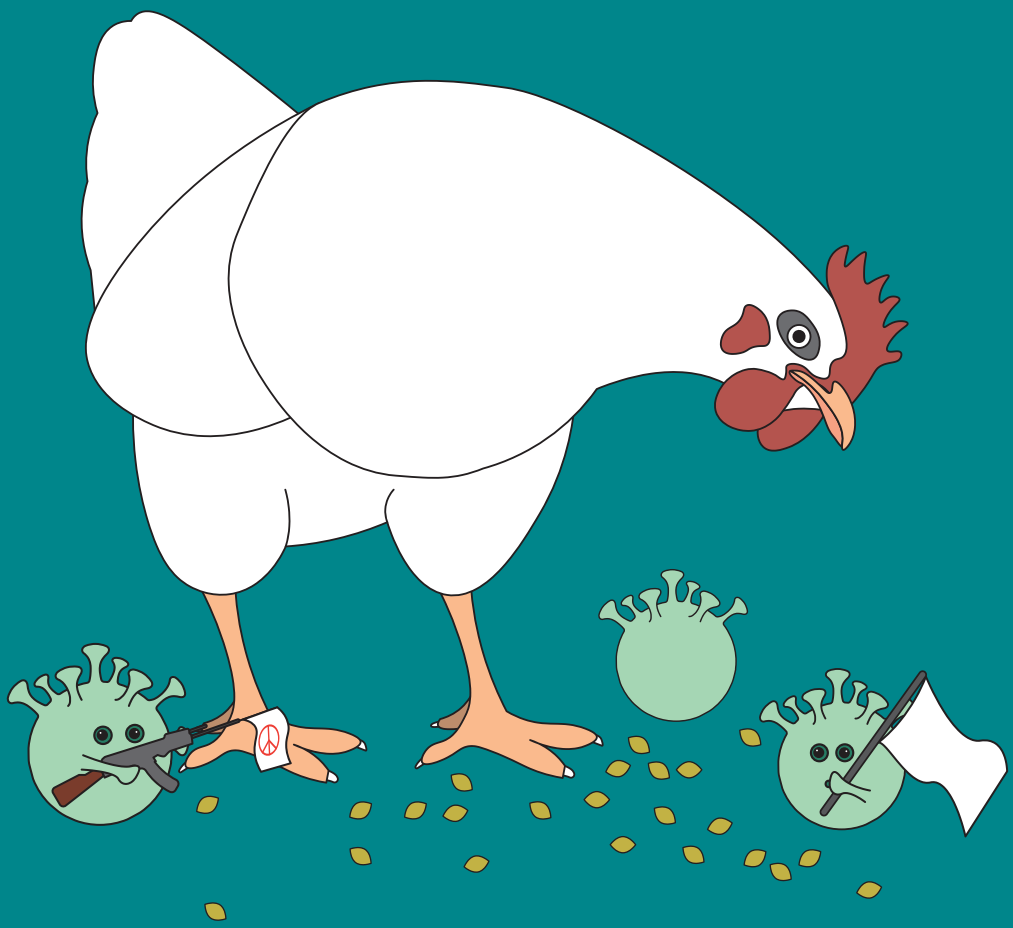
Avian chlamydiosis in chickens: from cell to population



Marloes Heijne

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Marloes Heijne
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Avian chlamydiosis in chickens: from cell to population

Aviaire chlamydiose in kippen: van cel naar populatie
(met een samenvatting in het Nederlands)

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Marloes Heijne

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Promotor:

Prof. dr. M. Nielen

Copromotoren:

Dr. A.P. Koets
Dr. J.A. van der Goot

Contents

CHAPTER 1

General Introduction

Background on *Chlamydia* research and avian chlamydiosis

Avian chlamydiosis refers to disease in birds caused by bacteria from the genus *Chlamydia*. The term avian chlamydiosis was initially introduced to replace the terms psittacosis (disease in psittacines and humans) and ornithosis (disease in pigeons and poultry) as these are specifically caused by the bacterium *Chlamydia psittaci* (1). *C. psittaci* is zoonotic and the term psittacosis is still used to describe the disease in humans.

First detailed descriptions about psittacosis date back to 1879, when Jacob Ritter identified newly imported birds as the source of human pneumonia cases (2). In 1895, Morange introduced the term psittacosis, which refers to the Greek word for parrot, when flu-like symptoms in humans were associated with parrots (3). In the winter of 1929-1930 a psittacosis outbreak, caused by the import of parrots, resulted in a total of 766 human cases and 112 case fatalities worldwide (4). Although the number of cases were relatively small compared to other pandemics such as the current COVID-19 pandemic, the "great parrot fever" led to press headlines and further research into the cause of this mysterious disease (5).

In 1930, the morphology and life cycle of the agent causing psittacosis was described (6). At that time, the agent was classified as a virus due to its filterable size and failure to cultivate it as a bacterium (7). A few years later, *C. psittaci* could be cultivated on the chorioallantoic membrane (8) and in the yolk sac of embryonated chicken eggs (9). Attempts to grow *Chlamydia* in cell culture also started in the 1930s, but it took until 1969 before cell culture became more successful than yolk sac culture (10). In 1965, *Chlamydia* was finally classified as a bacterium based on studies on its morphology with electron microscopy (11)

From 1930 to 1938, 174 cases of human psittacosis were reported from the Faroe Islands (12). The disease was transmitted via juvenile fulmars (*Fulmarus glacialis*) that were captured and subsequently prepared for cooking. This outbreak highlighted psittacosis could be transmitted by other bird species than psittacines, although parrots might have been the source of infection for the fulmars (13).

Later, Meyer showed that psittacosis had a wide host range in domestic and free-living birds; he listed 70 bird species where *Chlamydia* was detected (14). In 2003, this list had been extended to 469 domestic and free-living bird species comprising 30 avian orders (15). Since 2009, the taxonomy of *Chlamydia* has become more complex due to the discovery of new avian chlamydial species, such as *Chlamydia avium*, *Chlamydia buteonis* and *Chlamydia gallinacea* (16-18). Therefore, the term avian chlamydiosis is currently applied to all chlamydial infections in avian species (1).

Chlamydial bacteriology

Chlamydia are obligate intracellular Gram-negative bacteria belonging to the family of *Chlamydiaceae* and order of *Chlamydiales*. In 2015, the two members of the family, *Chlamydophila* and *Chlamydia*, were reunited in one genus: *Chlamydia* (19). The genus *Chlamydia* currently consists of 14 species and an extending number of *Candidatus* species (20). The recognized and candidate species with their main host are shown in figure 1. Zoonotic potential is described for *C. abortus, C. caviae, C. felis and C. psittaci* (21, 22), while *C. pneumonia and C. trachomatis are mainly known as human pathogens* (23). *C. avium, C. buteonis, C. gallinacea, C. psittaci and Ca. C. ibidis* are predominantly found in birds (24).

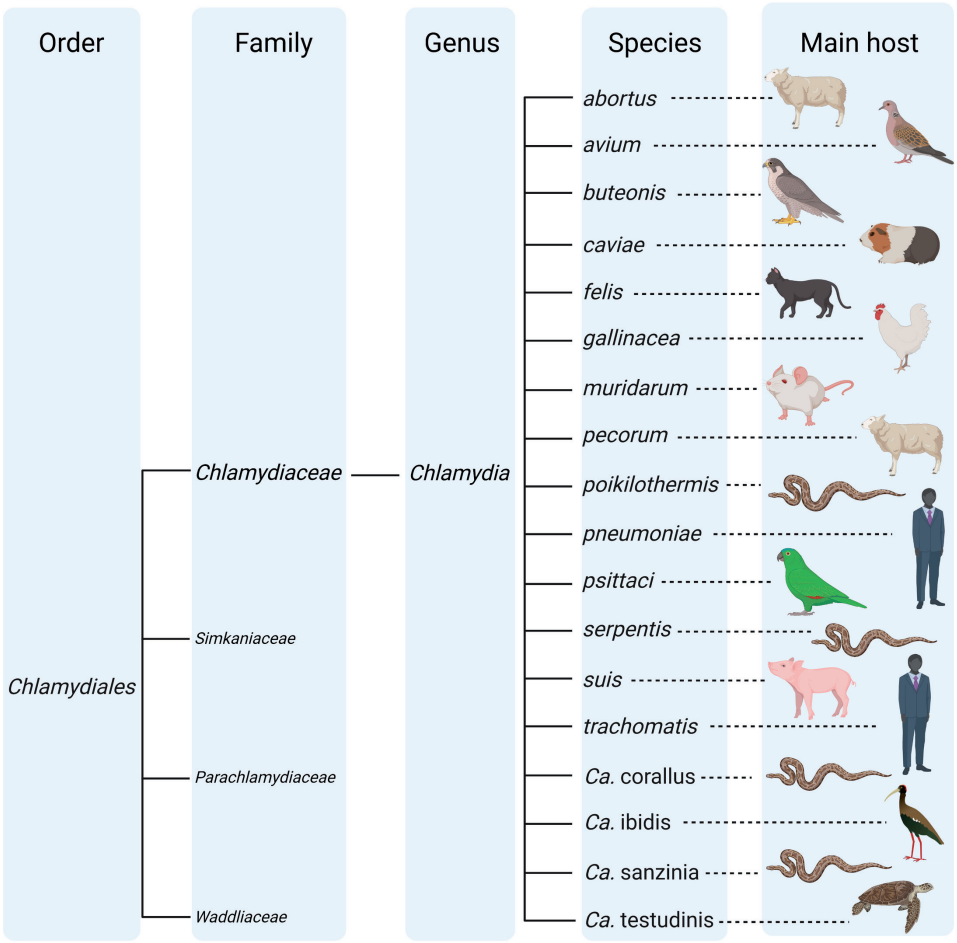

Fig. 1 *Chlamydia* taxonomy and host range
The current taxonomy of Chlamydia including candidate species is shown. Species are listed in alphabetical order instead of their phylogenetic relationship. The figure was created with BioRender.com.

All *Chlamydia* have a biphasic life cycle with elementary bodies (EBs) and reticulate bodies (RBs), where the RBs are always located intracellular in contrast to EBs (Fig. 2). EBs are non-replicative and infectious with a spore-like cell wall, which allows them to survive outside host cells under harsh conditions. They are relatively small particles that are electron-dense due to condensed chromatin, with a diameter ranging from 0.2-0.4 µm (25). After host cell entry, EBs transform into RBs, which is the replicative and non-infectious stage. RBs are less electron-dense and about 0.5 -1.5 µm in size. The entire life cycle lasts about 48–72 hours depending on the chlamydial species (26).

In host cells, *Chlamydia* create an intracellular niche (an inclusion) from which they acquire nutrients for their survival and evade the host immune response. Their exact strategy differs between chlamydial species and hosts (27). Replication mainly takes place in epithelial cells, but some species, for example *C. psittaci*, are also able to replicate inside macrophages (28, 29).

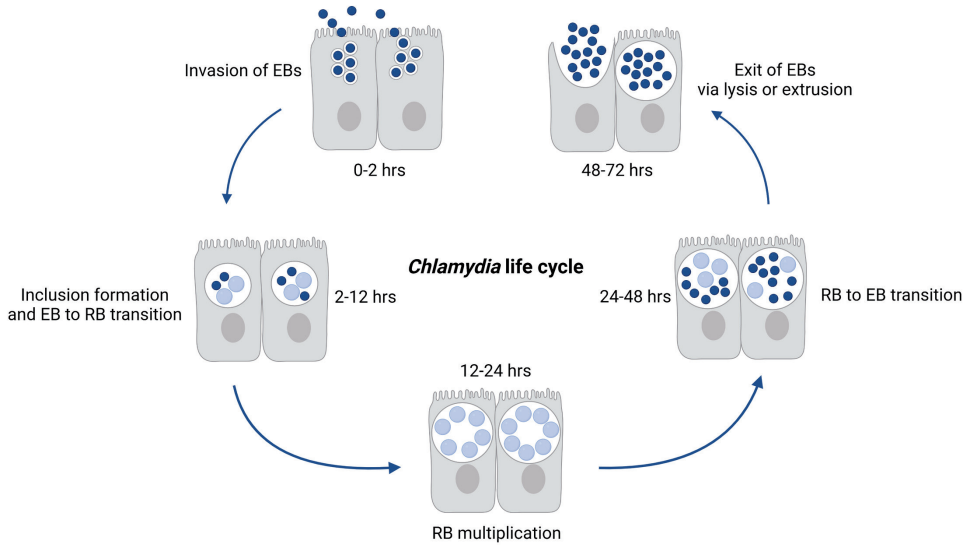

Fig. 2 *Chlamydia* life cycle
Elementary bodies (EBs) attach and invade host epithelial cells and form inclusions (within two hours after internalization). Between 2 and 12 hours EBs differentiate into reticulate bodies (RBs). After 12 hours RBs start to multiply by binary fission with a peak between 18 and 24 hours. Around 24 hours RBs differentiate back to EBs until their release via lysis of the host cells or extrusion between 48 to 72 hours (26). The duration of the life cycle depends on the chlamydial species. The figure was created with BioRender.com.

About 60% percent of the mass of the outer membrane of *Chlamydia* consists of the major outer membrane protein (MOMP), which is one of the major surface antigens (30). It is frequently used as target in serology, but also for subtyping within different chlamydial

species. In EBs MOMP probably functions as an adhesin, while in RBs it acts as a porin (27). Other important outer membrane constituents are the chlamydial lipopolysaccharide (LPS), OmcA and OmcB, polymorphic membrane proteins (pmps) and the Type III secretion system (T3SS). The T3SS forms a needle like structure allowing the export of several effector proteins into the host cell, and is considered one of the most important virulence factors (31).

Chlamydia have relatively small genomes compared to many other bacteria, with an average genome size of 1 mega base pairs (Mbp) carrying about 880 to 1050 coding sequences (CDS). Recent genomic comparison between 12 different chlamydial species and 33 strains revealed that all species share about 80% of their genome involving 784 genes (32). Outside this core genome, the most variable regions are genes encoding pmps, genes encoding T3SS effector proteins and the plasticity zone (33). The plasticity zone is a region near the replication terminus with genes that encode for a cytotoxin and membrane attack complex/perforin protein (MACPF) (23, 33). All these variable regions are related to virulence, host interaction and/or host tropism (23, 33). In addition, most strains carry plasmids that might contribute to their virulence (23).

Chlamydial infections in poultry

Until 2009, *C. psittaci* was considered the predominant chlamydial species in poultry. *C. psittaci* is most closely related to *C. abortus*, *C. caviae* and *C. felis* and can be subdivided in at least nine different genotypes (former serotypes) based on the gene sequence coding for MOMP, *ompA*. The genotypes are associated with different host species. Avian strains (genotype A to F) are considered to be more virulent than mammalian strains (genotype M56 and WC). In poultry, genotype C and E/B are mostly found in ducks and genotype D and E/B in turkeys (11). No specific genotype is associated with infections in chickens. Other more discriminatory typing schemes, such as Multi Locus Sequence typing (MLST), which is based on sequence analysis of seven housekeeping genes, identified at least 12 different *C. psittaci* sequence types (ST) that could also be associated with different host species (34).

C. psittaci is widespread, but its prevalence varies between genotypes and bird species (35). Chickens were thought to be relatively resistant to *C. psittaci* infection, but a Belgian study in 2014 reported 6/7 broiler breeder, 7/7 broiler and 5/5 layer farms PCR and culture positive for *C. psittaci* in pharyngeal swabs (36). Other studies in Belgium and Northern-France from 2010 and 2013 also reported a high prevalence of *C. psittaci* in chickens determined with PCR, culture (on pharyngeal swabs and tissues) and/or serology (37, 38).

In birds, *C. psittaci* is excreted in faecal droppings and nasal discharges (35). Faecal shedding is intermittent and can be activated through stress factors such as transport and overcrowding (35). Shedding can last for several months (35). The main route of transmission is the respiratory route via inhalation of dried faecal material or respiratory exudate (11). Transmission can also occur via ingestion. Vertical transmission and mechanical transmission via biting flies, lice or mites have been described, but are not considered important transmission routes (11).

In general, clinical signs and pathogenesis of *C. psittaci* depend on the strain, host, host age and (environmental) stressors (11). In turkeys, severe systemic infections and mortality have been described (39, 40). Ducks seem to be less susceptible to *C. psittaci* infection and are mainly subclinically infected (41, 42). Experimental infections with *C. psittaci* in chickens can remain asymptomatic, but can also result in severe systemic illness and mortality (38, 43, 44).

In 2009, a new chlamydial species was detected in poultry, later classified as *Chlamydia gallinacea* (16, 17). *C. gallinacea* is widespread, with reports from Asia (45), Australia (46), Europe (47-51), North-(52) and South-America(53). Most studies have been conducted in chickens in which *C. gallinacea* is highly prevalent. Since its discovery, *C. gallinacea* has also been found in other species such as pigeons, woodcocks, a parrot, an ultramarine grosbeak and cattle (1, 45, 46, 54-56).

Genome analysis showed that *C. gallinacea* is a separate species, based on an average nucleotide identity of less than 81 percent with *C. avium* as the most closely related species (17). Strain 08-1274/3^{T} is considered the type strain and was isolated from a chicken in France (17). Molecular studies using outer membrane protein A (*ompA*) genotyping or MLST showed *C. gallinacea* is diverse, with at least 13 different *ompA* types and 15 different sequence types (ST) in 25 strains and without any host or geographical association (45, 57). Whether this diversity contributes to differences in pathogenicity of *C. gallinacea* strains is currently unknown.

C. gallinacea is shed in faecal droppings in high quantities, thus contamination of the environment might play an important role in transmission. The main route of transmission is considered to be the faecal-oral route (56). In contrast to *C. psittaci,* airborne transmission via dried faecal material does not seem to occur (58). Vertical transmission has been proposed after detection of *C. gallinacea* in the albumen and yolk of embryonated eggs. However, environmental contamination via penetration through the egg shell could not be excluded (58). Cases of mechanical transmission via biting flies, lice or mites have not been described.

Infections with *C. gallinacea* in chickens appear to be mainly asymptomatic, but reduced weight gain in broilers has been observed (45, 47, 48). Experimental data on the pathogenesis of *C. gallinacea* in poultry are limited thus far, and show *C. gallinacea* is mainly residing in the gut, although it has been detected with PCR in blood, lung, heart, liver, trachea, kidney, pancreas and spleen samples (16, 45, 58). Additional research into the pathogenicity is required to assess the importance of *C. gallinacea* as a poultry pathogen.

One Health perspective

The Dutch Q fever outbreak from to 2007 to 2010 highlighted the possible impact of environmental transmission of zoonotic pathogens from farm animals to humans. After the outbreak, studies were initiated to assess the public health risks of intensive farming in densely populated areas. One observation was a higher incidence of human pneumonia cases with unknown aetiology in the direct proximity of poultry farms (59). About the same time, studies from surrounding countries showed *C. psittaci* was more prevalent in chickens than previously assumed (36, 38, 60). *C. psittaci* is a zoonosis and infection in humans can result in severe pneumonia (61), but around 2012 its occurrence in Dutch poultry farms was unknown. The annual number of notified human *C. psittaci* cases in the Netherlands varies between 25 and 85. However, disease burden calculations estimated that more than 1500 human cases might remain undiagnosed since awareness of psittacosis is generally low (61) and *C. psittaci* is often not included in routine diagnostic panels (62).

In addition, *C. gallinacea* was described as a new widespread chlamydial species in poultry with a possible zoonotic potential, since transmission to humans was suspected in slaughterhouse workers with signs of pneumonia (1, 16). The lack of understanding of the prevalence of (potential) zoonotic chlamydial species in Dutch poultry triggered the research in this thesis.

Scope and outline of this thesis

In this thesis we aimed to gain insight into the prevalence of *C. psittaci* and *C. gallinacea* in poultry, specifically chicken layers, in the Netherlands. Moreover, we aimed to further elucidate the pathogenicity of *C. gallinacea* and studied possible cross protection between chlamydial species in chickens.

In **Chapter 2** the prevalence of *C. psittaci* and *C. gallinacea* in Dutch layers was investigated as part of a surveillance program for zoonotic pathogens in farm animals. Furthermore,

potential risk factors were investigated that could be associated with the presence of *C. gallinacea*, such as the occurrence of clinical signs or a higher mortality rate.

In **Chapter 3** and **4** the pathogenicity of *C. gallinacea* was further analysed. First, two novel strains (NL_G47 and NL_F725) were isolated and compared using an *in vivo* infection model in embryonated chicken eggs and comparative genomics. Subsequently, the primary pathogenicity of *C. gallinacea* in chickens was investigated in three consecutive animal experiments. Chickens were inoculated orally with one of the Dutch isolates (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.

Chapter 5 describes the results of cross infection experiments with *C. psittaci* and *C. gallinacea* in chickens. We hypothesized that an infection with *C. gallinacea* might protect against an infection with *C. psittaci*. To investigate this hypothesis, chickens were inoculated with *C. gallinacea* NL_G47 and, after five weeks, inoculated with either a different strain of *C. gallinacea* (NL_F725) or with a strain of *C. psittaci*. These treatments were compared to single exposure with either *C. gallinacea* (NL_F725) or *C. psittaci.* Reduced shedding or tissue dissemination in the groups that had been pre-inoculated with *C. gallinacea* NL_G47 would be an indication of possible cross protection between *C. gallinacea* strains and/or *C. psittaci*.

The last chapter (**Chapter 6**) discusses whether results in layers might be extrapolated to broilers or other poultry species, whether *C. gallinacea* should be considered a pathogen and how the findings of this thesis should be interpreted from a One Health perspective.

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CHAPTER 2

A cross sectional study on Dutch layer farms to investigate the prevalence and potential risk factors for different *Chlamydia* species

Marloes Heijne[1], Jeanet A. van der Goot[1], Helmi Fijten[2], Joke W. van der Giessen[1,3], Eric Kuijt[2], Catharina B.M. Maassen[3], Annika van Roon[3], Ben Wit[4], Ad P. Koets[1,5], Hendrik I.J. Roest[1]

[1] Department of Bacteriology and Epidemiology, Wageningen Bioveterinary Research, Lelystad, the Netherlands
[2] Department of Infection Biology, Wageningen Bioveterinary Research, Lelystad, the Netherlands
[3] Center for Zoonoses and Environmental Microbiology, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
[4] Consumer and Safety Division, Food and Consumer Product Safety Authority (NVWA), Utrecht, the Netherlands
[5] Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

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Abstract

In poultry several *Chlamydia* species have been detected, but *Chlamydia psittaci* and *Chlamydia gallinacea* appear to be most prevalent and important. *Chlamydia psittaci* is a well-known zoonosis and is considered to be a pathogen of poultry. *Chlamydia gallinacea* has been described more recently. Its avian pathogenicity and zoonotic potential have to be further elucidated. Within the Netherlands no data were available on the presence of *Chlamydia* on poultry farms. As part of a surveillance programme for zoonotic pathogens in farm animals, we investigated pooled faecal samples from 151 randomly selected layer farms. On a voluntary base, 69 farmers, family members or farm workers from these 151 farms submitted a throat swab. All samples were tested with a generic 23S *Chlamydiaceae* PCR followed by a species specific PCR for *C. avium*, *C. gallinacea* and *C. psittaci*. *C. avium and psittaci* DNA was not detected at any of the farms. At 71 farms the positive result could be confirmed as *C. gallinacea*. Variables significantly associated with the presence of *C. gallinacea* in a final multivariable model were 'age of hens', 'use of bedding material' and 'the presence of horses'. The presence of *C. gallinacea* was associated with neither clinical signs, varying from respiratory symptoms,nasal and ocular discharges to diarrhoea, nor with a higher mortality rate the day before the visit. All throat swabs from farmers, family members or farm workers tested negative for *Chlamydia* DNA, giving no further indication for possible bird-to-human (or human-to-bird) transmission.

Introduction

Chlamydia avium, Chlamydia gallinacea and *Chlamydia psittaci* belong to the family of *Chlamydiaceae*, a group of obligate intracellular bacteria. *Chlamyia psittaci* is widespread and can infect over 465 bird species and several mammalian species, including humans (1). Pathogenicity in animals depends on host species and *C. psittaci* strain. Clinical symptoms in birds vary from asymptomatic to acute death. *Chlamydia psittaci* is a well-known zoonosis and the cause of psittacosis. Transmission from birds to humans occurs via aerosolised respiratory or faecal excretions. In the Netherlands, psittacosis is notifiable in humans and pet birds but not in poultry. In poultry, chickens appeared to be less sensitive to chlamydial infection and a sporadic source of human infection (1-3). However, in recent publications *C. psittaci* is regularly detected and chicken-to-human transmission is more frequently described (4-6).

Chlamydia avium and *C. gallinacea* have been detected in pet birds and poultry since 2009, first being classified as "atypical" and in 2014 added as new members of the genus *Chlamydia* (7-9). *Chlamydia avium* has been found in psittacines and pigeons, *C. gallinacea* in chickens, guinea fowl and turkeys (10). Recent studies hypothesised *C. gallinacea* to be endemic in chickens causing only mild clinical signs such as reduced weight gain in broilers (11). Its zoonotic potential was suggested, but conclusive evidence has not been presented yet (8).

The impact of transmission of zoonotic pathogens from farm animals to humans was highlighted by the Dutch Q fever outbreak (2007-2010). Due to this outbreak, studies were initiated to assess the public health risks of intensive farming in densely populated areas (12). One of the findings was a higher incidence of human pneumonia cases in the direct proximity of poultry farms (13, 14). The cause of this higher incidence was unknown. We therefore hypothesised that *C. psittaci* or *C. gallinacea* could play a role. However, no data were available on the presence of *C. psittaci* and *C gallinacea* on Dutch poultry farms.

We investigated 755 faecal samples from 151 layer farms for the presence of *Chlamydiacea* DNA. Per farm a questionnaire was completed to identify possible risk factors. To gather information on possible bird to human transmission, farmers, family members or farm workers were invited to participate on a voluntary basis in throat swab sampling.

Materials and methods

Sampling strategy

Between March 2015 and January 2016, a cross-sectional study on layer farms was performed as part of a surveillance programme for zoonotic pathogens in farm animals. From the 993 layer farms in the Netherlands, 154 farms were randomly selected, stratified on farming system (conventional n=79, free range n=34, organic n=22, enriched cages n=8, enriched colony n=6). Finally, 151 farms completed a questionnaire and were included in the analysis. For *Chlamydia* testing, five pooled faecal samples were collected from one barn per farm, resulting in 755 samples. Each pooled sample contained twelve scoops of fresh faeces. Additional information on farm characteristics, husbandry practices, biosecurity measures, clinical history and antibiotic usage was acquired via a questionnaire (Fig. 1). Farmers, family members and farm workers were asked to participate in the poultry-to-human transmission study by submitting two throat swabs (collected through self-sampling) for *Chlamydia* testing. In total 69 farmers, family members or farm workers from 41 farms participated in the study.

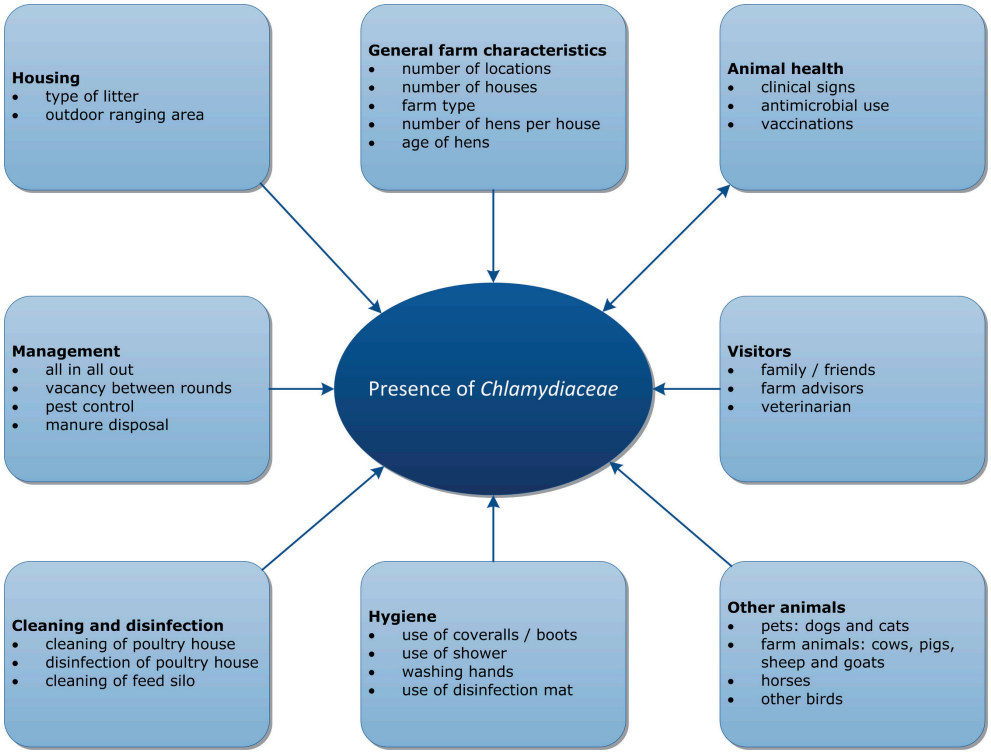

Fig. 1. Overview of risk factors

Overview of possible risk factors for the presence of Chlamydiaceae on which information was gathered via a questionnaire.

Ethics statement

The Medical Ethics Review Committee (Utrecht Medical Centre, Utrecht) stated that the Medical Research Involving Human Subjects Act (WMO) does not apply to this study and therefore no official approval for the study is required under the WMO. All volunteers gave their written consent for participation in the study.

Laboratory tests

DNA isolation of all pooled faecal samples was performed with a NucliSENS® easyMAG® (Biomerieux, Zaltbommel, the Netherlands). In brief, faecal material was taken from each sample with a dry swab, suspended in 1.5 ml Phosphate Buffered Saline (PBS) and thoroughly vortexed. From this suspension, 500 µl was added to 2 ml NucliSENS® lysis buffer for off-board lysis. After at least one hour of incubation at room temperature, the lysis buffer was added to 80 µl of silica and extracted according to manufacturer instructions for specific protocol B. Within this protocol an optimised washing protocol is used with extra and longer washing steps. The final elution volume was 100 µl. DNA isolation of human throat swabs was performed with a MagNA Pure® LC (Roche Diagnostics, Almere, The Netherlands) according to manufacturer instructions for off-board lysis. Of the sample 200 µl was processed to a final elution volume of 50 µl. *Chlamydiaceae*-DNA was detected using a generic PCR that targeted the 23S rRNA gene with primers and probes according to Ehricht et al (15). *Chlamydia psittaci* DNA was detected using a PCR that targeted the *ompA* gene with primers and probes according to Pantchev et al (16). For *C. avium* and *C. gallinacea* a duplex PCR was used targeting the *enoA* gene. For *C. avium* primer and probe sequences were used according to Zocevic et al (9). For *C. gallinacea* primer and probe sequences were used according to Laroucau et al (6). To validate the *C. avium* and *C. gallinacea* duplex PCR, 10-fold serial dilutions (single and in a mixture) of *C. gallinacea* strain 14DC0101 and *C. avium* strain 10DC97 were tested. The duplex PCR appeared to be as sensitive as the single PCR. No differences in Ct values were observed when *C. avium* and *C. gallinacea* were added in a single dilution or as a mixture. The final volume of the reaction mixture was 20 µl, including 5 µl of the DNA template, 10 µl TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Fisher Scientific, Landsmeer, the Netherlands), 1 µM of each primer, 0.2 µM of the probes, 0.2 µl UDG (5U/µl) and distilled PCR water to reach the final volume. Amplification was carried out in an ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Fisher Scientific, Landsmeer, the Netherlands) using the following cycling parameters: 5 min at 37 ◦C, 20 sec at 95 ◦C, 50 cycles of 95 ◦C for 3 sec and 60 ◦C for 30 sec. As a control for DNA extraction, a known *C. psittaci* positive faecal swab was used. In each 23S and *C. psittaci* PCR run a dilution series of three *C. psittaci* DNA isolates was used as positive controls. In the *C. gallinacea* and *C. avium* duplex PCR, DNA from *C. gallinacea* strain 14DC0101 and *C. avium* 10DC97 and a mix of both strains were used as positive controls. Each real-time PCR run included a non-template control using 5 µl distilled water as template, and during the extraction per 12 samples a negative sample

with 1.5 ml PBS was added. Samples with a Ct value up to 40 were considered positive and samples with a Ct value above 40 were considered negative. Farms were considered positive if at least one of five samples tested positive in the PCR.

GIS map

Chlamydia gallinacea positive and negative farms were plotted on a laying hen density map of the Netherlands (Fig. 2). Data were extracted from CBS Statline (http://statline.cbs.nl) and imported into QGIS version 2.18.

Statistical analyses

Farm prevalence was determined with an exact (Clopper-Pearson) 95 percent confidence interval (epitools.ausvet.com.au). Data from the questionnaires were collected via a digital form in Epi Info™ and analysed using IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., Armonk, N.Y., USA). Overlapping variables or small categories were merged or summarised when possible. Potential risk factors for the presence of *C. gallinacea* were initially examined with a univariable analysis using a Chi square test or a logistic regression for continuous variables. Variables associated ($p \leq 0.20$) with the outcome of interest (presence of *C. gallinacea*) were considered for inclusion in a stepwise, backward, multiple logistic regression analysis. The selected variables for the multivariable analysis were tested for mutual correlation. A likelihood ratio test was performed to eliminate variables from the multivariable model. Variables had to be significant ($p \leq 0.05$) to remain in the final model. The goodness of fit of the final model was tested using the Hosmer and Lemeshow test.

Results

Chlamydiaceae DNA was detected on 74 of the 151 farms and confirmed as *C. gallinacea* on 71 farms (farm prevalence 47%, 95% CI: 39-55%). Neither *C. psittaci* DNA nor *C. avium* DNA was detected in any of the samples from the 151 farms. The distribution of the number of positive samples per farm in the 23S *Chlamydiaceae* PCR and the *C. gallinacea* PCR is shown in Table 1. On 31 farms all five samples were positive in both the *Chlamydiaceae* PCR and *C. gallinacea* PCR, whereas on 67 farms all five samples were negative in both the *Chlamydiaceae* PCR and *C. gallinacea* PCR. At seven farms no *Chlamydiaceae* DNA was detected, but per farm one or two samples tested positive for C. *gallinacea* DNA with Ct values above 36. The 71 farms that had one or more positive samples in both the *Chlamydiaceae* PCR and the *C. gallinacea* PCR were included in the risk factor analysis.

Table 1. The distribution of the number of positive samples per farm in the *Chlamydiaceae* and *C. gallinacea* PCR

		Number of positive samples per farm in *C. gallinacea* PCR (n=5 per farm)						
		0	**1**	**2**	**3**	**4**	**5**	**total**
Number of positive samples per farm in *Chlamydiaceae* PCR (n=5 per farm)	**0**	67	6	1	0	0	0	74
	1	2	6	1	1	0	0	10
	2	2	3	1	2	2	0	11
	3	0	2	1	3	0	1	7
	4	0	0	2	1	3	1	6
	5	0	0	0	1	3	30	34
	total	71	17	6	8	8	32	142*

** The results of 142 farms are shown. From seven farms one or more samples showed inhibition. From two farms only four samples could be tested. The results of these nine farms are not shown in the table, but the farm level results were used in the analysis.*

The location of the positive and negative farms is shown in Fig. 2. *Chlamydia gallinacea* positive and negative farms appear to be equally distributed in the Netherlands.

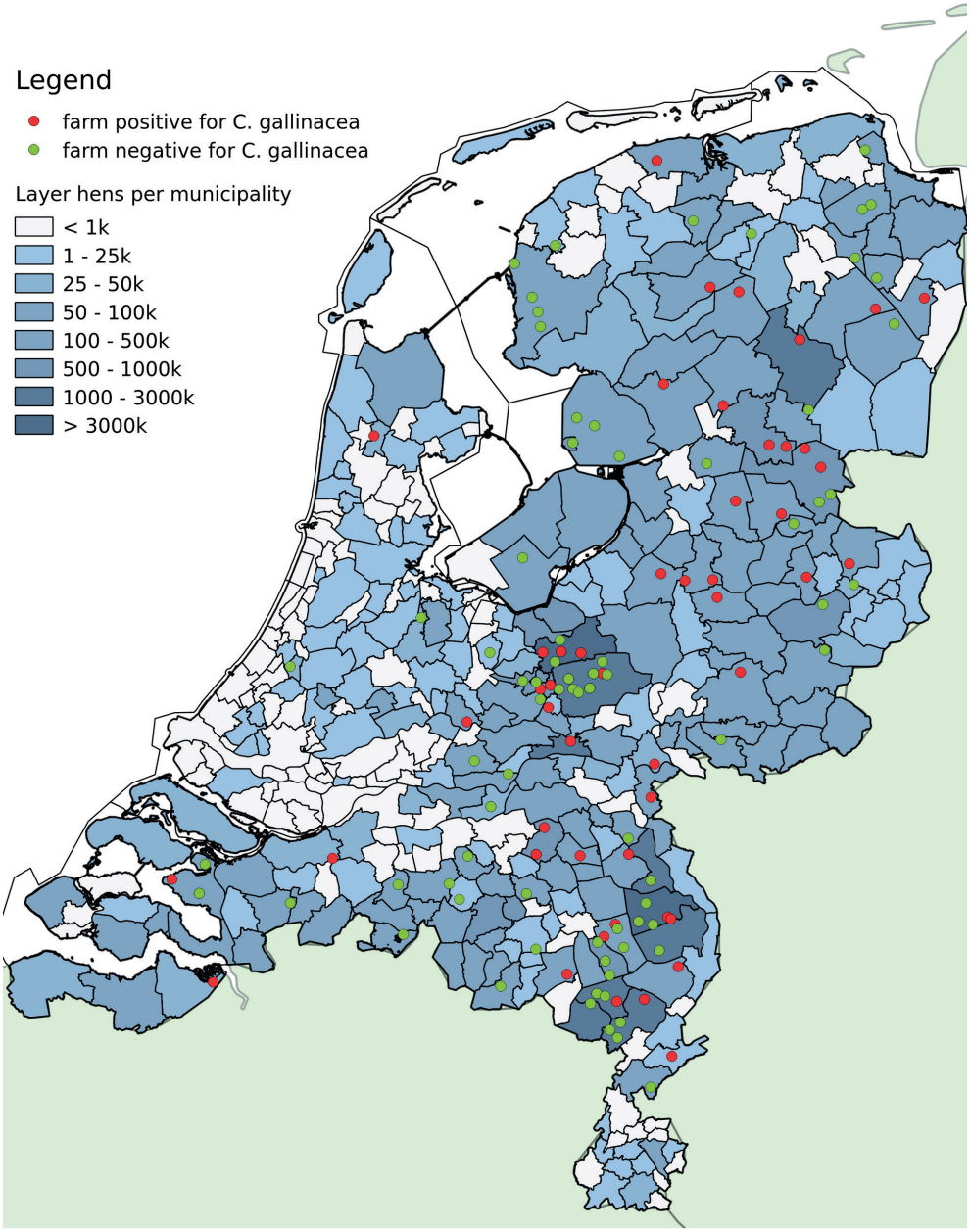

Fig. 2. Map with *C. gallinacea* positive and negative farms
Chlamydia gallinacea positive and negative farms plotted on a laying hen density map of the Netherlands.

General descriptors about farm type and the median farm size are shown in Table 2. Farm type is related to farm size. Farms with enriched cages and colony systems are larger than free range and organic farms. Due to the relation with farm type, farm size was excluded from the analysis. Background and coding information on the variables in the univariable and multivariable analyses are added in the S1 and S2 files. For the variables 'age of hens' and 'manure disposal' the smallest categories were merged. From the variable 'vacancy period' (period between two flocks, when the barn is empty), outliers with a vacancy period above 90 days were excluded from the analysis.

In the univariable analysis, ten variables met the criteria of $p \leq 0.2$, i.e. 'age of hens', 'use of bedding material', 'presence of horses', 'frequency of manure disposal', 'visitors have to shower before entrance', 'other birds', 'free range', 'vaccination against *Pasteurella multocida*' or 'Egg Drop Syndrome' and 'vacancy period' (Table 3). No mutual correlations were found between these ten variables and they were all included in the multivariable analysis. No associations were found between the presence of *C. gallinacea* and 'one or more locations', 'more than one poultry house', 'all in all out at farm level', 'fly control', 'visitors', 'disinfection method', 'frequency of cleaning of the feed silo', 'washing hands before entrance' and the 'presence of other farm animals or pets'. All farms reported that they controlled rats and mice. The variables 'use of disinfection mat before entrance' and 'use of tools in one or more houses' were not included in the analysis, due to inconsistent answers in the questionnaires.

In the multivariable analysis, three variables were significantly associated with the presence of *C. gallinacea* as shown in Table 4: 'age of hens', 'use of bedding material' and 'presence of horses'. The final model met the criteria of the Hosmer-Lemeshow goodness of fit test.

Table 2. General descriptors of farm type and farm size

Farm type	Number of farms	% of participating farms	Median farm size (range)
Conventional (Barn egg)	79	52.3	33,696 (1,000-239,000)
Free range	34	22.8	24,410 (900-117,000)
Enriched cages	8	5.3	97,693 (648-180,000)
Enriched colony system	6	3.9	182,600 (66,000-383,000)
Organic	22	14.5	11850 (500-32,800)
Missing information	2	1.3	n.a.
Total	151	100	28,750 (500-383,000)

Table 3. Variables from the univariable analysis with p ≤ 0.2 (ranked by p-value)

Variable	No. of infected farms n=71* (%)	No. of non-infected farms n=80 (%)	Odds Ratio (CI 95%)	p-value (Chi square)
Age of hens[#]				< 0.01
till 40 weeks	*15/70 (21.4)*	*32/77 (41.6)*	*Ref*	*Ref*
40-60 weeks	*28/70 (40.0)*	*13/77 (16.9)*	*4.6 (1.87-11.29)*	*< 0.01*
older than 60 weeks	*27/70 (38.6)*	*32/77 (41.6)*	*1.80 (0.81-4.00)*	*0.15*
Use of bedding material	64/69 (92.8)	60/80 (75.0)	4.27 (1.51-12.09)	<0.01
Horses present	26/71 (36.6)	14/80 (17.5)	2.72 (1.28-5.78)	<0.01
Manure disposal[#]				0.08
once or less than once a week	*6/70 (8.6)*	*18/77 (23.4)*	*Ref*	*Ref*
once every two weeks	*21/70 (29.6)*	*14/77 (18.3)*	*4.50 (1.43-14.14)*	*0.10*
once a month	*17/70 (23.9)*	*18/77 (23.4)*	*2.83 (0.91-8.83)*	*0.07*
less than once a month	*26/70 (38.0)*	*27/77 (35.1)*	*8.89 (0.99-8.4)*	*0.05*
Use of shower before entrance (visitors)	6/71 (8.5)	14/80 (17.5)	0.44 (0.16-1.16)	0.10
Other birds present[i]	5/71 (7.0)	1/80 (1.3)	5.99 (0.68-52.5)	0.10
Vaccination against *Pasteurella multocida*[i]	8/71 (11.3)	3/80 (3.8)	3.26 (0.83-12.80)	0.12
Vacancy period[ii]			/	0.14
Vaccination against Egg Drop Syndrome	41/71 (57.7)	37/80 (46.3)	1.59 (0.83-3.02)	0.16
Free range sampled house	39/69 (42.0)	25/80 (31.3)	1.60 (0.81-3.12)	0.17

**Due to missing values, the number of farms per variable can differ, [#]p-value was calculated with logistic regression, [i]Fisher exact p-value was used (cells with counts n<5), [ii]continuous variable*

Table 4. Results of multivariable analysis (ranked by p-value)

Variable	Odds Ratio (CI 95%)	p-value
Age of hens		< 0.01
till 40 weeks	*ref*	*ref*
40-60 weeks	*5.41 (2. 02-14.53)*	*< 0.01*
older than 60 weeks	*2.28 (0.94-5.53)*	*0.07*
Use of bedding material	4.22 (1.40-12.75)	0.01
Horses present	2.67 (1.16-6.12)	0.02

For the multivariable analysis 139 farms were selected, 12 had missing values for one or more of the selected variables.

No associations between the presence of *C. gallinacea* DNA and clinical signs, varying from respiratory symptoms and nasal and ocular discharges to diarrhoea, were found. The number of farms reporting clinical signs was low (n=11). Also no association was found between the mortality rate the day before the visit and the presence of *C. gallinacea*. A total of 83 farms reported a mortality rate per day of < 0.01%, 58 a mortality rate between 0.01% and 0.05%, and 7 farms a mortality rate > 0.05% (3 farms did not report the mortality rate the day before the visit).

The 69 human throat swabs all tested negative in the *Chlamydiaceae* PCR. A total of 26 human samples were collected from farmers, family members or workers from 17 *C. gallinacea* DNA positive farms and 42 samples from 24 *C. gallinacea* DNA negative farms. One human sample could not be related to a sampled farm.

In summary, *C. gallinacea* DNA is highly prevalent on Dutch layer farms (farm prevalence 47%, 95% CI 39-55%), while neither *C. psittaci* DNA nor *C. avium* DNA were detected in any of the samples from the 151 farms. In the multivariable model, the presence of *C. gallinacea* appears to be associated with the ' age of hens', 'presence of horses' and 'use of bedding material'. No association was found with clinical signs or mortality rate the day before the visit. All of the 69 human throat swabs collected from farmers, family members or workers tested negative for *Chlamydiaceae* DNA.

Discussion

Our cross-sectional study shows that *C. gallinacea* DNA is present on 47% (95% CI 39-55%) of layer farms in the Netherlands. The high prevalence of *C. gallinacea* DNA is in agreement with publications that postulate *C. gallinacea* to be the most important *Chlamydia* spp in chickens (11, 17). In 2012, Zocevic et al. detected mainly DNA of atypical *Chlamydias* (later

redefined as *C. gallinacea*) in 95 of 283 samples from different poultry flocks from France, Greece, Slovenia, Croatia and China (9). Guo et al. detected *C. gallinacea* DNA in about 20% (359/1791) of oral and cloacal swabs of chickens from different provinces in China (11). Hulin et al. reported a predominance of *C. gallinacea* in a poultry slaughterhouse where mainly chickens were slaughtered; in 52 / 129 flocks one or more samples were PCR positive for *C. gallinacea*.

C. psittaci and *C. avium* DNA were not detected at any of the 151 farms (95% CI 0- 2%). These results are in line with the findings of Guo et al., where 41 of 1791 (2.3%) chicken samples were PCR positive for *C. psittaci* (11), and with the study of Hulin et al. where only one of the 129 flocks (bird species not specified) from the chicken slaughterhouse was PCR positive (17). In contrast, Lagae et al. PCR detected and cultured *C. psittaci* from individual pharynx swabs from 7/7 broiler, 5/5 layer and 6/7 broiler breeder farms in Belgium. Differences in sampling methods might play a role. It has been shown that pharyngeal swabs are a more sensitive sampling method than cloacal swabs or faecal samples for the detection of *C. psittaci* (18). Culturing, however has proven to be a less sensitive detection method than PCR, so this might not fully explain the large difference in prevalence (19). The prevalence of *C. psittaci* might differ between countries. The absence of *C. avium* was expected. So far, this bacterium has only been found in psittacines and pigeons and not in poultry (7).

In the risk factor analysis ' age of hens', 'use of bedding material' and 'presence of horses' were associated with the presence of *C. gallinacea*. The age related risk for the presence of *C. gallinacea* peaks between 40 and 60 weeks (OR 5.41, $p < 0.01$). Factors that might influence this risk are the moment of introduction, the duration of *C. gallinacea* infections and the acquisition of immunity. However, this information is currently not available for *C. gallinacea* infections. Studies with a more longitudinal approach are therefore needed. The association with the 'use of bedding material' might be explained by the introduction of the bacterium via bedding material or the effect of this material on the persistence of the bacterium in the environment. It has been reported that the elementary bodies of other *Chlamydiaceae* can survive in litter for several months (20). There is no obvious explanation for the association with the 'presence of horses'. Several *Chlamydia* species have been detected in horses, but the presence of *C. gallinacea* has not been described (21). However, *C. gallinacea* has been detected in vaginal swabs from cattle in China suggesting it might not be restricted to poultry (22). There might be other associated factors as well, such as frequent movement of trailers, which explains the association with horses. More detailed studies are needed to confirm the relation between the risk factors in the final model and the presence of *C. gallinacea* DNA.

We did not observe an association between the presence of *C. gallinacea* and 'clinical signs', based on the results of the questionnaire. It should be noted that only 11 farms reported overt clinical problems, which varied from respiratory symptoms and nasal and ocular discharges to diarrhoea. Also no association was found with the mortality rate the day before the visit. An association with increased mortality cannot be excluded, because the mortality rate might have increased earlier in the infection and subsequently returned to a normal level. To study this we should have analysed for a period longer than 1 day before the visit. Furthermore, a possible clinical outcome of a *C. gallinacea* infection could be more subtle or subclinical. For example Guo et al. did not report any clinical signs, but did find a reduction in growth of broiler chicks (11). Reinhold et al. discussed the role of *Chlamydiaceae* in cattle and suggested subclinical and chronic chlamydial infections might be economically more important than a clinical outbreak (20). Further studies should also take into account subclinical or more economically important parameters, such as egg production during the entire production round.

All human samples collected, tested negative for *Chlamydiacea* DNA. Participants were not selected for clinical signs and 26 were working or living at a *C. gallinacea* positive farm. A positive sample would have given an indication of possible bird-to-human (or human-to-bird) transmission. To date *C. gallinacea* has only been suggested as a cause of human pneumonia (8), but in our study we could not confirm this. Sputum or bronchoalveolar lavage fluid (BAL) from patients with community acquired pneumonia (CAP) should be examined to further investigate whether *C. gallinacea* could be a cause of human pneumonia.

Our study adds to the hypothesis that *C. gallinacea* is the endemic *Chlamydia* of chickens. However, many questions still need to be answered. The most important of these is to elucidate the zoonotic potential of C. *gallinacea* and to investigate the pathogenesis of a *C. gallinacea* infection, as these could be of economic significance for the poultry sector.

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Supporting information

S1 File. Background data risk factor analysis. This file holds data on the variables used in the univariable and multivariable analysis and can be found at: https://doi.org/10.1371/journal.pone.0190774.s001

S2 File. Variable coding information. This file holds information on the coding of the variables and can be found at: https://doi.org/10.1371/journal.pone.0190774.s002

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CHAPTER 3

Genetic and phenotypic analysis of the pathogenic potential of two novel *Chlamydia gallinacea* strains compared to *Chlamydia psittaci*

Marloes Heijne[1*], Martina Jelocnik[2], Alexander Umanets[1], Michael S.M. Brouwer[1], Annemieke Dinkla[1], Frank Harders[3], Lucien J.M. van Keulen[3], Hendrik Jan Roest[1,#], Famke Schaafsma[1,#], Francisca C. Velkers[4], Jeanet A. van der Goot[5], Yvonne Pannekoek[6] and Ad P. Koets[1,4]

[1] Department of Bacteriology and Epidemiology, Wageningen Bioveterinary Research, Lelystad, the Netherlands
[2] Genecology Research Centre, University of the Sunshine Coast, Sippy Downs, Australia
[3] Department of Infection Biology, Wageningen Bioveterinary Research, Lelystad, the Netherlands
[4] Department of Population Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands
[5] Department of Diagnostics and Crisis Organisation, Wageningen Bioveterinary Research, Lelystad, the Netherlands
[6] Department of Medical Microbiology, Amsterdam UMC, University of Amsterdam, the Netherlands
[#] Current affiliation: Hendrik Jan Roest, Directorate Animal Supply Chain and Animal Welfare, Ministry of Agriculture, Nature and Food Quality, The Hague, the Netherlands
[#] Current affiliation: Famke Schaafsma, Department of Healthy Living, TNO, Zeist, the Netherlands

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Abstract

Chlamydia gallinacea is an obligate intracellular bacterium that has recently been added to the family of *Chlamydiaceae*. *C. gallinacea* is genetically diverse, widespread in poultry and a suspected cause of pneumonia in slaughterhouse workers. In poultry, *C. gallinacea* infections appear asymptomatic, but studies about the pathogenic potential are limited. In this study two novel sequence types of *C. gallinacea* were isolated from apparently healthy chickens. Both isolates (NL_G47 and NL_F725) were closely related to each other and have at least 99.5% DNA sequence identity to *C. gallinacea* Type strain 08-1274/3. To gain further insight into the pathogenic potential, infection experiments in embryonated chicken eggs and comparative genomics with *Chlamydia psittaci* were performed. *C. psittaci* is a ubiquitous zoonotic pathogen of birds and mammals, and infection in poultry can result in severe systemic illness. In experiments with embryonated chicken eggs, *C. gallinacea* induced mortality was observed, potentially strain dependent, but lower compared to *C. psittaci* induced mortality. Comparative analyses confirmed all currently available *C. gallinacea* genomes possess the hallmark genes coding for known and potential virulence factors as found in *C. psittaci* albeit to a reduced number of orthologues or paralogs. The presence of potential virulence factors and the observed mortality in embryonated eggs indicates *C. gallinacea* should rather be considered as an opportunistic pathogen than an innocuous commensal.

Introduction

Chlamydiaceae are a family of obligate intracellular bacteria containing one genus and 14 species, and comprising human and animal pathogens. In birds, infections are caused by *Chlamydia psittaci* or more recently recognized species such as *C. gallinacea* (1). *C. psittaci* is zoonotic and has been reported worldwide in more than 465 bird species belonging to at least 30 orders (2). Most human infections have been linked to contact with birds or their environments (3). *C. gallinacea* is mainly detected in poultry with reports from almost all continents (4-6). *C. gallinacea* has incidentally been found in wild birds and cattle as a possible result of infection spill-over (7, 8). Possible zoonotic transmission of *C. gallinacea* has been considered but could neither be confirmed nor ruled out in slaughterhouse workers that developed pneumonia after they were exposed to *C. gallinacea* infected poultry (9).

Infections with *C. psittaci* in birds are often asymptomatic, but can result in localized syndromes (e.g., conjunctivitis) or severe systemic illness. Chlamydial strain, avian host, host age and (environmental) stressors are important factors in the occurrence and severity of clinical signs (3). Studies investigating the pathogenesis of *C. gallinacea* in birds are currently limited. As yet, clinical signs of disease in *C. gallinacea* infections have not been reported in observational field studies (4, 9, 10). Under experimental conditions it has been demonstrated that infection in broilers results in reduced weight gain (4). In a transmission study, *C. gallinacea* was mainly present in rectal and cloacal samples without clinical signs of disease and transmission occurred via the faecal-oral route (11). Thereby, at present *C. gallinacea* is considered a rather non-pathogenic species.

Molecular studies using outer membrane protein A (*ompA*) genotyping or Multi Locus Sequence Typing (MLST) showed *C. gallinacea* is diverse, with at least 13 different *ompA* types and 15 different sequence types (ST) in 25 strains(4, 12). Fine detail comparative genomics revealed that the *C. gallinacea* genome is conserved, syntenic and compact, but possesses the hallmark of chlamydial specific virulence factors: inclusion membrane (Inc) proteins, polymorphic membrane proteins (Pmps), a Type III Secretion System (T3SS), a plasticity zone with a cytotoxin (*tox*) gene, and the chlamydial virulence plasmid (12, 13). Whether this genetic diversity and the presence of chlamydial virulence genes contributes to the pathogenicity of *C. gallinacea* remains a question, as clinical disease in infected chickens has not been reported in the limited number of field and experimental studies.

The aim of this study was to investigate the pathogenicity of two novel *C. gallinacea* strains by comparing them to a virulent *C. psittaci* strain using an *in vivo* infection model in embryonated chicken eggs and performing comparative genomics with inter- and intra-species genomes. In the eggs, *C. gallinacea* induced mortality was observed, but to a lower extent than *C. psittaci* induced mortality. Comparative genomics showed that both

novel *C. gallinacea* isolates possess the hallmark genes coding for known and potential virulence factors as found in *C. psittaci*, albeit to a reduced number of orthologs or alleles. The current results indicate *C. gallinacea* should be considered as an opportunistic pathogen rather than an innocuous commensal.

Methods

Ethical statement and biosafety

The cloacal and caecal sampling of the chickens was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee (permit number AVD108002016642) of Utrecht University (the Netherlands). All procedures were conducted in accordance with national regulations on animal experimentation and in compliance with the ARRIVE guidelines (14) where applicable. No ethical approval is required for work with embryonated chicken eggs until day 18 according to Dutch Law.

All culture work with *C. gallinacea* was performed under biosafety level 2 and all culture work with *C. psittaci* under biosafety level 3.

Sample collection, inoculum preparation and isolation of *Chlamydia*

Sample collection and inoculum preparation

Layer flocks at the Faculty of Veterinary Medicine in Utrecht, the Netherlands were monitored for the presence of *C. gallinacea* with boot sock sampling. The flocks were obtained from commercial laying hen rearing farms at 18-weeks of age and had an average size of 50 hens that were distributed evenly over two pens. Background data on the flock are supplied in Supplementary Fig.. S1 and Supplementary Data S1. From each pen, environmental boot sock samples (Poultry Boot Swabs, BioTrading) were collected monthly. After collection, the boot socks were suspended in 100 ml Dulbecco's Phosphate Buffered Saline (DPBS, Gibco, Life Technologies Limited). The suspension was centrifuged 15 minutes at 500 x *g* and 500 µl of the supernatant was used for DNA isolation. When the boot socks were PCR positive for *Chlamydia*, individual cloacal swabs and caeca were collected. Cloacal swabs were stored in one millilitre Sucrose Phosphate Glutamate (SPG) and caeca in ten percent weight per volume (w/v) according to standard protocols(15, 16). SPG contains sucrose (75 g/litre), KH_2PO_4 (0.52 g/litre), K_2HPO_4 (1.25 g/litre) and L-glutamic acid (0.92 g/litre). Before use, fetal bovine serum (0.1 ml/ml), amphotericin B (4 µg/ml), gentamicin (40 µg/ml and vancomycin (25 µg/ml) were added. Samples were stored at -80 °C.

To prepare the inoculum for the eggs, swabs were thawed at room temperature for approximately one hour. Swabs were centrifuged for ten minutes at 500 x *g* and 200 µl of the supernatant was used for inoculation. Caeca were prepared following two methods.

For the isolation of NL_G47 the caecum was cut lengthways in parts of approximately two cm. Subsequently the parts were washed in SPG and the epithelium was removed by scraping with a scalpel. The scrapings of epithelium were washed in two ml of SPG and the suspension was filtered over a 0.8 µm filter (Acrodisc Syringe Filter, Pall Life Sciences). After one hr of incubation at room temperature the suspension was used for inoculation. For the isolation of NL_F725, caeca were homogenized in a 10% w/v suspension in an ULTRA-TURRAX tube (BMT-20-S, IKA) on an ULTRA-TURRAX Tube Drive (IKA) at 6000 RPM for 90 seconds and switching direction every 30s. The suspension was centrifuged at 500 x *g* for 15 min and the supernatant was used for culturing as described below.

Inoculation of embryonated SPF chicken eggs

Specific-pathogen-free (SPF) embryonated chicken eggs were delivered after five days of incubation, candled to check viability and incubated overnight at 37.5 – 38 °C and 65% relative humidity in small egg incubators (Octagon 20 Advance, Brinsea). Inoculation was performed at day six of incubation (one day after delivery).

Before inoculation, the eggs were candled, and the air chamber was marked with a pencil. The eggs were cleaned with a wipe drenched in 70% ethanol. In the middle of the area of the marked air chamber, a hole was drilled with a 0.8 mm engraving bit (26150105JA, Dremel). Subsequently, the eggs were moved to a flow cabinet and sprayed with 70 percent ethanol. Per egg, 200 µl was inoculated in the yolk sac with a one millilitre syringe and a 22G x 40mm needle. The full needle was inserted perpendicularly into the drilled hole.

Per clinical sample, four eggs were inoculated. As a negative control, two eggs were inoculated with DPBS (Gibco, Life Technologies Limited) and, as a positive control, two eggs were inoculated with *C. gallinacea* strain 08DC65. Strain 08DC65 was obtained from the Friedrich Loeffler Institute in Jena, Germany.

After inoculation eggs were wiped with 70% ethanol and the hole was closed with a droplet of nail polish. The eggs were placed in the egg incubators and incubated until day 16 or until mortality. At day 16, eggs were chilled overnight at 4°C to euthanise the embryo non-invasively.

Candling of embryonated SPF chicken eggs

Mortality was monitored by daily candling. With candling, the appearance of vessels and movement of the embryo was monitored (17). The result of candling was graded:

- no abnormalities observed: vessels are visible, movement of the embryo
- abnormalities observed: congestion or bleeding from vessels, decreased movement of the embryo
- mortality: no or less vessels visible and no movement of the embryo

When abnormalities were observed an extra candling was performed on the same day. After mortality or an increase in the severity of the abnormalities, eggs were chilled overnight at 4 °C until harvesting.

Harvesting of embryonated SPF chicken eggs
Mortality within three days after inoculation (day nine of incubation) was considered as acute mortality inconsistent with a *Chlamydia* infection (18). These eggs were disinfected with 70% ethanol, opened at the air sac side and checked for any visual deformations. Furthermore, a sheep blood agar plate was inoculated with a loopful from the yolk sac and incubated overnight at 37 °C to check for bacterial contamination.

Eggs were harvested for the isolation of *C. gallinacea* when mortality occurred from day nine of incubation or when no mortality was observed at day 16 of incubation. At harvesting the part of the egg shell covering the air sac was removed, and subsequently the egg shell membrane and the allantois membrane were opened with disposable tweezers. The allantoic fluid was removed with a pipette, the egg was then emptied in a Petri dish to harvest the yolk sac membrane. The yolk sac membrane was weighed and transferred to an ULTRA-TURRAX tube (BMT-20-S, IKA). Depending on the volume of the yolk sac and the size of the tube, SPG buffer was added and the yolk sac membrane was homogenized on an ULTRA-TURRAX Tube Drive (IKA) for 90 s (switching between forward and reverse every 30 s) at 6000 RPM. The suspension was transferred to 50 ml Falcon tubes and SPG buffer was added until a 20 % w/v suspension.

The yolk sac membranes from eggs inoculated with the same sample and harvested at the same day, were pooled to create one homogenous batch of an isolate. A 10 µl droplet of the yolk sac suspension was spotted in duplo on glass slides and air dried. The glass slides were tested with the IMAGEN *Chlamydia* test kit (immunofluorescence test, IFT) according to manufacturer's instructions (Thermo Scientific). Two hundred µl of the suspension was used for PCR testing.

Isolation in cell culture
Isolation and propagation in cell culture was performed as described earlier (19). Briefly, Buffalo Green Monkey (BGM) cells were seeded with Dulbecco 's Modified Eagle Medium (DMEM, Gibco, Life Technologies Limited) and 10% serum in 24-well plates (Greiner Bio-One GmbH, Germany). The plates were incubated at 37 °C with 5% CO_2 in a humidified incubator until 80% confluency of the monolayer. After inoculation, the plates were centrifuged at 2450 × *g* and 37 °C for 60 min and subsequently incubated for two hours. The medium was then replaced with UltraMDCK serum-free medium (Lonza). At day one and day four, 200 µl of the supernatant was collected for PCR to monitor replication. Plates were harvested at day four for DNA isolation, further passaging or storage at -80 ˚C.

Titration experiments in embryonated SPF chicken eggs

The isolated *C. gallinacea* strains NL_G47 and NL_F725, and *C. psittaci* strain NL_Borg were tested in titration experiments. Strain NL_Borg was selected because it is genetically closely related to strain FalTex and NJ1, which are both isolated from outbreaks in poultry (turkeys) (20).

To standardise the inocula before the start of the titration experiments, all three strains were passaged three times in embryonated eggs under similar conditions. The third passage yolk sac membrane suspensions were used to prepare tenfold serial dilutions in DPBS (Gibco, Life Technologies Limited) for inoculation of the yolk sac of six-day incubated chicken eggs. The eggs were incubated at 37 °C and 65% relative humidity in egg incubators (Octagon 20 Advance, Brinsea). After mortality or six days after inoculation the eggs were chilled overnight at 4 °C and harvested as described earlier.

In a first experiment the range for the dilution series was defined by inoculating a limited number of eggs per dilution. In a subsequent experiment the range was limited to four dilution steps. Per dilution step, four or five eggs were inoculated with 200 µl suspension. Two or more eggs were inoculated with sterile DPBS (Gibco, Life Technologies Limited) as a negative control and, as a positive control, two eggs were inoculated with a lower dilution than the range that was used in the experiment.

After each titration experiment the 50% egg infective dose (EID_{50}) and, when possible, the 50% egg lethal dose (LD_{50}) per ml inoculum was calculated according the Spearman-Karber method (21, 22). The difference in EID_{50} between strains was assessed using the Wilcoxon-Mann-Whitney test.

Histology and immunohistochemistry

From infected and non-infected eggs, the chorioallantoic membrane, yolk sac and embryo were harvested for histology and immunohistochemistry. After fixation in 10% neutral buffered formalin, tissues were routinely processed into paraffin blocks. Four µm sections were cut and collected on coated glass slides. Sections were stained with haematoxylin-eosin (HE) or immuno-stained with a polyclonal anti-*Chlamydia* antibody (LS-C85741) and a monoclonal anti-*Chlamydia* antibody (MBS830551).

For the polyclonal antibody the antigen was retrieved by proteolysis-induced epitope retrieval (0.1% Trypsin in TBS for 30 min at 37 °C). For the monoclonal antibody heat-induced epitope retrieval was used (citrate buffer, pH 6.0, 21°C for five min). The primary antibody (dilution 1:100) was incubated for 60 min. HRP EnVision anti-Mouse or HRP Envision anti-Rabbit (Dakopatts) were used as a secondary antibody for 30 min, depending

on the nature of the first antibody. Subsequently, sections were incubated for five min in DAB+ substrate (Dakopatts) and then counterstained with Mayer's haematoxylin.

DNA extraction, PCR and genome sequencing

Five hundred µl of the sample suspensions, washing suspension, yolk sac suspension or cell culture supernatant 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). Samples were tested with a *Chlamydiaceae* PCR targeting the 23S rRNA and *C. gallinacea* PCR targeting the *enoA* gene or *C. psittaci* PCR targeting the *ompA* gene as described earlier (10, 23).

For genome sequencing, twenty-four-well cell culture plates were freeze-thawed twice and the cells were subsequently harvested for DNA extraction as described earlier (19). DNA was isolated according to the DNeasy Blood and Tissue kit (Qiagen GmbH, Germany).

The DNA samples were prepared for Illumina sequencing using the SMARTer ThruPLEX DNA-Seq kit (Takara Bio, USA) according to manufacturer protocol. Quality control of the library preparation was performed on a Tapestation 2200 (Agilent Technologies, Germany) and the DNA concentration was determined on a Clariostar (BMG Labtech, the Netherlands) with use of the Quant-IT PicoGreen dsDNA kit (Invitrogen Ltd, UK). Sequencing was performed on an Illumina MiSeq platform. The complete genome and plasmid sequences were assembled using SKESA 2.4.0 (24). Contigs containing sequences of BGM cells were removed prior to subsequent analysis.

Assembled contigs (from Illumina short reads) were annotated using the PGAP pipeline using *C. gallinacea* Type Strain 08-1274/3 (accession number NZ_CP015840.1) as the reference genome for the newly isolated *C. gallinacea* strains and *C. psittaci* NJ1 (accession number CP003798.1) for *C. psittaci* NL_Borg (25). All data are available in the NCBI database under BioProject number PRJNA687129 (including reads available under SRR15184193; SRR15184194 and SRR15212117) and the publicly available Bacterial Isolate Genome Sequence Database (BIGSdb) ((http://pubmlst.org/chlamydiales) (*C. gallinacea* isolates NL_G47 (id: 4548) and NL_725 (id: 4560) and *C. psittaci* NL_Borg (id: 4561)).

Molecular typing and phylogenetic analysis

Sequence types for our strains were determined using contigs deposited and queried against the *Chlamydiales* PubMLST database (http://pubmlst.org/chlamydiales). Phylogenetic trees were generated by exporting gene sequences from the *Chlamydiales* database (http://pubmlst.org/chlamydiales) as an XMFA file containing each locus as an aligned block. The XMFA file was converted to an aligned concatenated sequence

for Neighbor-Joining tree analysis using the Maximum Composite Likelihood model in MEGA7(26). Bootstrap tests were for 1000 repetitions (27-29).

For rMLST, complete sequences (~22.000 bp) of 52 genes encoding ribosomal proteins (*rps*) were analysed (30). The *rps* gene *rpmD*, encoding the 50S ribosomal protein L30 is absent in genomes of *Chlamydia* isolates analysed so far. For MLST, sequences of fragments (400 – 500 base pairs) from seven housekeeping genes (*enoA*, *fumC*, *gatA*, *gidA*, *hemN*, *hlfX*, *oppA*) were analysed (31). Isolates used for rMLST and MLST including provenance and allelic profile data are listed in Supplementary Data S6.

Comparative genome analyses

Average nucleotide identity (ANI) determination for the newly sequenced *C. gallinacea* genomes was performed using the ANI calculator available at enve-omics.ce.gatech.edu/ani/ (32), whilst the genome completeness based on the percent of bases aligned to the reference genome and quality of the assemblies was estimated using Quast (32-34). SNPs in contigs assembled from Illumina reads, were identified using Snippy v4.6.0(35).

C. gallinacea pairwise genome comparisons were performed using the Geneious Prime 2020.2 platform (https://www.geneious.com). Our strains were compared against *C. gallinacea* strain 08-1274/3 (accession number NZ_CP015840.1) and JX-1 (accession number CP019792). The genomic regions of interest and/or polymorphic loci were extracted from the analysed genomes and aligned with MAFFT and/or Clustal Omega (as implemented in Geneious Prime) for further nucleotide and/or translated protein sequence analyses performed using DNASp 6.0 (36). The total number of polymorphisms (and gaps), % nucleotide and amino acid sequence identity, number of haplotypes and haplotype diversity (Hd), and ratios of the rates of non-synonymous to synonymous nucleotide substitutions per site (dn/ds) averaged over the entire gene alignment were calculated.

As the Type 3 Secretion System (T3SS) play a key role in the interaction of *Chlamydia* and hosts, EffectiveDB (http://effectivedb.org) was used to predict the T3S secreted proteins of *C. gallinacea*. For prediction the standard Effective T3 classification module 2.0.1 was used with a cut-off score of 0.9999 (37). Similarly, to predict transmembrane *C. gallinacea* proteins, and identify inclusion membrane proteins characterised by bilobed hydrophobic domains, TMHMM 2.0 server (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) was used (38).

The visualisation of nucleotide BLAST comparisons of our newly sequenced draft *C. gallinacea* genomes to published *C. gallinacea* genomes 08-1274/3 and JX-1, and/or *C. psittaci* NJ1 (accession number CP003798.1) was performed with BLAST Ring Image

Generator (BRIG)(39). Visualisation of the BLAST comparison, sequence identity and genomic structure of the plasticity zone for *C. gallinacea* and those from other related species, was performed using EasyFig, with the -tblastx option with a minimum E-value of $1x10^{-3}$ used as BLAST parameters for EasyFig (40).

For the identification of orthologous genes in *C. gallinacea* and *C. psittaci*, an all-vs-all comparison of the translated coding sequences (CDSs) was performed using global sequence alignment of each CDS. Translated CDSs were aligned using DIAMOND v0.9.14 and the best hit for each query was selected (41). Only hits with an expect (E) value less than $1x10^{-3}$ were included. CDS with no hits or hits with an E-value above the threshold were further investigated and the annotation artefacts were removed. The remaining CDS were assigned unique. In addition, all CDS were investigated using both nucleotide and translated amino acid sequence blast analyses. Results of the alignment were structured and visualized using the *tidyverse* package and R v3.6.1 (42, 43).

Results

Isolation and pathology of *C. gallinacea* NL_G47 and NL_F725 in embryonated chicken eggs

Layer flocks at the Faculty of Veterinary Medicine in Utrecht, the Netherlands were monitored for the presence of *C. gallinacea* to isolate Dutch field strains. In these flocks, *C. gallinacea* strain NL_G47 could be isolated from a caecal scraping sample collected in January 2018 from a 40-week old clinically healthy layer hen. *C. gallinacea* strain NL_F725 could be isolated from a caecal suspension sample collected in August 2017 from a 34-week old layer hen. Both hens originated from different flocks, but were housed at the same location at different time points. About one month before the *C. gallinacea* positive caecal samples were collected, both flocks tested PCR positive for *C. gallinacea* in environmental boot sock samples as shown in the timeline of Supplementary Fig. S1. *C. gallinacea* positivity in the flock from strain NL_F725 preceded a coinciding Infectious Laryngotracheitis (ILT) infection. To prevent further spread of ILT the flock had to be culled. Background data of the flocks are added to Supplementary Data S1.

C. gallinacea NL_G47 and NL_F725 were isolated in the yolk sac of embryonated specific-pathogen-free (SPF) chicken eggs and replication was confirmed with positive immunofluorescence of the yolk sac membrane (see Supplementary Fig. S2) and a positive *Chlamydiaceae* PCR targeting the 23S rRNA gene. With the isolation of NL_G47 in the yolk sac of embryonated eggs, mortality was observed at day 10 after inoculation (incubation day 16) and at day 6 (incubation day 12) in the second passage. At primary isolation of NL_F725 no mortality of the embryos was observed, but eggs were harvested before day

10 after inoculation (day 8 after inoculation, incubation day 14) for logistical reasons. With the second passage of NL_F725, mortality of the embryos was observed at day 6 or day 7 after inoculation (incubation day 12 or 13). Based on egg candling, congestion of the blood vessels was observed prior to mortality of the embryos. At harvest the embryos were deep red (rubor), showed cyanotic toes and haemorrhaging of the skin (Supplementary Fig. S2).

To investigate any histological lesions NL_G47 infected eggs were harvested at day 10 of incubation when anomalies of the vessels were observed with candling. Granular basophilic intracellular inclusions were seen in the epithelial cells of both the chorioallantoic membrane and the yolk sac membrane (Figs 1A and C). These intracellular inclusions were strongly positive for chlamydial antigen labelling (Fig. 1B, D).

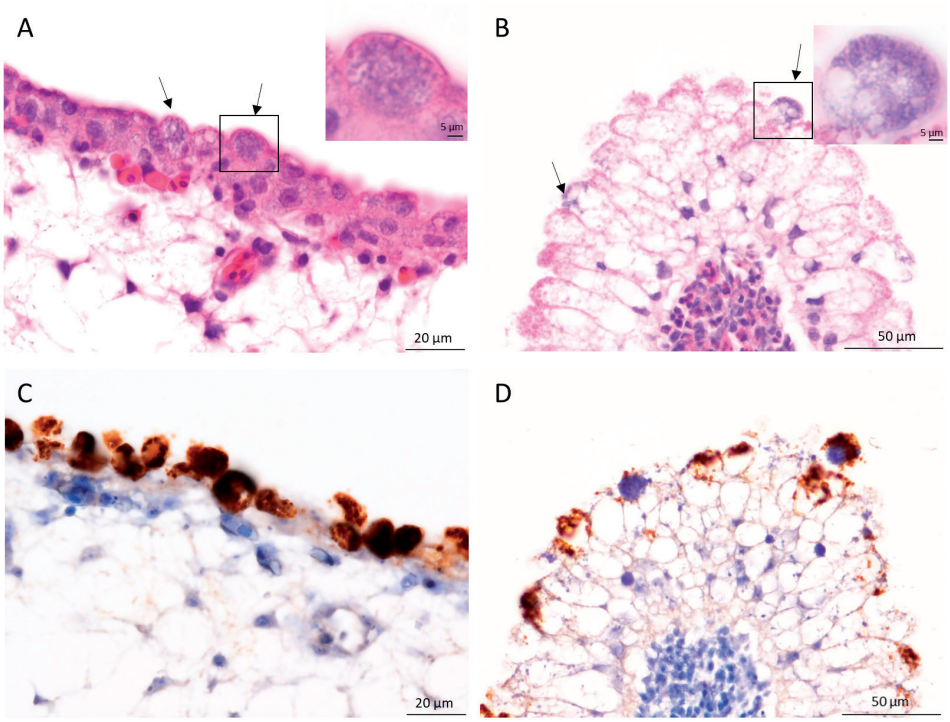

Fig. 1. Chorioallantoic membrane and yolk sac membrane of 10 days embryonated eggs infected with NL_G47

Intracellular inclusions (arrows) in the epithelial cells of the chorioallantoic membrane **(A)** *and yolk sac membrane* **(B)**. *Inset: higher magnification showing the granular basophilic inclusions in the HE staining. Positive immunolabelling of the intracellular inclusions for chlamydial antigen in the chorioallantoic membrane* **(C)** *and yolk sac membrane* **(D)**. *Sections were photographed with an Olympus BX51 microscope equipped with a high-resolution digital camera and using Olympus' cellSens software.*

Primary isolation and propagation of *C. gallinacea* NL_G47 and NL_F725 in Buffalo Green Monkey (BGM) cells initially failed, but after three passages in eggs the strains could be propagated in BGM cells.

Assessment of virulence of *C. gallinacea* in embryonated eggs

Titration experiments in embryonated chicken eggs were performed to quantify the infectious dose and gain further insight into the pathogenic potential of the novel isolates compared to *C. psittaci*. Ten-fold serial dilutions of third passage yolk sac cultures of *C. gallinacea* NL_G47 and NL_F725, and *C. psittaci* NL_Borg, were used to calculate the 50% egg infective dose (EID_{50}) based on positivity in the immunofluorescence test (IFT) of the yolk sac membrane (with or without mortality of the eggs). The experiments were repeated seven times for NL_G47 with a median EID_{50} of $10^{5.6}$, two times for NL_F725 with a median EID_{50} of $10^{5.9}$ and three times for NL_Borg with a median EID_{50} $10^{8.2}$. All negative control eggs that were inoculated with Dulbecco's Phosphate Buffered Saline (DPBS), remained viable until harvesting and tested *Chlamydia* negative by the IFT, except in one experiment with NL_G47 where aspecific mortality was observed in two of four eggs within three days after inoculation. As shown in Fig. 2A, the EID_{50} of *C. psittaci* strain NL_Borg was significantly higher than the EID_{50} ($P<0.05$, Wilcoxon-Mann-Whitney test) of *C. gallinacea* NL_G47. The EID_{50} of NL_F725 was in the same range as the EID_{50} of NL_G47, but could not be statistically assessed due the low number of observations.

For *C. psittaci* NL_Borg the 50% lethal dose (LD_{50}) could also be calculated from the experiments with a median LD_{50} of $10^{7.4}$. The LD_{50} of the experiments with *C. psittaci* NL-Borg showed overlap with the calculated EID_{50} (Fig 2A). The LD_{50} from the experiments with *C. gallinacea* NL_G47 and NL_F725 could not be calculated, because the number of eggs in the dilutions with observed mortality was too low to calculate the LD_{50}. To get further insight into differences in mortality and infectivity between *C. gallinacea* and *C. psittaci*, the data from all separate experiments were merged into one dataset (see Supplementary Table S1).

The percentage of eggs that was IFT positive with mortality, IFT positive without mortality and IFT negative is shown per dilution and per *Chlamydia* strain (Figs 2B-D). For *C. gallinacea* strain NL_G47, mortality was observed until the 10^{-2} dilution and IFT positivity until the 10^{-6} dilution (Fig. 2B). For *C. gallinacea* strain NL_F725 no mortality was observed in the dilutions that were tested (from 10^{-2} until 10^{-7}), but IFT positivity was seen until the 10^{-6} dilution similar to *C. gallinacea* NL_G47 (Fig. 2C). For *C. psittaci* strain NL_Borg, mortality was observed until dilution 10^{-7} and IFT positivity until 10^{-8} (Fig. 2D). These results indicate mortality in the *C. psittaci* infected eggs was relatively higher than in the *C. gallinacea* infected eggs and there might be a difference in mortality between *C. gallinacea* strains, although the number of observations was low.

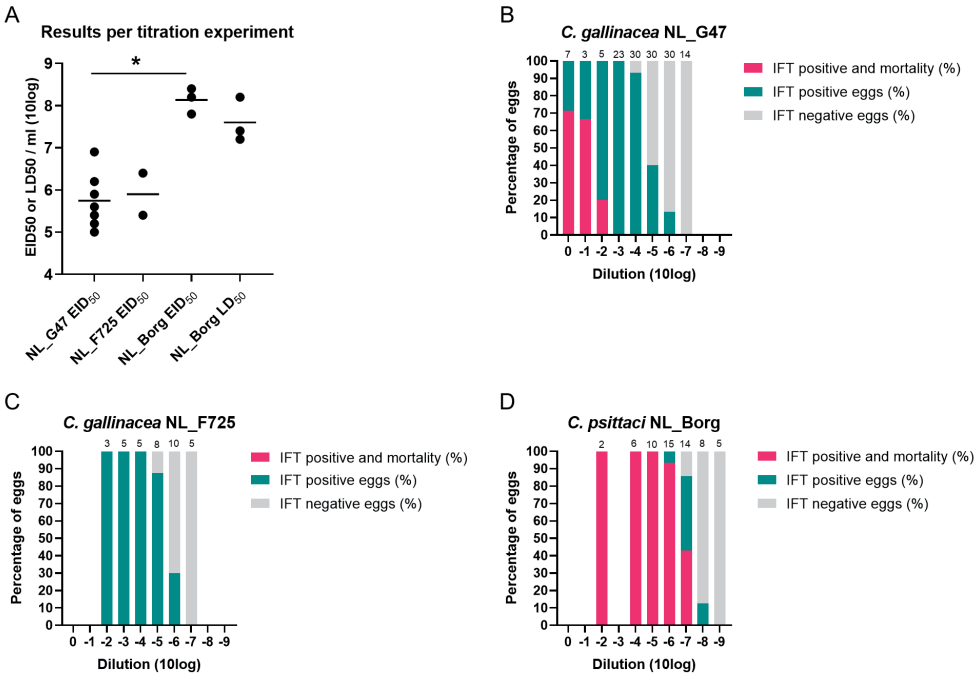

Fig. 2. Assessment of virulence of *C. gallinacea* in embryonated eggs
A *shows the 50% egg infective dose 50 (EID_{50}) of C. gallinacea NL_G47, NL_F725 and C. psittaci NL_Borg based on IFT of the yolk sac. The difference between EID_{50} of NL_G47 and NL_Borg was significantly different (*, P<0.05, Wilcoxon-Mann-Whitney test). For C. psittaci NL_Borg the 50% lethal dose (LD_{50}) was also calculated. The median EID_{50} or LD_{50} of the experiments is indicated with a bar.*
B, C *and* **D** *depict the cumulative results of the separate titration experiments per Chlamydia strain. Per dilution, the percentage of eggs that was positive for Chlamydia in the immunofluorescence test (IFT) with mortality, IFT positive without mortality and IFT negative are shown. The total number of eggs per dilution are presented at the top of every bar. These data are also included in Supplementary Table S1. The figure was created in GraphPad Prism 9.0.0.*

General characteristics of the genome sequences of Dutch *C. gallinacea* isolates

After isolation in eggs and one passage in BGM cells, DNA of both isolates was sequenced to confirm their genetic identity. The genomes of NL_G47 and NL_F725 have a total length of 1,066,007 and 1,064,097 bp, respectively, and include the ~1.059 Mbp chromosome and a 7.5 kbp chlamydial plasmid (Table 1). Ribosomal MLST (rMLST) (30) and phylogenetic analysis of concatenated rRNA genes confirmed that both isolates belong to *C. gallinacea* (Fig. 3A), whilst the MLST showed that both isolates are genetically diverse and assigned to unique sequence types (ST280 and ST284). Phylogenetically, these clustered in distinct clades, with NL_G47 forming a well-supported clade with the French isolate 08-1274/3, whilst NL_F725 clustered in a genetically diverse clade consisting of Chinese *C. gallinacea* strains (Fig. 3B).

Table 1. Genome descriptions of *C. gallinacea* NL_G47, *C. gallinacea* NL_F725 and *C. psittaci* NL_Borg

	***C. gallinacea* NL_G47**	***C. gallinacea* NL_F725**	***C. psittaci* NL_Borg**
Host	Chicken (*Gallus gallus*)	Chicken (*Gallus gallus*)	In-house reference strain
Anatomical site	Caecum	Caecum	Unknown
Clinical presentation	Asymptomatic	Asymptomatic	Unknown
Total No. of Illumina reads	1912918	1762101	3029302
Percent of mapped reads	72.92 %	73.06%	93.46%
No. of *de novo* contigs[a]	12	19	11
N50	114660	96259	254182
Average coverage depth	366X	181X	633X
%GC of *de novo* contigs	37.89%	37.89%	38.92%
Number of bp mapped against reference genome chromosome (% complete compared to reference strains[b])	1058515 bp (99.89%)	1057023 bp (99.75%)	1144332 bp (98.5%)
Number of bp mapped against the reference plasmid	7492 bp	7492 bp	7552 bp
Number of predicted CDS	916	919	989
% Average nucleotide identity[c]	99.63% (SD: 1.09%) to *C. gallinacea* 08_1274/3	99.50% (SD: 1.53% to *C. gallinacea* 08_1274/3	-
	99.42% (SD: 1.43%) to *C. gallinacea* JX-1	99.52% (SD: 1.31%) to *C. gallinacea* JX-1	-
	-	-	99.99% (SD: 0.04%) to *C. psittaci* NJ1
No of SNPs to reference strains[d]	2608 to *C. gallinacea* 08_1274/3	3328 to *C. gallinacea* 08_1274/3	65 to *C. psittaci* NJ1
Plasticity Zone length	15861bp	15845bp	29000bp
Accession numbers	JAEMHG000000000	JAEMHH000000000	-

a de novo chlamydial contigs; b Quast analyses using Short read assemblies where NL_G47 and NL_F725 were compared to 08_1274/3, and NL_Borg with NJ1; c Average nucleotide identity (ANI) determination was performed at enve-omics.ce.gatech.edu/ani/ (Goris et al., 2007) using both best hits (one-way ANI) and reciprocal best hits (two-way ANI) between two genomic datasets with C. gallinacea 08_1274/3, C. gallinaceae JX-1 or C. psittaci NJ1 as the reference genome; d SNPs identified using Snippy v4.6.0.

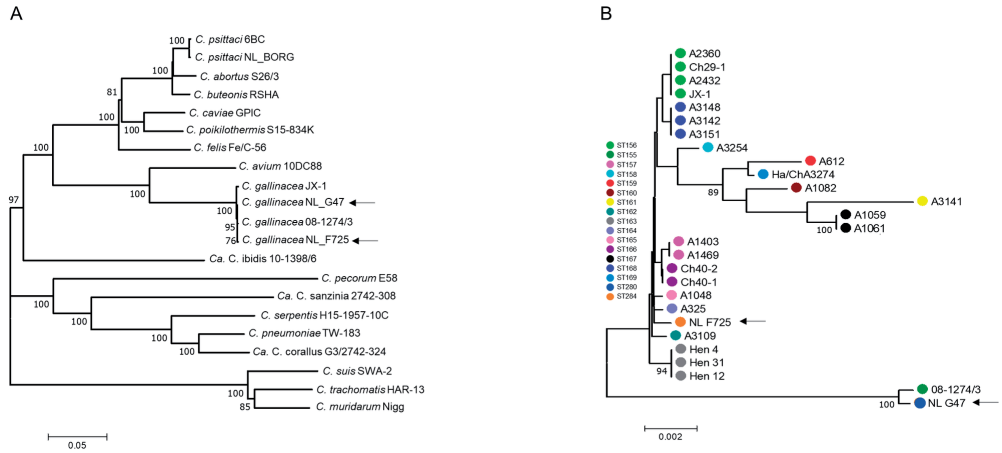

Fig. 3. Phylogenetic analyses of concatenated sequences of Chlamydia.
Concatenated sequences were aligned and analysed in MEGA7(26).
Numbers on tree nodes indicate bootstrap values over 75% of the main branches. Horizontal lines are scale for nucleotide substitutions per site.
***A** Neighbor-Joining tree of concatenated sequences of 52 ribosomal genes (rMLST)(30) of Chlamydia Type strains as well as three Candidatus species (Ca. C. corallus, Ca. C. ibidis and Ca. C. sanzinia), C. psittaci strain NL_Borg and two additional C. gallinacea strains. All C. gallinacea strains (Dutch strains indicated by an arrow) clustered together in a well-supported and distinct clade with Chlamydia avium as the closest relative.*
***B** Neighbor-Joining tree of concatenated sequences of 7 housekeeping genes fragments (MLST)(31) of 27 C. gallinacea strains. Shared Sequence types (ST) in clades are indicated by color and STs are denoted by the color key.*

Comparative genome analysis of *C. gallinacea* and *C. psittaci*

To investigate genomic differences that might be related to the observed differences in the degree of pathology and mortality in eggs, the *C. gallinacea* and *C. psittaci* genomes were analysed and compared. As evaluated by whole genome alignments, *C. gallinacea* genomes NL_G47 and NL_F725 are syntenic with the same gene number and order, sharing at least 99.4 % sequence identity with *C. gallinacea* strain 08-1274/3 (type strain; accession number NZ_CP015840.1) and JX-1 (accession number CP019792). All *C. gallinacea* genomes contain conserved hallmark chlamydial virulence genes coding for Incs, Pmps, T3SS and a Plasticity Zone (PZ) with a gene coding for the large cytotoxin (*toxB*) (Fig. 4A, C, Supplementary Fig. S3). Most sequence variation was found in several distinct chromosomal regions, namely in genes encoding the membrane proteins (e.g. *ompA* and *pmps*), a conserved hypothetical protein, a phage tail protein, heme (*hemE*, and *hemN*) and glycogen (*glgP*) metabolism genes (Supplementary Data S2). The PZ, a region of high genetic variability in chlamydial species, was conserved in number of genes and sequence among the four *C. gallinacea* genomes with 99.3 – 99.8 % nucleotide identity, but varied in

gene content, namely lack of hypothetical protein, MAC/Perforin (MAC/P) and nucleotide metabolism genes, compared to the related avian species (Fig. 4C). Although, the length of the PZ of *C. gallinacea* is reduced compared to *C. psittaci*, it does contain an intact CDS for the cytotoxin (*toxB*), in contrast to the PZ of *C. avium* that lacks this gene. As observed previously, this locus has a premature stop codon in strain JX-1 (Fig. 4C).

The genome sequence of our in-house reference strain *C. psittaci* NL_Borg was almost identical (99.99% sequence identity) to reference strain *C. psittaci* NJ1 (accession number CP003798.1) with only 65 synonymous Single Nucleotide Polymorphisms (SNPs), evenly distributed across the chromosome. In the whole genome alignment, it was observed that the *C. psittaci* genome is 101.85 Kbp longer than the genome of *C. gallinacea* and contains 73 more CDSs (Fig. 4A).

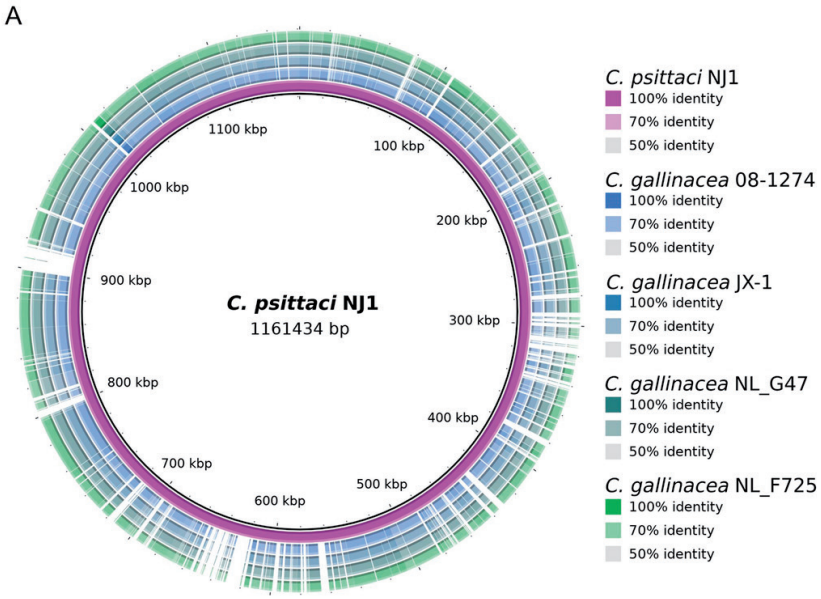

Fig. 4. Genome comparison of *C. gallinacea* and *C. psittaci*.

A *Whole genome BLAST comparison between C. psittaci NJ1 and four C. gallinacea genomes (including Type strain 08-1274/03). The image is created with BLAST Ring Image Generator (BRIG)(39) and the first ring corresponds to the genome that was used for the comparison.*

B *CDS for which no homologue (alignment E score higher than $1x10^{-3}$) could be identified in C. gallinacea 08-1274/03 or C. psittaci NJ1. Every colored block in the figure corresponds to a CDS. The different proteins are categorized and colored according to their function and location. The figure was created using the tidyverse package and R v3.6.1(42, 43)*

C *Graphical representation of the gene content of the PZs of representative Chlamydia species of avian origin including the Dutch C. gallinacea strains. Arrows represent PZ genes colored according to function (see key). Grey shading scale denotes % nucleotide identity. The image was created with Easyfig(40) .*

Given that our newly sequenced genomes are syntenic and almost identical to the comparator reference genomes, but only cover 98.51-99.75% of the reference chromosome lengths (Table 1), genomes of the type strains 08-1274/3 and NJ1 were used as representatives for *C. gallinacea* and *C. psittaci* species, respectively, in a translated coding sequences (CDSs) comparison. With a local alignment approach, all translated CDSs of *C. gallinacea* 08-1274/3 (n=913) and *C. psittaci* NJ1 (n=986) were compared to each other to identify unique and/or highly variable regions (Supplementary Data S3). The plasmids of *C. gallinacea* and *C. psittaci* were not included, because they are syntenic with both eight CDSs encoding the conserved chlamydial plasmid proteins.

As expected in closely related species and analysed by both amino acid and sequence similarity analyses, the majority of CDSs have orthologues in both species. In *C. gallinacea,* for only seven CDSs an orthologue could not be identified in *C. psittaci* (Fig. 4B, Supplementary Data S4). Of those, one belonged to the family of putative Incs, a second had a metabolic function related to chromosome partition and the remaining five were hypothetical proteins with unknown function. Fifty-three CDS were unique to *C. psittaci* relative to *C. gallinacea* (Supplementary Data S4). Ten of these CDSs were located at the PZ coding for proteins such as the Membrane Attack Complex/Perforin domain-containing protein (MAC/PF), proteins involved in purine metabolism (*gua*AB-ADA operon), adherence domain and a putative membrane protein.

Outside the PZ, 18 of the unique CDS of *C. psittaci* were related to previously characterised potential virulence factors (Fig. 4B, Supplementary Data S4). Most of these proteins belonged to the family of putative Inc proteins, membrane proteins and conserved hypothetical proteins. The remaining unique CDS were related to metabolism or to CDS coding for proteins of unknown function. Additional analysis of secretion signals of T3SS effector CDSs, important in *Chlamydia* virulence, revealed that a serine protease referred to as chlamydial protease-like activating factor (CPAF) is not predicted to be secreted in *C. gallinacea* in contrast to *C. psittaci* (44). However, *C. psittaci* orthologues of the recently described T3SS that associate with the host's inner nuclear membrane (SINC), and translocated actin-recruiting phosphoprotein (TARP) were identified and predicted to be secreted (Supplementary Data S5).

Overall, the analysis revealed the novel *C. gallinacea* genomes NL_G47 and NL_F725 have at least 99.5 % sequence identity to the Type strain 08-1274/3 and include the hallmark chlamydial virulence genes. However, *C. psittaci* has a larger set of genes that are related to virulence and metabolism, including more *inc*s, *pmp*s, T3SS effectors and additional genes in the PZ.

Discussion

In this study, the pathogenic potential of two new chicken-derived *C. gallinacea* strains (NL_G47 and NL_F725) were investigated combining classical *in vitro* methods using embryonated chicken eggs and whole-genome analyses. During isolation of NL_G47 and NL_F725, pathogenic changes were observed that also have been described for other *Chlamydia* species (18), such as deep red colour (rubor), cyanotic toes and skin haemorrhage of the embryo. Mortality in embryonated eggs after yolk sac inoculation with *C. gallinacea* has been reported by Guo et al.(4), but was not mentioned by Laroucau et al. (9).

The layer flocks from which the strains originated were apparently healthy, which is in line with observations from other field studies (4, 9, 10). It could not be evaluated if *C. gallinacea* infection led to impaired production as data on egg production were not collected in this teaching flock. The duration and frequency of shedding during *C. gallinacea* infection was only assessed to a limited extent due to the sampling strategy.

In the flock of strain NL_F725, the *C. gallinacea* infection preceded an infection with Infectious Laryngotracheitis (ILT) resulting in preventative culling to limit the spread of ILT. Whether a primary infection of *C. gallinacea* enhances infection with other pathogens or whether co-infection might exacerbate the disease outcome, is currently unknown. For *C. gallinacea*, only co-infections with *C. psittaci* have been reported in chickens without details about the clinical outcome (5, 45). For *C. psittaci*, it has been suggested that co-infections with respiratory pathogens might lead to a more severe disease outcome (46, 47). The effect of co-infection could be a topic for future investigations.

In titration experiments in embryonated eggs, the pathogenicity of *C. gallinacea* was compared to a virulent *C. psittaci* poultry strain. The infectious dose and mortality in *C. gallinacea* infected eggs was lower compared to *C. psittaci* infected eggs. Furthermore, although the observations were limited, a small difference in pathogenicity between both *C. gallinacea* strains was observed. *C. gallinacea* NL_G47 infection resulted in mortality up to the 10^{-2} dilution (1 of 5 eggs), while no mortality was observed in the 10^{-2} dilution with strain NL_F725 (0 of 3 eggs). As follow, this is a first indication of a possible difference in pathogenicity between genetically different *C. gallinacae* strains, but needs to be confirmed due the low number of observations.-

Furthermore, a higher mortality in *C. psittaci* infected eggs compared to *C. gallinacea* is in line with findings in available field and experimental studies. In these studies, *C. gallinacea* infection led to reduced weight gain in chickens and the absence of clinical symptoms, while exposure to a known high virulent *C. psittaci* strain can lead to severe systemic

infections in chickens and turkeys(4, 10, 48, 49). In contrast, exposure to a less virulent *C. psittaci* strain resulted in mild respiratory symptoms indicating the importance of detailed strain knowledge and infection conditions (48).

The difference in infectious dose and mortality between *C. gallinacea* and *C. psittaci* in embryonated eggs might be a result of a shorter development cycle of *C. psittaci*. The development cycle of *C. gallinacea* takes about 60 to 72 hours while that of *C. psittaci* about 50 hours (3, 50). In the experiments, all eggs were harvested at the same time point, which could mean *C. psittaci* was able to replicate to a higher number of bacteria. The difference in replication time could therefore contribute to the virulence of *C. psittaci*.

To get further insight into the genetic background of *C. gallinacea* in relation to pathogenicity, additional genomic comparisons were performed. Both *C. gallinacea* isolates were at least 99.4% identical to *C. gallinacea* Type strain 08-1274/3, with genetic diversity contained to several distinct chromosomal regions, and had a smaller set of potential virulence genes compared to *C. psittaci*. However, the question remains if a smaller set of virulence genes is a disadvantage for the particular isolate or species involved and determines the observed difference in pathogenicity. The closest genetic relative of *C. gallinacea*, *C. avium*, also has a reduced set of virulence genes compared to *C. psittaci*, and exhibits the smallest PZ region of all *Chlamydia*, but in cases involving pigeons and psittacines infection does lead to clinical signs and mortality (19, 51).

Moreover, *C. gallinacea* does contain all hallmark virulence factors such as Incs, Pmps, T3SS and an intact cytotoxin in the PZ, except in strain JX-1 (12). In addition, *C. gallinacea* has genes encoding for the well-known T3SS effectors TARP and SINC that play a role in the pathogenesis of *Chlamydia* spp. In *C. psittaci*, TARP influences the active uptake in the host cell and SINC targets the nuclear envelope where it is hypothesized to interact with host proteins that control nuclear structure, signaling, chromatin organization, and gene silencing(52, 53). Future studies need to confirm if both effectors are indeed secreted in *C. gallinacea,* with which host proteins they interact, and whether differences in gene expression can be identified that might play a role in pathogenicity.

Based on our current results in embryonated eggs and the genomic comparisons, it is too early to conclude if *C. gallinacea* is a phenotypical commensal. Although less pathogenic than the *C. psittaci* strains of avian origin, *C. gallinacea* does possess the hallmark *Chlamydia* virulence genes, and infection does lead to mortality in embryonated chicken eggs after yolk sac inoculation. Furthermore, there might be small differences in virulence between *C. gallinacea* strains. Additional pathogenesis studies in chickens, including predisposing conditions such as co-infections, are therefore needed to further elucidate the pathogenic

potential of *C. gallinacea* and possible strain differences. These future studies will help to assess the importance of this pathogen for poultry industry.

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Supporting information

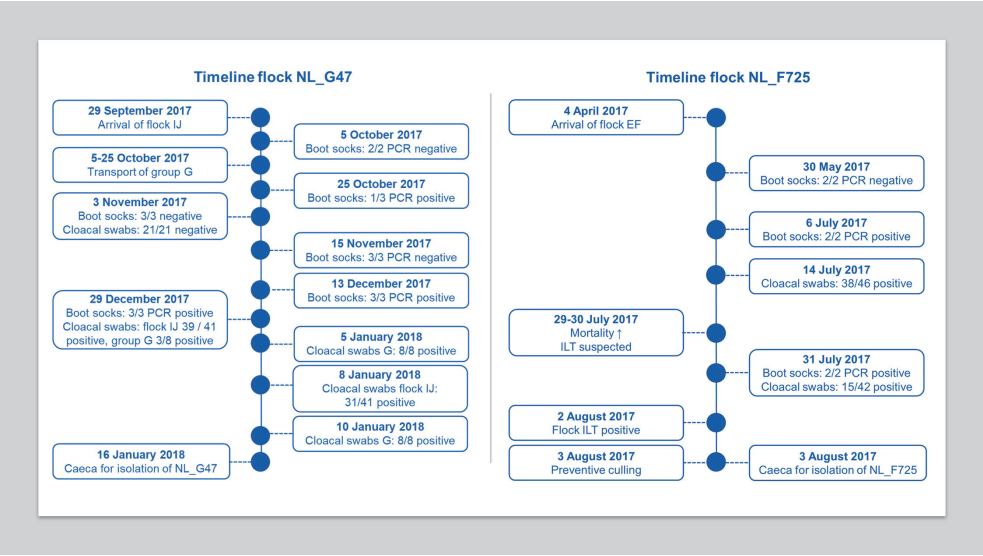

Fig. S1 . Timeline with flock information

In the timeline sampling data are provided of the flocks from which C. gallinacea strain NL_G47 and NL_F725 could be isolated. All boot sock and cloacal samples were tested with the 23S Chlamydiaceae PCR. In the caecal samples for isolation the presence of C. gallinacea was confirmed with the C. gallinacea PCR. ILT positivity of flock NL_F725 was confirmed with an antibody ELISA.

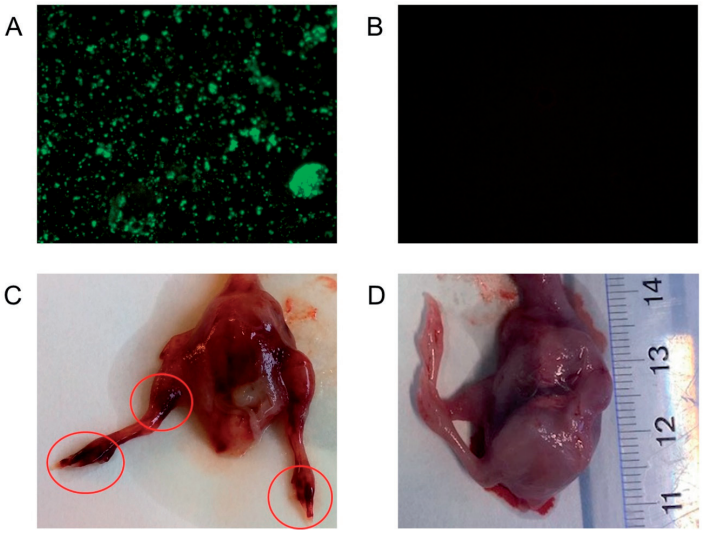

Fig. S2. IFT of the yolk sac membrane and pathologic lesions of the embryo

A and B show a positive and a negative IFT result of the yolk sac membrane. C and D show an embryo of an uninfected and a C. gallinacea infected egg with haemorrhages of the toes and upper leg.

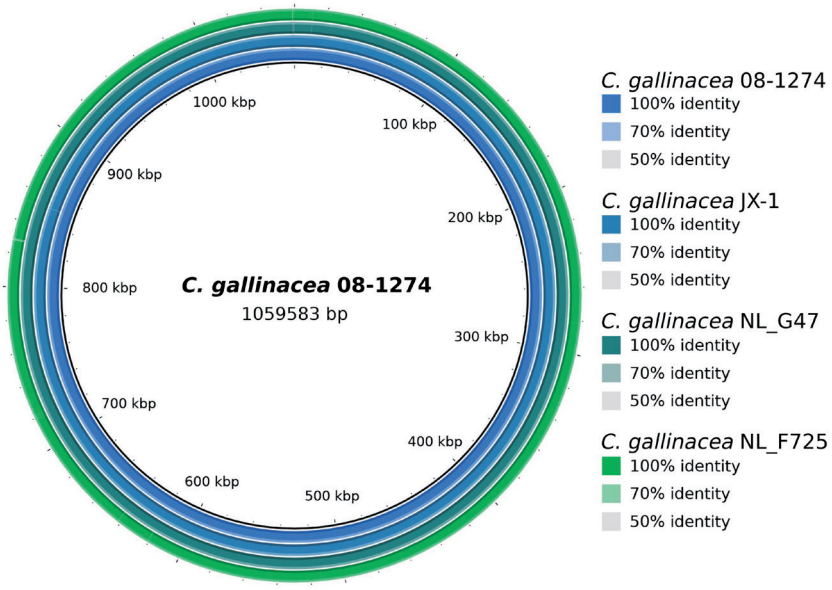

Fig. S3. Genome comparison of four different *C. gallinacea* strains
Whole genome BLAST comparisons between four C. gallinacea genomes created with BLAST Ring Image Generator (BRIG)(39)

Table S1. Results of titration experiments.
In the table the data from all the separate titration experiments were included.

Results per isolate	Undiluted	1E+01	1E+02	1E+03	1E+04	1E+05	1E+06	1E+07	1E+08	1E+09
C. gallinacea NL_G47*										
IFT negative	0	0	0	0	2	18	26	14	nt	nt
IFT positive, no mortality	2	1	4	23	28	12	4	0	nt	nt
IFT positive and mortality	5	2	1	0	0	0	0	0	nt	nt
Total number of eggs	7	3	5	23	30	30	30	14	nt	nt
% IFT positive, no mortality	29%	33%	80%	100%	93%	40%	13%	0%	nt	nt
% IFT positive and mortality	71%	67%	20%	0%	0%	0%	0%	0%	nt	nt
C. gallinacea NL_F725										
IFT negative	nt	nt	0	0	0	1	7	5	nt	nt
IFT positive no mortality	nt	nt	3	5	8	7	3	0	nt	nt
IFT positive and mortality	nt	nt	0	0	0	0	0	0	nt	nt
Total number of eggs	nt	nt	3	5	8	8	10	5	nt	nt
% IFT positive, no mortality	nt	nt	100%	100%	100%	88%	30%	0%	nt	nt
% IFT positive and mortality	nt	nt	0%	0%	0%	0%	0%	0%	nt	nt
C. psittaci NL_Borg										
IFT negative	nt	nt	0	nt	0	0	0	2	7	5
IFT positive no mortality	nt	nt	0	nt	0	0	1	6	1	0
IFT positive and mortality	nt	nt	2	nt	6	10	14	6	0	0
Total number of eggs	nt	nt	2	nt	6	10	15	14	8	5
% IFT positive, no mortality	nt	nt	0%	nt	0%	0%	7%	43%	13%	0%
% IFT positive and mortality	nt	nt	100%	nt	100%	100%	93%	43%	0%	0%

*nt = not tested; all isolates were passaged three times in eggs; the differences in number of eggs per dilution step are caused by the number of experiments, the dilution range used in every experiment, mortality before day three and the availability of surplus eggs; * For strain C. gallinacea G47 the titration results of two different batches are shown.*

Data S1. Background of flocks. The file provides information about the origin of the flocks, the number of chickens per pen, the breed and the date of hatch and arrival. The file can be found at: https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-021-95966-9/MediaObjects/41598_2021_95966_MOESM1_ESM.xlsx

Data S2. Details of loci with sequence variation in *C. gallinacea*.
The file can be found at: https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-021-95966-9/MediaObjects/41598_2021_95966_MOESM2_ESM.xlsx

Data S3. Comparison of translated CDSs. Results of the local alignment approach in which all translated CDSs of *C. gallinacea* 08-1274/3 and *C. psittaci* NJ1, and vice versa were compared to each other to identify regions with less or no homology. The file can be found at: https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-021-95966-9/MediaObjects/41598_2021_95966_MOESM3_ESM.xlsx

Data S4. Unique CDSs. List of CDS for which no homologue could be identified in *C. gallinacea* or *C. psittaci*. These data are graphically depicted in Fig. 4B.
The file can be found at: https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-021-95966-9/MediaObjects/41598_2021_95966_MOESM4_ESM.xlsx

Data S5. Results of analysis of predicted T3SS effectors.
The file can be found at: https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-021-95966-9/MediaObjects/41598_2021_95966_MOESM5_ESM.xlsx

Data S6. MLST and rMLST data. In the file isolates used for rMLST and MLST including provenance and allelic profile data are listed with their allele numbers.
The file can be found at: https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-021-95966-9/MediaObjects/41598_2021_95966_MOESM6_ESM.xlsx

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CHAPTER 4

Pathogenicity of *Chlamydia gallinacea* in chickens after oral inoculation

Marloes Heijne[1*], Jeanet van der Goot[2], Herma Buys[2], Annemieke Dinkla[1], Hendrik Jan Roest[3], Lucien van Keulen[1], Ad Koets[1,4]

[1] Department of Bacteriology, Host-Pathogen Interaction and Diagnostics Development, Wageningen Bioveterinary Research, Lelystad, the Netherlands
[2] Department of Diagnostics and Crisis Organisation, Wageningen Bioveterinary Research, Lelystad, the Netherlands
[3] Directorate Animal Supply Chain and Animal Welfare, Ministry of Agriculture, Nature and Food Quality, The Hague, the Netherlands
[4] Department of Population Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

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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.

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 (1-4). In poultry, transmission occurs via the fecal-oral route (5). 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 (4, 6). Zoonotic transmission of *C. gallinacea* has been considered, but there is no definite proof (2, 5, 7).

Genomic studies revealed *C. gallinacea* has the hallmark *Chlamydia* virulence genes, although to a lesser number than *C. psittaci* (6, 8, 9). 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 (9), but infections are associated with clinical disease and mortality in pigeons and parrots (1, 10). Furthermore, molecular typing provided evidence for substantial genetic diversity among *C. gallinacea* strains, which might result in differences in pathogenicity (8). Therefore, further research 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(6). Furthermore, strain NL_G47 is genetically different from strain JX-1 which was used in other published experimental studies (4, 5). 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 (6). 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.

Materials and methods

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).

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}) (6).

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).

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.

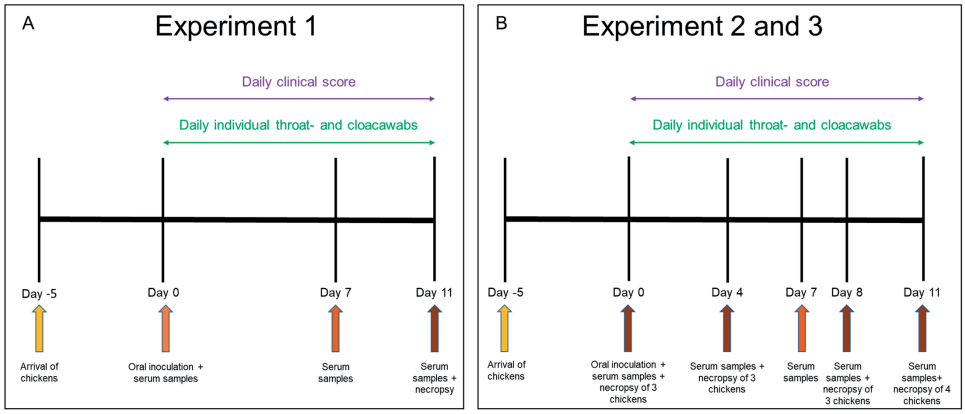

Fig. 1. Timeline of experiments with sampling moments

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).

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 (2x 20 sec at 4m/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.

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 (2, 11).

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

Subsequently, formalin fixed sections were incubated for 5 minutes 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.

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 0.5M 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).

Results

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.

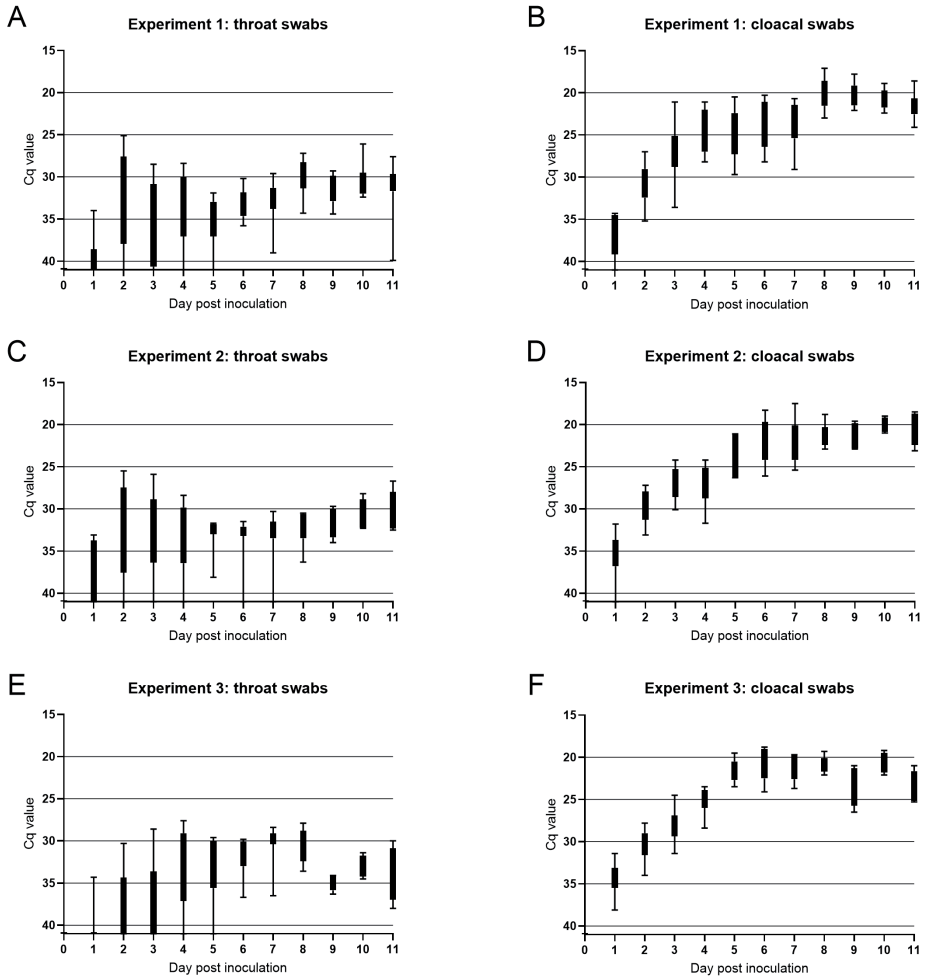

Fig. 2. PCR results of throat (A,C,E) and cloacal swabs (B,D,F)

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 B every day post infection (dpi) at the X-axis shows the PCR results of 13 chickens. In C,D,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.

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.

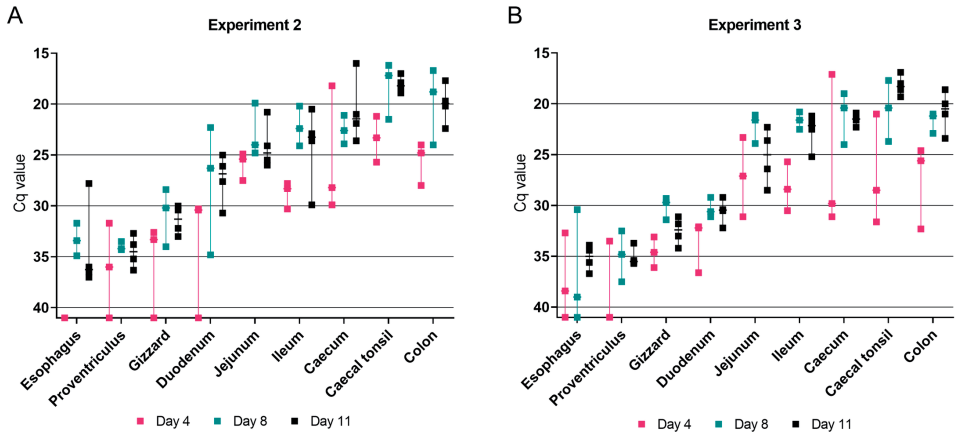

Fig. 3. PCR results of samples from the gastrointestinal tract

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.

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).

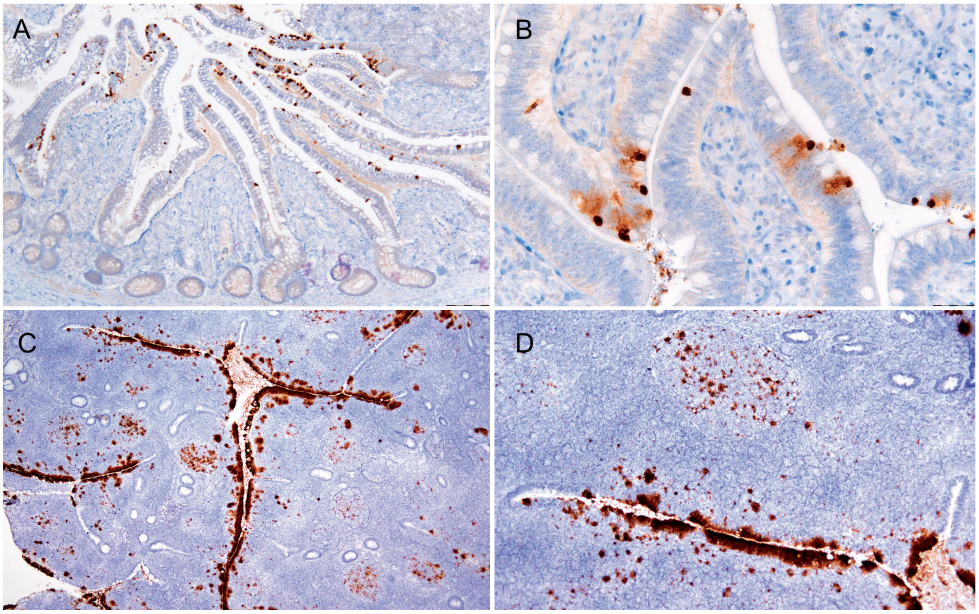

Fig. 4. Histology and immunohistochemistry of the ileum and caecum
A and B: IHC staining for Chlamydia in the ileum at day 4 post infection Bacteria are clearly visible in the epithelium either as apical located inclusion bodies or diffusely present in the cytoplasm. A bar = 100 µm, B bar = 20 µm. C 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

At day 8 and 11 after infection, the number of epithelial cells that stained positively for chlamydia had increased. Chlamydiae were most abundant in the epithelium of the caecum and caecal tonsil (Fig. 5C 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. 5 A-C) and mononuclear phagocytes within the lamina propria (Fig. 5 D-F).

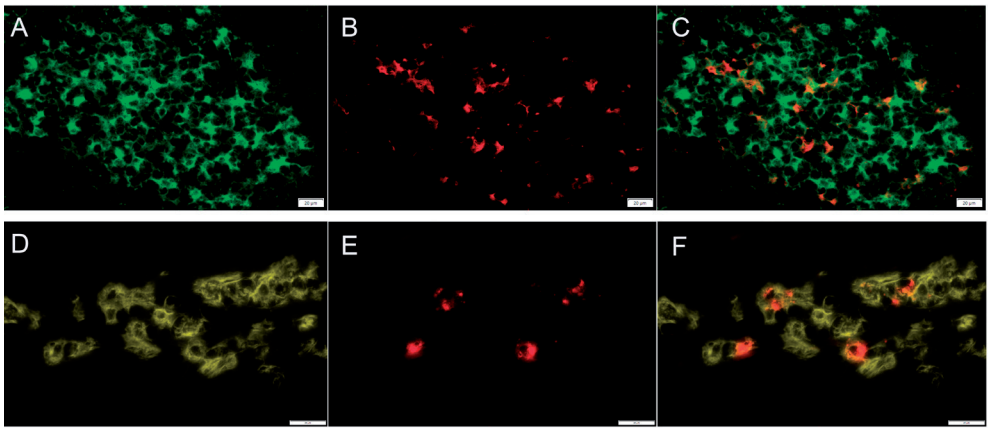

Fig 5. Co-localization of chlamydial antigen and follicular dendritic cells (A-C) or mononuclear phagocytes (D-F) in the caecal tonsil at 11 days post infection
A: Follicular dendritic cell staining with mAb 74.3, B: Chlamydia staining with pAb LS-C85741, C: merge of A and B, D: mononuclear phagocytes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

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

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.

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 (12) or recovery of viable bacteria from the colo-rectum (13), 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 (14). 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 (15). 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 (16). 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 (17). 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 (4, 5, 7). 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 (18, 19). 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* (4) and in contrast to *C. abortus* for which small ruminants are considered the predominant host (20).

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 (5), or for at least three months in cloacal swabs (4) suggesting a persistent infection of the gut. Persistent infections of *Chlamydia* in the gut can result in long term or chronic health effects (21), 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 (22). A negative effect of *C. gallinacea* infection on production has already been shown in broilers with reduced weight (4). 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 (2).

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 (2, 4, 23).

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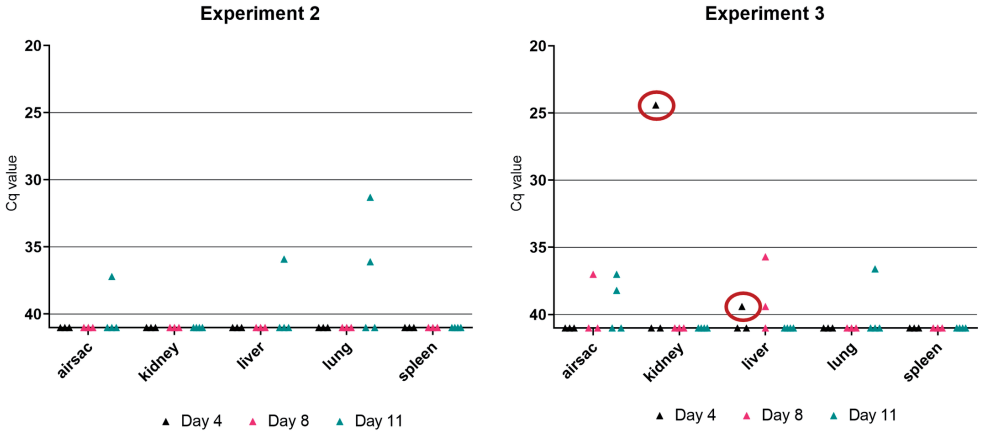
Supporting information

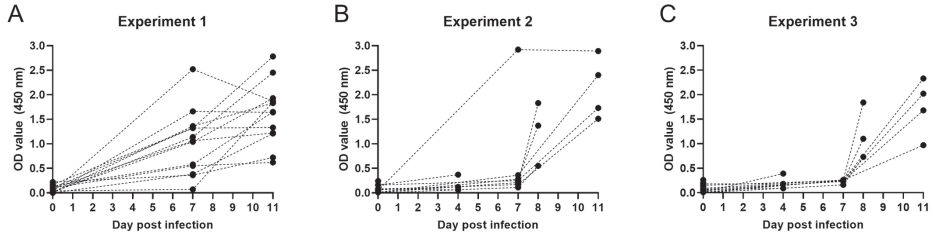
S1 Table. Scoring card clinical signs

	No signs (0)	Mild (1)	Severe (2)
Mental state	Active, makes noise, responds to environment or handling	Less active, bulging (with feathers upright), but responding to environment	No response to environment, lying, retreating, hardly to no response to handling, stopped eating and drinking
Head	No discharge from nose or eye, no red eyes	Watery to mucous discharge from eye and/ or nose (tear stripe), red eyes	Severe mucous or bloody discharge and/or dense red, swollen eyes
Upper airways	No sneezing or shaking with the head	Occasionally sneezing	Frequent sneezing and shaking of the head
Lower airways	No increased respiration frequency or symptoms of shortness of breath	Slightly increased respiration frequency and / or noises such as gargling and grating	Clearly increased breathing frequency, open mouth, stretched neck, symptoms of shortness of breath, noises such as gargling and rattling
Gait and balance	Normal gait, no uncoordinated movements or tremors	Difficulty with coordination when standing up, can walk but seems to have more difficulty with coordination of movements	Disturbed balance, difficulty walking or paralysis, twisted neck, walking in circles, severe muscle tremors
Feces	Normal chicken feces, no abnormal consistency or color	Feces with abnormal color (green to yellow) and/or consistency (wetter)	Feces with abnormal color, consistency and quantity, presence of blood



S2 Fig. PCR results of tissue samples outside the gastro-intestinal tract

A shows the results of experiment 2 and B of experiment 3. Per timepoint the results of the individual samples are shown. The results of day 0 are not presented. On the Y-axis the cycle treshold (Cq) value is depicted. The axis has been rotated in order to make the graph more intuitive. Red circles indicate false-positives (see text).



S3 Fig. ELISA results of experiment 1,2 and 3

In B and C the number of animals decreases due to sequential sacrificing. Every dot represents a sampling moment.

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CHAPTER 5

Host-pathogen interactions during experimental cross infection of *Chlamydia gallinacea* and *Chlamydia psittaci* in chickens

Marloes Heijne[1*], Jeanet van der Goot[2], Herma Buys[2], Annemieke Dinkla[1], Hendrik Jan Roest[1,3], Lucien van Keulen[1], Ad Koets[1,4]

[1] Department of Bacteriology, Host-Pathogen Interaction and Diagnostics Development, Wageningen Bioveterinary Research, Lelystad, the Netherlands
[2] Department of Diagnostics and Crisis Organisation, Wageningen Bioveterinary Research, Lelystad, the Netherlands
[3] Current affiliation: Directorate Animal Supply Chain and Animal Welfare, Ministry of Agriculture, Nature and Food Quality, The Hague, the Netherlands
[4] Department of Population Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

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Abstract

Chlamydia psittaci was considered the predominant chlamydial species in poultry until *Chlamydia gallinacea* was discovered in 2009. *C. psittaci* is a zoonotic obligate intracellular bacterium reported in more than 465 bird species including poultry. In poultry, infections can result in asymptomatic disease, but also in more severe systemic illness. The zoonotic potential of *C. gallinacea* has yet to be proven. Infections in poultry appear to be asymptomatic and in recent prevalence studies *C. gallinacea* was the main chlamydial species found in chickens. The high prevalence of *C. gallinacea* resulted in the question if an infection with *C. gallinacea* might protect against an infection with *C. psittaci*.

To investigate possible cross protection, chickens were inoculated with *C. gallinacea* NL_G47 and subsequently inoculated with either a different strain of *C. gallinacea* (NL_F725) or *C. psittaci*. Chickens that had not been pre-inoculated with *C. gallinacea* NL_G47 were used as a *C. gallinacea* or *C. psittaci* infection control. In the groups that were inoculated with *C. psittaci*, no difference in throat or cloacal shedding, or in tissue dissemination was observed between the control group and the pre-inoculated group. In the groups inoculated with *C. gallinacea* NL_F725, shedding in cloacal swabs and tissues dissemination was lower in the group pre-inoculated with *C. gallinacea* NL_G47.

These results indicate previous exposure to *C. gallinacea* does not protect against an infection with *C. psittaci*, but might protect against a new infection of *C. gallinacea*.

Introduction

Chlamydia gallinacea and *Chlamydia psittaci* belong to the *Chlamydiaceae*, a family of obligate intracellular bacteria that currently exists of one genus and 14 species (1). Until 2009, *C. psittaci* was considered the predominant chlamydial species in poultry. *C. psittaci* is ubiquitous and has been reported in several mammalian species and more than 465 bird species including poultry (2). Infections can remain asymptomatic, but also result in severe systemic illness and mortality depending on the chlamydial strain, host, host age and (environmental) stressors (3). Moreover, *C. psittaci* has a known zoonotic potential; infections in humans can ultimately result in severe pneumonia (3).

In 2009, a novel *Chlamydia* species was identified in poultry and later classified as *C. gallinacea* (4, 5). Soon after its discovery, it became clear that *C. gallinacea* is highly prevalent in chickens (6-8). Infections with *C. gallinacea* do not result in clinical signs of disease, but can lead to production loss such as reduced weight gain (8). There is currently no microbiological evidence of a zoonotic potential of *C. gallinacea*, although *C. gallinacea* has been considered the causative agent in cases of pneumonia in slaughterhouse workers (5, 6).

In a Dutch cross sectional study in 2018, *C. gallinacea* was detected by PCR in pooled faecal samples at 71 of the 151 investigated layer farms. *C. psittaci* was not detected in any sample from these farms (6). This was unexpected, since a Belgian study in 2014 reported 6/7 broiler breeder, 7/7 broiler and 5/5 layer farms PCR and culture positive for *C. psittaci* in pharyngeal swabs (9). Other studies in Belgium and Northern-France from 2010 and 2013 also reported a high prevalence of *C. psittaci* determined with PCR, culture (on pharyngeal swabs and tissues) and/or serology (10, 11). Culture with PCR confirmation is *Chlamydia* species specific, but with the current understanding of *C. gallinacea* in poultry, a high seroprevalence might also be explained by possible cross reactive antibodies as a major outer membrane protein (MOMP) based *C. psittaci* ELISA was used (11).

Cross reactive antibodies between chlamydial species are known to occur because of the close structural similarity among some of the major surface antigens such as MOMP (12) and, could potentially result in cross protection against multiple *Chlamydia* species. Apart from differences in methodology, this may offer an explanation why *C. psittaci* was not detected in the Dutch prevalence study (6) or, vice versa, *C. gallinacea* was not detected in the Belgian prevalence study (9). We therefore hypothesised that the high prevalence of *C. gallinacea* in Dutch layers resulted in herd immunity against *C. psittaci* due to possible cross protection.

To investigate the hypothesis of possible cross protection, chickens were inoculated with *C. gallinacea* NL_G47 and, after five weeks, inoculated with either a different strain of *C. gallinacea* (NL_F725) or with a strain of *C. psittaci*. These treatments were compared to single exposure with either *C. gallinacea* (NL_F725) or *C. psittaci.* Reduced shedding or tissue dissemination in the groups that had been pre-inoculated with *C. gallinacea* NL_G47 would be an indication of possible cross protection between *C. gallinacea* strains and/or *C. psittaci*. Cross protection between *C. gallinacea* and *C. psittaci* could be a beneficial scenario from a one health perspective, because infections with *C. gallinacea* seem relatively harmless for poultry and *C. gallinacea* has no proven zoonotic potential thus far.

Materials and methods

Ethical statement

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

Inocula

Chlamydia gallinacea NL_G47 and NL_F725 were isolated from caecal material from laying hens as described earlier (13). *Chlamydia psittaci* strain NL_Borg is an in-house reference strain closely related to the turkey outbreak strain *C. psittaci* NJ1 (13). All strains were passaged three times in the yolk sac of embryonated SPF chicken eggs and stored at -80°C in a 20% yolk sac suspension in Sucrose Phosphate Glutamate (SPG) until inoculation (14). The infectious dose of the suspensions was calculated via egg titration experiments and expressed as the Egg Infectious Dose 50 (EID_{50}) (13, 15, 16).

Animals and housing

A total of 48 five-week-old Specified Pathogen Free (SPF) White Leghorn layers were obtained from Royal GD (Deventer, the Netherlands). *Chlamdiaceae* are not included in standard SPF testing, therefore 10 cloacal swabs from layers of the mother flock were collected. All swabs tested PCR negative for *Chlamydia* spp. before the chickens were delivered. All chickens had a seven-day acclimatization period prior to the first inoculation.

After arrival the hens were housed in groups on sawdust bedding in temperature-controlled rooms under optimal light conditions and humidity. Feed and water were provided ad libitum. Control chickens or chickens infected with *C. gallinacea* were housed in veterinary biosafety level 2 (vBSL 2) facilities and chickens infected with *C. psittaci* were housed in biosafety level 3 (BSL 3) facilities at Wageningen Bioveterinary Research (WBVR, Lelystad, the Netherlands).

Experimental design

The experiment consisted of two parts as shown in Fig. 1A. In the first part 26 randomly selected chickens were orally inoculated with *C. gallinacea* NL_G47 seven days after arrival. The remaining twenty-two chickens were not inoculated and served as a control group. Both groups were housed seperately and chickens were numbered randomly. At day 28, after the first inoculation, the groups were transported to a new location with BSL-3 facilities. Both groups were transported separately to prevent cross contamination.

At the new location the control group and the infected group were further subdivided in two groups (resulting in four experimental groups, Fig. 1A). The chickens were allocated to the groups alternately by number. At day 35, chickens were either inoculated with *C. gallinacea* strain NL_F725 or with the *C. psittaci* NL_Borg strain. For *C. gallinacea* inoculation was performed orally, because the faecal-oral route is the main route of transmission (17). For *C. psittaci* inoculation was performed oro-nasally, because both the respiratory and oral route have been described (18). At day 42 the animals were sacrificed (see Fig. 1B).

The inoculations were performed with a 1 ml syringe (Terumo Europe N.V.) and an oral gavage needle (18Gx1,5", Terumo Europe N.V.). For the oro-nasal inoculation, chickens first received one droplet of the suspension in one nostril after which the remaining suspension was inoculated orally. At the first inoculation with *C. gallinacea* NL_G47 chickens received 0.5 ml of a yolk suspension of NL_G47 with an infectious dose of $10^{5.9}$ EID_{50} per ml. At the second round chickens were inoculated with either 0.5 ml of a yolk suspension of NL_F725 or with 0.5 ml of a yolk suspension of *C. psittaci* NL_Borg, both with an infectious dose of $10^{5.4}$ EID_{50} per ml. The inoculation dose was confirmed by back-titration and the infectious dose was within a range of one log step of the initial dose.

In the first part of the experiment the weight of the animals was recorded at day 0 (before inoculation) and day 28. During the whole experiment (42 days) clinical signs were recorded daily according to a clinical scoring card (Additional file 1). In the first part of the experiment, cloacal swabs (Puritan HydraFlock sterile swab, ITK Diagnostics BV) were collected at day 0, 4, 7, 14 and 28. In the second part cloacal swabs were collected daily from day 35 until day 42 in all groups. In the *C. psittaci* exposed groups, additional throat swabs were collected daily, because for *C. psittaci* throat swabs might be a more sensitive method to measure shedding (19). In the *C. gallinacea* groups no throat swabs were collected based on earlier results that showed shedding in cloacal swabs was higher (14). In the first part serum samples were collected at day 0, day 7, day 14, day 28 and, in the second part, at day 35 and day 42 at necropsy. All samples at day 0 were collected prior to inoculation to confirm the absence of a current *Chlamydia* infection. A timeline of the experiments including sampling moments is given in Fig. 1B.

At the end of the experiment the chickens were euthanized by maximum blood collection via heart puncture, under anesthesia by intramuscular injection of a mixture of 0.3 ml/kg ketamine (Ketamine 10%, Alfasan Diergeneesmiddelen B.V.) and 0.5 ml/kg xylazine (Sedamun, Dechra Veterinary Products).

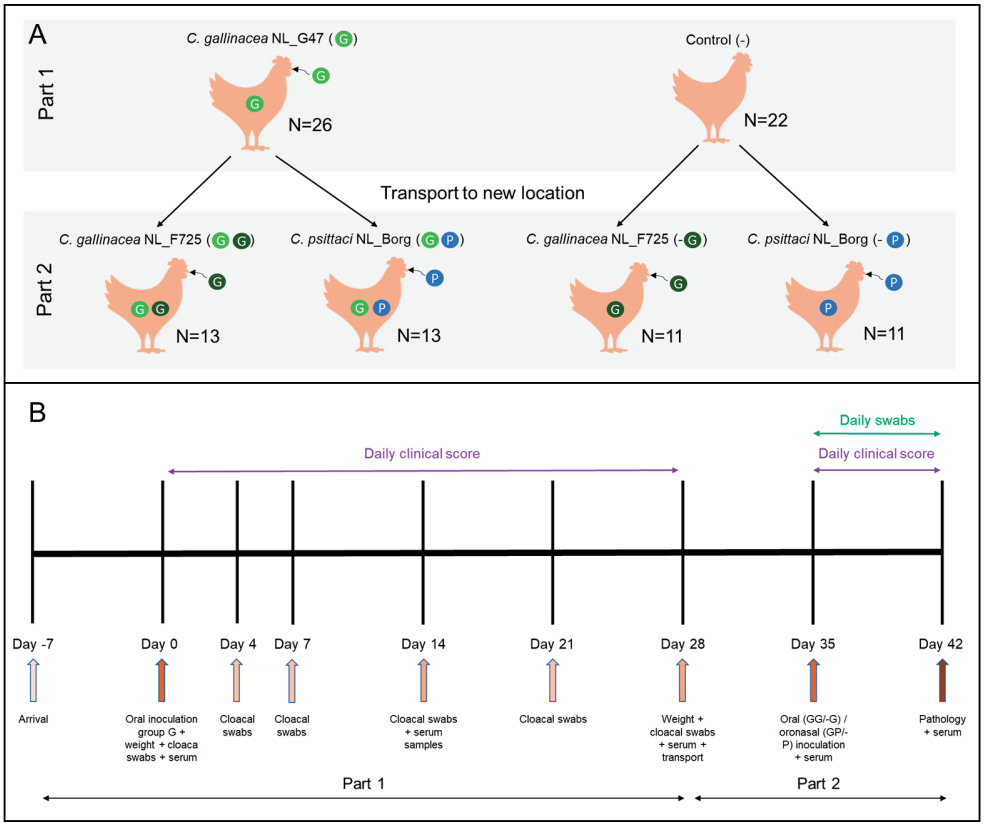

Fig. 1. Experimental design

A shows the experimental setup with group size and type of inoculum (coloured bacteria) and B the timeline of the experiment with sampling moments. From day 35 to day 42 daily throat and cloacal swabs were collected in the C. psittaci inoculated group GP and -P. In group GG and -G only daily cloacal swabs were collected.

Necropsy

At necropsy 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, 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 (2x 20 sec at 4m/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.

PCR analyses

Swabs were immersed in 1.5 ml PBS 13 (BM014, WBVR) and thoroughly vortexed (10s) to suspend the sample from the swab. From the swab or tissue suspension, 200 µl was used for DNA extraction. Swabs and tissues suspensions from the BSL-3 lab were heated for 30 minutes at 99˚C before cell lysis to prevent transfer of infectious material from the containment area. In a prior pilot experiment was established that the heating step did not influence the PCR outcome. DNA extraction was performed with a MagNA Pure LC total Nucleic Acid Isolation kit in the MagNA Pure® system (Roche Diagnostics) according to instructions provided by the manufacturer. All DNA samples were tested with a *Chlamydiaceae* PCR targeting the 23S rRNA as has been previously described in detail (6, 20). Samples from chickens that were exposed to *C. psittaci* were also tested with a specific *C. psittaci* PCR targeting the *ompA* gene according to methods published previously (21).

Histology and immunohistochemistry

Formalin fixed tissue samples were cut into 4 µm sections and collected on positively charged glass slides (SuperfrostPlus®, Thermo Scientific). Sections were then stained with haematoxylin-eosin (HE) or immunostained with a polyclonal anti-Chlamydia antibody (LS-C85741) or a monoclonal anti-*Chlamydia* antibody (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 anti-mouse HRP conjugated polymer was used as a secondary antibody (Invitrogen) and DAB+ as substrate (Dako, Agilent). Sections were counterstained with Mayer's hematoxylin and mounted permanently. Photographs were taken with an Olympus BX51 microscope equipped with a high-resolution digital camera.

Serology

Serum samples were tested with an in-house ELISA using a commercially available mix of *Chlamydia abortus* and *Chlamydia trachomatis* antigens (Institut Virion\Serion GmbH), because specific serological tests for *C. gallinacea* are currently not commercially available. An antigen coating solution was prepared with a final concentration of 4 µg / ml of each antigen in bicarbonate coating buffer with pH 9.6 (BM112, WBVR,). Ninety-six-well microtiter plates (Nunc MaxiSorp™, Thermo Fisher Scientific) were coated overnight at 37 °C with 100 µl per well in coating buffer. Following six washes with 0.05% Tween® 80, the plates were blocked with 190 µl per well of 5% skimmed-milk powder (Elk, Campina) in Tris-buffered saline with 0.1% Tween® 20 detergent (TBST, BM309, WBVR) for 60 min at room temperature (RT). The plates were washed as described above, subsequently

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), 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). The reaction was terminated after 10 min by the addition of 100 µl 0.5M sulfuric acid. The optical density (OD) was measured at 450 nm on a Thermo Labsystems Multiskan RC microplate reader (Thermo Fisher Scientific).

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).

Statistics

Groups were compared using a linear mixed model with Ct value as outcome, for the swabs Day and Group were fixed effects and Chicken a random effect. For the model with the organs, Organ and Group were fixed effects and Chicken a random effect. Models with and without Group were compared by the likelihood ratio test. Analyses were performed in R (22), using the package lme4.

Results

To investigate possible cross protection chickens were first inoculated with *C. gallinacea* NL_G47 (part 1 of the study) and after five weeks inoculated with *C. gallinacea* NL_F725 or *C. psittaci* NL_Borg (part 2 of the study). During part 1 the control group was not inoculated (see experimental design in Fig. 1).

Part 1: primary inoculation with *C. gallinacea* NL_G47

The group that was inoculated with C. *gallinacea* NL_G47 (group G) in part 1 of the experiment was successfully infected (Fig. 2A-C). All cloacal swabs tested positive in the *Chlamydiaceae* PCR at day 7 and after day 14 shedding declined as shown in Fig. 2A and B. Before transport at day 28, 19 / 26 cloacal swabs were PCR positive (i.e. Ct<40) with a mean Ct of 35.6. Furthermore, a rise in antibody titre in the ELISA was observed (Fig. 2C). The uninfected control group (-) remained PCR negative in cloacal swabs and seronegative in the ELISA (Fig. 2A-C). During the first 28 days after inoculation no clinical signs, nor a difference in weight was observed in the controls and *C. gallinacea* inoculated chickens (Additional file 2).

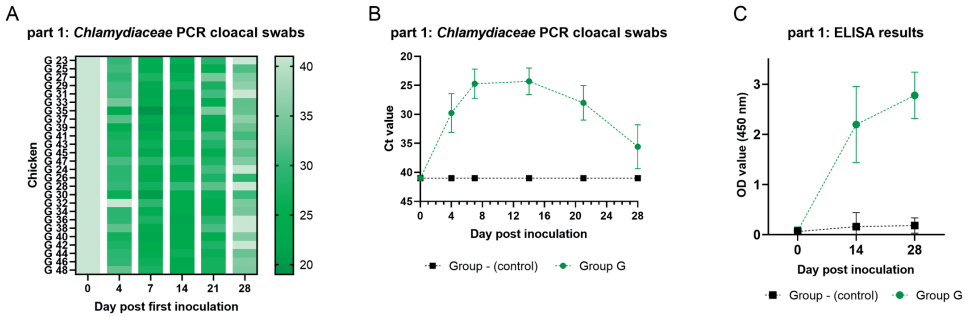

Fig. 2. PCR results of cloacal swabs (A and B) and ELISA results of serum samples (C)
In A results of individual cloacal swabs of group G in the Chlamydiaceae PCR per timepoint are depicted in a heatmap. The darker the colour, the lower the Ct value as shown in the colour scale at the right side. Ct values > 40 or no PCR signal are shown as Ct 41. The results of the negative control group (-) are not shown. B shows the mean Ct value of the cloacal swabs in time per group (of the Chlamydiaceae PCR). The error bar indicates the SD. On the Y-axis the cycle treshold (Ct) value is depicted. The Y-axis has been rotated and Ct values > 40 or no PCR signal are shown as Ct 41. In C the mean OD (450 nm) value of the serum samples per group per timepoint is shown. The error bar indicates the SD.

Part 2: secondary inoculations

In part 2 both groups were split resulting in four experimental groups. Two groups (GG and -G) were inoculated with *C. gallinacea* NL_F725 and two groups (GP and -P) were inoculated with *C. psittaci* (Fig. 1A).

Secondary inoculation with C. psittaci (group GP and -P)

The *C. psittaci* inoculated groups (GP and -P) were tested with a *Chlamydiaceae* PCR and a specific *C. psittaci* PCR, which does not cross react with *C. gallinacea*. Before inoculation at day 35, 6/13 throat (mean Ct 37.9) and 10/13 cloacal swabs (mean Ct 34.6) of group GP test positive in the *Chlamydiaceae* PCR, but negative in the *C. psittaci* PCR (Fig. 3A and C, Additional file 3A-B). This can be explained by the remaining presence of *C. gallinacea* NL_G47 and is in line with the findings at day 28 (Fig. 2A-B). From day 36 onwards, the mean Ct value in the *Chlamydiaceae* PCR in throat and cloacal swabs of group GP is lower than the mean Ct value in the *C. psittaci* PCR (Additional file 3A and B). This difference seems to be caused by the remaining presence of *C. gallincea* NL_G47 until day 37 in throat swabs and until day 38 in cloacal swabs, also when the results of group -P are taken into account. At the remaining days, a difference in sensitivity between both PCRs might also play a role (Additional file 3).

After inoculation, PCR based shedding of *C. psittaci* was higher in throat swabs as compared to cloacal swabs and based on the throat swabs, no significant difference between the groups was observed (Fig.. 3). At day 7, 11/13 throat swabs tested *C. psittaci* PCR positive in group GP as compared to 11/11 in group -P (Fig.. 3A). In cloacal swabs, 4 chickens tested *C. psittaci* PCR positive at day 7 in both groups (Fig. 3C).

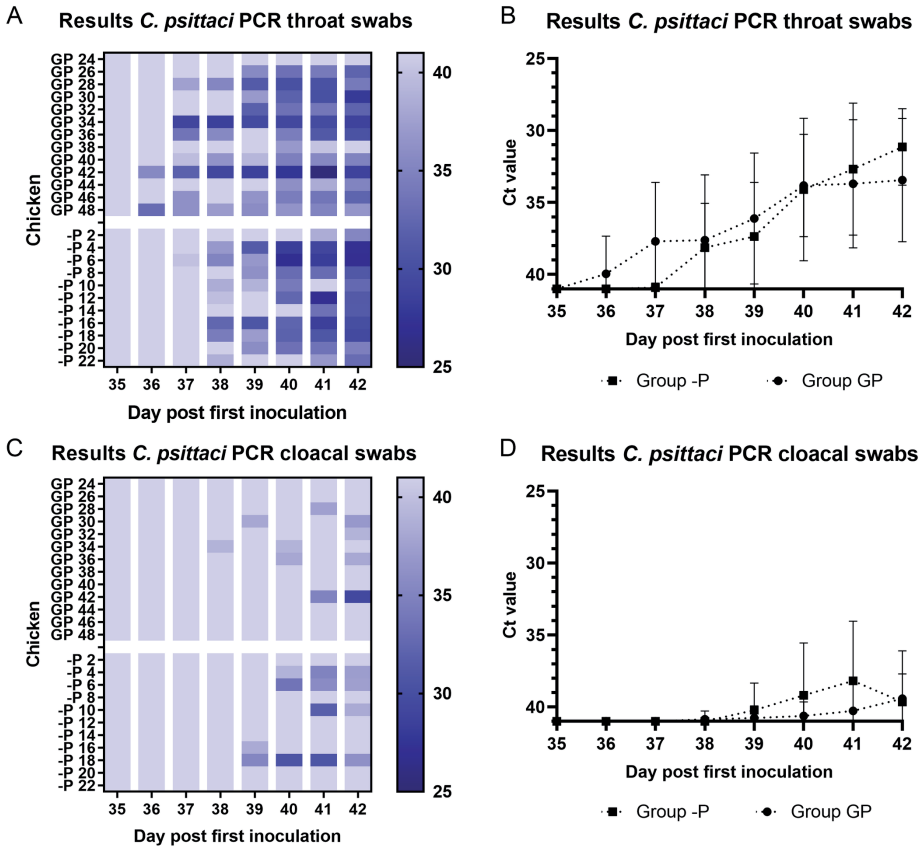

Fig. 3. *C. psittaci* PCR results of group GP and -P

In A individual results of the C. psittaci PCR of throat swabs per timepoint are depicted in a heatmap. The darker the colour, the lower the Ct value as shown in the colour scale at the right side. Ct values > 40 or no PCR signal are shown as Ct 41. B shows the mean Ct value of the throat swabs pet timepoint per group (of the C. psittaci PCR). The error bar indicates the SD. On the Y-axis the cycle treshold (Ct) value is depicted. The Y-axis has been rotated and Ct values > 40 or no PCR signal are shown as Ct 41. In C individual results of the C. psittaci PCR of the cloacal swabs per timepoint are depicted in a heatmap. The darker the colour, the lower the Ct value as shown in the colour scale at the right side. Ct values > 40 or no PCR signal are shown as Ct 41. D shows the mean Ct value of cloacal swabs in time per group (of the C. psittaci PCR). The error bar indicates the SD. On the Y-axis the cycle treshold (Ct) value is depicted. The Y-axis has been rotated and Ct values > 40 or no PCR signal are shown as Ct 41.

In both group GP and -P, no clinical signs were observed during part 2 of the experiment based on the scoring card criteria. At necropsy, enlarged spleens were observed in 12/13 chickens of group GP and 10/11 of group -P. (Fig. 4B) Histological examination of the spleen showed a marked hyperplasia of both white and red pulp. (Fig.. 4C) The hyperplasia of the white pulp included both the peri-arteriolar lymphocyte sheath (PALS) as well as

the peri-ellipsoid sheath (PELS). In group -P one chicken showed diffuse small white spots on the liver. Histological examination revealed a multifocal hepatitis with small foci of coagulation necrosis and influx of heterophils (Fig. S5). In another chicken the airsacs had a glazy appearance which was diagnosed by histopathology as an exsudative aerosacculitis. The presence of chlamydial antigen however could (not) be confirmed with immunohistochemistry in any of the tissues examined.

In group GP, *C. psittaci* was detected with PCR in 10/13 airsac samples (median Ct 36), 12/13 lung samples (median Ct 36.8), 12/13 liver samples (median Ct 33.7), 12/13 spleen samples (median Ct 30.1), 10/13 kidney samples (median Ct 36.7), 7/13 ileum samples (median Ct 38.9), 5/13 caecum samples (median Ct 41) and 6/13 samples of the caecal tonsil (median Ct 41). In group -P, *C. psittaci* was detected with PCR in 9/11 airsac samples (median Ct 34.4), 11/11 lung samples (median Ct 33.4), 11/11 liver samples (median Ct 32.3), 11/11 spleen samples (median Ct 29.4), 11/11 kidney samples (median Ct 35.9), 8/11 ileum samples (median Ct 36.4), 7/11 caecum samples (median Ct 37.6) and 8/11 samples of the caecal tonsil (median Ct 37.1). Fig. 4A shows the tissue dissemination patterns in group GP and -P overlapped. Overall there was a significant difference between the Ct values of the groups GP and -P ($\chi^2 = 5.83$, p=0.016).

In group GP, one chicken remained *C. psittaci* PCR negative in throat swabs, cloacal swabs, and tissue samples during the entire experiment.

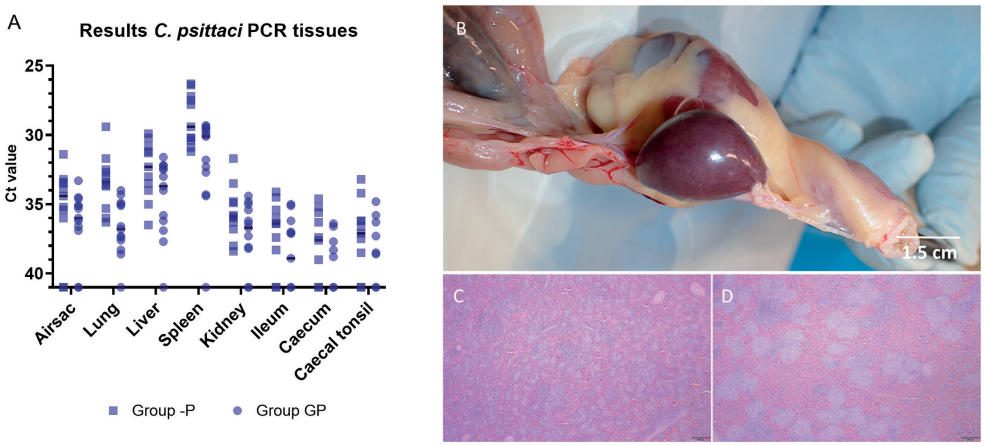

Fig. 4. Results of tissues in group GP and -P

A: the C. psittaci PCR results of tissue samples are shown. On the Y-axis the cycle treshold (Ct) value is depicted. The Y-axis has been rotated and Ct values > 40 or no PCR signal are shown as Ct 41. The bar indicates the median. B: macroscopic enlargement of the spleen in a C. psittaci infected animal. C and D: histological examination of the spleen, same magnification. Notice the pronounced hyperplasia of the red and white pulp in the C. psittaci infected animal (D) compared to the animal infected with C. gallinacea (C).

Secondary inoculations with C. gallinacea NL_F725 (group GG and -G)

Samples of the *C. gallinacea* NL_F725 infected groups (GG and -G) were only tested with the *Chlamydiacea* PCR, because no strain specific real-time PCR was available for *C. gallinacea* NL_F725 or NL_G47. In the group that was initially inoculated with *C. gallinacea* NL_G47 and subsequently inoculated with *C. gallinacea* NL_F725 (GG) significant reduced cloacal shedding was observed (Fig. 5A and B) as compared to group -G ($\chi^2 = 35.6$, $p<0.001$). In group GG PCR based cloacal shedding decreased in time, but in the control group (-G) shedding increased (Fig. 5B). At the end of the experiment at day 42, 2/13 cloacal swabs tested positive (Ct<40) in group GG, while all (11/11) cloacal swabs in group -G tested positive (Fig. 5A).

At necropsy no pathological lesions were observed in group GG and -G. In group GG 5/13 chickens tested PCR positive in the ileum (median Ct 41), 10/13 in the caecum (median Ct 34.2) and 12/13 in the caecal tonsil (median Ct 29.9). In group -G, 11/11 chickens tested positive in the ileum, caecum and caecal tonsil (median Ct 25.2, 23.7 and 22.5 respectively) (Fig. 5C). In both group GG and -G, one chicken tested positive in the airsac (Ct 32.5 and 37 respectively). All other tissue samples tested PCR negative.

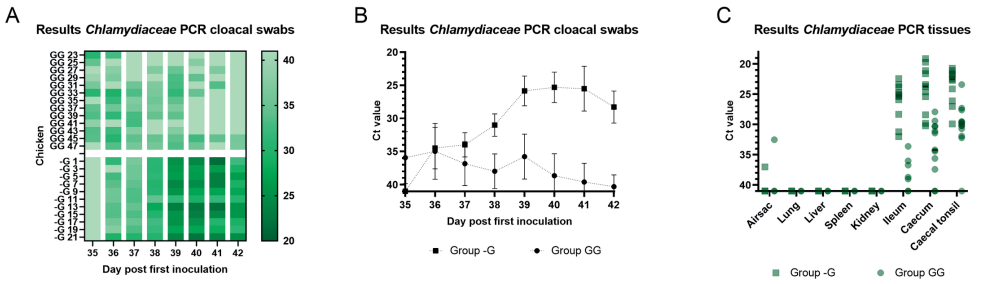

Fig. 5. *Chlamydiaceae* PCR results of group GG and -G

In A individual results of the Chlamydiacea PCR of the cloacal swabs per timepoint are depicted in a heatmap. The darker the colour, the lower the Ct value as shown in the colour scale at the right side. Ct values > 40 or no PCR signal are shown as Ct 41. B shows the mean Ct value of cloacal swabs in time per group (of the C. psittaci PCR). The error bar indicates the SD. On the Y-axis the cycle treshold (Ct) value is depicted. The Y-axis has been rotated and Ct values > 40 or no PCR signal are shown as Ct 41. In C the Chlamydiacea PCR results of tissue samples are shown. The Y-axis has been rotated and Ct values > 40 or no PCR signal are shown as Ct 41. The bar indicates the median.

5.4.2.3. Differences between C. psittaci and C. gallinacea inoculation

In the *C. gallinacea* inoculated control group (-G) and the *C. psittaci* inoculated control group (-P) the shedding and tissue dissemination pattern was different. In group -G cloacal shedding was higher than in group -P and the start of the excretion was different: the *C.*

gallinacea inoculated group started shedding on day 1 post inoculation (day 36), while the *C. psittaci* inoculated group started shedding only on day 4 after inoculation (day 39) (Fig. 6A). In group -P, shedding mainly occurred in throat swabs. In group -G throat swabs were not collected. In tissues *Chlamydia* was mainly detected in the ileum, caecum and caecal tonsil in all chickens of the -G group. In the -P group *Chlamydia* could be detected in all tissues, but the lowest Ct values were detected in the spleen and the highest in the gut in contrast to the results of the -G group (Fig. 6B).

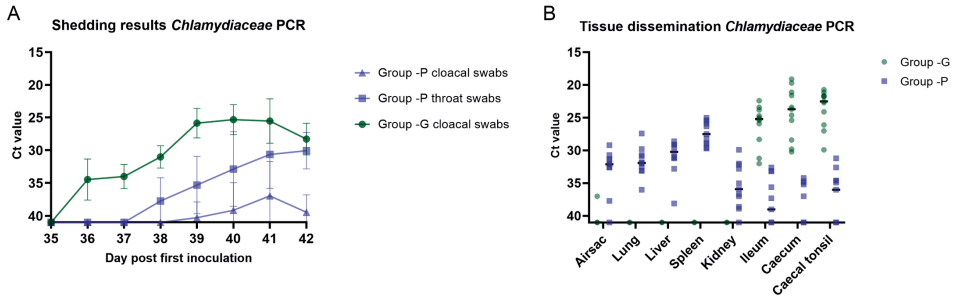

Fig. 6. *Chlamydiaceae* PCR results of group -G and -P

A shows the mean Ct value of swabs in time per group of the Chlamydiaceae PCR. The error bar indicates the SD. On the Y-axis the cycle treshold (Ct) value is depicted. The Y-axis has been rotated and Ct values > 40 or no PCR signal are shown as Ct 41. In B the Chlamydiaceae PCR results of tissue samples are shown of group -G and group -P. The Y-axis has been rotated and Ct values > 40 or no PCR signal are shown as Ct 41. The bar indicates the median.

Summarising, chickens were succesfully infected with *C. gallinacea* NL_G47 in part 1 of the experiment while the controls remained *Chlamydia* negative. In part 2 no difference in *C. psittaci* shedding was observed between the group that was initially inoculated with *C. gallinacea* NL_G47 and subsequently infected with *C. psittaci* (GP) as compared to the control group that was subsequently inoculated with *C. psittaci* (-P). In the group that was initially inoculated with *C. gallinacea* NL_G47 and subsequently inoculated with *C. gallinacea* NL_F725 (GG) significant reduced cloacal shedding was observed as compared to the control group (-G). Furthermore, *C. psittaci* was mainly excreted via the throat and detected in systemic organs such as the spleen, while *C. gallinacea* was mainly detected in the gut. In none of the four groups clinical signs were observed based on the scoring card criteria.

Discussion

In 2018 a high prevalence of *C. gallinacea* was detected on Dutch layer farms, but *C. psittaci* was absent in contrast to earlier studies in surrounding countries (6, 9, 11). We hypothesized that the absence of *C. psittaci* could possibly be explained by cross protection between *C. gallinacea* and *C. psittaci*. This idea was also driven by the fact that cross reactive antibodies between chlamydial species are known to occur, because of the close structural similarity among some of the major surface antigens (12). To investigate whether an infection with *C. gallinacea* could protect against an infection with *C. psittaci*, chickens were first inoculated with *C. gallinacea* NL_G47 and subsequently inoculated with either *C. gallinacea* NL_F725 or with *C. psittaci* NL_Borg. The inoculations did not result in a difference in shedding or tissue dissemination of *C. psittaci* between the group that did not receive a first inoculation and the group that did receive a first inoculation with *C. gallinacea*, so cross protection was not observed.

We did observe a difference in tissue dissemination and shedding pattern between the groups that were inoculated with *C. gallinacea* and *C. psittaci*. In both groups the inoculation route was slightly different: the *C. gallinacea* groups were inoculated orally which resulted in an infection of the gut, while the *C. psittaci* groups received an oro-nasal inoculation that caused a more systemic infection (i.a. of the spleen). In *C. gallinacea*, the oral route is the main route of transmission for *C. gallinacea* and transmission via the respiratory route could not be proven (17). Considering *C. psittaci,* infections via the respiratory route are more efficient than infections via the oral route (23). The different porte d'entree and subsequent localization of the infection is probably caused by a difference in tissue tropism of *C. gallinacea* and *C. psittaci*.

A difference in tissue tropism between *C. gallinacea* and *C. psittaci* could also partially explain why cross protection was not observed. The successful clearance of a *Chlamydia* infection probably depends on both a local and systemic, cell-mediated and humoral response with neutralizing antibodies that act either by inhibiting binding to epithelial cells or activation of complement, leading to lysis of the *Chlamydia* membrane (24, 25). In our study, a rise in anti-*Chlamydia* antibodies was measured after the first inoculation with *C. gallinacea*, but, after the second inoculation, *C. psittaci* could be detected in organs such as the spleen and liver, suggesting circulating neutralizing antibodies against *C. psittaci* were not elicited or could not prevent infection. In a *C. trachomatis* vaccine study, neutralizing antibodies against the variable domain 4 (VD4) of MOMP were very important in preventing infection in a mouse model, but this effect was also specific (24). Small differences in the amino acid sequence of the epitope could already prevent neutralization (26, 27). Additional in-vitro studies are therefore needed to investigate

if *C. gallinacea* infection elicits neutralizing antibodies and if these antibodies have a neutralizing effect on *C. psittaci*.

If neutralizing antibodies are not elicited or do no neutralize *C. psittaci*, (partial) cross protection against *C. psittaci* would depend on local immune responses. However, a local response might not be effective because of the observed difference in tissue tropism between *C. gallinacea* and *C. psittaci*. In the group that received a first inoculation with *C. gallinacea* NL_G47 and a subsequent inoculation with *C. gallinacea* NL_F725 cross protection was observed. This could support the possible role of the local immune response, although neutralizing antibodies could have an effect as well. This would require further investigation as already concluded.

In addition, the difference in shedding pattern between *C. gallinacea* and *C. psittaci* could cause differences in transmission, which might alternatively explain why *C. gallinacea* was highly prevalent and *C. psittaci* was not detected in the prevalence study (6). At first, the degree of shedding of *C. gallinacea* appeared to be higher which will facilitate transmission and, second, the main route of transmission is different. In chickens, the respiratory route is likely to be more important for *C. psittaci* based on previous studies that compared inoculation routes (23), and the higher degree of shedding via the throat, although this might depend on the stage of infection. Pharyngeal shedding is mainly higher during the early part infection until day eight (28); our experiment ended at seven days post inoculation with *C. psittaci*. To further understand how these differences in infection dynamics could affect prevalence, comparative transmission studies with *C. gallinacea* and *C. psittaci* in chickens would be of added value. These studies should also take into account sampling strategy regarding the differences in shedding pattern.

Before the second inoculation with *C. psittaci* or *C. gallinacea* NL_F725, 10/13 chickens where still shedding *Chlamydia* in both groups. It was expected shedding would decrease after transport to a clean environment, because it was thought part of the shedding might be explained by passive transfer of *C. gallinacea* (DNA) from the environment. However, this effect was not observed and the transport of the chickens as a stress factor might have had an enhancing effect on cloacal shedding as known for *C. psittaci* (29).

The remaining presence of NL_G47 at the start of the second part of the experiment could have underestimated the effect on shedding in the GG group, because NL_G47 and NL_F725 could not be differentiated with the *Chlamydiaceae* PCR. On the other hand, it could also have caused a type of competitive exclusion in which the local presence of NL_G47 prevented NL_F725 to enter gut epithelial cells (30). This kind of effect seems unlikely, because it has not been described before in *Chlamydia* and might have been observed in both the GG and GP group. However, cloacal shedding and colonization of the gut in *C.*

psittaci infection (group -P) was in general much lower than in *C. gallinacea* infection (-G) possibly due to a difference in tissue tropism as discussed above.

In conclusion, a prior *C. gallinacea* infection, does partially protect against a new *C. gallinacea* infection based on the PCR based results of cloacal shedding. However, a prior infection with *C. gallinacea* is not protective against a subsequent infection with *C. psittaci* based on shedding and tissue dissemination. The absence of *C. psittaci* in an earlier prevalence study (6) can therefore not be explained by such cross protection. The question remains how often *C. psittaci* is introduced in chickens flocks, how well infections can be transmitted and whether infections might go unnoticed as no clinical signs were observed during our experiment. This would require future comparative transmission studies.

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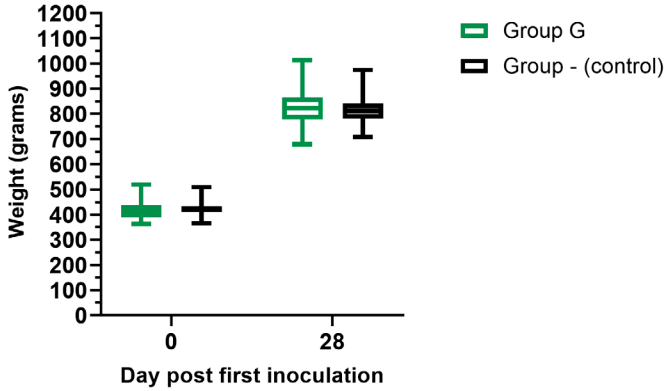
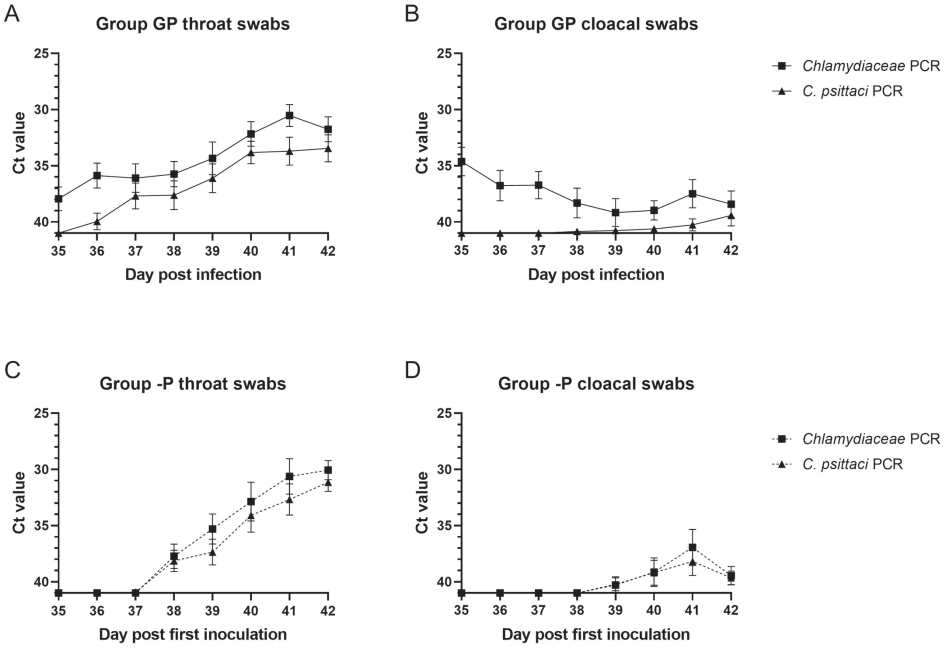
Supporting information

Supporting file 1. Scoring card clinical signs

	No signs (0)	Mild (1)	Severe (2)
Mental state	Active, makes noise, responds to environment or handling	Less active, bulging (with feathers upright), but responding to environment	No response to environment, lying, retreating, hardly to no response to handling, stopped eating and drinking
Head	No discharge from nose or eye, no red eyes	Watery to mucous discharge from eye and/ or nose (tear stripe), red eyes	Severe mucous or bloody discharge and/or dense red, swollen eyes
Upper airways	No sneezing or shaking with the head	Occasionally sneezing	Frequent sneezing and shaking of the head
Lower airways	No increased respiration frequency or symptoms of shortness of breath	Slightly increased respiration frequency and / or noises such as gargling and grating	Clearly increased breathing frequency, open mouth, stretched neck, symptoms of shortness of breath, noises such as gargling and rattling
Gait and balance	Normal gait, no uncoordinated movements or tremors	Difficulty with coordination when standing up, can walk but seems to have more difficulty with coordination of movements	Disturbed balance, difficulty walking or paralysis, twisted neck, walking in circles, severe muscle tremors
Feces	Normal chicken feces, no abnormal consistency or color	Feces with abnormal color (green to yellow) and/or consistency (wetter)	Feces with abnormal color, consistency and quantity, presence of blood



Supporting file 2. Differences in weight in group G and – (control)
Differences in weight (grams) in group G and group – (control) at the start of part 1 of the experiment and after 28 days is shown in a boxplot. The whiskers plot down to the smallest value and up to the largest and the box extends from the 25th to 75th percentile.



Supporting file 3. Differences between the *Chlamdyiaceae* PCR and *C. psittaci* PCR
A and B show the mean Ct value of throat and cloacal swabs in time of group GP in the Chlamydiaceae PCR and C. psittaci PCR. C and D show the mean Ct value of throat and cloacal swabs in time of group -P in the Chlamydiaceae PCR and C. psittaci PCR. The error bar indicates the SEM in all figures. On the Y-axis the cycle treshold (Ct) value is depicted. The Y-axis has been rotated and Ct values > 40 or no PCR signal are shown as Ct 41.

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CHAPTER 6

General discussion

Chlamydia gallinacea and *Chlamydia psittaci* are both obligate intracellular bacteria that can infect poultry. In poultry, *C. psittaci* was considered the main chlamydial species until the discovery of *C. gallinacea* in 2009 (1). *C. gallinacea* is widespread in chickens, but research into its pathogenic potential is limited thus far (2). Furthermore, the prevalence of *C. psittaci* in chickens might be higher than previously thought (3). Therefore, the major aims of this thesis focussed on the following research questions:

1. What is the prevalence of *Chlamydia* in Dutch poultry?
2. What is the primary pathogenicity of *C. gallinacea*?
3. Could a previous infection with *C. gallinacea* protect against an infection with *C. psittaci*?

The prevalence of *Chlamydia* in Dutch layers was investigated in **Chapter 2**. *C. gallinacea* DNA was detected in pooled faecal samples on 71 of the 151 layer farms, but *C. psittaci* DNA was not detected. No association between clinical signs (i.e. respiratory symptoms, nasal and ocular discharge, mortality) and the presence of *C. gallinacea* was found.

In **Chapter 3**, two novel *C. gallinacea* strains (NL_G47 and NL_F725) were isolated from the caeca of apparently healthy chickens. Genomic analysis showed both strains were unique and possessed the hallmark genes coding for known and potential virulence factors as found in *C. psittaci*, albeit to a reduced number of orthologs or alleles. Phenotypic analysis in embryonated specific pathogen free (SPF) eggs revealed *C. gallinacea* induced mortality, but to a lower extent than *C. psittaci*.

Subsequent experiments with *C. gallinacea* strain NL_G47 in six-week old SPF layers in **Chapter 4,** confirmed observations from field studies that *C. gallinacea* infection does not result in acute clinical disease. In this study 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, nor in macroscopic or histologic signs of inflammation. At day 11 post inoculation, chlamydial antigen was co-localized within macrophages in the lamina propria and follicular dendritic cells in the caecal tonsil and, from day 7 onwards, a rise in antibody titre was shown.

After the finding in **Chapter 2** that *C. gallinacea* was highly prevalent in Dutch layer farms, we hypothesized in **Chapter 5** that the absence of *C. psittaci* could be explained by cross protection between *C. gallinacea* and *C. psittaci*. Chickens were therefore first inoculated with *C. gallinacea* NL_G47 and subsequently inoculated with either *C. gallinacea* NL_F725 or with *C. psittaci* NL_Borg. The inoculations did not result in a difference in shedding or tissue dissemination of *C. psittaci* between the group that did not receive a first inoculation and the group that did receive a first inoculation with *C. gallinacea*. Thus, the absence of *C. psittaci* in the prevalence study could not be explained by cross protection. However,

a prior *C. gallinacea* infection did partially protect against a new *C. gallinacea* infection based on the PCR results of cloacal shedding.

Chlamydia infections in poultry other than layers

In this thesis, data were mostly collected in layers, but what do these data tell about broilers or other poultry species? Poultry is an umbrella term for domesticated bird species, such as chickens, ducks, geese and turkeys, and usually further subdivided into layers and broilers based on the production goal (eggs or meat). In the Netherlands, about 90% of poultry is kept in specialized poultry farms with a total number of about 100 million animal places in 2020 (CBS Statline). The majority of these animals are chickens with approximately 44 million layers, housed at 856 farms and 49 million broilers, housed at 637 farms (Table 1). Chickens are therefore the most important part of poultry industry in the Netherlands, but can data from layers be extrapolated to broilers?

Table 1. Poultry farms and number of animal places in the Netherlands in 2020 (CBS Statline)

Poultry and farm type	Number of farms (n)	Number of animals (n)
Chickens; layers	856	43,165,986
Chickens; layers; parent flocks	48	1,674,306
Chickens; broilers	637	49,228,507
Chickens; broilers; parent flocks	248	7,794,318
Turkeys	31	585,134
Ducks	50	819,191
Other poultry species	12	33,285

In chickens, the selection for two different production goals (meat versus eggs) has resulted in two different farming systems with two different animal types. Broilers are fast growing animals that are slaughtered at six weeks of age, while layers grow more slowly and are slaughtered at about 80 weeks of age. Therefore, prevalence data cannot readily be extrapolated, although a lower prevalence would be expected in broilers due to their shorter lifespan. This was already observed in a Mexican cross-sectional study, where layer flocks indeed had a higher risk of being *Chlamydia* positive than broiler flocks, although farming systems might differ between the Netherlands and Mexico (4). In a more recent Dutch surveillance study in broilers, *Chlamydiaceae* DNA could not be detected in pooled faecal samples at 90 investigated farms (unpublished results). Although exact prevalence data cannot be extrapolated, layers can be considered an indicator for the presence of *Chlamydia* in chickens in general.

Differences between broilers and layer systems might also result in differences in disease susceptibility and disease outcome in *Chlamydia* infection. Studies investigating these differences have not been performed, but both *C. gallinacea* and *C. psittaci* have been detected in broilers and layers (3, 4). Furthermore, in an experimental study with *C. gallinacea* in broilers, it was observed that infection might result in reduced weight gain (5). This effect was not observed in SPF layers between six to ten weeks of age (**Chapter 5**) and is most likely due to the difference in growth between broilers and layers. In layers, it would be more logical to investigate other production parameters such as egg production at a later stage.

Extrapolation of data to other poultry species is even more difficult. A French study investigating the prevalence of *Chlamydia* in different poultry species has shown *C. gallinacea* was mostly detected in chickens and guinea fowl, while *C. psittaci* was more prevalent in ducks (6). From *C. psittaci* infections in different bird species, it is known that susceptibility and disease outcome depends on both the host, *C. psittaci* strain and environmental factors (7). For example, in turkeys, severe systemic infections including mortality have been described (8, 9), while ducks seem to be less susceptible to *C. psittaci* infection and are mainly subclinically infected (10, 11). Hence, separate prevalence studies would be required to estimate the prevalence of *Chlamydia* in turkeys and ducks in the Netherlands. Moreover, based on the data about *C. psittaci*, results about the pathogenesis of *C. gallinacea* in chickens cannot be translated to other poultry species. Nonetheless, current prevalence data suggest that other poultry species are less susceptible to *C. gallinacea* infection (5, 6, 12).

C. gallinacea, a pathogen at all?

Although potential virulence factors are present in the *C. gallinacea* genome and infection of embryonated eggs can result in mortality, acute clinical disease was not observed in layers in the prevalence study nor in the oral infection experiments. From that perspective *C. gallinacea* does not behave like a pathogen. However, chlamydial infections are intracellular by nature and notorious for causing long term or chronic health effects due to persistent infections (13-15). Hence, is there evidence that *C. gallinacea* might cause persistent infections?

Persistence refers to infections that cannot be cleared either at cell, organ, organism or population level. In *Chlamydia* research the different levels of persistence are often confounded with each other (16). Furthermore, persistence can coincide at different levels. *Chlamydia* persistence at cell level means the replication cycle is interrupted and the bacteria turn into a viable but non-cultivable state (aberrant bodies) when facing

a stress stimulus such as non-bactericidal antibiotic treatment, cytokine exposure or nutrient deprivation (15). This phenomenon can be considered a kind of dormancy and is mainly observed in vitro, but there is some evidence it might occur in vivo as well (15). Persistency at cell level has not been investigated for *C. gallinacea*, but is likely to occur in vitro in line with findings for other *Chlamydia*. It will be much more challenging to detect this type of persistence in vivo as current methodology might not be sensitive enough.

Persistence at organ, organism or population level usually refers to an infection that cannot be cleared, because of the continuing presence of susceptible cells or animals that can be infected. In *Chlamydia*, this type of persistence is seen in chronic gastrointestinal infections in which it is thought that local downregulation of the immune system prevents clearance of the infection (14, 17). *C. gallinacea* also resides in the gastrointestinal tract. In chapter 5, *C. gallinacea* could be detected in 20/26 cloacal swabs for at least 35 days after inoculation. In other studies, *C. gallinacea* was detected in the rectum at day 26 post infection (18), or for at least three months in cloacal swabs (5). In a recent transmission experiment (unpublished results) *C. gallinacea* DNA could be detected until four to five months after first detection in cloacal swabs of chickens in the exposed flock (Fig. 1, unpublished data). In this experiment two groups of 25 *Chlamydia* PCR negative layers were exposed to three *C. gallinacea* positive layers. Within two weeks after the introduction of the positive layers all cloacal swabs of the contact animals tested PCR positive. During the experiment shedding appeared to be intermittent in individual animals. After five months all cloacal swabs tested PCR negative at three consecutive time points with two week intervals. These results indicate *C. gallinacea* infections might persist for several months, but are cleared based on cloacal shedding. It was not investigated whether *Chlamydia* could still be present in the gut and subsequent cloacal shedding could be below the detection level. Furthermore, no apparent health effects were observed, although more subtle signs would have remained unnoticed as a *C. gallinacea* negative control group was not included.

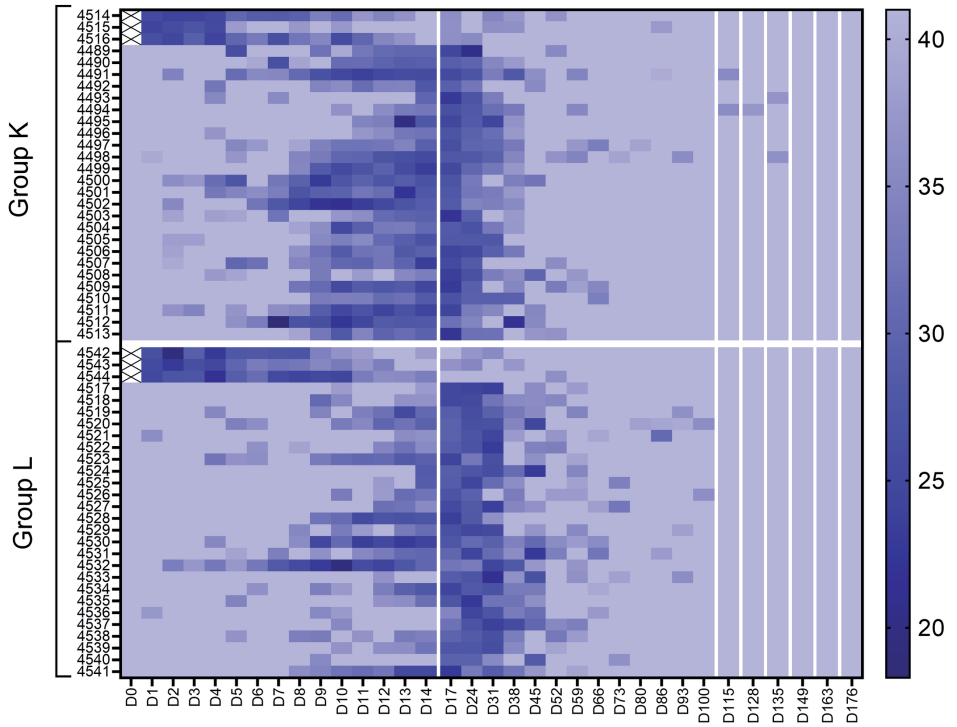

Fig. 1. PCR results of cloacal swabs in field transmission experiment

Chlamydiaceae PCR results of individual cloacal swabs per timepoint depicted in a heatmap. The darker the colour, the lower the Ct value as shown in the colour scale at the right side. Ct values > 40 or no PCR signal are shown as Ct 41. The chickens were housed in two separate pens (group K and L) in one room. The upper three chickens in group K and L are the C. gallinacea positive chickens that were added after the sampling at day 0. Chickens were sampled daily until day 14, once a week until day 100 and at more irregular time intervals of one to two weeks from day 100 onwards.

Chronic health effects or adverse effects on production parameters caused by persistent infections, might be the result of (metabolic) costs due to a possible higher turnover of gut epithelial cells or an increase in adaptive immune response (19). Such an effect has been observed in broilers with reduced weight gain, although this cannot be considered long term considering the slaughter age of broilers (5). It would be more relevant to investigate an effect on egg production, also because *C. gallinacea* spreads rapidly and is highly prevalent in Dutch layers. However, this will be hard to achieve under field conditions as it might be very difficult (or impossible) to include flocks that remain *C. gallinacea* negative throughout their production cycle.

Moreover, even if *C. gallinacea* does negatively affect production in layers, it could be questioned whether the costs of a control strategy would outweigh the possible benefit as long as there is also no clear evidence of a zoonotic potential. A *C. gallinacea* vaccine is

currently not available and might even have more negative side effects than an infection itself. Flock treatments with antibiotics are not easy to perform and very undesirable in view of the risks of development and spread of antibiotic resistance. In conclusion, it cannot be excluded that infections potentially have negative long term effects and *C. gallinacea* might still be considered a pathogen. However, based on current data in chickens it is not a pathogen of concern for which control or eradication seems indicated.

A one health perspective on *C. psittaci* infections in chickens

In our prevalence study, *C. psittaci* could not be detected in layers in the Netherlands (**Chapter 2**). In a more recent study (unpublished results), *C. psittaci* could also not be detected in 90 broiler flocks. Therefore, there is currently no evidence that the earlier reported increased risk of human pneumonia around poultry farms is associated with *C. psittaci* infections in chickens (20). Other studies investigating the association between poultry farms and human pneumonia could either not confirm the higher incidence, or, associated the higher incidence with more indirect causes such as exposure to air pollutants and endotoxins (21, 22).

The absence of *C. psittaci* in the prevalence study does not exclude any future introduction. Our study (**Chapter 5)** showed chickens can experimentally be infected with *C. psittaci* in line with findings of other experimental studies (23-27). Furthermore, we did not find an indication that chickens are protected against a *C. psittaci* infection due to a prior infection with *C. gallinacea*. *C. psittaci* is endemic in many bird species (7, 28-30), so introduction of *C. psittaci* might be possible via contact with infected wild birds. Transmission from wild birds to poultry has been experimentally shown in the past, when wild birds were considered a possible source of outbreaks in turkeys in the United States (31, 32). It is also likely that the risk of introduction will be higher on farms with outdoor housing as already known for Avian Influenza where wild birds play an important role in the epidemiology (33, 34). After introduction, *C. psittaci* infections might remain unnoticed as clinical signs are not always observed as shown in **Chapter 5**, but the question is how well *C. psittaci* can be transmitted between chickens.

C. psittaci can be transmitted from inoculated chickens to uninfected cagemates in an experimental setting (25). In this study, the infection in cagemates was less severe than in the inoculated chickens, raising the question whether the cagemates would have been able to transmit *C. psittaci* to other uninfected chickens. Unfortunately, data from field studies about *C. psittaci* infection dynamics in chickens are scarce. Most studies in chickens focus on the risks of zoonotic transmission at slaughterhouse level. The lack of field data might implicate *C. psittaci* infections are not efficiently transmitted between

chickens. Finally, it has always been assumed that chickens are less susceptible to infection than other bird species (11, 35). Nevertheless, it would be of added value to confirm this experimentally, also from a One Health perspective. If transmission of *C. psittaci* between chickens is limited, the risk that chickens will be a future reservoir for human *C. psittaci* infections is also very limited.

Final thoughts and future research

From a One Health perspective, *Chlamydiaceae* infections in Dutch chickens are currently not a problem, although some questions remain to be answered. Most important is to investigate how well *C. psittaci* can be transmitted between chickens, also because poultry husbandry might change to more outdoor systems in the future which can increase the risk of introduction of a *C. psittaci* infection. Transmission studies should focus on naturally infected animals and at least take into account *C. psittaci* genotype A, B and D. Genotype A is probably the most common *C. psittaci* genotype worldwide and detected in different animal species, including chickens, although it is classically associated with psittacine birds (36, 37). Genotype B is mostly found in homing and feral pigeons and shown to cause disease in chickens under experimental conditions (23, 24). In the Netherlands genotype A and B are the most frequently detected *C. psittaci* genotypes in humans and pet birds (38, 39). Genotype D should be included as it was used in our study in **Chapter 2** and **5**, caused severe outbreaks in turkeys and was shown to cause disease in chickens in other experimental studies (23, 24). Transmission studies will provide further insight into the susceptibility of chickens to *C. psittaci* infection and subsequent infection dynamics, which will help to determine whether chickens could be a potential reservoir for *C. psittaci* infections.

Furthermore, the prevalence of *C. psittaci* in ducks and turkeys in the Netherlands is still unknown. In turkeys, *C. psittaci* infections are mostly associated with severe clinical signs and high mortality (35). During an outbreak of *C. psittaci* on a Dutch turkey farm in the nineties, mortality up to 65% was reported (9). It is very unlikely that this type of infections will be missed. Some studies also indicate infections might be milder and can remain undetected (40, 41). Furthermore, it is important to note that there are no turkey slaughterhouses in the Netherlands. Most cases of poultry-to-human transmission are associated with slaughterhouses (11, 36).

In ducks infections are considered to be mainly subclinical (10, 11), but more severe infections have been reported as well (35). Currently, there are no indications from routine pathology at the Animal Health Service (Royal GD) that *Chlamydia* infections play a role in ducks in the Netherlands, but infections could be missed as *Chlamydia* will not be

detected with routine bacteriological culture. Furthermore, a weak spatial association has been found between duck farms and human psittacosis cases in the Netherlands (42). This association should be treated with caution as the same study also reported an association with chicken processing plants, which seems unlikely based on the results from the prevalence studies. These data show it would be worthwhile to investigate the prevalence of *C. psittaci* in turkeys and ducks, with ducks being the most relevant species. It would be most convenient to use surveillance studies that are already planned as was also done in the prevalence study in layers. However, there is no urgent need regarding the fact that the numbers of ducks and turkeys are relatively small in the Netherlands and the number of human psittacosis cases are low and without a clear association with poultry (39).

From the perspective of general preparedness, it would be helpful if serological tools would become available that at least can discriminate between *Chlamydia gallinacea* and *Chlamydia psittaci* in poultry. This topic was not further discussed in this thesis, but discriminatory diagnostic detection of different *Chlamydia* species relies on molecular tests, which only give information about the current status of an animal. To identify any previous exposure to *Chlamydia*, the availability of a discriminatory serological test would be of added value for any future surveillance studies. Some promising results in chickens have already been obtained with a multiplex serological assay that uses synthetic peptides developed for mammalian serology (43), but this assay needs further validation.

At last, it would be interesting to further investigate the host-pathogen interactions of *C. gallinacea* in the gut and to study co-infections with other poultry pathogens. This will not be easy and will require a more fundamental approach, but it is intriguing how *C. gallinacea* can survive in gut epithelial cells without causing any visible signs of inflammation. Most exciting is to speculate if *C. gallinacea* infections could have a beneficial effect for the host. For example, do *C. gallinacea* infections play a role in biological processes like trained immunity? This is the concept that certain stimuli can shape or adapt the innate immune system which results in better responsiveness and has been shown in exposure to bacillus Calmette-Guerin (BCG) vaccine (44). Trying to answer more fundamental questions about the interaction of *C. gallinacea* with its host might help us to better understand the grey area between health and disease especially in the gut with its huge microbiome.

Take home messages

- *Chlamydia* infections in Dutch chickens should not be considered a current One Health threat as *Chlamydia psittaci* could not be detected in a prevalence study, nor is there evidence that the highly prevalent *Chlamydia gallinacea* causes acute disease in chickens or has a zoonotic potential.

- Future introduction of *C. psittaci* at chickens farms via contact with wild birds cannot be ruled out and requires transmission studies to further investigate the susceptibility of chickens to *C. psittaci* infection and subsequent infection dynamics.

- It is intriguing how *C. gallinacea* is able to survive in gut epithelial cells without causing any visible signs of inflammation or acute disease. This requires more fundamental research into host-pathogen interactions of *C. gallinacea*, which may help to better understand the grey area between health and disease.

- The prevalence of *Chlamydia* in duck and turkey farms was not investigated in this thesis, but there is no urgent need regarding the fact that the number of duck and turkey farms are relatively small in the Netherlands.

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Summary

Chlamydia gallinacea and *Chlamydia psittaci* are intracellular bacteria belonging to the *Chlamydiaceae* family and are a cause of avian chlamydiosis in poultry. *C. psittaci* was considered the predominant chlamydial species in poultry until *Chlamydia gallinacea* was discovered in 2009. *C. psittaci* occurs worldwide, is zoonotic and has a wide host range. Depending on the *C. psittaci* strain, host, age of the host and environmental factors (stress), infections in poultry can be asymptomatic or result in more severe issues, such as acute respiratory distress and mortality. Infections in humans can result in severe pneumonia. *C. gallinacea* is widespread in chickens, and infections do not seem to result in disease, but reduced weight gain has been observed in broilers. Studies about the pathogenic potential of *C. gallinacea* are still limited, and any zoonotic potential has yet to be determined.

Prior observations in poultry that contributed to the questions addressed in this thesis were as follows. It was unknown if *C. gallinacea* and *C. psittaci* also occur in Dutch poultry, however, since 2010, *C. psittaci* infections in chickens were reported in surrounding countries. Moreover, in 2012, a Dutch study reported a higher number of pneumonia cases in residents living near poultry farms. At the time, the cause of these pneumonia cases was unknown, but *Chlamydia* was proposed as potentially playing a role.

The aim of this study was to gain insight into the prevalence of *Chlamydia* in Dutch chickens and to investigate the pathogenic potential of *C. gallinacea* in chickens. Finally, it was investigated whether a previous *C. gallinacea* infection in chickens could protect against a *C. psittaci* infection.

Chapter 2 investigates the prevalence of *Chlamydia* in Dutch layers. *C. gallinacea* DNA was detected in pooled faecal samples on 71 of 151 layer farms, but *C. psittaci* DNA was not detected. No association between clinical signs (i.e. respiratory symptoms, nasal and ocular discharge, mortality) and the presence of *C. gallinacea* was found.

Chapter 3 describes two novel *C. gallinacea* strains (NL_G47 and NL_F725) that were isolated from the caeca of seemingly healthy chickens. Subsequent genomic analysis showed both strains were unique and possessed the hallmark genetic coding for known and potential virulence factors found in *C. psittaci*, albeit to a reduced number of orthologs or alleles. Whether these genetic differences contribute to phenotypic differences is unclear. Phenotypic analyses in embryonated specific pathogen free (SPF) eggs revealed *C. gallinacea* induced mortality, but to a lesser extent than *C. psittaci*.

Subsequent experiments with *C. gallinacea* strain NL_G47 in six-week-old SPF layers, detailed in **Chapter 4**, confirmed observations from field studies that *C. gallinacea* infections do not result in acute clinical disease. In this study, 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, nor were there macroscopic or histologic signs of inflammation. On day 11 post inoculation, chlamydial antigen was co-localized within macrophages in the lamina propria and follicular dendritic cells in the caecal tonsil and, from day 7 onwards, a rise in antibody titre was shown.

After the finding in **Chapter 2**, that *C. gallinacea* was highly prevalent on Dutch layer farms, we hypothesize in **Chapter 5** that the absence of *C. psittaci* could be explained by cross protection between *C. gallinacea* and *C. psittaci*. Chickens were therefore first inoculated with *C. gallinacea* NL_G47 and subsequently inoculated with either *C. gallinacea* NL_F725 or *C. psittaci*. The inoculations did not result in a difference in shedding or tissue dissemination pattern of *C. psittaci* between the groups. Thus, the absence of *C. psittaci* in the prevalence study could not be explained by cross protection from previous *C. gallinacea* infections. However, a prior *C. gallinacea* infection did partially protect against a new *C. gallinacea* infection based on the PCR results of cloacal shedding.

The last chapter (**Chapter 6**) discusses whether the results in laying hens can be translated to broilers or other poultry species. Furthermore, it addresses whether *C. gallinacea* should be considered a pathogen, and how the absence of *C. psittaci* should be interpreted regarding animal and public health. The current conclusion is that *Chlamydia* infections in Dutch chickens cannot be considered a One Health problem. Infections with *C. gallinacea* do not lead to clinical disease in chickens, and *C. psittaci* could not be detected in a prevalence study in layers. However, these results do not exclude the future introduction of *C. psittaci* in chickens, nor its occurrence in other poultry species. These questions would require further research. It is also intriguing that *C. gallinacea* is able to replicate intracellularly without causing visible signs of inflammation, a phenomenon that is observed in other *Chlamydia* and intracellular bacterial infections as well. This requires more fundamental research into host-pathogen interactions, which may help to better understand the grey area between health and disease.

Samenvatting

Chlamydia gallinacea en *Chlamydia psittaci* zijn beide intracellulaire bacteriën die tot de familie van de *Chlamydiaceae* behoren en aviaire chlamydiose in pluimvee kunnen veroorzaken. Tot de ontdekking van *C. gallinacea* in 2009 werd aangenomen dat *C. psittaci* de belangrijkste *Chlamydia* soort in pluimvee was. *C. psittaci* komt wereldwijd voor, kan van dier op mens worden overgedragen (zoönose) en kent een breed scala aan gastheren. Infecties in pluimvee kunnen zonder verschijnselen verlopen, maar ook tot ernstige verschijnselen als benauwdheid en sterfte leiden. Dit hangt af van de *C. psittaci* stam, gastheer, leeftijd van de gastheer en omgevingsfactoren (stress). Infecties in mensen kunnen resulteren in een ernstige longontsteking. *C. gallinacea* komt vooral voor in kippen en infecties lijken niet tot ziekte te leiden. Wel is bij vleeskuikens verminderde groei waargenomen. Naar het ziekteverwekkend vermogen van *C. gallinacea* is echter nog maar beperkt onderzoek gedaan. Ook het zoönotisch potentieel is nog niet opgehelderd.

Voorafgaand aan het onderzoek in dit proefschrift was onbekend in hoeverre *C. gallinacea* en *C. psittaci* ook in Nederlands pluimvee vóórkomen, terwijl er na 2010 in omliggende landen wel degelijk *C. psittaci* in kippen werd aangetoond. Bovendien werd in een Nederlandse studie uit 2012 een hoger aantal gevallen van longontsteking bij omwonenden van pluimveebedrijven gerapporteerd. De mogelijke oorzaak van deze gevallen van longontsteking was op dat moment onbekend, maar een hypothese was dat *C. psittaci* of *C. gallinacea* wellicht een rol zouden kunnen spelen.

Het doel van dit onderzoek was om het vóórkomen van *Chlamydia* in kippen in Nederland in kaart te brengen, inzicht te krijgen in het ziekteverwekkend vermogen van *C. gallinacea* door Nederlandse stammen te kweken, te karakteriseren en te testen in een model met eieren. Daarnaast is experimenteel onderzoek in kippen uitgevoerd. Als laatste is onderzocht of een doorgemaakte *C. gallinacea* infectie in kippen, een infectie met *C. psittaci* kan voorkomen.

In het onderzoek naar het vóórkomen, dat beschreven is in **hoofdstuk 2,** werd op 71 van de 151 onderzochte leghennenbedrijven met een PCR test. *C. gallinacea* aangetoond in fecesmonsters. *C. psittaci* werd in geen van de onderzochte monsters gevonden. De aanwezigheid van *C. gallinacea* kon niet worden gerelateerd aan het optreden van klinische verschijnselen zoals neus- of ooguitvloeiing, benauwdheid of diarree, noch met verhoogde sterftecijfers.

In **hoofdstuk 3** is beschreven hoe uit het caecum van gezonde leghennen, afkomstig van twee verschillende koppels, twee genetisch verschillende isolaten van *C. gallinacea* werden gekweekt. Verder onderzoek aan het genoom liet zien dat *C. gallinacea* genen

heeft die in *Chlamydia* gerelateerd worden aan virulentie. *C. gallinacea* heeft wel minder van dit soort genen dan *C. psittaci*, maar de vraag is in hoeverre deze genetische verschillen samenhangen met het ontstaan van ziekte in dieren. In kippeneieren kan experimentele infectie met *C. gallinacea* via de dooierzak, sterfte van het embryo veroorzaken.

In **hoofdstuk 4** is verder onderzoek gedaan naar het ziekteverwekkend vermogen van *C. gallinacea* in kippen. Na orale toediening, leidde een infectie met *C. gallinacea* niet tot acute klinische ziekte. *C. gallinacea* kon vooral worden aangetoond in epitheelcellen van het jejunum, ileum en caecum zonder zichtbare verschijnselen van ontsteking. Daarnaast werd *C. gallinacea* in macrofagen en dendritische cellen van de caecale tonsil aangetoond en werd een toename van antilichamen gemeten. Verspreiding van *C. gallinacea* naar andere organen werd niet aangetoond.

Als laatste is in **hoofdstuk 5** onderzocht of een doorgemaakte *C. gallinacea* infectie de kans op een infectie met *C. psittaci* kon verlagen. De resultaten van de infectie experimenten lieten geen onderbouwing zien voor deze veronderstelling. De afwezigheid van *C. psittaci* in de prevalentiestudie uit **hoofdstuk 2**, kan dus niet worden verklaard door mogelijke kruisbescherming (door de hoge prevalentie van *C. gallinacea*). Na een doorgemaakte *C. gallinacea* infectie is de uitscheiding via de cloaca bij een nieuwe *C. gallinacea* infectie met een andere stam wel lager.

In de algemene discussie (**hoofdstuk 6**) wordt besproken in hoeverre de resultaten over het voorkomen van *Chlamydia* in leghennen ook vertaald kunnen worden naar vleeskuikens of andere pluimveesoorten. Daarnaast wordt ingegaan op de vraag of *C. gallinacea* nu een ziekteverwekker is of niet en wat het niet aantonen van *C. psittaci* betekent voor zowel de dier- als volksgezondheid. De eindconclusie is dat *Chlamydia* infecties in kippen in Nederland op dit moment geen *One Health* probleem vormen. De huidige resultaten sluiten toekomstige introductie van de zoönotisch *C. psittaci* in kippen echter niet uit. Daarvoor zou verder onderzoek nodig zijn. Ook is het interessant dat *C. gallinacea* in staat is om zich intracellulair te vermeerderen zonder dat dit tot zichtbare ziekte of ontsteking leidt. Dit geldt overigens voor meer *Chlamydia* infecties en vraagt om fundamenteel onderzoek naar de gastheer-pathogeen interacties om beter te begrijpen wat ziek en gezond is.

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About the author

Marloes Heijne (1982) started her degree in Veterinary Medicine at the Ghent University in Belgium. After finishing her first year in Ghent, she returned to the Netherlands to study Veterinary Medicine at Utrecht University. Besides her study in Veterinary Medicine, Marloes finished a minor in public administration and organisation science at the Utrecht University School of Governance. In 2005, she completed her Master's thesis, on avian influenza dynamics in poultry and waterfowl with a focus on China, as part of a three-month internship at the Food and Agricultural Organisation (FAO) in Rome. In 2008, Marloes graduated as a veterinarian specialising in Farm Animal Health.

From 2008 to 2011 she worked as a farm animal veterinarian at the 'Dierenartspraktijk van Waard tot Klif' in Friesland. In 2011, she returned to Utrecht to work as a lecturer at the Farm Animal Health department of the Faculty of Veterinary Medicine. In her current job at Wageningen Bioveterinary Research (WBVR) in Lelystad, she is about to finish her training as a veterinary microbiologist. The research part of this training has continued into her PhD. Marloes participated in several research projects, including a ZonMW funded research project 'Plat4m-2bt-psittacosis'. She also acquired funding from the Dutch Ministry of Agriculture for the work presented in Chapters 3, 4 and 5 of this thesis. She has been a project leader on statutory tasks for notifiable bacterial animal diseases and zoonoses including avian chlamydiosis at WBVR since 2019.

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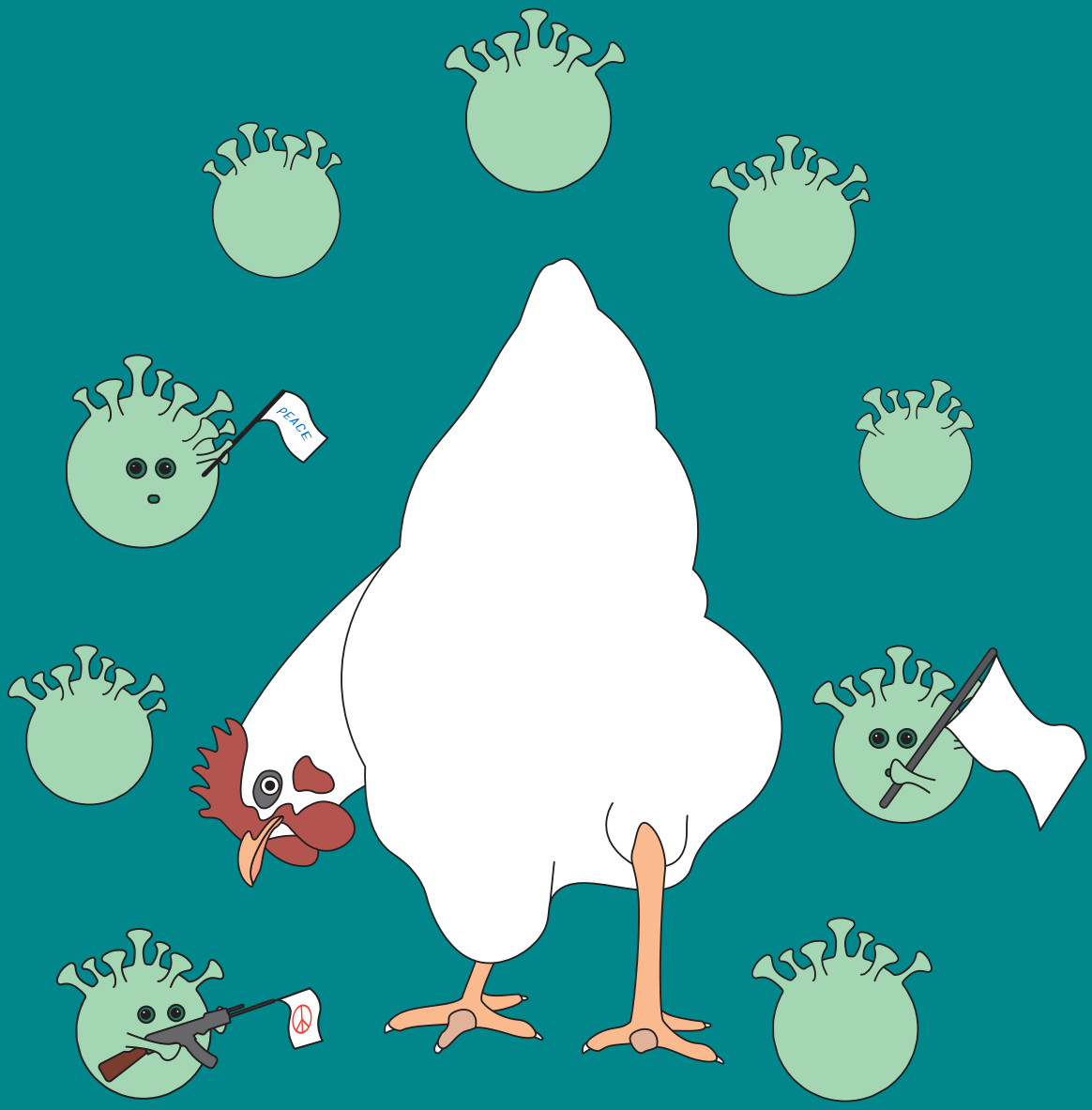
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