have relevant risk factors such as exposure to broad spectrum antibiotics and prolonged courses of vancomycin. Additional testing for detection may also be warranted in clinical specimens from patients with risk factors who have continuing evidence of infection with an *Enterococcus* that cannot be identified using automated systems such as the VITEK2. To determine the true burden of this organism, further large studies looking at the prevalence of VDE in surveillance rectal swabs of patients 'pre-colonised' with VRE are warranted. The clinical relevance and relative virulence of this organism is also currently unknown due to the lack of clinical data.

To complicate matters further, optimal treatment regimens for significant VDE infections is unknown. Cessation of vancomycin alone may not be sufficient to cure infections due to the rapid emergence of vancomycin-independent revertant mutants.[11] The efficacy of antimicrobials such as linezolid and daptomycin are yet to be determined in a clinically significant manner due to the low prevalence of invasive infections. In patients colonised with the organism, just like in VRE, it is important to adhere to strict infection control measures to prevent nosocomial spread.[8]

Though relatively rare, it is important to be aware of this phenomenon of vancomycin-dependence in patients at risk as well as the challenges associated with identification and susceptibility testing of the organism. The emergence of this pathogen also emphasises the importance of antimicrobial stewardship policies in preventing the evolution of increasingly resistant enterococci.

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Identification of *Fusobacterium nucleatum* in formalin-fixed, paraffin-embedded placental tissues by 16S rRNA sequencing in a case of extremely preterm birth secondary to amniotic fluid infection

Sir,

According to the World Health Organization, an estimated 15 million babies are born preterm (before 37 completed weeks of gestation) each year, with preterm birth rates increasing in almost all countries that collect reliable data.[1] Globally, complications resulting from preterm birth are the leading cause of death in children under 5 years of age, and those who do survive often have serious long-term health problems and disabilities.[2,3]

One of the most common aetiologies of preterm birth is amniotic fluid infection, caused by bacteria ascending from the vagina into the uterus and infecting the amniotic fluid.[4] Despite the high prevalence of preterm birth secondary to amniotic fluid infection, our understanding of the types and roles of bacteria involved is remarkably limited because in many cases, the causative microorganisms are not known.[5,6]

In order to elucidate the identity of the pathogenic organisms, one needs to perform microbiology studies on a suitable clinical specimen. However, tissues that might contain the causative organisms, such as tissue sample from the neonate or amniotic fluid sample from the mother, are not routinely available for testing in the clinical setting. In contrast, formalin-fixed, paraffin-embedded (FFPE) placental tissues are usually readily accessible for laboratory analysis since pathological examination of placentas has become standard of care in preterm birth cases, and therefore these placentas are routinely sent to the pathology department.

Here, we report the feasibility of performing 16S rRNA sequencing on FFPE placental tissues in bacterial identification in a case of extremely preterm birth secondary to amniotic fluid infection.

A 31-year-old, gravida 3, para 0 woman was admitted at the hospital at 20 4/7 weeks gestational age for fevers and chills. Previous obstetrical history was unavailable. On admission, she had elevated temperature and white cell

counts, and she went into preterm labour shortly after. Blood cultures were drawn and antibiotics were administered. Approximately 6 hours later, she had rupture of membranes and delivered a male neonate, who lived for 2 hours. The mother consented to a limited autopsy, restricted to external examination and X-rays only. The placenta was sent for pathological assessment.

Post-mortem examination revealed an extremely premature male neonate with no discernible developmental abnormalities.

The placenta was of normal size (144 g) and had a greenish tinge. Microscopic examination revealed acute chorioamnionitis, stage 3, grade 2, corresponding to severe maternal inflammatory response, and acute chorionic vasculitis and funisitis, stage 3, grade 2, corresponding to severe fetal inflammatory response.[7] Numerous Gram negative, fusiform bacteria were noted in the membranes and chorionic plate (Fig. 1).

In order to determine the identity of the bacteria, FFPE umbilical cord/membrane tissues (sample 1) and FFPE full-thickness placental tissue (sample 2) were submitted for 16S rRNA sequencing and phylogenetic analysis. Isolation of DNA was performed using the QIAmp DNA mini kit following the tissue protocol (Qiagen, USA). Briefly, 25 mg of tissue sample was placed into a 1.5 mL microcentrifuge tube and the tissue protocol began from step 1 (removal of paraffin with xylene not done). DNA was eluted with 50 µL AE buffer twice.

Amplification of 16S rRNA was done with primers 8F (AGAGTTTGATCCTGGCTCAG) and 926R (CCGTCAATTCATTTGAGTTT. 2 µL of the DNA extract was added to a 23 µL polymerase chain reaction (PCR) reaction containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl2, 0.2 mM each of dNTP (Invitrogen, USA), 0.4 µg each primer, and 1 unit of Platinum Taq DNA polymerase (Invitrogen). Thermocycling conditions were as follows: 94°C for 2 min, 32 cycles at 94°C 60 s, 56°C 60 s, and 72°C for 2 min, 72°C for 2 min, and held at 4°C. PCR products were run in a 2% agarose gel to confirm amplification, purified using the Qiagen MinElute gel extraction kit, then run in another 2% agarose gel to estimate DNA concentrations. Purified 16S rRNA PCR products were sequenced with the 8F primer, and the resulting sequence was assigned a tentative classification using the National Center for Biotechnology Information Basic Local Alignment Search Tool. A detailed phylogenetic analysis was performed for final confirmation of bacterial species through Cluster W analysis (DNASTAR, USA).

Both samples (FFPE umbilical cord/membranes and full thickness placental tissue) sent for 16S rRNA sequencing contained perfect sequence matches to *Fusobacterium nucleatum*, an oral pathogen (Fig. 2).

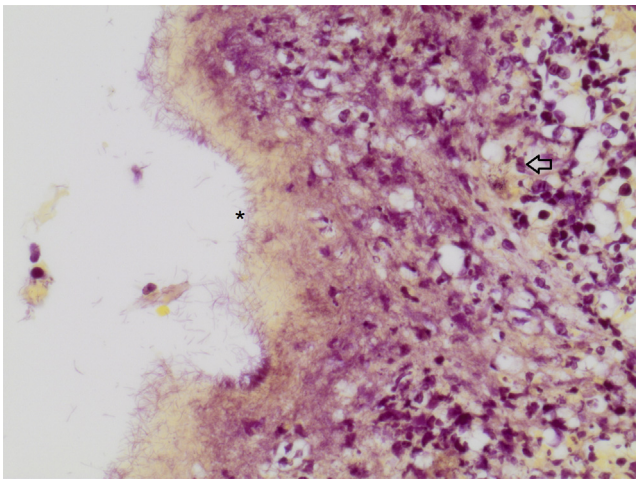

Fig. 1 Section of the placental membranes showing many neutrophils (arrow) and Gram negative fusiform bacteria (asterisk).

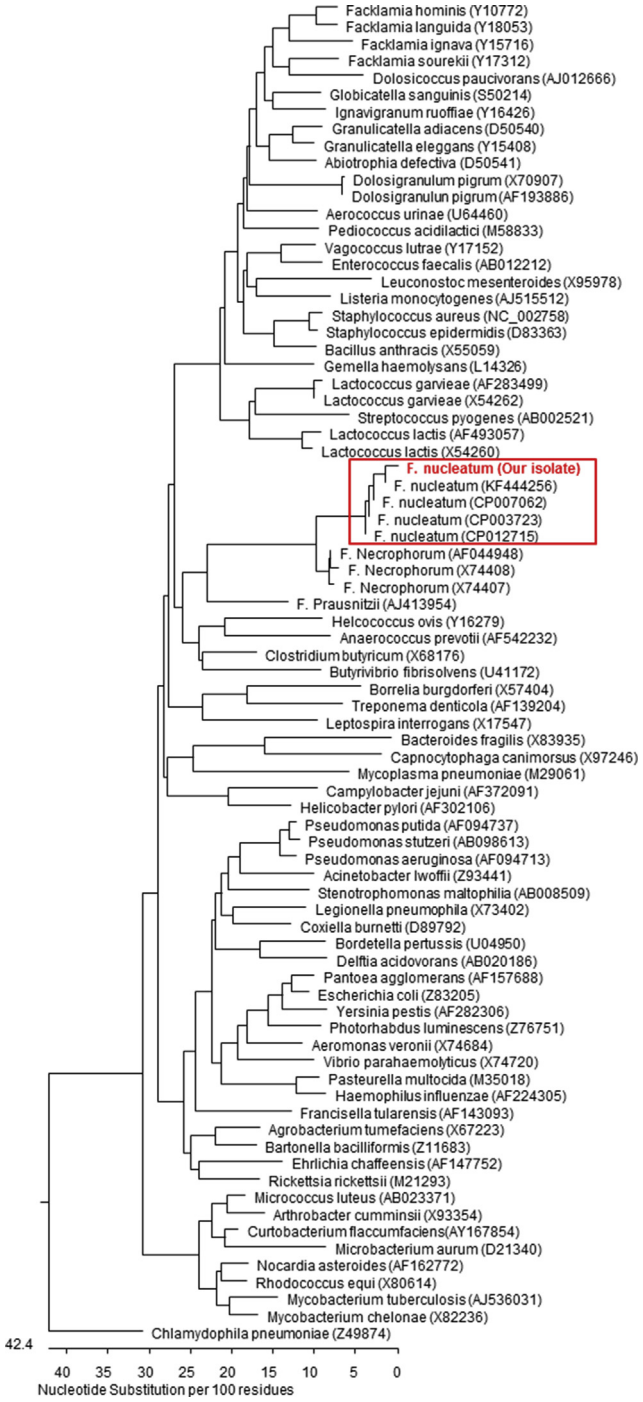

Fig. 2 Phylogenetic tree showing the 16S rRNA relationships of our *Fusobacterium nucleatum* isolate with other members of the genus *Fusobacterium* and other medically important bacteria. The tree was constructed by Clustal W analysis (DNASTAR, USA), based on the entire length of the 16S rRNA gene of our *F. nucleatum* isolate.

The maternal blood culture drawn prior to antibiotics administration showed no growth.

Fusobacterium nucleatum, a Gram negative anaerobe, is a common component of periodontal plaque.[8] In a study involving pregnant mice, Han *et al.*[9] demonstrated that *F. nucleatum* could be transmitted haematogenously to the placenta, resulting in premature delivery, stillbirth, and neonatal deaths. Han *et al.*[10] subsequently reported a case of term stillbirth caused by *F. nucleatum* that may have translocated from the mother's oral cavity to the uterus. Gauthier *et al.*[11] identified *F. nucleatum* in the amniotic fluid of three women with preterm labour and intact membranes by bacterial culture. The bacteria were found in the oral samples of two women, and in the oral sample of the partner of the third woman, who reported oral-genital contact during pregnancy.

Although amniotic fluid infection is a recognised major cause of preterm birth, bacterial identification is rarely performed in these cases. This is because tissues from preterm neonates and/or amniotic fluid samples are not routinely attainable for microbiology studies. Placentas, on the other hand, are frequently sent for pathological assessment. A pathological diagnosis of amniotic fluid infection is made retrospectively when acute chorioamnionitis, a maternal inflammatory response, is identified. While most pathologists have no difficulty diagnosing acute chorioamnionitis by light microscopy, attempt to identify the bacterial species by light microscopy proves to be significantly more challenging because many bacterial species are morphologically indistinguishable from one another. Additionally, in most cases of amniotic fluid infection, bacterial colonies are not apparent in the histological sections of placenta.

One might argue that, instead of performing 16S rRNA sequencing on FFPE placental tissues, one can perform bacterial culture using fresh placental tissues for bacterial identification. Indeed, bacterial cultures can be useful in the majority of cases. Queiros da Mota *et al.*[12] demonstrated that in cases of suspected chorioamnionitis, the histological and bacteriological results were concordant in 70% of the examined placentas, with 61.1% negative cases (negative histological chorioamnionitis and negative bacterial cultures), and with 7.4% positive cases (positive histologic chorioamnionitis and positive bacterial cultures). Nonetheless, in actual clinical practice, amniotic fluid infection is not always suspected; therefore, fresh placental tissues are not always routinely sent for bacterial culture. Furthermore, placentas associated with complications secondary to preterm birth are frequently sent to tertiary hospitals for consultation, and in those cases, the placentas are often placed in formalin for transport.

Even if bacterial cultures using fresh placental tissues were performed, the causative pathogen could still elude detection. In fact, microbes could not be recovered by bacterial culture in a significant proportion of placentas with acute chorioamnionitis, possibly owing to fastidious growth requirements.[5,6,12]

A number of studies pertaining to preterm birth demonstrated the feasibility of performing 16S rRNA sequencing on culture-negative amniotic fluid in bacterial identification.[13,14] However, amniocentesis, being an invasive procedure, is rarely performed on mothers during labour; hence, amniotic fluid is not normally available as a clinical specimen.

To conclude, this is the first reported case to demonstrate the feasibility of performing 16S rRNA sequencing on FFPE placental tissues in bacterial identification, and also the first to demonstrate the use of this molecular technique in neonatal death investigation. With the identification of the responsible pathogen, parents can be appropriately counselled and treated to avoid future preterm birth and pregnancy losses. In this case, the couple was counselled and a dental referral was arranged. The mother subsequently became pregnant and gave birth to a healthy baby boy at 37 gestational weeks approximately 1 year later.

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