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Pathogenicity of *Chlamydia gallinacea* in chickens after oral inoculation

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ABSTRACT

Chlamydia gallinacea is a recently discovered and widespread obligate intracellular bacterium in chickens. In chickens, infections appear to be asymptomatic, but can result in reduced weight gain in broilers. Molecular typing revealed *C. gallinacea* is genetically diverse which might lead to differences in pathogenic potential between strains. However, studies about the pathogenesis of different *C. gallinacea* strains are still limited. In this study, the pathogenesis of *C. gallinacea* strain NL_G47 was investigated in three consecutive animal experiments. The first experiment served as a pilot in which a maximum culturable dose was administered orally to 13 chickens. Excretion of chlamydial DNA in cloacal swabs was measured during 11 days post infection, but no clinical signs were observed. The second and third experiment were a repetition of the first experiment, but now chickens were sacrificed at consecutive time points to investigate tissue dissemination of *C. gallinacea*. Again excretion of chlamydial DNA in cloacal swabs was detected and no clinical signs were observed in line with the results of the first experiment. PCR and immunohistochemistry of tissue samples revealed *C. gallinacea* infected the epithelium of the jejunum, ileum and caecum. Furthermore, *C. gallinacea* could be detected in macrophages in the lamina propria and in follicular dendritic cells (FDCs) of the B cell follicles in the caecal tonsil. Results of serology showed a systemic antibody response from day seven or eight and onward in all three experiments. The experiments with strain NL_G47 confirmed observations from field studies that *C. gallinacea* infection does not result in acute clinical disease and mainly resides in the epithelium of the gut. Whether the presence of *C. gallinacea* results in chronic persistent infections with long term and less obvious health effects in line with observations on other infections caused by *Chlamydiae*, needs further investigation.

1. Introduction

Chlamydia gallinacea is an obligate intracellular bacterium belonging to the family of *Chlamydiaceae*. This family comprises important pathogens including the zoonotic *Chlamydia psittaci* and the strictly human pathogen *Chlamydia trachomatis*. Since the proposal of *C. gallinacea* as a new species in 2014, high prevalences are reported in poultry in different countries around the world (Guo et al., 2016; Heijne et al., 2018; Ornelas-Eusebio et al., 2020; Sachse et al., 2014). In poultry, transmission occurs via the fecal-oral route (You et al., 2019). Infections appear to be asymptomatic, although yolk sac inoculation of embryonated chicken eggs caused mortality and experimental infection of broilers resulted in reduced weight gain (Guo et al., 2016; Heijne et al., 2020). Zoonotic transmission of *C. gallinacea* has been considered, but there is no definite proof (Heijne et al., 2018; Laroucau et al., 2009; You et al., 2019).

Genomic studies revealed *C. gallinacea* has the hallmark *Chlamydia* virulence genes, although to a lesser number than *C. psittaci* (Guo et al., 2017; Heijne et al., 2020; Holzer et al., 2020). However, the relation between the number and type of virulence genes and the phenotypical outcome is not straightforward. *Chlamydia avium*, for example, the closest relative of *C. gallinacea*, probably has the lowest number of virulence associated genes compared to other chlamydial species (Holzer et al., 2020), but infections are associated with clinical disease and mortality in pigeons and parrots (Kik et al., 2020; Sachse et al., 2014). Furthermore, molecular typing provided evidence for substantial genetic diversity among *C. gallinacea* strains, which might result in differences in pathogenicity (Guo et al., 2017). Therefore, further research

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into the pathogenicity of various *C. gallinacea* strains is needed.

Here, we investigated the primary pathogenicity of *C. gallinacea* strain NL_G47 in chickens. Previous Multi Locus Sequence Typing (MLST) revealed strain NL_G47 has an unique sequence type (ST 280) and forms a well-supported clade with Type strain 08-1274/3(Heijne et al., 2020). Furthermore, strain NL_G47 is genetically different from strain JX-1 which was used in other published experimental studies (Guo et al., 2016; You et al., 2019). Strain NL_G47 was isolated from an asymptomatic laying hen from a Dutch flock in 2018, and, after inoculation in the yolk sac of embryonated chicken eggs, mortality was observed (Heijne et al., 2020). In the present study, chickens were inoculated orally with NL_G47 and shedding was measured in throat and cloacal swabs during 11 days post infection. In addition, tissue dissemination was investigated through sequentially sacrificing of animals and blood was collected to measure a serologic response. The results from this study will help to assess if *C. gallinacea* infection causes acute disease in chickens and if *C. gallinacea* should be considered a threat to poultry health.

2. Materials and methods

2.1. Ethical statement

The animal experiment was conducted in accordance with the national regulations on animal experimentation. The project license was approved by the Dutch Central Authority for Scientific Procedures on Animals (CCD) (permit number AVD4010020173926).

2.2. Inoculum

Chlamydia gallinacea NL_G47 was isolated from caecal material from a clinically healthy laying hen rom a Dutch flock in 2018 as described earlier. The isolate was passaged three times in the yolk sac of SPF chicken eggs and stored at −80 °C as a 20 % yolk sac suspension in Sucrose Phosphate Glutamate (SPG) until inoculation. The infectious dose of the suspension was determined by egg titration experiments and expressed as the Egg Infectious Dose 50 (EID_{50}) (Heijne et al., 2020).

2.3. Animals and housing

A total of 39 five-week-old Specified Pathogen Free (SPF) White Leghorn hens were obtained from MSD Animal Health (Boxmeer, the Netherlands). *Chlamdiaceae* are not included in standard SPF testing, therefore three additional drag swabs of the incubators of the parent flock were collected. All drag swabs tested PCR negative for *Chlamydia* spp. At arrival a pooled fecal sample taken from the transport boxes of the five-week old hens also tested PCR negative for *Chlamydia* spp. All chickens had a 6-day acclimatization period prior to inoculation.

At arrival the hens were housed as a group on sawdust bedding in temperature-controlled rooms under optimal light conditions and humidity. Feed and water were provided ad libitum. The experiment was performed in biosafety level 2 (BSL 2) facilities at Wageningen Bioveterinary Research (WBVR, Lelystad, the Netherlands).

2.4. Experimental design

Three subsequent experiments were performed with thirteen chickens per experiment. In every experiment chickens were assigned a number randomly. The first experiment was a pilot experiment to test the inoculation route and dose. If shedding could be shown and the experiment would not lead to severe clinical signs or mortality, the second and third experiment would be repeated with the same dose and inoculation route as the first experiment.

In the second and third experiment chickens were sequentially sacrificed: three chickens at day zero (before inoculation), three at day four, three at day eight and four at day 11 after inoculation. The chickens that were sacrificed at day zero served as a negative control group.

In every separate experiment inoculation was performed orally with a 1 ml syringe and an oral gavage needle. All chickens, except the control groups were inoculated with 0.5 ml of a 20 % yolk suspension in SPG with an infectious dose of $10^{5.2}$ EID_{50} per bird. The inoculation dose was confirmed by back-titration, for each experiment and the infectious dose was found to be within a range of 0.7 log10 EID_{50}/mL of the initial dose.

Clinical signs were recorded daily according to a clinical scoring card (Table S1). Throat and cloacal swabs were collected daily. Serum samples were collected at day zero, day seven and at euthanasia at day four, eight or 11. A timeline of the experiments including sampling moments is given in Fig. 1. All samples at day zero were collected prior to inoculation to confirm the absence of a current *Chlamydia* infection. All experiments finished 11 days after inoculation.

The chickens in the first experiment were euthanized by intraperitoneal administration of one ml pentobarbital (Euthasol 50 % solution, AST Farma, Oudewater, the Netherlands). In the second and third experiment the chickens were euthanized by maximum blood collection via heart puncture under generalised anesthesia by intramuscular injection of a mixture of 0.3 mL/kg ketamine (Ketamine 10 % Alfasan) and 0.5 ml/kg xylazine (Sedamun, Dechra).

2.5. Necropsy

In the first experiment all animals were sacrificed at day 11 and samples were collected from airsac, lung, liver, spleen, ileum, caecum and colon. In the second and third experiment all carcasses were opened on a clean plastic sheet which was replaced after each necropsy. To prevent cross contamination new sterile instruments and petridishes were used for every tissue sample. Tissue samples (approximately 0,5 cm^3) were collected from the airsac, lung, liver, spleen, kidney, esophagus, proventriculus, ventriculus, duodenum, jejunum, ileum, caecum, caecal tonsil and colon. Samples for PCR were collected in 1 ml SPG in Lysing Matrix D tubes (MP Biomedicals) and ribolysed (2 × 20 s at 4 m/sec) before storage at −80 °C. Tissue samples for histology and immunohistochemistry were collected in 10 % neutral buffered formalin and routinely processed into paraffin blocks. In the third experiment additional tissue samples were collected from jejunum, ileum, caecum and caecal tonsil, embedded in OCT compound using cryomoulds (TissueTek®, Sakura Finetek, USA), snap frozen with liquid nitrogen and stored at −80 °C.

2.6. PCR analyses

Swabs were suspended in 1.5 ml PBS and thoroughly vortexed. From swab or tissue suspension, 200 µl was used for DNA extraction. DNA extraction was performed with a MagNA Pure LC total Nucleic Acid Isolation kit in the MagNA Pure® system (Roche Diagnostics, Almere, the Netherlands). DNA was tested with a *Chlamydiaceae* PCR targeting the 23S rRNA (Ehricht et al., 2006; Heijne et al., 2018).

2.7. Histology and immunohistochemistry

Frozen and formalin fixed tissue samples were cut into 4 µm sections and collected on positively charged glass slides (SuperfrostPlus®, Thermo Scientific). Frozen samples were fixed for 10 min in acetone and air dried. Sections were then stained with haematoxylin-eosin (HE) or immunostained with a polyclonal anti-Chlamydia antibody (LifeSpan BioSciences, Cat# LS-C85741-1000, RRID:AB_1813851) or a monoclonal anti-Chlamydia antibody (MyBioSource, Cat# MBS830551). Epitope retrieval of the formalin fixed sections consisted of proteolysis induced epitope retrieval for the polyclonal antibody (0,1% protK in TBS for 30 min at 37 °C) and heat induced epitope retrieval (citrate buffer, pH 6.0, 121 °C for 5 min) for the monoclonal antibody. Anti-rabbit or

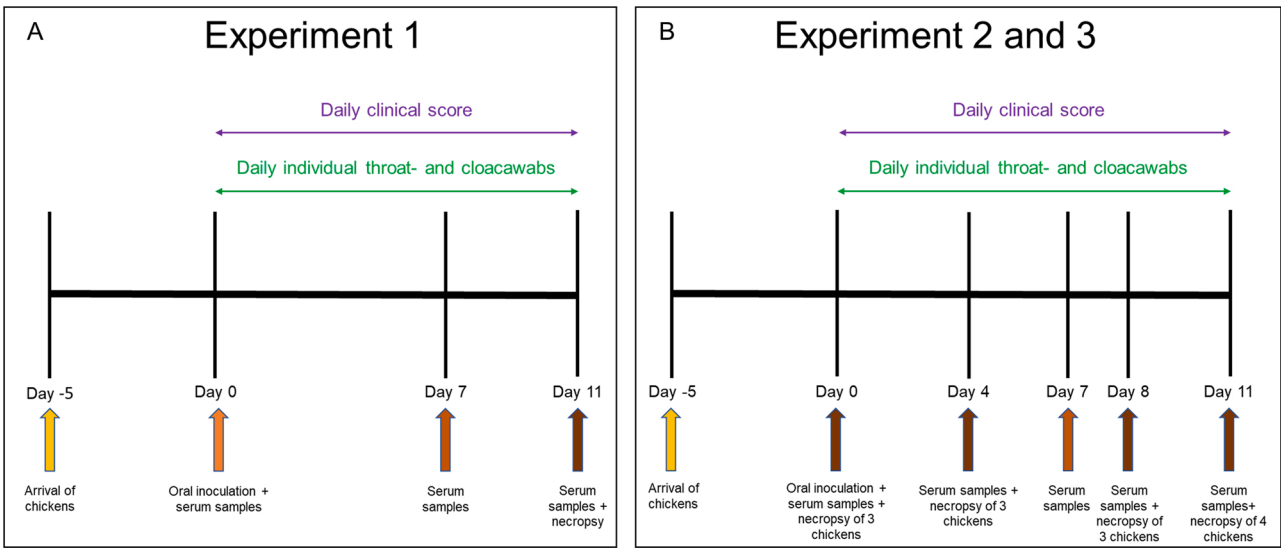

Fig. 1. Timeline of experiments with sampling moments.

anti-mouse HRP conjugated polymer was used as a secondary antibody (Invitrogen, Carlsbad, USA).

Subsequently, formalin fixed sections were incubated for 5 min in DAB + substrate (Dako, Agilent, Santa Clara, USA), counterstained with Mayer's hematoxylin and mounted permanently. Cryo sections were incubated with Alexa Fluor™ 488, 546, or 647 tyramide reagent (Invitrogen) and mounted in antifading mounting medium containing DAPI (Vector laboratories, Peterborough, UK). Co-localisation of chlamydial antigen was assesed by double immunofluoresence staining using a mouse anti-chicken monocyt/macrophages monoclonal (Clone KUL01, Southern Biotech, Birmingham, USA) or a mouse anti-chicken FDCs monoclonal (Clone 74.3, WBVR, Lelystad, the Netherlands). Sections were photographed with an Olympus BX51 (fluorescence) microscope equipped with a high-resolution digital camera. Monochromatic digital photographs for immunofluorescence were false colored using CellSense® software.

2.8. Serology

Serum samples were tested with an in-house ELISA coated with a commercial mix of *Chlamydia abortus* and *Chlamydia trachomatis* antigen (Institut Virion\Serion GmbH, Würzburg, Germany), because specific serological tests for *C. gallinacea* are currently not available. Ninety-six-well microtiter plates (Nunc MaxiSorp™, Thermo Fisher Scientific, Landsmeer, the Netherlands) were coated overnight at 37 °C with 100 µl per well with a concentration of 4 µg/ml of each antigen in coating buffer BM112 (WBVR, Lelystad, the Netherlands). Following six washes with 0.05 % Tween® 80, the plates were blocked with 190 µl per well of 5 % skimmed-milk powder (Campina Elk, the Netherlands) in TBST (BM309, WBVR, Lelystad, the Netherlands) for 60 min at room temperature (RT). The plates were washed as described above, then 100 µl of chicken serum per well (diluted 1:500 in 5 % skimmed milk powder-TBST) was added and the plates were incubated for 60 min at 37 °C. After further washing, 100 µl of goat anti-chicken IgY(H + L)-HRP (Southern Biotech, Birmingham, USA, diluted 1:6,000 in 5 % skimmed milk powder-TBST) was added per well, and the mixture was incubated for 60 min at 37 °C. Again six washes with 0.05 % Tween® 80 were performed and one wash with Super-Q® water. Bound antibody was detected with TMB One component HRP Microwell substrate (TMBW-1000-01, SurModics, Minnesota, USA). The reaction was terminated after 10 min by the addition of 0.5 M sulfuric acid. The optical density (OD) was measured at 450 nm on a Thermo Labsystems Multiskan RC microplate reader (Thermo Fisher Scientific, Landsmeer, the Netherlands).

Per plate, two plate controls were included with two wells per control. In one control, no serum and no conjugate was added to the wells, in the other control no serum was added. All obtained chicken sera were tested in one batch and the individual OD values were corrected for plate differences by subtracting the mean OD value of the plate control (without serum but with conjugate).

3. Results

3.1. Clinical signs and shedding

In the first experiment no clinical signs were observed and shedding was shown in both throat and cloacal swabs (Fig. 2A and D). The second and third experiment were therefore performed with the same inoculation dose and route. During experiment two and three no clinical signs were reported, all chickens appeared clinically healthy at necropsy and no pathological lesions were observed. The PCR results of shedding in throat and cloacal swabs of the second an third experiment are shown in Fig. 2B, E, C and F.

In all three experiments, a similar shedding pattern in both throat and cloacal swabs was observed. Overall shedding was higher in cloacal swabs than in throat swabs. In cloacal swabs shedding increased in the first four to five days and then flattened.

3.2. Dissemination in the gastro-intestinal tract

In Fig. 3, PCR results of dissemination of *C. gallinacea* per timepoint in the gastrointestinal tract in the second and third experiment are depicted. The results of experiment 2 and 3 show that the load of chlamydiae increases towards the more distal parts of the gut, i.e. jejunum, ileum, caecum and colon. The load also increases in time from day 4 to day 8 in all sample types, and appears to be in the same range at day 8 and day 11.

In the HE sections of the gut, chlamydiae were not clearly discernible in any of the tissues. In addition, no inflammatory response was seen in the lamina propria or submucosa (Fig. 4A and B). However, using immunohistochemistry, chlamydial antigen was detected from day 4 onward in the epithelium of the jejunum, ileum and caecum but not in the colon (Fig. 4A). Chlamydiae were seen in rounded structures at the luminal side of the cells (inclusion bodies) or located diffusely in the cytoplasm (Fig. 4B).

At day 8 and 11 after infection, the number of epithelial cells that

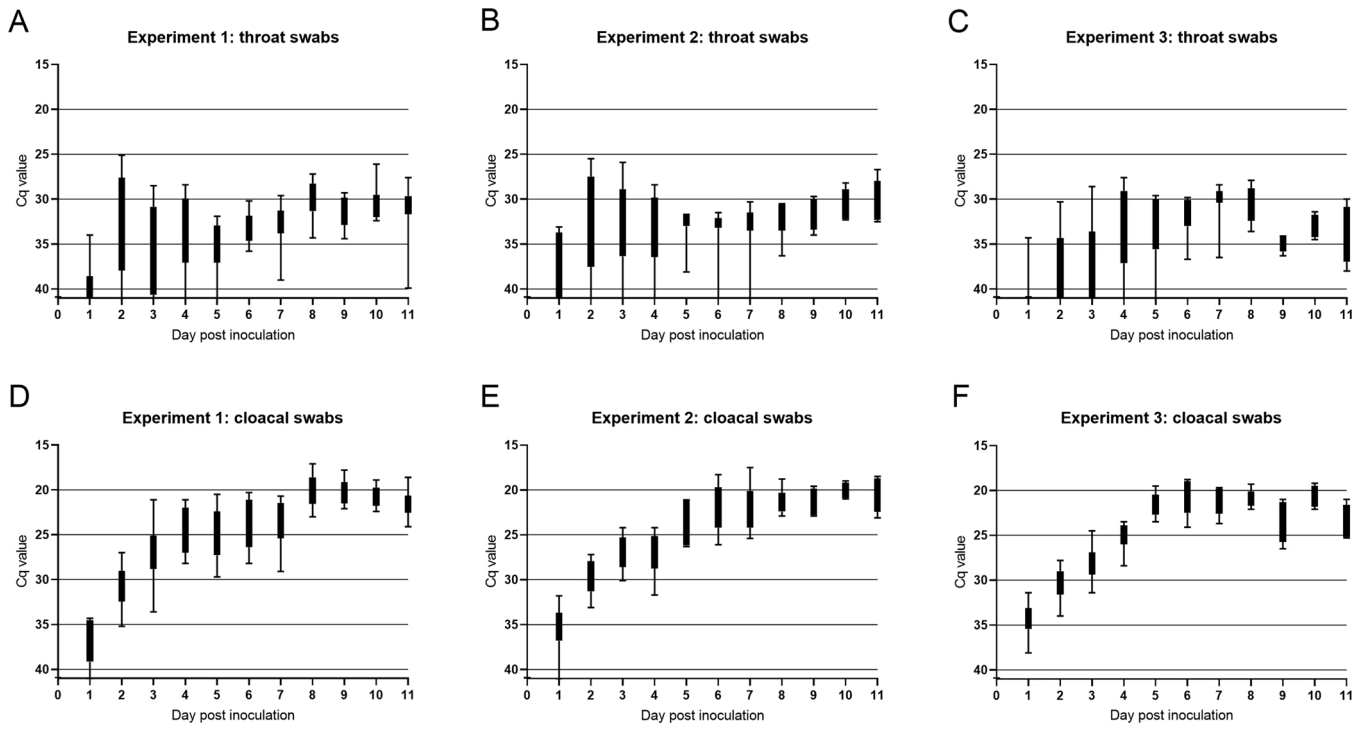

Fig. 2. PCR results of throat (A,B,C) and cloacal swabs (D,E,F).
The results are shown per experiment. On the Y-axis the cycle treshold (Cq) value is depicted. The Y-axis has been rotated and Cq values >40 are shown as Ct 41. The whiskers plot down to the smallest value and up to the largest and the box extends from the 25th to 75th percentile. In A and D every day post infection (dpi) at the X-axis shows the PCR results of 13 chickens. In B,C,E and F dpi 0 shows the results of 13 chickens, dpi 1–4 of 10 chickens, dpi 5–8 of 7 chickens and dpi 9–11 of 4 chickens.

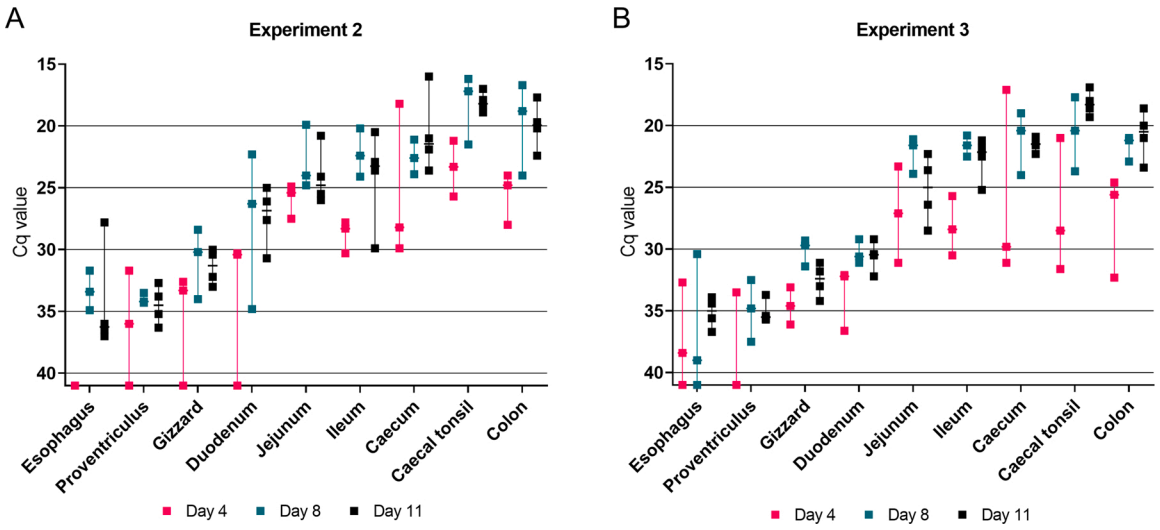

Fig. 3. PCR results of samples from the gastrointestinal tract.
A shows the results of experiment 2 and B of experiment 3. Per timepoint the median and range of the individual samples are shown. The results of day 0 are not presented as all samples tested PCR negative. On the Y-axis the cycle treshold (Cq) value is depicted. The axis has been rotated and Cq values >40 are shown as Cq 41.

stained positively for chlamydia had increased. Chlamydiae were most abundant in the epithelium of the caecum and caecal tonsil (Fig. 4C and D). In addition to the staining of the epithelium, chlamydial antigen was seen in single cells within the lamina propria and within the lymphoid follicles of the ileum and caecal tonsil.

Double immunostaining for chlamydial antigen and chicken cell markers showed that chlamydia co-localized with follicular dendritic cells in the lymphoid follicles (Fig. 5A–C) and mononuclear phagocytes within the lamina propria (Fig. 5D–F).

3.3. Dissemination to other organs

In the second and third experiment dissemination of chlamydia to tissues outside the gastro-intestinal tract was investigated (Fig. S2). In both experiments no chlamydial DNA was detected in spleen samples. In experiment 3 at day 4, one kidney sample had a Cq value of 25 and one liver sample a Cq value of 39 (Fig. S2 B, red encircled). This was most probably a result of contamination, because a part of the gut ruptured during necropsy. Chlamydial DNA could only be detected scarcely in

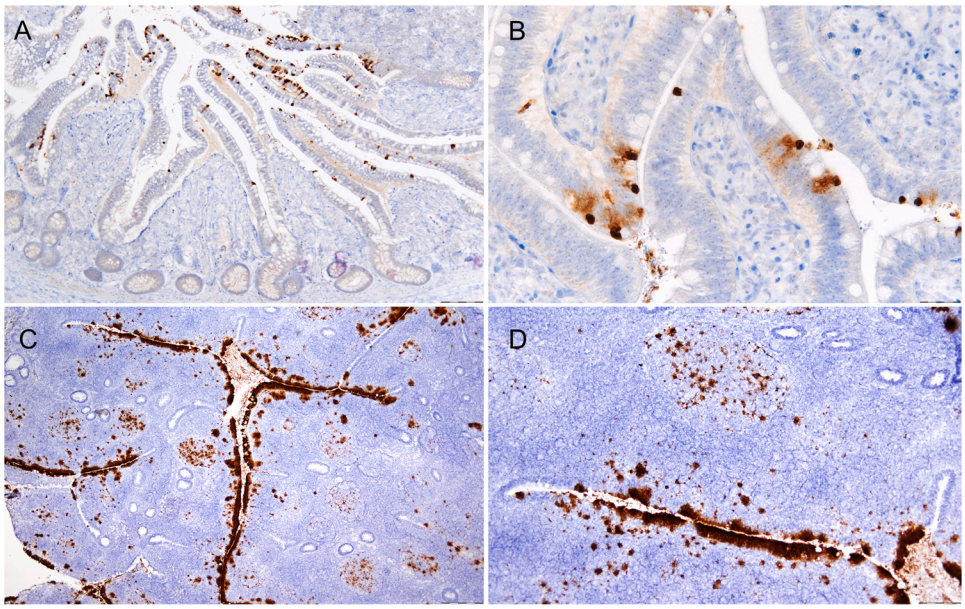

Fig. 4. Histology and immunohistochemistry of the ileum and caecum.
A and B: IHC staining for *Chlamydia* in the ileum at day 4 post infection Bacteria are clearly visible in the epithelium either as apical located inclusion bodies or diffusely present in the cytoplasm. A bar =100 μm, B bar =20 μm. C and D: IHC staining for *Chlamydia* in the caecal tonsil at 11 days post infection increased bacterial load in the epithelium compared to day 4 resulting in an almost continuous lining of the gut lumen. Chlamydial antigen is also present within single cells in the lamina propria (arrow) and in the lymphoid follicles (arrowhead). C. bar =200 μm, D. bar =100 μm.

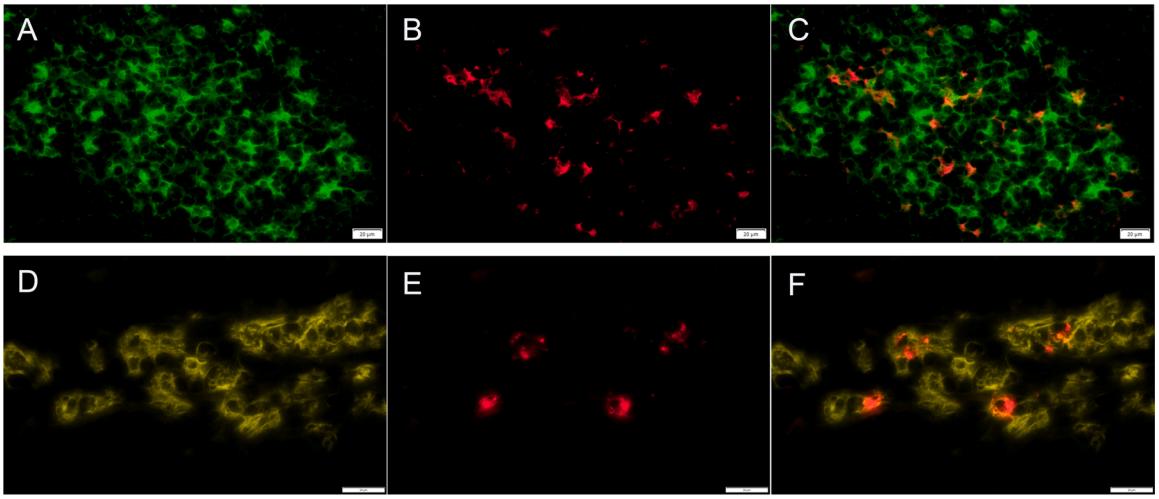

Fig. 5. Co-localization of chlamydial antigen and follicular dendritic cells (A-C) or mononuclear phagocytes (D-F) in the caecal tonsil at 11 days post infection.
A: Follicular dendritic cell staining with mAb 74.3, B: *Chlamydia* staining with pAb LS-C85741, C: merge of A and B, D: mononuclear phagocytes staining with mAb KUL01, E: *Chlamydia* staining with pAb LS-C85741, F: merge of D and E.

airsac, liver and lung with Cq values above 30, and the presence of chlamydia antigen in these tissues could not be confirmed with IHC.

3.4. Serologic response

The ELISA results indicate the development of a serologic response against *Chlamydia* in all three experiments (Fig. S3). At day zero all serum samples had a corrected OD value below 0.3. From day seven or eight an increase in OD (450 nm) was observed in all experiments. The level of response varied between animals but each individual animal displayed increased antibody response in the course of infection.

4. Discussion

C. gallinacea is a relatively recently discovered and widespread pathogen in poultry, but studies investigating the pathogenicity of *C. gallinacea* are still limited. Here, the pathogenicity of *C. gallinacea* strain NL_G47 was investigated in six-week old SPF layers. The layers were orally inoculated which resulted in throat- and cloacal shedding and infection of epithelial cells of the jejunum, ileum and caecum without signs of clinical disease, and macroscopic or histologic signs of inflammation. At day 11, chlamydial antigen was co-localised within macrophages in the lamina propria and FDCs in the caecal tonsil and, from day 7 onwards, a rise in antibody titre was shown. The presence of chlamydial antigen in epithelial cells of the gut, macrophages in the lamina propria and FDCs in the caecal tonsil, in combination with the development of an antibody response, has not been shown before for *C. gallinacea*.

Examination of the gut showed that the chlamydial load increased over time and towards the more distal parts, i.e. jejunum, ileum, caecum and colon, based on the results of qPCR. In the epithelial cells of the jejunum, ileum and caecum, the presence of chlamydial antigen was confirmed with immunohistochemistry. In contrast, the presence of chlamydial antigen in the epithelial cells of the colon could not be confirmed, although Cq values in the PCR overlapped at day 4, 8 and 11 in the jejunum, ileum, caecum and colon (see Fig. 3). Reisolation of viable *C. gallinacea* from these tissues was not performed, because it would be very difficult to discriminate whether bacteria were present in

epithelium or faecal content. However, the absence of chlamydial antigen in the colon epithelium suggests chlamydiae were only present in the lumen (residing in the fecal content) of the colon and replication in epithelial cells occured in the more proximal parts of the gut, i.e. jejunum, ileum and caecum.

Studies investigating the infection of *Chlamydia* in different parts of the poultry gut are limited. Experimental studies with *C. psittaci* in chickens reported the presence in the jejunum (Yin et al., 2013) or recovery of viable bacteria from the colo-rectum (Takahashi et al., 1988), but did not mention the presence of chlamydial antigen in the epithelium of the colon. In oral infections with *C. psittaci* in ducks, chlamydial antigen was detected in the caecum, but no data were presented about the colon (Thierry et al., 2016). Therefore, we cannot conclude if this difference has been observed in other chlamydial infections in poultry as well.

Furthermore, the possible cause of the observed difference in chlamydial infection of the epithelium in the jejunum, ileum and caecum and colon, is unknown. Perhaps that differences in the microbiome or mucin layer related to the function of the various parts of the gut might facilitate or prevent epithelial infection. For example in mice and humans the epithelium of the colon mainly secretes peptides that bind and aggregate bacteria, while the ileum mainly produces antibacterial peptides that kill bacteria reaching proximity to the epithelium (Schroeder, 2019). Aggregation of bacteria could be a more successful barrier for chlamydial infection than killing by antimicrobial peptides. Further research into the role of the microbiome and mucin layer in chlamydial infection would help to understand how *C. gallinacea* infects the gut epithelium.

The asymptomatic presence of *Chlamydia* in the gut is regarded as a typical feature of *Chlamydiae* and has been described in virtually all hosts (Rank and Yeruva, 2014). In a murine model with *C. muridarum,* oral infection resulted in an adaptive immune response, but infections in the caecum were not resolved and did lead to pathologic changes, probably due to the downregulation of the local immune response (Yeruva et al., 2013). These findings are in line with the results of our study. We did measure an increase in antibody response from day 7 onward, which might be an underestimation of the response against *C. gallinacea* as a mix of *C. abortus* and *C. trachomatis* antigen was used. Furthermore, we did not observe macroscopic or histological signs of inflammation in the gut, although chlamydial antigen was present in jejunum, ileum and caecum. We could also co-localise chlamydial antigen within macrophages in the lamina propria and FDCs in the caecal tonsil, which probably reflects the successful probing or uptake of chlamydiae by macrophages/dendritic cells from the intestinal lumen and subsequent presentation of antigen to FDCs in the B cell follicles resulting in the increase of the adaptive immune response.

A successful adaptive (systemic) immune response could also explain the limited systemic dissemination of *C. gallinacea* and might be a consequence of its relatively non-pathogenic nature in chickens. In our study chlamydial DNA was only detected incidently in airsac, liver and lung and the presence of chlamydial antigen was not confirmed with IHC. Reisolation of viable *Chlamydia* was not performed, but the limited systemic dissemination of *C. gallinacea* is in line with findings in other studies (Guo et al., 2016; Laroucau et al., 2009; You et al., 2019). Studies investigating the pathogenic potential of *C. abortus* and *C. psittaci* in comparative chicken models, revealed expression of both immunologically relevant and bacterial relevant factors was higher in *C. psittaci* infection (Braukmann et al., 2012; Kalmar et al., 2015). These differences could explain why *C. psittaci* is more invasive than *C. abortus* in avian hosts. It would be useful to perform similar studies with *C. gallinacea* to further understand its pathogenic nature and host-pathogen interaction. In particular, because chickens are considered the natural host of *C. gallinacea* (Guo et al., 2016) and in contrast to *C. abortus* for which small ruminants are considered the predominant host (Essig and Longbottom, 2015).

Although our study focused on the short term health effects, *C. gallinacea* could cause persistent infections in gut epithelium due to the possible local downregulation of the immune response as hypothesised earlier. In our experiments, *C. gallinacea* was still highly present at the end of the experiments at day 11. In other studies *C. gallinacea* was detected in the rectum at day 26 post infection (You et al., 2019), or for at least three months in cloacal swabs (Guo et al., 2016) suggesting a persistent infection of the gut. Persistent infections of *Chlamydia* in the gut can result in long term or chronic health effects (Reinhold et al., 2011), because an infection in gut epithelial cells (due to a possible higher cell turnover) and an increase in adaptive immune response will result in (metabolic) costs that might have an adverse effect on production parameters (Kogut et al., 2018). A negative effect of *C. gallinacea* infection on production has already been shown in broilers with reduced weight (Guo et al., 2016). In layers, this effect, on for example egg production, deserves further investigation. Though, this type of field research would require a rigorous design considering the high prevalence of *C. gallinacea* at farm level (Heijne et al., 2018).

In conclusion, our study confirms *C. gallinacea* infection mainly resides in the gut and results in asymptomatic cloacal shedding. The combination of asymptomatic shedding and possible persistent infection of the gut could result in adverse long term health effects. Furthermore, persistent cloacal shedding of *C. gallinacea* facilitates orofecal transmission and probably explains why it is highly endemic in poultry (Guo et al., 2016; Heijne et al., 2018; Hulin et al., 2015).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2021.109166.

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