Case Report

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New insight into the analysis of amniotic fluid microflora using 16S rRNA gene sequencing

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Introduction: Intra-uterine infection is a major cause of spontaneous pre-term birth before 30 weeks of gestation. In addition, intra-amniotic inflammation is present in approximately 80 % of patients with acute cervical insufficiency. The microbiome is known to cause intra-amniotic infection causing pre-term birth. Recently, surveys of the microbial communities in the vagina or in amniotic fluid using sequencing of the 16s rRNA gene have been carried out. Here, we reported the microbiome compositions in mid-trimester amniotic fluids in two pregnant women with cervical insufficiency identified using 16S rRNA sequencing.

Case presentation: A 35-year-old primipara and a 37-year-old nullipara were admitted to Hallym University Medical Center, Korea. They were negative for nitrazine and Actim PROM (Medix Biochemica) tests, and their amniotic membranes had prolapsed beyond the external os. A physical examination indicated that cerclage using a uniconcave balloon should be carried out, and amnioreduction was performed. Thereafter, they delivered at very early pre-term birth of below 24 weeks and the birth outcomes were pre-natal death. Analysis of mid-trimester amniotic fluids in these two pregnant women with cervical insufficiency identified *Sneathia sanguinegens* and *Fusobacterium nucleatum*. These bacteria were not identified using vaginal or amniotic cultures.

Conclusion: These results suggest that 16S rRNA gene sequencing can be used to identify the predominant microbiome causing pre-term birth in pregnant women with cervical insufficiency. Further studies are needed in large samples to improve our understanding of microbes causing pre-term birth and to prevent pre-term birth in pregnant women with cervical insufficiency.

Keywords: cervical insufficiency with bulging membranes; intra-uterine infection/abdominal discomfort; vaginal discharge; physical examination-indicated cerclage.

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Introduction

Intra-uterine infection is a major cause of spontaneous pre-term birth before 30 weeks of gestation (Gravett *et al.*, 1986; Yoon *et al.*, 2001). In addition, intra-amniotic inflammation is present in approximately 80 % of patients with acute cervical insufficiency (Lee *et al.*, 2008). The microbiome is known to cause intra-amniotic infection causing pre-term birth. PCR analysis of amniotic fluid of pregnant women who delivered pre-term detected 11 % bacteria in culture-negative samples (Gardella *et al.*, 2004). PCR also revealed 45 % bacteria in the culture-negative amniotic fluid of participants with pre-term premature rupture of

membranes (DiGiulio *et al.*, 2010). Recently, surveys of the microbial communities in the vagina have been carried out using sequencing of the 16S rRNA gene (MacIntyre *et al.*, 2015; Baldwin, *et al.*, 2015). Here, we report that *Sneathia sanguinegens* and *Fusobacterium nucleatum* were identified using 16S rRNA gene sequencing in mid-trimester amniotic fluid from two women with cervical insufficiency who delivered before 24 weeks.

Case report

Case 1

A 35-year-old primipara was admitted to Hallym University Medical Center, Korea, for vaginal spotting at 22 6/7

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weeks of gestation. Speculum examination revealed 5 cm cervical dilation and amniotic membranes prolapsed beyond the external os. The patient was afebrile and stable haemodynamically, and had no persistent uterine contractions or vaginal bleeding. A sample was taken from the posterior vaginal fornix. A nitrazine and an Actim PROM test (Medix Biochemica), performed to rule out pre-term rupture of the membranes, were negative. Ultrasonography was performed to examine fetal biometry and anomalies, the amount of amniotic fluid, the status of the cervix and the location of the placenta. The patient was observed for 6 h to exclude pre-term labour and chorioamnionitis before cerclage placement. The serum white blood cell count was $14\,070\text{ cells }\mu\text{l}^{-1}$ and the C-reactive protein was 26.1 mg l^{-1} . Vaginal exudate cultures were positive for *Ureaplasma* sp. Physical examination indicated that cerclage using a uniconcave balloon should be performed. To reduce the amount of amniotic fluid and evaluate possible subclinical chorioamnionitis, transabdominal amniocentesis was performed under ultrasonographic guidance before the cerclage procedure. A portion of the amniotic fluid was submitted for Gram staining and culture for anaerobic and aerobic bacteria; the amniotic fluid cultures were negative. The patient was given cephalosporin intravenously in the operating room and for the next 3 days. For 3 days post-operatively, she was given 500 mg azithromycin orally and treated with bed rest and continued tocolysis with intravenous magnesium sulfate. She developed labour pains 6 days after surgery at 23 6/7 weeks of gestation and the cerclage suture was removed. She delivered a 510 g female baby vaginally; the baby died in the delivery room. The patient was treated conservatively and discharged 2 days after delivery (Table 1).

Case 2

A 37-year-old nullipara was admitted to Hallym University Medical Center for abdominal discomfort and vaginal

discharge at 22 3/7 weeks gestation. Speculum examination revealed 2 cm cervical dilation and the amniotic membranes were visible at the external os. The patient was afebrile and haemodynamically stable, and had no persistent uterine contractions or vaginal bleeding. A nitrazine and an Actim PROM test were negative. The patient was observed for 6 h before cerclage placement. The serum white blood cell count was $13\,920\text{ cells }\mu\text{l}^{-1}$ and the C-reactive protein was 49.5 mg l^{-1} . Vaginal exudate cultures were positive for *Ureaplasma* sp. Physical examination indicated that cerclage should be carried out using a uniconcave balloon, and amnioreduction was performed. The patient was given cephalosporin intravenously in the operating room. She developed a fever of $38.1\text{ }^{\circ}\text{C}$ and frequent uterine contractions 1 day after the cerclage. Clinical chorioamnionitis was suspected and the suture was removed. The next day, the patient delivered a 520 g male baby vaginally at 22 5/7 weeks gestation; the baby died in the delivery room. Three days later, amniotic fluid obtained from the patient was positive for *Gardnerella vaginalis*. The patient was treated with antibiotics (second-generation cephalosporin antibiotics) and discharged 2 days after delivery (Table 1).

Investigations

16S RNA gene sequencing

DNA in amniotic fluid was amplified in triplicate reactions, pooled, and purified using MO BIO 96-htp PCR clean-up kits. Briefly, genomic DNA was amplified using the 16S amplicon PCR forward primer 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and 16S amplicon PCR reverse primer 5'-GTCTCGTGGGCTCGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'. The amplicons were sequenced on an Illumina MiSeq, and clusters for the cDNA libraries were generated on a MiSeq flow cell and sequenced for 300 bp paired-end reads (2×300) with a MiSeq Reagent kit v.3 (Illumina). Raw image data were transformed by base calling into sequence data and stored in FASTQ format. Raw reads were generated by the MiSeq platform and initially filtered for quality $Q30 > 80\%$. The resulting 10 000 high-quality sequence reads were analysed using CL community.

Fig. 1 shows the bacterial phyla and species found in the amniotic fluid of each case. Although fusobacteria comprised 96.3 and 78.3 % of the phyla in cases 1 and 2, respectively, the predominant bacterial species differed: in case 1, 75.2 % was *Sneathia sanguinegens*, while in case 2, 69.2 % was *Fusobacterium nucleatum*.

Discussion

Amniotic fluid infection occurs in 10–15 % of pregnancies complicated by pre-term labour (Hitti *et al.*, 2001; Cao *et al.*, 2014). Notably, intra-amniotic inflammation is present in approximately 80 % of patients with acute cervical insufficiency (Lee *et al.*, 2008). Here, we report the micro-

Table 1. Clinical characteristics of the two women who delivered pre-term

	Case 1	Case 2
Age	37	37
GA at cerclage (weeks)	22.6	22.3
Serum WBC (cells ml^{-1})	14070	13920
C-reactive protein	26.1	49.5
Placenta pathology	Chorioamnionitis	Chorioamnionitis
AF WBC (cells ml^{-1})	1120	470
AF volume (ml)	80	25
AF culture	Negative	<i>Gardnerella vaginalis</i>
Vaginal swap	<i>Ureaplasma</i>	<i>Ureaplasma</i>
GA at delivery (weeks)	23.6	22.5
Birth weight (g)	510	520

GA, gestational age; AF, amniotic fluid; WBC, white blood cells.

Fig. 1. Prevalent phyla and species in mid-trimester amniotic fluid samples of the two cases. All phyla and species were identified by 16S rRNA gene sequencing. The inner circles indicate the abundance of phylotypes and the outer circles indicate the abundance of species types. (a) Relative abundances of phyla and species in case 1. The major phylum in the amniotic fluid microbiome profiles was *Fusobacteria* (96.3 %) and the major species was *Sneathia sanguinegens* (75.2 %). (b) Relative abundances of phyla and species in case 2. The major phylum was *Fusobacteria* (78.3 %) and the major species was *Fusobacterium nucleatum* (69.2 %). Uc, Unclassified.

biome compositions in mid-trimester amniotic fluid from two women who delivered pre-term. The bacterial cultures were negative, but *S. sanguinegens* and *Fusobacterium* spp. were identified using 16S rRNA gene sequencing. These infections caused very early pre-term birth (before 24 weeks) and resulted in pre-natal death.

S. sanguinegens and other members of the phylum *Fusobacteria* in amniotic fluid are associated with pre-term birth and stillbirth (Hassan *et al.*, 2006; Thiesen *et al.*, 2007). *S. sanguinegens* forms long, Gram-negative, non-motile rods that sometimes exhibit bulbous protrusions (Collins *et al.*, 2001; Goto *et al.*, 2007). The species was previously named *Leptotrichia sanguinegens*, but was reassigned to a separate genus. *S. sanguinegens* has been associated with infections of the female reproductive tract, as has *Leptotrichia amnionii* (Thiesen *et al.*, 2007). Moreover, *S. sanguinegens* is part of the vaginal flora and causes ascending infections of the uterus, fetal membranes and fallopian tubes (Gauthier *et al.*, 2011). Recently, Romero *et al.* (2014) reported that the abundance of *S. sanguinegens* in the vaginal microbiota did not differ between pre-term and term births. However, their results included diverse phylotypes in low numbers, while in our case 1, 16S rRNA gene sequencing identified only a few species in the amniotic fluid, and 75.2 % of organisms were *S. sanguinegens*, supporting the notion that overgrowth of one or a few strains is harmful, whereas a diverse community with low numbers of individuals of each species does not seem to interfere with pregnancy (Wassenaar and Panigrahi, 2014). In our cases, the amniotic fluid cultures were negative.

F. nucleatum is a non-spore-forming, Gram-negative anaerobe (Bolstad *et al.*, 1996) that is common in the human

oral cavity (Moore and Moore, 1994; Copenhagen-Glazer *et al.*, 2015). Periodontal disease is a risk factor for pre-term labour (Han *et al.*, 2009; Gauthier *et al.*, 2011), and *F. nucleatum* has been associated with pre-term birth (Hill, 1998; Cahill *et al.*, 2005), stillbirth (Han *et al.*, 2010) and early-onset neonatal sepsis (Wang *et al.*, 2013). In pregnant mice, *F. nucleatum* injected intravenously invaded the placenta, proliferated in peripheral tissues, spread to the amniotic fluid and resulted in pre-term birth (Han *et al.*, 2004). In humans, some researchers have reported that *F. nucleatum* was part of the mother's or father's oral flora but was undetected in the mother's vaginal or rectal samples (Han *et al.*, 2009; Gauthier *et al.*, 2011). Gauthier *et al.* (2011) reported intra-amniotic *F. nucleatum* in pregnant women who delivered pre-term, and postulated that it could originate from the father's oral flora. In case 2, there were only a few species in the amniotic fluid, and *F. nucleatum* comprised 69.2 % of the bacteria. Again, amniotic fluid cultures were negative.

There have been several studies on the microbiome in the vagina or in amniotic fluid associated with pre-term birth (DiGiulio, 2012; Wang *et al.*, 2013; Hyman *et al.*, 2014). However, in patients with cervical insufficiency, to the best of our knowledge this is the first study using 16S rRNA gene sequencing to identify the predominant microbiome in amniotic fluid. *S. sanguinegens* and *F. nucleatum* detected in our study were also detected in previous studies on pre-term birth (DiGiulio, 2012; Wang *et al.*, 2013; Hyman *et al.*, 2014). *S. sanguinegens* and *F. nucleatum* appeared to be highly prevalent in patients with pre-term delivery, which was unexpected based on their near absence in culture-based studies (DiGiulio, 2012).

This molecular microbiological approach enables the detection of uncultivated microbes, thereby improving our understanding of the diversity of microbes causing pre-term birth.

In conclusion, our 16S rRNA gene sequencing results identified *S. sanguinegens* and *F. nucleatum* in amniotic fluid as causes of pre-term birth. These bacteria were not detected in amniotic fluid cultures, while *Ureaplasma* sp. was detected in both vaginal swabs and *Gardnerella* sp. was detected in the amniotic fluid culture of case 2. This suggests that 16S rRNA gene sequencing can be used to identify the predominant microbiome in pregnant women with cervical insufficiency. Further studies are needed with larger sample numbers to improve our understanding of microbes causing pre-term birth and to prevent pre-term birth in pregnant women with cervical insufficiency.

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References

- Baldwin, E. A., Walther-Antonio, M., MacLean, A. M., Gohl, D. M., Beckman, K. B., Chen, J., White, B., Creedon, D. J. & Chia, N. (2015). Persistent microbial dysbiosis in preterm premature rupture of membranes from onset until delivery. *PeerJ* 26, e1398.
- Bolstad, A. I., Jensen, H. B. & Bakken, V. (1996). Taxonomy, biology, and periodontal aspects of *Fusobacterium nucleatum*. *Clin Microbiol Rev* 9, 55–71.
- Cahill, R. J., Tan, S., Dougan, G., O'Gaora, P., Pickard, D., Kennea, N., Sullivan, M. H., Feldman, R. G., Edwards, A. D. & Universal, D. N. A. (2005). Universal DNA primers amplify bacterial DNA from human fetal membranes and link *Fusobacterium nucleatum* with prolonged preterm membrane rupture. *Mol Hum Reprod* 11, 761–766.
- Cao, B., Stout, M. J., Lee, I. & Mysorekar, I. U. (2014). Placental microbiome and its role in preterm birth. *Neoreviews* 15, e537–e545.
- Collins, M. D., Hoyles, L., Tornqvist, E., von Essen, R. & Falsen, E. (2001). Characterization of some strains from human clinical sources which resemble “*Leptotrichia sanguinegens*”: description of *Sneathia sanguinegens* sp. nov. *gen. nov. Syst Appl Microbiol* 24, 358–361.
- Copenhagen-Glazer, S., Sol, A., Abed, J., Naor, R., Zhang, X., Han, Y. W. & Bachrach, G. (2015). Fap2 of *Fusobacterium nucleatum* is a galactose-inhibitable adhesin involved in coaggregation, cell adhesion, and preterm birth. *Infect Immun* 83, 1104–1113.
- DiGiulio, D. B. (2012). Diversity of microbes in amniotic fluid. *Semin Fetal Neonatal Med* 17, 2–11.
- DiGiulio, D. B., Romero, R., Kusanovic, J. P., Gómez, R., Kim, C. J., Seok, K. S., Gotsch, F., Mazaki-Tovi, S., Vaisbuch, E. & Other authors (2010). Prevalence and diversity of microbes in the amniotic fluid, the fetal inflammatory response, and pregnancy outcome in women with preterm pre-labor rupture of membranes. *Am J Reprod Immunol* 64, 38–57.
- Gardella, C., Riley, D. E., Hitti, J., Agnew, K., Krieger, J. N. & Eschenbach, D. (2004). Identification and sequencing of bacterial rDNAs in culture-negative amniotic fluid from women in premature labor. *Am J Perinatol* 21, 319–323.
- Gauthier, S., Tétu, A., Himaya, E., Morand, M., Chandad, F., Rallu, F. & Bujold, E. (2011). The origin of *Fusobacterium nucleatum* involved in intra-amniotic infection and preterm birth. *J Matern Fetal Neonatal Med* 24, 1329–1332.
- Goto, M., Hitomi, S. & Ishii, T. (2007). Bacterial arthritis caused by *Leptotrichia amnionii*. *J Clin Microbiol* 45, 2082–2083.
- Gravett, M. G., Hummel, D., Eschenbach, D. A. & Holmes, K. K. (1986). Preterm labor associated with subclinical amniotic fluid infection and with bacterial vaginosis. *Obstet Gynecol* 67, 229–237.
- Han, Y. W., Redline, R. W., Li, M., Yin, L., Hill, G. B. & McCormick, T. S. (2004). *Fusobacterium nucleatum* induces premature and term stillbirths in pregnant mice: implication of oral bacteria in preterm birth. *Infect Immun* 72, 2272–2279.
- Han, Y. W., Shen, T., Chung, P., Buhimschi, I. A. & Buhimschi, C. S. (2009). Uncultivated bacteria as etiologic agents of intra-amniotic inflammation leading to preterm birth. *J Clin Microbiol* 47, 38–47.
- Han, Y. W., Fardini, Y., Chen, C., Iacampo, K. G., Peraino, V. A., Shamonki, J. M. & Redline, R. W. (2010). Term stillbirth caused by oral *Fusobacterium nucleatum*. *Obstet Gynecol* 115, 442–445.
- Hassan, S., Romero, R., Hendler, I., Gomez, R., Khalek, N., Espinoza, J., Nien, J. K., Berry, S. M., Bujold, E. & other authors (2006). A sonographic short cervix as the only clinical manifestation of intra-amniotic infection. *J Perinat Med* 34, 13–19.
- Hill, G. B. (1998). Preterm birth: associations with genital and possibly oral microflora. *Ann Periodontol* 3, 222–232.
- Hitti, J., Hillier, S. L., Agnew, K. J., Krohn, M. A., Reisner, D. P. & Eschenbach, D. A. (2001). Vaginal indicators of amniotic fluid infection in preterm labor. *Obstet Gynecol* 97, 211–219.
- Hyman, R. W., Fukushima, M., Jiang, H., Fung, E., Rand, L., Johnson, B., Vo, K. C., Caughey, A. B., Hilton, J. F. & other authors (2014). Diversity of the vaginal microbiome correlates with preterm birth. *Reprod Sci* 21, 32–40.
- Lee, S. E., Romero, R., Park, C. W., Jun, J. K. & Yoon, B. H. (2008). The frequency and significance of intraamniotic inflammation in patients with cervical insufficiency. *Am J Obstet Gynecol* 198, e1–e8.
- MacIntyre, D. A., Chandiramani, M., Lee, Y. S., Kindinger, L., Smith, A., Angelopoulos, N., Lehne, B., Arulkumaran, S., Brown, R. & other authors (2015). The vaginal microbiome during pregnancy and the postpartum period in a European population *Sci Rep* 11, 8988.
- Moore, W. E. & Moore, L. V. (2000). *The bacteria of periodontal disease. Periodontol* 5, 66–77.
- Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Bieda, J., Chaemsathong, P., Miranda, J., Chaiworapongsa, T. & Ravel, J. (2014). The vaginal microbiota of pregnant women who subsequently have spontaneous preterm labor and delivery and those with a normal delivery at term. *Microbiome* 2, 18.
- Thielsen, C. M., Nicolaidis, M., Lökebö, J. E., Falsen, E., Jorde, A. T. & Müller, F. (2007). *Leptotrichia amnionii*, an emerging pathogen of the female urogenital tract. *J Clin Microbiol* 45, 2344–2347.
- Wang, X., Buhimschi, C. S., Temoin, S., Bhandari, V., Han, Y. W. & Buhimschi, I. A. (2013). Comparative microbial analysis of paired amniotic fluid and cord blood from pregnancies complicated by preterm birth and early-onset neonatal sepsis. *PLoS One* 8, e56131.
- Wassenaar, T. M. & Panigrahi, P. (2014). Is a foetus developing in a sterile environment? *Lett Appl Microbiol* 59, 572–579.
- Yoon, B. H., Romero, R., Moon, J. B., Shim, S. S., Kim, M., Kim, G. & Jun, J. K. (2001). Clinical significance of intra-amniotic inflammation in patients with preterm labor and intact membranes. *Am J Obstet Gynecol* 185, 1130–1136.