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To cite this article: Marian Kacerovsky, Roberto Romero, Lenka Pliskova, Radka Bolehovska, Helena Hornychova, Adela Matejkova, Hana Vosmikova, Ctirad Andrys, Martina Kolackova, Piotr Laudański, Vera Pelantova, Bo Jacobsson & Ivana Musilova (2019): Presence of *Chlamydia trachomatis* DNA in the amniotic fluid in women with preterm prelabor rupture of membranes, The Journal of Maternal-Fetal & Neonatal Medicine, DOI: [10.1080/14767058.2019.1640676](https://doi.org/10.1080/14767058.2019.1640676)

To link to this article: <https://doi.org/10.1080/14767058.2019.1640676>



Accepted author version posted online: 04 Jul 2019.
Published online: 15 Jul 2019.



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Presence of *Chlamydia trachomatis* DNA in the amniotic fluid in women with preterm prelabor rupture of membranes

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ABSTRACT

Objective: The primary aim of this study was to assess the rate and load of amniotic fluid *Chlamydia trachomatis* DNA and their associations with intra-amniotic infection and intra-uterine inflammatory complications in women with preterm prelabor rupture of membranes (PPROM). The secondary aim was to assess the short-term morbidity of newborns from PPRM pregnancies complicated by amniotic fluid *C. trachomatis* DNA.

Methods: A retrospective study of 788 women with singleton pregnancies complicated by PPRM between 24+0 and 36+6 weeks of gestation was performed. Transabdominal amniocenteses were performed at the time of admission. *C. trachomatis* DNA in the amniotic fluid was assessed by real-time polymerase chain reaction using a commercial AmpliSens® *C. trachomatis*/*Ureaplasma*/*Mycoplasma hominis*-FRT kit, and the level of Ct DNA was quantified.

Results: Amniotic fluid *C. trachomatis* DNA complicated 2% (16/788) of the PPRM pregnancies and was present in very low loads (median 57 copies DNA/mL). In addition to amniotic fluid *C. trachomatis* DNA, other bacteria were detected in 62% (10/16) of the *C. trachomatis* DNA-complicated PPRM pregnancies. Amniotic fluid *C. trachomatis* DNA was associated with intra-amniotic infection, histologic chorioamnionitis (HCA), and funisitis in 31%, 47%, and 33%, respectively. The presence of *C. trachomatis* DNA accompanied by *Ureaplasma* species in the amniotic fluid was associated with a higher rate of HCA than the presence of amniotic fluid *C. trachomatis* DNA alone. The composite neonatal morbidity in newborns from PPRM pregnancies with amniotic fluid *C. trachomatis* DNA was 31%.

Conclusion: The presence of *C. trachomatis* DNA in the amniotic fluid is a relatively rare condition in PPRM. Amniotic fluid *C. trachomatis* DNA in PPRM is not related to intensive intra-amniotic and intra-uterine inflammatory responses or adverse short-term neonatal outcomes.

ARTICLE HISTORY

Received 26 March 2019
Revised 30 June 2019
Accepted 3 July 2019

KEYWORDS

Amniotic fluid; intra-amniotic inflammation; intra-cellular bacteria; preterm delivery

Introduction

Preterm prelabor rupture of membranes (PPROM) is characterized by the rupture of fetal membranes and amniotic fluid leakage before the onset of regular

uterine activity prior to a gestational age of 37 weeks. PPRM affects ~3% of all pregnancies and leads to significant neonatal morbidity [1–3]. Despite intensive progress in the research on PPRM, its pathophysiology

and complications have not been fully elucidated. Therefore, PPROM is still associated with several dilemmas.

Microbial invasion of the amniotic cavity (MIAC) has been a complication in PPROM pregnancies for more than three decades [4–16]. There are two aspects associated with MIAC from a clinical viewpoint that are based on the concomitant presence or absence of intra-amniotic inflammation, namely, intra-amniotic infection and colonization [14,17]. MIAC is a very heterogeneous condition in terms of the types of bacteria involved, the microbial loads, and the intensity of the host's inflammatory response [7,12,18–21].

Aside from *Ureaplasma* species, which is the most common bacteria associated with MIAC in PPROM, a plethora of different extracellular cultivable, difficult to cultivate, or not-cultivable bacteria can be responsible for MIAC [5,12,18,20]. Even bacteria that cannot reproduce outside their host cell (obligatory intra-cellular bacteria) such as *Chlamydia trachomatis* have been revealed in amniotic fluid from PPROM [22,23].

Chlamydia trachomatis, which belongs to the family Chlamydiaceae, is the most commonly reported sexually transmitted bacteria that lead to infections of the human genital tract [24–26]. *Chlamydia trachomatis* infects the columnar epithelial cells and causes endocervical infection, which is mainly asymptomatic [24]. There is a solid body of evidence that has demonstrated a relationship between maternal genital *C. trachomatis* infection and an increased risk of spontaneous preterm labor with intact membranes and PPROM [27–32].

The direct presence of *C. trachomatis* is primarily evaluated by non-cultivation approaches, because *C. trachomatis* cultivation requires time-consuming and expensive tissue culture techniques [33,34]. The gold standard to assess MIAC in PPROM is aerobic and anaerobic cultivations of amniotic fluid along with a special cultivation for genital mycoplasma, and therefore information about amniotic fluid *C. trachomatis* as a cause of MIAC in PPROM is sparse [22,35]. In addition, there is a gap of knowledge relating to the role of amniotic fluid *C. trachomatis* in the development of intra-amniotic infection and acute histologic chorioamnionitis (HCA), funisitis, and subsequent neonatal outcomes.

Therefore, the primary aim of this study was to assess the rate and load of amniotic fluid *C. trachomatis* DNA and their association with intra-amniotic infection and intra-uterine inflammatory complications in women with PPROM. The secondary aim was to assess short-term morbidity of newborns from PPROM

pregnancies complicated by amniotic fluid *C. trachomatis* DNA.

Materials and methods

Patient population

A retrospective study of pregnant women with PPROM between the gestational ages of 24+0 and 36+6 weeks, and who were admitted to the Department of Obstetrics and Gynecology, University Hospital Hradec Králové between May 2008 and July 2018, was conducted. Women with singleton pregnancies who were at least 18 years of age were included in the study. Women with diabetes mellitus, gestational diabetes mellitus, preeclampsia, hypertension (either chronic or pregnancy-induced), chromosomal or structural fetal abnormalities, signs of hypoxia of the fetus, or significant vaginal bleeding were excluded from the study.

PPROM was diagnosed by an examination of the women using a sterile speculum to verify the pooling of amniotic fluid in the vagina. In clinically challenging diagnoses, the leakage of amniotic fluid was confirmed by the presence of insulin-like growth factor binding proteins (Actim PROM test; Medix Biochemica, Kauniainen, Finland) in the vaginal fluid.

Women with PPROM at <34 weeks of gestation were treated using tocolytics for 48 h, antibiotics, and corticosteroids to accelerate lung maturation, while those with PPROM beyond 34 weeks of gestation were treated with antibiotics only. The women with PPROM in this study were managed with two different approaches. Between May 2008 and December 2013, transabdominal amniocenteses were performed for research purposes only, and these results were not used for clinical management. During this period, women with PPROM were treated actively (except for those at <28 gestational weeks). Labor was induced, or an elective cesarean section was performed no later than 72 h after the rupture of the membranes, depending on the gestational age of the pregnancy, fetal status, and maternal serum C-reactive protein concentrations. Beginning in January 2014, the performance of transabdominal amniocentesis procedures and collection of information on the presence of MIAC and/or intra-amniotic inflammation became a routine part of the clinical management of women with PPROM at our department. Women with both intra-amniotic infection beyond the 28th gestational week were managed actively (labor was induced, or an elective cesarean section was performed after finalizing corticosteroid treatment within 72 h of membrane

rupture for pregnancies before 34 weeks of gestational age and within 24 h of membrane rupture for those beyond 34 weeks). The remaining women with PPROM were managed expectantly.

This study was approved by the institutional review board committee (July 2014; No. 201407 S14P). All women provided written informed consent and were self-reported as Caucasians. Amniotic, cervical, vaginal, cervicular fluid, and umbilical cord blood samples from the women in this cohort were used in our previously published studies.

Sample collection

Ultrasound-guided transabdominal amniocentesis was performed on admission before the administration of corticosteroids and antibiotics; ~1–2 mL of amniotic fluid was obtained. The aliquot of non-centrifuged amniotic fluid was immediately transported to the microbiology laboratory for real-time polymerase chain reaction (PCR) testing for the presence of *Ureaplasma* species, *Mycoplasma hominis*, and *C. trachomatis* DNA and the 16S rRNA gene. Remaining amniotic fluid was used for research purposes. Amniotic fluid sample was centrifuged for 15 min at 2000g to remove the cells and debris, the supernatant divided into aliquots and stored at -80°C until analysis. From February 2012, a total of 100 μL of non-centrifuged amniotic fluid was also used for the bedside assessment of interleukin (IL)-6 concentration.

Umbilical cord blood samples were obtained by venipuncture of the clamped umbilical cords immediately after the delivery of the neonates and prior to the delivery of the placenta using a Vacutainer blood collecting system. The samples of umbilical cord blood were immediately centrifuged and aliquoted, and the supernatants were stored at -70°C until assayed. The aliquots of umbilical cord blood serum from the women with *C. trachomatis* DNA in amniotic fluid were used to assess umbilical cord blood IL-6 concentrations.

The placenta, fetal membranes, and umbilical cord were fixed in 10% neutral buffered formalin at delivery. Tissue samples were obtained from the placenta (at least two samples), umbilical cord (usually one sample), and fetal membranes (at least two samples) and then routinely processed and embedded in paraffin. Sections of the tissue blocks were stained with hematoxylin and eosin. Four tissue blocks (two with the placenta, one with free fetal membranes, and one with basal decidua and chorionic villi) from

pregnancies complicated by amniotic fluid *C. trachomatis* DNA were used for further analysis of *C. trachomatis* status.

Chlamydia trachomatis detection in the amniotic fluid and placenta

DNA was isolated from the amniotic fluid and extracted from the paraffin-embedded placental and fetal membrane tissues with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and the MagCore Genomic DNA FFPE one-Step Kit (RBC Bioscience, Taipei, Taiwan) according to the manufacturer's instructions, respectively.

Real-time PCR was performed with a Rotor-gene 6000 instrument (Qiagen, Hilden, Germany) using a commercial AmpliSens[®] *C. trachomatis/Ureaplasma/M. hominis*-FRT kit (Federal State Institution of Science, Central Research Institute of Epidemiology, Moscow, Russia) to detect DNA from *Ureaplasma* species, *M. hominis*, and *C. trachomatis* in a single PCR tube. A PCR run for β -actin, which is a housekeeping gene, was included as a control for the presence of PCR inhibitors.

AmpliRun[®] *C. trachomatis* DNA control with a concentration of 16,000 copies/ μL (MBC012, vircell MICROBIOLOGISTS, Spain) and AmpliSens[®] *C. trachomatis/Ureaplasma/M. hominis*-MULTIPRIME-FRT kit (Federal State Institution of Science, Central Research Institute of Epidemiology) were used to create a calibration curve. The level of *C. trachomatis* DNA in copies/mL was determined by an absolute quantification technique employing an external calibration curve. The detection limit was 1–10 copies DNA/mL.

Amniotic fluid IL-6 concentrations

The stored amniotic fluid samples from women with positive *C. trachomatis* DNA were used for the assessment of IL-6 in amniotic fluid. IL-6 concentrations were assessed with a lateral flow immunoassay, Milenia[®] QuickLine IL-6, using the Milenia[®] POCScan Reader (Millenia BioTeC, GmbH, Giessen, Germany). The measurement range was 50–10,000 pg/mL, and the intra-assay and inter-assay variations were 12.1% and 15.5%, respectively.

Umbilical cord blood IL-6 concentrations

IL-6 levels were assessed by enzyme-linked immunosorbent assays using the Human IL-6 Quantikine kit (R&D Systems Inc, Minneapolis, MN, USA). The

sensitivity of the test was <0.70 pg/mL, and the inter-assay and intra-assay coefficients were $<10\%$.

Diagnosis of intra-amniotic infection, colonization, and fetal inflammatory response syndrome

Intra-amniotic infection was defined as an amniotic fluid IL-6 concentration ≥ 745 pg/mL measured by a point-of-care test (intra-amniotic inflammation) [36,37] along with the presence of bacteria in the amniotic fluid. Colonization was defined as the presence of bacteria in the amniotic fluid without intra-amniotic inflammation. Fetal inflammatory response syndrome (FIRS) was defined as the presence of an umbilical cord blood IL-6 concentration >11.0 pg/mL [38].

Diagnosis of histologic chorioamnionitis

The degree of polymorphonuclear leukocyte infiltration was assessed separately in the free membranes (amnion and chorion-decidua), chorionic plate, and umbilical cord according to the criteria given by Salafia et al. [39]. The diagnosis of HCA was based on the presence of inflammatory changes in the chorion-decidua (grades 3–4), chorionic plate (grades 3–4), umbilical cord (grades 1–4), and/or amnion (grades 1–4). The diagnosis of funisitis was based on the presence of inflammatory changes in the umbilical cord (grades 1–4).

Diagnosis of severe neonatal morbidity

Maternal and perinatal medical records were reviewed by two investigators (MK and IM). The data regarding short-term neonatal morbidity were reviewed for all newborns from PPRM pregnancies complicated by amniotic fluid *C. trachomatis*. “Composite neonatal morbidity” was defined in this study as follows: the need for intubation; and/or the need for nasal continuous positive airway pressure; and/or respiratory distress syndrome (defined by the presence of two or more of the following criteria: evidence of respiratory compromise, a persistent oxygen requirement for more than 24 h, administration of exogenous surfactant, and radiographic evidence of hyaline membrane disease); and/or transient tachypnea of the newborn (defined as any supplemental oxygen requirement during the first 6 h that does not increase during the subsequent 18 h, improvement in clinical conditions within 3–6 h, and chest radiographs that are either normal or show reduced translucency, infiltrates, and hyperinflation of the lungs); and/or bronchopulmonary

dysplasia (defined as an infant oxygen requirement at 28 days of age); and/or pneumonia (diagnosed by abnormal findings on chest X-rays); and/or retinopathy of prematurity (identified using retinoscopy); and/or intra-ventricular hemorrhage (diagnosis made using cranial ultrasound examinations according to the procedure of Papile et al. [40]); and/or necrotizing enterocolitis (defined as radiologic findings of either intramural gas or free intra-abdominal gas); and/or early (during the first 72 h of life) or late-onset (between the ages of 4 and 120 days) sepsis (either proven by bacterial culture or clinically strongly-suspected sepsis); and/or neonatal death before hospital discharge.

Statistical analysis

The demographic and clinical characteristics were compared using the non-parametric Mann–Whitney *U* test and presented as medians (interquartile range [IQR]) for continuous variables. Categorical variables were compared using the Fisher’s exact test or chi-square test, as appropriate, and presented as numbers (%). The normality of the data was tested using the D’Agostino and Pearson omnibus normality test. A Spearman correlation was used to assess the association between microbial loads and amniotic fluid IL-6 levels. A partial Spearman correlation was used to adjust the results for a potential confounder. Differences were considered statistically significant at $p < .05$. All *p*-values were from two-tailed tests, and all statistical analyses were performed using SPSS 19.0 for Mac OS X (SPSS Inc., Chicago, IL, USA) and with GraphPad Prism 8.0.0 for Mac OS X (GraphPad Software, La Jolla, CA, USA).

Results

Demographic and clinical characteristics of the study population

A total of 814 women with singleton pregnancies complicated by PPRM at gestational ages between 24+0 and 36+6 weeks were recruited during the study period. Twenty-six women were excluded because of amniocentesis failure. As a result, 788 women were included in the study. MIAC was found in 28% (223/788) of the study population. The most common bacterium found in the amniotic fluid was *Ureaplasma* species, which was identified in 18% (144/788) of the women.

Amniotic fluid *C. trachomatis* rate and microbial load

Amniotic fluid *C. trachomatis* DNA was present in 2% (16/788) of the women with PPROM between the gestational ages of 24 + 0 and 36 + 6 weeks. The maternal and clinical characteristics of the study group, according to the presence and absence of *C. trachomatis* DNA or other bacteria in the amniotic fluid, are shown in Table 1. *Chlamydia trachomatis* DNA alone was found in 38% (6/16) of the women with amniotic fluid *C. trachomatis* DNA. In the remaining 62% (10/16) women with amniotic fluid *C. trachomatis* DNA, the concomitant presence of other bacteria (eight with *C. trachomatis*+*Ureaplasma* species, one with *C. trachomatis*+*Ureaplasma* species+*Lactobacillus crispatus*, and one with *C. trachomatis*+*Leptotrichia amnionii*) was observed. The median load of amniotic fluid *C. trachomatis* DNA was 57 copies DNA/mL (IQR 21–200).

Placental and fetal membrane samples were available for 94% (15/16) of the women with amniotic fluid *C. trachomatis* DNA, the presence of *C. trachomatis* DNA in the placenta or fetal membranes was identified in 20% (3/15) of the women with amniotic fluid *C. trachomatis* DNA (one woman had the presence of *C. trachomatis* DNA in both the placenta and the fetal membranes, two women had the presence of *C. trachomatis* DNA just in the fetal membranes). The clinical characteristics of the women with amniotic fluid *C. trachomatis* DNA are shown in Table 2.

Amniotic fluid *C. trachomatis* DNA and intra-amniotic and intra-uterine complications

The presence of intra-amniotic infection, HCA, funisitis, and FIRS was found in 31% (5/16), 47% (7/15), and 24% (4/16) of the women with amniotic fluid *C. trachomatis* DNA, respectively.

No correlation was observed between the microbial loads of *C. trachomatis* DNA and levels of IL-6 in the amniotic fluid ($\rho=0.36$; $p=.17$). A positive correlation was identified between the microbial load of *Ureaplasma* spp. and amniotic fluid IL-6 levels ($\rho=0.88$, $p=.007$) in the women with both *C. trachomatis* DNA and *Ureaplasma* spp. in their amniotic fluid.

We divided the women with amniotic fluid *C. trachomatis* DNA into the following two subgroups: women with the amniotic fluid *C. trachomatis* DNA alone and women with amniotic fluid *C. trachomatis* DNA and concomitant bacteria. No differences in the rate of intra-amniotic infection, levels of amniotic fluid

IL-6, and loads of amniotic fluid *C. trachomatis* DNA were observed between the two groups (intra-amniotic infection: *C. trachomatis* alone, 17% [1/6] vs. *C. trachomatis* with other bacteria, 40% [4/10], $p=.59$; amniotic fluid IL-6: *C. trachomatis* alone median 226 pg/mL, IQR 94–934 vs. *C. trachomatis* with other bacteria, median 318 pg/mL, IQR 95–10 000, $p=.51$; load: *C. trachomatis* alone, median 81 copies DNA/mL, IQR 20–683 vs. *C. trachomatis* with other bacteria, median 57 copies DNA/mL, IQR 20–443, $p=.99$).

The women with amniotic fluid *C. trachomatis* DNA alone had lower rates of HCA and funisitis, but not FIRS, than the women with amniotic fluid *C. trachomatis* DNA and concomitant bacteria in the crude analysis (HCA: *C. trachomatis* alone 0% [0/6] vs. *C. trachomatis* with other 77% [7/9], $p=.007$; funisitis: *C. trachomatis* alone 0% [0/6] vs. *C. trachomatis* with other 56% [5/9], $p=.04$; FIRS: *C. trachomatis* alone 0% [0/6] vs. *C. trachomatis* with other 40% [4/10], $p=.23$) and after adjustment for the interval between PPROM and delivery (HCA: $p=.005$; funisitis: $p=.05$).

Amniotic fluid *C. trachomatis* DNA and selected aspects of short-term neonatal morbidity

Respiratory distress syndrome (19%, 3/16), intra-ventricular hemorrhage grades I–II (13% 2/16), and bronchopulmonary dysplasia (7%, 1/16) were observed among the newborns from PPROM pregnancies complicated by amniotic fluid *C. trachomatis* DNA. Consequently, composite neonatal morbidity was 31% (5/16). Neither early- nor late-onset sepsis was identified among the newborns from these pregnancies (Table 3).

Discussion

This retrospective study includes almost 800 women with PPROM between the gestational ages of 24 and 37 weeks, and all pregnancies with amniotic fluid *C. trachomatis* were scrutinized. The main findings of this study are as follow: (i) amniotic fluid *C. trachomatis* DNA, which had very low loads, complicated 2% of the PPROM pregnancies; (ii) amniotic fluid *C. trachomatis* DNA was accompanied by other bacteria (mainly *Ureaplasma* spp.) in more than half of the cases; (iii) *C. trachomatis* DNA in the placenta or fetal membranes was found in 20% of the women with amniotic fluid *C. trachomatis* DNA; (iv) amniotic fluid *C. trachomatis* DNA was associated with intra-amniotic infection and HCA in 31% and 47%, respectively; (v) amniotic fluid *C. trachomatis* DNA accompanied by other bacteria

Table 1. Maternal and clinical characteristics of pregnancies complicated by preterm prelabor rupture of membranes with respect to the presence and the absence of *C. trachomatis* DNA or other bacteria in the amniotic fluid.

Characteristic	<i>C. trachomatis</i> DNA in amniotic fluid (n = 16)	Other bacteria in amniotic fluid (n = 207)	No bacteria in amniotic fluid (n = 565)	p-value1	p-value2	p-value3
Maternal age [years, median (IQR)]	25 (22–34)	31 (26–34)	31 (28–35)	0.07	0.02	0.10
Primiparous [number (%)]	7 (44%)	86 (42%)	310 (55%)	1.00	0.45	0.01
Prepregnancy body mass index [kg/m ² , median (IQR)]	23.7 (19.0–27.2)	22.3 (19.9–25.0)	23.0 (20.7–26.7)	0.87	0.50	0.002
Smoking [number (%)]	6 (38%)	55 (27%)	71 (13%)	0.39	0.01	0.0001
Interval between PPROM and amniocentesis [hours, median (IQR)]	7 (3–10)	5 (3–10)	5 (3–10)	0.77	0.58	0.43
Gestational age at admission [weeks, median (IQR)]	32 + 5 (31 + 1 – 34 + 4)	32 + 4 (29 + 2 – 35 + 5)	33 + 6 (31 + 3 – 36 + 1)	0.69	0.28	<0.0001
Gestational age at delivery [weeks, median (IQR)]	33 + 5 (31 + 3 – 35 + 3)	32 + 6 (29 + 5 – 36 + 0)	34 + 1 (32 + 1 – 36 + 2)	0.40	0.47	<0.0001
Latency between PPROM and delivery [hours, median (IQR)]	38 (24–79)	43 (17–85)	41 (17–89)	0.65	0.60	0.80
CRP levels at admission [mg/L, median (IQR)]	7.7 (4.0–10.9)	7.3 (3.1–14.1)	5.0 (2.4–8.7)	0.99	0.12	<0.0001
WBC count at admission [$\times 10^9$ L, median (IQR)]	11.9 (11.0–14.0)	12.8 (10.8–15.6)	12.0 (10.0–14.4)	0.44	0.75	0.0009
Administration of antibiotics [number (%)]	16 (100%)	203 (98%)	550 (97%)	1.00	1.00	0.79
Administration of corticosteroids [number (%)]	13 (81%)	145 (70%)	364 (64%)	0.41	0.19	0.17
Spontaneous vaginal delivery [number (%)]	13 (81%)	148 (71%)	383 (68%)	0.57	0.42	0.34
Forceps delivery [number (%)]	0 (0%)	2 (1%)	5 (1%)	1.00	1.00	1.00
Cesarean delivery [number (%)]	3 (19%)	58 (28%)	170 (30%)	0.57	0.42	0.59
Birth weight [grams, median (IQR)]	1975 (1578–2280)	1910 (1310–2380)	2200 (1740–2530)	0.70	0.14	<0.0001
Apgar score < 7; 5 minutes [number (%)]	0 (0%)	18 (9%)	19 (3%)	0.37	1.00	0.004
Apgar score < 7; 10 minutes [number (%)]	0 (0%)	8 (4%)	9 (2%)	1.00	1.00	0.09

Abbreviations: CRP: C-reactive protein; IQR: interquartile range; PPROM: preterm prelabor rupture of membranes; WBC: white blood cells.

Continuous variables were compared using a non-parametric Mann–Whitney *U* test. Categorical variables were compared using the Fisher's exact test. Continuous variables are presented as median (IQR) and categorical as number (%). Statistically significant results are marked in bold.

p-value1 – the comparison between the women with *C. trachomatis* DNA in amniotic fluid and the women with other bacteria in amniotic fluid.

p-value2 – the comparison between the women with *C. trachomatis* DNA in amniotic fluid and the women without bacteria in amniotic fluid.

p-value3 – the comparison between the women with other bacteria than *Chlamydia trachomatis* DNA in amniotic fluid and the women without bacteria in amniotic fluid.

Table 2. Clinical characteristics of women with PPROM complicated by the presence of *C. trachomatis* DNA in the amniotic fluid.

Women	GA at admission	AF microbial load of Ct (copies DNA/mL)	AF IL-6 (pg/mL)	Bacteria found in AF	UCB IL-6 (pg/mL)	Ct in placenta and/or fetal membranes	HCA	Funisitis
1.	32 + 1	1.9×10^7	10,000	Ct++ <i>Ureaplasma</i> spp. (4.1×10^7 copies DNA/mL)	333.6	Yes	Yes	Yes
2.	36 + 2	2.1×10^3	134	Ct	1.7	No	No	No
3.	31 + 5	1.2×10^3	449	Ct++ <i>Ureaplasma</i> spp. (5.3×10^5 copies DNA/mL)	2.6	*	*	*
4.	34 + 0	2.0×10^2	108	Ct	2.2	Yes	No	No
5.	25 + 1	2.0×10^2	10,000	Ct++ <i>Ureaplasma</i> spp. (2.0×10^6 copies DNA/mL)	2.2	Yes	Yes	Yes
6.	35 + 3	1.7×10^2	158	Ct++ <i>Ureaplasma</i> spp. (7.0×10^4 copies DNA/mL)	42.6	No	Yes	Yes
7.	31 + 0	1.4×10^2	318	Ct	1.9	No	No	No
8.	28 + 0	6.9×10^1	10,000	Ct++ <i>Ureaplasma</i> spp. (4.0×10^4 copies DNA/mL)	67.0	No	Yes	Yes
9.	32 + 4	4.5×10^1	72	Ct++ <i>Ureaplasma</i> spp. (2.8×10^3 copies DNA/mL)	44.4	No	Yes	No
10.	33 + 1	2.1×10^1	740	Ct	0.3	No	No	No
11.	30 + 5	2.1×10^1	2166	Ct++ <i>Leptotrichia amnionii</i>	5.7	No	Yes	No
12.	31 + 6	2.1×10^1	1516	Ct	8.3	No	No	No
13.	32 + 6	2.1×10^1	50	Ct++ <i>Ureaplasma</i> spp. (1.2×10^3 copies DNA/mL)	2.4	No	No	No
14.	35 + 3	1.7×10^1	50	Ct	0.8	No	No	No
15.	32 + 5	1.4×10^1	186	Ct++ <i>Ureaplasma</i> spp. (4.0×10^4 copies DNA/mL)	4.1	No	Yes	Yes
16.	34 + 2	1.2×10^1	103	Ct++ <i>Ureaplasma</i> spp. (1.2×10^2 copies DNA/mL)+ <i>Lactobacillus crispatus</i>	2.7	No	No	No

Abbreviations: AF: amniotic fluid; Ct: *C. trachomatis*; HCA: acute histological chorioamnionitis; IL-6: interleukin-6; UCB: umbilical cord blood.

*Placenta was not available.

was related to a higher rate of HCA and funisitis; and (vi) composite neonatal morbidity affected one-third of the newborns from PPROM pregnancies complicated by amniotic fluid *C. trachomatis* DNA.

There is a solid body of evidence that amniotic fluid *C. trachomatis* can complicate PPROM [14,22,41]. The first observation of the rate of amniotic fluid *C. trachomatis* in women with PPROM was published more than 20 years ago [22]. Ville et al. [22] identified amniotic fluid *C. trachomatis* in 7% of the women with PPROM. The rate of women with amniotic fluid *C. trachomatis* DNA in our study was lower (2%). However, Lee et al. found amniotic fluid *C. trachomatis* in 1.2% of their mixed cohort that consisted of women with PPROM, spontaneous preterm labor with intact membranes, cervical incompetence with bulging membranes, and oligo- or polyhydramnios [35].

The rate of MIAC decreases with advancing gestational age [12,14]. However, found that amniotic fluid *C. trachomatis* DNA, irrespective of the presence of concomitant bacteria, was mainly detected in pregnancies with higher gestational ages ($6 \times$ and $4 \times$ between the gestational ages of $32 + 0$ and $33 + 6$ weeks, and $34 + 0$ and $36 + 6$ weeks, respectively). Only two women with amniotic fluid *C. trachomatis* DNA had a gestational age $<28 + 0$ weeks.

The use of specific real-time PCR to assess amniotic fluid *C. trachomatis* DNA provided an opportunity to reveal the burden of *C. trachomatis* DNA in the amniotic fluid samples. In a vast majority of the samples, the load of amniotic fluid *C. trachomatis* DNA was very low. The amount of *C. trachomatis* DNA $>10,000$ copies/mL was noted in only one woman, and only three woman had amounts of *C. trachomatis* DNA >1000 copies/mL. The median amniotic fluid microbial loads of *Ureaplasma* species in women with and without intra-amniotic infection were 3.2×10^7 and 4.0×10^4 , respectively [14]. Therefore, the loads of *C. trachomatis* DNA in amniotic fluid seem to be much lower than the average microbial load of *Ureaplasma* species in amniotic fluid. This finding is consistent with the fact that *Ureaplasma* species is an extracellular bacterium, while *C. trachomatis* is an obligate intra-cellular bacterium with a unique biphasic developmental cycle: infectious, metabolically active, extracellular elementary body and non-infectious, metabolically inert, and intra-cellular reticular body [24,26]. Therefore, the identification of only the elementary body in the amniotic fluid is highly likely.

Chlamydia trachomatis is a tryptophan auxotroph, because it is unable to synthesize tryptophan and must obtain this essential amino acid from the host [42,43].

Table 3. Clinical characteristics of newborns from PPROM pregnancies complicated by the presence of *C. trachomatis* DNA in the amniotic fluid.

Women	AF microbial load of Ct (copies DNA/mL)	Ct in placenta and fetal membranes	Composite neonatal morbidity	RDS	Need for intubation	n-CPAP (days)	IVH (grades 1–2)	BPD	EOS	LOS	ROP
1.	1.9×10^7	Yes	Yes	No	No	0	Yes	No	No	No	No
2.*	2.1×10^3	No	No	No	No	0	No	No	No	No	No
3.*	1.2×10^3	#	No	No	No	0	No	No	No	No	No
4.	2.0×10^2	Yes	No	No	No	0	No	No	No	No	No
5.	2.0×10^2	Yes	Yes	Yes	No	39	No	Yes	No	No	No
6.	1.7×10^2	No	No	No	No	0	No	No	No	No	No
7.	1.4×10^2	No	Yes	Yes	No	26	No	No	No	No	No
8.	6.9×10^1	No	Yes	Yes	No	21	No	No	No	No	No
9.	4.5×10^1	No	No	No	No	0	No	No	No	No	No
10.*	2.1×10^1	No	Yes	No	No	0	Yes	No	No	No	No
11.	2.1×10^1	No	No	No	No	0	No	No	No	No	No
12.	2.1×10^1	No	No	No	No	0	No	No	No	No	No
13.	2.1×10^1	No	No	No	No	0	No	No	No	No	No
14.	1.7×10^1	No	No	No	No	0	No	No	No	No	No
15.	1.4×10^1	No	No	No	No	0	No	No	No	No	No
16.	1.2×10^1	No	No	No	No	0	No	No	No	No	No

Abbreviations: AF: amniotic fluid; BPD: bronchopulmonary dysplasia; Ct: *C. trachomatis*; EOS: early-onset sepsis; IVH: intraventricular hemorrhage; LOS: late-onset sepsis; n-CPAP: nasal continuous positive airway pressure; RDS: respiratory distress syndrome; ROP: retinopathy of prematurity.

*Newborns from the PPROM pregnancies managed actively.

#The placenta was not available.

The induction of tryptophan catabolizing enzymes (indoleamine-2,3-dioxygenase) by interferon gamma ($\text{INF-}\gamma$) restricts the growth and development of *C. trachomatis* in human epithelial cells [42,43]. Therefore, $\text{INF-}\gamma$ is considered a major anti-chlamydial effector cytokine [44]. *Chlamydia trachomatis* strains that infect the genital tract have developed a mechanism to evade this defense strategy by using a subset of genes that enable the synthesis of tryptophan from indole [45,46]. As a result, the presence of indole in the genital tract, which is produced by some bacteria that belong under the umbrella of bacterial vaginosis (e.g. *Peptoniphilus*, *Porphyromonas*, and *Fusobacterium*), might help *C. trachomatis* survive regardless of $\text{INF-}\gamma$ driven tryptophan depletion [47]. In this study, we found the concomitant presence of *Ureaplasma* species in more than half of the cases with amniotic fluid *C. trachomatis* DNA. This observation is consistent with a previous study that showed a high prevalence of mixed *C. trachomatis* and *Ureaplasma parvum* infection in the genital tracts of healthy women attending their first prenatal visit [48]. However, based on the data from that experimental study, we could not elucidate whether the concomitant presence of *Ureaplasma* species was friend or foe. On one hand, *U. parvum* prompted and increased *C. trachomatis* growth under $\text{INF-}\gamma$ exposure with no impact on indoleamine-2,3-dioxygenase expression, which might suggest that the concomitant presence of *Ureaplasma* appears to be a *C. trachomatis* strategy to escape $\text{INF-}\gamma$ responsible tryptophan starvation [49]. On the other hand, the

concomitant presence of *U. parvum* in the absence of $\text{INF-}\gamma$ diminished *C. trachomatis* growth [49].

Chlamydia trachomatis has been found in the human placenta as early as the first trimester [50–52]. In addition, *C. trachomatis* is able to infect trophoblast cells and modulate their production of inflammatory mediators [53,54]. Rours et al. [52] detected *C. trachomatis* in 25% of the placental samples from pregnancies complicated by preterm labor at a gestational age of <32 weeks. However, there is a gap in information on the prevalence of *C. trachomatis* in the placenta from women with PPROM complicated by the presence of amniotic fluid *C. trachomatis* DNA. Therefore, we investigated whether women with amniotic fluid *C. trachomatis* DNA had bacteria in the placenta and fetal membranes. The presence of *C. trachomatis* DNA in the placenta and the fetal membranes was found in 7% (1/15; the woman with amniotic fluid *C. trachomatis* DNA load of 1.9×10^7 copies DNA/mL) and 20% (3/15; one woman with an amniotic fluid *C. trachomatis* DNA load of 1.9×10^7 copies DNA/mL, and two women with *C. trachomatis* DNA loads of 2.0×10^2 copies DNA/mL each) of women with amniotic fluid *C. trachomatis* DNA. The rate of placental *C. trachomatis* DNA in this study was much lower than that in the Rours et al. study [52]. However, our results should be interpreted with caution, because we assessed only 16 samples compared to 304 placentas in the above mentioned study [52].

Intra-amniotic infection, defined as MIAC accompanied with an elevation of the amniotic fluid levels of a selected inflammatory mediator (e.g. IL-6, matrix

metalloproteinase 8) owing to an intra-amniotic inflammatory response, is responsible for 52%–70% of cases of MIAC in PPRM [14,17]. However, the rate of intra-amniotic infection among women with amniotic fluid *C. trachomatis* DNA was much lower (31%), regardless of the presence or absence of concomitant bacteria. Similarly, the rates of HCA and FIRS in the women with amniotic fluid *C. trachomatis* DNA were lower than those observed in PPRM pregnancies complicated by MIAC [14,38,55,56]. In addition, no correlation between the loads of *C. trachomatis* DNA and IL-6 levels in amniotic fluid was identified. Taken together, these findings suggested that amniotic fluid *C. trachomatis* DNA is merely colonization that is not related to an intensive intra-amniotic, intra-uterine, and fetal inflammatory response measured by IL-6 levels in the amniotic fluid, rate of HCA, and rate of FIRS, respectively. However, IL-6 level cannot be an optimal marker to assess the intra-amniotic inflammatory response to amniotic fluid *C. trachomatis* DNA because *C. trachomatis* infection activates the inflammasome [51,57,58]. The inflammasome is a multiprotein complex that consists of cytoplasmic-based pattern recognition receptors, an adaptor protein, and caspase-1. The activation of the inflammasome, which plays an important role in intra-amniotic inflammation and preterm and term delivery, leads to the production of IL-1 β and IL-18 [59–65]. Models from the H8 and Sw 71 first trophoblast cell lines showed that *C. trachomatis* infection of the trophoblast cells led to the elevation of IL-1 β levels in both models; however, IL-6 levels were elevated in only the H8 cell line model, and the IL-6 levels were even lower in the Sw.71 cell line model [53]. Nevertheless, the complete absence of HCA, funisitis, and FIRS in the women with amniotic fluid *C. trachomatis* DNA alone supports the hypothesis that amniotic fluid *C. trachomatis* DNA predominantly represents amniotic fluid colonization without any intensive inflammatory response.

The gestational age of delivery has been considered a main determinant of short-term neonatal morbidity in PPRM [66–68]. However, knowing whether the presence of amniotic fluid *C. trachomatis* DNA is related to adverse neonatal outcomes is very important from a clinical point of view. The prevalence of composite neonatal morbidity, respiratory distress syndrome, and intra-ventricular hemorrhage grades I–II in the subgroup of newborns from the PPRM pregnancies with amniotic fluid *C. trachomatis* DNA was similar to the prevalence previously found in the subgroup of women with PPRM without MIAC or intra-amniotic inflammation (composite neonatal morbidity: 31% vs.

33%, respiratory distress syndrome: 19% vs. 25%, intra-ventricular hemorrhage: 13% vs. 12%) [14]. Only the rate of bronchopulmonary dysplasia was slightly higher (6% vs. 2%) [14]; however, this one case of bronchopulmonary dysplasia was identified in a newborn delivered at a gestational age of 27 + 0 weeks.

The first strength of this study is the large and homogeneous (all women were Caucasian) cohort of women with PPRM in whom amniotic fluid was assessed to identify the presence of *C. trachomatis* DNA. The second strength is the assessment of amniotic fluid *C. trachomatis* DNA by specific real-time PCR with a detection limit between 1 and 10 copies DNA/mL, which makes it possible to identify women with amniotic fluid *C. trachomatis* DNA at very low loads. This study also had some limitations. First, the presence of *C. trachomatis* DNA in the placenta and the fetal membranes, as well as short-term neonatal outcomes, were only evaluated in the pregnancies with the presence of amniotic fluid *C. trachomatis* DNA and not in the whole cohort of women. Second, data regarding long-term outcomes of the newborns from PPRM complicated by amniotic fluid *C. trachomatis* DNA were not investigated. Third, the intra-amniotic inflammatory response to *C. trachomatis* DNA was described by amniotic fluid IL-6 only; a broader panel of inflammatory cytokines and chemokines would provide a more precise description of host intra-amniotic inflammatory response to amniotic fluid *C. trachomatis* DNA. Last, the presence of MIAC was identified using techniques which did not include microbial culture. Since previous study has shown that amniotic fluid test results can be negative using non-cultivation approaches but positive by culture [12], the size of the group of the women with other bacteria than *C. trachomatis* in amniotic fluid might have been underestimated, while the group of the women with no bacteria in amniotic fluid might have been overestimated. In conclusion, amniotic fluid *C. trachomatis* DNA is relatively rare in PPRM. Amniotic fluid *C. trachomatis* DNA in PPRM is not associated with intensive intra-amniotic and intra-uterine inflammatory responses, or adverse short-term neonatal outcomes.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the project PERSONMED – Center for the Development of Personalized Medicine in

Age-Related Diseases [Reg. Nr. CZ.02.1.01/0.0/0.0/17_048/0007441].

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