Effects of inoculating dose on the kinetics of *Chlamydia muridarum* genital infection in female mice

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Abstract

Chlamydia trachomatis infections have been implicated in problems such as pelvic inflammatory disease and infertility in females. While there have been some studies examining the kinetics of ascending infection, there is limited information on the kinetics of pathology development and cellular infiltrate into the reproductive tissues in relation to the effects of inoculating dose, and a better understanding of these are needed if an efficacious vaccine is to be developed. The murine model of female genital tract *Chlamydia muridarum* infection is frequently used as a model of human *C. trachomatis* reproductive tract infection. To investigate the kinetics of ascending genital infection and associated pathology development, female BALB/c mice were intra-vaginally infected with *C. muridarum* at doses ranging from 5×10^2 to 2.6×10^6 inclusion forming units. We found that the inoculating dose affects the course of infection and the ascension of bacteria, with the highest dose ascending rapidly to the oviducts. By comparison, the lowest dose resulted in the greatest bacterial load in the lower reproductive tract. Interestingly, we found that dose did not significantly affect the degree of inflammatory cell infiltrate in the various regions. Overall, this data demonstrates the effects of infectious dose on the kinetics of ascending chlamydial infection and associated inflammatory infiltration in BALB/c mice.

Keywords

Chlamydia infection, female reproductive tract, inflammation.

Introduction

Chlamydia trachomatis is the most common sexually transmitted disease worldwide, causing a large socio-economic burden on health care systems. The World Health Organisation (WHO) estimates that 92 million new chlamydial infections are detected each year [1]. It is estimated that up to 70% of infections in females and 50% in men are asymptomatic, causing sequelae such as pelvic inflammatory disease (PID) [2] and epididymitis [3], respectively. The rise in genital chlamydial infections has coincided with the rise in not only PID, but also ectopic pregnancy, tubal infertility and salpingitis [4]. The health care cost associated with infections are estimated to be between $US2-10 billion each year [5], with PID alone estimated to cost $US5.5 billion annually [6].

There has been a significant amount of research into development of an efficacious vaccine in animal models (reviewed in [2, 7]). However there is little consistency in the infectious challenge dose used, with the doses ranging from 1.5×10^3 to 1×10^7 inclusion forming units (ifu) [8, 9]. Rank *et al.* [10] estimated the transmission dose of *Chlamydia caviae* in guinea pigs to be 10^2 ifu, after examining the levels and progression of infection in female guinea pigs acquired through mating experiments. The variation in inoculating dose is a problem as it is unclear how the infectious dose will alter the disease outcomes and the response of the animal.

There is a basic understanding of the cellular pathogenesis of *Chlamydia* (reviewed in [11]), with limited information about the kinetics of ascending infection and the associated pathology development. A chlamydial infection induces an influx of

inflammatory cells including neutrophils, T cells, B cells and macrophages that are stimulated by the production of proinflammatory cytokines and chemokines [11]. Studies show that even low levels of infection, induce a profound immune response [12]. *Ex vivo* studies using human fallopian tube tissues have indicated that interleukin-1 (IL-1) is the initial proinflammatory cytokine activated by a chlamydial infection and confirm that this cytokine is involved in tissue destruction [13]. Acute and chronic/persistent infections can promote foci of inflammatory responses along with promoting tissue remodelling, cellular proliferation and healing that, if persist, lead to scarring [11]. Although there is a role for an adaptive immune response in chlamydial disease, it is secondary to the secretion of pro-inflammatory cytokines and chemokines from infected non-immune cells [11].

However, the exact time-frame of chlamydial ascension along the female reproductive tract and the level of infection required to induce this response is not known. Knowledge of the kinetics of infection is essential to aid in the development of a vaccine, and would demonstrate how the challenge dose affects the final outcomes of the disease. A study by Maxion *et al.* [14] has shown that the infectious dose modulates the innate immune response and that an increased level of infection correlates with a decrease in oviduct sequelae. In a murine model the effects of infection can vary depending not only on the inoculating dose and the serovar of *Chlamydia* [15], but also on the age of the animal [16], the mouse strain used [17] and the hormone levels present [18].

As there is limited information on the effects of inoculating dose on the kinetics of *C. muridarum* genital infection and its associated pathology development, this study aimed to examine these in a murine model.

Results

The infectious dose affects the course of vaginal shedding in mice.

To monitor the course and degree of infection in the mice, vaginal swabs were collected every 3 days and were cultured on McCoy cells; the cut-off for a productive infection was set at 300 ifu. The mid (5×10^4 ifu) and high (2.6×10^6 ifu) dose infections caused an initial infection 3-fold greater ($p<0.001$) than the low dose, that dropped rapidly by day 9 post infection (p.i.) (Fig. 1). The animals that received these two doses reached the cut off level by day 35 p.i. In contrast, the low dose (5×10^2 ifu) infection group shed significantly greater ($p<0.05$) levels of *Chlamydia* 9 days p.i. On day 35 p.i. this group was still infected, secreting 18-fold more IFU than the mid and high dose animals, but was not significantly different. By day 42 p.i. the low dose group reached the cut-off level.

The infectious dose affects the ascension of *Chlamydia* in the murine female reproductive tract.

While swab collection and analysis allows the level of bacterial shedding in the lower genital tract to be determined, the removal and culture of tissues allows the level of viable bacteria present within the submucosal layers of all regions of the genital tract to be examined. Culture of the cervico-vaginal region revealed that although the difference between the mid and high inoculating dose is quite large, the level of infection that occurred in the tissues was not significantly different (Fig. 2A). In both groups, infection was not detected in the cervico-vaginal tissues from day 21 p.i.

However, the low dose group had a significantly higher initial infection (day 6 p.i.) in this region than the mid ($p<0.01$) and high dose ($p<0.05$) groups.

In the uterine horn tissues (Fig. 2B) the low dose group had a similar course of infection as the mid and high doses, with the exception of a peak in infection at day 9 ($p<0.001$), which was not observed with the other doses. The chlamydial burden within the uterine horns for all 3 doses was much lower than that observed in the cervico-vaginal region and oviducts.

The oviduct tissues had the greatest chlamydial burden of any of the regions. The high dose had a 7.5 – 13 fold greater infection ($p<0.001$) than the mid and low doses, respectively, on day 6 p.i. (Fig. 2C). The mid and low dose groups had a similar degree of infection. From day 9 p.i. all three groups had the same pattern of infection. Interestingly, the chlamydial burden within the oviducts was higher than that seen in the uterine horn and cervico-vaginal tissues for all 3 doses during the early stages of infection.

Overall, the infectious dose affects the degree of ascending infection in the murine FRT, with rapid ascension to the oviducts observed in mice that received the high dose, despite the low dose causing greater infections in the lower regions of the reproductive tract.

The infectious dose affects the development of pyosalpinx but not hydrosalpinx.

Pyosalpinx is defined as the oviduct containing pus and is often a result of acute salpingitis. Hydrosalpinx is defined as dilation of the oviducts and luminal filling with a clear serous fluid and is used as an indicator of infertility [19]. The animals that received the low dose did not appear to develop gross visual pathology as severe as the two higher doses by 42 days p.i., despite similar infection levels (Fig. 3A). This group was therefore extended, with animals examined at days 49 and 70 p.i, and showed that hydrosalpinx developed to the same degree as seen with the 2 higher doses, but at later time-points. The low dose group had very low levels of pyosalpinx until day 15 p.i. In contrast, the mice that received the mid dose of infection developed pyosalpinx by day 6 p.i., with it being most severe at 9 days p.i. (Fig 3B). By day 35 p.i. hydrosalpinx was present and quite severe and continued to day 42 p.i., when the experiment was terminated. Similarly, the mice that received the high dose of infection also developed pyosalpinx, but the severity peaked at day 12 p.i. By day 35 p.i., hydrosalpinx had developed in these mice (Fig. 3C).

Overall it was found that the low dose group had the least severe pyosalpinx development and all 3 groups developed very similar degrees of hydrosalpinx, albeit the low dose group was at a later time-point.

The infectious dose does not significantly alter the level of inflammatory cell infiltration throughout the course of infection.

The histopathological changes in the 3 regions of the reproductive tracts were measured using H&E stain and the levels of acute (neutrophils) and chronic (lymphocytes) inflammation were measured.

Acute Inflammation:

An increased presence of neutrophils is indicative of an acute infection at the site and they are important in this model as they are required for the clearance of chlamydial infections [20]. The greatest levels of infiltrate were seen during the acute phase of infection in all 3 groups in all the regions (Fig. 4). Surprisingly, within the cervico-vaginal region there was no significant difference in the level of neutrophils observed between any of the groups or in comparison to the control (Fig. 4A). In the uterine horn tissues all 3 groups had significantly greater levels of neutrophil infiltrate than the controls on day 6 p.i. ($p<0.001$, $p<0.05$, $p<0.01$ respectively), but dropped to very low levels by day 15 p.i. in all 3 groups (Fig. 4B). On day 9 p.i. the low dose group had significantly greater infiltrate than the mid and high dose ($p<0.05$ and $p<0.001$ respectively), correlating with the greater chlamydial burden seen in the uterine tissues at this time-point. Interestingly, the numbers of neutrophils present in the uterine horn tissues during the very early stages of infection were much greater than that seen in the cervico-vaginal regions. In the oviducts the level of neutrophils was greatest on days 9-15 p.i., after which, they dropped, coinciding with the clearance of *Chlamydia* from the oviduct tissues (Fig. 4C). Neutrophil presence in the mid and high dose, at these time-points correlates with the presence, of pyosalpinx (Fig. 3).

Chronic Inflammation:

The presence of lymphocytes within the tissues can represent chronic inflammation and in these experiments all 3 regions of the reproductive tract had elevated levels of lymphocyte infiltration compared to the negative and progesterone controls (Fig. 5). Specifically, in the mice that received the mid dose, the levels of infiltrate in the

cervico-vaginal region were significantly greater than controls on days 9 and 12 p.i. ($p<0.01$ and $p<0.05$ respectively; Fig. 5A). Within the uterine horn tissues both the low and mid dose groups demonstrated a trend of increased levels of lymphocyte infiltration compared to both control groups at numerous time-points (Fig. 5B)., but overall, it was the mid dose that induced the greatest lymphocyte infiltrate present at all time-points until day 35 p.i. In the oviducts (Fig. 5C) the overall level of infiltrate was much lower than that observed in the cervix/vagina and uterine horns. All lymphocyte infiltrate in the oviduct had subsided by day 35 p.i., the time at which hydrosalpinx was observed. There was an overall trend, with the mid dose having the greatest levels of lymphocytic infiltrate in all 3 regions of the reproductive tract during the acute stages of infection (days 6 – 21 p.i.).

The levels of neutrophils were only elevated for a short period of time that coincided with the peak infection levels within the tissues. In contrast, even after the infection had cleared from the tissues, the level of lymphocyte infiltration remained elevated. Overall, the level of inflammatory cell infiltrate was not significantly affected by the levels of infection seen in the 3 regions of the reproductive tract

Discussion

In this study we have demonstrated the kinetics of ascending genital tract chlamydial infection and the associated inflammation kinetics in female BALB/c mice. We have shown that the infectious dose of *C. muridarum* alters both the rate and level of clearance and ascension in the female reproductive tract and the development of gross pathology. However the overall level of inflammatory cell infiltrate was not significantly affected by the infectious dose administered, highlighting that inoculum that is 5000 fold less than the highest used here is sufficient to cause inflammatory infiltrate to a similar, if not greater degree.

The pattern of chlamydial shedding from the cervico-vaginal region was similar to that reported previously [14, 21]. Interestingly, there were only two time-points where the levels of shed bacteria significantly differed to each other. However, examination of the chlamydial burden within the tissues of the 3 regions of the mouse FRT highlighted differences between the groups, with the low dose able to ascend to infect the uterine horns to a greater degree than the mid and high dose. In contrast, it was the high dose that ascended to the oviducts to the greatest degree. This difference within the oviducts may have been because the lower reproductive tract was overloaded with *Chlamydia* and canalicular spread has allowed *Chlamydia* that were not bound to epithelial cells to migrate to areas where there were uninfected cells, including the oviducts. This overloading of epithelium with *Chlamydia* has previously been suggested by Kelly *et al.* [22].

The infectious dose modulates the innate immune response in relation to the infection [14], with the level of chlamydial burden being directly related to the development of oviduct pathology. Maxion [14] reported that there were differences in oviduct dilation and trends in the ability of increasing doses to cause a greater infiltration of both adaptive and innate immune cells such as polymorphonuclear cells (PMN) [14]. This was not seen in our study, with the high dose causing the greatest infection in the oviducts and no significant differences in the levels of infiltrating cells. This may be linked to different variants of *C. muridarum* being used. There are 2 naturally occurring isolates of *C. muridarum*, Weiss and Nigg II. Recently it has been found that while these two variants are identical in their patterns of infection, they differ in their virulence [23]. Maxion *et al.* [14] do not state which strain of *C. muridarum* has been used, but based on their obtained ID50 (2.5×10^3 ifu) and all of the animals in this study becoming infected at 5×10^2 ifu, using the same strain of mice, this suggests that different variants may have been used by Maxion *et al.* [14] and our group and therefore could explain the differences found.

The chlamydial burden seen in the uterine horns was much lower than that observed in the oviducts. Recruitment of $CD45^+$ major histocompatibility complex class II (MHC II) cells limit the level of infection in uterine tissues early in infection [24], suggesting that an early MHC class II response may have limited the level of infection within the uterine tissues in this case. The cervico-vaginal regions had a greater overall chlamydial burden than the uterine and oviduct tissues and a more prolonged infection. This may be because the immune system in that region of the reproductive tract may be dampened due its continual contact with natural flora and other potential

pathogens, allowing the bacteria to initially infect epithelial cells to a greater degree [22].

We have also demonstrated that the development of gross pathology is not necessarily dose dependent, with the low dose developing a similar level of hydrosalpinx as the mid and high dose groups but at a later time-point. Pyosalpinx is defined as the presence of PMN's, or pus, within the oviduct, and in these experiments was found to occur during the acute infection stages. Hydrosalpinx occurs when the oviducts are occluded and clear serous fluid accumulates causing oviduct dilation [19]. Many [19, 25] have used the presence of hydrosalpinx as a marker for infertility in the mouse model, with Shah *et al.* [19] demonstrating that oviduct occlusion directly correlates to infertility in mice. The presence of hydrosalpingeal fluid in women undergoing in vitro fertilisation has been linked to decreased implantation rates [26]. The exact reasons behind this are unclear, but it is believed that the fluid contains cytokines such as IL-2, that are involved in the development of pathology, and it is this pathology development that decreases the rate of successful pregnancy outcomes [26]. Here we found that the mid dose had the greatest overall level of hydrosalpinx development, possibly related to greater levels of chronic pathology (lymphocytes) and also a more prolonged presence of neutrophils. Whilst the development of pyosalpinx and hydrosalpinx have been examined in both C57BL/6 and C3H/HeN mice [19], this is the first time, to our knowledge, that the kinetics of their development has been examined in the BALB/c model, at multiple time-points and at varying infectious doses.

Importantly, we have demonstrated that the infectious dose administered did not significantly affect the overall level of inflammatory cell infiltration. We have shown

that neutrophils are present in the early stages of infection in all three regions of the mouse reproductive tract. Treatment of animals with granulocyte-depleting monoclonal antibodies has shown that neutrophils play a critical role in the clearance of early stage chlamydial infections from the reproductive tract [20], but too intense a neutrophil response may promote pathology development [27]. Here we have seen that the mid dose had a more prolonged elevation of neutrophil infiltration in the oviducts and the greatest level of hydrosalpinx development at the earlier timepoints. It is believed that the actual chlamydial infection is not the cause of the inflammation or pathology development, but rather the host immune response [12]. This is supported by findings from fallopian tube samples of hysterectomy patients, where infection levels were disproportional to the severity of tissue destruction [13]. Upon infection an epithelial cell secretes various pro-inflammatory cytokines and it is believed that this cytokine secretion triggers a cascade of events that leads to the development of chronic pathology, scarring and tubal infertility [11, 28].

With the transmission rates of *C. trachomatis* on the rise, 50-60% of infections being asymptomatic and potential sequelae such as PID, there is a need to understand the mechanisms of ascending infection leading to pathology development and to develop ways of preventing the damage. This work highlights that a low level inoculum can cause a similar level of damage as one more than 5000 times greater, suggesting that using a high inoculum to establish infection is unnecessary and in fact may result in an under estimation of the effectiveness of experimental vaccines. Importantly, we have demonstrated the kinetics of not only ascending genital tract infection in mice, but also the development of infection related inflammation and pathology in relation to varying infectious doses.

Materials and Methods

Chlamydia Strain

Chlamydia muridarum (Weiss; ATCC VR-123, Virginia, USA), formerly the mouse pneumonitis biovar of *C. trachomatis* (MoPn), was grown by inoculation of McCoy cell monolayers in Dulbecco's minimal essential medium supplemented with 5% fetal calf serum (FCS), 2mM L-glutamine, 100µg/mL Streptomycin sulfate, 2µg/mL Gentamycin and 20mM HEPES. Elementary bodies were purified using a discontinuous Renografin gradient as previously described [29].

Mice

Female BALB/c mice, 6-8 weeks of age, were obtained from The Animal Resource Centre, Perth (Australia), and housed in an accredited laboratory animal care facility under specific-pathogen free conditions. Animals received food and water *ad libitum*. All procedures were approved by the Queensland University of Technology Animal Research Ethics Committee.

Infection

Mice were given 2.5mg of medroxyprogesterone acetate (Depo-Provera, Pfizer, NSW, Australia) subcutaneously, seven days prior to infection. The mice were anaesthetised intraperitoneally using ketamine (Parnell Laboratory, NSW, Australia) and xylazine hydrochloride (Bayer, NSW, Australia) and infected intra-vaginally with 20µl of

sucrose-phosphate-glutamate (SPG) containing one of the 3 infectious doses, 5×10^2 inclusion forming units (ifu; low), 5×10^4 ifu (mid) or 2.6×10^6 ifu (high). Mice were then sacrificed on days 6, 9, 12, 15, 21, 35 and 42 post-infection. The low dose group also had animals sacrificed on days 49 and 70 post infection to examine the extended pathology development.

Detection of *C. muridarum* Infection

Infection was monitored by collecting cervico-vaginal swabs (Copan, CA, USA) on the days mentioned above. Swabs were placed in tubes containing 500µl SPG and glass beads and were stored at -80°C. To monitor the infection individual wells of McCoy cell monolayers in 48 well plates were inoculated with 10µl of swab specimen and media. Plates were incubated for 4 hours at 37°C, after which the swab solution was removed and replaced with fresh supplemented media containing 1µg/mL cycloheximide. The wells were incubated for a further 24-30 hours, then fixed with methanol. The inclusions were visualised by staining with rabbit anti-*C. trachomatis* antibody (Pierce/Progen, Richlands, Australia) and Immunopure ABC/DAB Staining Kit (Pierce/Progen, Richlands, Australia), as described elsewhere [21]. An animal was classed as having a productive infection if there were greater than 300 ifu's per swab.

Assessment of Ascending Infection

To monitor the progress of the infection along the reproductive tract the cervico-vaginal region, uterine horns and oviducts representing the upper reproductive tract, were removed upon sacrifice, placed in 100µl SPG and stored at -80ºC. To determine

the chlamydial burden within the tissues, individual cervico-vaginal, uterine horns and pooled oviducts were weighed and homogenised, and 10-25µl of homogenate was placed onto individual wells of McCoy cell monolayers and cultured and stained as above. Inclusions present in 20 fields (× 40 magnification) were counted and the ifu/1 mg of tissue was calculated.

Assessment of Gross Pathology

Upon sacrifice the reproductive tracts were examined *in situ* for macroscopic changes. The presence of pyosalpinx and hydrosalpinx was recorded and the level of fluid retention was also scored, with those having small amounts of fluid present in the oviducts given a score of 1, those with moderate amounts a score of 2 and those with large amounts of fluid given a score of 3.

Histopathology Assessment

Tissues were removed at sacrifice, fixed in 10% formaldehyde and embedded in paraffin wax. Five µm sections were cut, dewaxed and rehydrated through graded ethanol solutions to PBS. Haematoxylin and eosin staining was performed. Regions of the reproductive tract, cervix/vagina, uterine horn and oviducts, were counted separately to each other. Ten random fields (× 1000 magnification) of each were counted ensuring to include both epithelium and sub-mucosa, with the observer blinded to the time-point and dose being examined.

Statistics

All data are presented as mean ± standard error of mean (SE). All statistics were performed using GraphPad Prism version 5.00 (GraphPad Software, CA, USA). Significant differences in the swab clearance data, tissue ifu and inflammatory cell infiltration was determined using a two-way ANOVA with Bonferroni's post test with significance set for $p<0.05$. All experiments contained 5 mice and were repeated twice.

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Figure Legends

Figure 1: Course of infection determined by vaginal swabs after vaginal inoculation with varying doses of *C. muridarum*. Vaginal swabs were collected days 6, 9, 12, 15, 21 and 35 post vaginal infection to determine levels of viable organisms by McCoy cell culture. Data are mean ± SE of mean for 10 mice, from two separate experiments, with a productive infection classified as greater than 300 ifu. Two-way ANOVA was performed with Bonferroni's post test. †: $p<0.05$ (low compared to high dose); #: $p<0.001$ (low compared to mid dose).

Figure 2: Kinetics of infection in (A) cervico-vaginal, (B) uterine horn and (C) oviduct tissue homogenate culture after vaginal inoculation with varying doses of *C. muridarum*. Tissues collected at various time-points were homogenised, equal amounts cultured on McCoy cell monolayers and ifu/1 mg of tissue was determined. Data are mean ± SE for 10 mice. Two-way ANOVA with Bonferroni's post test was performed. †: $p<0.05$; *: $p<0.01$; #: $p<0.001$.

Figure 3: Course of gross pathology development, including pyosalpinx and hydrosalpinx, over the time-points examined. (A) Low dose; (B) Mid dose; (C) High dose. Visual observations were made at the time of sacrifice. Scoring system was: 1: low level of fluid present in oviduct; 2: moderate amount of fluid present; and 3: large level of fluid present. The data represents two separate experiments, each containing 5 mice, and is the mean ± SE of values obtained. ND: Not determined at these timepoints.

Figure 4: The kinetics of neutrophil (acute) infiltration within the (A) cervix/vagina; (B) uterine horn; (C) oviduct of the murine female reproductive tract. Data are mean ± SE for 10 mice. Two-way ANOVA was performed with Bonferroni's post test. N: Negative animals, P: Progesterone treated only animals. †: $p<0.05$; *: $p<0.01$; #: $p<0.001$.

Figure 5: The kinetics of lymphocyte (chronic) infiltration within the (A) cervix/vagina; (B) uterine horn; (C) oviducts of the murine female reproductive tract. Data are mean ± SE for 10 mice. Two-way ANOVA was performed with Bonferroni's post test. N: Negative animals, P: Progesterone treated only animals. †: $p<0.05$; *: $p<0.01$; #.