Role of Waddlia chondrophila Placental Infection in Miscarriage

Baud, D; Goy, G; Osterheld, M C; Croxatto, A; Borel, N; Vial, Y; Pospischil, A; Greub, G

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PRESENCE OF BOVINE *WADDLIA CHONDROPHILA* IN HUMAN PLACENTA SUPPORTS AN ASSOCIATION OF THIS EMERGING PATHOGEN WITH MISCARRIAGE

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RUNNING TITLE

Bovine *Waddlia* and human miscarriage

BIOGRAPHICAL SKETCH

David Baud, MD-PhD is an obstetrician specialised in materno-fetal medicine (MFM). His current research focuses on emerging infectious causes of adverse pregnancy outcomes and fetal therapy.
ABSTRACT

*Waddlia chondrophila* is an intracellular bacterium suspected to cause human and bovine abortion. We confirmed an association between *Waddlia* antibodies and human miscarriage, and demonstrated by PCR and immunohistochemistry its presence in placenta and/or genital tract of patients with miscarriages. These results suggest a possible role of *Waddlia* in miscarriage.
Approximately 25% of pregnant women will experience at least one miscarriage (1,2). However, a cause is identified in only 50% of cases (3,4). Intracellular bacteria, which do not grow on media routinely used to isolate human pathogens from clinical samples, represent possible agents of miscarriage of unexplained etiology (4,5).

*Waddlia chondrophila*, a *Chlamydia*-related bacteria first identified in bovine abortion, has been associated with human miscarriages (6,7). In a previous work based on 438 sera from women attending a Recurrent Miscarriage Clinic, *Waddlia* seroprevalence was higher for women who had sporadic (31.9%) and recurrent (33%) miscarriages than for women who had uneventful pregnancies (7.1%, p<0.001)(6).

To further investigate the role of *Waddlia* in human miscarriage, we studied 386 new patients with miscarriage or uneventful pregnancies. In addition to serology, we also performed PCR and immunohistochemistry to detect *Waddlia* in the placenta and vaginal samples.
THE STUDY

From 2006-2009, 386 women were prospectively enrolled at the obstetrical ward of the University Hospital of Lausanne (Table 1, adapted from (8)). The “miscarriage” group (n=125) included women diagnosed in the emergency gynaecology unit with an acute episode of miscarriage. The “control” group (n=261) included women attending labour ward with an uneventful pregnancy and no previous history of miscarriage, stillbirth or preterm labour. Age, black ethnicity and number of lifetime sexual partners were significantly different between both groups.

Immunofluorescence was performed using *W. chondrophila* as antigen as described earlier (6). Eighty-four women exhibited anti-*Waddlia* reactivity demonstrated by positive immunofluorescence against total immunoglobulin (Table 1). Among them, 67 and 6 women exhibited anti-*Waddlia* IgG titer $\geq 1:64$ or IgM $\geq 1:32$, respectively (FluolineG or FluolineM, BioMérieux, Marcy l'Etoile, France). IgG seroprevalence was higher in patients with miscarriage (23.2%) than that observed in women with an uneventful pregnancy (14.6%, p=0.044, Table 2). When women with and without anti-*Waddlia* antibody reactivity were compared, maternal age, contact with animals, education, number of previous partner, previously used contraceptive, living place (countryside/city) were not associated with a positive *Waddlia* serology. However, women from black ethnicity were more likely to exhibit anti-*Waddlia* antibodies (Odds ratio (OR) 3.15, 95% Confidence Interval (CI) 1.39-7.16) in the multivariate logistic regression model.

As previously reported, a significant association between miscarriage and *Chlamydia trachomatis* IgG seropositivity was observed (8). The association between *Waddlia* seropositivity and miscarriage remained significant even when adjusted for *C. trachomatis* serostatus and vice-versa. Indeed, in a multivariate logistic regression adjusted for both
variables, *C. trachomatis* and *W. chondrophila* seropositivity remained independently associated with miscarriage (OR 2.42, CI 1.22-4.79 and OR 1.87, CI 1.08-3.22, respectively).

After DNA extraction with QIAamp DNA Mini kit (Qiagen, Hilden, Germany), all vaginal swabs and placenta samples were tested by a 16S rRNA *Waddlia* specific real-time PCR as described previously (9). No PCR inhibition was observed. Thirty-two cases were positive, either in the vaginal swab (n=20) or in the placenta (n=12), no sample being positive in both types of samples. Ten of these positive PCR cases were miscarriages and, except one, all these cases were found positive by PCR in the vaginal swabs (Table 1&2). Two of these 10 “miscarriage” patients had positive IgG serologies against *Waddlia* (patient 36 and 140).

Patient 36 exhibited the highest IgG titer (1:1024) of all 386 subjects. Among control cases, 3 patients exhibited anti-*Waddlia* IgG titer ≥ 1:64. Among them, one showed both IgG and IgM positivity and one exhibited only anti-*Waddlia* IgM positivity with a titer of 1:32.

All placenta specimens were examined by a pedopathologist (Table 2, Figure 1A-D). The 10 miscarriage cases positive by PCR presented various histological features including deciduitis, chorioamnionitis, and presence of plasmocytes in the decidua compatible with chronic endometritis. Two of them exhibited a normal histology.

Placentas from the 32 PCR positive cases, as well as from 10 negative controls, were tested by immunohistochemistry for the presence of *Waddlia* using specific rabbit polyclonal antibody, as described elsewhere (10). Three placentas showed positive cells (Table 2, Figure 1E-H). Patients 523 and 535 were miscarriage cases positive by serology for total Ig but negative for IgG and IgM (Table 2). Patient 250 was a woman with an uneventful pregnancy with a positive PCR in vaginal swab but a negative serology.

Immunohistochemistry showed that *Waddlia* apparently infects mainly cells of the glandular epithelium. *Waddlia* was never found in endothelial cells (Figure 1).
Altogether, 5 cases presented strong evidences of a *Waddlia* infection, confirmed by at least two different diagnostic tests (Figure 2). Thus, two miscarriage cases presented a positive IgM and IgG serology (titer of 1:32) as well as positive PCRs. Three other cases (2 miscarriages and 1 control) were positive by PCR and presented a positive immunohistochemistry. Moreover, 31 additional cases presented some evidence of acute infection (i.e. either a positive PCR (n=27) or anti-*Waddlia* IgM reactivity (n=4)).
CONCLUSIONS

A higher seroprevalence in the miscarriage group confirmed the association between miscarriage and *Waddlia* seropositivity already observed in a previous work investigating a Londonian population (6). We also demonstrated here the presence of *Waddlia* DNA in the placenta and the vagina (vaginal swabs) of 32 patients, including 10 with miscarriage. Among these 10 miscarriage cases positive by PCR, 4 were considered as confirmed cases of infection since being also positive with another independent *Waddlia*-specific diagnostic test, i.e. serology (n=2) or immunohistochemistry (n=2). Presence of *Waddlia* in a human tissue indicates that this intracellular bacteria may grow and/or persist within placental cells and might damage the placenta (11).

The underlying mechanism of *Waddlia*-associated miscarriage may involve bacterial proteins, such as the heat-shock protein 60 (HSP60) or production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) (5).

The fact that *Waddlia* was detected in the vagina indicates that the infection might be of ascending origin and may follow vaginal colonization. However, no association between sexual activity, use of condom and positive *Waddlia* serology could be documented in a recent study, despite a 8.3% seroprevalence among 517 young Swiss men (12).

In this study, we mainly studied the presence of *Waddlia* in the genital region. However, entry could occur at another site. Indeed, DNA from *Waddlia* was also detected in sputa of patients suffering from pneumonia (9,13), and respiratory tract infection could disseminate to the uterus through the bloodstream. Contrarily to what was observed in our previous study (4), *Waddlia* seropositivity was not associated with contact with animals.

In conclusion, this prospective study confirmed an association between *Waddlia* seropositivity and miscarriage. Moreover, 4 among 125 subjects with miscarriage (3.2%) were found positive either by serology and PCR or by PCR and immunohistochemistry and were thus
considered as confirmed cases of infection, whereas a single *Waddlia* infection (0.4%) was
documented by two independent diagnostic tests in the control group without miscarriage
(p=0.04). Overall, these results suggest a strong association of *Waddlia* with human
miscarriage (6,7). When suspecting a *Waddlia*-associated miscarriage, we recommend
performing PCR on placenta and vaginal swabs as well as serology.
ACKNOWLEDGMENTS

We thank all midwives and doctors who participated to the sampling of this study. Their involvement was essential to the whole process, and they enthusiastically gave their time to provide information and samples. We thank Sebastien Aeby for technical help, Francoise Damnon, Karine Lepigeon and Andre Baud for computer assistance.

FUNDING

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CONFLICT OF INTERESTS

There are no conflicts of interest.
Reference List


Table 1. Characteristics of patients according to their miscarriage history. Adapted from (7,8).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control (n=261)</th>
<th>Miscarriage (n=125)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age in years (±SD)</strong></td>
<td>31.5 ± 5.0</td>
<td>33.3 ± 6.1</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Origin</strong></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>White</td>
<td>217 (84.8%)</td>
<td>69 (71.9%)</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>20 (7.8%)</td>
<td>21 (21.9%)</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>19 (7.4%)</td>
<td>5 (5.2%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>0 (0%)</td>
<td>1 (1.0%)</td>
<td></td>
</tr>
<tr>
<td><strong>Lifelong sexual partners</strong></td>
<td></td>
<td></td>
<td>0.031</td>
</tr>
<tr>
<td>1</td>
<td>58 (22.2%)</td>
<td>37 (29.6%)</td>
<td></td>
</tr>
<tr>
<td>2-3</td>
<td>43 (16.5%)</td>
<td>24 (19.2%)</td>
<td></td>
</tr>
<tr>
<td>4-6</td>
<td>45 (17.2%)</td>
<td>10 (8.0%)</td>
<td></td>
</tr>
<tr>
<td>&gt;6</td>
<td>36 (13.8%)</td>
<td>10 (8.0%)</td>
<td></td>
</tr>
<tr>
<td>Not answered</td>
<td>79 (30.3%)</td>
<td>44 (35.2%)</td>
<td></td>
</tr>
<tr>
<td><strong>Waddlia positive serology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ig total ≥ 1/64</td>
<td>47 (18.0%)</td>
<td>37 (29.6%)</td>
<td>0.010</td>
</tr>
<tr>
<td>IgG ≥ 1/64</td>
<td>38 (14.6%)</td>
<td>29 (23.2%)</td>
<td>0.044</td>
</tr>
<tr>
<td>IgM ≥ 1/32</td>
<td>5 (1.9%)</td>
<td>1 (0.8%)</td>
<td>0.669</td>
</tr>
<tr>
<td><strong>Waddlia PCR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal swab</td>
<td>11 (4.2%)</td>
<td>9 (7.2%)</td>
<td>0.226</td>
</tr>
<tr>
<td>Placenta</td>
<td>11 (4.2%)</td>
<td>1 (0.8%)</td>
<td>0.113</td>
</tr>
<tr>
<td><strong>Waddlia immunohistochemistry</strong></td>
<td>1 (0.4%)</td>
<td>2 (0.8%)</td>
<td></td>
</tr>
<tr>
<td><strong>C. trachomatis serology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG positive</td>
<td>19 (7.3%)</td>
<td>19 (15.2%)</td>
<td>0.018</td>
</tr>
<tr>
<td>IgA positive</td>
<td>10 (3.8%)</td>
<td>10 (8.0%)</td>
<td>0.091</td>
</tr>
<tr>
<td>Both IgG and IgA positive</td>
<td>7 (2.7%)</td>
<td>9 (7.2%)</td>
<td>0.037</td>
</tr>
</tbody>
</table>

*Defined as regular contact with an animal in the present and/or past (i.e pets)

*Not included in the statistical analysis
### Table 2. Characteristics of the cases of miscarriage positive for *Waddlia* by real-time PCR

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age</th>
<th>Ethnicity</th>
<th>Gravida</th>
<th>Parity</th>
<th>Number of pregnancy weeks</th>
<th>α-<em>Waddlia</em> total Ig titer</th>
<th>α-<em>Waddlia</em> IgG titer</th>
<th><em>Waddlia</em> PCR in vaginal swab</th>
<th><em>Waddlia</em> PCR in placenta</th>
<th>Histology</th>
<th><em>Waddlia</em> Immunohistochemistry</th>
<th>Possible other etiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>37</td>
<td>white</td>
<td>4</td>
<td>3</td>
<td>11.2</td>
<td>0</td>
<td>0</td>
<td>Negative</td>
<td>Positive</td>
<td>No inflammation</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>36</td>
<td>37</td>
<td>white</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>1:64</td>
<td>1:1024</td>
<td>Positive</td>
<td>Negative</td>
<td>Presence of PMN in the decidua</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>140</td>
<td>34</td>
<td>black</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>1:64</td>
<td>1:128</td>
<td>Positive</td>
<td>Negative</td>
<td>Presence of PMN and plasmocytes in the decidua compatible with a chronic endometritis</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>183</td>
<td>42</td>
<td>white</td>
<td>3</td>
<td>1</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>Positive</td>
<td>Negative</td>
<td>Presence of PMN in the decidua and glandular epithelium compatible with an early infection</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>305</td>
<td>29</td>
<td>white</td>
<td>5</td>
<td>0</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>Positive</td>
<td>Negative</td>
<td>Chorioamnionitis (presence of PMN in the chorion and extension of these inflammatory cells to the amnios)</td>
<td>Negative</td>
<td>Ureaplasma urealyticum in vaginal swab</td>
</tr>
<tr>
<td>357</td>
<td>19</td>
<td>asian</td>
<td>2</td>
<td>1</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>Positive</td>
<td>Negative</td>
<td>Rare lymphocyte in the decidua</td>
<td>Negative</td>
<td><em>B. abortus</em> antibodies</td>
</tr>
<tr>
<td>409</td>
<td>42</td>
<td>other</td>
<td>3</td>
<td>1</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>Positive</td>
<td>Negative</td>
<td>Presence of PMN in the subchorial fibrin and in glandular epithelium compatible with an early infection</td>
<td>Negative</td>
<td>Hyperthyroid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>459</td>
<td>29</td>
<td>white</td>
<td>3</td>
<td>1</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>Positive</td>
<td>Negative</td>
<td>Presence of PMN and of hemorrhagic necrosis</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>523</td>
<td>34</td>
<td>other</td>
<td>3</td>
<td>1</td>
<td>10.5</td>
<td>1:64</td>
<td>0</td>
<td>Positive</td>
<td>Negative</td>
<td>No inflammation</td>
<td>Positive</td>
<td>C. trachomatis antibodies (PCR negative)</td>
</tr>
<tr>
<td>535</td>
<td>35</td>
<td>white</td>
<td>3</td>
<td>1</td>
<td>10</td>
<td>1:64</td>
<td>0</td>
<td>Positive</td>
<td>Negative</td>
<td>Presence of PMN in the fibrin of the decidua compatible with an early infection</td>
<td>Positive</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE LEGEND

Figure 1.
Haematoxylin and eosin stained histological sections of the placentas: (A) patient n° 140: chronic endometritis with various inflammatory cells in the deciduas including plasmocytes (arrows), magnification 600x. (B) patient n°183: presence of polymorphonuclear cells (PMN) in a an endometrial gland, magnification 400x, (C) patient n°305: chorioamnionitis with PMN extending from the chorion to the amnios, magnification 200x, (D) patient n°535: Presence of PMN in the subchorial fibrin near the gestational sac, magnification 400x.

Immunohistochemistry positive cases showing the presence of W. chondrophila in placental tissue. A rabbit polyclonal antibody directed against Waddlia at a dilution of 1:12,000 was used. Detection was performed with the detection Kit (Dako ChemMate, Dako, Glostrup, Denmark) according to the manufacturer's instructions. By using the antibody diluent instead of the primary antibody, a negative control of each section was performed. Negative and positive control pellets were included as described previously (10). All highly positive cells were found in endometrial glands epithelium: (E) Patient n°535 (miscarriage), magnification 400x. (F) Patient n°535, magnification 600x (G) Patient n°523 (miscarriage), magnification 600x, (H) Patient n°250 (control), magnification 600x. AEC/peroxidase method, haematoxylin counterstain

Figure 2. Overall results and decision tree on which samples to screen. Please note that as many as 5 patients presented a confirmed infection, defined as a minimum of two independent positive Waddlia-specific tests. In addition, among all 386 patients, as many as 31 patients presented evidence of acute current Waddlia infection.
Figure 1.
Figure 2.