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Original article

Intrauterine inoculation of minipigs with *Chlamydia trachomatis* during diestrus establishes a longer lasting infection compared to vaginal inoculation during estrus

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Abstract

Advanced animal models, such as minipigs, are needed for the development of a globally requested human *Chlamydia* vaccine. Previous studies have shown that vaginal inoculation of sexually mature Göttingen minipigs with *Chlamydia trachomatis* resulted in an infection lasting only 3–5 days.

The aim of this study was to evaluate the effect of targeting the upper porcine genital tract by transcervical and transabdominal intrauterine inoculation, compared to previously performed vaginal inoculation. Furthermore, we investigated the effect of the hormonal cycle, estrus vs. diestrus, on the establishment of a *C. trachomatis* infection in the minipig.

Targeting the upper genital tract (transcervical inoculation) resulted in a longer lasting infection (at least 7 days) compared to vaginal inoculation (3–5 days). When comparing intrauterine inoculation during estrus and diestrus, inoculation during diestrus resulted in a longer lasting infection (at least 10 days) compared to estrus (3–5 days). Furthermore, we found a significant *C. trachomatis* specific IFN-γ response in pigs inoculated during estrus correlating with the accelerated clearance of infection in these pigs.

These findings suggest that for implementation of an optimal model of *C. trachomatis* in minipigs, inoculation should bypass the cervix and preferable be performed during diestrus.

Keywords: Minipig model; *Chlamydia trachomatis*; Sexually transmitted disease

1. Introduction

Chlamydia trachomatis is worldwide the most frequent sexually transmitted bacterial infection [1]. It causes severe complications such as tubal infertility, and it is internationally acknowledged that a vaccine would be the best preventive strategy [2]. For development of safe and effective vaccines,

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proper animal models are essential, however, a proper advanced animal model for testing *Chlamydia* vaccines is still missing [3].

Traditionally, mice and guinea pigs are the animal models of choice, but pigs and especially minipigs have become increasingly popular as models in preclinical research due to their high level of anatomical, physiological and immunological similarities to humans [4–6]. These similarities suggest that pigs have major predictive value as preclinical models of vaccine efficacy. Both conventional pigs and minipigs are found to be suitable models of genital *C. trachomatis* infection, but minipigs are favored due to their small size at sexual maturity and specific pathogen free (SPF) status [4,6,7].

Initial studies with vaginal inoculation of sexually mature Ellegaard Göttingen minipigs with *C. trachomatis* Serovar D (*Ct* SvD) during estrus found that an infection lasting only 3–5 days could be established [8,9] thus making it challenging to evaluate the efficacy of vaccine induced antibody and cell-mediated immune responses. Focus has therefore been on establishing a longer lasting infection in the porcine female genital tract to enable better evaluation of vaccine efficacy/protection.

The genital tract target cells for *C. trachomatis* are the columnar epithelial cells found in the endocervix of women [10]. Deposition of *C. trachomatis* during sexual intercourse at the external opening of cervix in women is therefore hypothetically sufficient for *C. trachomatis* to reach the target cells. In pigs, however, the cervix is dominated by stratified squamous epithelium [11] and the bacteria have to reach the uterine mucosa to establish an infection of the columnar target cells.

Murine models of genital *Chlamydia* are generally treated with progesterone before inoculation to enable inoculation during diestrus, and achieve a higher infection rate [4,12]. This is consistent with studies in pigs experimentally infected with *Escherichia coli*, where intrauterine inoculation during metestrus caused endometritis and inoculation during estrus did not [13]. Overall the findings indicate that inoculation during estrus is unfavorable for the establishment of a genital infection.

We hypothesized that transcervical inoculation in the minipig would allow the *C. trachomatis* bacteria more efficient access to their target cells, thereby allowing a more efficient colonization. Furthermore, we hypothesized that inoculation during diestrus rather than estrus would allow for establishment of a longer lasting infection. The aim of this study was therefore to evaluate the effect of transcervical inoculation during estrus and intrauterine inoculation during estrus and diestrus, on the course of genital *C. trachomatis* infection in minipigs and to compare the results to vaginal inoculation.

2. Materials and methods

2.1. C. trachomatis

The *C. trachomatis* serovar D (*Ct* SvD; UW-3/Cx, ATCC® VR-885™) strain used was originally isolated from a female patient with an asymptomatic genital infection. It has been propagated in HeLa-229 cells, harvested, and purified as previously described [14,15].

2.2. Minipigs and experimental design

This study was performed with two groups of each 12 SPF (http://minipigs.dk/the-goettingen-minipig/health-status/) sexually mature female Göttingen Minipigs (Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark) of approximately 10 months. One group ($n = 12$) was inoculated by a transcervical route in estrus (TC-group), and the other group ($n = 12$) had laparotomy and direct intrauterine inoculation performed (IU-group) in estrus or diestrus. Upon arrival at the study site, the minipigs were allowed one week of acclimatization and were under all circumstances treated in accordance with the Danish law on animal experiments. The experiments were approved by the Danish Animal Experiments Inspectorate (license number: 2013-15-2934-00978). Details on the experimental setup are summarized in Fig. 1.

Before inoculation the minipigs were treated orally for 18 days with Regumate Equine® (Altrenogest, 20 mg/animal and day, MSD Animal Health, Ballerup, Denmark) for estrous cycle synchronization. Following Regumate® treatment, the minipigs were monitored for clinical evident estrus (behavior, vaginal hyperemia, and edema of the vulva). Inoculation during estrus was performed on the first day of evident estrus and inoculation during diestrus was performed 5 days after the end of clinical evident estrus. Serum progesterone levels (Central Laboratory, University Hospital for Companion Animals, University of Copenhagen) were determined at the day of inoculation to verify that minipigs in the IU-group were either in estrus (all pigs < 0.3 ng/ml) or diestrus (range 12.6–35.6 ng/ml) (Supplementary file 1).

The pigs were during all procedures anesthetized with Zoletil® mixture (1 vial Zoletil®50 Vet (125 mg tiletamin and 125 mg zolazepam) resuspended in 6.25 ml xylazine (20 mg/ml), 1.25 ml ketamine (100 mg/ml), and 2.5 ml butorphanol (10 mg/ml)) [16]. The following doses were used: 1 ml/12 kg body weight (BW) for non-invasive TC inoculation, 1 ml/17 kg BW for vaginal swabbing, 1 ml/15 kg BW for blood collection and vaginal swabbing. See Supplementary file 2 for the anesthesia protocol for laparotomy.

In the TC-group, the minipigs were inoculated transcervically through a combination of a Spirette insemination catheter (17102/9067, Minitube, Tiefenbach, Germany) and a urinary catheter (273410, Kruuse, Langeskov, Denmark) with $4*10^9$ *Ct* SvD inclusion forming units (IFUs) in 10 ml sterile SPG buffer (0.2 M sucrose, 20 mM sodium phosphate, and 5 mM glutamic acid buffer). The minipigs were placed with the hindquarters elevated for 20 min following inoculation to avoid reflux. All 12 minipigs were inoculated utilizing the same method, but divided into three groups euthanized on days 3, 7 and 14 post infection (pi) (Fig. 1).

The transcervical catheterization was only performed during estrus, where the cervix is under estrogen influence and therefore edematous and soft (http://extension.missouri.edu/p/

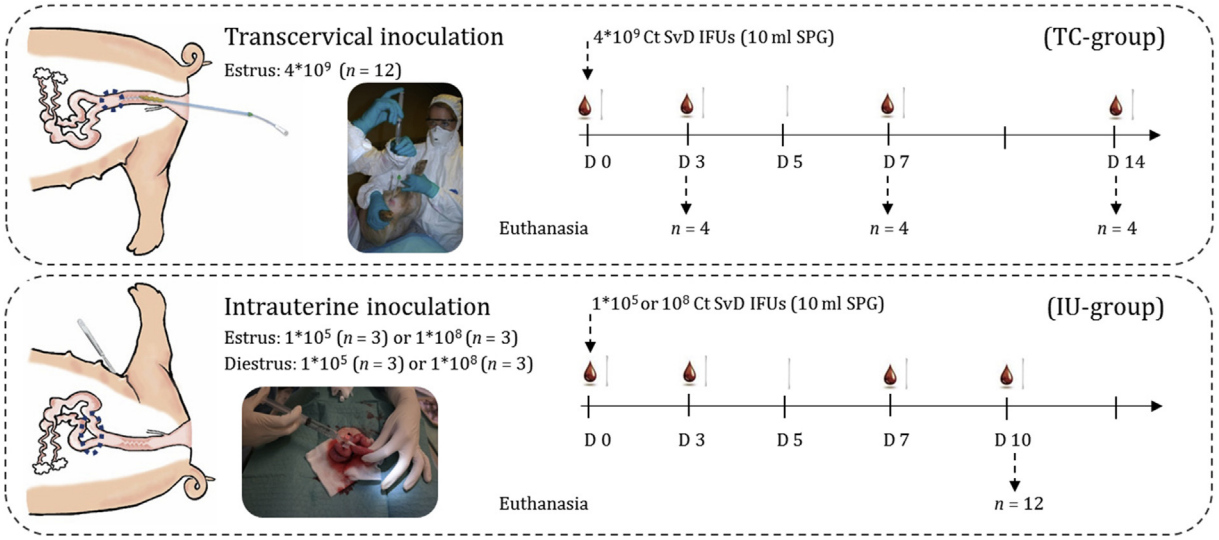

Fig. 1. **Experimental setup**. This study was performed with two groups of each 12 Ellegaard Göttingen Minipigs (EGM). In the transcervical group (TC-group) 12 EGM were inoculated during estrus by transcervical catheterization with $4*10^9$ *Chlamydia trachomatis* Serovar D (Ct SvD) Inclusion Forming Units (IFUs). The pigs were euthanized in three groups on days 3, 7 or 14 post infection (pi). In the intrauterine group (IU-group) the 12 EGM had laparotomy and intrauterine inoculation performed. 6 EGM were inoculated during estrus and 6 EGM during diestrus. Furthermore, 3 pigs in each group were given $1*10^5$ IFUs and three pigs were given $1*10^8$ IFUs giving 4 groups with three EGM in each. All 12 EGM were euthanised on day 10 pi. Symbols indicate collection of blood (blood drop) and vaginal swab samples (swab). On the day of euthanasia, thorough sampling from the genital tract was performed.

G2312) allowing catheterization. Cervical catheterization has not been successful during diestrus in minipigs (the authors' personal experience) and laparotomy was therefore performed to access the uterus independently of estrous cycle stage enabling comparison of inoculation during diestrus and estrus.

In the IU-group, laparotomy and intrauterine inoculation were performed following the detailed description in Supplementary file 2. Six minipigs were inoculated during estrus and six minipigs during diestrus. Furthermore, within each group of six minipigs, three minipigs were inoculated with $1*10^5$ IFUs and three minipigs with $1*10^8$ IFUs. All minipigs in the IU-group were euthanized on day 10 pi (Fig. 1).

2.3. Sampling

The rectal temperature was taken daily starting two days prior to the inoculation until euthanasia, and furthermore 6–9 h after inoculation. A rectal temperature of 39.5 °C was set as fever limit [9].

Blood samples were collected from the *vena jugularis externa* with a vacutainer system. Serum was isolated by centrifugation for 15 min at 3500 rpm and stored at −20 °C.

Vaginal swabs were collected with sterile regular size Copan FLOQswab (Statens Serum Institut, Copenhagen, Denmark) in 1 ml SPG. A vaginoscope was utilized during sampling, and aseptic procedures were followed. Following sampling, the swabs were vortexed for 1 min with three sterile glass-beads and stored at −80 °C until further analyses.

A detailed scheme for blood- and vaginal swab sampling is shown in Fig. 1.

2.4. Antibody ELISA

IgG and IgA ELISAs to determine antibodies against *C. trachomatis* were performed essentially as described previously [8], with the exception that Polysorp® plates (NUNC A/S, Roskilde, Denmark) were coated with whole inactivated *Ct* SvD elementary bodies (EB) 4 μg/ml over night at 4 °C. Detection was performed with a goat anti-pig IgG antibody (1:10.000, AAI41P, Serotec, UK) and a goat anti-pig IgA antibody (1:2000, AAI40P, Serotec, UK). A positive control (serum from a previous study [8]) was included as an internal standard to correct for plate-to-plate variation and a negative control was included on each plate (two wells without serum). Difference in optical density (OD) during the study was calculated as $\Delta OD = OD_{450nm-650nm\ (day\ x)} - OD_{450nm-650nm\ (day\ 0)}$.

2.5. Interferon-γ release assay

Isolation and stimulation of peripheral blood mononuclear cells (PBMCs) were performed as described earlier [8], with the exception that stimulation was performed with whole *Ct* SvD EBs 1 μg/ml. The IFN-γ ELISA on the supernatant was also performed as previously described [8]. On each plate, a standard with a predetermined concentration of porcine IFN-γ was added in duplicate serial dilutions together with two wells without substrate as a negative control. A log–log transformed standard curve was made from the serial dilutions of the standard, and an equation was made correlating the measured optical density (OD) value with the amount of IFN-γ in the sample. The IFN-γ concentration in each sample was calculated with this equation.

2.6. Vaginal chlamydial load

The vaginal load of *C. trachomatis* was evaluated by cultivation of swab material in the TC-group, whereas swabs from the IU-group were evaluated by qPCR due to practical circumstances and the increased sensitivity by qPCR. Cultivation was performed as described elsewhere [7]. In summary, a monolayer of McCoy cells was inoculated with 100 µl swab material in duplicates, centrifuged for 1 h at 750× g and then incubated 2 h at 35 °C followed by a replacement of the media with fresh cycloheximide media and incubation for approximately 30 h at 37 °C. Inclusions were then visualized with rabbit-anti recombinant major outer membrane protein (rMOMP) serum (from our own lab) and an Alexa 488 conjugated goat-anti-rabbit IgG secondary antibody (Life Technologies, Paisley, UK). Inclusions were counted in 20 fields of view (40× objective magnification) with an Olympus IX71 fluorescence microscope (Olympus, Ballerup, Denmark) and the total number of inclusions per swab was calculated.

In the IU-group, detection of *C. trachomatis* was performed by qPCR. Swab samples were pretreated by mixing 600 µl swab material with 350 µl Bacteria lysis buffer (Roche, Hvidovre, Denmark), 50 µl Proteinase K (Sigma-Aldrich, Brøndby, Denmark) and 5 µl internal amplification control (Goffin Molecular Technologies, Maurik, The Netherlands). The mixture was incubated 10 min at 65 °C followed by 10 min at 90 °C, where after DNA was extracted with a MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche, Denmark) on a MagNA pure 96 instrument with the Pathogen Universal protocol. DNA was eluted in 50 µl.

Detection of chlamydial DNA was performed with a Presto CT-NG PCR test kit (Goffin Molecular Technologies) [17] according to the manufacturer's protocol with 10 µl DNA and 15 µl master mix run on a LightCycler® 96 instrument (Roche). A standard curve was prepared with a *Ct* SvD batch made in our lab with a known concentration of viable bacteria.

2.7. Euthanasia

The minipigs were euthanized by intramuscular (IM) injection of Zoletil® mixture [16] 1 ml/7 kg followed by exsanguination and necropsy. Tissue samples and swabs were taken from the cervix, uterine body, uterine horns (bilaterally sampled both proximally and distally), and the uterine tubes (Fallopian tubes). Regular size Flock swabs were used for all swabs except the uterine tubes, where minitip size was used.

2.8. Histopathology

Macroscopic evaluation of the genital tract was performed during necropsy. Tissue samples from vagina, cervix, uterine horns and uterine tubes were fixed in 10% neutral buffered formalin and processed by standard procedures [9]. Tissue sections were stained with hematoxylin and eosin (HE) for histopathology.

Chlamydia immunohistochemistry (IHC) was performed as described elsewhere [9] with a mouse anti-*Chlamydia* antibody (clone AC1-P; Progen, Heidelberg, Germany) at a dilution of 1:100 and a Vectastain ELITE ABC Mouse kit detection system (VWR Bie og Berntsen A/S, Søborg, Denmark). Blinded evaluation was performed. Negative control sections were made with non-sense serum replacing the primary antibody.

2.9. Statistical analysis

All statistics were performed with GraphPad Prism 6 (GraphPad Software Inc., CA, USA). Data were analyzed with the non-parametric Friedman's test and Dunn's multiple comparison test. P values lower than 0.05 ($P < 0.05$) were considered statistically significant. Further levels of significance are indicated with asterisks $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

3. Results

3.1. Vaginal chlamydial load

The current study was performed with two groups of each 12 sexually mature female SPF Göttingen Minipigs. The TC-group was inoculated transcervically during estrus, while the IU-group underwent direct intrauterine inoculation via laparotomy during either estrus or diestrus (Fig. 1). Since cervical catheterization of minipigs has not been successful during diestrus, laparotomy was performed to be able to compare intrauterine inoculation during diestrus and estrus.

Culturing of vaginal swabs from the TC-group showed a stable number of at least 10^4 viable *C. trachomatis* bacteria until day 7 pi. On day 14 pi no viable bacteria were detected (Fig. 2). Within the two IU-groups given the low dose inoculum ($1*10^5$ IFUs) only few *Chlamydia* bacteria could be detected by qPCR in 1/3 minipigs on day 3 pi in the group inoculated during diestrus and in 0/3 minipigs inoculated during estrus (data not shown). Therefore, a dose of $1*10^5$ IFUs was considered too low to establish an infection in this model and consequently these two groups have been omitted in other parts of this article.

Within the two IU-groups inoculated with $1*10^8$ IFUs, the group inoculated during estrus had a high vaginal load of *Chlamydia* bacteria on day 3 pi and 1/3 minipigs had detectable amounts of bacteria on day 5 pi, while all samples day 7 and 10 were negative. In the IU-group inoculated with $1*10^8$ IFU during diestrus, 3/3 pigs had a high load of bacteria until day 7 pi, 1/3 minipigs a high level in the vagina on day 10 pi and 2/3 minipigs a high level in the uterus on day 10 pi. Furthermore, 1/3 minipigs had *Chlamydia* in the uterine tubes on day 10 pi (Fig. 2).

3.2. Immunohistochemistry

To identify true infection of the porcine genital tract cells, *In situ C. trachomatis* inclusions were detected by immunohistochemical staining. No inclusions were detected in the vagina and cervix. In the TC-group 3/4 minipigs on day 3 pi

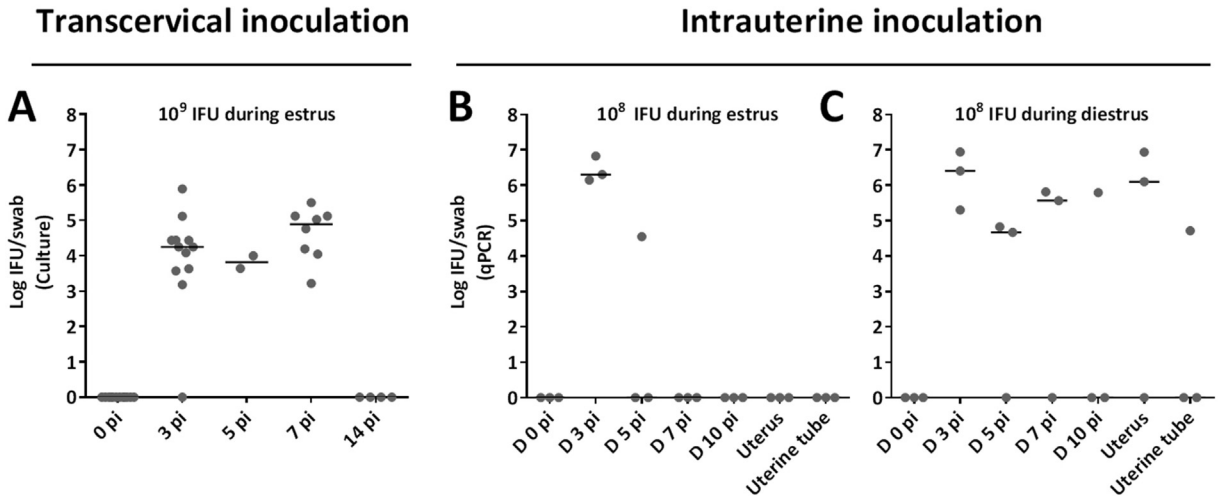

Fig. 2. **Chlamydial load following inoculation**. (A) Detection of *C. trachomatis* from vaginal swabs on day 0, 3, 5, 7 and 14 pi in McCoy cell culture following transcervical inoculation during estrus. (B + C) qPCR detection of *C. trachomatis* in vaginal swabs on day 0, 3, 5, 7 and 10 pi and swabs from uterus and uterine tubes on day 10 pi following intrauterine inoculation during estrus (B) and diestrus (C) with 10^8 IFUs.

and 7 pi had distinct inclusions in the endometrial columnar epithelial cells (Fig. 3) but inclusions were no longer present on day 14 pi. In the IU-group, inclusions were only detected in one pig inoculated during diestrus with $1*10^8$ IFUs (Fig. 3). Negative controls with no primary antibody were performed on the positive sections and confirmed true positivity.

3.3. Antibody response

To characterize the infection-induced antibody response, levels of IgG and IgA antibodies against *C. trachomatis* EBs were determined in serum with an in-house sandwich ELISA. In the TC-group on day 14 pi, 3/4 minipigs had a serum IgG response ($\Delta OD > 0$) and 1/4 minipigs had a serum IgA response. In the IU-group, all minipigs irrespectively of estral inoculation time had a serum IgG response on day 10 pi, 3/3 inoculated during estrus had a serum IgA response and 2/3 inoculated during diestrus had a serum IgA response on day 10 pi.

3.4. Cell-mediated immune response (CMI)

IFN-γ producing CD4 T cells are an important part of the CMI against *Chlamydia* and the IFN-γ response was therefore evaluated in this study by an IFN-γ release assay on PBMCs. In the TC-group a significant IFN-γ response was detected on day 7 (Dunn's multiple comparison; $p < 0.01$) and a further significantly increased response had developed at day 14 pi (Dunn's multiple comparison; $p < 0.01$) (Fig. 4). In the IU-group a significant IFN-γ response was found in the minipigs inoculated during estrus on day 7 pi (Dunn's multiple comparison; $p < 0.05$), however, the change in response was no longer significant on day 10 pi (Fig. 4). The IFN-γ response in the minipigs inoculated during diestrus was not significantly increased at day 7 or 10 pi (Friedman; $p = 0.19$) (Fig. 4).

3.5. Clinical signs and necropsy findings

Only a few minipigs showed clinical signs such as decreased activity following inoculation. In the TC-group, two pigs were given analgesic (20 μg/kg IM buprenorphine) due to a reduced appetite 8 h after inoculation, and normal eating behavior was restored the following day. In the IU-group all minipigs were supplied with 20 μg/kg buprenorphine for the first three days following the laparotomy.

The rectal temperature was measured daily. In the TC-group one pig had a temperature of 39.2 °C approaching the fever limit of 39.5 °C following the catheterization and

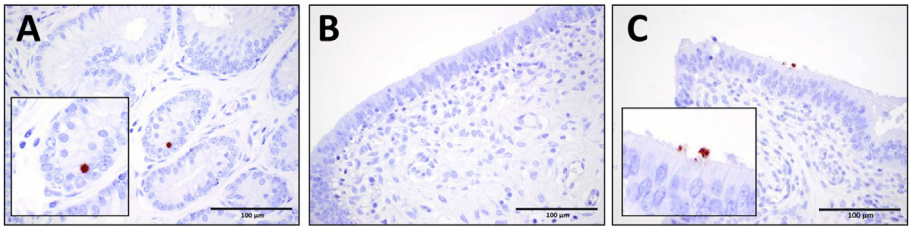

Fig. 3. **Photomicrographs showing immunostained *C. trachomatis* antigen in uterine epithelial cells**. (A) Inclusion from day 7 post inoculation (pi) after transcervical (TC) inoculation. In the TC-group 3/4 minipigs had distinct inclusions on day 7 pi. (B) In the intrauterine (IU) group inoculated during estrus no inclusions were found on day 10 pi. (C) A newly bursted inclusion from day 10 pi in the IU-group inoculated during diestrus with $1*10^8$ IFUs. In this group, inclusions on day 10 pi were detected in 1/3 minipigs.

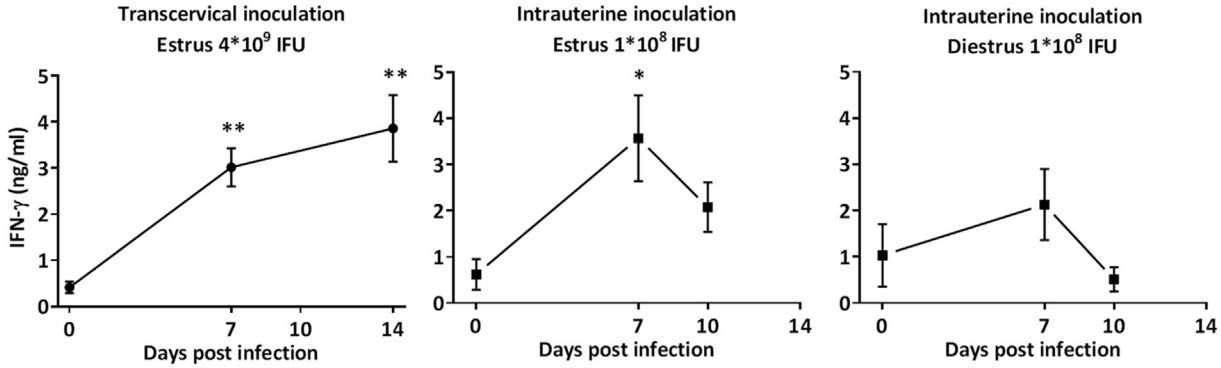

Fig. 4. **IFN-ɣ release assay**. Peripheral blood mononuclear cells (PBMCs) were isolated and re-stimulated with *C. trachomatis* elementary bodies (EBs) (4 μg/ml) and the IFN-ɣ levels in the supernatant were determined. Statistical comparisons were performed comparing the responses on day 7, 10 and 14 to day 0.

inoculation. In the IU-group four minipigs experienced 39.4 °C following the laparotomy and inoculation, but the increase in temperature was in all cases transient with the temperature returning to a normal level the following day.

In the TC-group, one pig had clinically visible mucoid vaginal discharge on day 2 pi, one minipig on day 3 pi (Fig. 5) and two minipigs had clinically visible bloody mucoid discharge on day 3 pi. These findings were consistent with the necropsy findings at day 3 pi in the TC-group, where 3/4 minipigs had bloody or purulent vaginal content (Fig. 5). Furthermore, one pig had purulent exudate in the uterus on day 3 pi (Fig. 5). The minipigs euthanized on days 7 and 14 pi in the TC-group only had mild mucosal edema and hyperemia (Supplementary file 3).

In the IU-group, all minipigs were euthanized on day 10 pi. Macroscopically, one pig had serous fluid in the uterus (Fig. 5), but otherwise only mild edema and hyperemia was found in the uterine mucosa (Supplementary file 3).

3.6. Histopathology

In the TC-group, the histological findings at day 3 pi was characterized by a luminal content with neutrophils, corresponding to the macroscopic finding of pus, and subepithelial

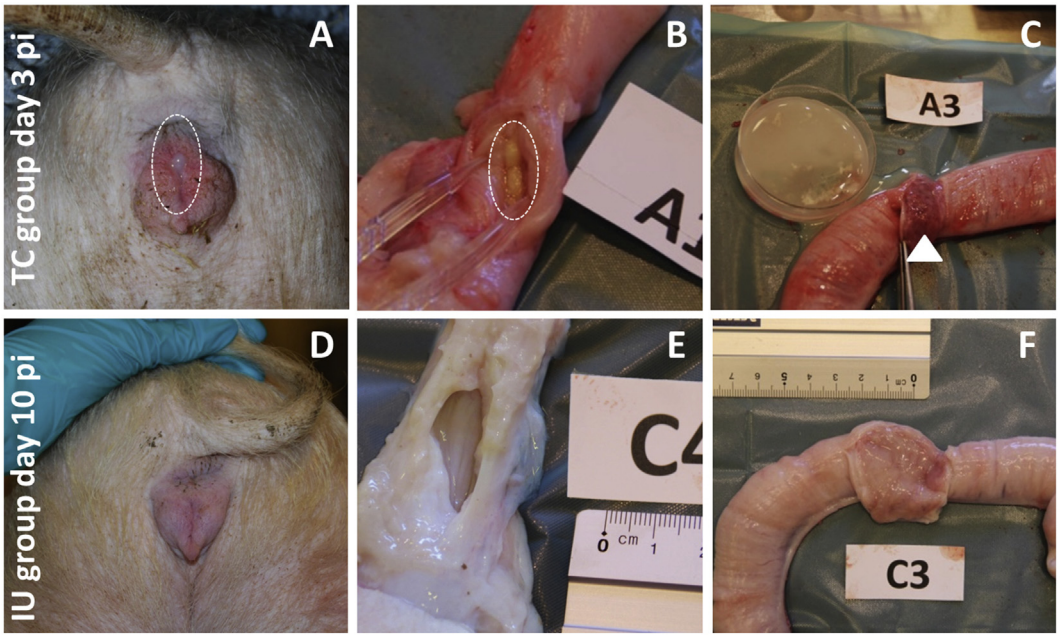

Fig. 5. **Clinical and macroscopic findings**. The top row represents the transcervical (TC) group on day 3 pi. The bottom row represents the intrauterine (IU) group on day 10 pi as well as the TC-group on day 7 pi and 14 pi. A) Vaginal discharge on day 3 pi after transcerivcal inoculation with $1*10^9$ IFUs (TC-group). Vaginal swabs from this animal showed $1.78*10^4$ IFUs. B) Pus in the lumen (encircled) could be seen after opening of the dorsal part of the vagina on day 3 pi after transcervical inoculation. Vaginal samples from this animal showed $1.78*10^4$ IFUs. C) Uterus from day 3 pi after transcervical inoculation filled with purulent exudate (Petri dish) and an inflamed hyperemic mucosa (arrowhead). Vaginal swabs from this animal showed $2.72*10^4$ IFUs. D) Vulva with no vaginal discharge from the IU group on day 3 pi. E) Vagina with no pus in the IU group on day 10 pi. F) Uterus of a pig euthanised on day 14 pi after IU inoculation showing a normally colored (light red) and moist mucosa (samples from this uterus had no viable *Chlamydia*).

infiltration with leukocytes (Fig. 6). On day 7 pi, the leukocytic infiltration was milder and only few neutrophils were detected in the lumen. On day 14 pi, the infiltration was almost absent, except for a few randomly distributed leukocytes. Neutrophils were not detected in the lumen. In the IU-group, all minipigs were euthanized on day 10 pi and the only changes seen were stromal edema and none to mild infiltration with leukocytes. A few minipigs also had few neutrophils in the uterine lumen. Lesions were not observed in the uterine tubes of either group.

4. Discussion

In this study we evaluated the effects of inoculating minipigs with *C. trachomatis* either by transcervical catheterization or directly into the uterus following laparotomy. Transcervical inoculation during estrus resulted in an approximately two log units higher bacterial load (IFUs) than vaginal inoculation during estrus, performed in a previous study [9] (10^3 IFUs/swab vs. 10^5 IFUs/swab) along with an infection that lasted longer (at least 7 days vs. 3–5 days). This is consistent with studies in mice, showing that transcervical inoculation induces a more efficient infection with colonization of the upper genital tract and a higher vaginal level of bacteria within the first 6 days following inoculation [18].

The transcervical inoculation during estrus resulted in a longer lasting infection (viable bacteria 7 days pi, Fig. 2) compared to intrauterine inoculation during estrus (chlamydial DNA 3–5 days pi, Fig. 2). This might be due to the mechanical damage caused to the cervical mucosa during catheterization, and thereby an increased vulnerability of the mucosa to infection during transcervical inoculation.

When comparing intrauterine inoculation during estrus vs. diestrus, it was clear that inoculation during diestrus favored colonization. Inoculation during diestrus induced an infection with a duration of at least 10 days in 2/3 minipigs, whereas intrauterine inoculation during estrus gave infections with a duration of 3–5 days. The more efficient infection, when inoculated during diestrus compared to estrus, is in line with earlier observations in pigs experimentally inoculated with *E. coli* where intrauterine inoculation during metestrus resulted in a robust infection and endometritis, whereas inoculation during estrus did not [13]. These observations might be explained by the more active porcine innate genital immune system during estrus, with for example a significantly higher number of neutrophils in the endometrium [6,19]. It is furthermore consistent with observations from murine models where progesterone treatment before inoculation, corresponding to inoculation during diestrus, is important for successful establishment of *C. trachomatis* infection [4,18]. In addition, studies in rats have shown similar results with progesterone treatment allowing the establishment of *C. trachomatis* infection in the uterus [20]. These findings from animal models support the findings from women indicating that the use of oral contraceptives is associated with an increased risk of *C. trachomatis* infection [21].

The above-described findings are slightly contradictory to *in vitro* studies showing that estrogen prone porcine and human endometrial cells are more susceptible to *Chlamydia in vitro* [22,23]. *In vitro* studies, however, do not include the impact of the genital immune system. It is known that especially the innate part of the genital immune system is more active during estrus in pigs [6,19] and therefore likely to be involved in the fast clearance of infections during estrus. Hence, the faster clearance of infection during estrus, might be due to the thicker epithelium [24] and a faster clearance by the innate immune cells – either directly by the innate cells [25,26] or through an increased activation of the adaptive immune response [27,28].

In general, the low numbers of animals in each group make statistical comparisons challenging, and it cannot be excluded that the differences found in this study relied on biological variation. However, the clear correspondence with findings from other animal models and other microbial agents in pigs adds validity to the presented differences.

Earlier studies in prepubertal conventional pigs have shown the presence of *Chlamydia* spp. until day 25 pi [29], however, as shown by Lorenzen et al. [19] prepubertal minipigs have almost no neutrophils and other leukocytes in the vaginal mucosa, which is likely to explain the longer lasting infection in prepubertal minipigs.

In the present studies *in situ* inclusions of *C. trachomatis* were detected by IHC in the columnar epithelial cells in the endometrial mucosa on days 3 and 7 pi in the TC-group,

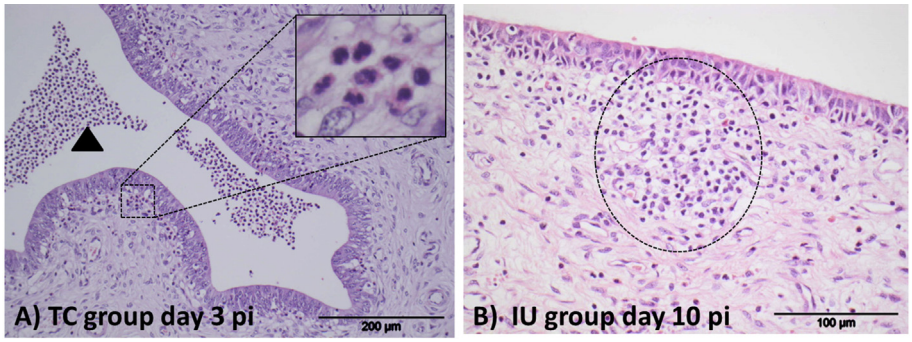

Fig. 6. **Photomicrographs showing genital tract inflammation**. Hematoxylin and eosin stained tissue sections from the genital tract. A) Endometrium on day 3 pi (pig from the transcervical group) with a purulent luminal content (arrowhead) and subepithelial infiltration with neutrophils (insertion). B) Cervicitis on day 10 pi (intrauterine group) with mild to moderate leukocytic infiltration and a small lymphoid follicle (encircled).

whereas only a single inclusion was detected in one minipig in the IU-group inoculated during diestrus. The presence of inclusions confirms that an infection was established. However, the low number of inclusions detected did not correlate with the numbers detected by qPCR, which was higher. This discrepancy might be due to the detection of DNA from non-viable bacteria by the qPCR, but also, and most likely, due to the low sensitivity of the IHC related to the very limited area examined by histology. In a previous study using vaginal inoculation [9] *Chlamydia* antigen was detected by IHC on days 3 pi and 5 pi in the vagina and cervix while *Chlamydia* antigen was not found in the uterus. Hence, vaginal inoculation is more likely to establish a shorter lasting vaginal/cervical infection, whereas transcervical and intrauterine inoculation induces colonization of the uterine mucosa.

On day 3 pi, during necropsy, we observed vaginal discharge and purulent exudate in the genital tract of 2/4 minipigs in the TC-group, which did not occur after vaginal inoculation of *C. trachomatis* in minipigs [9]. This corresponds to findings by Gondek et al. [18] in mice using the *C. trachomatis* LGV strain, where approximately 15% of the mice inoculated transcervically developed pathology, whereas vaginal inoculation with this strain did not lead to significant pathology.

The cervical catheterization is rather harsh for the complex porcine cervix with winding cervical pulvini [6] and it is inevitable to induce mechanical trauma and subsequent inflammation in the cervix during this procedure. It is therefore likely that the bloody discharge on day 3 pi was due to the catheterization procedure and not the *Chlamydia* infection. A mock inoculated group could have clarified this uncertainty. The laparotomy on the other hand is minimally traumatic to the genital tract but the minipigs might be slightly affected by the surgical wound, despite being supplied with opioid analgesia the first three days after surgery. The opioid analgesia was chosen over NSAID therapy, due to the strong analgesic effect and to minimize the effect on the infection-induced inflammation and immune response. However, it cannot be excluded that the opioid therapy has influenced the inflammation in the IU-group [30].

As stipulated above, neither of the methods are without side effects. Laparoscopic inoculation could be a less traumatic alternative to laparotomy, however, placing the syringe into the lumen of the uterus can be difficult without sufficient fixation of the uterus and laparoscopy furthermore requires rather expensive equipment and a surgeon with experience in using the equipment.

Different methods were used to determine the chlamydial load following inoculation; culturing in the TC-group and qPCR in the IU-group. Cultivation of swab material will only detect viable *C. trachomatis* EBs. The quantitative PCR targets the *C. trachomatis* cryptic plasmid DNA and thereby detects all forms of *C. trachomatis* – both RBs and EBs, viable and non-viable. Due to practical circumstances, the vaginal swab samples were frozen (−80 °C) before analysis in the IU-group. Therefore, the qPCR assay with the higher sensitivity was preferred for the IU-group, despite the risk of detecting non-viable *Chlamydia*. Furthermore, vaginal swabs were performed throughout the studies to evaluate the chlamydial load and duration of ongoing infection. However, the detected vaginal level of *Chlamydia* might not exactly reflect the uterine infection, since bacterial flow across the cervix varies through the reproductive cycle.

The immune responses evaluated in this study showed that 3/4 minipigs in the TC-group and 6/6 in the IU-group seroconverted in terms of a positive serum IgG response against *C. trachomatis*. The timing of seroconversion is in line with studies from mice, showing detectable levels of antibodies 10–14 days after a primary infection [31].

The CMI response, in terms of IFN-γ levels from re-stimulated PBMCs, was significant in the TC-group and in the IU-group inoculated during estrus, but not in the IU-group inoculated during diestrus, showing the longest lasting infection. Hence, there seems to be a correlation between a significant IFN-γ response and faster clearance of infection, when comparing the IU-group inoculated during estrus and the IU-group inoculated during diestrus. This correlation corresponds well with studies in mice, showing that IFN-γ producing T cells are important for protection against and clearance of *C. trachomatis* infection [31].

In summary, this study shows that by bypassing the porcine cervix, a longer lasting infection with *C. trachomatis* can be established when compared to vaginal inoculation; Furthermore that intrauterine inoculation during diestrus induces a longer lasting infection than intrauterine inoculation during estrus, in the absence of a significant IFN-γ response. These results indicate that inoculation of pigs should be performed by bypassing the cervix either using transcervical or intrauterine route during diestrus for establishment of the longer lasting *Chlamydia* infection and to improve evaluation of the efficacy of vaccine candidates.

Conflict of interest

All authors confirm that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micinf.2017.01.008.

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