Validation of the *Chlamydia trachomatis* genital challenge pig model for testing recombinant protein vaccines

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Chlamydia trachomatis is a Gram-negative obligate intracellular bacterial pathogen that is the leading cause of bacterial sexually transmitted disease in humans in developing countries. A vaccination programme is considered to be the best approach to reduce the prevalence of *C. trachomatis* infections. However, there are still no commercial *C. trachomatis* vaccines. In order to develop effective *C. trachomatis* vaccines, it is important to identify those antigens that elicit a protective immune response, and to develop new and adequate methods and adjuvants for effective vaccine delivery, as conventional methods have failed to induce protective immunity. In order to test different vaccine candidates, animal models are needed. Former studies have used non-primate monkeys, mice or guinea pig infection models. The present study used a pig model for testing recombinant protein vaccines. Two recombinant proteins, polymorphic membrane protein G (PmpG), and secretion and cellular translocation protein C (SctC), were tested for their ability to create protection in a pig *C. trachomatis* challenge model. The vaccines were administered subcutaneously with GNE adjuvant. Six weeks later, animals were challenged intravaginally with *C. trachomatis* serovar E. After a further 4 weeks, the pigs were euthanized. PmpG-immunized pigs were better protected than pigs immunized with the less promising SctC candidate vaccine antigen. Interestingly, significant protection was apparently not correlated with a strong humoral immune response upon subcutaneous immunization. In conclusion, the pig model is useful for studying the efficacy of vaccine candidates against genital human *C. trachomatis* infection.

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INTRODUCTION

Chlamydia trachomatis is a Gram-negative obligate intracellular bacterial pathogen that is the leading cause of bacterial sexually transmitted disease. If the infection is recognized, it can easily be treated with antibiotics, but once infection and pathology are established, treatment may be less effective. Unfortunately, up to 75 % of women and 50 % of men infected with *C. trachomatis* are asymptomatic (Gonzales *et al.*, 2004; Stamm, 1999). If the infection remains untreated, it often results in pelvic inflammatory disease, ectopic pregnancy and chronic pelvic pain in women that can lead to infertility, epididymitis in men, and infant pneumonia in children (Ibrahim *et al.*, 1996; Taylor-Robinson & Thomas, 1980;

Washington & Katz, 1991; Weström *et al.*, 1992). A vaccination programme is considered to be the best approach to reduce the prevalence of *C. trachomatis* infections, as it would be much cheaper and have a greater impact on controlling *C. trachomatis* infections worldwide than a screening programme or treating infections with antibiotics.

In order to test different vaccine candidates, animal models are needed. Former studies have used non-human primates, mice or guinea pig infection models (reviewed by Vanrompay *et al.*, 2006). Sustained genital tract infections with oculo-genital serovars of *C. trachomatis* have been established in a number of animal species such as baboons (Thygeson, 1936), other non-human primates (Johnson *et al.*, 1980; Möller & Mårdh, 1980; Ripa *et al.*, 1979) and seldom-used models, such as rabbits, rats and cats (Woodland *et al.*, 1983). Mice became and continue to be the small animal of choice from 1981, when it was shown that they are susceptible to genital tract infection

Abbreviations: EB, elementary body; HRP, horseradish peroxidase; MOMP, major outer-membrane protein; p.i., post-infection; Pmp, polymorphic membrane protein; RB, reticulate body; SctC, secretion and cellular translocation protein C; T3SS, type 3 secretion system; TCID₅₀, 50 % tissue culture infective dose.

with human isolates of *C. trachomatis* (Tuffrey & Taylor-Robinson, 1981) and with the mouse pneumonitis agent (Barron *et al.*, 1981). None of these models perfectly mimics the anatomy, histology and endocrinology of the human reproductive system or the pathogenesis and immune responses that occur during a human genital infection. There is a need for an intermediate animal model between the mouse and primate animal models to play a role in successful vaccine development. This animal model should contribute to the process where interesting findings in the mouse can be translated into possible vaccine candidates to test in primates for ultimate use against human genital tract infections.

Recently, Vanrompay *et al.* (2005) demonstrated that pigs may be useful as a large-animal model for studying the pathology, pathogenesis and immune response of human *C. trachomatis* genital infections. Pigs are physiologically and genetically closely related to humans (Bray *et al.*, 2003; Tuggle *et al.*, 2003). Moreover, Tuggle *et al.* (2003) demonstrated that the majority of genes expressed in the major porcine female reproductive tissues are ubiquitously expressed in human genital tissues. Dawson *et al.* (2007) calculated homology within the immune system of humans, pigs and mice, and found 65% homology for the mucosal immune system between humans and pigs and only 35% homology between humans and mice. In addition, sampling is easy in this large-animal model. Thus, the pig model may function as either an intermediate animal model between mouse and non-human primates or as a substitute for the latter during the development of vaccines for use in human clinical trials.

To validate the pig model for screening of candidate vaccine antigens, two recombinant protein vaccines were tested. One was based on the polymorphic membrane protein (Pmp) G, and one on the secretion and cellular translocation protein C (SctC). PmpG is a member of the Pmp family, which is unique to the order Chlamydiales. For *C. trachomatis*, nine Pmp proteins have been identified (Gomes *et al.*, 2006; Stephens *et al.*, 1998), the genes for which are located in two clusters, *PmpA–C* and *PmpE–I*, except for the *PmpD* gene. Different Pmp subtypes of *C. trachomatis* have recently become leading candidates in the development of a component vaccine against chlamydial infection (Crane *et al.*, 2006; Karunakaran *et al.*, 2008). Mygind *et al.* (2000) identified PmpG and PmpH as major constituents of the *C. trachomatis* L2 outer-membrane complex. Furthermore, PmpG is one of the most conserved Pmp proteins (Mygind *et al.*, 2000; Stothard *et al.*, 2003).

SctC is a structural protein of the chlamydial type 3 secretion system (T3SS) (Beeckman & Vanrompay, 2010). This protein is part of the secretin of the T3SS and pierces the outer membrane of the bacteria. Furthermore, it is one of seven T3SS proteins that are highly conserved among *C. trachomatis* serovars (Subtil *et al.*, 2000). Goodin *et al.* (2005) assessed the immunogenicity and potential protective efficacy of the YscC protein of *Yersinia pestis* against

lethal plaque challenge. Different forms of YscC protein failed to elicit a protective immune response in the mouse model for lethal plaque infection, probably due to incorrect protein folding, inaccessibility of the YscC protein to the host immune system *in vivo* or the nature of the immune response generated. Also, the presence of the single recombinant protein alone may not accurately represent the conformation of YscC in combination with additional proteins from the T3SS.

The purpose of this study was to evaluate a pig model for testing vaccine candidates that might protect against *C. trachomatis* genital infection. Recombinant PmpG and SctC proteins were tested as vaccine candidates, representing a promising and less promising candidate vaccine antigen, respectively.

METHODS

C. trachomatis. *C. trachomatis* serovar E strain 334 was used to challenge pigs after vaccination. This strain was isolated from a male and a female patient with a symptomatic clinical course of infection (Lyons *et al.*, 2004). The female patient reported lower abdominal pain and mucopurulent vaginal secretion, whilst the male patient suffered from dysuria and reported mucous secretion. The bacteria were grown in McCoy cells using standard techniques (Vanrompay *et al.*, 1992). The 50% tissue culture infective dose (TCID₅₀) of the bacterial stock was determined by the method of Spearman and Kärber (Mayr *et al.*, 1974).

Proteins. His-labelled PmpG and SctC proteins (both from *C. trachomatis* serovar D strain UW-3/Cx) were expressed in *Escherichia coli* strain C41-pET28a and subsequently purified under denaturing conditions by immobilized metal affinity chromatography using Talon beads (IMAC), followed by dialysis in PBS. The purified recombinant proteins were revealed by SDS-PAGE followed by Coomassie blue staining (Fig. 1). The recombinant proteins were

Fig. 1. Coomassie blue stained SDS-PAGE gel of purified recombinant SctC (102 kDa) and PmpG (115 kDa) proteins (lanes 3 and 4, respectively). The protein bands of PmpG and SctC were clearly visible at the expected position. However, additional bands, probably *E. coli* protein, were also present in the purified protein fractions. Lane 1, size marker (kDa); lane 2, negative control (PBS).

formulated in GNE adjuvant. The adjuvant GNE (Intervet) is a non-bacterial water-in-oil adjuvant based on sorbitan mono-oleate (Span-20), Tween 80 and paraffin. This adjuvant is frequently used for veterinary purposes and elicits a strong antibody response.

Immunization experiment. Fifteen 6-week-old conventionally bred female pigs (Belgian Landrace), seronegative for antibodies against family *Chlamydiaceae* as determined by a recombinant major outer-membrane protein (MOMP)-based ELISA, were randomly divided into three groups of five. The pigs were treated orally with colistine [15 000 U (kg body weight)⁻¹] (Colivet; Prodivet Pharmaceuticals) for the first 4 days upon arrival in order to prevent *E. coli* infection.

At day 0, pigs of group 2 (PmpG) and group 3 (SctC) were immunized with 2 ml recombinant PmpG or SctC, respectively. The concentration of both antigens in GNE was 0.075 µg µl⁻¹. Vaccines were administered subcutaneously. Pigs of the placebo group (group 1) received PBS with GNE adjuvant and functioned as non-vaccinated controls. At 6 weeks post-vaccination (day 41), all pigs were infected by intravaginal injection of a TCID₅₀ (1 × 10⁸ cells) of *C. trachomatis* serovar E strain 334. Pigs were sacrificed at 5 days post-infection (p.i.). Euthanasia was performed by intravenous injection of an overdose of (70 mg kg⁻¹) pentobarbital (Nembutal; Ceva Santé Animale), followed by exsanguination.

Following challenge, clinical signs were monitored every other day and body temperature was measured twice a week. Vaginal swabs were also collected in 2 ml 2-sucrose phosphate transport medium for chlamydial isolation and in 2 ml PBS (pH 7.4) for mucosal antibody detection. Sera were obtained from blood collected from the jugular vein of the pig and used for antibody detection. Samples for chlamydial isolation were stored at -80 °C and sera for antibody detection at -20 °C until analysis. At euthanasia, all pigs were examined for gross lesions. The scoring system for macroscopic lesions is presented in Table 1. Samples of the spleen, liver, vagina, cervix, corpus uteri, uterine tubes, oviducts, ovaries and urethra were embedded in methylcellulose medium, frozen in liquid nitrogen and stored at -80 °C until preparation for cryostat tissue sections. A sample of all tissues was also fixed in 10 % phosphate-buffered formalin for histopathology. The vaccination scheme and the experimental set-up are presented in Tables 2 and 3. The Ghent University Ethical Commission approved the experimental design.

***C. trachomatis* culture.** Vaginal swabs were shaken (300 r.p.m.) for 1 h at 4 °C to release the bacteria from the rayon tip into 2-sucrose phosphate transport medium. The transport medium containing the bacteria was inoculated onto McCoy cells that had been cultured on 13 mm coverslips inserted in Chlamydia Trac bottles (International Medical) as described previously by Vanrompay *et al.* (1992). In addition to the described protocol, cells were washed twice with 1 ml phosphate buffer (36 mM CaCl₂·2H₂O, 2 mM MgCl₂·6H₂O, 34 mM NaCl, 0.68 mM KCl, 1.8 mM NaH₂PO₄·2H₂O and 0.4 mM KH₂PO₄) supplemented with 0.003 % DEAE/dextran before the inoculum was added to the cells. Chlamydial growth was monitored using a Mikrotrak immunofluorescence test (Kordia) based on detecting *C. trachomatis* MOMP.

***C. trachomatis* antigen detection in tissue sections.** Cryostat tissue sections (5 µm) were also examined by use of Mikrotrak immunofluorescence staining (Kordia). All slides were examined microscopically (Nikon Eclipse TE 2000-E; magnification × 600) and bacteria were quantified as follows: positive cells were counted in five different randomly chosen microscopic fields. The calculated mean was presented as a score ranging from 0 to 4. A score of 0 indicated that no antigen was present. Scores of 1 and 2 indicated mean values of 1–5 and 6–10 elementary bodies (EBs) per five microscopic fields in the absence of inclusions, respectively. Scores of 3 and 4 represented 1–5 and 6–10 inclusion-positive cells, respectively.

ELISA for PmpG and SctC IgA and IgG antibodies in serum and mucosal secretions. ELISAs were performed on serum and vaginal swabs in PBS. Sera were heat inactivated (56 °C) for 30 min and pre-treated with kaolin to reduce background activity (Novak *et al.*, 1993). Vaginal swabs were shaken (300 r.p.m.) for 1 h at room temperature and then centrifuged for 10 min at 360 g and the supernatant collected. Serum and mucosal antibody titres were determined using the following protocol. Briefly, 96-well ELISA plates (MaxiSorb; Nunc) were coated with 1 µg recombinant PmpG or SctC antigen per well for 3 h at 37 °C. Coating buffer consisted of 0.04 M NaHCO₃ and 0.006 M Na₂CO₃ dissolved in double-distilled water. Non-specific binding sites were blocked overnight at 4 °C using PBS supplemented with 5 % BSA. Serial twofold dilutions of sera and mucosal samples were assayed starting from a dilution of 1 : 100. The dilution buffer consisted of PBS with 3 % BSA and 0.05 % Tween 20. To determine IgG antibody titres, plates were incubated with a 1 : 5000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-pig IgG (H + L) antibody (Bethyl Laboratories) for 1 h at 37 °C. For the determination of IgA-specific antibody titres, plates were incubated with a 1 : 50 dilution of mouse anti-swine IgA mAb (clone 27.8.1; Van Zaane & Hulst, 1987) and a 1 : 5000 dilution of HRP-conjugated anti-mouse IgG (H + L) (Dako). Both incubations steps were performed for 1 h at 37 °C. Finally, the substrate and chromogene ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] (KPL) was added. Anti-PmpG and anti-SctC immunoglobulin titres were presented as the reciprocal of the highest serum dilution that gave an absorbance value at 405 nm above the cut-off value (mean A₄₀₅ of seronegative specific-pathogen-free pigs ± 2 × SD). Sera from a previous experimental infection were used as positive controls. Sera of colostrum-free piglets and dilution buffer served as negative controls. Results were presented as A₄₀₅ ± 2 × SD.

Whole EB ELISA. Anti-*Chlamydia* serum IgG titres were also measured in an ELISA using whole EBs (serovar D strain UW-3/Cx) as antigen. ELISA plates (Maxisorp) were coated overnight at 4 °C with purified EBs in PBS. Non-specific binding sites were blocked in blocking buffer. Sera were diluted in ELISA buffer (PBS with 1 % BSA, 0.1 % kaolin, 0.05 % Tween 80) and tested at twofold serial dilutions starting at 1 : 16. The plates were then incubated with HRP-conjugated goat anti-swine IgA (Southern Bioscience) or IgG (KPL) diluted in EIA buffer. Subsequently, plates were incubated with tetramethylbenzidine substrate solution (Sanquin). The reaction was stopped by the addition of 1 M H₂SO₄ and absorbance was read at 450 nm (Titertek Multiscan Plus; ICN). Negative and positive control

Table 1. Scoring system for *C. trachomatis* macroscopic lesions in pigs

Tissue	Score 0 (0)	Score 1 (+)	Score 2 (++)	Score 3 (+++)
Vagina, cervix, corpus uteri, uterine tubes, oviducts, ovaries, urethra	Normal	Slightly congested	Moderately congested	Severely congested
Spleen and liver	Normal	Slightly enlarged	Moderately enlarged	Severely enlarged

Table 2. Vaccination scheme

Group	Name	Vaccination	Dose (µg)	Route
1 (n=5)	Placebo group	GNE adjuvant	–	Subcutaneous
2 (n=5)	PmpG group	PmpG recombinant protein in GNE adjuvant	150	Subcutaneous
3 (n=5)	SctC group	SctC recombinant protein in GNE adjuvant	150	Subcutaneous

sera from a previous *C. trachomatis* experimental infection experiment were included on each plate and used to calculate the cut-off value. The mean $\pm$ SD was calculated for every group. End-point dilutions (limiting dilution titres) were calculated using the MultiCalc program (Pharmacia). The cut-off value for each plate was calculated by the program using $MA \times 1.5$, where MA was the mean absorbance for the negative control.

Western blotting. Chlamydial proteins (*C. trachomatis* serovar D strain UW-3/Cx), as well as recombinant purified PmpG and SctC, were analysed by SDS-PAGE (4–12 % gradient; Invitrogen) and blotted onto a 0.2 µm nitrocellulose membrane (Invitrogen) using an iBlot dry blotting system (Invitrogen). After blotting, the membrane was incubated in blocking buffer [1 % skimmed milk in 0.04 M PBS (pH 7.2) with 0.05 % Tween 20] for 30 min at 37 °C followed by incubation with primary antibody for 1 h at 37 °C. Subsequently, the blotting membrane was incubated with HRP-conjugated goat anti-swine IgG (Nordic) diluted 1:1000 in blocking buffer for 1 h at 37 °C. Next, the blots were developed with tetramethylbenzidine substrate.

Histopathology. Samples were fixed in 10 % phosphate-buffered formalin, dehydrated and embedded in paraffin, sectioned at 5 µm and stained with haematoxylin and eosin. All slides were examined microscopically (Leitz). The microscopic findings were either graded as minimal histological change (0) or slight (1), moderate (2), marked (3) or severe (4) histological change, or indicated as present/absent without a grade. For the uterus tubes and oviduct, a mean grade was determined for the samples from the left and right sides.

Statistical analyses. All statistical analyses were performed using the non-parametric Mann–Whitney *U* test. Results were considered significantly different if $P < 0.05$.

RESULTS

Clinical signs and macroscopic lesions

Elevated body temperatures, up to 40 °C, were observed in all three groups 2 days after infection with *C. trachomatis*. After 4 days, the body temperatures of vaccinated pigs

decreased gradually to normal values (38.5 °C). After 21 days, pigs of the placebo group still had fever with a mean body temperature of 39.8 °C. However, the differences were not statistically significant.

At necropsy, severe gross lesions were noticed in the placebo group. The mean scores for *C. trachomatis* macroscopic lesions are presented in Table 4. These non-vaccinated pigs showed hyperaemia of the vulva and vagina, congestion and oedema of the uterine wall, large amounts of clear watery uterine contents, congestion of the mucosae of the upper genital tract and enlarged local draining lymph nodes. The PmpG-vaccinated group showed only mild to no lesions. Two of the five pigs had small amounts of watery uterine contents and showed very mild hyperaemia of the cervix and the uterus, and mild mucosal congestion of the uterine tubes. One had a slightly congested mesovarium. No additional macroscopic lesions were observed. The SctC-vaccinated group displayed severe gross lesions. Surprisingly, lesions in the vagina were more pronounced than for the placebo group. The vaginal and cervical mucosae of all SctC-immunized pigs were clearly congested and oedematous. The uterus of all animals contained clear watery fluid, some more than others. Four of the five pigs showed congested uterine horns and one pig had a slightly enlarged spleen.

C. trachomatis detection in the urogenital tract

The mean immunofluorescence staining scores on cryostat sections of the reproductive tract are presented in Fig. 2. Strong *C. trachomatis* replication in the urogenital tract of the placebo animals was seen but with rather high variations for pigs within a group. The staining scores demonstrated the presence of both infectious EBs and replicating organisms [reticulate bodies (RBs)] inside intracellular inclusions. We could detect inclusions in tissues of both the lower and upper reproductive tract and the urethra. In the reproductive tract of the SctC-vaccinated animals, both

Table 3. Experimental set-up

Time	Manipulation	Sample
Day 0	Vaccination	Blood
Day 41	Challenge	Blood/vaginal swabs
Days 41–64		Vaginal swabs taken every 2 days
		Blood taken on day 55
		Clinical signs observed every other day
		Rectal temperature measured twice a week
Day 64	Euthanasia	Blood/vaginal swabs/tissues for histology/tissues for cryostat tissue sections

Table 4. Mean scores per group $\pm$ SD (percentage of positive pigs) for *C. trachomatis* macroscopic lesions at 25 days post-challenge

Means with different superscript letters within a row are significantly different ($P < 0.05$).

Tissue	Group 1 (placebo)	Group 2 (PmpG)	Group 3 (SctC)
Vagina	0.4 ± 0.9^a (20)	0 ± 0^b (0)	2.0 ± 0.0^c (100)
Cervix	2.2 ± 0.5^a (100)	0.4 ± 0.5^b (40)	2.2 ± 0.5^a (100)
Corpus uteri	1.8 ± 1.1^a (80)	0.4 ± 0.6^b (40)	1.4 ± 1.1 (80)
Uterine tubes	2.0 ± 0^a (100)	0.4 ± 0.6^b (40)	1.4 ± 1.1 (80)
Oviducts	0 ± 0 (0)	0 ± 0 (0)	0.4 ± 0.6 (40)
Ovaries	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)
Urethra	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)
Liver	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)
Spleen	0 ± 0 (0)	0 ± 0 (0)	0.40 ± 0.44 (20)

EBs and RBs could be demonstrated, but compared with the placebo group, fewer inclusions were found. Also, only EBs were found in the ovaries of the SctC-immunized animals, and this was also noted in the placebo group. EBs were present in the lower and upper genital tract of the PmpG-immunized group. If inclusions were found in a specific tissue, they could only be detected in one or two animals out of the five. In the urethra, both EBs and RBs could be detected. Chlamydial antigens were absent in the spleen and liver of all animals. We found significant differences ($P < 0.05$) only in the mean antigen scores of the vagina. The PmpG-vaccinated group revealed less antigen detection in vaginal tissue when compared with the SctC-immunized group and controls.

C. trachomatis excretion

Table 5 presents the results of *C. trachomatis* vaginal shedding from the day of the challenge until euthanasia.

Fig. 2. Mean scores for the detection of *C. trachomatis* in the urogenital tract at day 25 p.i. The asterisk indicates statistically significant differences ($P < 0.05$) between the PmpG-vaccinated group and the SctC-vaccinated group, and between the PmpG-vaccinated group and the placebo-vaccinated group. Statistical analysis was carried out with the Mann-Whitney *U* test. Results are shown as mean scores $\pm$ SD; no line indicates that there was no variation. White bars, placebo group; black bars, PmpG group; grey bars, SctC group.

No vaginal shedding was seen at day 0 and *C. trachomatis* was detected from day 2 p.i. onwards. Vaccinated and non-vaccinated placebo animals consistently excreted *C. trachomatis* during the experiment. The mean excretion scores of the PmpG-vaccinated group were always lower than those of the placebo group. At days 4, 6 and 22, the differences were statistically significant. The mean excretion scores of the PmpG-vaccinated group were always (except on days 10 and 14) lower than those of the SctC-vaccinated group. At the day 10 and 14 time points, excretion was detected in only three and two of the five SctC-immunized animals, respectively, whilst on the other days, *C. trachomatis* excretion was detected in all five animals. The mean secretion scores for the PmpG- and the SctC-immunized groups did not differ significantly during the experiment, with the exception of day 6 when a significant lower mean score for the PmpG-immunized group could be observed. The mean excretion scores of the placebo group and the SctC-vaccinated group were

Table 5. Mean scores for *C. trachomatis* vaginal excretion at different time points following challenge

Means with different superscript letters within a row are significantly different ($P < 0.05$).

Time p.i.	Group 1 (placebo)	Group 2 (PmpG)	Group 3 (SctC)
Day 0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Day 2	2.2 ± 1.1	1.2 ± 1.1	2.2 ± 1.1
Day 4	2.2 ± 1.1^a	1.0 ± 0.0^b	1.8 ± 1.1
Day 6	2.2 ± 1.1^a	1.0 ± 0.0^b	2.2 ± 1.1^a
Day 8	2.2 ± 1.1	1.4 ± 1.5	1.4 ± 0.9
Day 10	2.6 ± 0.9^a	1.4 ± 0.9	0.6 ± 0.6^b
Day 12	2.6 ± 0.9	1.8 ± 1.1	2.2 ± 1.1
Day 14	2.6 ± 0.9^a	1.8 ± 1.1^a	0.4 ± 0.6^b
Day 16	2.6 ± 0.9	1.6 ± 1.3	2.4 ± 1.3
Day 18	0.8 ± 1.3	0.8 ± 0.5	1.0 ± 0.0
Day 20	2.6 ± 0.9	2.2 ± 1.1	3.0 ± 0.0
Day 22	2.6 ± 0.9^a	1.0 ± 0.0^b	2.0 ± 1.0^a
Day 25	3.0 ± 0.0	2.2 ± 1.1	2.6 ± 0.9

statistically the same during the experiment except for days 10 and 14. At the end of the experiment, the mean scores for all three groups were statistically the same.

Serum and mucosal antibody responses measured by SctC ELISA

At day 0 (6 weeks of age), mean anti-SctC IgG-specific antibodies could be demonstrated in all pigs of the placebo group and in all sera (100%) of the pigs of the SctC-immunized group. This was not tested in the PmpG-vaccinated pigs. Serum antibody titres at day 0 did not differ significantly for the two groups. At day 49, the day of the *C. trachomatis* challenge, mean anti-SctC IgG-specific serum antibody titres of the placebo group and the SctC-vaccinated group were 140 ± 54 and $20\,400 \pm 0$, respectively, which was statistically different. We also measured anti-SctC IgA-specific serum antibody titres. At day 0, IgA-specific serum antibodies were absent. At day 49, anti-SctC IgA-specific serum antibody titres for the placebo group were still zero, whilst the SctC-vaccinated group showed a titre of 6656 ± 2804 .

Prior to immunization, mucosal antibodies could not be demonstrated in either the placebo group or the SctC-immunized group. At day 49 (day of challenge), anti-SctC IgG-specific mucosal antibody titres were 0 ± 0 and 120 ± 44 for the placebo and vaccinated groups, respectively. Mucosal anti-SctC IgA-specific antibodies could not be demonstrated in either the placebo or the SctC-vaccinated group.

Serum and mucosal antibody responses measured by PmpG ELISA

Surprisingly, anti-PmpG IgG and IgA antibodies could not be detected in either the placebo group or the PmpG-vaccinated group using an ELISA with recombinant PmpG coated on the plates. The positive-control serum gave an A_{405} value of 0.350, which is low. Mucosal antibodies could not be demonstrated in either the placebo or the PmpG-vaccinated group.

Serum antibody responses measured by whole EB ELISA and Western blotting

Fig. 3 shows the mean EB-specific antibody response for the three groups, prior to immunization (day 0), on the day of challenge (day 41) and 3 weeks post-challenge (day 64). All control pigs, PmpG-vaccinated pigs and SctC-vaccinated pigs

Fig. 3. Mean IgG titre measured by ELISA using whole EBs. Sera were taken at day 0 (vaccination, $t=0$) (white bars), day 41 (challenge, $t=41$) (black bars) and day 64 (3 weeks after challenge, $t=64$) (grey bars). Results are shown as means $\pm$ SD.

were ELISA positive with an antibody titre ranging from 45 to 180. This was confirmed by Western blotting for pigs 1 (placebo group), 14 (PmpG group), 18, 19 and 20 (SctC group) (Fig. 4). In Western blotting, serum of the placebo group was tested against whole EBs and several protein bands were visualized in all five pigs, 23 days after challenge ($t=64$) (Fig. 4a). According to the Western blotting results, PmpG immunization was not very successful, as only the serum of pigs 11, 14 and 15 showed a specific band of 115 kDa, when tested against whole EBs on the day of challenge (Fig. 4b). However, non-specific (non-PmpG) protein bands were also observed in four of the five immunized pigs (not in pig 12). Following challenge, proteins bands were visualized for all animals. Western blotting revealed successful immunization with SctC. On the day of challenge ($t=41$), serum of all SctC-vaccinated animals reacted strongly with SctC of whole EBs (Fig. 4c; lanes 2). The SctC bands were different in size (range 75–102 kDa), which could be due to post-translational modification. The sera of SctC-immunized pigs also reacted strongly with the His-purified recombinant SctC fraction (lane 3). However, the latter reaction clearly demonstrated the presence of contaminating *E. coli* fragments in the recombinant SctC fraction used for immunization, as many non-SctC bands were visualized after reaction with pig serum.

Histopathology

The mean scores for the severity of histopathological lesions are presented in Table 6. The findings in the urogenital tract in this study consisted of: (i) the presence

Fig. 4. (a) Analysis of total IgG (H+L) antibody responses measured by Western blotting, using sera (diluted 1:100) from placebo-immunized pigs. Sera were taken on the day of immunization ($t=0$), 2 weeks p.i. ($t=55$) and 3 weeks p.i. ($t=64$). Lane 1, size marker (kDa); lane 2, EBs (UW-3/Cx); lane 3, purified recombinant protein. (b) Analysis of total IgG (H+L) antibody responses measured by Western blotting, using sera (diluted 1:100) from pigs immunized with recombinant PmpG (115 kDa). Sera were taken on the day of immunization ($t=0$), the day of challenge ($t=41$) and 3 weeks p.i. ($t=64$). Lane 1, size marker (kDa); lane 2, EBs (UW-3/Cx); lane 3, purified recombinant protein. (c) Analysis of total IgG (H+L) antibody responses measured by Western blotting, using sera (diluted 1:100) from pigs immunized with purified recombinant SctC (102 kDa). Sera were taken on the day of immunization ($t=0$), challenge ($t=41$), 2 weeks p.i. ($t=55$) and 3 weeks p.i. ($t=64$). Lane 1, size marker (kDa); lane 2, EBs (UW-3/Cx); lane 3, purified recombinant protein, except for the SctC blot at $t=64$. For the SctC blot at $t=64$: lane 1, purified recombinant protein; lane 2, EBs; lane 3, size marker (kDa).

Table 6. Mean severity scores ($\pm$ SD) of histopathological lesions in urogenital tissues

Tissue	Lesion	Group 1 (placebo)	Group 2 (PmpG)	Group 3 (SctC)
Urethra	Superficial layer of exfoliated and/or inflammatory cells	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	Interepithelial inflammatory cells*	0.6 $\pm$ 0.5	0.5 $\pm$ 0	0.7 $\pm$ 1.2
	Intraepithelial oedema	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.6 $\pm$ 1.7
	Infiltration of polymorphonuclear cells in lamina propria	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	Infiltration of lymphocytes and plasma cells in lamina propria	1.0 $\pm$ 0.5	0.8 $\pm$ 0.7	1.4 $\pm$ 1.7
Vagina	Superficial layer of exfoliated and/or inflammatory cells	0.2 $\pm$ 0.5	0.4 $\pm$ 0.5	0.6 $\pm$ 1.7
	Interepithelial inflammatory cells*	1.6 $\pm$ 1.5	1.4 $\pm$ 0.5	0.8 $\pm$ 0.6
	Intraepithelial oedema	1.6 $\pm$ 0.9	1.0 $\pm$ 1.2	0.4 $\pm$ 0.6
	Infiltration of polymorphonuclear cells in lamina propria	1.2 $\pm$ 1.6	0.4 $\pm$ 0.5	0.0 $\pm$ 0.0
	Infiltration of lymphocytes and plasma cells in lamina propria	1.6 $\pm$ 1.9	1.4 $\pm$ 0.5	0.6 $\pm$ 1.7
Cervix	Superficial layer of exfoliated and/or inflammatory cells	0.6 $\pm$ 1.3	0.6 $\pm$ 0.5	0.0 $\pm$ 0.0
	Interepithelial inflammatory cells*	1.0 $\pm$ 1.2	1.6 $\pm$ 0.8	0.6 $\pm$ 0.5
	Intraepithelial oedema	1.0 $\pm$ 0.7	1.0 $\pm$ 0.5	1.4 $\pm$ 0.9
	Infiltration of polymorphonuclear cells in lamina propria	0.8 $\pm$ 1.3	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	Infiltration of lymphocytes and plasma cells in lamina propria	1.6 $\pm$ 1.5	0.4 $\pm$ 0.5	1.0 $\pm$ 1.8
Corpus uteri	Superficial layer of exfoliated and/or inflammatory cells	0.0 $\pm$ 0.0	0.3 $\pm$ 0.6	0.0 $\pm$ 0.0
	Interepithelial inflammatory cells*	1.0 $\pm$ 0.7	0.7 $\pm$ 1.1	0.4 $\pm$ 0.0
	Intraepithelial oedema	1.0 $\pm$ 0.7	0.5 $\pm$ 0.5	0.4 $\pm$ 1.4
	Infiltration of polymorphonuclear cells in lamina propria	0.4 $\pm$ 0.5	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	Infiltration of lymphocytes and plasma cells in lamina propria	1.6 $\pm$ 0.5	0.3 $\pm$ 0.6	0.8 $\pm$ 1.4
Uterine horns	Superficial layer of exfoliated and/or inflammatory cells	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	Interepithelial inflammatory cells*	0.6 $\pm$ 0	1.2 $\pm$ 0.8	0.8 $\pm$ 0.4
	Intraepithelial oedema	0.2 $\pm$ 0.6	1.0 $\pm$ 0.7	0.6 $\pm$ 0.5
	Infiltration of polymorphonuclear cells in lamina propria	0.2 $\pm$ 0.6	0.2 $\pm$ 0.4	0.2 $\pm$ 0.4
	Infiltration of lymphocytes and plasma cells in lamina propria	0.8 $\pm$ 0.6	1.0 $\pm$ 0.0	1.0 $\pm$ 0.0
Oviduct	Interepithelial inflammatory cells*	0.0 $\pm$ 0.0	0.2 $\pm$ 0.4	0.4 $\pm$ 0.5
	Intraepithelial oedema	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	Oedema in the lamina propria	0.6 $\pm$ 1.3	0.0 $\pm$ 0.0	0.2 $\pm$ 0.4
	Infiltration of polymorphonuclear cells in lamina propria	0.4 $\pm$ 0.5	0.4 $\pm$ 0.5	1.4 $\pm$ 1.5
	Infiltration of lymphocytes and plasma cells in lamina propria	0.6 $\pm$ 1.3	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0

*Lymphocytes and polymorphonuclear cells.

of a superficial layer of exfoliated (epithelial) cells and/or inflammatory cells: cells and debris lying on the surface of the epithelium; (ii) interepithelial inflammatory cells, thus indicating the presence of migrating lymphocytes or polymorphonuclear cells in the epithelium; (iii) intraepithelial oedema characterized by swollen epithelial cells

sometimes associated with degenerative features and/or cell death (apoptosis); (iv) infiltration of inflammatory cells in the lamina propria with infiltration of the connective tissue layer beneath the epithelium with polymorphonuclear cells (neutrophilic and/or eosinophilic), a feature of more acute inflammation, or with lymphocytes and/or plasma cells, a

feature of more subacute to chronic inflammation, which was sometimes associated with the formation of lymphoid follicles; (v) infiltration of lymphocytes in the lamina propria; and (vi) hyperaemia.

Lesions related to the chlamydial infection were found especially in the lower genital tract, namely the vagina, cervix and corpus uteri, but also in the urethra, uterus tubes and oviduct. No lesions were found in the ovaries. In the urethra, the mean severity of the lesions for the PmpG group was comparable to the placebo group, but there was a slight mean increase in severity score for the SctC group in comparison with the non-vaccinated placebo group. In the vagina, cervix and corpus uteri, a slight increase in severity of the lesions (especially for infiltration of lymphocytes and plasma cells in the lamina propria) could be observed for the placebo group. In the vagina, cervix and corpus uteri, a slight decrease in mean severity of the lesions was noted for the vaccinated groups, especially for the less protected SctC group, compared with the placebo group. However, histological differences at euthanasia were too small to conclude a protective action from either of the vaccines.

DISCUSSION

In this study, the main objective was to evaluate the pig *C. trachomatis* genital challenge model developed by Vanrompay *et al.* (2005) for testing candidate vaccine antigens. To evaluate the pig model, two recombinant protein vaccines based on PmpG or SctC were administered subcutaneously to pigs. After 6 weeks, animals were challenged with *C. trachomatis* serovar E. After a further 4 weeks, pigs were euthanized.

Seronegative pigs were selected by use of a *C. trachomatis* serovar E recombinant MOMP-based antibody ELISA. However, all MOMP-seronegative animals ($n=15$) gave a positive reaction in the SctC-based ELISA and some also reacted positively in the whole EB ELISA. We previously demonstrated that a MOMP-based ELISA is highly sensitive and specific (Verminen *et al.*, 2006) and serological cross-reactions between the chlamydial MOMP and proteins of other pathogens have not been described so far. In contrast, serological cross-reactions between the chlamydial SctC protein and SctC proteins of other Gram-negative organisms might occur, as the amino acid consensus similarity between the SctC of *C. trachomatis* and for example *Yersinia enterocolitica*, *E. coli* and *Aeromonas hydrophila* is 27.1, 25.3, and 26.1 %, respectively. All these bacteria infect pigs. The presence of epitopes that are cross-reactive with other Gram-negative bacteria such as *E. coli*, *Salmonella typhimurium* and *Coxiella burnetii* has been described for the chlamydial LPS (10 kDa) (Caldwell & Hitchcock, 1984; Nurminen *et al.*, 1984; Schramek *et al.*, 1980) and the chlamydial hsp60 (Yuan *et al.*, 1992). Cross-reactive epitopes might explain the high number of SctC positives before immunization, especially as the recombinant SctC

fraction still contained *E. coli* proteins as visualized by Coomassie blue staining. Non-SctC and non-PmpG bands on the blots were probably also due to antibodies against contaminating *E. coli* fragments, especially when using an adjuvant as strong as GNE.

PmpG immunization appeared not to be very successful in generating antibodies, despite the use of GNE adjuvant. This could be a false-negative ELISA result, due to an insufficient binding (coating) capacity of recombinant PmpG or a conformational change (globular structure) during binding, thereby blocking the accessibility of B-cell epitopes, as the positive controls also generated low absorbance values (range 0.3–0.35). Western blotting under reducing conditions confirmed the ELISA results. However, Western blotting needs to be performed under non-reducing conditions to examine this more carefully.

At euthanasia, the PmpG-immunized group appeared to be the best protected followed by the placebo group and then the SctC-immunized group. PmpG-vaccinated pigs showed minor or no macroscopic lesions, whilst SctC-immunized pigs and control pigs showed significant gross lesions. For the PmpG-immunized group, the mean chlamydial antigen detection scores for different tissues were, with the exception of the urethra, uterine horns and oviducts, always lower than for the controls. Mean chlamydial antigen detection scores for urogenital tissues and mean vaginal shedding scores confirmed the autopsy observations, as these scores were in general lowest for the PmpG-vaccinated group.

The histopathological findings noted in the urogenital tract of the placebo and the less-protected SctC group were comparable to those described by Vanrompay *et al.* (2005). Remarkably, and perhaps not logically, protection against severe macroscopic lesions was correlated with more prominent histological changes such as infiltration of lymphocytes and plasma cells in the lamina propria and the presence of intraepithelial inflammatory cells (lymphocytes and polymorphonuclear cells), as the worst protected group (SctC) showed fewer histological changes at autopsy. However, the differences were not statistically significant.

So far, we have studied only the humoral antibody response. High antibody titres obtained by SctC immunization were apparently not correlated with higher protection. As postulated above, antibodies against the recombinant SctC might not recognize the natural conformation of SctC within the secretin of the T3SS. Protection in the PmpG-immunized group was much better, although chlamydial replication in the urogenital tract and vaginal shedding could not be prevented. Eradication of the infection was not observed. In future, it would be interesting to monitor the animals for longer and to include the cellular immune response. Furthermore, administration routes other than subcutaneous injection should be investigated.

Pmps are believed to be very promising candidate vaccine antigens and they are incorporated into the Tracvax vaccine, which has been tested in randomized phase I trials (WHO,

2006). Indeed, the recombinant PmpG used in our pig challenge model created (albeit not always) significant protection against severe macroscopic lesions, bacterial replication in the urogenital tract and bacterial excretion. However, at the moment we do not know the underlying immune mechanisms involved. In this study, recombinant proteins were expressed in a prokaryotic system, whereas *in vivo* the host cell might be responsible for post-translational modifications of chlamydial proteins. We cannot rule out the possibility that recombinant proteins expressed in a eukaryotic system could induce more significant protection against genital *C. trachomatis* infection. Nevertheless, we do not feel confident in using recombinant proteins for future vaccine development, especially as they are poor inducers of cell-mediated immunity (van Drunen Littel-van den Hurk *et al.*, 2000; Verminnen *et al.*, 2005), which is very important in the defence against chlamydial infections.

As expected, differences in protection were observed for the promising (PmpG) and less promising (SctC) candidate vaccine antigens. Interestingly, significant protection was apparently not correlated with a strong humoral immune response upon subcutaneous immunization with recombinant *C. trachomatis* proteins. In conclusion, the pig model is useful for studying the efficacy of vaccine candidates against genital *C. trachomatis* infection and should be further extended incorporating T-cell proliferation tests with flow cytometric characterization of different T- and B-cell subsets and antigen-presenting cells, enzyme-linked immunosorbent spot assays for quantification of IgM-, IgG- and IgA-secreting B cells in blood, spleen and local draining lymph nodes, and finally T helper 1 (Th1), Th2 and Th0 cytokine detection.

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