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Ureaplasma urealyticum and *Mycoplasma hominis* Presence in Umbilical Cord is Associated with Pathogenesis of Funisitis

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Ureaplasma urealyticum* (*U. urealyticum*) and *Mycoplasma hominis* (*M. hominis*) are known to cause an intrauterine infection for preterm deliveries, but it is not known whether they are actually pathogenically involved in the development of funisitis, chorioamnionitis (CAM), and chronic lung disease (CLD) in preterm infants. Our purpose was to identify *U. urealyticum* and *M. hominis* in the umbilical cord, placenta, and tracheal aspirate (TA) or gastric fluid (GF) of preterm infants, and to clarify whether they contribute to funisitis, CAM, and CLD. Of 128 preterm infants, 86 umbilical cords, 83 placentas, and 84 TA or GF samples obtained postnatally from preterm infants were examined. *U. urealyticum* and *M. hominis* were detected by polymerase chain reaction and prospectively analyzed to determine whether the presence of *U. urealyticum* or *M. hominis* can lead to the development of funisitis, CAM, and CLD. *U. urealyticum* or *M. hominis* was isolated in nine (10.5%) of the umbilical cords, five (6.0%) of the placentas, and fifteen (17.9%) of the TA or GF samples. Funisitis was identified in all umbilical cords with *U. urealyticum* or *M. hominis*, but in only 13% of the umbilical cords without *U. urealyticum* and *M. hominis* ($p < 0.001$). Placentas and TA or GF with or without *U. urealyticum* and *M. hominis* did not show significant differences with regard to the development of CAM or CLD. Our results suggest that *U. urealyticum* and *M. hominis* presence is associated with the pathogenesis of funisitis, but not of CAM or CLD.

Ureaplasma urealyticum (*U. urealyticum*) and *Mycoplasma hominis* (*M. hominis*) are common bacteria found in the genitourinary tract of women during pregnancy (5, 15). Recently it was reported that an intrauterine *U. urealyticum* and *M. hominis* infection is a risk factor for the onset of spontaneous preterm delivery (5, 7, 10, 13, 17, 32). Funisitis and chorioamnionitis (CAM) are inflammations of the umbilical cord and placentas, respectively, and are both regarded as histological markers of intrauterine infections and recognized as the causes of preterm labor/delivery (5, 8, 32). However, it is not yet completely clear whether *U. urealyticum* and *M. hominis* are actually pathogenically involved in the development of funisitis and CAM.

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Chronic lung disease (CLD) is the most common morbidity among preterm infants. The pathogenesis of CLD is multi-factorial (1), including immaturity, patent ductus arteriosus (23), volutrauma, oxygen toxicity, excess fluid intake (20, 27), and intrauterine infection. Particularly, the intrauterine lung injury to the fetus that occurs as a result of *U. urealyticum* or *M. hominis* infection has attracted much interest as a potent risk factor for the development of CLD (4, 6, 24, 30). On the other hand, a report by Heggie et al. (9) suggests that respiratory *U. urealyticum* colonization does not appear to be a contributory cause of CLD, as was demonstrated by the detection of *U. urealyticum* in tracheal aspirates (TA) of preterm infants soon after birth. No definite conclusion has thus been reached regarding the association between intrauterine *U. urealyticum* infection and the development of CLD.

The purpose of this study was therefore to determine the detection rates of *U. urealyticum* and *M. hominis* in the umbilical cord, placenta, and TA or gastric fluid (GF) of preterm infants in order to clarify the association between *U. urealyticum* and *M. hominis* presence in and the development of funisitis, CAM, and CLD, prospectively.

MATERIALS AND METHODS

Patient population. With the approval of the Ethics Committee of Kobe University Graduate School of Medicine, 128 infants who were born at < 32 wks of gestational age at Kobe University Hospital or Kobe Children's Hospital Perinatal Center between April 2004 and March 2006 were enrolled in this prospective study. Infants with chromosomal or congenital anomalies and outborn infants were excluded. For this study, 86 umbilical cords, 83 placentas, and 84 TA or GF samples from 128 preterm infants were collected after informed consent had been obtained from their parents, and tried to detect *U. urealyticum* and *M. hominis*.

Sample preparation. After delivery of the infants, approximately 1 cm^3 of the umbilical cord and placenta were immediately placed in a sterilized container and stored at -20ºC until extraction of DNA from the tissues. TA was obtained from infants requiring mechanical ventilation at 10 days of age or on a day when an infant was extubated. GF was obtained with a nasogastric tube from infants within several hours of delivery. TA and GF were immediately placed in a sterilized tube, and stored at -20ºC until extraction of DNA. Genomic DNA from the archived umbilical cord, placenta, TA, and GF, was extracted by using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI). The obtained DNA solutions were stored at −80ºC until the polymerase chain reaction (PCR) assay.

Detection of *U. urealyticum* and *M. hominis* by PCR. PCR was used for all samples to detect the presence of *U. urealyticum* and *M. hominis* with specific primers for the Multiple-Banded antigen gene of *U. urealyticum* (Forward: GTATTTGCAATCTTTATATG TTTTCG, Reverse: CAGCTGATGTAAGTGCAGCATTAAATTC), and for the 16S rRNA gene of *M. hominis* (Forward: CAATGGCTAATGCCGGATACGC, Reverse: GGTACCGT CAGTCTGCAA) as described previously (3, 26, 29). Amplification by PCR using these primers resulted in a 403 bp or 448 bp product fragment for *U. urealyticum* detection and in a 334 bp product fragment for *M. hominis* detection. When these primers for *U. urealyticum* detection are used, the amplified fragment is either of 403 bp and 448 bp, because *U. urealyticum* is classified under 14 serotypes, and serotype 1, 3, 6, or 14 is detected as a 403 bp fragment and serotype 2, 4, 5, 7, 8, 9, 10, 11, 12, or 13 is detected as a 448 bp fragment (11). For amplification, TaqDNA polymerase at 95ºC was used for 4 min followed by 35 cycles of 95ºC for 50 sec, 56ºC for 50 sec, and 72ºC for 50 sec, followed by a 3-min post-extension at 72ºC. The reaction PCR products were separated by agarose gel

electrophoresis and the presence or absence of the above-mentioned bands was identified under UV illumination (Fig. 1).

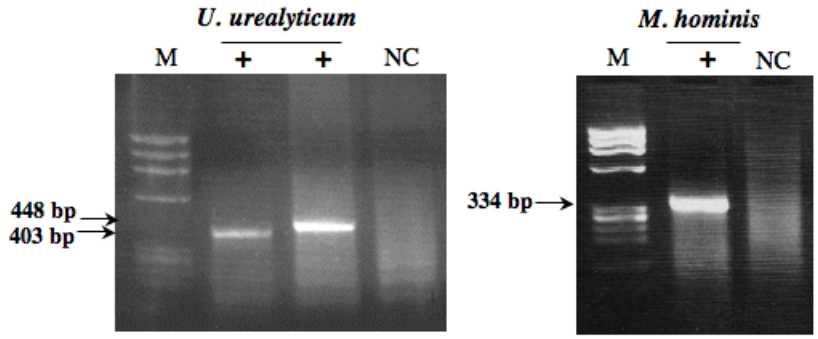

FIG. 1 Electrophoretic analysis by PCR to detect *U. urealyticum* and *M. hominis*. When *U. urealyticum*, or *M. hominis* is present in tissue specimens, the band of 403 bp or 448 bp, or 334 bp in size appear on an ethidium bromide-stained agarose gel under UV illumination, respectively. M: Marker, NC: Negative control.

Definition of funisitis, CAM, and CLD. All umbilical cords and placentas were histologically examined by pathologists who were blinded to the clinical histories of the patients. Funisitis was defined as the presence of neutrophil infiltration into the umbilical walls or Wharton's jelly, and CAM as the presence of an acute inflammatory infiltration in the fetal membrane (Blanc classification ≥ Stage 2) (2). CLD was diagnosed when the infants required oxygen therapy at 36 wks of gestational age and showed abnormalities on their chest X-ray film.

Statistical analysis. Statistical analyses were performed with Fisher's exact test to determine association between the presence of *U. urealyticum* or *M. hominis* in the umbilical cord, placenta, and TA or GF and the development of funisitis, CAM, and CLD. Differences were deemed statistically significant when $p < 0.05$.

RESULTS

Clinical characteristics. As seen in Table 1, the 128 preterm infants enrolled in this study were born between 23 and 31 wks (median: 28 wks) of gestational age with a birth weight of 478 to 1810 g (median: 1006 g). Thirty-three of them (26%) were delivered from mothers with premature rupture of the membrane, and 95 (74%) received antenatal treatment with antibiotics, and 59 (46%) received antenatal steroids. Funisitis was diagnosed in 17% (22/128) of these infants, CAM in 57% (73/128), and CLD in 33% (40/121). Mortality in this infant population was 5%.

Detection rates of *U. urealyticum* or *M. hominis* in umbilical cord, placenta and TA or GF. PCR was used to examine 86 umbilical cords, 83 placentas, and 84 TA or GF samples from 128 preterm infants for the presence of *U. urealyticum* or *M. hominis.* Four (4.7%) of the umbilical cord tissues were positive for *U. urealyticum*, and five (5.8%) were positive for *M. hominis*. Four (4.9%) of the placentas were positive for *U. urealyticum* and only one (1.2%) was positive for *M. hominis* (1.2%). Finally, 12 (14.3%) of the TA or GF samples were positive for *U. urealyticum* and four (4.8%) were positive for *M. hominis* (4.8%). Both *U. urealyticum* and *M. hominis* were detected in one TA sample. TA or GF showed the highest detection rate of *U. urealyticum* or *M. hominis* (17.9%), followed by umbilical cord (10.5%) and placenta (6.0%) (Table 2).

TABLE 1. Clinical characteristics of 128 preterm infants.

Gestation (wks)	28 (23~31)
Birth weight (g)	1006 (478~1810)
Premature rupture of the membrane	33/128 (26%)
Antenatal antibiotics	95/128 (74%)
Antenatal steroid	59/128 (46%)
Funisitis	22/128 (17%)
CAM	73/128 (57%)
CLD	40/121 (33%)[a]
Mortality	7/128 (5%)

[a] Seven infants died before 36 wks of gestational age.
CAM: chorioamnionitis, CLD: Chronic lung disease

TABLE 2.
Detection rates of *U. urealyticum* or *M. hominis* in umbilical cord, placenta, and TA or GF.

	U. urealyticum	*M. hominis*	*U. urealyticum* or *M. hominis*
Umbilical cord (n=86)	4 (4.7%)	5 (5.8%)	9 (10.5%)
Placenta (n=83)	4 (4.9%)	1 (1.2%)	5 (6.0%)
TA or GF (n=84)	12 (14.3%)	4 (4.8%)	15 (17.9%)[a]

[a] Both *U. urealyticum* and *M. hominis* were detected in one sample of TA

Association between *U. urealyticum* or *M. hominis* presence in umbilical cord, placenta, and TA or GF and the development of funisitis, CAM, and CLD. All umbilical cords which were positive for *U. urealyticum* or *M. hominis* developed funisitis. In contrast, only 13% of the umbilical cords without *U. urealyticum* or *M. hominis* developed it. Statistically highly significant differences were identified between the presence and absence of *U. urealyticum* and *M. hominis* in terms of the development of funisitis ($p < 0.001$). Although there appeared to be 100% progression to CAM in the positive *U. urealyticum* placenta, there was no significant difference compared to the placenta without *U. urealyticum* ($p = 0.10$) because 55% of the placentas, which were negative for *U. urealyticum* developed CAM due to other causes. Only one placenta with *M. hominis* did not develop CAM. The development of CLD showed no significant differences between the presence and absence of *U. urealyticum* and *M. hominis* (Table 3).

TABLE 3. Association between *U. urealyticum* or *M. hominis* presence in umbilical cord, placenta, and TA or GF and the development of funisitis, CAM, and CLD.

A. Umbilical cord (n = 86)

	Number of funisitis cases	p value
Positive for *U. urealyticum* (n = 4)	4 (100%)	p < 0.001
Positive for *M. hominis* (n = 5)	5 (100%)	s p < 0.001
Negative for both (n = 77)	10 (13%)	

B. Placenta (n = 83)

	Number of CAM cases	p value
Positive for *U. urealyticum* (n = 4)	4 (100%)	p = 0.10
Positive for *M. hominis* (n = 1)	0 (0%)	p = 0.46
Negative for both (n = 78)	43 (55%)	

C. TA or GF (n = 82)

	Number of CLD cases	p value
Positive for *U. urealyticum* (n = 11) [a, b]	7 (64%)	p = 0.09
Positive for *M. hominis* (n = 4) [b]	1 (25%)	p = 0.54
Negative for both (n = 68) [a]	25 (37%)	

[a] The data for two samples of TA with *U. urealyticum* and without *U. urealyticum* could not be obtained because of death before 36 wks of gestational age.

[b] Both *U. urealyticum* and *M. hominis* were detected in one sample of TA

DISCUSSION

We used the PCR method to detect *U. urealyticum* and *M. hominis*, which have been suggested to be associated with intrauterine inflammation, to identify their specific genes, although the culture method is a useful tool to obtain much more information than the PCR method, such as the amount of the microbe, the life or death of the microbe, and the drug sensitivity of the microbe. Some studies have shown that this PCR method has high sensitivity and specificity for the detection of *U. urealyticum* and *M. hominis* (19, 26). We could obtain the result less than 5 hours, the time needed for extraction of DNA from tissue samples was around 90 minutes, amplification by PCR took around 2 hours and 30 minutes, and 30 minutes was needed for the separation of the reaction PCR products by agarose gel electrophoresis. This is less than the time needed to detect *U. urealyticum* and *M. hominis* with the culture method. We therefore chose PCR analysis for rapid detection of *U. urealyticum* or *M. hominis* in umbilical cord, placenta, and TA and GF samples from preterm infants (See MATERIALS AND METHODS and Fig).

As it has been demonstrated that intrauterine inflammations such as CAM are present in most cases in which birth occurs at less than 30 wks of gestational age (7), infants born at < 32 wks of gestation were enrolled in this study. Not surprisingly, CAM was detected in 57% of the enrolled infants, who comprised our patient population and were born at 2 perinatal centers in Japan. Since only 17% had funisitis, it was clear that CAM and funisitis do not occur simultaneously, although both of them are recognized as histological markers of intrauterine inflammation (12, 18, 21, 22, 28). We speculated from this finding that CAM and funisitis may be caused through different routes. According to our hypothesis, vaginal organisms ascended first into the choriodecidual space, where inflammation of the chorioamuniotic membrane would progress to CAM. For the development of funisitis, on the other hand, the umbilical cord would become inflamed after organisms had crossed the chorioamuniotic membrane into the amniotic fluid.

U. urealyticum and *M. hominis* are common genital bacteria recognized as the cause of preterm spontaneous labor (7, 13). In a number of studies, *U. urealyticum* was isolated from placentas, and TA or GF, that is, amniotic fluids, of preterm infants (4, 13, 24, 25, 30, 31, 32), but no reports have been published yet about the isolation of *U. urealyticum* from umbilical cords of preterm infants. Furthermore, little is known about the involvement of *M. hominis* in the development of CAM and funisitis. We therefore determined the detection rates of *U. urealyticum* and *M. hominis* not only in placentas, and TA or GF, but also umbilical cords. *U. urealyticum* was isolated in 4.9% of all samples from the placentas and in 14.3% of those from TA or GF. These detection rates were low in comparison to the 10 to 60% rates of previous studies (4, 24, 30, 32). The exact reasons for this difference are not known, but race may be one of the reasons, because black women have CAM and preterm delivery more frequently than others (4, 7, 23, 24). *M. hominis* was detected in both placenta and TA or GF, but at a lesser frequency than that for *U. urealyticum*. To the best of our knowledge, ours is the first to detect *U. urealyticum* and *M. hominis* at almost the same rates (4.7% and 5.8%, respectively) in umbilical cords associated with preterm delivery.

Our results shown in Table 2 demonstrate that *U. urealyticum* and *M. hominis* were very rarely detected together in the three types of specimens we studied, because both of them were detected in only one TA sample. Wang et al. recovered both *U. urealyticum* and *M. hominis* in the TA and GF of only 3/43 preterm infants with CLD (30). These findings lead us to speculate that *U. urealyticum* and *M. hominis* might be colonized independently, although the sample size of our and other studies was too small to draw any definite conclusions.

There is increasing evidence implicating antenatal intrauterine infection in the pathogenesis of CLD of prematurity (4, 6, 24, 25, 30, 31). We hypothesized that intrauterine infection would affect not only the fetus, but also the umbilical cord and placenta. In order to determine the association between *U. urealyticum* and *M. hominis* presence and the development of funisitis, CAM, and CLD, we therefore prospectively investigated whether the presence of *U. urealyticum* or *M. hominis* leads to the development of funisitis, CAM, and CLD. We found that umbilical cords with *U. urealyticum* or *M. hominis* were significantly associated with the development of funisitis (Table 3). We believe that infected umbilical cords are the source of inflammation because it has been clarified that funisitis is associated with fetal inflammatory response syndrome (12, 18, 21, 22), but there have been no reports to this effect. Our data are thus the first to suggest that *U. urealyticum* and *M. hominis* may constitute a source for the development of funisitis. As funisitis is one of the causes of preterm labor (8, 12, 32), the antibiotic treatment of *U. urealyticum* and *M. hominis* may be useful for prevention of preterm deliveries. The results shown in Table 3 demonstrate

there was no significant correlation between the presence of *U. urealyticum* and *M. hominis* in placenta and the development of CAM, which may therefore be caused by pathogens other than *U. urealyticum* and *M. hominis*. However, we detected only four cases positive for *U. urealyticum* and one for *M. hominis* in the placentas, while all placentas positive for *U. urealyticum* developed CAM. Larger study populations are needed to yield a definite clarification of this association.

To examine the association between *U. urealyticum* and *M. hominis* infection and CLD, we used both TA and GF of preterm infants because TA could only be obtained by from the fetal lung fluid of infants who were ventilated, whereas GF was readily obtained even from infants that were not ventilated. In contrast to a number of previous studies including two major meta analyses (25, 31), our results showed no significant association between *U. urealyticum* and *M. hominis* presence in TA and GF and the development of CLD. We suggest that *U. urealyticum* and *M. hominis* infection may not be a dominant cause of CLD, which is caused by multiple factors (1, 20, 23, 27). However, the detection rates of *U. urealyticum* in this study were low compared to those previously reported. A larger Japanese study population is therefore needed to make this association clear.

In conclusion, we used the PCR method to identify the specific gene of *U. urealyticum* and *M. hominis* and detected *U. urealyticum* and *M. hominis* in the umbilical cord, placenta, or TA or GF of only 5-20% of Japanese preterm infants. Ours is the first study to demonstrate that *U. urealyticum* and *M. hominis* presence in uterus is associated with the pathogenesis of funisitis, but that *U. urealyticum* and *M. hominis* infection are not related with CAM or CLD. More large-scale studies will be needed to confirm our results and to clarify the implications of intrauterine infection by *U. urealyticum* and *M. hominis* for preterm delivery and the development of neonatal diseases such as CLD.

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