Veterinary Microbiology 162 (2013) 740–749

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Emerging *Chlamydia psittaci* infections in the chicken industry and pathology of *Chlamydia psittaci* genotype B and D strains in specific pathogen free chickens

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ARTICLE INFO

Article history:
Received 28 June 2012
Received in revised form 25 September 2012
Accepted 27 September 2012

Keywords:
Chlamydia psittaci
Chickens
Poultry
Broilers
Pathology

ABSTRACT

Sera of 30 Belgian and 10 Northern French chicken farms were tested by a *Chlamydia* (*C.*) *psittaci* major outer membrane protein (MOMP) based ELISA. Ninety-six percent, 93% and 90% of the Belgian broilers, broiler breeders and layers were seropositive. Ninety-one percent of the French broilers were seropositive. In addition, tissues of 5 Belgian and 5 French broiler farms were examined at slaughter. All French farms were culture positive while *C. psittaci* was cultured from the lungs of 80% of examined Belgian farms. *C. psittaci* infections are apparently emerging in chickens raised in Belgium and Northern France. We could proof Hill–Evans postulates for chicken-derived *C. psittaci* genotype B and D strains. Chicken-processing plant employees should be considered a risk group for human psittacosis. There is a need for higher awareness and for efficient risk assessment and management of *C. psittaci* infections in chickens as chlamydiosis in broilers seems to be underdiagnosed and infections with highly virulent strains do occur.

1. Introduction

Chlamydia (*C.*) *psittaci* is an obligate, intracellular Gram-negative bacteria causing respiratory disease in poultry, mainly in turkeys and ducks. Reports on *C. psittaci* outbreaks on chicken farms or on zoonotic transmissions linked to contact with *C. psittaci*-infected chickens are extremely rare. So, far genotypes B, C, F and E/B have been found in chickens (Gaede et al., 2008; Zhang et al., 2008; Dickx et al., 2010; Zhou et al., 2010). Maybe, chickens seldom become infected and/or strains infecting chickens are less virulent, presenting a minor risk for humans. We therefore investigated the occurrence of *C. psittaci* by performing a retrospective study on 300 serum samples collected in 2005 from 10 randomly selected Belgian broiler breeder, broiler and layer farms. Ninety-eight, 95, and 95% of the layers, broilers, and broiler breeders were seropositive (Dickx et al., 2010) by our major outer membrane protein (MOMP) based enzyme-linked immunosorbent assay (ELISA) (Verminnen et al., 2006). Seropositive chickens were present on all farms. Laroucau et al. (2009), recently reported on chlamydia infection in broilers raised in France. Chlamydial DNA was detected in 12 of 18 (67%) investigated chicken flocks. Characterization of the agents indicated the presence of atypical *Chlamydia*. All chicken flocks appeared healthy. Thus, *Chlamydia* infections are apparently common in Belgian and French chickens, but Hill–Evans postulates (Evans, 1976; Fredricks and Relman, 1996) for establishing

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http://dx.doi.org/10.1016/j.vetmic.2012.09.026

microbial disease causation remains to be full-filled for the chicken infectious *Chlamydia* strains. This study aims at gathering information on the current epidemiological status of *Chlamydia* infections in Belgian and French (Northern France) industrial chickens. In addition, we examined the pathology of the most frequently found *C. psittaci* genotypes by performing experimental infections in specific pathogen free (SPF) chickens. To our knowledge, we are the first to examine the pathology of chicken-derived *C. psittaci* strains in SPF chickens.

2. Materials and methods

2.1. Prevalence study

2.1.1. Chlamydia seroprevalence in broilers, broiler breeders and layers

Six-hundred chickens from 10 randomly chosen Flemish broiler, broiler breeder and layer farms (20 chickens per farm) were examined at slaughter for the presence of serum antibodies against *Chlamydia*. The chickens were randomly selected in one abattoir in West-Flanders (Belgium). Also, 200 chickens from 10 randomly chosen French (Northern France) broiler farms (20 chickens per farm) were randomly selected at slaughter for serological examination. We received no information on the clinical status of the examined farms, or on antibiotic treatments. Blood samples were stored overnight at room temperature. Sera were collected after centrifugation (325 × *g*, 10 min, 4 °C) and stored at −20 °C. Antibody titres were determined by use of a recombinant *C. psittaci* MOMP-based antibody ELISA as described by Verminnen et al. (2006). The MOMP of *C. psittaci* possesses genus-specific epitopes, allowing the detection of antibodies against all *Chlamydia* species.

2.1.2. Isolation of Chlamydia from broilers

We took organ samples from broilers being slaughtered in the same abattoir in West-Flanders. The broilers came from other farms than the ones used for the seroprevalence study. One-hundred and two lungs, 94 livers and 91 spleens from 5 French (Northern France) broiler farms were randomly collected at slaughter. Each organ sample came from a different chicken. Thus, 287 chickens were sampled in total. All farms had experienced respiratory disease (rhinitis, dyspnoea) during the brood with the need for doxycycline treatment. We were unable to obtain detailed information on diagnoses performed, age of the chickens while being sick, dose and duration of treatments.

Additionally, 50 lungs from 5 Flemish broiler farms (10 lungs/farm) were selected at slaughter based on the presence of pneumonia. Farms had experienced respiratory disease (rhinitis, dyspnoea) with the need for doxycycline treatment. Information on age of the chickens while being sick, dose and duration of treatments was not provided by the farmers (Table 1).

Finally, 9 randomly selected Flemish broiler farms were visited by colleagues for a study on the prevalence of multi-resistent *Staphylococcus aureus* in broilers. Forty randomly selected chickens per farm were also sampled for *Chlamydia* diagnosis using pharyngeal swabs provided with *chlamydia* transport medium. Information on sickness during the brood, age, dose and duration of treatments was absent.

All organ samples and pharyngeal swabs were transported on ice, delivered to the laboratory within 12 h and stored at −80 °C until tested. Culture was performed using buffalo green monkey (BGM) cells, identifying the organism by a direct immunofluorescence staining (IMAGEN™, Oxoid, United Kingdom) at 6 days post-inoculation. *C. psittaci* positive cells were enumerated in five randomly

Table 1
Information on the sampled Belgian broiler farms.

Farm Nr.	Animals Per barn	Age at sampling	Coccidiostats		Antibiotic treatments
			Starter feed	Grower feed	
A	30,000	Slaughter age	Nicarbazin & Narazin	Salinomycin	Doxycyclin (tetracycline)
B	25,000	Slaughter age	Nicarbazin & Narazin	Salinomycin	Doxycyclin (tetracycline)
C	25,000	Slaughter age	Nicarbazin & Narazin	Salinomycin	Doxycyclin (tetracycline)
D	30,000	Slaughter age	Nicarbazin	Salinomycin	Amoxicillin
E	30,000	Slaughter age	Nicarbazin & Narazin	Salinomycin	Amoxicillin
F	42,000	5 weeks	Nicarbazin & Narazin	Salinomycin	None
G	25,000	4 weeks	Nicarbazin & Narazin	Salinomycin	Tylan for 2 days (1 g/10L and 4200L/day)
H	20,000	6 weeks	Nicarbazin & Narazin	Salinomycin	Oxacillin (beta-lactam) for 3 days (300 g/day)
I	18,000	4 weeks	None	None	Cosumix (sulpha- trimethoprim) when needed
J	14,000	4 weeks	Nicarbazin & Salinomycin	Salinomycin	None
K	15,000	6 weeks	Salinomycin	Salinomycin	None
L	30,000	29 days	Nicarbazin & Narazin	None	Amoxicillin (β-lactam) for 3 days (2 g/10L/day)
M	25,000	6 weeks	Nicarbazin & Narazin	Salinomycin	Doxycyclin (tetracycline) for 4 days (1 kg/day)
N	30,000	29 days	Monensin	Salinomycin	Emdotrim (sulpha-trimetoprim) for 3 days (2L/1000L and 8000 L)

Farms A to E were sampled (lungs) in the slaughterhouse. Farms F to N were visited for sampling (pharyngeal swabs) in the barn.

selected microscopic fields (600×, Nikon Eclipse TE2000-E, Japan) and results were scored from 0 to 6. Score 0 indicated that no *C. psittaci* was present; score 1 was given when a mean of 1–5 non-replicating elementary bodies was present; scores 2, 3, 4, 5 and 6 were given when a mean of 1–5, 6–10, 11–15, 16–20 and >20 inclusion (elementary and replicating reticulate bodies) positive cells was present.

2.1.3. Molecular characterization of Chlamydia strains

Species identification of isolates was performed by the 23S rRNA-based ArrayTube DNA microarray (Sachse et al., 2005). A sample was considered "chlamydia-negative" when all signal intensities except the internal staining control (biotin marker) were below NI (normalized signal intensity) = 0.07. *C. psittaci* strains were further characterized by the outer membrane protein A (*ompA*) genotype-specific real-time PCR (Geens et al., 2005), by the *ompA*-based genotyping ArrayTube DNA microarray (Sachse et al., 2008) and by *ompA* sequencing (VIB Genetic Service Facility Antwerp, Belgium) using CTU/CTL primers [3′-ATGAAAAAACTCTTGAAATCGG-5′/3′-CAAGATTTTCTA-GA(T/C)TTCAT(C/T)TTGTT-5′], generating an amplicon of 1098 bp (Denamur et al., 1991) or primers CPsittGenoFor [3′-GCTACGGGTTCCGCTCT-5′] and CPsittGenoRev [3′-TTTGTTGATYTGAATCGAAGC-5′] (Heddema et al., 2006) generating an amplicon of 1041 bp.

2.2. Animal experiment

2.2.1. Experimental design, animals, Chlamydia strains

The experimental design was evaluated and approved by the Ethical Commission for Animal Experiments of Ghent University (EC 2010/054). Briefly, 4 groups of 22-day-old SPF chickens (Lohman, Cuxhaven, Germany) were individually tagged and housed in separate negative pressure isolators (IM1500, Montair, Sevenum, The Netherlands). At the age 1 week, groups 1–3 were exposed for 1 h to an aerosol of 10^6 $TCID_{50}$ *C. psittaci* suspended in PBS (5 μm droplets; Cirrus™ nebulizer; Lameris, Aartselaar, Belgium). A forth 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 (from a Belgian broiler showing pneumonia), *C. psittaci* genotype B strain 10/525 (from a Belgian broiler showing pneumonia) and with *C. psittaci* genotype D strain 10/298 (from a French broiler showing pneumonia), respectively. Both Belgian *Chlamydia* strains came from different farms. Molecular characterization of the *Chlamydia* strains is described in detail in the results.

Contaminating organisms in both the lungs and the inocula used to infect the SPF chickens were absent as demonstrated by: (i) bacterial isolation attempts (F. Boyen; Laboratory for Bacteriology and Mycology, Fac Veterinary Medicine, UGhent), (ii) a genus-specific PCR for mycoplasma (Lierz et al., 2007), (iii) a PCR for the avian metapneumovirus (aMPV) subtypes A, B, C and D (Guionie et al., 2007), (iv) a PCR for the infectious bronchitis virus (IBV) (Jones et al., 2011), and (v) a PCR for the infectious laryngotracheitis virus (ILTV) (Creelan et al., 2006). P. Butaye (CODA, Brussels, Belgium) and H. Nauwynck (Ghent University, Faculty of Veterinary Medicine, Department of Virology, Parasitology and Immunology) provided a *Mycoplasma gallisepticum*, aMPV, ILTV (U76/1035) and IB (strain M41) control for PCR.

2.2.2. Clinical parameters and macroscopic lesions

Clinical signs were daily recorded until 34 days post-infection (dpi). It was our purpose to euthanize two birds per group at 2, 4, 6, 8, 10, 14, 17, 21, 24, 28 and 34 dpi for detailed examination. However, dead birds would be examined immediately regardless the dpi. Macroscopic lesions were scored according to Table 2.

2.2.3. C. psittaci replication in tissues, examination of lungs and histopathology

At euthanasia, tissue samples of the conjunctiva, conchae, sinus, trachea, lungs, abdominal and thoracic airsacs, pericardium, spleen, liver, kidney, jejunum and ovary/testis were immersed in methocel (Methocel MC, Sigma), snap frozen in liquid nitrogen and stored at −80 °C until processed. Deceased birds were also sampled. Cryostat tissue sections (5 μm) were examined for the presence of *Chlamydia* by the IMAGEN™ immunofluorescence staining. The presence of *Chlamydia* was enumerated as for the field samples. A small part of the lungs, taken at 4 and 8 dpi, was examined for the presence of contaminating bacteria and viruses as described above.

The lungs, the thoracic airsac and the spleen of euthanized birds at 4, 6, 10, 14, 21, 24 and 34 dpi were taken for histopathology. They were fixed in 10%

Table 2
Macroscopic lesion scoring system.

Tissue	Lesion score 1	Lesion score 2	Lesion score 3
Conjunctiva	Congestion unilateral	Congestion bilateral	Petechiae
Conchae	Slightly congested	Severely congested	Severely congested + viscous mucus
Lung	Congestion bilateral	Congestion + grey foci unilateral	Congestion + grey foci bilateral
Thoracic airsac	Diffuse opacity	Focal fibrinous airsacculitis	Diffuse fibrinous airsacculitis
Abdominal airsac	Diffuse opacity	Focal fibrinous airsacculitis	Diffuse fibrinous airsacculitis
Pericardium	Serous pericarditis	Serous pericarditis	Serous adhesive pericarditis
Spleen	Slightly enlarged	Moderately enlarged	Severely enlarged + petechiae
Liver	Slightly congested	Moderately congested	Moderately congested + petechiae
Kidney	Slightly enlarged	Moderately enlarged	Severely enlarged
Intestine	Slightly congested	Moderately congested and fluid inside	Severely congested and fluid inside

Tissues with no lesions were scored 0.

phosphate-buffered formalin, processed, embedded in paraffin, sectioned at 5 μm, and stained with haematoxylin and eosin. All slides were examined microscopically (Leitz, New York, USA). Histopathology was performed by I. Debyser (Covetop; Consultancy in Veterinary and Toxicological Pathology; Edingen, Belgium). Microscopic findings were graded [minimal histological change (1), slight (2), moderate (3), marked (4) or severe (5)].

2.2.4. Chlamydia excretion

Pharyngeal and cloacal excretion were determined by examining rayon-tipped, aluminium-shafted swabs (Colpan; Fiers, Kuurne, Belgium) provided with *C. psittaci* transport medium and used for sampling at euthanasia and for sampling deceased birds. Swabs were stored at −80 °C until processed. *Chlamydia* excretion was monitored using standard procedures for culture, bacterial identification (IMAGEN™ immunofluorescence staining) and quantification (scoring system as for the field samples) of bacteria in BGM cells.

3. Results

3.1. Prevalence study

3.1.1. Chlamydia seroprevalence in broilers, broiler breeders and layers

All Belgian broiler, broiler breeder and layer farms and all French broiler farms were seropositive. Ninety-six percent, 93% and 90% of the Belgian broilers, broiler breeders and layers were seropositive. Ninety-one percent of the French broilers were seropositive. The percentage of seropositive chickens on the Belgian broiler, broiler breeder and layer farms was 10% (1 broiler farm), 100% (9 broiler farms), 20% (1 broiler breeder farm), 30% (1 broiler breeder farm), 90% (1 broiler breeder farm), 100% (7 broiler breeder farms), 20% (1 layer farm), 90% (1 layer farm) and 100% (8 layer farms). Antibody titres on the Belgian farms with a high number of seropositive animals (90–100%) ($n = 26$) ranged from 1/400 till 1/6400 while 1/100 till 1/800 for the 4 remaining farms.

The percentage of seropositive chickens on the French broiler farms was 30% (2 farms), 90% (1 farm) and 100% (7 farms). Antibody titres on the French farms with a high number of seropositive animals (90–100%) ($n = 8$) ranged from 1/200 till 1/3200 while 1/100 till 1/400 for the 2 remaining farms.

3.1.2. Isolation of Chlamydia from broilers

Results of culture are summarized in Table 3. All five (100%) examined French broiler farms were culture positive. Culture positive lungs, spleens and livers were found on all farms. Cultures scores ranged from 1 to 4. Overall, the lung was more often culture positive than the spleen, and positivity rate was lowest in liver samples. Also, culture scores were always the highest for the lungs (max score 4) followed by the spleen (max score 2) and the liver (max score 1), respectively. That is why we only sampled lungs during the following sampling round for examining Belgian broilers. Lungs of 4 of 5 (80%) Belgian broiler farms were culture positive. The culture scores ranged from 1 to 4.

We found culture positive pharyngeal swabs on all visited farms. For all farms, the maximum culture score was 2. When testing 40 broilers per farm, the percentage of positives was 30% (2 farms), 37% (4 farms), 45% (1 farm), 52% (1 farm) and 70% (1 farm).

3.1.3. Molecular characterization of Chlamydia strains

Only lung isolates were molecularly characterized, as culture scores were the highest, making molecular characterization more feasible. We could only characterize 50 of 69 (72%) *Chlamydia* isolates. They all reacted with the *C. psittaci* specific probes in the 23S rRNA-based ArrayTube DNA microarray. Forty-five of 50 *C. psittaci* strains could be successfully genotyped by both the genotype-specific real-time PCR and the *ompA*-based ArrayTube DNA microarray. However, the genotype-specific real-time PCR detected one mixed infection, while the microarray did not. PCR amplification for *ompA* sequencing was successful in 35 of 50 *C. psittaci* DNA samples. Molecular characterization revealed the presence of *C. psittaci* genotype B and D in, respectively, 1 (40%) and 3 (60%) of 5 *Chlamydia* positive French broiler farms. The remaining fifth farm was dealing with a mixed infection, as genotypes B and D were discovered. Genotype B was detected on all 5 *C. psittaci* positive Belgian farms.

3.2. Animal experiment

3.2.1. Molecular characterization of Chlamydia strains used

Strains 10/298, 10/423 and 10/525 were all *C. psittaci* according to the 23S rRNA-based ArrayTube DNA microarray. Genotyping identified strains 10/298 and 10/423 as genotype D and B, respectively, by all methods used.

Table 3
Culture results for organs sampled from French and Belgian broilers.

French broilers								Belgian broilers			
Farm	Farm size[a]	Lung		Liver		Spleen		Farm	Farm size[a]	Lung	
No.	*N*	*N*	Positives (%)	*N*	Positives (%)	*N*	Positives (%)	No.	*N*	*N*	Positives (%)
1	25,000	10	5 (50)	19	4 (21)	14	5 (36)	A	60,000	10	2 (20)
2	30,000	22	5 (23)	16	9 (56)	16	8 (50)	B	30,000	10	0 (0)
3	50,000	30	15 (50)	20	2 (10)	20	8 (40)	C	60,000	10	5 (50)
4	40,000	20	12 (60)	20	5 (25)	20	9 (45)	D	25,000	10	8 (80)
5	25,000	20	12 (60)	19	4 (21)	21	6 (28)	E	25,000	10	6 (60)
Total		102	49 (48)	94	24 (25.5)	91	36 (39.5)			50	20 (40)

[a] Number of broilers per farm.

However, strain 10/525 reacted with the micro array probe for the proposed provisional genotype YP84 (Sachse et al., 2008), while the genotype-specific real-time PCR identified this strains as genotype B. Subsequent sequencing of the *ompA* gene using the primers CPsittGenoFor and CPsittGenoRev revealed 100% identity with the *ompA* gene of the *C. psittaci* genotype B reference strain CP3 (pigeon strain), while only 96 and 93% nucleic acid and amino acid homology, respectively, with strain YP84 (parrot strain). Multi locus sequence typing (MLST) was performed for double-checking (performed by Y. Pannekoek; Amsterdam University). MLST revealed exactly the same Sequence Type as for CP3. Strain 10/525 was therefore assigned as genotype B.

3.2.2. Clinical signs and macroscopic lesions

Non-infected controls remained healthy throughout the experiment. Chickens in groups 1 and 2, which were infected with the genotype B strains, showed slight apathy and conjunctivitis, rhinitis and mild dyspnoea from 4 till 16 dpi and had mild intermittent diarrhoea (green watery droppings) from 10 till 16 dpi. The number of affected animals gradually decreased with only 2 of 10 (20%) and 4 of 10 (40%) remaining chickens of groups 1 and 2, respectively, showing clinical signs at 16 dpi. Chickens infected with the genotype D strain (group 3) were severely ill and 11 of 22 (50%) animals died between 7 and 10 dpi. Symptoms started at 2 dpi in 8 of 22 (36%) chickens and from 4 dpi onwards, all animals showed marked apathy and anorexia, severe conjunctivitis and rhinitis, exacerbating dyspnoea (mostly sitting on the floor, eyes closed, heads hanging down, sometimes scratching their eyes, gasping and head shaking, wings removed from the body to breath simpler) and intermittent severe diarrhoea (green watery droppings).

Non-infected controls showed no macroscopic lesions. Macroscopic lesions were most severe for group 3 and total mean scores per tissue over all dpi were always higher than for groups 1 and 2, except for the jejunum. Mean lesion scores for groups 1 and 2 were comparable, although

Table 4
Mean scores for the macroscopic lesions in euthanized or deceased chickens infected with the genotype B strain 10/423, genotype B strain 10/525 or genotype D strain 10/298 till 34 dpi.

	dpi	N[a]	Conj	Conc	Sinus	Trach	Lung	T. airs	A. airs	Peric	Liver	Spleen	Kidn	Jejun	O/T
Group 1	2[a]	2 + 0	0	0	0	0	0	0	0	0	0	0	0	0	0
(genotype	4	2 + 0	2	1	0	0	1	1	1	0	0	0	0	0	0
B-10/423)	6	2 + 0	1	1	0	0	1	1	1	0	0	0	0	0	0
	8	2 + 0	1	1	0	1	2	2	2	1	1	1	1	0	0
	10	2 + 0	2	2	1	1	3	3	3	2	1	1	1	1	0
	14	2 + 0	1	2	1	2	3	3	3	2	1	1	1	1	0
	17	2 + 0	1	1	1	1	3	2	2	1	1	1	1	1	0
	21	2 + 0	1	1	1	0	2	1	1	0	0	1	0	1	0
	24	2 + 0	1	0	0	0	2	1	1	0	0	2	0	1	0
	28	2 + 0	1	0	0	0	2	1	1	0	0	2	0	1	0
	34	2 + 0	1	0	0	0	2	1	1	0	0	2	0	1	0
	T[b]	22 + 0	12	9	4	5	21	16	16	6	4	11	4	7	0
Group 2	2	2 + 0	0	0	0	0	0	0	0	0	0	0	0	0	0
(genotype	4	2 + 0	1	1	0	0	1	1	1	0	0	0	0	0	0
B-10/525)	6	2 + 0	2	1	0	0	1	1	1	0	0	0	0	1	0
	8	2 + 0	2	2	1	1	2	2	3	1	1	1	1	1	0
	10	2 + 0	2	2	1	1	3	3	3	2	2	2	1	1	0
	14	2 + 0	1	2	1	1	3	3	3	2	1	2	1	1	0
	17	2 + 0	1	1	1	1	2	3	3	2	1	1	1	1	0
	21	2 + 0	1	1	1	0	2	3	3	1	0	1	1	1	0
	24	2 + 0	1	0	0	0	2	1	1	0	0	1	1	1	0
	28	2 + 0	1	0	0	0	2	1	1	0	0	0	0	1	0
	34	2 + 0	1	0	0	0	2	1	1	0	0	0	0	1	0
	T[b]	22 + 0	13	10	5	4	20	19	20	8	5	8	6	9	0
Group 3	2	2 + 0	0	0	0	0	0	0	0	0	0	0	0	0	0
(genotype	4	2 + 0	3	1	1	2	1	2	2	0	0	1	0	0	0
D-10/298)	6	1 + 1	3	1	1	2	3	3	3	2	0	1	3	0	0
	7	0 + 3	3	2	2	2	3	3	3	3	3	3	3	1	0
	8	0 + 5	2	2	2	2	3	3	3	2	3	3	3	1	1
	9	0 + 1	2	2	2	2	3	3	3	2	3	3	2	1	1
	10	0 + 2	2	2	2	3	3	3	3	3	2	3	3	1	1
	14	2 + 0	1	2	1	2	3	3	3	3	2	3	2	1	1
	17	2 + 0	2	2	2	1	2	3	3	2	2	3	1	1	1
	21	1 + 0	1	2	2	0	1	2	2	1	1	2	1	1	1
	T[b]	10 + 12	19	16	15	16	22	25	25	18	16	22	18	7	6

dpi: days post-infection; Conj: conjunctiva; Conc: conchae; Trach: trachea; T. Airs: thoracic airsac; A. airs: abdominal airsac; Peric: pericardium; Kidn: kidney; Jejun: jejunum; O/T: ovary/testes.

[a] Number of chickens (euthanized + deceased).

[b] Total score over all dpi.

Table 5
Culture scores[a] for pharyngeal and cloacal *C. psittaci* excretion in euthanized (n) or deceased chickens (n⁺).

dpi	Group 1 (10/423)		Group 2 (10/525)		Group 3 (10/298)	
	Pharyngeal	Cloacal	Pharyngeal	Cloacal	Pharyngeal	Cloacal
2	1 (2)	0 (2)	1 (2)	0 (2)	1 (2)	0 (2)
4	2 (2)	1 (2)	4 (2)	1 (2)	3 (1)/5 (1)	1 (2)
6	5 (2)	1 (2)	4 (2)	3 (2)	5 (1)/6 (1⁺)	3 (1)/5 (1⁺)
7	NA	NA	NA	NA	5 (3⁺)	5 (3⁺)
8	5 (2)	2 (2)	5 (2)	4 (2)	5 (5⁺)	5 (5⁺)
9	NA	NA	NA	NA	5 (1⁺)	5 (1⁺)
10	4 (1)/5 (1)	3 (1)/4 (1)	4 (2)	3 (1)/4 (1)	5 (2⁺)	5 (2⁺)
14	4 (1)/5 (1)	1 (1)/2 (1)	4 (1)/5 (1)	1 (1)/2 (1)	6 (2)	4 (2)
17	1 (2)	3 (2)	3 (2)	1 (2)	4 (1)/5 (1)	4 (2)
21	2 (2)	3 (2)	3 (2)	4 (2)	2 (1)	2 (1)
24	1 (2)	1 (1)/2 (1)	3 (1)/5 (1)	1 (2)	–	–
28	1 (2)	1 (2)	3 (2)	4 (2)	–	–
34	0 (1)/1 (1)	1 (2)	1 (2)	1 (1)/2 (1)	–	–
Total score	55 (22)	37 (22)	73 (22)	49 (22)	99 (10 + 12⁺)	83 (10 + 12⁺)

[a] *C. psittaci* positive cells were enumerated in five randomly selected microscopic fields (600×, Nikon Eclipse TE2000-E, Japan) and results were scored from 0 to 6. Score 0 indicated that no *C. psittaci* was present; score 1 was given when a mean of 1–5 non-replicating elementary bodies was present; scores 2, 3, 5 and 6 were given when a mean of 1–5, 6–10, 11–15, 16–20 and >20 inclusion (elementary and replicating reticulate bodies) positive cells was present. dpi: days post-infection; NA: not applicable as no euthanasia was planned and no chickens deceased.

the total mean scores per tissue over all dpi were the highest for group 2, with the exception of the trachea, the lung and the spleen (Table 4).

3.2.3. *C. psittaci* excretion

Non-infected controls shed no *C. psittaci*. Pharyngeal excretion in all 3 infected groups was the same at 2 dpi (Table 5). Cloacal excretion was not observed at that time. Pharyngeal excretion augmented in all infected groups, but especially for group 3, followed by groups 2 and 1, respectively. Pharyngeal excretion for group 3 reached a maximum score of 6, while the maximum pharyngeal excretion score for groups 1 and 2 was 5. Cloacal excretion was observed in all infected groups from 4 dpi onwards and at that time scores were the same for all infected groups. Cloacal excretion gradually increased during the experiment and was the highest for group 3 (max score of 5) followed by groups 2 and 1 (both max score of 4), respectively. At 34 dpi, all animals left in groups 1 and 2, were still excreting *C. psittaci* in their faeces. The last remaining chicken of group 3, being euthanized at 21 dpi, was also excreting *C. psittaci*.

3.2.4. *C. psittaci* replication in tissues and examination of the lungs for contaminants

Lungs of animals examined at 4 and 8 dpi contained no contaminating bacteria or viruses. *C. psittaci* was absent in tissues of non-infected controls. *Chlamydia* was present in all infected birds with the exception of birds of groups 1 and 2, euthanized at 2 dpi (Table 6). At that time, *C. psittaci* could already be detected (albeit low scores) in the upper (conchae) and lower respiratory tract (lung and thoracal airsac) of 1 of 2 euthanized birds of group 3.

Euthanized chickens of groups 1 and 2, both infected with genotype B, became positive at 4 dpi. The upper respiratory tract of birds of both groups remained positive till 17 dpi and scores were albeit comparable as they ranged between 0.5 and 1. For group 2, scores for tissues of the lower respiratory tract increased more rapidly than for group 1 and they remained high till 21 dpi, while high scores for group 1 were only noticed till 14 dpi. The pericardium in both groups became positive at 8 dpi and remained positive for 17 and 21 dpi for group 1 and 2, respectively. Scores were identical. At 8 dpi, a systemic infection was observed in both groups as liver, spleen and kidneys became positive, which lasted till the end of the experiment at 34 dpi. Maximum scores for these tissues were higher for group 2 than for group 1. The jejunum in both groups remained positive till 34 dpi and scores were the same for both groups.

For group 3, the conjunctiva and the upper respiratory tract were still positive at 21 dpi, which was not the case for groups 1 and 2. Replication was more intense as the maximum score noticed for the upper respiratory tract was 3 (conchae and trachea), while it was only 1 for groups 1 and 2. The same was true for the lower respiratory tract, with a very intense *Chlamydia* replication between 6 and 21 dpi. Replication in the pericardium also lasted longer (from 4 till 21 dpi), but scores were comparable to the ones noticed for groups 1 and 2. At 6 dpi, a systemic infection was observed as liver, spleen, kidney and jejenum became positive. Scores for the liver, kidney and especially the spleen were higher than the ones observed in groups 1 and 2. Remarkably, *C. psittaci* was also discovered in the testes and ovaria, which was not the case in groups 1 and 2.

3.2.5. Histopathology

Microscopic lesions were absent in the lungs, thoracic airsac and spleen of the control chickens. Histopathological findings were present in all infected groups and are summarized in Table 7. Bronchitis was characterized by: (i) formation of follicles in the bronchus associated lymphoid tissue (BALT), (ii) lymphocytic infiltrates in the bronchial wall, sometimes with some heterophils, and (iii)

Table 6
Mean scores for the presence of *C. psittaci* in tissues of euthanized or deceased chickens infected with the genotype B strain 10/423, genotype B strain 10/525 or genotype D strain 10/298.

	dpi	N[a]	Conj	Conc	Sinus	Trach	Lung	T. airs	A. airs	Peric	Liver	Spleen	Kidn	Jejun	O/T
Group 1	2	2 + 0	0[a]	0	0	0	0	0	0	0	0	0	0	0	0
(genotype	4	2 + 0	0.5	0.5	0	0	0.5	1	1	0	0	0	0	0	0
B-10/423)	6	2 + 0	0.5	0.5	0	0.5	1	1	1	0	0	0	0	0	0
	8	2 + 0	1	0.5	0.5	0.5	2	2	2	1.5	0.5	0.5	0.5	0	0
	10	2 + 0	1	1	0.5	0.5	3	4	4	3	0.5	1.5	0.5	1	0
	14	2 + 0	0.5	1	1	1	1.5	3.5	3.5	3	0.5	0	0.5	1	0
	17	2 + 0	0	0	1	1	1	2	2	2	0.5	1	0.5	1	0
	21	2 + 0	0	0	0	0	1	0.5	1	0	0	0.5	0	0.5	0
	24	2 + 0	0	0	0	0	1	1	1	0	0	1	0	0.5	0
	28	2 + 0	0	0	0	0	1	0	1	0	0	1	0	0.5	0
	34	2 + 0	0	0	0	0	1	0	1	0	0	2	0	0.5	0
	T[b]	22 + 0	3.5	3.5	3	3.5	13	15	17.5	9.5	2	7.5	2	5	0
Group 2	2	2 + 0	0	0	0	0	0	0	0	0	0	0	0	0	0
(genotype	4	2 + 0	0.5	0.5	0	0	0.5	1	1	0	0	0	0	0	0
B-10/525)	6	2 + 0	0.5	0.5	0	0.5	1.5	1	1	0	0	0	0	0.5	0
	8	2 + 0	1	1	0.5	1	2.5	2	3	1.5	0.5	2	0.5	1	0
	10	2 + 0	1	1	1	1	2	2.5	4	3	1	3	1	1	0
	14	2 + 0	0.5	1	1	1	3	3	3	3	1	2	1	1	0
	17	2 + 0	1	1	1	1	3	3	3	2	0.5	1	0.5	1	0
	21	2 + 0	0	0	0	0	2	3	3	1	0	1	0.5	0.5	0
	24	2 + 0	0	0	0	0	0.5	1	1	0	0	2	0	0.5	0
	28	2 + 0	0	0	0	0	1	1.5	1.5	0	0	0	0	0.5	0
	34	2 + 0	0	0	0	0	1	1.5	1.5	0	0	0	0	0.5	0
	T[b]	22 + 0	4.5	5	3.5	4.5	17	19.5	22	10.5	3	11	3.5	6.5	0
Group 3	2	2 + 0	0	0.5	0	0	0.5	0.5	0	0	0	0	0	0	0
(genotype	4	2 + 0	0.5	2	1	2	1	2	2	1	0	0	0	0	0
D-10/298)	6	1 + 1	1	3	1	2	4	5	5	3	1	1	3	1	0
	7	0 + 3	1	2	2	2	2	5	5	3	2	2	3	1	1
	8	0 + 5	1.5	2	2	2	2	5	5	2	2	4	1	1	1
	9	0 + 1	1.5	2	2	2	3	5	5	2	1	3	0	1	1
	10	0 + 2	1.0	2	2	3	3	5	5	2	1	3	2	1	0.5
	14	2 + 0	0.5	1	1	2	3	5	5	2	1	3	2	1	1
	17	2 + 0	0	0	1	1	3.5	3	4	1	1	2	2	1	0
	21	1 + 0	1	0	2	0	3	5	5	1	1	4	1	1	0
	T[b]	10 + 12	8	14.5	14	16	25	40.5	41	17	10	22	14	8	4.5

dpi: days post-infection; Conj: conjunctiva; Conc: conchae; Trach: trachea; T. Airs: thoracic airsac; A. airs: abdominal airsac; Peric: pericardium; Kidn: kidney; Jejun: jejunum; O/T: ovary/testes.
[a] Number of chickens (euthanized + deceased).
[b] Total score over all dpi.

serofibrinous exudate in the bronchial lumen associated with heterophils. Bronchitis was most severe in group 3 at day 4 dpi and still moderately present at 21 dpi. Inflammation was more severe in group 2 compared to group 1, but was absent from 24 dpi in group 2, whilst still slightly present at 34 dpi in group 1 at 10 and 14 dpi.

Pneumonitis was characterized by: (i) lymphocytic infiltrates in the lung tissue, (ii) serofibrinous exudate

Table 7
Histopathological lesions in *C. psittaci* infected chickens.

	Average score for histopathological lesions in 2 chickens of group																				
Lesion[a]	Group 1 (10/428) on dpi								Group 2 (10/525) on dpi								Group 3 (10/298) on dpi					
	4	6	10	14	21	24	34	Total	4	6	10	14	21	24	34	Total	4	6[b]	10	14	21[b]	Total
Bronchitis	0	0	2	1	1.5	1.5	0.5	6.5	0	0.5	3	3	2	0	0	8.5	3.5	3	NT	1.5	3	11
Pneumonitis	0	1	3	3	2	2.5	3	14.5	0	1	1.5	3	3	1.5	1	11	3.5	3	NT	0	1	7.5
Pleuritis	0	0	1.5	1	1.5	1.5	0.5	6	0	0	1.5	1.5	1	1	0	5	0.5	1	NT	0	0	1.5
Aerosacculitis	0	1	3.5	4.5	3	3	2	17	0	1	3.5	3.5	4.5	3	2	17.5	3.5	3	NT	3.5	0	10
Splenitis	0	0	4	4	4	4	4.5	20.5	0	0	4	4	4.5	4	4	20.5	4.5	4	NT	3.5	4	16
Serositis	0	0	1	1	0	1	0	3	0	0	0	0	0	0	0	0	0	0	NT	0	0	0

[a] Histological changes: 1, minimal; 2, slight; 3, moderate; 4, marked; 5, severe.
[b] Only 1 instead of 2 chickens examined, as one animal was already dead at 6 dpi and only one animal was left at 21 dpi. NT not tested.

associated with heterophils in the lumen of the tertiary bronchus and or atria/infundibula and (iii) epithelial hyperplasia of atria/infundibula. Pneumonitis was also most severe in group 3 at 4 and 6 dpi. Later on, pneumonitis was more severe in groups 1 and 2, in which inflammation was still present at 34 dpi. Pleuritis was present in all infected groups and was characterized by: (i) lymphocytic infiltrates, (ii) serofibrinous exudation and (iii) mesothelial hyperplasia with proliferation of the serosal lining. Pleuritis was most severe in group 1, in which inflammation was still present at 34 dpi. In contrast, inflammation was absent at 14 and 34 dpi in groups 3 and 2, respectively.

Aerosacculitis was characterized by: (i) lymphocytic infiltrate, sometimes associated with follicle formation, (ii) serofibrinous exudation, (iii) mesothelial hyperplasia, (iv) mesothelial necrosis with formation of fibrino-necrotic membranes on the surface and (v) proliferation of fibroblastic tissue as a repair reaction after exudation and/or necrosis. Aerosacculitis was more severe in groups 1 and 2 compared to group 3, especially at 14 dpi (Fig. 1) in group 1 and 21 dpi in group 2. For group 3, aerosacculitis was prominent in all chickens examined before 21 dpi.

Splenitis was characterized by: (i) hyperplasia of the reticulo-endothelial system with proliferation of the macrophages present in the spleen, signifying aspecific stimulation of the immune system, (ii) hyperplasia of the lymphoid system with proliferation of lymphoid cells characteristic for specific stimulation of the immune system. Splenitis was moderate to severe in all 3 infected groups from 10 dpi onwards in groups 1 and 2 and from 4 dpi in group 3. Additionally, in group 1, a minimal to slight inflammation of the splenic capsule or serosa was noted until 24 dpi. Serositis of the splenic capsule was characterized by lymphocytic infiltration of the serosal surface with exudation and fibroblastic proliferation.

4. Discussion

Limited epidemiological data on *C. psittaci* infections in chickens from 1960 to 2000 indicate that chickens are relatively resistant to disease. Acute infection progressing to disease and mortality was believed to occur only in young birds, and the incidence of actual epidemics was very low (Andersen and Vanrompay, 2008). However, diagnosis at that time was suboptimal and mainly focused on outbreaks linked to increased mortality, as occurred in those days in commercially raised turkeys, ducks and pigeons.

However, natural infections in chickens are still believed to be unapparent and transient although *C. psittaci* strains isolated from turkeys did cause similar pathology and mortality in chickens as in turkeys (Suwa et al., 1990). But, in fact, we only have limited information on the occurrence of *C. psittaci* in chickens. Etiological diagnosis of respiratory disease in poultry is mostly only performed if the initiated antibiotic treatment fails. Although antibiotic usage decreased the last years, antibiotics are still frequently used and among them are the ones being active against *C. psittaci*. A recent report on veterinary antibiotic consumption in Belgium (BelVet-SAC report 2012; http://www.belvetsac.ugent.be/) concluded that the three most applied antimicrobial classes are: (1) sulphonamides and trimethoprim, followed by (2) tetracyclines (most effective against *C. psittaci*) and (3) penicillins, which induce persistent *Chlamydia* infections. In France, tetracylines are most frequently used (Moulin et al., 2008). Thus, respiratory disease due to *C. psittaci* might often be solved without the need for a proper diagnosis.

Although a pathogen of humans, little is known on the current epidemiology and pathology of *C. psittaci* in chickens. One of the reasons for our ignorance is that *C.*

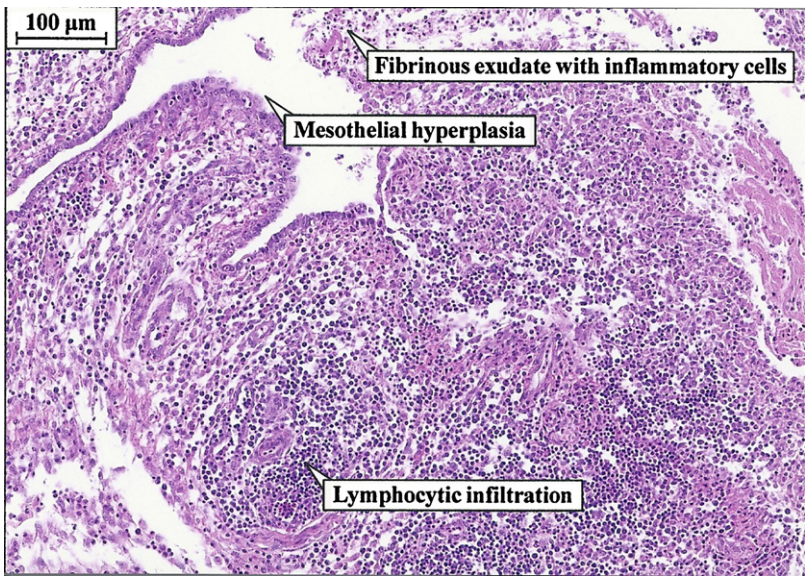

Fig. 1. Haematoxylin and eosin staining of the thoracic airsac of a chicken infected with strain 10/423 at 14 dpi. Note the infiltration of lymphocytes, the presence of fibrinous exudate with inflammatory cells and the mesothelial hyperplasia (20×).

psittaci is not included in routine diagnosis as culture requires biosafety level 3 and the sensitivity and/or specificity of commercial antigen or antibody detection kits is not as it should be (Vanrompay et al., 1994; Sting et al., 2006; Verminnen et al., 2006). More recently, nucleic acid amplification techniques (NAAT's) have given us the opportunity to detect *C. psittaci* in a fast, sensitive and specific way (Sachse et al., 2009). Moreover NAAT's allowed molecular characterization of *C. psittaci* and rapid tracing of zoonotic sources (Branley et al., 2008) and made it possible to detect a new chlamydial agent in French and Australian commercially raised chickens (Laroucau et al., 2009; Robertson et al., 2010).

Ever since applying NAAT's, *C. psittaci* has been detected more often in chickens. Virulent *C. psittaci* strains were detected by NAAT's and isolated from diseased chickens raised in Australia, France, China and Germany (Gaede et al., 2008; Zhang et al., 2008; Laroucau et al., 2009; Robertson et al., 2010; Zhou et al., 2010). Recently, *C. psittaci* was detected in a Belgian chicken slaughterhouse and also in a Belgian chicken hatchery. Zoonotic transmission occurred (Dickx et al., 2010; Dickx and Vanrompay, 2011).

The present study demonstrates the occurrence of highly and less virulent *C. psittaci* strains in broilers raised in Belgium and Northern France. Therefore, the statement that *C. psittaci* infections occur less frequently in chickens has to be reconsidered, as our data suggest that *C. psittaci* is (re)-emerging in chickens. Diagnosis has been improved, but *C. psittaci* strains might also have adapted for replication and survival in chickens. Moreover, as our results clearly demonstrate the high prevalence of *C. psittaci* genotype B and D strains, the finding of *C. psittaci* might not be regarded as a curiosity.

Surprisingly, we are the first to study the pathology of chicken-derived *C. psittaci* strains in chickens. We could only find 5 reports on experimental infections in chickens, but these either used strains isolated from: (i) a budgerigar (Izawa-1; genotype A), a parrot (GCP-1) or a pigeon (P-1041) (Takahashi et al., 1988a,b), (ii) a turkey (strain C-1, Bankowski et al., 1967; no strain or genotype specified, Suwa et al., 1990), or (iii) ruminants (B-577, Bo-Yokohama, SPV-789) (Takahashi et al., 1988b). Also, they did not use the natural route of infection, namely inhalation of aerosols. *Chlamydiae* were directly injected into the airsac or trachea, or chickens were infected orally. The avian strains (10^5 ELD_{50}) used by Takahashi et al. (1988b), induced a generalized infection within 10 dpi followed by death in 8-day-old White Leghorn chickens. Strains isolated from *Psittacidae* were more virulent than the one pigeon strain used, as they caused higher mortality in chickens. Strains derived from ruminants were far less pathogenic to chickens than avian strains.

The presently obtained strains from Belgian and French broilers were *C. psittaci* genotype B or D and created disease in experimentally infected SPF chickens. Thus, *C. psittaci* is a primary pathogen for chickens. Bacterial isolation attempts and PCR for mycoplasma species, aMPV, IB and ILTV indicated that no contaminating microorganisms contributed to the high mortality in group 3 and the pathology observed in all infected SPF chickens. Differences in pathology were observed: genotype D was more virulent than genotype B. The same has been observed for SPF turkeys (Vanrompay et al., 1995). It is impossible to compare the natural infections with the results of an experimental infection in SPF chickens, as data on mortality, macroscopic lesions, cloacal excretion and histology in natural infected chickens are lacking. Data on pharyngeal excretion are available. However, the SPF chickens were infected at the age of 1 week and sampling of conventional chickens occurred at 4, 5 or 6 weeks of age. Also, we do not know: (1) when conventional broilers became infected, (2) the immune status of conventional broilers before contracting the infection, or (3) the influence of possible concurrent infections.

Indeed, 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 and Schulze, 1995; Shi et al., 2003). However, Beeckman et al. (2010) determined the cytokine responses following *C. psittaci* infection of chicken macrophages. High IL-10 and no TGF-β4 responses were observed. This could induce macrophage deactivation and NF-κB suppression and thereby, could dampen innate immunity, rendering the birds more susceptible to other pathogens.

In conclusion, *C. psittaci* infections are apparently emerging in chickens. We could proof Hill-Evans postulates for chicken-derived *C. psittaci* genotype B and D strains. Chicken-processing plant employees should be considered a risk group for human psittacosis. There is a need for higher awareness and for efficient risk assessment and management.

Conflict of interest statement

None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper.

Acknowledgements

The study was funded by Ghent University (grant IOF10/STEP/002) and by MSD Animal Health (Boxmeer, The Netherlands). Lizi Yin has a PhD fellowship from the China Scholarship Council (CSC grant; 01SC2812) and from the Special Research Fund of Ghent University (co-funding of the CSC grant). We gratefully thank A. Dumont (Department of Molecular Biotechnology), G. De Smet, S. Brabant and R. Cooman (Department of Virology, Parasitology and Immunology) for their technical assistance.

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