Chlamydia psittaci strains from broiler chickens induce histopathological lesions and mortality in SPF chickens

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Abstract

A detailed study on histopathological lesions induced by two C. psittaci outer membrane protein A (ompA) genotype B strains (10/423 and 10/525) and one genotype D strain (10/298) in experimentally infected (aerosol) specific pathogen free (SPF) chickens was performed. The strains were derived from Belgian and French commercially raised broilers with pneumonia. Both genotype B and D strains induced conjunctivitis, rhinitis, sinusitis, tracheitis, bronchitis, pneumonitis, airsacculitis, splenitis, hepatitis, nephritis, and enteritis in sequentially (days 2 to 34 post infection) euthanized chickens. Inflammation of the ovaries was only observed in genotype D infected chickens. Overall, the genotype D strain caused more severe gross and histopathological lesions and mortality (54.5%) early upon infection. The genotype D strain seemed to replicate faster as severity of the lesions increased more quickly. C. psittaci is a primary pathogen in chickens, and efficient monitoring and control of this emerging zoonotic pathogen is urgently needed.

Keywords: chicken, Chlamydia psittaci, histopathology.

Introduction

Chlamydia (C.) psittaci is an obligate, intracellular Gram-negative bacteria causing respiratory disease in birds. Recently, new Chlamydia species have been found in birds (13, 16, 21). In the past five years, several reports on C. psittaci infections in chickens have been published (5-7, 23, 24, 26). However, data on pathology of C. psittaci in chickens is still scarce. By the time this study was started, only six reports on experimental infections in chickens could have been found. Strains from the following birds and mammals have been used in these studies: a budgerigar (Izawa-1; genotype A), a parrot (GCP-1; no genotype specified), a pigeon (P-1041; no genotype specified) (17, 18), turkeys (strain C-1; no strain or genotype specified and the Turkey/California/181 strain; no genotype specified) (1, 2, 14, 15), and ruminants (B-577, Bo-Yokohama and SPV-789) (17). The avian strains (1013 Egg Lethal Dose 50) used by Takahashi et al. (17) induced a generalised infection in White Leghorn chickens within 10 d post infection (dpi) followed by death. Psittacine strains were more virulent than the pigeon strain, as they caused higher mortality. Ruminant strains were far less pathogenic to chickens than avian strains. A ruminant strain (DC15) created respiratory disease in bovine model of respiratory C. psittaci infection (11). Thus, chicken-derived strains were not used and only one of the used avian strains was genotyped. Avian C. psittaci is currently classified into the well-characterised outer membrane protein A (ompA) genotypes A-F and E/B. In the above experiments, chickens were not infected by the natural route, namely by inhalation of aerosols. Instead, Chlamydiaceae were directly injected into the air

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sac or trachea, or chickens were infected orally. Only Suwa et al. (14, 15) used specific pathogen free (SPF) chickens.

Until now, genotypes B, C, D, F, and E/B have been found in chickens (5-7, 23, 24, 26, 27). Genotypes B and D have been found most often, but less is known on their pathology in chickens. The purpose of this study was to perform a detailed analysis of the histopathological lesions induced by chicken-derived C. psittaci genotype B and D strains in experimentally infected (aerosol) SPF chickens. It would allow us to examine the general assumption that C. psittaci is not pathogenic, or less pathogenic to chickens, and therefore is of no importance in industrial raised chickens.

Material and Methods

Experimental design. Samples for the histopathological study were collected during an experiment to full-fill Hill-Evans postulates for chicken infectious C. psittaci strains. Results on the latter have been published (24).

C. psittaci strains. The following strains were used: C. psittaci, genotype B, strain 10/423 (isolated from a broiler with pneumonia from a Belgian farm), C. psittaci, genotype B, strain 10/525 (originated from another Belgian broiler farm, also associated with pneumonia), and C. psittaci, genotype D, strain 10/298 (originated from a broiler with pneumonia from a French farm) (24). Molecular characteristics was performed by a genotype-specific real-time PCR, allowing the identification of the outer membrane protein A (ompA) genotypes A to F and E/B (8), and by Multi Locus Sequence Typing (MLST) using seven housekeeping genes (enoA, fumC, gatA, gidA, hemN, hlfX, oppA) (12). The strains were grown in Buffalo Green Monkey (BGM) cells, as previously described (19). Bacterial titration was performed by the method of Spearman and Kaerber (9) to determine the 50% tissue culture infectious dose (TCID<sub>50</sub>) per mL.

Experimental infection. The experimental design was evaluated and approved by the Ethical Commission for Animal Experiments of Ghent University. Briefly, four groups of 22 one-day-old SPF chickens (Lohman, Cuxhaven, Germany) were individually tagged and housed in separate negative pressure isolators (IM1500, Montair, the Netherlands). At the age of one week, groups 1 to 3 were exposed for 1 h to an aerosol of 10<sup>6</sup> TCID<sub>50</sub> C. psittaci suspended in PBS (5 µm droplets; Cirrus™ nebulizer; Lameris, Belgium). The fourth group received an aerosol of PBS and served as non-infected control. Groups 1, 2, and 3 were infected with C. psittaci genotype B strain 10/423, C. psittaci genotype B strain 10/525, and C. psittaci genotype D strain 10/298 respectively.

Sampling for C. psittaci detection and histopathology. At 2, 4, 6, 8, 10, 14, 17, 21, 24, 28, and 34 d post infection (dpi), two chickens from each group were euthanized and subjected to a necropsy and gross pathological examination. The presence of C. psittaci was confirmed by examination (600x, Nikon Eclipse TE2000-E, Japan) of 5 µm thick frozen tissue sections (CM1950 cryotome; Leica Microsystems, Belgium) of the lungs, liver, and spleen using the IMAGEN™ Chlamydia immunofluorescence staining (Oxoid, United Kingdom), as previously described (20). Tissue samples of the conjunctiva, conchae, sinus, trachea, lungs, abdominal and thoracic air sacs, pericardium, spleen, liver, kidneys, jejunum, and ovary/testes of euthanized birds were taken for histopathology. They were fixed in 10% phosphate-buffered formalin, processed, embedded in paraffin, sectioned at 5 µm, and stained with haematoxylin and eosin. All slides were examined microscopically (Leitz, USA).

Results

Direct immunofluorescence (DIF) staining of frozen tissue sections confirmed the presence of C. psittaci in all infected animals, while C. psittaci was absent in controls. Replicating C. psittaci were detectable in lung tissue from 2 dpi and 4 dpi onwards in genotype D and B infected birds respectively. Progression to a systemic infection as demonstrated by replicating C. psittaci in the spleen and liver was evident as soon as 6 dpi and 8 dpi for genotype D and B strains respectively. The most severe gross and microscopic lesions were located at the lower respiratory tract, pericardium, and spleen. Twelve out of 22 (54.5%) genotype D infected chickens died between 7 and 10 dpi, while all genotype B infected chickens survived the infection. The high mortality rate in genotype D infected chickens early upon infection was concomitant to diffuse fibrinous thoracic and abdominal airsacculuiis, congested lungs with bilateral grey foci, fibrinous adhesive pericarditis, a severely enlarged spleen and kidneys, and petechiae on conjunctivae, liver, and spleen. Genotype B infected chickens also showed severe gross pathology in the lungs and air sacs early upon infection, but pericarditis was serous and not adhesive, spleen and kidneys were only slightly enlarged, and petechiae were not observed. In general, the nature of induced histopathological lesions in assessed tissues was similar for all three C. psittaci infection strains. However, lesions appeared quicker and conjunctivitis, tracheitis, bronchitis, pneumonitis, pericarditis, hepatitis, and nephritis were more severe in chickens of group 3 (genotype D) compared to groups 1 and 2 (genotype B). Due to high mortality in genotype D infected chickens, the course of infection with strain 10/298 could be only monitored until 21 dpi instead of 34 dpi, as was done for both genotype B strains. Necropsy of control birds did not reveal features of gross pathology. Histopathology of tissues of controls also did not reveal inflammation or lesions described for C. psittaci infected birds.
Conjunctivae. Gross pathological lesions of the conjunctivae in genotype B infected chickens were limited to congestion from 4 dpi onwards. Genotype D infected chickens revealed petechiae on the conjunctivae from 4 dpi until 7 dpi, later on lesions on the conjunctivae were also limited to congestion. At 6 dpi, focal infiltration of lymphocytes, macrophages, and heterophils was noticed in the lamina propria of conjunctivae of genotype B infected chickens. From 14 dpi onwards, epithelial hyperplasia was observed and infiltration of immune cells was extensive and diffuse. At 34 dpi, infiltrating immune cells were mainly lymphocytes, but epithelial erosion was observed in one of two euthanized chickens. Upon infection with genotype D, conjunctivitis was more severe and occurred sooner. Extensive diffuse infiltration of lymphocytes, macrophages, and heterophils in the lamina propria of conjunctivae was already observed at 6 dpi. From 14 dpi onwards, plasma cells infiltrated the lamina propria of conjunctivae, and epithelial vacuolisation and erosion were present.

Conchae. The conchae were slightly to severely congested in all infected birds between 4 dpi and 21 dpi. However, severe congestion was more often observed in genotype D infected birds. Starting from 6 dpi, the microscopic image in all groups revealed rhinitis characterised by infiltration of mainly lymphocytes and some heterophils in the lamina propria, which was diffuse on 6 dpi but focal from 14 dpi onwards.

Sinuses. On gross pathology, sinuses of groups 1, 2, and 3 were congested from 10 dpi to 21 dpi, from 8 dpi to 21 dpi, and from 4 dpi to 21 dpi respectively. Severe congestion was only observed in genotype D infected birds. Histopathology revealed sinusitis, characterised by a mild subepithelial infiltration of lymphocytes and heterophils in the lamina propria of sinuses of all infected animals. In group 3, these changes were present from 4 dpi until the end of the trial at 21 dpi, whereas in groups 1 and 2 sinusitis was only present from 10 dpi to 17 dpi and 21 dpi, respectively.

Tracheae. Slight congestion of the trachea was present from 8 to 17 dpi following infection with genotype B strains, whereas genotype D infection induced moderate to severe congestion of the trachea from 4 dpi until 17 dpi. Tracheitis was characterised by a moderate infiltration of heterophils from 4 dpi until 17 dpi (group 3), from 8 dpi until 17 dpi (group 1), and from 10 dpi until 21 dpi (group 2). In group 3, infiltrating immune cells were also present at 21 dpi, but at this time point these were mainly lymphocytes.

Bronchi. Bronchitis was observed from 8 dpi until the last euthanasia to 34 dpi in group 1, from 6 dpi until 21 dpi in group 2, and from 4 dpi until the last euthanasia on 21 dpi in group 3. Overall, bronchitis was most severe in group 3. The general features of bronchitis in all infected groups were characterised by lymphocytic infiltration in the lamina propria of the primary, secondary, and tertiary bronchi, sometimes associated with some heterophils, and activation of the bronchus associated lymphoid tissue (BALT) with formation of lymphoid follicles (follicular bronchiolitis). In genotype D infected chickens euthanized at 6 dpi, diffuse and moderate infiltration of lymphocytes in the lamina propria of bronchi was observed. Moreover, lymphocytic infiltration was also present in the epithelial layers. At 14 dpi, the histopathological lesions were characterised by activation of BALT, presence of lymphocytes and some heterophils in the bronchial walls, as well as serofibrinous exudate with heterophils in the bronchial lumen. Lesions in the tertiary bronchi were less severe at 21 dpi, with only minimal multifocal infiltration of lymphocytes and epithelial hyperplasia. In group 2, slight to mild follicular bronchiolitis was present at 6 dpi. At 14 dpi, moderate infiltration of lymphocytes and some heterophils in the lamina propria of bronchi was multifocal (n = 1) or diffuse (n = 1). BALT was activated (n = 2). At 34 dpi, infiltration of lymphocytes and some heterophils in the lamina propria was diffuse and mild in the primary and secondary bronchi, but multifocal and minimal in the tertiary bronchi. In group 1, no lesions were observed at 6 dpi. At 14 dpi, infiltration of mainly lymphocytes and some heterophils was diffuse and slight in the primary and secondary bronchi, but multifocal and mild in the tertiary bronchi. One of the euthanized chickens had luminal fibrinous exudate with heterophils in the bronchial lumen, while the other indicated follicle formation in the tertiary bronchus in the absence of exudate. At 34 dpi, follicular bronchiolitis and minimal luminal serofibrinous exudate with heterophils was present in both euthanized birds.

Lungs. Except for 2 dpi, the lungs of all infected chickens were bilaterally congested or displayed unilateral or bilateral grey foci. Grey foci in the lungs of genotype D infected birds were bilaterally present from 6 dpi until 14 dpi and unilaterally at 17 dpi. A genotype B infection resulted in unilateral grey foci at 8 dpi, which were manifested bilaterally from 10 to 14 dpi (group 2) or to 17 dpi (group 1), and again unilaterally until the end of the trial at 34 dpi. As for bronchitis, pneumonitis became evident upon infection with each of the three *C. psittaci* infection strains, but was most severe and occurred sooner in genotype D infected chickens. Pneumonitis was present from 4 dpi in group 3 and from 6 dpi in groups 1 and 2, and the lasted for all strains until the end of the trial. Within genotype B infected birds, pneumonitis was more severe following infection with strain 10/428 (group 1) as compared to strain 10/525 (group 2). Common histopathological lesions were infiltration of lymphocytes and heterophils in lung parenchyma, epithelial hyperplasia of-atra/infundibula, and formation of follicles in the BALT (Fig. 1). Groups 1 and 3 also showed serofibrinous exudate in the pleura and air capillary parenchyma respectively. Birds of group 1 suffered from severe pneumonitis at 6 dpi. Lesions were characterised by marked multifocal
(n = 1) or diffuse (n = 1) inflammation of lung parenchyma and presence of serofibrous exudate in the pleura. At 14 dpi and 21 dpi, minimal epithelial hyperplasia was observed and inflammation of lung parenchyma was minimal and diffuse. At 21 dpi, BALT was activated as shown by follicle formation. From 6 dpi until 14 dpi, the lungs of genotype B infected chickens presented multifocal moderate inflammation of lung parenchyma and formation of follicles in the BALT. Inflammation was absent in group 2 from 24 dpi onwards, but hyperplasia of the BALT was still observed until 34 dpi. In contrast, in group 1, inflammation of lung parenchyma was still present at 34 dpi. Multifocal infiltration of lymphocytes and some heterophils in lung parenchyma was moderate to mark. In addition, minimal multifocal epithelial hyperplasia was observed, as well as minimal to significant luminal serofibrous exudate with heterophils, and minimal multifocal infiltration of lymphocytes and heterophils in the pleura.

**Air sacs.** Gross pathology revealed diffuse opacity of thoracic and abdominal air sacs in all infected groups early upon infection (4 dpi). Later on, focal to diffuse fibrinous airsacculitis occurred (Fig. 2). In groups 1, 2, and 3, diffuse fibrinous airsacculitis was present from 10 to 17 dpi, from 10 to 14 dpi, and from 6 to 14 dpi, respectively. The nature of histopathological changes was similar between infected groups. However, likewise gross pathology, severe airsacculitis occurred much earlier upon the infection. The entire microscopic lesions were characterised by lymphocytic infiltrate, sometimes associated with follicle formation, serofibrous exudation, mesothelial hyperplasia, mesothelial necrosis with formation of fibrino-necrotic membranes on the surface, and proliferation of fibroelastic tissue as a repair reaction after exudation and/or necrosis (Fig. 3).

**Pericardium.** Aerogenous infection with *C. psittaci* caused serous pericarditis (genotype B strains) and fibrinous adhesive pericarditis (genotype D strain). Pericarditis was macroscopically evident from 8 dpi until 17 dpi in group 1, from 8 dpi until 21 dpi in group 2, and from 6 dpi until the last bird was euthanized at 21 dpi in group 3. Similar to the gross pathology, histopathological lesions of the pericardium were most severe in group 3 and occurred much earlier upon infection compared to groups 1 and 2. In group 3, severe fibrinous pericarditis was observed at 6 and 14 dpi, while in groups 1 and 2, moderate serofibrous pericarditis was present at 14 dpi. Pericarditis in group 3 was characterised by a severe thickening of the epicardium and diffuse infiltration of lymphocytes, macrophages, and heterophils in the epicardium. Thickening of the epicardium and infiltration of inflammatory cells was less pronounced in groups 1 and 2.

**Spleen.** Macroscopic lesions of the spleen in genotype B infected birds were evidenced by a slight to moderate splenomegaly from 8 dpi until 24 dpi (group 2) or 34 dpi (group 1). Genotype D infection resulted in slight splenic enlargement early upon infection (4 to 6 dpi), which later on progressed to severe splenomegaly (until 21 dpi) (Fig. 4) and presence of petechiae (until 17 dpi). Histopathological changes were severe in all groups and were observed in each infection group until the end of the trial. However, severe splenitis in group 3, occurred much earlier upon infection (4 dpi) compared to groups 1 and 2 (10 dpi). The histopathological image was characterised by the following lesions: hyperplasia of the reticulo-endothelial system with proliferation of macrophages, hyperplasia of the lymphoid system with proliferation of lymphoid cells, marked infiltration of lymphocytes, presence of fibrin thrombi, and epithelial hyperplasia. In addition, chickens of group 1 showed minimal to slight inflammation of the splenic capsule or serosa until 24 dpi.

**Liver.** Hepatomegaly was not noticed at gross pathology, but the liver was slightly congested from 8 dpi until 17 dpi in groups 1 and 2. In group 3, the liver was moderately congested from 7 dpi until 17 dpi, and slightly congested at 21 dpi. In addition, petechiae were present in group 3 from 7 dpi until 9 dpi. Hepatitis was present in all groups, but it was more severe and occurred earlier in group 3 (at 4 dpi) compared to groups 1 and 2 (at 8 dpi). From 24 dpi onwards, no hepatic lesions were observed in groups 1 and 2. At this time, all birds of group 3 were already euthanized or dead. At 6 dpi, hepatitis in group 3 was characterised by mild reticulosis (abnormal increase in histiocytes), moderate infiltration of heterophils in the space of Disse (perisinusoidal space), slight proliferation of bile ducts, and moderate periporal infiltration of lymphocytes and macrophages.

**Kidneys.** The kidneys in groups 1 and 2 were slightly enlarged from 8 dpi until 17 dpi and 24 dpi respectively. In group 3, the kidneys were moderately to severely enlarged from 6 dpi until 14 dpi, and slightly enlarged at 21 dpi. Histopathological changes were similar in nature in all groups, but were more pronounced in group 3. In group 3, nephritis was already observed at 6 dpi, when inflammation was still absent in the kidneys of other groups. Nephritis was characterised by minimal (groups 1 and 2) to moderate (group 3) infiltration of mainly lymphocytes and heterophils throughout the parenchymatous tissue. In group 3, inflammation was observed from 6 dpi until 21 dpi, while in groups 1 and 2, only from 10 dpi until 14 dpi.

**Jejuna.** The jejunum was slightly congested from 10 dpi, 6 dpi, and 7 dpi onwards in groups 1, 2, and 3 respectively. Mild enteritis was in group 1 only at 14 dpi, in group 2 at 14 dpi and 17 dpi, and in group 3 from 6 dpi until the end of the trial. In all infected groups, enteritis was characterised by focal, slight infiltration of the serosa by lymphocytes, macrophages, heterophils, and eosinophils.

**Ovaries/testes.** No gross pathological lesions were observed. Only in group 3, mild infiltration of lymphocytes and heterophils was found in the ovaries at 6 dpi and 14 dpi.
Fig. 1. Haematoxylin and eosin staining of the lung of an uninfected SPF chicken (photo 1) and SPF chickens aerogenously infected with C. psittaci with genotype D strain 10/298 at 4 dpi (photo. 2), genotype B strain 10/423 at 24 dpi (photos 3 and 4) and genotype B strain 10/525 at 24 dpi (photo 5). Histopathological lesions of pneumonitis were similar in nature between infection strains, but were more severe and occurred sooner in C. psittaci genotype D infected chickens. Photo 2: early occurrence of bronchial infiltration with lymphocytes and heterophils (black arrows). Photo 3: luminal serofibrinous exudate (dotted white line) with heterophils (black arrows). Photos 4 and 5: activation of the bronchus associated lymphoid tissue (BALT) with formation of lymphoid follicles (white arrows).

Fig. 2. Diffuse fibrinous airsacculitis in a C. psittaci genotype D infected SPF chicken at 9 dpi (7), compared to the healthy translucent thoracic air sacs in a non-infected SPF chicken (6).
Fig. 3. Haematoxylin and eosin staining of chicken thoracic air sacs of an uninfected SPF chicken (photo 8) and SPF chickens aerogenously infected with *C. psittaci* genotype D strain 10/298 at 4 dpi (photos 9, 10, and 11), genotype B strain 10/423 at 14 dpi (photos 12 and 13) and genotype B strain 10/525 at 14 dpi (photos 14 and 15). Photo 8: normal mesothelial lining (white line) and lamina propria (black line). Photos 9 to 15: severe aersacculitis occurring much earlier following genotype D infection. Overall, histopathologic lesions were for all infection strains characterised by mesothelial hyperplasia (11 - thick white line) and necrosis (9 - white arrow) with formation of sero-fibrinous exudation (10 - dotted white line) to fibro-necrotic membranes (12-15 - dashed with line). Lymphocytic infiltration in the lamina propria (9, 12-15 - dashed black line) with follicle formation (12, 14 - black open arrows), infiltration of heterophils (10 - black arrows), and proliferation of fibroblastic tissue (9-13 - thick black line) with neovascularisation (13 - white arrow).

Fig. 4. Enlarged and congested spleen in an SPF chicken following aerogenous infection with *C. psittaci* genotype D at 9 dpi (right), compared to the spleen of a non-infected SPF chicken (left).

Discussion

The obtained results demonstrated that aerogenous infections of SPF chickens with chicken-derived *C. psittaci* strains induced a clinical course of infection with complications outside the respiratory tract. Gross pathology did not differ in nature or less severe as described for naturally infected psittacine birds, which are often assumed to be particularly prone to develop severe pathology upon a *C. psittaci* infection. Gross lesions were not pathognomonic and varied widely. The most typical findings in parrots include fibrinous airsacculitis, pericarditis, splenomegaly, and/or hepatomegaly (4). Only hepatomegaly was not observed in the current trial.

Lesions in genotype D (strain 10/298) infected chickens occurred earlier upon infection and were more severe than the ones observed in chickens infected with the genotype B strains 10/432 or 10/525. The observed histopathological lesions were similar to those described in chickens, turkeys, or quail infected with non-chicken derived *C. psittaci* strains (3, 14, 19). Airsacculitis seems to be more pronounced in turkeys, as fibrinous necrotising airsacculitis was observed in turkeys infected with the virulent turkey-derived Texas Turkey and genotype D 92/1293 strains. The virulent chicken-
derived genotype D strain 10/298 induced fibrinous airsacculitis in the present trial. The latter seems to be host-related, as an infection of SPF chickens with strain 92/1292 also induced fibrinous airsacculitis (24). Further research should focus on host-related factors, such as early immune responses upon a C. psittaci infection in chickens and turkeys.

Genotype D strain 10/298 caused inflammation in the ovaries. Interestingly, C. psittaci antigen was demonstrated by DIF in the ovaries of genotype D infected chickens (23). Vertical transmission of C. psittaci occurs in chickens (22), but the extent or impact of vertical transmission of C. psittaci infections in chickens is yet to be examined.

Genotype D and especially genotype D strains cause severe respiratory disease in SPF chickens (24, 25). Until recently, it was commonly assumed that C. psittaci is less pathogenic for chickens than for turkeys and ducks. Possibly, this is why avian chlamydiosis is under investigation in chickens. However, recent epidemiological studies demonstrated its high prevalence in industrial environment. Thus, a systematic C. psittaci examination in case of respiratory disease in chickens is justified, in particular, when diseased birds display gross pathological and histopathological lesions as described here. Replication of C. psittaci is the highest in the air sacs and to a lesser extent in lung tissue (24, 25). The liver and spleen, which are frequently examined for differential diagnosis, are thus less desirable organs to detect C. psittaci.

In conclusion, C. psittaci is a primary pathogen for chickens, causing marked gross pathological and histopathological lesions, as it does in other avian species. C. psittaci is therefore of importance in industrial raised chickens.

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