Longitudinal monitoring for respiratory pathogens in broiler chickens reveals co-infection of *Chlamydia psittaci* and *Ornithobacterium rhinotrapehale*

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*Chlamydia psittaci* is prevalent in broiler chicken production. However, the role of *C. psittaci* in the respiratory disease complex needs to be clarified. Our aim was to identify the time point when a *C. psittaci* infection appeared on a broiler farm and to examine the presence of other respiratory pathogens at that time. We focused on the ‘major’ respiratory pathogens occurring in Belgian broilers, namely infectious bronchitis virus (IBV), avian metapneumovirus (aMPV), *Ornithobacterium rhinotrapehale*, *Mycoplasma gallisepticum* and *Mycoplasma synoviae*, and examined their co-occurrence with *C. psittaci* on three commercial broiler farms. For all farms, 1-day-old broilers showed high maternal antibody titres against *C. psittaci* in the presence of viable *C. psittaci*. Maternal antibodies seemed to protect against respiratory signs. Maternal antibodies declined and clinical outbreaks could be identified serologically even before maternal antibodies completely disappeared. Mixed infections with genotypes B/C and B/C/D were observed. Broilers with *C. psittaci* antibody increases showed conjunctivitis, signs of upper respiratory disease and dyspnoea. *C. psittaci* always preceded an *O. rhinotrapehale* infection. Infections with aMPV, IBV or *Mycoplasma* spp. were not observed. Evidence was provided that *C. psittaci* could occur at an early age in broilers without a predisposing respiratory infection. Both *C. psittaci* and *O. rhinotrapehale* should be considered when developing prevention strategies for respiratory disease in broilers.

†These authors contributed equally to this work.

**Abbreviations:** aMPV, avian metapneumovirus; IBV, infectious bronchitis virus; MOMP, major outer membrane protein; NAAT, nucleic acid amplification technique; NDV, Newcastle disease virus; URTD, upper respiratory tract disease.

**INTRODUCTION**

Nucleic acid amplification techniques (NAATs) have given us the opportunity to detect *Chlamydia psittaci* in a fast, sensitive and specific way. Moreover, NAATs have allowed the molecular characterization of *C. psittaci*. Ever since applying NAATs, *C. psittaci* has been detected more often in chickens. Virulent *C. psittaci* strains were detected by NAATs, and isolated from diseased chickens raised in Australia, Belgium, China, France and Germany (Gaede et al., 2008; Zhang et al., 2008; Laroucau et al., 2009; Robertson et al., 2010; Zhou et al., 2010; Yin et al., 2013a). Recently, *C. psittaci* was detected in a Belgian chicken slaughterhouse and in a Belgian chicken hatchery. Zoonotic transmission occurred at both locations (Dickx et al., 2010; Dickx & Vanrompay, 2011). Yin et al. (2013a, b) demonstrated the occurrence of highly and less virulent *C. psittaci* strains in broilers raised in Belgium and Northern France, and they were able to prove the Hill–Evans postulates for chicken-derived *C. psittaci* genotype B and D strains. Moreover, Lagae et al. (2013), found *C. psittaci* in broiler breeders, broilers and layers, and their transmission to farmers. Humans (*n*=2) on the *C. psittaci*-negative farm never had respiratory complaints, whilst 25 of 29 (86.2 %) humans, all working on *C. psittaci*-positive farms, reported yearly medical complaints potentially related to psittacosis. Four (12.5 %) of 31 farmers mentioned in the questionnaire that they had pneumonia after starting to raise chickens, which was higher than the yearly rate of 8 : 1000 pneumonia cases in Belgium. *C. psittaci* seems to be emerging or re-emerging in broiler chicken production. However, the role of *C. psittaci* in the respiratory disease complex is unclear and needs to be clarified.

When *C. psittaci* infects chickens, it is often considered to co-infect with a virus, another bacterium or even fungi, although only three such case reports have been described (Malkinson et al., 1987; Reetz & Schultze, 1995; Shi et al., 2003). Beeckman et al. (2010) determined the cytokine responses following *C. psittaci* infection of chicken macrophages. High IL-10 and no transforming growth factor-β responses were observed. This could induce macrophage
deactivation and NFκB suppression, and thereby could
dampen innate immunity, rendering the birds more
susceptible to other pathogens.

Virulent *C. psittaci* strains are found more often in broilers
and recently Sachse & Laroucau (2014) presented evidence
for the existence of a new member of the order *Chlamydiales*,
namely *Chlamydia gallinacea* sp. nov., comprising strains from
poultry. The present study focuses on the 'major' well-
recognized respiratory pathogens occurring in Belgian
broilers, namely infectious bronchitis virus (IBV), avian
metapneumovirus (aMPV), *Ornithobacterium rhinotragale*,
*Mycoplasma gallisepticum* and *Mycoplasma synoviae*, and
evaluates their co-occurrence with *C. psittaci*. Belgian chicken
farms are like those in the rest of Western European and US
chicken production units, i.e. modern industrial farms. We
do implement Sanitel – a traceability system that tracks
products through the entire supply chain. It is a prerequisite
to an effective supply chain and quality management. The
present study identified the time point when a *C. psittaci*
infection appeared on three broiler chicken farms and
examined the presence of other respiratory pathogens from
production onset until slaughter in order to find concurrent
respiratory pathogens.

**METHODS**

**Farm management.** Farm selection was based on (1) only broiler
chickens present, (2) minimum of 10 000 chickens per barn and (3)
the farmer’s cooperation. The study was conducted in one, ad
randomly chosen barn of two medium size (total capacity 60 000
broilers; farms A and B) and one large size (total capacity 120 000
broilers; farm C) Belgian farms located in East Flanders.

All farms applied an all-in/all-out management schedule with a
sanitary service period of 2 weeks between slaughter and restocking
(six broods per year) during which the barns were cleaned and
discharged. Farms tested negative for *Salmonella*. *Salmonella* testing
was routinely performed by the Federal Agency for Food Safety upon
arrival of the chickens and 3 weeks before slaughter according to EU
regulations (2160/2003, frequency; 646/2007, broilers). Samples were
examined by culture and serotyping was performed if positive.

During the first week, broilers were raised at an ambient temperature
of 29–32 °C, reducing the temperature slowly to 23–21 °C by the
time the birds were shipped. Mechanical ventilation was regulated as
required. The farmers checked the climate four times a day. The
climate was regulated using a computer-controlled cooling/heating/
ventilation system (Barn computer installation; Stalinrichting A & J
De Jaeger). Hens and toms were raised in the same climate-controlled
barn with a soft floor covered with crushed flax cores. The lighting
schedule was 24 h light during the first 36 h (24 h light:0 h dark),
18 h light:6 h dark at age 3–14 days, followed by a gradual increase
in hours of light (+15 min per day) to reach 20 h light:4 h dark at
age 21 days, which was maintained until slaughter age. At age 35–
36 days, broiler density was reduced by removal of approximately
one-third of the remaining birds, for which the rearing period was
thus ~1 week shorter. All birds were vaccinated against Newcastle
disease virus (NDV), IBV and infectious bursitis virus (Gumboro
disease). All animals were treated according to the advice of the
company veterinarian. Additional details on farm management and
vaccination against respiratory disease in breeders (origin of the
broilers under study) and broilers are presented in Table 1.

**Study concept.** One-day-old broiler chickens were monitored until
slaughter according to a previously described study concept for
broiler turkeys (Verminnen et al., 2006; Van Droogenbroeck et al.,
2011). Observations, sampling age, specimens and tests used are
presented in Table 2. The occurrence of conjunctivitis, signs of upper
respiratory tract disease (URTD) and dyspnoea was scored weekly at
barn level. Mortality and antibiotic use were recorded daily at barn
level. At age 1 day, 20 randomly selected 1-day-old toms were
individually tagged with a leg number and their back was marked
with blue ink to allow on-site, rapid identification, as they were
allowed to move freely throughout the barn. Broilers were sampled
at age 1 day, and at 7, 14, 21, 28, 35 and 40 days. Bio-aerosol
monitoring was performed at the same times plus just before
stocking, in a cleaned and disinfected barn. The following samples
were collected: (1) blood for *C. psittaci*, *O. rhinotragale*, *M.
gallisepticum*, *M. synoviae* and aMPV serology; (2) pharyngeal swabs and
air samples for *C. psittaci* isolation, and subsequent molecular
characterization; and (3) pharyngeal swabs for (i) *O. rhinotragale*
16S rDNA PCR, (ii) *M. gallisepticum/M. synoviae* real-time PCR, (iii)
real-time reverse transcription (RT)-PCR for aMPV subtypes A–D
and (iv) RT-PCR for IBV; samples were examined retrospectively,
when the animals were already slaughtered. First, ELISAs were
performed in order to test all sera for a three- to fourfold rise in
antibody titre in order to detect any ongoing respiratory infection. At
the seroconversion time point, attempts were made to detect the
respiratory pathogens. The latter study concept was also used by Van
Loock et al. (2005).

**Sample processing prior to analyses.** Blood samples were
collected by venipuncture of the cutaneous ulnar vein (vena ulnaris)
and stored overnight at room temperature. Sera were collected after
centrifugation (300 g, 10 min, 48 °C), pre-treated with kaolin to
remove ELISA background activity (Novak et al., 1993) and stored at
−20 °C until tested for the presence of antibodies.

Rayon-tipped, aluminium shaft swabs (Copan; Fiers) were used to
sample the pharynx. Swabs for *C. psittaci* culture and subsequent
molecular typing of isolates contained 2 ml *Chlamydia* transport
medium (Vanrompay et al., 1992) and were transported at 4 °C.
Upon arrival in the laboratory, swabs were shaken for 1 h at 4 °C and
centrifuged (10 min, 2790 g, 4 °C). Supernatants were stored at
−80 °C until inoculation on cell culture. Swabs for different PCRs
contained 2 ml RNA/DNA stabilization reagent (Roche) and were
transported at 4 °C. Swabs were shaken for 1 h at room temperature.
DNA extraction was performed as described by Van Loock et al.
(2005). DNA extracts were stored at −80 °C until tested. The amount
of live *C. psittaci* in the air was determined by the use of a MAS-100
EcoSampler (Merck) in combination with ChlamyTrap 1 air
collection medium, as described by Van Droogenbroeck et al.
(2009). Samples were stored at −80 °C until inoculation on cell
culture.

**ELISAs.** Sera were analysed by a major outer membrane protein
(MOMP)-based *C. psittaci* ELISA (Verminnen et al., 2006). Anti-
MOMP IgG (H + L) titres were determined using a standard protocol
twofold dilutions starting at 1/100 and micro-well plates coated with
recombinant *C. psittaci* MOMP prepared in transient transfected
CO-7 cells (Vanrompay et al., 1998). In addition, *O. rhinotragale*,
*M. gallisepticum*/M. synoviae, IBV and aMPV antibody titres were
determined using four indirect commercial ELISAs (FlockChek ORT,
MG/MS, APV and IBV Antibody Test kits; IDEXX). All tests were
performed according to the manufacturer’s guidelines.

**Culture and genotyping of *C. psittaci*.** Pharyngeal swabs and air
samples were examined for the presence of viable *C. psittaci*. Culture
was performed using BGM (buffalo green monkey) cells, identifying
the organism by direct immunofluorescence staining (IMAGEN;
Table 1. Information on farm management and vaccination against respiratory pathogens in breeders and broilers (WVPA- België, 2010).

<table>
<thead>
<tr>
<th>Farm</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of breeders (weeks)</td>
<td>52.5</td>
<td>53</td>
<td>49</td>
</tr>
<tr>
<td>Antibiotic treatment of breeders</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Respiratory vaccines: breeders</td>
<td>NDV: Nobilis ND clone 30 day 12; Nobilis IB M41 + D274 and NC clone 30 day 56; Nobilis RT + IB Multi + ND + EDS day 119</td>
<td>Nobilis ND clone 30 day 14; Nobilis IB Multi + ND day 119</td>
<td>Hipraviar La Sota day 12; Nobilis RT + IB Multi + ND + EDS day 119</td>
</tr>
<tr>
<td></td>
<td>IBV: Poulvac IB primer day 1; Nobilis IB 4-91 day 12; Nobilis IB Multi + ND day 56; Poulvac IB QX day 70; Nobilis RT + IB Multi + ND + EDS day 119</td>
<td>Nobilis IB Ma5 day 28; Nobilis IB Ma5 day 84; Nobilis IB Multi + ND day 119</td>
<td>Poulvac IB primer day 1; Nobilis IB 4-91 day 12; Poulvac IB QX day 70; Nobilis RT + IB Multi + ND + EDS day 119</td>
</tr>
<tr>
<td>aMPV</td>
<td>Nobilis RTV 8544 day 84</td>
<td>None</td>
<td>Nobilis RTV 8544 day 84</td>
</tr>
<tr>
<td>Infectious laryngotracheitis virus</td>
<td>Nobilis IB Multi + ND + EDS day 119</td>
<td>None</td>
<td>Nobilis RT + IB Multi + ND + EDS day 119</td>
</tr>
<tr>
<td>Disinfection of broiler barn</td>
<td>Poulvac ILT day 42</td>
<td>Poulvac ILT day 70</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Walls and floor: 1000 l H2O + 50 l formol + 5 l Hi-Logic</td>
<td>Walls and floor: 2 l Virocid in 400 l H2O</td>
<td>Walls and floor: 25 l formol in 250 l H2O; fumigation: 25 l formol + 5 l MS Megades</td>
</tr>
<tr>
<td>Dimension of broiler barn (m²)</td>
<td>900</td>
<td>800</td>
<td>1600</td>
</tr>
<tr>
<td>No. of broilers in barn</td>
<td>18 000</td>
<td>14 000</td>
<td>30 000</td>
</tr>
<tr>
<td>Stocking density of broilers (m⁻²)</td>
<td>20</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>Slaughter age (days)</td>
<td>40</td>
<td>42</td>
<td>41</td>
</tr>
<tr>
<td>Respiratory vaccines: broilers</td>
<td>NDV: Nobilis ND C2 day 1</td>
<td>Nobilis ND C2 day 1</td>
<td>Nobilis ND C2 day 1</td>
</tr>
<tr>
<td></td>
<td>IBV: Poulvac IB primer day 1</td>
<td>Poulvac IB primer day 1</td>
<td>Poulvac IB primer day 1</td>
</tr>
</tbody>
</table>

Suppliers of vaccines: Nobilis vaccines (MSD Animal Health), Poulvac vaccines (Zoetis), Hipraviar vaccine (Hipra Benelux). Suppliers of disinfectants: Hi-Logic (Agro 2000), Virocid (CID Lines) and MS Megades (Schippers).
Clinical signs, mortality and antibiotic use

Clinical signs, mortality and antibiotic use are presented in Figs 1, 2 and 3. Conjunctivitis, signs of URTD and dyspnoea were present on all farms. On all farms, clinical signs were first observed at age 7 days. At that time, 5% (farms B and C) and 10% (farm A) of the broilers showed mild dyspnoea. For farm A, conjunctivitis, signs of URTD and dyspnoea were present at age 21 days, leading to amoxicillin (Octacillin; Eurovet) treatment (days 23–24 and 27–28) and clinical improvement towards age 28 days. However, clinical signs reappeared after treatment stopped and even worsened towards the end of the rearing period (40 days). Nevertheless, broilers were not treated again. For farms B and C, conjunctivitis, signs of URTD and dyspnoea were present at age 35 days. However, by that time broilers on both farms had already been treated with amoxicillin (days 27–29 on both farms) for signs of URTD and severe dyspnoea (farm B) or signs of URTD and conjunctivitis (farm C). In both farms, respiratory disease worsened towards the end of the rearing period (42 days, farm B; 41 days, farm C), but broilers were not treated. At the end of the rearing period, cumulative mortality was comparable for all farms (3.17, 3.54 and 3.17% for farms A, B and C, respectively).

Serology

Results are presented in Figs 1, 2 and 3. Mycoplasma spp. antibodies were only detected at age 1 day in four of 20 (20%) broilers of farm C. Antibodies against O. rhinotracheale were detected on farms B and C. For farm B, antibodies against O. rhinotracheale were present at age 1 day (three of 20 broilers; 15%) and 28 days (one of 20 broilers; 5%). For farm C, antibodies against O. rhinotracheale were present at age 35 days (four of 20 broilers; 20%). Antibodies against aMPV were noticed on all farms, in 1- as well as 7-day-old broilers. For farms A, B and C, eight of 20 (40%), one of 20 (5%) and 14 of 20 (70%) 1-day-old broilers were, respectively, seropositive for aMPV. C. psittaci antibodies were found on all farms. The curves for the percentage of seropositives were similar, showing almost identical numbers of seropositives at age 1 day and at slaughter, with a clear decrease in the number of seropositives in between.

Identification of respiratory pathogens

Results are presented in Figs 1, 2 and 3. IBV RT-PCR was not performed, as all sera were negative. PCR assays for aMPV and for M. gallisepticum and M. synoviae were negative. Broilers in farms B and C became infected with O. rhinotracheale. The 1- and 28-day-old seropositive broilers of farm B were, respectively, negative and positive (two of 20; 10%) in the O. rhinotracheale PCR. The 35-day-old seropositive broilers of farm C became positive (eight of

![Table 2. Experimental setup](image)

**Table 2. Experimental setup**

<table>
<thead>
<tr>
<th>Farm</th>
<th>Clinical signs and mortality</th>
<th>Air in barns* (days)</th>
<th>Pharyngeal swabs† [age (days)]</th>
<th>Blood‡ [age (days)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n=20)</td>
<td>Daily</td>
<td>0, 1, 7, 14, 21, 28, 35, 40</td>
<td>1, 7, 14, 21, 28, 35, 40</td>
<td>1, 7, 14, 21, 28, 35, 40</td>
</tr>
<tr>
<td>B (n=20)</td>
<td>Daily</td>
<td>0, 1, 7, 14, 21, 28, 35, 40</td>
<td>1, 7, 14, 21, 28, 35, 40</td>
<td>1, 7, 14, 21, 28, 35, 40</td>
</tr>
<tr>
<td>C (n=20)</td>
<td>Daily</td>
<td>0, 1, 7, 14, 21, 28, 35, 40</td>
<td>1, 7, 14, 21, 28, 35, 40</td>
<td>1, 7, 14, 21, 28, 35, 40</td>
</tr>
</tbody>
</table>

*For C. psittaci culture and molecular characterization.
†For (1) C. psittaci culture and molecular characterization, (2) O. rhinotracheale 16S rDNA PCR, (3) M. synoviae/M. gallisepticum real-time PCR, (4) real-time RT-PCR for aMPV subtypes A–D and (5) RT-PCR for IBV.
‡For antibody determination (ELISAs) against C. psittaci, O. rhinotracheale, M. synoviae, M. gallisepticum, aMPV and IBV.
Fig. 1. Farm A. (a) Serology of *C. psittaci*, and its presence in pharyngeal swabs and air samples (culture and genotype-specific PCR). d, Day. (b) Serology of *M. gallisepticum/M. synoviae* (Ms/Mg), aMPV and *O. rhinotracheale* (ORT), and their presence in pharyngeal swabs (PCR). (c) Mortality, clinical observations and ambient temperature.
Fig. 2. Farm B. (a) Serology of *C. psittaci*, and its presence in pharyngeal swabs and air samples (culture and genotype-specific PCR). d, Day. (b) Serology of *M. gallisepticum/M. synoviae* (Ms/Mg), aMPV and *O. rhinotracheale* (ORT), and their presence in pharyngeal swabs (PCR). (c) Mortality, clinical observations and ambient temperature.
20; 40%) in the *O. rhinotracheale* PCR and three of them (15%) were still positive at slaughter. Thus, an *O. rhinotracheale* infection was first noticed towards the end of the rearing period, at 4 (farm B) or 5 (farm C) weeks of age.

Broilers in all three farms became infected with *C. psittaci*. In fact, *C. psittaci* was present in 1-day-old broilers, but seroconversion only occurred ~3–4 weeks of age, just before the occurrence of an *O. rhinotracheale* infection. Viable *C. psittaci* genotypes B and D (farm A), B and C (farm B), and C (farm C) were found in the empty, cleaned and disinfected barns. For farm A, genotype B was no longer discovered at age 14 days. However, genotype C had appeared at age 1 day and stayed, together with genotype D, present until slaughter. Genotypes B and C were present in broilers and in the air of farm B until the animals reached age 35 and 42 days, respectively. Genotype C was present during the whole rearing period in both broilers and air of farm C. The number of culture-positive broilers ranged from 87.5 to 100% (farm A), from 26.1 to 100% (farm B) and from 37.5 to 91.3% (farm C). Thus, *C. psittaci* culture-positive chickens were transported to the slaughterhouse at the end of all rearing periods, which is an important finding regarding the zoonotic potential of *C. psittaci*.

**DISCUSSION**

NDV, avian influenza virus, aMPV and IBV are the viruses that most frequently affect the respiratory tract of broilers, and thus might interact with *C. psittaci*. Vaccination of broilers and/or broiler breeders is the main approach to control these economically important viral pathogens (Jones, 2010; Jackwood, 2012; Ou & Giambrone, 2012). *O. rhinotracheale* is a primary bacterial pathogen of broilers (van Veen et al., 2000). Currently, 18 *O. rhinotracheale* serotypes designated A–R have been identified, with serotype A being highly prevalent in most broiler-producing countries (Numee et al., 2012). Clinical disease in broilers is less severe than in turkeys, and involves sneezing, rhinitis and facial oedema. Nevertheless, infections in broiler chickens can cause economic losses due to increased mortality, growth retardation and increased slaughterhouse condemnation rates. An *O. rhinotracheale* serotype A vaccine has been registered for use in broiler breeders, but is still not widely used, although De Herdt et al. (2012) presented evidence for the improved performance of broilers originating from *O. rhinotracheale*-vaccinated breeders.

We found antibody titres against aMPV on all farms. These were most likely maternal antibodies as: (i) broilers were not vaccinated against aMPV, (ii) no viral RNA could be detected at that time in broilers and (iii) breeders were immunized twice against aMPV. Maternal antibodies against aMPV had disappeared at age 14 days and broilers remained uninfected until slaughter. All examined broilers were seronegative for IBV, although they received an IBV primer containing the attenuated IBV strains H120 and D274 at age 1 day. They remained seronegative throughout the rearing period. Thus, they must have been protected or IBV did not occur on these farms. On farm C, we found maternal antibody titres against Mycoplasma, as no Mycoplasma DNA could be detected at that time. Thus, breeders must have been infected by Mycoplasma spp. However, the infection caused no clinical disease in breeders and they remained untreated. Maternal antibodies had disappeared at age 7 days and broilers remained seronegative until slaughter. On farm B, we found maternal antibody titres against *O. rhinotracheale*, as no *O. rhinotracheale* DNA could be detected at that time. Broilers received no *O. rhinotracheale* vaccine. Thus, brooders must have been infected by *O. rhinotracheale*. However, the infection caused no clinical disease in breeders and they remained untreated. Maternal antibodies against *O. rhinotracheale* had disappeared at age 7 days, and broilers experienced an *O. rhinotracheale* infection 3 weeks later as demonstrated by both ELISA (seroconversion) and PCR. Around the same time, broilers of farm C also experienced an *O. rhinotracheale* infection, as revealed by ELISA (seroconversion) and PCR.

Recently, we found virulent *C. psittaci* strains in Belgian and French broilers, and, as in turkeys, *C. psittaci* could be involved in the multi-factorial aetiology of respiratory infections in commercial chickens (Van Loock et al., 2005). For all farms, 1-day old broilers showed relatively high maternal antibody titres against *C. psittaci* in the presence of viable *C. psittaci*. This indicates vertical transmission, which is known to occur in chickens (Wittenbrink et al., 1993), and/or insufficient cleaning/disinfection, as *C. psittaci* was found in all empty barns, even before stocking. However, maternal antibodies seemed to protect against respiratory signs. Protection by maternal antibodies against *C. psittaci* has been demonstrated previously during an experimental infection in turkeys (Van Loock et al., 2004).

Maternal antibodies declined and clinical outbreaks could be identified serologically noticed even before maternal antibodies completely disappeared. Mixed infections with genotypes B/C and B/C/D were observed, which is not uncommon in poultry (Dicks & Vanrompay, 2011; Lagae et al., 2013). When a mixed infection occurred, the cumulative mortality augmented faster (quicker above 2%). However, we have no proof that this observation was due to the mixed *C. psittaci* infection.

Broilers with antibody increases showed conjunctivitis, signs of URTD and dyspnoea, and according to the company veterinarian had to be treated with amoxicillin. However, this penicillin antibiotic is certainly not the choice for treating *C. psittaci*. Moreover, *Chlamydia* enters a viable, non-dividing and non-infectious state (historically termed persistence and more recently referred to as the chlamydial stress response) when exposed to penicillin G in culture. Notably, penicillin G-exposed *Chlamydiae* can re-enter the normal developmental cycle upon drug removal (Goellner et al., 2006; Kintner et al., 2014). The latter
(a) Prevalence (%) of Culture score (x10) over Age (days) with letters indicating genotypes in air.

(b) Prevalence (%) of seroprevalence over Age (days) with PCR results indicated.

(c) Mortality (%) and Temperature (°C) over Age (days) with various clinical signs and symptoms.

*Letters indicate genotypes in air.
apparently happened as demonstrated by the reaugmenting pharyngeal culture scores upon drug removal, resulting in an ongoing infection until slaughter.

There might be a pathogenic interaction between C. psittaci and other viral and/or bacterial respiratory pathogens. Interactions between respiratory pathogens have already been demonstrated for M. gallisepticum and NDV in experimentally infected chickens (reviewed by Kleven, 1998) and for C. psittaci and aMPV in experimentally infected turkeys (Van Loock et al., 2006). On one farm, respiratory signs appeared together with a proven C. psittaci infection. We could not detect an O. rhinotracheale infection on farm A, perhaps due to the antibiotic treatment at days 23–24. On the other two farms, respiratory signs always appeared together with a proven C. psittaci and O. rhinotracheale infection. C. psittaci always preceded O. rhinotracheale. This might suggest an association between C. psittaci and O. rhinotracheale. C. psittaci infections could weaken the health of broilers, making them more susceptible to O. rhinotracheale infections. The same has been observed in turkeys (Van Loock et al., 2005; Van Droogenbroeck et al., 2011). Thus, both C. psittaci and O. rhinotracheale should be considered when developing prevention strategies for respiratory disease in broilers.

The presence of C. psittaci in empty barns and in 1-day-old broilers remains intriguing. Vertical transmission occurs in chickens (Wittenbrink et al., 1993) and bio-aerosol monitoring during hatching revealed increasing numbers of C. psittaci (Dickx & Vanrompay, 2011). Breeders possibly had experienced a C. psittaci infection, as maternal antibodies were present. Vaccination of broiler breeders might reduce these early infections. In addition, C. psittaci could also be brought into the barns by (1) contaminated (wild birds) flax cores, although no one has yet examined this possible route, (2) insufficiently cleaned equipment/machines and/or (3) blood-sucking ectoparasites. The presence of Chlamydial DNA in ticks suggests that they are indeed carriers of Chlamydiae (Croxatto et al., 2014). However, ectoparasites were not observed during our study. We did not select for a farm with a negative air sample at day 0, as our studies in turkey breeders prove that it makes no difference if the air is negative or positive at day 0 – a C. psittaci infection outbreak always occurs during the brood and the timing is independent of the air being positive or negative at day 0 (Van Droogenbroeck et al., 2011). One-day-old animals are the main source of incoming infections.

In conclusion, more attention should be paid to the prevention of the zoonotic pathogens C. psittaci and O. rhinotracheale. Evidence was provided that C. psittaci can occur at an early age in broilers without a predisposing respiratory infection. Our results on the kinetics of C. psittaci could assist in determining an optimal vaccination time point in broilers. Vaccination of breeders and/or broilers against these pathogens together with perhaps bio-aerosol monitoring as a non-invasive infectious disease monitoring system could improve broiler performance.

ACKNOWLEDGEMENTS

The study was funded by the Federal Public Service of Health, Safety of the Food Chain and Environment (convention RF-11/6245 MINSPEC-PRO), Ghent University (IOF10/STEP/002) and MSD Animal Health (Boxmeer, The Netherlands).

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