Experimental enteric infection of gnotobiotic piglets with *Chlamydia suis* strain S45

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ABSTRACT

Enteric chlamydial infections of pigs with *Chlamydia* (*C.*) *suis* are frequent and often subclinical. The enteric pathogenicity of *C. suis* strain S45 was investigated in gnotobiotic piglets. Piglets from three litters (*n* = 31) were inoculated with egg-grown chlamydiae at 2–3 days of age (*n* = 17) or used as controls (*n* = 14). They were observed for clinical signs, killed and necropsied sequentially at 2–13 days postinoculation (DPI). Feces were collected daily and investigated with an ELISA for chlamydial antigen. At necropsy, specimens were collected for histopathology and for immunohistochemical, PCR-based, and serological (complement fixation test, ELISA) detection of chlamydiae.

Chlamydial replication and associated symptoms and lesions were observed from 2 to 13 DPI and were particularly pronounced within the first week PI. Clinical symptoms consisted of moderate-to-severe diarrhea, slight and transient anorexia, weakness and body weight loss. Immunohistochemistry and ELISA revealed that chlamydial replication was particularly marked at 2–4 DPI and primarily located in the small intestinal villus enterocytes. Further sites of replication included large intestinal enterocytes, the lamina propria and Tunica submucosa, and the mesenteric lymphnodes. Histopathological changes included moderate-to-severe villus atrophy with flattened enterocytes and focal villus tip erosions, and moderate mucosal inflammatory cell infiltrates and lymphangitis in the small intestine. PCR of spleen tissue and blood was mostly negative for chlamydiae, indicating that they did not substantially disseminate into the host up to 13 DPI. All sera were negative for anti-chlamydial antibodies. In conclusion, *C. suis* strain S45 elicited significant enteric disease and lesions in gnotobiotic piglets indicating its pathogenic potential for swine.

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1. Introduction

Chlamydiae are obligate intracellular pathogens with a broad host spectrum and they have been isolated from a great variety of organs and diseases in swine (Szeredi et al., 1996). Chlamydia species detected in natural infections in swine include *Chlamydyophila* (*Cp.*) *abortus*, *Cp. pectorum* and a recently proposed species, *Chlamydia* (*C.*) *suis*, which consists of porcine strains phylogenetically related to human *C. trachomatis* (Kaltenboeck et al., 1993; Everett et al., 1999). In previous immunohistochemical and molecular biological studies in Switzerland, involvement of *C. suis* was reported in the vast majority of chlamydial intestinal infections in swine, while *Cp. abortus* was rarely detected (Zahn et al., 1995; Szeredi et al., 1996; Schiller et al., 1997a). A study in Germany reported a high...
prevalence of mixed infections with C. suis and Cp. abortus in the lung and gut of pigs (Hoelzle et al., 2000). Another investigation revealed that C. suis was the most prevalent chlamydial species in intestinal samples from boars, while Cp. abortus was less prevalent and Cp. pecorum occurred only sporadically (Kauffeld et al., 2006).

Prevalences of enteric chlamydial infections ranging from 15% in suckling pigs to 30% in finishing pigs have been reported (Zahn et al., 1995; Szeredi et al., 1996; Nietfeld et al., 1997). Nearly the same prevalence of diarrhea was found in infected and non-infected animals, suggesting that chlamydiae do not always cause diarrhea (Zahn et al., 1995; Nietfeld et al., 1997). However, in some cases no other enteropathogenic organisms were detected (Nietfeld et al., 1993, 1997). Variations in virulence between strains, in particular between C. suis strains, cannot be excluded as a reason for this incongruence, especially considering the relatively high degree of genetic diversity observed in this chlamydial species when compared to other chlamydial species (Everett et al., 1999; Bush and Everett, 2001). A synergistic effect of chlamydiae with other enteropathogenic organisms has been suggested (Pospisilch and Wood, 1987), and can be regarded as a further possible explanation for the inconsistent association of enteric chlamydiae with diarrhea.

Experimental infections have so far indicated that two porcine isolates classified as C. suis (Everett and Andersen, 1997), originating from diarrheic pigs, were enteropathogenic for gnotobiotic piglets (Rogers and Andersen, 1996). In contrast, a Cp. abortus strain was virtually apathogenic for the gastrointestinal tract of gnotobiotic piglets (Guscetti et al., 1998), while a Cp. psittaci isolate of avian origin elicited only mild intestinal lesions and symptoms (Guscetti et al., 2000). The aim of this study was to investigate enteric pathogenicity and dynamics of replication of the porcine C. suis strain S45 in gnotobiotic piglets. This isolate was harvested in the late 1960s from feces of an asymptomatic pig in Austria (Koelbl, 1969) and has been designated as the C. suis type strain (Everett et al., 1999). C. suis strains previously used in experimental infections of gnotobiotic piglets have been shown to significantly differ from strain S45 in the intergenic spacer region, the 23S and the 16S rRNA gene regions (Everett and Andersen, 1997; Everett et al., 1999). Moreover, they apparently belong to different serovars (Everett and Andersen, 1997). In the present investigation, pathogenicity was assessed based on clinical and histological observations, while the dynamics of chlamydial replication was studied by monitoring chlamydial antigen excretion in the feces using an ELISA, and by immunohistochemically labeling chlamydial antigen in the tissues of the experimental animals.

2. Materials and methods

2.1. Inocula

The inocula for the piglets were prepared from a lyophilized preparation of the porcine C. suis S45 strain (kindly donated by Prof. J. Storz, Baton Rouge, Louisiana). The isolate had been passaged in chicken eggs between 25 and 35 times. The lyophilisate was resuspended in sucrose–phosphate–glutamate (SPG) medium and propagated in the yolk sac of embryonated specific-pathogen-free chicken eggs as previously described (Guscetti et al., 1998). All procedures for harvesting the inocula, determining the relative titres, preparing sham-inocula from uninfected eggs, and excluding presence of bacterial contamination were done as previously described (Guscetti et al., 1998).

2.2. Animals and experimental design

Thirty-one colostrum-deprived landrace piglets from three litters were derived by closed hysterotomy and maintained in microbiological isolators as previously described (Guscetti et al., 1998). The animals were fed four times a day according to a restrictive feeding regimen with commercially available sterile evaporated cow milk (Migros Genossenschaft, Zürich, Switzerland) mixed 1:1 with water (Miniats and Jol, 1978). During episodes of watery diarrhea, the single rations were reduced, and the piglets were fed more frequently (up to eight times per day), and, on a few occasions, water was additionally made available to the piglets. Microbiological sterility monitoring of the isolators and of the animals has been previously described; after necropsy, a sample of the small and large intestine from every animal was investigated for contaminating bacteria or fungi (Sydler et al., 1996). Due to the limited number of facilities and devices available, each litter was subdivided into an inoculated and a control subgroup in all experiments. The relative size of the subgroups was decided during hysterotomy as soon as the total number of piglets was known. The schedule for inoculation and euthanasia of each animal is depicted in Fig. 1. Piglets exposed to chlamydiae were called principal piglets or principals and they were numbered consecutively in each subgroup according to the sequence decided for euthanasia. The principal piglets of groups 1 and 2 were inoculated intragastrically (IG) with 5 × 10^6 inclusion forming units (IFU) and 5 × 10^6 IFU of chlamydiae, respectively, by using a flexible stomach tube as previously described (Guscetti et al., 1998). The principals of group 3 were inoculated per os (PO) with 5 × 10^6 IFU, and, since it was not possible to feed the piglets separately, each individual piglet received the inoculum in the mouth by means of a syringe. All groups were inoculated between 47 and 55 h postpartum.

All animals were observed clinically for symptoms of intestinal disease, for loss of appetite and body condition, and for changes in behaviour. During the first 4–5 days postinoculation (DPI) the animals were observed every 4 h, later four times a day. Body weight was measured daily using a spring balance, and the mean daily body weight gain was calculated for each group of piglets. Rectal body temperature was measured daily using a digital thermometer. The severity of diarrhea within each individual subgroup was scored as indicated in Table 1. The animal experiments were performed according to the relevant Swiss laws and the experiment design was approved by an ethical
committee (authorization no. 229/93, granted by the Veterinary Office of the Canton Zurich).

2.3. Chlamydial antigen detection in feces (ELISA)

After inoculation of the animals, and as soon as expulsion of meconium ceased, fecal samples were collected at least once daily directly from the living piglets with a few exceptions where collecting was not possible. The specimens were transferred from the isolators once a day, diluted 1:10 in SPG medium, homogenized, aliquoted, and stored at \( -70 \) °C until examination. After thawing at room temperature, the samples were subjected to a purification procedure through repeated centrifugation as previously described (Guscetti et al., 1998) which resulted in a final 1:40 dilution of the original sample. A commercially available enzyme-linked immunosorbent assay (IDEIA\textsuperscript{®}, DAKO Diagnostics, Zug, Switzerland) was run on 1:40, 1:400, and 1:1600 dilutions of the original samples following the instructions of the manufacturer. Optical density (OD) values were measured at 490 nm. The cut off values for each dilution (0.4, 0.18 and 0.16 OD\textsubscript{490}, for dilutions 1:40, 1:400, and 1:1600, respectively) were determined by calculating the mean absorbance of 86 fecal samples of negative control gnotobiotic piglets plus three times the standard deviation of the mean.

2.4. Samples at necropsy

The piglets were killed as previously described (Guscetti et al., 1998). They were exenterated immediately after euthanasia and the intestine was spread out on the necropsy table. Specimens from the intestine intended for histopathology and immunohistochemistry were immersed within approx. 10 min after euthanasia in 4% neutral buffered formaldehyde. Sites sampled included seven equidistant sites between pylorus and ileocaecal valve designated as duodenum (2 cm caudally from the pylorus), jejunum 1–5 and ileum (2 cm proximally from the ileocaecal valve), furthermore caecum (corpus and apex), colon ascendens (mid-portion), and colon descendens (mid-portion). Further organs fixed in formaldehyde included conjunctiva, nasal mucosa, cervical lymph node, tonsils, trachea, lungs, stomach, mesenteric lymph node, liver, spleen, kidney, knee joint synovial membrane, uterus or testicles. In addition, in all piglets of group 3 at least three lymph nodes from the laryngo-pharyngeal region were sampled.

Samples of the spleen intended for detection of Chlamydia by polymerase chain reaction (PCR) were collected from all inoculated piglets and from six control piglets (representing each group) using sterile instruments; they were wrapped into sterile aluminium foils and stored at \( -70 \) °C until investigated. In addition, blood samples from all piglets of group 3 were collected shortly before euthanasia and stored at \( -70 \) °C until investigated.

### Table 1

**Diarrhea scoring system.**

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>All piglets showing increased frequency of defection of normal or only slightly softened feces (when compared to the controls)</td>
</tr>
<tr>
<td>2</td>
<td>Some piglets with symptoms as described for score 1, some with softened feces</td>
</tr>
<tr>
<td>3</td>
<td>All piglets with softened feces</td>
</tr>
<tr>
<td>4</td>
<td>Some piglets with softened feces, and some with watery feces</td>
</tr>
<tr>
<td>5</td>
<td>All piglets with watery feces</td>
</tr>
</tbody>
</table>

Fig. 1. Experimental design. i.g. = intragastric; p.o. = per os; IFU = inclusion forming units; vertical arrows indicate time of inoculation; crosses indicate time of euthanasia and necropsy for each individual piglet.
2.5. Immunohistochemistry for chlamydial antigen

Paraffin sections from all tissues collected were investigated for the presence of chlamydial lipopolysaccharide (LPS) using a genus-specific monoclonal antibody (clone AC-I, Progen, Heidelberg, Germany) and an immunoperoxidase method as previously described (Szeredi et al., 1996). Consecutive sections were incubated with phosphate-buffered saline (pH 7.4) instead of the primary antibody as negative controls. Occasional sections yielding questionable results and all sections of the tonsils were retested using two serial sections incubated for 1 h with the same antibody diluted 1:200 in antibody diluent (ChemMate®, Code No. S2022, DAKO, Zug, Switzerland). In these cases, the reaction was visualized using a labeled streptavidin-biotin method (ChemMate® Detection Kit, Code No. K 5003, DAKO, Zug, Switzerland) according to the manufacturer’s instructions; in addition, a third section was incubated with antibody diluent alone as a negative control.

The number of chlamydial LPS-positive inclusions in intestinal tissues, mesenteric lymph nodes and tonsils was determined semiquantitatively and assigned to one of the categories indicated in Table 2.

Table 2

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>++++</td>
<td>A large number of labeled inclusions (average &gt; 20) in &gt;50% of the villi</td>
</tr>
<tr>
<td>+++</td>
<td>A moderate number of labeled inclusions (average of 10–20) in &gt;50% of the villi or a large number of labeled inclusions (average &gt; 20) in &gt;50% of the villi</td>
</tr>
<tr>
<td>++</td>
<td>A small number of labeled inclusions (average &lt; 10) in &gt;50% of the villi or a moderate number of labeled inclusions (average of 10–20) in &lt;25% of the villi</td>
</tr>
<tr>
<td>+</td>
<td>&lt;100 inclusions in total</td>
</tr>
<tr>
<td>-</td>
<td>No chlamydial antigen detected</td>
</tr>
</tbody>
</table>

2.6. Histopathology

After 24 h fixation in formaldehyde, all tissue specimens were embedded in paraffin by routine methods, sections were stained with hematoxylin and eosin (HE). Sections of each site in the intestine consisted of two cross-sections and two longitudinal sections, with exception of the duodenum, where only one section of each was embedded. In addition to the histopathological investigation, villus and crypt lengths were determined morphometrically in jejunum sections 3–5 using an interactive morphometric analyzer (Videoplan Rel. 2.1., Kontron, Eching, Germany) as previously described (Guscetti et al., 1998). The villus/crypt ratios were calculated for each site and piglet. The remaining tissue samples were subjected to routine histopathology.

2.7. Polymerase chain reaction for chlamydial sequences

DNA was extracted from the samples using a commercial DNA extraction kit (QIAamp Tissue Kit, QIAGEN, Basel, Switzerland). A nested PCR was performed as previously described (Schiller et al., 1997b). Briefly, four genus-specific primers recognizing conserved regions of omp1 were used. The first set of primers, 191CHOMP (sense) and CHOMP336 (antisense), amplified a 470 base pairs (bp) product. In the secondary amplification step the primers 201CHOMP (sense) and CHOMP271 (antisense) yielded a 257 bp product. The PCR fragments were separated by agarose gel electrophoresis and stained with ethidium bromide.

2.8. Chlamydial serology

Serum samples were collected from each piglet at birth (umbilical cord blood) and before euthanasia, and were stored at −70 °C until examination. All sera of all groups were investigated using a complement fixation test (CFT), all sera of groups 1 and 2 were additionally investigated using three enzyme-linked immunosorbent assays (ELISA) as described below.

2.8.1. Complement fixation test (CFT)

This test was performed in microtiter plates using Ornithosis® CFT-reagents (Behringwerke, Marburg, Germany) as previously described (Szeredi et al., 1996). A reaction was first considered as positive by 100% binding of the complement in the test system at a serum dilution of 1:10.

2.8.2. Enzyme-linked immunosorbent assay (ELISA)

The serum samples were tested for porcine anti-chlamydial IgG in a previously described ELISA (Szeredi et al., 1996) using three different antigens: elementary bodies (EB) of porcine C. pecorum (strain CHL2PHS) and of human C. trachomatis (strain L2), and chlamydial lipopolysaccharide (LPS). Ninety-six-well microtiter plates (Immunonol M129A, Dynatech, Denkendorf, Germany) were coated with the respective EB purified from buffalo green monkey (BGM) cell cultures by differential centrifugation or with uninfected BGM cells serving as negative control antigen. For the detection of anti-LPS antibodies, microtiter plates coated with commercially available recombinant chlamydial LPS were used (Medac, Hamburg, Germany). Remaining nonspecific binding sites were blocked with 0.5% (w/v) blocking reagent (Roche Diagnostics, Mannheim, Germany). Duplicate samples were incubated at a dilution of 1:100. Sera positive and negative for anti-chlamydial antibodies served as controls. Specific antibodies were detected by alkaline phosphatase-conjugated anti-porcine IgG (anti-IgG) antibodies (Kirkegaard and Perry, Gaithersburg, MD, USA) using 4-nitrophenylphosphate (Roche Diagnostics, Mannheim, Germany) as a substrate. The resulting color reaction was quantified at 405 nm. Extinctions were standardized by the results of a positive control serum originating from a pig immunized with a chlamydial vaccine.

3. Results

3.1. Microbiological sterility monitoring

Group 1: Staphylococcus sp. was isolated from the gut of the principal piglet killed at 5 DPI. Group 2: Lactobacillus sp.
was isolated from the gut of all principals and controls. Group 3: *Bacillus* sp. was isolated from the gut of the principals killed at 12 and 13 DPI. *Staphylococcus epidermidis* was isolated from the gut of the control piglet killed at 9 DPI. No contaminant bacterial or fungal microorganisms were isolated from the intestines of any of the remaining animals.

### 3.2. Clinical and necropsy findings

All inoculated piglets showed signs of enteric disease starting between 2 and 4 DPI and lasting until the end of the respective observation period, whereas all controls were asymptomatic. Some degree of anorexia was observed in the principals of all groups, especially at the height of diarrhea or thereafter, while dehydration was not seen in any of the piglets. Principals in all groups showed a transient loss of weight or stop of weight gain starting at 3–4 DPI and lasting for 1–3 days, while the control animals gained weight daily after 3 days of age. All inoculated and control piglets had body temperatures ranging mainly between 39.0 and 39.5 °C.

*Fig. 2* shows the evolution of mean diarrhea scores for each inoculated subgroup. The principals of group 1, which were inoculated IG with $5 \times 10^6$ IFU of *C. suis* S45, showed overall the most severe clinical outcome. The peak of symptoms was between 2 and 3 DPI. At this time, all inoculated piglets had watery feces containing flecks of undigested curd, they showed a slight loss of condition, some degree of weakness, and they were less active and showed reduced appetite when compared to the controls. These piglets showed a marked loss of weight at 3 DPI (mean: ~200 g), while they gained weight at the remaining DPI. A slight appetite reduction was observed when compared to the controls. The principals of group 2, inoculated with $5 \times 10^4$ IFU of *Chlamydia* IG, showed less severe but more protracted symptoms than those in group 1. Feces were mostly softened in all piglets between 3 and 8 DPI, the mean weight loss was ~100 g, ~50 g, and 0 g at 4, 5, and 6 DPI, respectively. From 4 to 9 DPI, the appetite of these piglets was at times slightly reduced when compared to the controls. The principals of group 3, inoculated PO with $5 \times 10^6$ IFU, showed the peak of diarrhea between 3

![Diarrhea scores during the respective observation periods in three groups of piglets inoculated with *C. suis*. The scores were assigned according to the descriptions in Table 1.](image-url)
and 5 DPI, with often watery feces between 4 and 5 DPI, and a slight weight loss (mean: ~30 g) observed only at 4 DPI. In this group there was some degree of heterogeneity between the individual piglets in the severity of the clinical signs. Some of the piglets occasionally showed coughing.

At necropsy, all inoculated piglets were thin. Examination of the large intestine revealed watery or softened contents, with remnants of undigested curd, especially in the piglets necropsied around the height of diarrhea. No lesions were visible macroscopically in the intestinal mucosa. Frequently, the mesenteric lymph nodes were slightly to moderately enlarged when compared to the controls. Consolidation of large portions of the cranial and cardiac lung lobes was observed in three principals (group 2: 12 DPI; group 3: 8 and 9 DPI). No relevant lesions were seen in any of the remaining organs and piglets.

### 3.3. Chlamydial antigen detection in fecal samples

Fecal specimens were investigated with an ELISA to monitor the course of chlamydial antigen shedding. All inoculated animals excreted chlamydial antigen with some differences between the groups (Fig. 3), whereas all samples of control piglets tested resulted negative. The main findings in the inoculated piglets were as follows: within 2 DPI, the principals of group 1 started shedding antigen at a high level and for a short period (peak within 3 DPI). The two piglets alive until 6 and 7 DPI resumed antigen shedding after 5 DPI. In group 2, chlamydial antigen shedding was delayed when compared to group 1 (start from 2 to 4 DPI, peak between 6 and 9 DPI), and the piglets continuously shed a medium level of antigen until the end of their observation period. Group 3 showed an intermediate pattern: shedding started and peaked relatively early (at approx. 2 DPI, and from 3 to 4 DPI).

![Fig. 3. Semiquantitative analysis of fecal chlamydial antigen shedding as detected by ELISA in piglets inoculated with C. suis. Combined results from 1:40, 1:400, and 1:1600 sample dilutions are presented for each individual piglet. Each box represents all samples examined during the time frame covered by the box and yielding the indicated result. ■ Fecal dilutions 1:1600 strongly positive (OD490 value < 1.0); □ fecal dilutions 1:1600 weakly positive (OD490 value ≥ 1.0); □□ fecal dilutions 1:400 positive; □□□ fecal dilutions 1:40 positive; □□□□ all fecal dilutions examined negative.

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**Fig. 3.** Semiquantitative analysis of fecal chlamydial antigen shedding as detected by ELISA in piglets inoculated with *C. suis*. Combined results from 1:40, 1:400, and 1:1600 sample dilutions are presented for each individual piglet. Each box represents all samples examined during the time frame covered by the box and yielding the indicated result. ■ Fecal dilutions 1:1600 strongly positive (OD490 value < 1.0); □ fecal dilutions 1:1600 weakly positive (OD490 value ≥ 1.0); □□ fecal dilutions 1:400 positive; □□□ fecal dilutions 1:40 positive; □□□□ all fecal dilutions examined negative.
respectively), high-level excretion was maintained a little longer than in group 1 (between 1 and 3 days). After 8 DPI, a second wave of excretion appeared to occur in the three piglets still alive at this time.

### 3.4. Chlamydial antigen immunohistochemistry of intestinal tissues

This investigation was aimed at detecting the sites and the intensity of chlamydial replication in the gut relative to the time PI. Chlamydial antigen was detected in all inoculated piglets predominantly in the small intestine (Table 3), while the large intestine was less frequently and less strongly affected. All sections from the controls were negative. The results of the semiquantitative assessment of chlamydial antigen in inoculated groups can be summarized as follows. In all groups the heaviest colonization occurred in the first few days after inoculation. In group 1, a high level of immunoreactivity was detected throughout the jejunum and ileum at 2 DPI, followed by a marked drop at 3 DPI and by a slight increase again predominantly in the distal small intestine at 5–7 DPI. The lower dose of inoculum in group 2 resulted in a moderate level of immunoreactivity in the intestinal tissues at 4 DPI, and a medium-to-low level at

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 3 4 5 6 7</td>
<td>2 3 4 9 12 13</td>
<td>2 3 4 8 9 12 13</td>
</tr>
<tr>
<td>Duodenum</td>
<td>- - - - -</td>
<td>- - - -</td>
<td>- - - -</td>
</tr>
<tr>
<td>Jejunum 1</td>
<td>++++ + + +</td>
<td>++ +</td>
<td>+ +</td>
</tr>
<tr>
<td>Jejunum 2</td>
<td>+++ + + +</td>
<td>+++</td>
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<td>Jejunum 4</td>
<td>+++ + + +</td>
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<td>++ +</td>
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<tr>
<td>Jejunum 5</td>
<td>+++ + + +</td>
<td>+++ +</td>
<td>++ +</td>
</tr>
<tr>
<td>Ileum</td>
<td>+++ + + +</td>
<td>+++ +</td>
<td>+++</td>
</tr>
<tr>
<td>Colon asc.</td>
<td>- - - -</td>
<td>- - - -</td>
<td>- - - -</td>
</tr>
<tr>
<td>Colon desc.</td>
<td>- - - -</td>
<td>- - - -</td>
<td>- - - -</td>
</tr>
<tr>
<td>Rectum</td>
<td>- - - -</td>
<td>- - - -</td>
<td>ND ND</td>
</tr>
<tr>
<td>Tonsils</td>
<td>- - - -</td>
<td>- - - -</td>
<td>ND ND</td>
</tr>
<tr>
<td>Mes. lymph node</td>
<td>+ + + + +</td>
<td>+ + + + +</td>
<td>+ +</td>
</tr>
</tbody>
</table>

Immunoreactivity scoring as indicated in Table 2; ND, not done; colon asc., colon ascendens; colon desc., colon descendens; mes. lymph node, mesenteric lymph node

![Fig. 4. Immunohistochemically labeled chlamydial inclusions in inoculated piglets: (a) inclusions in villus enterocytes are mostly rounded, well-delimited, and located sub- or paranuclearly; rarely apically within the cell cytoplasm; proximal jejunum, piglet 1–1 (bar = 20 \( \mu \text{m} \)); (b) cluster of medium-to-small inclusions mostly within macrophages in the lamina propria; ileum, piglet 2–3 (bar = 20 \( \mu \text{m} \)); (c) large inclusions within macrophages in the lamina propria, distal jejunum, piglet 1–4 (bar = 25 \( \mu \text{m} \)); (d) scattered, large-sized inclusions within inflammatory cell focus in the Tunica submucosa largely composed of neutrophils and some macrophages, distal jejunum, piglet 2–2 (bar = 30 \( \mu \text{m} \)); peroxidase anti-peroxidase immunostain against chlamydial lipopolysaccharide antigen, hemalum counterstain.](image-url)
9–13 DPI. In group 3, a medium-to-high level of immunoreactivity was present in the piglets killed between 2 and 4 DPI, with the strongest reaction observed at 3 DPI. Thereafter, chlamydial antigen was detectable at a low level in the tissues during the whole observation period in this group.

The villus enterocytes were the cell type most often colonized, thus representing the primary target for chlamydial infection. Within these cells, labeled *C. suis* inclusions were finely granular, of variable size, mainly ellipsoid or round, often well delimited (Fig. 4a); they were mostly located either basally or paranuclearly, less frequently apically in the cytoplasm. Enterocytes of the crypts were only rarely infected in the small intestine and large intestine. A noticeable number of the positive sections also exhibited cells in the lamina propria (tentatively identified as macrophages, fibroblasts and endothelial cells based on morphological criteria) yielding labeled inclusions. The relative number of such inclusions was small when compared to the number of inclusions within enterocytes at early DPI, but it increased progressively, and equaled or outnumbered the latter in piglets examined during the second week PI. Inclusions in cells other than enterocytes were usually small and pleomorphic, sometimes they were large (Fig. 4b–d). Cells in the lamina propria containing labeled inclusions were often isolated, but in some instances they occurred in small clusters. Occasionally, chlamydial antigen was also detected within inflammatory cell foci in the submucosa.

### 3.5. Histopathology

Histopathological lesions observed in the intestinal mucosa of the inoculated piglets comprised changes associated with an accelerated loss of villus enterocytes, collectively described here as villus atrophy, as well as inflammatory changes. Lesions related to villus atrophy included shortening and blunting of the villi, elongation of the crypts, fusions of villi, flattening of the villus enterocytes occasionally associated with erosions of the villus tips, as well as loss of the typical intracytoplasmic vacuoles of the villus enterocytes in the mid to distal small intestine. Inflammatory changes consisted of elevated numbers of intraepithelial leukocytes, and of inflammatory cell infiltrates in the lamina propria and the Tunica submucosa. Inflammation frequently involved lymph vessels, which were engorged with inflammatory cells (mainly macrophages and neutrophils), and their surroundings. Figs. 5 and 6a–f highlight the most striking histopathological findings. Occurrence of the lesions in relation to the time PI was as follows. In general, the lesions appeared sooner in the proximal to mid portion of the small intestine than in the distal jejunum and ileum, but they subsequently extended rather quickly to the distal sites. Here, the lesions were more severe and, in general, they persisted longer than proximally. Values of the villus-to-crypt ratios reported in Fig. 5 best illustrate the sequential evolution of villus atrophy. Changes in the villus lengths evolved similarly to the villus-to-crypt ratios, but the differences between inoculated piglets and controls were less pronounced. In inoculated piglets, mean values of villus length ranged from 300 to 600 μm in the mid jejunum and between 300 and 500 μm in jejunum 4 and 5. Occasionally, in principals killed at 2–4 DPI or at 12 and 13 DPI, mean villus length values above the indicated range were observed. The corresponding values for control piglets were 600–1300 μm in the mid jejunum (as an exception, in one control animal killed at 4 days of age the mean calculated value was 480 μm), 600–1400 μm for jejunum 4, and 600–1300 μm for jejunum 5. The mean values of crypt lengths were for most principals between 110 and 150 μm. A few inoculated
piglets killed at 2–4 DPI or at 12 and 13 DPI had mean crypt length values below 110 μm. The corresponding values for the controls ranged from 60 to 95 μm. Flattening of the villus epithelium and occasional erosions of villus tips (Fig. 6b), both signs of a recent, extensive loss of enterocytes, were seen in the small intestine mostly up to 6 DPI, and in one piglet (group 2) at 9 DPI. They preferentially occurred proximally at 2–3 DPI, and distally thereafter. A complete, or, in some cases, partial loss of cytoplasmic vacuoles, which can be considered as an expression of a loss of mature villus enterocytes, developed within the first 4 DPI, and persisted thereafter. The numbers of intraepithelial neutrophils were elevated from 2 to 5 DPI in inoculated piglets (Fig. 6a), later, intraepithelial neutrophils were only found in low numbers in occasional tissue sections. Neutrophils were almost never seen within the epithelial layer in the controls. The numbers of neutrophils in the lamina propria of inoculated piglets were strongly elevated from 2 to 7 DPI, and they decreased to a medium-to-low level thereafter. Neutrophils were present occasionally and in small numbers in the lamina propria of control piglets. The numbers of intraepithelial lymphocytes appeared to be elevated when compared to the controls between 8 and 13 DPI, while the numbers of lymphocytes in the lamina propria were variably increased between 4 and 8 DPI and
clearly increased thereafter (Fig. 6f). Macrophages were present at all times in inoculated piglets in infiltrates in the lamina propria and within lymphatic vessels that appeared engorged with such cells (Fig. 6e).

The dynamics of histopathological changes for the principals in the individual groups were as follows: Group 1: lesions appeared soon in this group, were extensive, and peaked in the piglet killed at 3 DPI. In the piglet killed at 2 DPI, there were large numbers of enterocytes released into the intestinal lumen, and erosions of villus tips were more numerous than in any other piglet. After 3 DPI there appeared to be a slow and steady recovery in this group. Group 2: lesions developed slowly in this group, according to the findings in the piglet killed at 4 DPI, but were comparatively severe at 9–13 DPI. Group 3: lesions also developed slowly in this group, but were more pronounced than in group 2 in the piglet killed at 4 DPI. In contrast, they were less severe than in group 2 at 12–13 DPI.

There were no histopathological changes in the guts of any control piglet. Enlarged mesenteric lymph nodes in the inoculated piglets contained increased numbers of lymphocytes, granulocytes, and macrophages when compared to the controls. With the exception of a broncho-interstitial pneumonia in the three piglets with consolidated lungs, there were no relevant histopathological lesions in the other organs investigated.

3.6. Detection of chlamydiae in extraintestinal sites

Systemic spread of chlamydiae within the host organism was monitored by means of immunohistochemistry on a large range of tissues, and using PCR with DNA isolated from spleen tissue and, in group 3, additionally from blood samples. Dissemination from the gut would be expected to result primarily in colonization of the liver, or of the mesenteric lymph nodes. While no antigen was found within the liver tissue, small to moderate numbers of labeled chlamydial inclusions were detected in the mesenteric lymph nodes of almost all inoculated piglets (Table 3). The inclusions were mostly located within macrophages, and usually were small sized. The tonsils represent a further organ theoretically relevant for chlamydial dissemination within the organism. Here, labeled inclusions were found only in very small numbers in two piglets inoculated IG. Despite peroral inoculation, no infection of this organ was detected in piglets of group 3. Although immunohistochemistry of the spleen was negative in all piglets, PCR detected chlamydiae-specific sequences in the spleen of two principal piglets of group 1 (at 3 and 4 DPI). All other spleen samples and all blood samples from inoculated and control piglets were negative for chlamydiae when tested using PCR.

Chlamydial antigen was detected in moderate amounts also in lung tissues of the three piglets with pneumonia. The inclusions were mostly located in bronchial epithelial cells and in macrophages within areas of inflammation. In two of these piglets, small numbers of labeled inclusions were also detected in the epithelium of the nasal mucosa.

With the exception of small numbers of labeled inclusions within epithelial cells of the gastric mucosa of one piglet (group 1, 2 DPI), chlamydial antigen was detected in none of the remaining organs of principals examined. All sections of the control piglets and all negative controls were immunohistochemically negative.

3.7. Serology

All sera were negative for specific complement-fixing antibodies as determined by using a CFT. All sera investigated also were negative for specific anti-chlamydial IgGs when tested in ELISAs using elementary bodies of Cp. pecorum or of C. trachomatis, or using chlamydial lipopolysaccharide as the antigen (data not shown). In summary, none of the serological tests used detected seroconversion in any of the piglets during the study period.

4. Discussion

The data presented in this study indicate that the porcine C. suis strain S45, although originating from a pig without clinical disease, is enteropathogenic for gnotobiotic piglets. In a previous study, diarrhea and similar histopathologic lesions had been reported upon inoculation of gnotobiotic piglets with two further C. suis strains isolated from pigs with diarrhea (Rogers and Andersen, 1996; Everett and Andersen, 1997). In addition to confirming the findings of these previous experiments, we measured a transient loss of weight in inoculated groups concomitant to the height of the enteric symptoms. The significance of the bacterial contaminants detected in some of the principals in the present study is unknown, but it is most likely minor, since they are not recognized enteropathogens, and they did not cause symptoms or lesions in the controls. In addition, the presence of transmissible gastroenteritis virus, porcine epidemic diarrhea virus, and rotavirus group A was excluded immunohistochemically by using appropriate antibodies in tissues from all piglets killed at 2–4 DPI (data not shown). In general, C. suis appears to be clearly more virulent in gnotobiotic piglets than the Cp. psittaci and Cp. abortus strains previously tested (Guscetti et al., 1998, 2000).

In the present work, the course of chlamydial replication in the gut was studied by means of immunohistochemistry and the course of chlamydial antigen shedding in the feces was monitored with an ELISA. The test results were comparable. In addition, the histopathological findings were most severe in the distal small intestine, where chlamydial replication appeared to be most intense. This is in accordance with previous findings in piglets inoculated with related strains (Rogers and Andersen, 1996). The findings in the present work suggest a dose-dependent variation in the pattern of chlamydial replication and associated lesions. A high dose of inoculum in group 1 resulted in early, high-level, and short-timed chlamydial replication within villus enterocytes, as shown by an early detection of high levels of antigen. This was associated with early, severe villus atrophy, which resulted...
in a moderately severe clinical disease for a relatively short period soon after infection. Some residual chlamydial replication was present later, probably due to a low number of remaining enterocytes susceptible for infection or chlamydial replication. A lower dose of inoculum in group 2 resulted in prolonged, medium-level chlamydial replication. Correspondingly, the associated events were delayed. Interestingly, after a delayed onset of villus shortening, the damage to the mucosa was relatively severe at late DPI, indicating that infection of susceptible enterocytes steadily progressed. The piglets in the third group, inoculated with a high dose of inoculum but per os instead of intragastrically, showed an intermediate pattern. It is likely that these animals ingested a smaller amount of inoculum than the piglets in group 1. Technical difficulties associated with PO inoculation resulted in aspiration of inoculum and ensuing pneumonia in two piglets and in a somewhat heterogeneous clinical response of the animals in this group. With all reservations ensuing from applying results of such a model to the situation in the field, it appears that a low level of infection is likely to result in a relatively mild or clinically inapparent disease, but in prolonged replication and shedding of the agent. Thus, while most natural enteric infections with chlamydiae in piglets are likely subclinical, a significant spreading of the agent within the gut of infected animals and among littermates must be assumed. Once infected even with a small dose of chlamydiae, piglets may develop significant lesions of the gut mucosa, which may not result in clinical disease. In this context it should be noted that lesions similar to those reported in the gnotobiotic piglets were observed in weaned pigs experimentally inoculated with *C. suis* in the absence of clinical signs (Rogers and Andersen, 2000). The chlamydiae-borne lesions may alter the susceptibility of the intestine for other enteropathogenic agents. Such a synergistic effect has been suggested for chlamydiae and *Salmonella* in a previous report (Pospischil and Wood, 1987). In another study, piglets with diarrhea naturally infected with chlamydiae were found to mostly harbor mixed infections with other enteropathogenic agents (Zahn et al., 1995).

As detected by immunohistochemistry, *C. suis* strain S45 produced inclusions located preferentially para- or subnuclearly, a feature occurring independently of the time PI and of the level of antigen load within a given tissue section. In contrast, a porcine *Cp. pecorum* strain (17105) inoculated under similar conditions mostly developed inclusions located in the apical cytoplasm of the enterocytes (unpublished observations). This difference between the two strains in vivo is consistent with the respective intracellular location of their inclusions observed in polarized CaCo cell cultures (Schiller et al., 2004). The pattern of S45 infections was similar to that described for *C. trachomatis* serovar L2, a chlamydial subtype associated with lymphogranuloma venereum in humans (Schramm and Wyrick, 1995). Contrary to the non-invasive *C. trachomatis* serovar E strain, isolate L2 showed propensity to invade the subepithelial tissue, since mature inclusions were preferentially discharged at the basolateral zone of infected epithelial cells. Invasion of the lamina propria and inflammatory changes of lymphatic vessels were a striking feature of *C. suis* S45 in the present experiment and of related chlamydial isolates in previously reported experiments (Rogers and Andersen, 1996).

Colonization of the mesenteric lymph nodes frequently occurred in *C. suis* inoculated piglets as assessed immunohistochemically in the present study. Further dissemination throughout the piglet organism, however, was not prominent up to 13 DPI, as concluded from results of immunohistochemical and PCR analyses. Colonization of the lungs was most likely due to inhalation at the time of dosing. In the spleen, the rare occurrence of positive PCR results concomitant with a negative immunohistochemical result possibly points to transient dissemination without significant replication, as immunohistochemistry is not well-suited for detecting low numbers of isolated elementary bodies. In contrast to the results in the present study, where all livers were immunohistochemically negative, chlamydiae were isolated from this organ in a large number of the gnotobiotic piglets inoculated with *C. suis* in a previous experiment (Rogers and Andersen, 1996). In agreement with the low grade of dissemination no seroconversion was detected in any of the principals in the present study. This fact may be a consequence of the short study period after inoculation and the use of very young, naïve gnotobiotic animals. In a previously described experimental respiratory *C. suis* infection of conventionally raised pigs an increase of IgG titres was measured as soon as after 5–6 days PI (Sachse et al., 2004). In that study PCR examination of pulmonary lymph node, liver, and spleen tissues pointed to a rather limited systemic dissemination as well.

The antigen ELISA used may not be suited for detection of chlamydiae in the feces of conventional piglets, although it showed appreciable results in the present study, where conditions were standardized, and only a small number of different known contaminating bacterial species were present at times. Various bacterial species commonly occurring in the normal gut have been reported as potentially cross-reactive in such tests (Andersen, 1994). The optical densities measured point to a high overall quantity of chlamydial antigen excretion compared to previous experiments with an avian *Cp. psittaci* strain and with an ovine *Cp. abortus* strain (Guscetti et al., 1998, 2000). Isolation attempts in Vero cell cultures in order to quantify the number of infectious *C. suis* organisms shed in the feces gave results between $10^2$ and $10^4$ IFU/ml in samples collected between 2 and 4 DPI in group 1 (data not shown).

In conclusion, in the present setting the *C. suis* type strain S45, similarly to other porcine *C. suis* isolates previously tested by others, was clearly enteropathogenic for gnotobiotic piglets. Chlamydial replication in the intestinal tissues and shedding of chlamydial antigen was extensive with some minor variations in dependence of the doses and route of inoculation. The findings in gnotobiotic piglets suggest that *C. suis* strain S45 bears the potential to cause lesions in the intestines of conventionally reared pigs.

**Conflict of interest statement**

None of the authors has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the present paper.
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References


