These findings suggest a possible association between bacteria like *Fusobacterium nucleatum* and complications such as miscarriage, intrauterine death, neonatal death, neonatal sepsis, preterm delivery, and premature rupture of membranes (27, 28, 29).

Since the adverse effect of microorganisms on pregnancy outcomes was revealed, this opinion is being supported by several reviews and research (2, 10, 14, 30, 31, 32). However, such information is limited in Nigeria. Looking at the prevalence of oral infection estimated to be 15% - 58% in females of reproductive age and the concern over poor oral health hygiene (11, 33) as well as a high rate of preterm delivery (6, 7, 8), it would not be out of place to investigate the involvement of microbial pathogens in maternal infection that could lead to preterm birth. This research and diagnostic investigation aspect

may have been neglected during antenatal care. Creating awareness and demonstrating the involvement of microbial agents will provide more insight into the need to consider microbial infection of the placenta as a risk factor for preterm birth and therefore guide strategies to mitigate its associated adverse pregnancy outcomes.

Materials and Methods

This is a pilot cross-sectional study of consenting pregnant women with preterm labour and delivery between 28 and 36 weeks gestation from April to August 2018.

Study site

Lagos University Teaching Hospital is located in Mushin, Lagos State, Nigeria. It is one of the largest hospitals in the country and provides maternal care mostly to people in the middle and higher socio-economic classes, with referrals that cut across other socio-economic classes. The Department of Obstetrics and Gynaecology was established in 1967. They provide antenatal care and delivery management for booked patients. It also accepts unbooked patients and treats obstetric and gynaecological emergencies admitted through the Accident and Emergency Unit.

Study procedure and data collection

Pregnant women with preterm labour who gave informed consent for participation were enrolled. Women who had induced preterm delivery, antepartum haemorrhage or those delivered before 28 weeks and after 36 weeks of gestation were excluded. The women's demographic information was obtained and knowledge of oral hygiene was assessed with a staff-assisted structured questionnaire that was administered individually to the participants. This was sectioned into demographics, dental care and history, pregnancy information and assessment of knowledge of preterm labour.

Sample collection

Amniotic fluid samples were collected from the posterior fornix after inserting a sterile Cusco's speculum into the vaginal cavity to visualise the cervix and detect amniotic fluid or during caesarean section using a sterile Pasteur pipette as described by Adeyeba *et al.* (34) and then transported to the laboratory in a sterile universal bottle. There was minimal risk of discomfort, cross-contamination and infection due to the process of collecting amniotic fluid and the strict observance of asepsis throughout this process. Samples that failed to meet the criteria for DNA extraction as a result of heavy

contamination from blood and meconium stains during sampling were not processed further.

Detection of *F. nucleatum* species-specific and *fad* genes by PCR

DNA Extraction

To detect the presence of *F. nucleatum* species-specific and *fad* genes in the amniotic fluid, DNA extraction and purification were done using the Norgen Genomic DNA Isolation Kit (Model 24700, Norgen Biotek, Corp, Canada). Briefly, 150 μ L of Amniotic fluid was transferred to a 1.5 mL microcentrifuge tube followed by the addition of 150 μ L of Digestion Buffer A. Then, 12 μ L of Proteinase K was added to the suspension. This was mixed by gentle vortexing and incubated at 55 °C for 1 hour on a dry bath Incubator (Model 190, Thermo Scientific) then 300 μ L of Buffer SK and 300 μ L of 96 - 100% ethanol were added to the Lysate and the mixture was vortexed. Moreso, 600 μ L of the mixture was thereafter transferred into a spin column in a 2 ml collection tube and centrifuged at 8000 rpm for 3 minutes. The flow through in the collection tube was discarded and reassembled and the spinning was repeated for the remaining Lysate. Furthermore, 500 μ L of Wash Solution A was added and the mixture was centrifuged at 14000 rpm for 1 min. The flow-through was discarded and the column was reassembled. Then 500 μ L of Wash Solution A was added again and centrifuged at 14000 rpm for 2min. The extracted DNA was purified by placing the Norgen spin collection column into a sterile 1.7 mL Elution tube and the collection tube containing the filtrate was discarded. 200 μ L of Elution Buffer B was added to the centre of the resin bed and then centrifuged at 6000 rpm for 1min and at 14000 rpm for an additional 2min to collect the total elution volume (DNA). The quality of the extracted DNA was analysed using 1.5% agarose gel electrophoresis in Tris-acetate EDTA (TAE) buffer (Containing 40 mM Tris-acetate, 2 mM EDTA (Ph 8.3) performed at 70 V for 2.5 hours using 100 bp DNA ladder (Solis BioDyne 07-11-00050) as standard molecular weight marker. Before then, the DNA was stained with 6X loading dye (Thermo Scientific) for visual tracking of DNA migration during electrophoresis before loading the mixture into the gel tank. Gels were stained with 0.5 μ g/ml of ethidium bromide for 45 minutes and were stained with water for 20 minutes. Stained gels were examined and photographed using a Digital Photo documentation System (Clinix, 1000).

DNA amplification

This was performed using *F. nucleatum* primers of 16S rRNA-F (5' -AGA GTT TGA TCC TGG CTC AG -3') and 16S rRNA-R (5'-GTC ATC

GTG CAC ACA GAA TTG CTG-3') to amplify a 360-bp region of the 16S rRNA gene (35) The presence of virulence *fadA* gene was detected using the primer pair *fadA*-F (5' -CAC AAG CTG ACG CTG CTA GA -3') and *fadA* -R (5' -TTA CCA GCT CTT AAA GCT TG -3' 9) to amplify a 232-bp region of the *fadA* gene (15) from positive samples of *F. nucleatum*. Amplifications were carried out using final volumes of 20 µL, with the reaction mix containing 12 µL sterile Milli-Q water, 1 X PCR buffer, 7.5 mM of dNTPs, Taq DNA polymerase, 0.16 µmol/L of each primer, and 3 µg of DNA. Amplification was carried out in thermocycler 005 (A& E Lab, UK) programmed to run the following cycling conditions; initial denaturing at 95 °C for 3 min; 30 cycles of denaturing at 95 °C for 30secs, annealing at 53 °C for 1 min, extension at 72 °C for 45 secs, followed by a final extension at 72 °C for 5 min. As a positive control, a reference strain *F. nucleatum* ATCC 10953 and Sterile Water was included as a negative control.

Gel Electrophoresis and visualization

In all PCR assays, 20 µl of the amplified products were separated using 1.5% agarose gel electrophoresis in Tri-acetate EDTA (TAE) buffer (40 mM Tris-acetate, 2 mM EDTA (Ph 8.3) performed at 70 V for 2.5 hours using 100 bp

DNA ladder (Solis BioDyne) as standard molecular weight marker. Gels were stained with 0.5 µg/ml of ethidium bromide for 45 minutes and were stained with water for 20 minutes. Stained gels were examined and photographed using a Digital Photo Documentation System (Clinix Model 1000).

Statistical analysis

The questionnaire was analysed using the Statistical Package for Social Sciences (SPSS) version 20.0. Descriptive statistics using frequencies, cross-tabulations and calculation of mean values for the variables were determined. Amplicon analysis was done by gel visualisation to observe and measure the presence or absence of amplification bands in base pairs.

Results

Out of a total of 40 participants who had preterm deliveries in LUTH from April to August 2018, 34/40 (85%) were enrolled and 25 amniotic fluid samples (AF) were collected (Figure 1). Nine of the 25 samples collected did not meet the criteria for DNA extraction. Thus, 16 samples went through PCR amplification processes and were analysed. Two of the amniotic fluids tested were cloudy and one was offensively purulent.



Figure 1: Gel analysis of amplicon from single plex –PCR. Gel Image showing electrophoresis bands. Lane 1: DNA Standard Marker, Lane (+) Positive control, Lane (-) negative control, Lanes 1-14. sample lanes show no amplification band that corresponds to the expected band sizes of **360 bp** for *F. nucleatum* 16S rRNA.

Table 1 shows the mode of delivery among women with preterm labour. Of the 16 women whose amniotic fluids were collected, 2 were by vaginal collection and 14 were during caesarean sections. The majority of the participants were

between the ages of 31 and 35 years (Table 2), with a mean age of 30.5 years. Ten (29.4%) of the participants were primigravida, and 24 (70.6%) were multigravida.

Table 1: Mode of Delivery among Women with Preterm Labour

Mode of delivery	Eligible (%)	Non-eligible (%)
Vagina	2 (5.88)	9 (26.47)
Caesarean section	14 (41.18)	9 (26.47)

Table 2: Age and Gravidity level of Women presenting with Preterm Labour

VARIABLE	FREQUENCY (n = 34)	Percentage
Age range		
20 – 25	7	20.6
26 - 30	9	26.5
31 – 35	18	52.9
Gravidity		
Primigravida	10	29.4
Multigravida	24	70.6

The overall assessment of the health history and knowledge of these women on causes of preterm labour shows that 6 (17.6%) of the

women had a history of diabetes, 4 (11.8%) had hypertension, and 5 (14.7%) had other pregnancy complications (Table 3).

Table 3: Health History and Knowledge of Women on the Causes of Preterm Labour (n=34)

Medical history and oral health risk factors	Responses (%)	
	Yes	No
The number who had a history of diabetes	6 (17.6)	28 (82.4)
The number who had a history of Hypertension	4 (11.8)	32 (88.2)
The number who had pregnancy complication(s)	5 (14.7)	29 (85.3)
Do you experience any toothache or pain around your gum?	15 (44.1)	19 (55.9)
Did you visit a dentist during this pregnancy?	11(32.4)	23 (67.4)
Do you know that germs (bacteria) in your mouth can cause preterm labour?	4 (11.8)	32 (88.2)

Only 4 (11.8%) were aware that bacteria could cause preterm labour, and 11 (32.4%) of the participants had visited the dentist during pregnancy. Of the 16 samples amplified none

had amplification corresponding to expected band sizes of 360 bp for *F. nucleatum* 16SrRNA (Plate 1) and 232 bp for the *fadA* gene (Table 4).

Table 4: PCR analysis of Amniotic Fluid from women with Preterm Labour at LUTH

S/n	DNA Code	Macroscopy	PCR result	
			<i>FN16S</i> gene	<i>FadA</i> gene
1	K001	Meconium stained	No amplification	No amplification
2	K002	Purulent, Cloudy yellow	"	"
3	K003	Cloudy	"	"
4	K004	Cloudy	"	"
5	K005	Clear pale yellow	"	"
6	K006	Clear pale yellow	"	"
7	K007	Clear pale yellow	"	"
8	K008	Clear	"	"
9	K009	Clear	"	"
10	K010	Clear	"	"
11	K011	Clear	"	"
12	K012	Clear pale yellow	"	"
13	K013	Clear pale yellow	"	"
14	K014	Clear	"	"
15	K015	Clear pale yellow	"	"
16	K016	Clear pale yellow	"	"
17	Positive Control (<i>F. nucleatum</i> ATCC 10953)		Amplification	-
18	Negative Control (PCR mix and Sterile Water)		No amplification	No amplification

Table 4 shows that none of the 16 samples was amplified except for the control.

Discussion:

Preterm birth is a worrisome complication of pregnancy that incurs short to long-term medical

and financial burdens on affected children, their families, and the health care system. For the first time in Lagos University Teaching Hospital, a

study was conducted to assess the contribution of a microbial pathogen, *F. nucleatum*, to preterm delivery. In modern-day laboratory medicine, genetic biomarkers are used on direct clinical specimens to detect the presence of microbial species and their virulent characteristics. The set of oligonucleotide primers for the two targeted genes used showed no amplification, indicating that there was no presence of *F. nucleatum* species in the uterus. However, our finding is at variance with the previous studies by You *et al.* (36) in Korea and that of Vander Haar *et al.* (28) in which *F. nucleatum* was detected in amniotic fluid samples.

None of the 16 amniotic fluids screened had *F. nucleatum* even though some of these samples were purulent and cloudy as opposed to the normal pale, clear yellow appearance of the amniotic fluid which is meant to be sterile. The primer used has been demonstrated to be species-specific for *F. nucleatum*, however, this is not enough to conclude the absence of these species as there are five (5) different subspecies of *F. nucleatum* (five: *F. nucleatum*, *animalis*, *polymorphum*, *fusiforme*, *vincentii*) (37). This has opened up the need to conduct further studies using a wide range of species-specific primers.

To ensure rapid and specific identification of *F. nucleatum* and its novel virulent *FadA* adhesin gene, these invasive samples were processed using a conventional polymerase chain reaction (PCR). This technique detects and characterizes the presence of DNA from both viable and non-viable organisms. Thus, the popular cultural method that supports the isolation and characterization of only the viable bacteria in the clinical sample was not utilized in this study. Moreover, our target species is an anaerobe and anaerobic cultures are known to be quite challenging. In addition, preterm deliveries have been linked to the virulent actions of other microbial species such as *S. sanguinegens*, and, *Gardnerella* sp. which have been detected in addition to *F. nucleatum* in amniotic fluid using more advanced 16S rRNA gene sequencing (36). Thus, species such as *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Gardnerella vaginalis*, and *Bacteroides* (36) need to be examined in future studies exploring both cultural and genetic-based laboratory procedures.

The *FadA* virulence gene is specific to pathogenic strains of *Fusobacterium* species. This gene has been studied and linked as a major virulent potential of these species (5). The species have been detected in the sampling of dental plaque, high vaginal swabs, amniotic fluid, and chorioamniotic tissue. The presence

of *F. nucleatum* in the amniotic fluid from women with caesarean section at full term or placenta tissues has been demonstrated using Polymerase Chain Reaction (PCR) and sequencing-based technique (26, 27, 28). Other genes are coding for virulence other than *Fusobacterium* adhesin A (*fadA*). For example, the adhesin molecules (virulence) have been identified as Fatty Acid Binding Protein 2 (*Fap2*) (2, 25). Primer specific for this gene can also be used to evaluate the presence of an *F. nucleatum* virulence gene. We hope to utilize these primers in our future study. In addition, the small sample size collected and analysed might have contributed to the result. It is therefore important for future studies to examine a larger population, especially of women from other hospital environments in Nigeria as species of interest may be observed.

Age is an important risk factor for many adverse events in pregnancy (38). The study observed that most of the participants were in the age range of 31 - 35 years. This seems to be in line with the study of Mokuolu *et al.* in 2010 (7) who from their study on prevalence and determinants of pre-term deliveries in Nigeria showed that this pregnancy complication is common among women aged 20 – 34 years. Most of the pregnant women presenting with preterm labour in this study were multigravida. This is also unexpected as pregnancy complication such as preterm delivery has not been strongly linked with many previous pregnancies and deliveries (9, 39, 40). This was further corroborated by our findings; however, the absence of a link may have been due to the small sample size in our study.

Study limitations

There were a few limitations in this study. The study assayed a small population and thus may not be used to make any statistical inference. There is a need to conduct a future study that involves a larger population of women with preterm labour to comprehensively establish if there is the involvement of microbial pathogens in preterm delivery. The sites of amniotic fluid sample collection (vaginal versus abdominal) could potentially create some differences in the yield and characteristics of the results obtained in our study. However, very strict aseptic techniques were ensured during the procedures regardless of the site of sample collection. The study did not include the evaluation of the oral health status and microbial evaluation to study the occurrence of the target species in the oral cavity. There are about five subspecies of *Fusobacterium nucleatum*. The study used one primer for *F. nucleatum* species and virulence primers. It would be good to conduct future

research using more species, subspecies and virulence-specific primers.

Conclusion

F. nucleatum species-specific and virulent *fadA* genes were not detected in amniotic fluid samples of pregnant women presenting with preterm delivery in LUTH. The absence of the oral commensal suggests the need for future studies to utilize more samples as well as multiplex primers capable of detecting more species of *Fusobacterium* and other commonly implicated pathogens in preterm delivery in Nigerian women living in the urban city of Lagos.

List of Abbreviations

DNA: Deoxyribonucleic acid
EDTA: Ethylenediaminetetraacetic Acid
fadA: Fusobacterium adhesion A
HIV: Human Immunodeficiency Virus
LUTH: Lagos University Teaching Hospital
PCR: Polymerase Chain Reaction
PGE2: Prostaglandin E2
PTB: Preterm Birth
SPSS: Statistical Package for Social Sciences
WHO: World Health Organization

Declarations

Ethics approval and consent to participate

Ethics approval and consent to participate Responsible conduct of research was employed- during the study by obtaining Ethical approval with Reference no: ADM/DCST/HREC/APP/2289 from the Health Research Ethics Committee, Lagos University Teaching Hospital, before embarking on the research. To obtain consent and ensure ethical research the study was explained to the participants in the language they understood. They were assured that participation was free and that they could withdraw from the study whenever they wanted. Neither would their withdrawal interfere with their treatment.

Consent for publication

All the authors gave consent for the publication of the work under the Creative Commons Attribution-Non-Commercial 4.0 license. We otherwise convey all copyright ownership, including all rights incidental thereto, exclusively to the journal when published.

Availability of data and materials

Data generated in this study are contained in this manuscript.

Conflict of interests

The authors have declared that they have no competing interests.

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Authors' contributions

This work was carried out in collaboration with all authors. Authors FON, KO, OIS, and OE designed the study, collected the data and wrote a draft of the manuscript. Authors KSO, FON and OIS, recruited and examined the patients. Authors FON and OIS performed the laboratory experiments and KSO performed the clinical studies. FON and- OU provided technical advice and revised the manuscript. All authors read and approved the final manuscript.

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