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Original Article

Human miscarriage and infection in Tunisia: Role of *Mycoplasma hominis* and high *Waddlia* seroprevalence

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Abstract

Introduction: Miscarriage is one of the most common adverse pregnancy outcomes. The aim of this study was to investigate the relationship between miscarriage in humans and infections caused by zoonotic bacteria and genital pathogens.

Methodology: Cervicovaginal swabs and placenta samples from 132 women with miscarriage (patient group: PG), and cervicovaginal swabs from 54 women with normal pregnancy (control group:CG), were subjected to bacteriological culture and real time PCRs detecting *Coxiella burnetii*, *Brucella* spp, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma urealyticum*, *Chlamydia trachomatis*, *Waddlia chondrophila* and *Parachlamydia acanthamoebeae* DNA. Serology of *C. burnetii*, *C. trachomatis* and *W. chondrophila* was also performed.

Results: Placenta samples were positive for *E. coli*, *S. agalactiae*, *U. urealyticum*, *M. hominis* and *C. trachomatis* in 4.7%, 3.1%, 3.1%, 0.7% and 0.7% of cases, respectively. For cervicovaginal swabs, *M. hominis* was more frequently detected among PG than CG with a significant statistical difference (p = 0.02). *C. trachomatis* was detected in 3.3% and 5.5% among PG and CG, respectively. *U. urealyticum* DNA was detected with high percentages in the two groups. Samples from both groups showed negatives results for *C. burnetii*, *Waddlia*, and *Brucella* qPCRs. A high rate of *W. chondrophila* seroprevalence (42%) was noted with significant difference among women with early miscarriage.

Conclusions: *C.trachomatis*, *S. agalactiae* and *M. hominis* may play a role in miscarriage. However, the full characterization of the vaginal flora using other technologies such as NGS-based metagenomics is needed to clarify their role in miscarriage. Finally, further investigations should be performed to explain high *W. chondrophila* seroprevalence.

Key words: miscarriage; genital infection; zoonosis; genital mycoplasmas; *Waddlia*.

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Introduction

Miscarriage is the most common complication of pregnancy [1], it refers to pregnancy loss at less than 20 weeks' gestation. Several etiologies such as genetic, anatomic, endocrine, hematological and immunological factors have been reported for this devastating complication of pregnancy. Nevertheless, over half of the cases remain unexplained [1].Thus, 15% of early miscarriages and 66% of late miscarriages have been related to infections [2].

Zoonoses and genital infections in pregnancy are considered as risk factors for adverse pregnancy outcomes [2,3]. Pathogens may gain access to the placenta by two principal routes: ascending from the genital tract, and hematogenous dissemination through the placenta [4]. *Coxiella burnetii* and *Brucella* spp, two zoonotic agents have both been related to human miscarriage and may cause systemic infections in humans [3]. In Tunisia, although zoonotic infections are

frequently reported, no study has addressed their relation to adverse pregnancy outcome.

Various agents causing genital infections are associated with miscarriage. *Chlamydia trachomatis* is the most common sexually transmitted bacterial disease in the world, with an increasing prevalence [5]. In Tunisia *C. trachomatis* infection was previously reported with high prevalence among the general population (7.7%) [6]. Among the health problems caused by *C. trachomatis it* is well stablished that the bacteria can provoke pelvic inflammatory disease and tubal factor of infertility [5,7]. Additionally, in the final stage of pregnancy, it is also known that *C. trachomatis* can be an agent of preterm birth and premature rupture of membrane, but its role in miscarriage is suspected. Genital mycoplasmas were also reported as the agents of ascending uterine infections associated with adverse pregnancy outcomes such as Premature delivery and miscarriage [2,8,9]. Ultimately, their prevalence in Tunisia remains unknown among women, but they were reported with low rate among infertile men. *M. homnis, U. urealyticum, M. genitalium* were detected in 5.8%, 3.5% and 1.1% male partners of infertile couples, respectively using real time PCR [10].

In recent years, new *Chlamydia*-like organisms, *Waddlia chondrophila* and *Parachlamydia acanthamoebae*, were reported to be implicated in adverse pregnancy outcomes such as miscarriage, stillbirth and preterm labour [11,12]. Initially *W. chondrophila* was isolated from bovine fetuses [13]. Latera serological study supported an association with bovine abortion [12,14]. Inhuman pathogenicity, *W. chondrophila* seropositivity was correlated with miscarriage in pregnant women [13,15]. *P. acanthamoebae* might also represent an agent of miscarriage in humans, since parachlamydial sequences have been detected in human cervical smears as well as bovin genital tracts [16]. *Chlamydia*-like bacteria were not studied in human in Tunisia. However, a recent study carried out in cattle from different geographical regions of Sfax in Tunisia have shown that *P. acanthamoebeae* and *W. chondrophila* may cause bovine abortion [17].

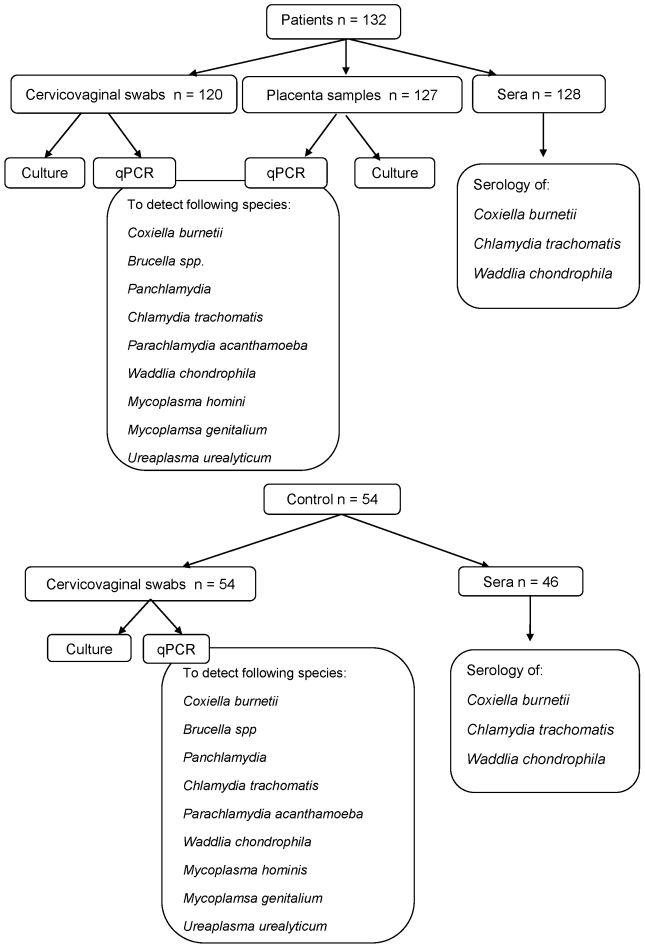
The aim of this study was to determine whether there is a relationship between miscarriage in humans and infections caused by some zoonotic bacteria and genital pathogens in patients from Tunisia.

Methodology

Patients

From March 2013 to April 2015, a total of 186 patients that agreed to participate were included in the study. The patient group (PG) included 132 women presenting with miscarriage or intra-uterine death during the first and second trimester of pregnancy. Patients were enrolled at maternity departments at Hedi Chaker Tertiary Health-care Hospital of Sfax, and Djebenianaand Mahres Secondary Health-care Hospitals of Sfax. Cervicovaginal swabs (n = 120), placenta samples (n = 127) and sera (n = 128) were obtained. The control group (CG) was composed of 54 women with normal pregnancy. They were recruited among women consulting for routine prenatal care in Family Planning Office in Sfax and Maternity Department at Hedi Chaker Tertiary Hospital of Sfax. Cervicovaginal swabs (n = 54) were collected for all patients while sera were obtained from 46 patients (8 sera were missing).

Figure 1. Flowchart of methodolgy.



The flowchart demonstrates the microbial investigations of both groups in this study.

Methods

Culture and qPCR of different samples were performed for both groups as described in Figure 1. From each patient, three cervicovaginal swabs were obtained: one was used for a smear test of vaginal fluid, the second was cultured for aerobic bacteria and yeasts on blood agar, blood-chocolate agar, VCN agar and Sabouraud agar incubated at 37°C for 24 hours and the third was eluted in 2-sucrose-phosphate based transport medium (2SP) transport medium. Placental samples were minced, homogenized and then cultured for aerobic and anaerobic bacteria on blood agar, blood-chocolate agar incubated in oxic and anoxic condition at 37°C for 48 hours. The identification of microorganisms was based on colony morphology and biochemical characterization using BioMerieux API kits (Marcy l'Etoile, France). Vaginal flora was evaluated on the smear using the nugent score [18].

Placenta sample and 2SP medium were stored at -80°C until their analysis by real time PCR (qPCR). DNA was extracted from those samples using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Initially, the extracted DNA was tested for human beta-globin gene to check for PCR inhibitors [19]. Each beta-globin positive DNA sample was subjected to several specific qPCR assays to detect the DNA of *Coxiella burnetii*, *Brucella* spp, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma urealyticum*. Chlamydial DNA was detected using a pan-*Chlamydiales* qPCR [20] and positive samples were subjected to qPCR specific for *Chlamydia trachomatis*, *Waddlia chondrophila* and *Parachlamydia acanthamoebeae*. The Ct cut-off values were fixed at 35 for all qPCRs. Primers and probes are listed in Table 1. The qPCR assays were performed on a CFX96TM real-time PCR cycler (Biorad, Hercules, USA) in a 20 µL final volume with Premix Ex Taq™ Probe qPCR (Takara, Shiga Japan). Positive samples in the pan-*Chlamydiales* qPCR were confirmed using an ABI7900 thermocycler in the center for Research on Intracellular Bacteria (CRIB), Institute of Microbiology, University of Lausanne, Switzerland. Positive controls for PCRs were provided from the Institute of Microbiology, Lausanne.

Table 1. Primers and probes used for qPCR.

Bacteria	Primers and probes	Oligonucleotide sequence (5' 3')	Target gene	Product size (bp)	Reference
C. burnetii	Forward	GTCTTAAGGTGGGCTGCGTG	IS1111	295	[33]
	Reverse	CCCCGAATCTCATTGATCAGC			
	Probe	**FAM** AGCGAACCATTGGTATCGGACGTT **BHQ**			
Brucella spp	Forward	TGCCGGAGCCTATAAGGACG	BCSP31	139	[34]
	Reverse	CGAGTGCCTTGCGTGTATCC			
	Probe	**FAM** ACCGACCCTTGCCGTTGCCGC **BHQ**			
M. hominis	Forward	TTTGGTCAAGTCCTGCAACGA	16S rRNA-encoding gene	101	[7]
	Reverse	CCCCACCTTCCTCCCAGTTA			
	Probe	**ROX** TACTAACATTAAGTTGAGGACTCTA **BHQ1**			
M. genitalium	Forward	GAGAAATACCTTGATGGTCAGCAA	MgPa	80	[35]
	Reverse	GTTAATATCATATAAAGCTCTACCGTTGTTATC			
	Probe	**HEX** ACTTTGCAATCAGAAGGT **BHQ1**			
U. urealyticum	Forward	CATTGATGTTGCACAAGGAG	Urease (UreD Subunit)	146	[7]
	Reverse	CGTGATTTTAATGTATCGGCTTTC			
	Probe	**FAM** TTGACCACCCTTACGAG **BHQ1**			
C. trachomatis	Forward	AACCAAGGTCGATGTGATAG	Cryptic plasmid	149	[36]
	Reverse	TCAGATAATTGGCGATTCTT			
	Probe	**ROX** CGAACTCATCGGCGATAAGG **BHQ2**			
Pan-Chlamydiales	Forward	CCGCCAACACTGGGACT	16S rRNA gene	207 to 215	[15]
	Reverse	GGAGTTAGCCGGTGCTTCTTTAC			
	Probe	**FAM** CTACGGGAGGCTGCAGTCGAGAATC **BHQ**			
W. chondrophila	Forward	GGCCCTTGGGTCGTAAAGTTCT	16S rRNA gene	101	[37]
	Reverse	CGGAGTTAGCCGGTGCTTCT			
	Probe	**FAM** CATGGGAACAAGAGAAGGATG **BHQ**			
P. acanthamoebaea e	Forward	CTCAACTCCAGAACAGCATTT	16S rRNA-encoding gene	103	[38]
	Reverse	CTCAGCGTCAGGAATAAGC			
	Probe	**FAM** TTCCACATGTAGCGGTGAAATGCGTAGATATG**BHQ**			

Sequences, target genes and the sizes of the different primers and probes used for qPCR in this study.

The serology of *C. burnetii* and *C. trachomatis* was performed in a Microimmunoflorescence assay (MIF) [21,22]. *C. burnetii* antigens were kindly provided by the Unité des Rickettsies Marseille. *C. trachomatis* antigens were prepared in our laboratory as previously described [21].

W. chondrophila antibodies were detected by enzyme-linked immunosorbent assay (ELISA) as described previously [23] in the Institute of Microbiology, Lausanne.

Statistical analysis

Data were analysed using SPSS 15.0 software. Student T-test was used to compare means, and χ^2 to compare proportions. p value < 0.05 was considered as significant.

Results

The mean age of patients was 31 years for PG [(18 – 43); V = 41.39] and 29 years for CG [19 – 45; V = 35.1]. Gestational period was from 5 weeks to 23 weeks in PG and from 5 weeks to 24 weeks in CG. All our patients were from the district of Sfax. A total of 174 cervicovaginal swabs were obtained from PG and CG and examined by Gram-staining. When compared to women of CG, women of PG had more frequently an intermediate vaginal flora with a significant statistical difference (Table 2).

Table 2. Microbiological characteristics of patient and control groups.

	Patient group n = 120	**Control group n = 54**	***p***
Flora			
Normal flora (score < 4)	51 (42.5)	43 (79.6)	< 0.001
Intermediate flora (score ≥ 4)	55 (45.8)	5 (9.2)	< 0.001
Bacterial vaginosis (score ≥ 7)	14 (11.6)	6 (11.1)	NS
Culture			
Streptococcus agalactiae (group B)	4 (3.3)	3 (5.5)	NS
Streptococcus pyogenes (group A)	1 (0.8)	0	NS
Streptococcus group D	2 (1.7)	0	NS
Streptococcus anginosus	1 (0.8)	0	NS
Enterococcus faecalis	1 (0.8)	3 (5.5)	NS
Escherichia coli	5 (4.2)	2 (3.7)	NS
Klebsiella pneumoniae	3 (2.5)	0	NS
Proteus mirabilis	1 (0.8)	0	NS
Providencia stuartii	1 (0.8)	0	NS
Pseudomonas aeruginosa	1 (0.8)	0	NS
Acinetobacter baumannii	1 (0.8)	0	NS
Staphylococcus aureus	1 (0.8)	0	NS
Candida albicans	8 (6.7)	5 (9.2)	0.05
Candida spp.	10 (8.3)	9 (16.6)	NS
qPCR			
C. burnetii	0	0	
Brucella spp	0	0	
M. hominis	11 (9.2)	1 (1.8)	**0.02**
M. genitalium	1 (0.8)	1 (1.8)	NS
U. urealyticum	46 (38.3)	22 (40.7)	NS
Pan-chlamydiales	4 (3.3)	3 (5.5)	
Chlamydia trachomatis	4 (3.3)	3 (5.5)	NS
Waddlia chondrophila	0	0	
Parachlamydia acanthamoebaeae	0	0	
Serology (126)			
C. burnetii	0	0	
C. trachomatis	1	1 (1.8)	NS
W. chondrophila	52 (43.3)	18 (33.3)	NS
≤ 12	43 (35.8)	9 (16.6)	**0.01**
13-24	9 (7.5)	9 (16.6)	

Statistical analyses of both groups. Intermediate flora was significantly higher in PG than CG. The statistical analyses of the microorganisms isolated from cervicovaginal swabs using culture and PCR. In the serology analyses prevalence of *Waddlia chondrophila antibodies* was significantly higher among patients with early miscarriage.

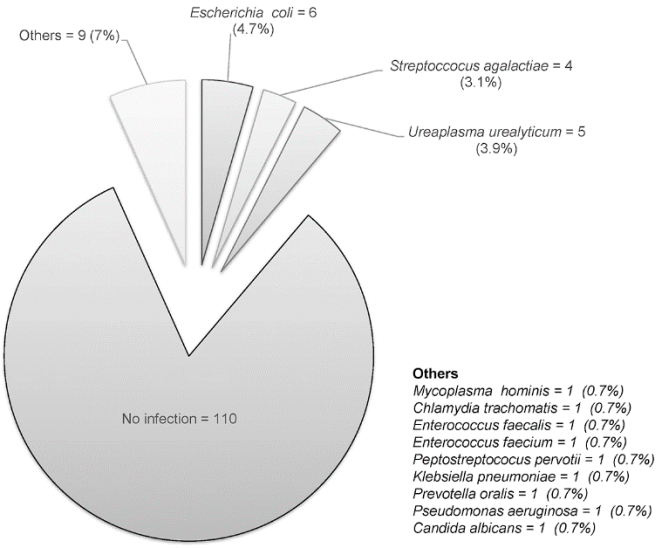
Cultures of cervicovaginal swabs (n = 120) were positive in 35 cases (29.2%) from PG and 20 cases (37%) from CG. All microorganisms isolated are listed in Table 2. The presence of *Streptococci*, Gram-negative bacilli and *Staphylococcu saureus* was not significantly higher in cervicovaginal swabs of PG compared to CG. However, *C. albicans* was more prevalent in the PG with $p = 0.05$.

Using qPCR, *U. urealyticum* was the most commonly detected bacterium in both groups without a statistical significant difference, whilst *M. hominis* infection rate was significantly higher in PG than CG. When studied according to Nugent score [18], *M. hominis* and *U. urealyticum* positivity was not associated with any type of flora in both groups. All positive samples for pan-*chlamydia* qPCR showed positive *C. trachomatis* qPCR. All other specific qPCRs were negative.

Placental samples were obtained by aspiration in 58 cases (43 %) among PG. For the 62 remaining cases, a vaginal curettage was performed. Overall positivity was 13.3% (17 cases were positive either by culture or by PCR). Microorganisms recovered are shown in Figure 2. When comparing to cervicovaginal swabs, culture positivity to the same microorganism was noted in 3 cases for *E. coli*, 2 cases for *Streptococccus agalactiae*, 1 case for *P. aeruginosa* and 4 for *U. urealyticum*. For the most detected pathogens using qPCR generally threshold cycles obtained for cervicovaginal swabs were less than those for placenta samples. All results of patients with positive placenta samples are shown in Table 3.

Regarding serological results, *C. burnetii* serology was negative in all cases. *C. trachomatis* IgG were detected in only one case from each group. The

Figure 2. Microorganisms isolated from placenta samples.



E. coli, *S. agalactiae* and *U. urealyticum* were the most frequent pathogens detected in the placenta samples using qPCR and standard culture.

Table 3. Details of patients with positive placenta sample either by culture and qPCR showing association with cervicovaginal swabs results.

N°	Gestational week	Aspiration	Vaginal curettage	Placenta culture	Placenta qPCR	Culture from cervicovaginal swabs	qPCR of cervicovaginal swabs
13	6	-	+	*E. coli*		*E. feacalis*	*C. trachomatis*
19	12	-	+	*Peptostreptococcus perovtii*			
22	10	+	-		*U. urealyticum/ C. trachomatis*		
30	8	-	+		*M. hominis*		
47	9	-	+	*E. coli*		*E.coli*	
52	10	+	-	*E. coli*		*E.coli*	*U. urealyticum*
72	7	+	-	*E. coli*			
76	17	-	+		*U. urealyticum*		*U. urealyticum*
88	21	-	+	*E. coli / E. faecium / P. oralis / candida spp*			
99	15	-	+	*S. agalactiae*		*S. agalactiae*	
100	9	-	+	*S. agalactiae*			
113	14	-	+		*U. urealyticum*	*K. pneumoniae*	*U. urealyticum*
118	17	-	+	*S. agalactiae/*	*U. urealyticum*		*U. urealyticum*
128	11	-	+		*U. urealyticum*	*Candida spp*	*U. urealyticum*
134	19	-	+	*P. aeruginosa / E. Feacalis / K. pneumoniae*		*P. aeruginosa / A. baumannii / C. albicans*	
139	9	+	-	*S. agalactiae*		*S. agalactiae*	

Comparison of microorganisms detected in placental samples and cervicovaginal swabs of all positives placenta samples. The culture positivity to the same microorganism was noted in 3 cases for *E. coli*, 2 cases for *S. agalactiae*, 1 case for *P. aeruginosa* and the PCR positivity to the same microorganism was detected in 4 cases for *U. urealyticum*.

seroprevalence of *W. chondrophila* was elevated in both groups, but it was significantly higher among patients with early miscarriage (Table 2).

Discussion

Several types of bacterial infections have been linked to adverse pregnancy outcomes [2,3,11,12]. The histological examination of fetal and placental tissues, and isolation by culture or genomic detection of the suspected infectious agent are significant argument in their implication in miscarriage [5,24]. In our study, women with miscarriage were compared to women with normal pregnancy outcome. Various pathogens were detected using different techniques as qPCR and culture.

Zoonotic infections, including *Brucella* and *C. burnetii* may cause severe complications during pregnancy [12]. In endemic regions, increasing rates of spontaneous abortion have been reported in pregnant women with clinical evidence of brucellosis [25] and Q fever [12]. In Tunisia, brucellosis is endemic in some regions but not Sfax [26]. Concerning Q fever, its epidemiology is unknown. In our laboratory, only 7 cases of Q fever were diagnosed during seven years (data not published). These findings cannot estimate the effects of *Coxiella burnetii* on miscarriage because of scarcity of data in Tunisia. So further studies are necessary to estimate it affects on miscarriage.

Many studies established an association between vaginal flora and different pregnancy complications. According to Hey *et al.*, intermediate microflora was associated with pregnancy loss between 16 and 24 weeks' gestation [8]. Guerra *et al.*, have demonstrated that the risk of adverse pregnancy outcome was in fact significantly increased in patients with abnormal flora or in those with bacterial vaginosis [27]. In our study, only intermediate flora, but not vaginosis, was more common among patient with miscarriage.

In our study, the culture results of placenta samples and cervicovaginal swabs were positive for many microorganisms. *Candida albicans* was more frequently detected in vaginal specimens from PG than CG with $p = 0.05$. In the literature, vaginal *Candida* colonization among pregnant women was not associated with adverse pregnancy outcome. Cotch et al., found an association of *Candida* colonization with *Trichomonas vaginalis*, group B streptococci, and aerobic *Lactobacillus* in pregnant women and not associated with adverse pregnancy outcome [28]. Furthermore, in placenta samples*C. albicans* was detected in only one case and it was associated to other bacterial pathogens which could be a vaginal contamination. In this study, *S. agalactiae* was isolated from placenta sample in 3.1% of cases, although, it was detected similarly in cervicovaginal swabs of the two groups which corroborates with the literature [29]. *Escherichia coli* was the most frequent bacteria isolated from placenta (4.7%) in our study. According a previous study, *E. coli* was isolated in 22 cases of midgestation fetal and placenta samples [29]. Several studies were interested to the vaginal colonization by *E. coli* and its role in preterm delivery essentially. Krohn *et al.*, showed that vaginal *E. coli* colonization was associated with delivery at < 34 weeks (RR = 1.7; 95%) and very low birth weight (RR = 1.9; 95%) [30]. The presence of *S. agalactiae* and *E. coli* in placenta samples may also prove that these pathogens play a significant role in adverse pregnancy outcomes.

A direct causal relationship between urogenital mycoplasmas and adverse pregnancy outcomes is difficult to demonstrate due to their high prevalence and the polymicrobial colonization of the genital tract. Previous studies found that genital mycoplasmas may play a causative role in spontaneous abortion and early pregnancy loss [31,32]. On another hand mycoplasmas were demonstrated to be more prevalent in patients with bacterial vaginosis. However, in our study, *M. hominis* and *U. urealyticum* were not associated with abnormal flora. Thus, significant high prevalence of *M. hominis* in cervicovaginal swabs of PG could be considered as a risk factor for pregnancy loss. Many studies showed that the colonization rates of *M. hominis* and *U. urealyticum* were most frequent in women with miscarriage and could be one of the causes leading to pregnancy loss [32]. According to Tavo *et al.*, the prevalence of *M. hominis* and *U. urealyticum* was highest among women who had undergone spontaneous abortions [2]. Other reports demonstrated that *U. urealyticum* and *M. hominis* were isolated from 48% of women with early pregnancy loss at the first prenatal consultation, compared with 8.2% of control women ($p < 0.0001$) [8]. *U. urealyticum* and *M. hominis* have been demonstrated to be associated to embryonic death in a study comparing 58 women with spontaneous abortion to 50 normal pregnant women [33]. The detection of bacteria from the placenta is the only approach to really differentiate true infections from vaginal contamination, as bacteria from the typical vaginal flora are rarely recovered from placental specimens [34]. In our study, *M. hominis* and *U. urealyticum* were the most common organisms detected in placenta samples in 0.7% and 3.9% respectively. For *M. genitalium*, our results are congruent with those previously reported. Although this bacterium could infect the upper genital

tract of women, it may not have a direct impact on the pregnancy outcome [2].

There is an increasing evidence that *Chlamydia* spp. and *Chlamydia*-like organisms may result in adverse pregnancy outcomes in humans and/or animals [11]. In this study, pan-*Chlamydiales*-PCR was used, which was specific to the detection ofspecies in the order, *Chlamydiales*. Positive samples were tested with specific qPCR for the detection of *Chlamydia trachomatis, Waddlia chondrophila* and *Parachlamydia acanthamoebea.* Infection with *C. trachomatis* in pregnancy has been associated with many adverse outcomes not only for mother but also for baby [11,35]. Starting as cervicitis, Chlamydial infection may ascend and infect the placenta or amniotic fluid [11,36]. The association of *C. trachomatis* and miscarriage was recently reported by the field [5]. In a study including 145 cases of human abortion and 261 controls, a positive association of *C. trachomatis* with miscarriage was observed either by serology or by qPCR on vaginal swabs as well as specific immunohistochemical analysis (IHC) performed in placenta [5]. The association of different techniques for the detection of the pathogens and/or their antibodies may confirm their role in miscarriage. In this study, MIF and qPCR were used to demonstrate the association between miscarriage and *C. trachomatis*. In cevicovaginal swabs, *C. trachomatis* was more frequently detected by qPCR in PG (3.3 %) than in CG (5.5%). Moreover, *C. trachomatis* DNA was detected in a placenta sample. However, *C. trachomatis* IgG were detected with the same proportion in PG and CG. The miscarriage is probably correlated to acute than chronic infection of *C. trachomatis.*

During the past decade, the order *Chlamydiales* has been enriched by the discovery of five new families: *Parachlamydiaceae, Simkaniaceae, Criblamydiaceae, Rhabdochlamydiaceae* and *Waddliaceae*. Although *Waddlia chondrophila* and *Parachlamydiaacanthamoebae* were associated to adverse pregnancy outcomes in many studies, they were not detected in our patients, neither in cervicovaginal swabs nor in placenta samples. The latest study reported by Baud *et al.*, in 2014 showed an association between *W. chondrophila* and miscarriage regardless of the duration of gestational. This positivity was confirmed by PCR and immunohistochemical analysis [37]. The same authors had already demonstrated this association using serology and early miscarriage in 2007 [12,15]. In our study, only early miscarriage was associated with high seroprevalence of *Waddlia*. Indeed, women with early miscarriage were more likely to have positive serology than women in their first trimester with uneventful pregnancy. The discrepant findings between high rates of seropositivity and negative qPCR results could be explained by a different hypothesis. Firstly, our patients could have their initial infections early in their pregnancy followed by miscarriage a few weeks later. With the exception of *Waddlia* DNA being detected in cattle in Sfax [17]. The epidemiological characteristics of *Waddlia* such as route of transmission, and mechanism of pathogenesis, are not fully known. Thus, a systemic infection via another portal of entry in our patients could not be ruled out. Further histological investigations of placenta samples could confirm these hypotheses. Finally, a possible cross reactivity between *W. chondrophila* and another species, explaining the qPCR negative results, could not be excluded. Since the specificity of this test was previously evaluated and has shown no interaction with *Chlamydia* and *Parachlamydia* [23], our findings suggest the possibility of a different circulating strain of *Waddlia* in Tunisia.

Conclusion

C. *trachomatis, M. hominis, U. urealyticum, S. agalactiae,* and *E. coli* were all detected in placental samples. Thus, we hypothesize that vaginal colonization may be a risk factor for fetus infection leading to spontaneous abortion. The presence of pathogens in the placenta may induce a maternal immune response to infection that could result to a miscarriage. The primary mechanism of pregnancy loss seems to be related to inflammatory process. Several vaginal infections were associated with vaginal flora disorder such us *M. hominis*. Thus, the evaluation of the vaginal flora composition using other technologies will help to clarify the relationship between the vaginal microbiome and adverse pregnancy outcomes and to establish the role of some pathogens in miscarriage.

The high seroprevalence of *W. chondrophila* found in our patients was unexpected. Other populations should be tested for *Waddlia* antibodies to explore further the epidemiology and pathogenic role of this bacterium.

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Authors' contributions
MS collected samples, performed culture, molecular testing, serology and data analysis and drafted the manuscript; KC performed serology of *Waddlia chondrophila* and data analysis; HS participated in molecular testing and in manuscript preparation; SK,LM, DL, MD, KC, KhC and HM : gynecologists that enrolled patients, collected samples and clinical data and participated in manuscript preparation; SA performed molecular analyses and data analysis in the Institute of Microbiology, Lausanne; AH supervised the study; GG supervised and supported the molecular analyses and serology of *Waddlia* in the Institute of Microbiology, Lausanne, and reviewed of the manuscript; AZ supervised the project, drafted the manuscript and reviewed the manuscript. All authors read and approved the final manuscript.

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