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Correlation between *Ureaplasma* spp. sub-group 1 and preterm pre-labour rupture of membranes revealed by an eMLST scheme

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ABSTRACT

Ureaplasma spp. is gaining recognition as an important pathogen associated with preterm birth (PTB) and preterm pre-labour rupture of membranes (PPROM). The aim of this study was to investigate the clonality of this organism in maternal/neonatal pairs with PTB or pre-labour rupture of membranes (PROM) or PPROM and the association between sub-groups and PPROM. In total, 50 of 93 maternal/neonatal pairs that were diagnosed with PTB, PROM or PPROM were identified with *Ureaplasma* spp. colonized in the amniotic fluid or umbilical cord or placenta. All 104 clinical *Ureaplasma* spp. samples (50, 30, and 24 cultured from amniotic fluid, umbilical cord, and placenta, respectively) were included for analysis of the genetic lineages using the eMLST scheme. A total of 34 eSTs were revealed, with two predominant eSTs (eST16 and eST41). Interestingly, six maternal/neonatal pairs displayed eST differences in the above three specimen sources. In addition, phylogenetic analysis showed two genetically significant distant clusters, and cluster I included the most clinical strains. Interestingly, there was a significant difference in the prevalence of sub-group 1 of cluster II between women with PPROM and those with PROM. In conclusion, the distribution of cluster I was predominately higher than that of cluster II in maternal/neonatal pairs. In addition, sub-group 1 was prone to associated PPROM through the specific epidemic clonal lineages.

1. Introduction

Rupture of membranes (ROM) refers to breakage of the amniotic sac and leakage of amniotic fluid before the onset of labour. When ROM occurs after 37 weeks' gestation, it is called pre-labour rupture of membranes (PROM), and there is minimal risk to the foetus, and labour typically starts soon after. However, with preterm pre-labour rupture of membranes (PPROM), in which rupture occurs prior to 37 weeks' gestation, the mother and foetus are at a greater risk of having complications (Frenette et al., 2013; Kacerovsky et al., 2013). PPROM is a precursor to approximately one-third of preterm births (PTB), and it is an important contributor of perinatal morbidity and mortality worldwide (Kacerovsky et al., 2014). The exact mechanisms underlying PTB and PPROM are not well understood, however, microbial invasion of the amniotic cavity and intra-amniotic infection have been implicated as the leading aetiological factor. Using cultivation techniques, approximately one-third of patients with PROM have microbial invasion in the amniotic cavity (Romero et al., 1992). Moreover, microbial invasion of the amniotic cavity occurs in 50% patients with PPROM, according to cultivation and molecular microbiologic techniques (DiGiulio et al., 2010). Specifically, bacteria are more abundant in foetal membranes collected from PPROM subjects compared to bacteria collected from term and preterm subjects (DiGiulio et al., 2010; Fortner et al., 2014; Leitich et al., 2003).

Ureaplasma parvum (serovars 1, 3, 6, 14) and *Ureaplasma urealyticum* (serovars 2, 4, 5, 7–13) are generally regarded as commensal microorganisms in the lower genital tract, but they can cause ascending invasive infections of the upper genital tract and are implicated in a variety of clinical manifestations, including bacterial vaginosis, nongonococcal urethritis, chorioamnionitis, adverse pregnancy outcomes, infertility, bronchopulmonary dysplasia in neonates (Murtha and Edwards, 2014; Viscardi, 2014; Waites et al., 2005). Compared to *U. urealyticum*, *U. parvum* is more frequently isolated from the amniotic fluid of preterm gestations (Kim et al., 2003). Although amniotic fluid

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can be colonized by *Ureaplasma* spp. without clinical manifestations, increasing evidence suggests that these bacteria, isolated from the amniotic fluid, cord blood, respiratory tract and the cerebrospinal fluid of preterm neonates, cause inflammation and can even lead to spontaneous PTB and PPROM, as well as post-delivery infectious complications and neonatal infections (Kafetzis et al., 2004; Hassan et al., 2006; Kasper et al., 2010; Senthamaraikannan et al., 2016; Viscardi, 2010). In the amniotic cavity, *Ureaplasma* spp. can trigger an inflammatory response that is more serve than that of other organisms in patients with PPROM (Oh et al., 2010). *U. urealyticum* colonization of the placenta is significantly associated with chorioamnionitis, and neonates colonized with *U. urealyticum* are found to be related to infection-related chronic lung disease and even mortality (Kafetzis et al., 2004). Moreover, *U. parvum* has been found to colonize in the amniotic fluid and cause uterine inflammation (Senthamaraikannan et al., 2016). Additionally, there is evidence that *Ureaplasma* spp. undergoes extensive horizontal gene transfer and may exist as “quasi-species” (Paralanov et al., 2012). Thus, in pregnant women, sufficient attention should be directed toward the pathogenic role of *Ureaplasma* spp., particularly separated by the specific pathogenic lineages. Furthermore, to the best of our knowledge, few studies have focused on the association between PTB or PPROM and the presence of specific *Ureaplasma* spp. genotypes or subgroups.

Because the expanded Multilocus sequence typing (eMLST) scheme for *Ureaplasma* spp. has been recently developed, few research studies have focused on the population structure of this organism (Zhang et al., 2014). Moreover, the clonality of *Ureaplasma* spp. isolated from maternal/neonatal pairs, along with PTB or PPROM, are largely unknown and require further investigation. Therefore, the aims of the present study were to first determine the population distribution of *Ureaplasma* spp. in maternal/neonatal pairs along with PTB or PPROM, using the eMLST analysis, and then to compare the clonality of *Ureaplasma* spp. in PPROM and PROM.

2. Material and methods

2.1. Bacterial strains and clinical specimens

A total of 104 clinical *Ureaplasma* spp. strains, collected at Yiwu Maternity and Child Care Hospital in China, between September 2015 and May 2016, were isolated from 93 maternal/neonatal pairs, who suffered from PTB or PROM. Among the 93 women that participated in this study, 47 (50.54%) were treated with antibiotics before the onset of labour. All of the neonates were delivered spontaneously between 35 and 41 weeks of gestation. The mean maternal age was 30 years (range 19–41 years; SD ± 4.84 years). Among the 104 isolates, 50 were obtained from the amniotic fluid, and 30 and 24 were cultured from the umbilical cord and corresponding placenta of neonates, respectively.

2.2. Ureaplasma spp. culture

Ureaplasma species were cultured and discriminated from *Mycoplasma hominis* using a commercially available Mycoplasma IST 2 kit (bioMérieux, Marcy l'Etoile, France). Using amniocentesis, approximately 5 mL of amniotic fluid was added in a sterilized plastic tube and immediately centrifuged at 5000 rpm. After the infants were delivered, approximately 1 cm^3 of the umbilical cord and placenta were immediately placed in a sterilized plastic tube and homogenated using a glass homogeniser within 2 h of sample collection. Then, the sediment of amniotic fluid or placenta homogenate or umbilical cord homogenate was placed in R1 medium and vortexed rapidly; then, 3 mL of above mixture was added to R2 medium. The Mycoplasma IST 2 strip was inoculated with the rehydrated R2 medium to separate *M. hominis* and *Ureaplasma* spp. and provided information about the antibiotic susceptibility after 24 to 48 h incubation. All broth cultures that were positive for *Ureaplasma* spp. were stored at −80 °C for further testing.

2.3. DNA extraction

To prepare the template for PCR, a total of 0.5 mL of *Ureaplasma* spp. broth culture of each strain was harvested through centrifugation at 12,000 × *g* for 10 min. A total of 50 μL of lysis buffer (10 mM Tris-HCl, pH 8.0; 50 mM KCl; 2.5 mM $MgCl_2$; and 0.5% Tween 20) and proteinase K (100 μg/mL) was used to resuspend the cells, and the mixture was incubated at 55 °C for 1 h. Then, the sample was heated at 95 °C for 10 min and centrifuged at 10,000 × *g* for 1 min to remove debris. The supernatant was utilized immediately or stored at −20 °C for future use.

2.4. Expanded multilocus sequence typing (eMLST)

For the 104 isolates tested in the present study, an eMLST scheme, using primers targeting four housekeeping loci (*ftsH*, *rpl22*, *valS*, and *thrS*) and two putative virulence loci (*ureG* and *mba-np1*), was performed according to the method described in our previous study (Zhang et al., 2014).

Novel alleles for each locus were assigned a new allele number, and distinct allelic profiles were assigned a new eST (expanded Sequence type). Based on the number of nucleotide differences in the concatenated partial sequences (total of 2814 bp) of six genes in the eMLST scheme, a neighbour-joining tree was constructed using Molecular Evolutionary Genetic Analysis software (MEGA 6.0), with 1000 resampling for bootstrapping, to establish clonality and determine potential relationships between isolates.

2.5. Statistical analysis

In the present study, IBM SPSS Statistics 19.0 was used to analyse all data, based on the Chi-square test. Statistical significance was accepted at $P < .05$.

2.6. Ethics statement

All enrolled patients provided written informed consent prior to data collection. The study and enrolment procedures were approved by the Institutional Ethics Committee of Yiwu Maternity and Child Care Hospital.

3. Results

3.1. Ureaplasma spp. prevalence among different specimen sources

A total of 93 maternal/neonatal pairs participated in this study. Of these, 82 women experienced PROM (62/93, 66.7%) or PPROM (20/93, 21.5%), and 31/93 (33.3%) experienced PTB. *Ureaplasma* spp. were detected in samples collected from 33/62 (53.2%) women with PROM and 13/20 (65.0%) women with PPROM. A total of 104 *Ureaplasma* spp. isolates were obtained from the amniotic fluid ($n = 50$ isolates), umbilical cord ($n = 30$ isolates) and placenta ($n = 24$ isolates). All data are shown in Table 1.

3.2. Genetic lineages of 104 Ureaplasma spp. strains

All the 104 clinical *Ureaplasma* spp. isolates were recovered to analyse the genetic lineages by using the eMLST scheme. For the 50 *Ureaplasma* spp. isolates cultured from the amniotic fluid of women, 29 eSTs were found, with eST16 (9 isolates) and eST41 (10 isolates) being the most predominant eSTs. Among the 30 cultured from the umbilical cord of neonates, 22 eSTs were revealed, with eST16 (5 isolates) and eST41 (4 isolates) as the most frequent eSTs. Similarly, among the 24 cultured from the placenta of neonates, 18 eSTs were revealed, with eST16 (4 isolates) and eST41 (4 isolates) as the most predominant eSTs. Moreover, 19 new eSTs (eSTs 214–232) were discovered in the present

Table 1
Comparison of *Ureaplasma* spp. prevalence among different specimen sources.

Specimen sources		PTB	ROM	PPROM	PROM
Total number of maternal/neonatal pairs (n = 93)		31	82	20	62
Amniotic fluid + umbilical cord + placenta	*Ureaplasma* spp. positive (n = 50)	17	46	13	33
	Ureaplasma spp. negative (n = 43)	14	36	7	29
Amniotic fluid	*Ureaplasma* spp. positive (n = 50)	17	46	13	33
	Ureaplasma spp. negative (n = 43)	14	36	7	29
Umbilical cord	*Ureaplasma* spp. positive (n = 30)	10	27	7	20
	Ureaplasma spp. negative (n = 63)	21	55	13	42
Placenta	*Ureaplasma* spp. positive (n = 24)	6	22	4	18
	Ureaplasma spp. negative (n = 69)	25	60	16	44

Abbreviations: PTB, Pretermbirth; ROM, rupture of membranes; PPROM, preterm pre-labour rupture of membranes; PROM, pre-labour rupture of membranes.

study.

3.3. STs/eSTs difference between the Ureaplasma spp. strains among different specimens

For 44/50 (88%) of maternal/neonatal pairs colonized with *Ureaplasma* spp., identical eSTs were detected within the amniotic fluid, umbilical cord and placenta. In contrast, different eSTs were detected within the amniotic fluid, umbilical cord and/or placenta in 6/50 (12%) of maternal/neonatal pairs (Table 2). In terms of the species, clusters, and sub-groups, they were highly consistent among the isolates collected from the amniotic fluid, umbilical cord, and placenta. However, when focusing on the allelic types and eSTs, six pairs of the isolates showed at least an allelic type difference. Of the six gene fragments, five maternal/neonatal pairs showed differences in rpL22 gene, and two pairs and one pair showed differences in *thrS* and *ureG*, respectively. No differences were observed between the *ftsH*, *valS*, *mba-np1* gene fragments.

3.4. Phylogenetic analyses

Based on the concatenated sequences of six gene fragments of the eMLST scheme, a neighbour-joining tree was constructed to illuminate the genetic relationship of 50 *Ureaplasma* spp. strains isolated from amniotic fluid and 14 reference strains of *Ureaplasma* spp. serovars, and two genetically significantly distant clusters were revealed (Fig. 1). Among the 50 clinical isolates, cluster I included most of the isolates (41/50), and cluster II contained only 9/50 isolates. In cluster I, five sub-groups (sub-group A, B, C, D, and E) were observed. Sub-group A, B, and C comprised the majority strains, with 15, 12, and 9 strains, respectively. Sub-group D contained 4 strains, and sub-group E contained only one strain. Within cluster II, sub-group 1 included 8 isolates, while only one strain existed in sub-group 2.

Table 2
Allele numbers, clusters, sub-groups, and expanded sequence types (eSTs) in 6 maternal/neonatal pairs.

Strains	ftsH	rpL22	valS	thrS	ureG	mba-np1	Cluster	Sub-group	eST
10AF	2	1	1	2	2	49	I	C	226
10 UC	2	1	1	2	2	49	I	C	226
10 P	2	3	1	2	2	49	I	C	227
12 AF	6	3	2	27	5	29	II	1	229
12 UC	6	3	2	11	5	29	II	1	228
16AF	1	2	1	1	3	2	I	A	41
16 UC	1	2	1	1	3	2	I	A	41
16 P	1	1	1	1	3	2	I	A	225
31 AF	1	2	1	1	3	2	I	A	41
31 UC	1	1	1	8	2	2	I	A	26
31 P	1	2	1	1	3	2	I	A	41
50 AF	13	2	9	1	2	7	I	C	231
50 UC	13	1	9	1	2	7	I	C	113
50 P	13	2	9	1	2	7	I	C	231
79 AF	2	1	1	8	2	2	I	C	74
79 UC	2	3	1	8	2	2	I	C	232
79 P	2	1	1	8	2	2	I	C	74

AF, amniotic fluid; UC, umbilical cord; P, placenta.

3.5. Relationship with PPROM or PROM

Five sub-groups (sub-group A–E) in cluster I and sub-group 1 in cluster II were selected to analyse the association with PPROM and PROM (Table 3). Analysis of the 13 *Ureaplasma* spp. strains isolated from women with PPROM revealed that 2 (15.4%), 2 (15.4%), 2 (15.4%), 1 (7.7%) and 1 (7.7%) isolates clustered in sub-group A, B, C, D and E of cluster I, respectively. Five (38.5%) strains were clustered in cluster II sub-group 1; however, no isolates from women with PPROM clustered in cluster II sub-group 2. Cluster II sub-group 1 isolates were detected at a significantly higher rate in women with PPROM compared to those with PROM (p = .031).

4. Discussion

Numerous previous studies have described a significant association between the isolation of *Ureaplasma* spp. in pregnant women and PTB or PPROM (Kafetzis et al., 2004; Kasper et al., 2010; Senthamaraikannan et al., 2016; Viscardi, 2010). In recent years, the differences (in the genome sizes and in the correlations with clinical manifestation) between *U. parvum* and *U. urealyticum* have received increasing attention. In this study, the analysis of 50 *Ureaplasma* spp. strains recovered from maternal/neonatal pairs, suffered from PTB or PROM, showed that there was a statistically significant difference in the detection of cluster II sub-group 1 in women with PPROM compared to PROM.

Herein, 50 maternal/neonatal pairs were identified as being colonized by *Ureaplasma* species in the amniotic fluid, umbilical cord or placenta. Our results showed the colonization of *Ureaplasma* spp. was uneven. *Ureaplasma* spp. was isolated in 50 (100%) specimens of amniotic fluid, while it was isolated in only 30 (60%) and 24 (48%) umbilical cord and placenta specimens, respectively. The low sensitivity of culture methods for *Ureaplasma* spp. could be responsible for this phenomenon, and we may have missed a group of low-titre infected people using broth culture to identify *Ureaplasma* spp. in the amniotic fluid, umbilical cord or placenta.

Analysis of 104 *Ureaplasma* spp. isolates (50 amniotic fluid specimens, 30 umbilical cord specimens, and 24 placenta specimens) identified 34 eSTs and the two most predominant cases (i.e., eST16 and eST41). Additionally, eST82, one of the most prevalent eSTs in male patients, was less common in maternal/neonatal pairs, which aligned with the findings in female patients analysed in our previous study (Ruan et al., 2017; Zhang et al., 2014). The predominant sequence types (i.e., ST1, ST9, eST16 and eST41) also corresponded to the known prototype serovars, according to the phylogenetic analysis in our previous study (Zhang et al., 2014). Our data presented here suggest that eMLST is a valuable tool that is capable of identifying genetic clusters of

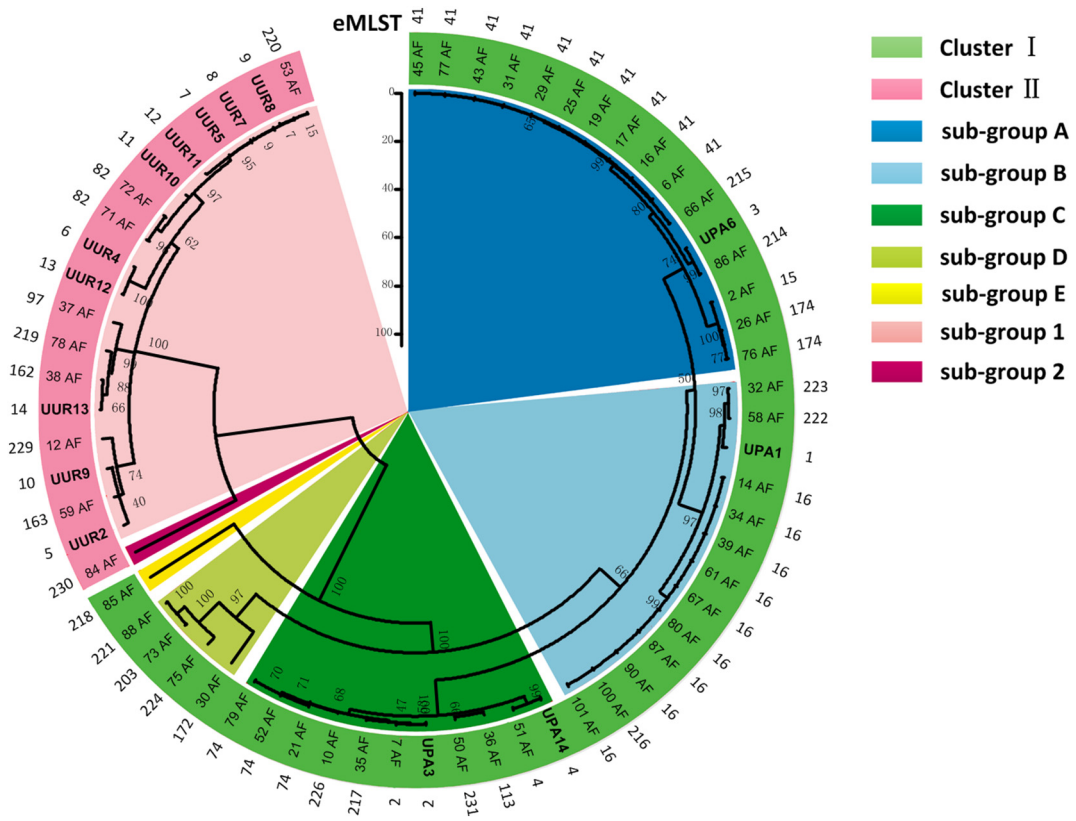

Fig. 1. Neighbour-joining tree, based on concatenated nucleotide sequences from the six loci, was constructed by MEGA 6.0 software. Two genetically significant distant clusters (cluster I and cluster II) were revealed among the 50 *Ureaplasma* spp. strains isolated from amniotic fluid. Cluster I included the reference sequence of serovars 1, 3, 6, and 14 (*U. parvum*) whereas cluster II included reference sequence of serovars 2, 4, 5, 7–13 (*U. urealyticum*). Additionally, five and two sub-groups were found in the cluster I and cluster II, respectively. The strains and the corresponding eSTs are given at the tip of each branch. Bootstrap values for 1000 replicates are indicated on the nodes of the tree (UPA, *U. parvum*; UUR, *U. urealyticum*).

Ureaplasma spp. and elucidating the relationships between the expected serovars in the absence of actual sequence analysis. Our results also showed that the differences among the specimens of amniotic fluid, umbilical cord, and placenta were not only the positive rate of *Ureaplasma* spp. but also the eSTs analysed by eMLST. Although high consistency was established in the levels of species, cluster, and sub-groups, 6 maternal/neonatal pairs showed eST differences. This might be due to the mixture of *Ureaplasma* species or serovars within a sample, and only one species or serovar was eventually separated and sequenced in the eMLST analysis.

To better understand the genetic relationship, a neighbour-joining tree was constructed for the 50 *Ureaplasma* species isolated from amniotic fluid. Two genetically significant distant clusters (cluster I and cluster II) were observed, and cluster I included the most (41, 82.0%) isolates, which was consistent with the finding in female patients but inconsistent with the finding in male patients (Ruan et al., 2017; Zhang et al., 2014). Note that the assortment of the clusters showed a high correlation with the two *Ureaplasma* species from cluster I (*U. parvum*) to cluster II (*U. urealyticum*), which was consistent with previous finding Zhang et al., 2014 (Ruan et al., 2017; Zhang et al., 2014). Five and two sub-groups were found in clusters I and II, respectively, and the distribution was uneven. When compared with the data of clinical *Ureaplasma* spp. collected from symptomatic and asymptomatic female patients in our previous study (Zhang et al., 2014), statistically significant differences in the frequency of sub-group 1 were observed ($P < .05$) (data not shown). Thus, the population distribution and dynamics of *Ureaplasma* spp. showed a considerable difference, not only between genders but also among the specimens from different disease sources.

To date, there has been great controversy regarding the virulence of

Table 3
Sub-groups of *Ureaplasma* spp. among PTB and ROM (PPROM and PROM).

Cluster	Sub-group	PTB	ROM	PPROM	PROM	P^a
Cluster I	Sub-group A	3 (17.6%)	14 (30.4%)	2 (15.4%)	12 (36.4%)	0.187
	Sub-group B	4 (23.5%)	10 (21.7%)	2 (15.4%)	8 (24.2%)	0.700
	Sub-group C	3 (17.6%)	8 (17.4%)	2 (15.4%)	6 (18.2%)	1.000
	Sub-group D	1 (5.9%)	4 (8.7%)	1 (7.7%)	3 (9.1%)	1.000
	Sub-group E	1 (5.9%)	1 (2.2%)	1 (7.7%)	0 (0)	0.283
Cluster II	Sub-group 1	5 (29.4%)	8 (17.4%)	5 (38.5%)	3 (9.1%)	**0.031***
	Sub-group 2	0 (0)	1 (2.2%)	0 (0)	1 (3.0%)	/
Total		17	46	13	33	

Abbreviations: PTB, preterm birth; ROM, rupture of membranes; PPROM, preterm pre-labour rupture of membranes; PROM, pre-labour rupture of membranes.
P^a, comparison between PPROM and PROM; /, not done.
* $P < .05$ was displayed in **bold**.

Ureaplasma spp. and the relationships between serovars or subtypes and PTB and PPROM (Kafetzis et al., 2004; Kasper et al., 2010; Senthamaraikannan et al., 2016). In the present study, sub-group 1, a sub-group of cluster II, was more prone to colonize in women with PPROM than that in those with PROM. Due to the limited number of strains, we cannot correlate sub-group 2 and PPROM, and further studies are necessary to expand the knowledge. In our previous study, sub-group 2, a sub-group of cluster II, seemed to be a potential risk factor in symptomatic female patients compared to asymptomatic female patients (Zhang et al., 2014). Additionally, the ratios of cluster II/cluster I for maternal/neonatal pairs with PPROM and PROM were 5/8 (62.5%) and 4/29 (13.8%), respectively. The ratios of symptomatic female patients and asymptomatic female patients were 18/116 (15.5%) and 6/129 (4.6%), respectively. Thus, our observations suggest that cluster II is more likely to act as pathogenic bacteria, and eMLST classification can provide a clinical treatment reference.

In conclusion, this study indicated that the prevalence of cluster I was predominately higher than that of cluster II in maternal/neonatal pairs. Our data demonstrated that sub-group 1 was more likely associated with PPROM through the specific epidemic clonal lineages.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.12.025.

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