ORIGINAL ARTICLE

Impact of *Waddlia chondrophila* infection on pregnancy in the mouse

D. Baud[1], A. Ammerdorffer[1,2], Y. Buffe[1], M. Vouga[1], G. Greub[3] and M. Stojanov[1]

1) *Materno-fetal and Obstetrics Research Unit, Department Woman-Mother-Child, Lausanne University Hospital, Switzerland,* 2) *Department of Medical Microbiology and Infection Control, Laboratory of Immunogenetics, Vrije Universiteit Amsterdam, the Netherlands and* 3) *Institute of Microbiology, Lausanne University Hospital and University of Lausanne, Switzerland*

Abstract

The intracellular bacterium *Waddlia chondrophila*, which belongs to the *Chlamydiales* order, was found to be associated with miscarriage in humans. There is little to no knowledge regarding the mode of infection, impact on the neonate and pathophysiology of this emerging bacterium. We have previously shown that *W. chondrophila* induces a systemic infection, organ pathology and elicits T helper type 1-associated humoral immunity in a murine model of genital infection. In the present study, we took advantage of this model of infection to evaluate the impact of this bacterium on the mouse pregnancy. We used two routes of inoculation, vaginal and intrauterine, to introduce infection before and after mating. Our results show that genital infection by *W. chondrophila* did not have any significant impact on gestation length and maternal weight gain, nor on the number of offspring and their weight. This observation indicates that the mouse model of infection is not suitable to study the effect of *W. chondrophila* on pregnancy and alternative models of infection, including *in vitro* ones, should be used. Moreover, an indirect immunopathological mechanism activated by this bacterium should be further explored.

Keywords: Adverse pregnancy outcomes, chlamydiales, genital model, mouse, *Waddlia chondrophila*
Original Submission: 24 April 2019; **Revised Submission:** 14 November 2019; **Accepted:** 18 November 2019
Article published online: 23 November 2019

Corresponding authors: M. Stojanov, Department of Woman-Mother-Child, Lausanne University Hospital, Switzerland.
Corresponding authors: D. Baud, Department of Woman-Mother-Child, Lausanne University Hospital, Switzerland.
E-mails: **david.baud@chuv.ch (D. Baud), milos.stojanov@chuv.ch (M. Stojanov)**

Introduction

Waddlia chondrophila is an obligate intracellular bacterium that has previously been associated with miscarriage in humans and cattle [1,2]. Several studies performed in different geographical regions showed that women with high antibody titres against *W. chondrophila* were at a higher risk of miscarriage compared with control women [3,4]. Moreover, *W. chondrophila* was identified by quantitative PCR and immunohistochemistry in the genital tract and placenta of women with miscarriages [5]. Similar findings were identified in cattle [6]. Nevertheless, the exact mechanism of action of *W. chondrophila* that leads to this increased rate of miscarriage is still unknown.

Wheelhouse *et al.* have recently tried to imitate *W. chondrophila*-associated abortion in a bovine model of infection using intravenous inoculation of this bacterium into gravid cows [7]. Although the study did not confirm the role of *W. chondrophila* as an abortive agent, one animal showed pathogen-associated lesions in the placenta, suggesting a tropism towards reproductive organs. To study the role of *W. chondrophila* in miscarriages, we have previously developed a genital mouse-model of infection [8]. Intrauterine infection by *W. chondrophila* was able to induce a systemic infection and organ pathology, while eliciting a T helper type 1-associated humoral immunity, typical of *Chlamydia*-like bacteria.

Infection with *W. chondrophila* (later leading to persistent infection) may occur much earlier than conception. Pregnancy is characterized by a relative deficit in T helper type 1 cell-mediated immunity and so may lead to the reactivation of an induced persistent *W. chondrophila* infection, cell damage and miscarriage [9]. An autoimmune response to an epitope

https://doi.org/10.1016/j.nmni.2019.100619

(HSP60; 60-kDa heat-shock protein) shared by a fetal and chlamydial antigen is another hypothesis that may explain how *W. chondrophila* may be involved in abortion [9]. Finally, persistent infection may induce the secretion of pro-inflammatory cytokines, which have an abortigenic effect. To investigate these hypotheses, an animal model of infection is needed.

With this study, we investigate whether pre- and post-mating infection with *W. chondrophila* induces adverse pregnancy outcomes in mice.

Materials and methods

Waddlia chondrophila inocula

Waddlia chondrophila was cultured and purified as described previously [10]. In short, *Acanthamoeba castellanii* were used to grow *W. chondrophila* ATCC VR-1470 in 75-cm^2 culture flasks (Corning, Corning, NY, USA) at 32°C in peptone–yeast–glucose medium. Cell cultures were harvested and filtered through a 5-µm filter (Merck Millipore, Darmstadt, Germany). The concentration of the culture was determined by quantitative PCR as previously described [11]. For the mock control, the same procedure was performed without *W. chondrophila*.

Mice

All animal experiments were approved by the Office Vétérinaire du Canton de Vaud, Lausanne, Switzerland (authorizations 2090.0 and 2090.1) and performed according to our institutional guidelines for animal experiments.

Female and male C57BL/6 mice (8–12 weeks old) were obtained from Charles River Laboratories (L'Arbresle, France) and housed under pathogen-free conditions at the animal facility of the University of Lausanne (UNIL, Epalinges, Switzerland). Upon mating, mice were housed in single-use filter-top cages, and water and food were provided *ad libitum*.

One week before infection with *W. chondrophila*, all female mice were treated subcutaneously 0.1 µg oestradiol (Sigma-Aldrich Chemie, Buchs, Switzerland) and 2 mg medroxyprogesterone acetate (Depo-Provera, Pfizer, New York, NY, USA) to induce oestrus, during which mice are more susceptible to infections [12].

Infection before pregnancy: intrauterine infection procedure

Infection by the intrauterine route was performed as previously described [8]. Infections were performed in three separate experiments, for a total of 22 mice per group (*W. chondrophila* and mock controls). Infection was performed by injecting 100 µL mock control or *W. chondrophila* inoculum (~10^7 bacteria/µL). Two weeks after the introduction of the infection, two females were placed in a cage housing a male mouse. This was chosen according to our previous experiments, which demonstrated a maximum immune response and organ pathology at 2 weeks post-infection [8]. Vaginal plug assessments were completed on a daily basis and once observed, female mice were housed in separate cages to avoid cross-contamination. Blood samples (~50 µL) were collected from the tail before infection, 2 days before mating and at days 7 and 14 post-mating.

Infection during the pregnancy: vaginal infection procedure

In total, six experiments were performed, including 26 mock controls and 26 *W. chondrophila*-infected mice. Mating was carried out by placing two females with one male per cage. Vaginal plug assessments to identify sexual activity were completed daily. One week after the appearance of the vaginal plug, mice were infected vaginally with 20 µL mock or *W. chondrophila* inoculum (~5 × 10^7 bacteria/µL) via manual restraint and infection using a pipette and sterile filter tip. No anaesthesia was used for this procedure. fter infection, females were housed separately to prevent cross-contamination. Blood samples (~50 µL) were collected from the tail before infection, 2 days before mating and at days 7 and 14 post-mating.

Microimmunofluorescence assay

All blood samples collected before and during the infection, as well as at euthanasia, were used to isolate plasma using Microvette 500 µL Lithium-Heparin (Sarstedt, Numbrecht, Germany). Samples were centrifuged for 5 min at 2000 ***g*** and plasma was collected in a separate tube and stored at −20°C until further processing. Microimmunofluorescence was used to detect anti-*W. chondrophila* antibodies using a formalin-inactivated (0.3%) *W. chondrophila* strain ATCC VR-1470 as an antigen. Serum samples were used at dilutions of 1/32 and 1/64 in phosphate-buffered saline supplemented with 1% bovine serum albumin (Serva, Heildeberg, Germany). Goat anti-mouse IgG secondary antibodies conjugated to Alexa Fluor 488 dye (Thermo Fisher Scientific, Allschwil, Switzerland; diluted 1/1000) were used to detect the presence of IgG. Microimmunofluorescence analyses were evaluated by two independent readers. Weighted Cohen's κ coefficient was determined for each experiment to judge the agreement of the results and always ranked between 'good' and 'very good'.

Statistical analysis

GraphPad Prism 7 software (GraphPad Software Inc., San Diego, CA, USA) was used to analyse and present data. The data are expressed as mean (±standard error of the mean) unless

indicated otherwise. Differences between experimental groups were tested using the Mann–Whitney *U* test, and a p value of <0.05 was considered statistically significant.

Results

Vaginal versus intrauterine infection with *W. chondrophila*

We have previously used the intrauterine route of infection to evaluate the pathogenicity of *W. chondrophila*. Although this route is useful for pre-mating infection, it is not compatible with infection during gestation, as it would independently induce miscarriages. For this reason, pregnant mice were infected via the vaginal route.

Schematic representations of the pre- and post-mating infection procedures are depicted in Fig. 1(a). After co-habitation with male mice, female mice were evaluated daily for the presence of mating plugs. In our experimental scenario, the pregnancy rate of vaginally infected mice was 92.3% (n = 52), with no significant difference between mock and *W. chondrophila*-infected mice. The pregnancy rate was lower for intrauterine-infected mice, reaching 70.5% (n = 44). The observed difference could be explained by the difference in the invasiveness of the two routes of infection, as the catheter used for the intrauterine inoculation might cause damage to the reproductive organs. This reasoning is supported by the fact that mice failing to become pregnant were equally distributed between the mock-infected (n = 6) and *W. chondrophila*-infected (n = 7) groups.

Impact of pre- and post-mating infection with *W. chondrophila* on pregnancy

Similar serological results were seen at delivery for the two different routes of inoculation (Fig. 2(a)). We avoided blood sampling during gestation to minimize the stress on the pregnant mice. All mock-infected mice had a negative serology. Exposure to *W. chondrophila* did not affect the duration of the gestation compared with mock controls for both routes of infection (Fig. 2(b)). There was a significant difference in length of gestation between the two routes of infection, which could be again explained by the increased invasiveness of the intrauterine infection procedure, as mentioned above. The weight gain of the pregnant mice was similar for both mock-infected and infected mice infected via the intrauterine route (Fig. 2(c)). Moreover, infection by *W. chondrophila* did not affect the average number of pups per pregnant mouse (Fig. 2(d)), nor the average weight of pups from the same litter (Fig. 2(d)), regardless of the route of infection.

Discussion

Waddlia chondrophila has been linked to adverse pregnancy outcomes in humans and bovines [2,6]. To study the abortive role of this bacterium, we have previously developed a female mouse model of genital infection in which intrauterine inoculation of the bacterium caused a strong immune response and disseminated infection [8]. The present study aimed at evaluating the impact of *W. chondrophila* infection on reproductive success using the above-mentioned murine model of infection. We used the intrauterine route of inoculation to study the impact of infection before mating and the vaginal route to study the influence of post-mating infection. The latter was used to avoid the potential higher risk of abortion induced by the technical aspects of intrauterine inoculation in already pregnant mice. The intrauterine route of infection was used before mating to increase the number of bacteria reaching the uterine cavity, which we previously demonstrated to induce a

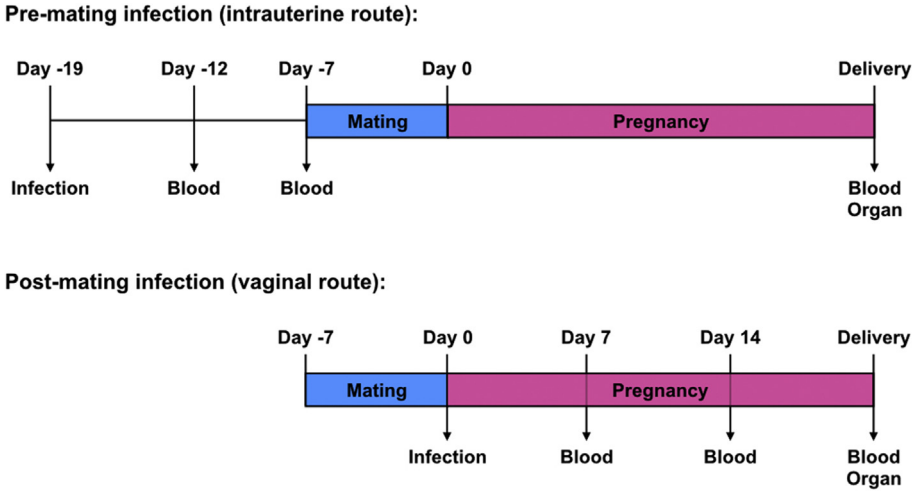

FIG. 1. Time course of vaginal and intrauterine infection models. Comparison of the two models of *Waddlia chondrophila* inoculation before mating (intrauterine infection) and after mating (vaginal infection). Time-points at which blood samples were collected are indicated by the arrows.

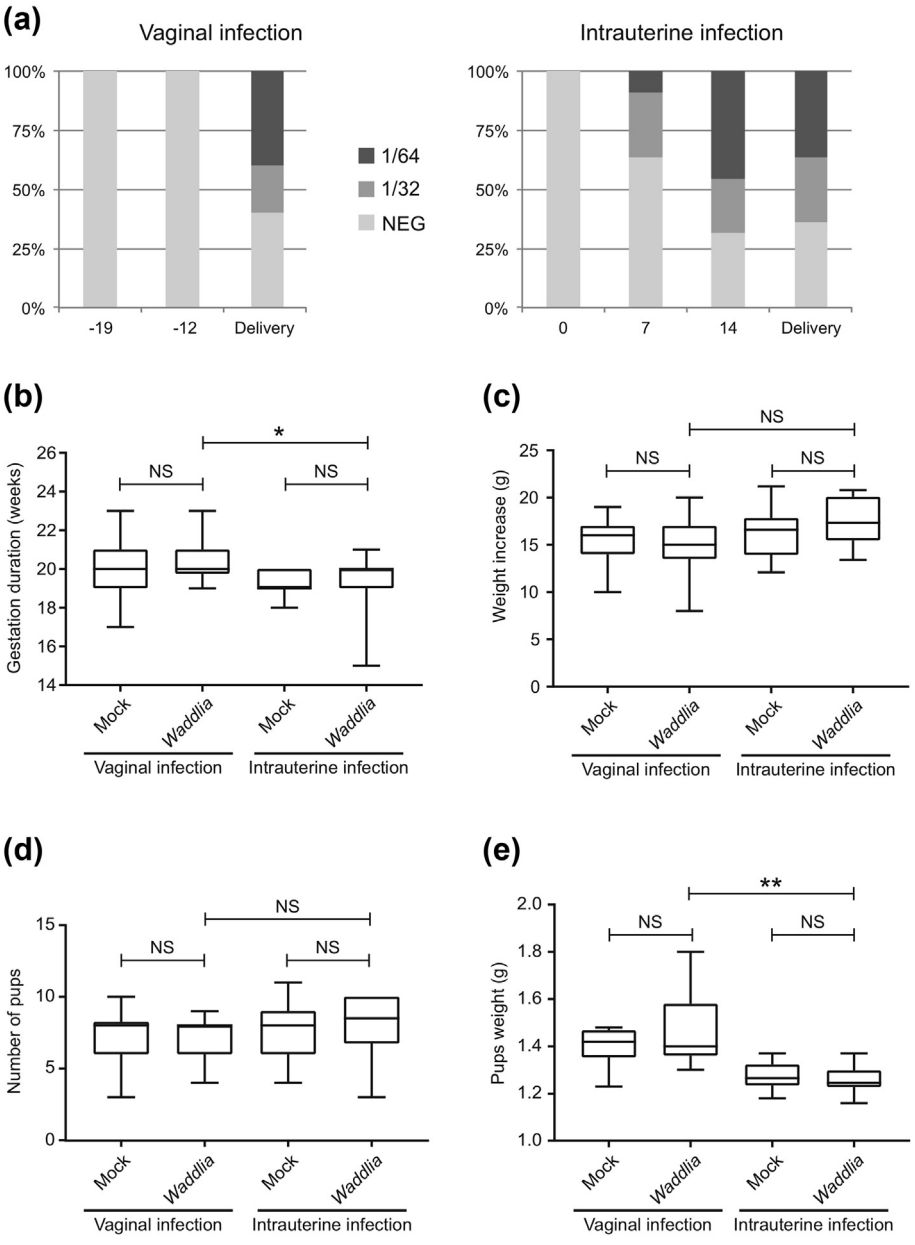

FIG. 2. Impact of *Waddlia chondrophila* infection on mouse pregnancy. (a) Serological analysis of infected female mice during the study (refer to Fig. 1 for the time-points). (b, c) Impact of *W. chondrophila* on length of gestation and maternal weight gain, respectively. (d, e) Impact of *W. chondrophila* on the number of pups and their weight, respectively.

significant immune response and local, as well as distant pathology 2 weeks post-infection [8].

Our results indicate that in our murine model of genital infection, *W. chondrophila* does not result in adverse pregnancy outcomes despite eliciting an immune response. Both routes of inoculation produced a strong immune response that peaked between days 14 and 21 post-infection. The infection did not impact any of the outcomes presented in the study, including the litter size and weight of the pups, duration of the gestation and maternal weight gain.

The fact that *W. chondrophila* was isolated from human and bovine samples, but never in rodents, may indicate that the mouse model of infection is not suitable to study the impact of *W. chondrophila* on gestation. There are some similarities with *Chlamydia trachomatis*, which also does not induce pathologies in a mouse model of genital infection. When inoculated vaginally or into the uterus, the bacterium is quickly cleared by the mouse immune system [13]. Conversely, this infection is closely related to *Chlamydia muridarum*, the murine counterpart of *C. trachomatis*, which causes similar obstetric issues as *C. trachomatis* in humans [14].

Recently, a bovine model of infection has been used to address this issue [7]. Although the infection did not produce a significant increase in miscarriage, in one heifer (out of nine),

W. chondrophila was successfully re-isolated from the diseased placenta. Although this suggests that there may be a tropism towards the reproductive tract, it also indicates that the bacterium is not systematically associated with adverse pregnancy outcomes.

In conclusion, infection with *W. chondrophila* was not associated with an increased rate of miscarriage in our mouse model of genital infection. Given the strong serological link in humans and the genital tropism of this bacterium, future research should focus more on immunopathological mechanisms as direct infection by *W. chondrophila* might not be the main cause of adverse pregnancy outcomes.

Conflicts of interest

The authors have no conflicts of interest to declare.

Author contributions

MS, AA and DB conceived the experimental design. AA, YB, MV and MS performed the experiments. MS, AA, YB, MV and DB interpreted the data. MS and DB prepared the figures and wrote the manuscript. GG reviewed the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by the Department of Obstetrics and Gynaecology, Lausanne University Hospital, Switzerland and by the Swiss National Science Foundation grant numbers 310030-156169/1, 320030-169853/1 and 320030-169853/2 attributed to David Baud. David Baud is also supported by the Fondation Leenaards through the Bourse pour la relève académique, by the Fondation Divesa and by the Loterie Romande. No conflicts of interest to declare.

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