Exacerbation of *Chlamydophila psittaci* pathogenicity in turkeys superinfected by *Escherichia coli*

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Abstract – Both *Chlamydophila psittaci* and *Escherichia coli* infections are highly prevalent in Belgian turkeys and therefore they both might contribute to the respiratory disease complex observed in turkeys. *C. psittaci* can infect turkeys within the first week of age, even in the presence of maternal antibodies. However, the first *C. psittaci* outbreaks occur mostly at the age of 3 to 6 weeks, the period when also *E. coli* infections appear on the farms. Therefore, we examined in this study the pathogenicity of an *E. coli* superinfection on *C. psittaci* predisposed turkeys. Turkeys were infected with *C. psittaci*, *E. coli* or with *C. psittaci* followed by *E. coli*. Simulating the impact of an *E. coli* infection during the acute phase or the latent phase of a *C. psittaci* infection, turkeys received *E. coli* at 1 or 5 weeks post *C. psittaci* infection, respectively. *E. coli* superinfection during the acute phase of *C. psittaci* infection increased *C. psittaci* excretion and stimulated chlamydia replication in the respiratory tract resulting in exacerbated clinical disease. Interestingly, *E. coli* superinfection during the latent phase of *C. psittaci* infection induced chlamydial replication, leading to increased *C. psittaci*-specific antibody titres. In addition, chlamydial predisposition gave higher *E. coli* excretion compared with turkeys that had only been infected with *E. coli*. Overall, the present study clearly demonstrates the pathogenic interplay between *C. psittaci* and *E. coli* resulting in more severe respiratory disease.

1. INTRODUCTION

Worldwide, turkey production suffers from the negative economical impact of respiratory disease. Nearly all turkey flocks experience one to multiple periods of respiratory disease leading to expensive med-
with respiratory disease [4, 7, 8, 12, 19, 25]. At present, *C. psittaci* is commonly found in Belgian, German and probably French turkeys\(^1\) [22, 28]. Devastating outbreaks with high mortality rates, similar to those between 1950 and 1970 in the USA occur occasionally, but respiratory signs without mortality mostly characterise present outbreaks (reviewed in [1]). Nevertheless, *C. psittaci* causes important economical losses and is a threat to public health since this zoonotic agent is able to infect poultry workers [5, 10, 15].

*E. coli* strains colonise the intestinal tract and protect the host against other pathogenic bacteria, but some strains induce respiratory disease in poultry [4]. The avian pathogenic *E. coli* (APEC) strains mostly isolated from broilers cause airsacculitis, pericarditis, peritonitis and necrotic foci in the liver [23]. APEC infections in broilers are mostly observed between 4 and 9 weeks of age and result in extensive economical losses [4]. In Belgium (1997–2000), APEC infections have an incidence of 17.7% in randomly selected broilers [24]. Nevertheless, APEC infections play only a complicating role, requiring predisposing environmental or host factors. Several researchers have demonstrated the exacerbation of respiratory disease in avian pneumovirus-, *Bordetella avium-* and *Mycoplasma meleagris*-infected turkeys by an *E. coli* superinfection\(^2\) [9, 14, 17, 18, 20].

*C. psittaci* is highly prevalent in Belgian turkeys and although the *E. coli* presence has not been intensively examined, occasional outbreaks with mortality do occur\(^3\). Thus, *E. coli* as well as *C. psittaci* may contribute to the turkey respiratory disease complex. *C. psittaci* can infect turkeys within the first week of age, even in the presence of maternal antibodies [28]. However, the first *C. psittaci* outbreaks in turkeys mostly occur at the age of 4 to 8 weeks, a period when also *E. coli* may appear. The present study tries to contribute to the elucidation of the turkey respiratory disease complex by examining the role of *E. coli* as a possible complicating factor during the acute and latent phase of a *C. psittaci* infection in experimentally infected specific pathogen-free (SPF) turkeys.

2. MATERIALS AND METHODS

2.1. *Chlamydophila psittaci* strain 92/1293

In the present study, *C. psittaci* genotype D strain 92/1293, isolated from a pooled homogenate of the lungs, cloaca and spleen of diseased Dutch broiler turkeys was used [25].

2.2. *E. coli* strain O2:K1

*E. coli* strain O2:K1 was kindly provided by H. Nauwynck (Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Belgium). The strain was isolated during a colibacillosis outbreak on a Belgian turkey farm [19].

2.3. Experimental design

The experimental design was evaluated and approved by the Ethical Commission for Animal Experiments of K.U.

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\(^3\) Van de Zande S., personal communication.
Table I. Experimental design.

<table>
<thead>
<tr>
<th>Group</th>
<th>C. psittaci infection</th>
<th>E. coli infection</th>
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</thead>
<tbody>
<tr>
<td>Acute C. psittaci infection</td>
<td>Week 1</td>
<td>Week 2</td>
</tr>
<tr>
<td>Acute C. psittaci infection</td>
<td>Week 2</td>
<td>/</td>
</tr>
<tr>
<td>Latent C. psittaci infection</td>
<td>Week 1</td>
<td>Week 6</td>
</tr>
<tr>
<td>Latent C. psittaci infection</td>
<td>Week 6</td>
<td>/</td>
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</tbody>
</table>

Leuven. The experimental design, simulating an E. coli superinfection during the acute phase (diseased turkeys, one week post infection) or latent phase (non-diseased turkeys, 5 weeks post infection) of a C. psittaci infection is presented in Table I. One-day-old turkeys (AFSSA, Ploufragan, France), claimed to be SPF, were divided into 8 groups of 13 turkeys. Turkeys were raised in negative pressure isolation units on wired floors (Montair, Sevenum, The Netherlands). At one week of age, groups 1, 3, 5 and 7 were aerosol-infected (Cirrus™ Nebulizer) with 10^4 tissue-culture-infective-doses50 (TCID50) per mL of C. psittaci genotype D strain 92/1293. Subsequently, groups 1 and 2 and groups 5 and 6 were oculo-nasally infected with 2.5 10^8 colony forming units (CFU) of E. coli strain O2:K1 at 1 and 5 weeks post C. psittaci infection, respectively. Groups 3 and 7 served as C. psittaci-infected, E. coli non-infected controls. Groups 2 and 6 only received E. coli at the ages of 2 and 6 weeks, respectively and served as E. coli-infected, C. psittaci non-infected controls. Groups 4 and 8 served as non-infected controls. In all groups, two turkeys were sacrificed at 3, 5, 7 and 10 days post E. coli infection. The remaining five turkeys of each group were sacrificed at 12 days post E. coli infection.

2.4. Samples

Turkeys were daily observed and clinical signs were scored as follows: score 0 = no clinical signs, score 1 = conjunctivitis, score 2 = score 1 + rhinitis, score 3 = score 2 + dyspnoea. Pharyngeal C. psittaci and E. coli excretion were monitored weekly and at 3, 5, 7, 10 and 12 days post E. coli infection. The samples were collected with cotton-tipped aluminium shafted swabs (Fiers, Kuurne, Belgium) in 2 mL complete C. psittaci transport medium [22]. Swabs were stored at –80 °C until tested.

Blood samples were collected by venipuncture (v. ulnaris) for the determination of C. psittaci major outer membrane protein (MOMP)-specific antibody titres immediately prior to C. psittaci infection and subsequently on a weekly basis. E. coli antibodies were not monitored due to the lack of an antibody detection test specific for avian pathogenic E. coli (APEC). Blood samples were stored overnight at room temperature, centrifuged (325 g, 10 min, 4 °C) and serum was collected and frozen at –20 °C until being tested.

The turkeys were examined for macroscopic lesions at autopsy. The samples of lungs and thoracic- and abdominal airsacs were stored at –70 °C for E. coli isolation, or fixed in 10% phosphate buffered formalin for histopathological examination, or imbedded in methocel and snap frozen in liquid nitrogen to assess the presence of C. psittaci by immunofluorescence staining.

2.5. C. psittaci isolation

Pharyngeal swabs were examined for the presence of C. psittaci by isolation in Buffalo Green Monkey (BGM) cells and IMAGEN™ direct immunofluorescence (DakoCytomation, Denmark), as previously described [26]. Chlamydophilae-positive cells were scored between 0 and
5: score 0 indicated no chlamydophilae-positive cells present; score 1 was given when a mean of 1 to 5 elementary bodies was present per slide; score 2 was given when a mean of 6 to 10 elementary bodies was present; scores 3, 4 and 5 were given when a mean of 1–5, 6–10 and >10 inclusion-positive cells, respectively were present.

2.6. Indirect immunofluorescence staining of C. psittaci in lungs and airsacs

The lungs and thoracic airsacs were analysed for the presence of C. psittaci MOMP by in situ immunohistochemical staining. Cryostat sections (10 µm) were examined by indirect immunofluorescence using a monoclonal antibody (MAb) directed against a family-specific epitope of the C. psittaci MOMP. Briefly, all dilutions were made in PBS (pH 7.3). Acetone-fixed cryostat tissue sections were washed in PBS for 5 min. The slides were incubated with 25 µL undiluted chlamydophilae-negative goat serum for 1 h at 37 °C. Subsequently, the slides were washed in PBS (2 × 5 min) and incubated for 45 min at 37 °C with 25 µL of the diluted family-specific MAb (1:200). The sections were washed in PBS (2 × 5 min) and incubated for 30 min at 37 °C with 25 µL of 1:30 diluted goat anti-mouse immunoglobulins labelled with fluorescein isothiocyanate (FITC; DakoCytomation, Denmark). Finally, the slides were washed in PBS (2 × 5 min) and in distilled water (2 × 30 s) and analysed with a fluorescence microscope (Leitz, Wetzlar, Germany). Our attention was especially focussed on the presence of inclusions demonstrating active C. psittaci replication versus elementary bodies in the absence of inclusions indicating that replication no longer occurred. The results were scored as mentioned for the isolation in BGM cells. Staining controls consisted of sections from non-infected SPF turkeys as well as from C. psittaci positive SPF turkeys from previous experiments [30].

2.7. C. psittaci antibody response

The enzyme-linked immunosorbent assay (ELISA) using recombinant MOMP was performed on turkey sera as previously described [29]. Anti-MOMP immunoglobulin titres were presented as the reciprocal of the highest serum dilution that gave an optical density (OD$_{450}$) above the cut-off value. The cut-off value was the mean absorbance of three seronegative turkeys ± three times the standard deviation (SD). Negative control sera were obtained from one-week-old SPF turkeys (AFSSA, Ploufragan, France). Positive control sera originated from experimentally infected SPF turkeys of previous experiments [30].

2.8. E. coli isolation

Pharyngeal swabs were shaken at 4 °C for 1 h and centrifuged (10 min, 2790 g, 4 °C). Tissue suspensions of lungs and airsacs (10%, w/v) were centrifuged (200 g, 20 min) and subsequently used for bacterial quantification. The presence of E. coli in pharyngeal excretions or tissues was determined by incubating ten-fold serial dilutions of pharyngeal swab supernatant on MacConkey agar (OXOID, Great-Britain). Dark red/purple colonies with typical E. coli morphology were counted and titres were expressed as log$_{10}$ CFU/g mucus.

The E. coli O-serotype was determined by sero-agglutination. Individual colonies were inoculated in 4 mL Luria-Bertani medium and grown overnight at 37 °C in a shaking incubator. Subsequently, E. coli were centrifuged (5000 g, 10 min, room temperature) and re-suspended in 1 mL sterile PBS (pH 7.3). Twenty-five microlitres of this suspension were incubated
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Figure 1. Mean score for clinical signs during the acute phase of a C. psittaci infection for the dual infected turkeys (group 1, □) and the single C. psittaci infected turkeys (group 3, ▲). a Score 0 = no clinical signs, score 1 = conjunctivitis, score 2 = score 1 + rhinitis, score 3 = score 2 + dyspnoea. b Day of E. coli infection. Days on which clinical signs are significantly different between the two groups are boxed.

with 25 µL of O1, O2 and O78 E. coli antisera in a 96 well agglutination test plate for 1 h at room temperature and subsequently for 24 to 48 h at 4 °C.

2.9. Histopathology

Formalin-fixed samples were dehydrated, embedded in paraffin and 5 µm sections were obtained. The sections were stained with hematoxylin-eosin and Periodic Acid Shiff.

2.10. Statistics

All statistical analyses were performed using the SAS software, version 8.2 (the SAS Institute, Cary, NC, USA). Due to non-normalised distribution of chlamydial excretion scores, the Kruskal-Wallis analysis was used to determine differences. Antibody titres in the serum were analysed using Proc GLM (multiple two-tailed t-test). \( P \leq 0.05 \) was considered significant.

3. RESULTS

3.1. Clinical signs and gross lesions

Non-infected turkeys (groups 4 and 8) and E. coli infected, C. psittaci non-infected turkeys (groups 2 and 6), remained free of any clinical signs of respiratory disease throughout the experiment. Moreover, these animals were free of any gross lesion on post mortem examination performed in week 4 or 8.

First clinical signs appeared in the C. psittaci-infected turkeys (groups 1 and 3, as well as 5 and 7) at 4 to 5 days post-infection (Fig. 1). Eight days post-infection, all C. psittaci-infected animals were clinically affected, showing conjunctivitis, rhinitis and/or dyspnoea. In the absence of E. coli superinfection these clinical signs stabilised and disappeared by day 14 to 16 post C. psittaci-infection (groups 3, 5 and 7). In contrast, more severe clinical signs were observed from days 12 to 16 post C. psittaci-infection (\( \alpha = 0.05 \)) in C. psittaci infected, E. coli superinfected turkeys and were completely resolved by day 17 (Fig. 1).
Table II. Pharyngeal C. psittaci excretion during the acute phase of C. psittaci infection.

<table>
<thead>
<tr>
<th>dpea</th>
<th>Mean scores of pharyngeal C. psittaci shedding ± standard deviation (% positive turkeys)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>group 1</td>
<td>2.4 ± 0.89</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
</tr>
<tr>
<td>group 3b</td>
<td>2.2 ± 0.84</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
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</tbody>
</table>

a dpe: days post E. coli infection. b Group 3 only received C. psittaci. c Score 0: no chlamydophilae present; score 1: a mean of 1–5 positive cells, containing C. psittaci elementary bodies, per slide; score 2: a mean of 6–10 positive cells, containing elementary bodies; scores 3, 4 and 5: a mean of 1–5, 6–10 and > 10 positive cells inclusion respectively.

During the acute phase of C. psittaci infection, no marked differences in macroscopic lesions could be detected between the C. psittaci infected turkeys (group 3) and the E. coli superinfected turkeys (group 1); all those turkeys, sacrificed between 3 and 10 days post E. coli infection, showed conjunctivitis, sinusitis, rhinitis, pneumonia, airsacculitis and/or enlargement of the spleen. Two turkeys of group 5, which had been superinfected with E. coli 5 weeks post-C. psittaci infection, showed airsacculitis and serous to fibrinous pericarditis and one turkey showed a strongly enlarged spleen. Conversely, the turkeys of the C. psittaci infected control group 7 showed no macroscopic lesions on post-mortem examination 7 weeks post-infection.

3.2. C. psittaci excretion

Pharyngeal swabs of the C. psittaci non-infected control groups 2, 4, 6 and 8 remained chlamydophilae-negative throughout the experiment, as pharyngeal swabs of groups 1, 3, 5 and 7 prior to C. psittaci infection. Upon infection, overall C. psittaci shedding in group 1, superinfected with E. coli, was higher than in group 3 (only C. psittaci infection), although statistically not significant (Tab. II). Pharyngeal C. psittaci excretion in groups 1, 3, 5 and 7 lasted for about 12 days. Thereafter, excretion was no longer observed, not even in the turkeys of groups 1 and 5, which had been superinfected with E. coli.

3.3. C. psittaci isolation from lungs and airsacs

In groups 1, 3, 5 and 7, replicating chlamydophilae could be detected in air sacs and/or lungs until 14 days post C. psittaci infection (Tab. III). From that day on, air sacs and/or lungs showed a mean of 1 to 5 elementary bodies per five microscopic fields. Interestingly, the turkeys superinfected with E. coli during acute C. psittaci infection (group 1) showed chlamydial replication until the end of the experiment.

Five weeks post C. psittaci infection, chlamydophilae could not be detected anymore in the respiratory tract of groups 5 and 7. However, superinfection with E. coli (group 5) reinitiated significant C. psittaci replication in both lungs and airsacs.

3.4. C. psittaci antibody response

At one week of age, all turkeys (claimed SPF) showed high antibody titres (log2 7 to log2 12) against C. psittaci (exemplified by turkeys of group 5 and 7 in Fig. 2). However, since no chlamydophilae could be isolated and a decrease in antibody titres
### Table III. Presence of C. psittaci in frozen tissue sections of lungs and airsacs during the acute and latent phase of C. psittaci infection.

<table>
<thead>
<tr>
<th>Group</th>
<th>Lung 3 (n = 2)</th>
<th>Airsacs 3 (n = 2)</th>
<th>Lung 7 (n = 2)</th>
<th>Airsacs 7 (n = 2)</th>
<th>Lung 10 (n = 2)</th>
<th>Airsacs 10 (n = 2)</th>
<th>Lung 12 (n = 5)</th>
<th>Airsacs 12 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
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<tr>
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</table>

a dpe: days post E. coli infection. b n: number of turkeys. c Scores: score 0 indicated no chlamydophilae positive cell; score 1: mean of 1 to 5 elementary bodies in the absence of replicating reticulate bodies; score 2: mean of 6 to 10 elementary bodies in the absence of replicating reticulate bodies; scores 3, 4 and 5: mean of 1–5, 6–10 and >10 inclusion positive cells, respectively.

### 3.5. E. coli isolation

Regardless of the time point of E. coli infection (2 or 6 weeks of age), the highest pharyngeal E. coli excretion was always observed 3 days post E. coli infection and at that time excretion was significantly higher in C. psittaci predisposed turkeys (groups 1 and 5, $P < 0.05$) than in non-predisposed turkeys (groups 2 and 6, Fig. 3). At all time points, a tendency of higher E. coli excretion was observed in the dual infected groups compared to the single E. coli infected groups. Importantly, all randomly selected E. coli colonies had serotype O2, identical to the serotype of the challenging strain.

E. coli was never isolated from any of the internal tissues of the E. coli non-infected control turkeys (groups 3, 4, 7 and...
Figure 3. Pharyngeal E. coli excretion (log_{10}CFU/g mucus) during the acute phase (A; groups 1 (■) and 2 (□)) and latent phase of a C. psittaci infection (B; groups 5 (■) and 6 (□)).

3.6. Histopathology

The trachea, lungs and airsacs of turkeys showing gross lesions (i.e. C. psittaci-infected groups 1, 3, 5 and 7) were examined. Turkeys of groups 2 and 6, which only received E. coli, did not develop macroscopic lesions and were therefore not examined histologically. Dual infected turkeys (group 1), showed no distinct histopathological differences with turkeys only infected with C. psittaci (group 3), except for lymphocyte infiltrations in the tracheal mucosae of group 3. Lungs and airsacs were comparable for both groups, showing capillary congestion and infiltration of heterophils. Histopathological lung lesions were clearly more severe in latently C. psittaci infected, E. coli superinfected turkeys (group 5),
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compared to single C. psittaci infected turkeys (group 7). The lungs showed capillary congestion and multiple lymphoid aggregates with central necrosis and giant cells. Yet, the airsacs of groups 5 and 7 showed similar lesions: epithelial desquamation, heterophilic infiltration and multiple lymphoid aggregates.

4. DISCUSSION

The outcome of respiratory disease in turkeys receiving a superinfection with E. coli O2:K1 during the acute or latent phase of a C. psittaci infection was studied. Notwithstanding the presence of C. psittaci-specific maternal antibodies, the chlamydophila infection alone caused conjunctivitis, rhinitis and dyspnoea for about 15 days resulting in pneumonia, airsacculitis, enlargement of the liver and spleen and chlamydial excretion until 12 days post infection. Thus, maternal antibodies did not block the C. psittaci infection and did not interfere with the experimental setup. Indeed, it has been demonstrated that one-week-old turkeys can become infected with C. psittaci, regardless of the presence of maternal antibodies [21, 28]. Nevertheless, we cannot rule out that the maternal antibodies might have reduced the severity of the C. psittaci infection, since antibodies can contribute to host protection, although CD4+ T helper 1 lymphocytes and interferon-γ are considered more important for protective immunity [11, 16]. Indeed, serovar-specific mucosal antibodies can impede the infection at the mucosal entrance gate, neutralizing C. psittaci before cellular adherence [3]. In addition, antibodies can intervene during extracellular dissemination of the bacteria.

Turkeys infected at one week of age with C. psittaci, received at 2 or 6 weeks of age an E. coli superinfection to simulate field situations [22, 25]. The C. psittaci-E. coli dual infection resulted in a respiratory disease with more severe clinical signs than infections with either agent alone. Severe conjunctivitis, rhinitis and dyspnoea were only observed in the dual infected groups and correlated well with the observed macroscopic lesions such as pneumonia, airsacculitis and pericarditis, and with the presence of replicating chlamydophilae in the pharyngeal swabs. Interestingly, due to the E. coli infection in the latently C. psittaci infected turkeys of group 5, anti-MOMP antibody titres increased further, whereas they decreased significantly to almost undetectable levels in the E. coli non-infected group 7. This indicates that reactivation of C. psittaci in E. coli superinfected turkeys took place. Indeed, C. psittaci replication in the lungs and air sacs was detected in the E. coli challenged turkeys (group 5), whereas not in the E. coli non-infected turkeys (group 7).

In accordance with other experimental infections in SPF or conventional turkeys [20], infection with E. coli alone produced no clinical signs or macroscopic lesions. In both latently or acutely C. psittaci infected turkeys, it was clear that a predisposing C. psittaci infection enhances colonisation and invasion of E. coli in the respiratory tract as well as pharyngeal E. coli excretion. The results of this study are consistent with observations in Bordetella avium-, avian pneumovirus-, Ornithobacterium rhinotraceale- or Mycoplasma meleagridis-infected turkeys receiving an E. coli superinfection [9, 14, 17, 18, 20]. However, E. coli excretion titres were not as high as those observed by Van de Zande et al. [20] in an APV-E. coli dual infection. C. psittaci replicates in mucosal epithelial cells and macrophages causing epithelial deciliation, desquamation and lysis. Consequently, epithelial degeneration during intracellular C. psittaci replication may allow extensive E. coli colonisation of the respiratory tract. Moreover, defence mechanisms of macrophages might have
been reduced by the *C. psittaci* infection of the macrophages themselves [13, 27].

In conclusion, the present study examined the pathogenic interplay between *C. psittaci* and *E. coli* O2:K1 in experimentally infected turkeys. A superinfection with *E. coli*, 1 or 5 weeks (acute and latent phase, respectively) after the *C. psittaci* infection, exacerbated the respiratory disease with a resurgence of *C. psittaci* replication. This study contributes to the unravelling of the multifactorial respiratory disease complex in turkeys, illustrating the pathogenic interplay between *C. psittaci* and *E. coli*.

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