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Role of *Chlamydia trachomatis* and emerging *Chlamydia*-related bacteria in ectopic pregnancy in Vietnam

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1 *Short Report - Epidemiology and Infection*

2 **ROLE OF *CHLAMYDIA TRACHOMATIS* AND EMERGING *CHLAMYDIA*-RELATED**
3 **BACTERIA IN ECTOPIC PREGNANCY IN VIETNAM**

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31 **ABSTRACT**

32 In this case-control study, we investigated the seroprevalence and molecular evidence of
33 *Chlamydia trachomatis* and *Waddlia chondrophila* in ectopic pregnancies (EP) and uneventful
34 control (C) pregnancies in 343 women from Vietnam. Whereas presence of *Chlamydia*
35 *trachomatis* IgG was strongly associated with EP (adjusted Odds Ratio [aOR] 5.41; 95%
36 Confidence Intervals [95%CI] 2.58-11.32), its DNA remained undetected in all tubal lesions. We
37 confirmed an independent association between antibodies against *Waddlia* and previous
38 miscarriage (aOR 1.87, 95%CI 1.02-3.42). Further investigations are needed to understand the
39 clinical significance of *Waddlia* high seroprevalence (25.9% in C) in this urban population.

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42 **KEYWORDS**

43 *Chlamydia*-related bacteria, adverse pregnancy outcome, genital tract infection, intracellular
44 bacteria, ectopic pregnancy.

45

46 **SHORT REPORT**

47 Chlamydiae are obligate intracellular bacteria belonging to the *Chlamydiales* order ¹. *C.*
48 *trachomatis* is the most common bacterial cause of sexually transmitted infections worldwide ².
49 In women, 90% of *C. trachomatis* infections remain asymptomatic. However, if left untreated,
50 chlamydial infection can lead to scarring of uterine tubes, PID (pelvic inflammatory disease),
51 ectopic pregnancy and adverse pregnancy outcomes ^{2, 3}. *C. trachomatis* induced pathogenesis is
52 largely a result of chronic immunopathological reactions, most likely caused by persistent
53 infections ³.

54 *Waddlia chondrophila*, a *Chlamydia*-related bacterium, has recently been associated with both
55 animal and human adverse pregnancy outcomes, such as miscarriage ^{2, 4-6}. Its mode of
56 transmission and pathogenesis remains to be explored.

57 Since several *Chlamydia* spp. and *Chlamydia*-related bacteria colonize the cervicovaginal mucosa
58 ⁵⁻⁷, which may lead to tubal scarring and have been associated with adverse pregnancy outcomes
59 in humans, we thus investigated their role in ectopic pregnancies. Ectopic pregnancy, a condition
60 in which a fertilized egg settles and grows in a location other than the inner lining of the uterus,
61 occurs in 2% of all pregnancies and remains the leading cause of pregnancy-related death in the
62 first trimester of gestation ^{8, 9}.

63 A total of 347 patients were recruited at Tu Du Hospital, Hô Chi Minh City (Vietnam). The
64 "Ectopic Pregnancy" group (EP) included 177 women with an ectopic pregnancy treated by
65 laparoscopic salpingectomy. The "Control" group (C) included 166 women without any history
66 of previous ectopic pregnancy and who experienced an uneventful pregnancy. Blood samples,
67 fallopian tubes or placental biopsies were collected for each EP and C patient. Local ethical
68 committees of both hospitals (clinical part in Vietnam & experimental part in Switzerland)
69 approved the study protocol and all patients included in the study gave their written consent.

70 Serological status and epidemiological data were compared between patients with and without
71 ectopic pregnancies, or between patients with and without *Waddlia* positive serology by the
72 Pearson χ^2 test (or the Fisher exact test when indicated) for categorical variables. For continuous
73 variables, medians were compared by the Wilcoxon-Mann-Whitney test. Multivariate logistic
74 regressions were performed to identify factors independently associated with ectopic pregnancies
75 and miscarriages. All statistical analyses were performed using STATA 13.0 (Stata Corporation,
76 College Station, USA).

77
78 Sociodemographical data are presented in Table 1. All sera were tested for antibodies against
79 *Chlamydia trachomatis* (Table 1), as previously described^{1, 4, 6, 7, 10, 11}. *C. trachomatis* IgG
80 seroprevalence was 6.6% in the present Asian control population. Similar prevalence has been
81 described by others^{7, 12, 13}. *C. trachomatis* seroprevalence was higher for women who
82 experienced an ectopic pregnancy (24.9%) than for women with an uneventful pregnancy (6.6%,
83 $p < 0.001$).

84 For *Waddlia* and other *Chlamydia*-related bacteria Micro-immunofluorescence (MIF) were
85 performed as previously described^{1, 4, 6}. All immunofluorescence samples were read by two
86 independent observers and only congruent results were considered positive. Sera that exhibited
87 total immunoglobulin (Ig) titer $\geq 1:64$ were tested for IgG and IgM reactivity using corresponding
88 anti-human Fluorescein-labelled Ig (FluolineG or FluolineM, BioMerieux, Marcy l'Etoile,
89 France) and serial two-fold dilutions of sera. *Waddlia* IgG and IgM positivity cut-offs were $\geq 1:64$
90 and $\geq 1:32$, respectively¹. There was a significant association between total anti-*Waddlia*
91 antibodies detected by microimmunofluorescence and ectopic pregnancy ($p = 0.04$). However,
92 there was no statistical association with EP when anti-*Waddlia* IgG, or anti-*Waddlia* IgM, were
93 considered. *Waddlia* ELISA was performed as previously described¹⁴ and confirmed the

94 association between *Waddlia* seropositivity and ectopic pregnancies (p=0.046). Serological
95 evidence of human exposure to other *Chlamydia*-related bacteria, such as *Parachlamydia*
96 *acanthamoebae*, *Estrella lausannensis* and *Criblamydia sequanensis* were not associated with
97 ectopic pregnancies (Table 1). When all variables from Table 1 were considered (stepwise
98 logistic regression analysis), the only three independent factors associated with ectopic pregnancy
99 were a positive *C. trachomatis* serology (adjusted Odds Ratio [aOD] 5.41; 95% Confidence
100 Intervals [95%CI] 2.58-11.31), number of sexual partners (aOR 9.34; 95%CI 1.95-44.66) and
101 parity (aOR 2.69; 95%CI 1.94-3.75), which are well known risk factors for ectopic pregnancy^{8,9}.
102 Patients' characteristics according to their *C. trachomatis* serological status are shown in
103 Supplementary Table 1.

104 Women seropositive for *Waddlia* (n=100, 29.2%) were older (p=0.007) and experienced previous
105 miscarriages more frequently (p=0.005) than *Waddlia* negative women (Table 2). Association
106 between *Waddlia* seropositivity and miscarriage remains significant (aOR 1.87; 95%CI 1.02-
107 3.42) even after adjustment for age, parity, comorbidity and other serologies including *C.*
108 *trachomatis*. There was no statistical association between *Waddlia* positive serology and medical
109 comorbidities, gynaecological complaints during pregnancy, work status, number of lifelong
110 sexual partners or presence of pets at home.

111 There was no cross-reaction between *Waddlia* and *C. trachomatis* serologies, since 77 patients
112 (23.1%) were positive only for *Waddlia* IgG and 37 (11.1%) were positive only for *C.*
113 *trachomatis* IgG (Table 2). Only 18 patient (5.4%) were positive for both bacteria (p= 0.513).

114 Presence of *Waddlia*^{15, 16} and/or *C. trachomatis*⁷ DNA was tested in IgG positive patients. DNA
115 extraction was performed from a 2-centimeter piece of fallopian tube (EP) or placental (C) tissue
116 using Wizard SV genomic DNA purification kit (Promega Corporation, USA), and a pan-
117 *Chlamydiales* PCR was performed as previously described¹⁷. This Pan-*Chlamydiales* PCR is

118 able to detect up to 5 DNA copies per reaction and demonstrated similar performance compared
119 to specific *Chlamydiales* PCRs. Neither the 50 fallopian tubes nor the 43 placental samples with a
120 positive *Waddlia* and/or *C. trachomatis* serology were positive for *Waddlia* or *C.trachomatis*
121 DNA. All 20 control patients with a negative serology (10 from the "EP" group and 10 from the
122 "C" group) were also negative by PCR.

123 In summary, our data showed a strong association between *C. trachomatis* seropositivity and
124 ectopic pregnancy. However, neither the fallopian tubes nor placenta of women with positive
125 *Chlamydia* or *Waddlia* serologies demonstrated presence of respective bacteria, as also shown by
126 others¹². Moreover, IgG but not IgM antibodies were detected during ectopic pregnancies. Thus,
127 these results suggest that the persistence of the bacteria is not necessary to induce tubal damage,
128 and reinforces the role of an immuno-pathological process due to a previous chlamydial infection
129^{18, 19}. However, the physiopathology mechanism by which tubal scarring occurs without the
130 presence of bacteria is not yet fully understood^{12, 19}.

131 *Waddlia* IgG seroprevalence in the control group (25.9%) was higher than previously described
132 in other asymptomatic patients: 14.6% in Switzerland⁶, and 7.1% in London⁴. This difference
133 could be explained as a result of higher genetic susceptibility of Vietnamese to *Waddlia* infection
134 or greater exposure to the yet unknown source of *Waddlia* infection^{2,4,6}.

135 Whereas our study only identified a limited association of *Waddlia* with ectopic pregnancies
136 ($p=0.04$), we observed a strong correlation between previous history of miscarriage and positive
137 *Waddlia* serology ($p=0.005$). This was expected since *Waddlia* was previously reported as an
138 abortigenic agent in both animal and human populations^{2,4-6,19}.

139 A major limitation of the study was the absence of data concerning other potential confounding
140 factors for ectopic pregnancy (i.e. other infectious agent) and miscarriage (i.e. chromosomal
141 anomalies).

142 In conclusion, this study confirmed the serologic association of *C. trachomatis* with ectopic
143 pregnancy ⁸ and of *Waddlia* with miscarriage ^{4, 6}. Moreover, we showed an association between
144 anti-*Waddlia* antibodies and ectopic pregnancy using both immunofluorescence and ELISA.
145 Absence of *C. trachomatis* and *W. chondrophila* DNA in the fallopian tubes or placental tissues
146 suggests that immunopathological mechanisms rather than bacterial infection are involved in
147 ectopic pregnancy. Further investigations are needed to understand the high prevalence of
148 *Waddlia* in this Asian population and to precise its role in ectopic pregnancy.

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163 **CONFLICT OF INTERESTS**

164 There are no conflicts of interest.

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236 **Table 1:** Sociodemographical data and serologies according to pregnancy outcome.

237 * MOMP-R, CT pELISA (R-Biopharm, Darmstadt, Germany)

238 ** Similar p-value when doubtful were excluded

239 OD: optical density

240 MIF: micro-immunofluorescence

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243	Characteristics	Control (n=166)	Ectopic pregnancy (n=177)	p value
245	Age in years (years \pm SD)	28 \pm 5.2	30.3 \pm 6.4	0.0003
246	≥ 40	6 (3.6%)	17 (9.6%)	0.031
247	Nulliparity	111 (66.9%)	72 (40.7%)	<0.001
248	Comorbidity	19 (11.5%)	7 (4%)	0.013
249	Pets at home	53 (31.93%)	54 (30.51%)	0.777
250	Lifelong sexual partners (≥ 2)	2 (1.2%)	12 (6.8%)	0.012
251	<i>Chlamydia trachomatis</i> ELISA*			
252	Negative	153 (92.2%)	126 (71.2%)	<0.0001
253	Positive	11 (6.6%)	44 (24.9%)	
	Doubtful**	2 (1.2%)	7 (4%)	
254	<i>Waddlia</i> MIF			
	Total Ig $\geq 1/64$	49 (29.5%)	71 (40.1%)	0.04
255	IgG $\geq 1/64$	43 (25.9%)	57 (32.2%)	0.2
	IgM $\geq 1/32$	2 (1.2%)	3 (1.7%)	1
256	<i>Waddlia</i> ELISA OD	0.35 \pm 0.097	0.371 \pm 0.092	0.046
257	<i>Parachlamydia</i> IgG MIF	10 (6.0%)	19 (10.7%)	0.125
	<i>Estrella</i> IgG MIF	21 (12.7%)	35 (19.8%)	0.081
258	<i>Criblamydia</i> IgG MIF	4 (2.4%)	5 (2.8%)	1.0

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260 **Table 2:** Patient`s characteristics according to their *Waddlia* serological status

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Characteristics	Waddlia IgG negative (n= 243)	Waddlia IgG positive (n=100)	p value
Age in years (years \pm SD) ≥ 40	28.7 \pm 5.7 10 (4.1%)	30.4 \pm 6.3 13 (13%)	0.02 0.007
Nulliparity	137 (56.4%)	46 (46%)	0.08
Previous miscarriage	34 (14%)	24 (24%)	0.005
Comorbidity	18 (7.4%)	7 (7%)	1
Pets at home	166 (68.31%)	70 (70%)	0.759
Lifelong sexual partners (≥ 2)	11 (4.5%)	3 (3%)	0.765
<i>Chlamydia trachomatis</i> ELISA	37 (15.2%)	18 (18%)	0.513
<i>Parachlamydia</i> IgG MIF	19 (7.8%)	10 (10%)	0.525
<i>Estrella</i> IgG MIF	38 (15.6%)	18 (18%)	0.63
<i>Criblamydia</i> IgG MIF	5 (2.1%)	4 (4%)	0.292

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279 **Supplementary Table 1:** Patient`s characteristics according to their *C. trachomatis* serological
 280 status. *C. trachomatis* doubtful results were excluded from the analysis.

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283	Characteristics	<i>C.trachomatis</i> IgG negative (n= 279)	<i>C.trachomatis</i> IgG positive (n=55)	p value
285				
286	Age in years (years \pm SD) ≥ 40	29.2 \pm 5.8 16 (5.7%)	29.4 \pm 6.5 6 (10.9%)	0.768 0.228
287	Nulliparity	126 (45.2%)	29 (52.7%)	0.375
288	Previous miscarriage	40 (14.3%)	15 (27.3%)	0.027
289	Comorbidity	21 (7.9%)	3 (5.5%)	0.778
290	Pets at home	82 (29.4%)	22 (40%)	0.151
291	Lifelong sexual partners (≥ 2)	12 (4.3%)	2 (3.6%)	1
293	<i>Waddlia</i> IgG MIF	77 (27.6%)	18 (32.7%)	0.513
294	<i>Parachlamydia</i> IgG MIF	24 (8.6%)	5 (9.1%)	1.0
294	<i>Estrella</i> IgG MIF	36 (12.9%)	15 (27.3%)	0.012
294	<i>Criblamydia</i> IgG MIF	6 (2.2%)	3 (5.5%)	0.171

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ANNEX

METHOD

Subject

A total of 343 patients were recruited at Tu Du Hospital, Hô Chi Minh (Vietnam) in 2007. The "Ectopic pregnancy" group (EP) included 177 women with an ectopic pregnancy which was treated by laparoscopy. The "Control" group (C) included 166 women who experienced a normal pregnancy, without any history of previous ectopic pregnancy, preterm labor or miscarriage. One blood sample, respectively affected fallopian tube or placental biopsy were collected for each EP and C patient. Local ethical committees of both hospitals have approved the study protocol and all patients included in the study gave their written consent.

All blood samples have been centrifuged as soon as possible and only sera have been kept and stored frozen (at - 20 C). Both ectopic pregnancy product and placenta have also been stored frozen (at -20 C). Each sample has been anonymized with a code, according to the patient's group. (EP1, EP2, EP3,... for ectopic pregnancies or C1, C2, C3, ... for controls)

For each patient, a case report form has been filled to investigate for potential risk factors (date of birth, number of pregnancies, number of children, animals at home, number of previous sexual partner,...).

All the samples collected at Tu Du Hospital (Vietnam) have been sent by express frozen courier to the CHUV (Lausanne, Switzerland) where the experimental part of this research has been performed. Local ethical committees of both hospitals have approved the study protocol and all patients included in the study gave their written consent.

W. chondrophila and *C. trachomatis* micro-immunofluorescence assay

Immunofluorescence test were performed by using *W. chondrophila* strain ATCC VR-1470 as antigen¹. All immunofluorescence were read blindly by two independent observers and only congruent results were considered positive. Sera that exhibited total immunoglobulin (Ig) titer \geq 1:64 were tested for IgG and IgM reactivity using corresponding anti-human Ig fluorescein (FluolineG or FluolineM, BioMerieux, Marcy l'Etoile, France) and serial two-fold dilutions of serum. IgG and IgM positivity cut-offs were \geq 1:64 and \geq 1:32, respectively, as proposed for other chlamydia-like organisms. The sera collected for a previous study from two women identified to be positive, respectively negative, for chlamydia were used as positive and negative controls². All sera were also tested for IgG antibodies against *Chlamydia trachomatis* with the MOMP-R, CT pELISA (R-biopharm, Darmstadt, Germany). These ELISA use a recombinant peptid of the major outer membrane protein (MOMP) of *C. trachomatis* and showed a good sensitivity/specificity ratio in previous studies³.

DNA extraction and PCR

DNA extraction was performed for Women with *Waddlia chondrophila* IgG title $>$ 1:64. Practically, a two centimeter piece of ectopic or placental tissue was dissected and DNA extraction was performed using Wizard SV genomic DNA purification kit (Promega Corporation, USA).

A PAN-CHLAM was performed on the DNA extract from ectopic or placental tissues for the women with an IgG title $>$ 1:64 in order to correlate a positive serology against *Waddlia* with the presence of this bacterium in the tissues⁴. This Pan-*Chlamydiales* PCR is able to detect up to 5 DNA copies per reaction and demonstrated similar performance compared to specific *Chlamydiales* PCRs.

Waddlia chondrophila ELISA

ELISA was performed for *Waddlia chondrophila* based on a recent study protocol⁵.

Statistical analysis

Serological status and epidemiological data were compared between patients with and without ectopic pregnancies, or between patients with and without *Waddlia* positive serology by the Pearson χ^2 test (or the Fisher exact test when indicated) for categorical variables. For continuous variables, medians were compared by the Wilcoxon-Mann-Whitney test. Multivariate logistic regressions were performed to identify factors independently associated with ectopic pregnancies and miscarriages. All statistical analyses were performed using STATA 13.0 (Stata Corporation, College Station, USA).

RESULTS

1. Socio-demographic data and pregnancy outcomes:

Women experienced ectopic pregnancy (n=177) were compared with women experienced an uneventful pregnancy (n=166) according to their epidemiological information (Table 1). Risks factors for an ectopic pregnancy were maternal age (p = 0.031), parity (p < 0.001) and number of sexual partner (p=0.012). They were no statistical difference between our two groups in term of medical comorbidity, gynecological complains during pregnancy, work activity or animal possession.

When all variables from Table 1 were considered (stepwise logistic regression analysis), the only three independent factors associated with ectopic pregnancy were a positive *C. trachomatis* serology (adjusted Odds Ratio [aOR] 5.41; 95% Confidence Intervals [95%CI] 2.58-11.31), number of sexual partners (aOR 9.34; 95%CI 1.95-44.66) and parity (aOR 2.69; 95%CI 1.94-3.75)

2. Chlamydial serologies:

Waddlia chondrophila :

There was a borderline significant association between total anti-*Waddlia* antibodies There was a significant association between total anti-*Waddlia* antibodies detected by microimmunofluorescence and ectopic pregnancy (p=0.04). However, there was no statistical association with EP when anti-*Waddlia* IgG, or anti-*Waddlia* IgM, were considered. *Waddlia* ELISA was performed as previously described¹⁴ and confirmed the association between *Waddlia* seropositivity and ectopic pregnancies (p=0.046). and ectopic pregnancy (p=0.04 ; Table 2). However, there was no statistical difference when anti-*Waddlia* IgG or IgM were considered. A total of 36 and 5 women exhibited high anti-*Waddlia* IgG titers $\geq 1/256$ and anti-*Waddlia* IgM \geq

1/32, respectively. *Waddlia* ELISA and confirm the borderline association between *Waddlia* seropositivity and ectopic pregnancies ($p=0.046$).

Women positive for *Waddlia* ($n=100$, 29.2%) were older ($p= 0.007$) and experienced previous miscarriages more frequently ($p=0.005$) than *Waddlia* negative women (Table 2). There was no statistical difference between both groups in terms of medical comorbidity, gynecological complains during pregnancy, work activity, lifelong sexual partner or presence of pets at home.

Chlamydia trachomatis and other *Chlamydia* related bacteria :

C.trachomatis seroprevalence (Table 1) was higher for women who experienced an ectopic pregnancy (24.9%) than for women with an uneventful pregnancy (6.6%, $p < 0.001$). Serological evidence of human exposure to other *Chlamydia*-like organisms, such as *Parachlamydia acanthamoebae* ($p=0.125$), *Estrella lausannensis* ($p=0.081$), *Criblamydia sequanensis* ($p=0.187$) were not associated with ectopic pregnancies.

There was no cross-reaction between *Waddlia* and *C.trachomatis* serologies, since 77 patients (23.1%) were positive only for *Waddlia* IgG and 37 (11.1%) positives only for *C.trachomatis* IgG. Only 18 patient (5.4%) were positive for both bacteria ($p= 0.512$).

3. Tissues identification of bacteria :

None of the 50 Fallopian tube and 43 placenta with a positive *Waddlia* and/or *C.trachomatis* serology demonstrated presence of *Waddlia* or *Chlamydia* DNA. All 20 control patients with a negative serology (10 EP and 10 C) were also negative by PCR.

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