Chlamydia psittaci in a chicken and turkey hatchery results in zoonotic transmission

Running title: Zoonotic transmission of C. *psittaci* in hatchery.

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Abstract

Chlamydia psittaci (*C. psittaci*) is an obligate intracellular Gram-negative bacterium causing respiratory disease (chlamydiosis) or asymptomatic carriage in birds. *C. psittaci* is a zoonotic agent causing psittacosis or parrot fever in humans. Vertical and/or horizontal transmission via eggs might have serious repercussions on the *C. psittaci* infection status of poultry flocks and thus on zoonotic risk for all workers along the poultry supply chain. We therefore studied the presence of *C. psittaci* in a hatchery. In addition, we examined all (N = 4) employees of the hatchery to evaluate the zoonotic risk. We could not detect *C. psittaci* on either eggs shells or eggshell membranes. However, different *C. psittaci* outer membrane protein (*ompA*) genotypes were cultured from the air of both turkey (genotypes A and C) and chicken (genotype D) hatching chambers. Zoonotic transmission occurred in all employees and mixed infection with up to 3 different genotypes (A, D and C), also found in air samples, were discovered. Diagnostic monitoring and reporting of *C. psittaci* infections in poultry workers should be promoted. Additionally, an efficient veterinary vaccine and information campaigns on zoonotic risk and preventive measures against *C. psittaci* transmission would be beneficial to public health.

Introduction.

Chlamydia psittaci (*C. psittaci*) is an obligate intracellular Gram-negative bacterium, causing respiratory disease (chlamydiosis) or asymptomatic carriage in birds (Vanrompay *et al.*, 1995a). It is a zoonotic agent causing psittacosis in humans. Zoonotic transfer occurs through inhalation of contaminated dust particles or contaminated aerosols created from nasal and/or eye secretions or from dried faeces (Beeckman & Vanrompay, 2009). In humans symptoms may include high fever accompanied by a relatively low pulse, chills, headache, myalgia, non-productive coughing

and difficult breathing. The incubation period is 5 to 14 days. The disease is rarely fatal in properly treated patients.

C. psittaci infections are nearly endemic in poultry (Laroucau *et al.*, 2009;Sting *et al.*, 2006;Van Loock *et al.*, 2005a;Verminnen *et al.*, 2006) and zoonotic transfer of *C. psittaci* is a threat to poultry workers all over the world (Chahota *et al.*, 2000;Dickx *et al.*, 2010;Gaede *et al.*, 2008;Laroucau *et al.*, 2008;Verminnen *et al.*, 2008).

Vertical or transovarial transmission of *C. psittaci* during formation of the egg in the ovarium/oviduct of the breeder has been described for chicken (Wittenbrink *et al.*, 1993) and turkey eggs (Lublin *et al.*, 1996). It leads to infection of one-day-old birds. Nevertheless, vertical transmission of *C. psittaci* is thought to be rare (Harkinezhad *et al.*, 2008b). Thus, it might not be the main origin of infection for one-day-old birds. As far as we know, horizontal infection of embryo's or other egg contents by eggshell penetrating of *C. psittaci* has not been examined. Fecal contamination of eggshells by infected hens or during egg passage in the cloaca might occur, as *C. psittaci* resides in the gut and is excreted through the feces (Harkinezhad *et al.*, 2008a). *C. psittaci* (∅ 0.2 µm) is very small compared to the well-known fecal egg contaminant *Salmonella* (∅ 0.7-1.5 µm, length 2-5 µm). Thus, *C. psittaci* could even be more easily internalized in eggs.

Vertical and/or horizontal transfer might have repercussions on the *C. psittaci* infection status of poultry flocks and thus on zoonotic risk for all workers along the poultry supply chain. We therefore studied the presence of *C. psittaci* in a hatchery. Additionally, we examined all employees of the hatchery to evaluate the zoonotic risk.

Material and methods

Background

The study was conducted in a Belgian hatchery located in West-Flanders. The hatchery had two hatching facilities in separate corridors; one used for both turkey and guinea fowl eggs and the other used solely for hatching chicken eggs. The hatchery was selected based on the willingness to participate in this study.

In June and September 2010, we performed a *C. psittaci* study in the turkey/guinea fowl and chicken hatching facilities, respectively. Eggshells and eggshell membranes were sampled. Additionally, *C. psittaci* bioaerosol monitoring was performed, sampling air from the turkey/guinea fowl or chickens hatching chambers. The employees, being 2 men (M1 and M2) and 2 women (F1 and F2), having daily contact with eggs and hatchlings, voluntarily participated (informed consent) in this study and provided us a self-taken pharyngeal swab for chlamydial diagnosis.

Sampling details and processing of samples prior to analyses.

During the study in the turkey/guinea fowl and chicken facilities, at the beginning (T0), two subsequent air samples were taken from a cleaned, disinfected (formaldehyde fumigation) hatching chamber (17.12 m^3; P13 Petersime, Zulte, Belgium), subsequently to be used for turkey or chicken eggs. Next, 26-days-old or 19-days-old embryonated turkey or chicken eggs, respectively, were brought in the hatching room, fumigated with formaldehyde and incubated at hatching conditions (turkey eggs, 37.4°C and 90% relative humidity (RH); chicken eggs, 37°C and 90% RH). Two days later the hatching process started. Two subsequent air samples were taken at several time points during this hatching process: i) in the morning when only a few animals (10%) had hatched (T1), ii) at noon (T2) and ii) in the evening when most animals (90%)

had hatched (T3). Bioaerosol monitoring was performed using the MAS-100 Eco Sampler (Merck, Darmstadt, Germany) together with the in-house made air collection medium (ChlamyTrap) at an air flow rate of 100 L/min during 10 min (Van Droogenbroeck *et al.*, 2008). At each time point, two samples were taken. After air sampling, petri dishes with 20 ml ChlamyTrap were transported on ice and stored at -80°C until tested. Samples taken on the same time were pooled for further processing. Next, 40 ml of the pooled air collection medium was divided in two equal parts. All samples were ultracentrifuged (45,000 x g, 45 min, 4 °C). Pellets for culture were suspended in 500 µL chlamydia transport medium (Vanrompay *et al.*, 1992) while the ones for *C. psittaci* genotyping were suspended in 198 µL DNA extraction buffer (Van Loock *et al.*, 2005b). Samples were stored at -80 °C until tested.

Rayon-tipped aluminum shafted swabs (Copan, Fiers, Kuurne, Belgium) were used to sample in twofold the employees, the eggshell of 20 randomly selected turkey (0.15%) or chicken (0.10%) eggs before hatching as well as the eggshell membranes of 20 additional, randomly selected turkey (0.15%) or chicken (0.10%) eggs after hatching. Eggs in a hatching room came from the same batch and one single parental flock. Swabs for chlamydial culture contained chlamydia transport medium, while dry swabs were used for *C. psittaci* genotyping. Swabs were transported on ice and stored at -80°C until processed.

***C. psittaci* culture.**

The presence of viable *C. psittaci* in air samples or swabs was examined by bacterial culture in Buffalo Green Monkey (BGM) cells identifying *C. psittaci* by use of a direct immunofluorescence staining at day 6 post inoculation (IMAGEN^{TM}, Oxoid, Drongen, Belgium) (Vanrompay *et al.*, 1994). The number of *C. psittaci* positive cells was counted in five randomly selected microscopic fields (600x; Nikon Eclipse TE2000-E, Japan). A score from 0 to 4 was

given. Score 0 indicated the absence of *C. psittaci*. Score 1 was given when a mean of 1–5 non-replicating elementary bodies were present. Scores 2, 3 and 4 were given when a mean of 1–5, 6–10 and >10 inclusion positive cells could be observed (Vanrompay *et al.*, 1994). Subsequently, *C. psittaci* in positive samples were titrated according to the method of Spearman and Kaerber determining the $\log_{10}$ 50% Tissue Culture Infective Dose ($TCID_{50}$) per ml chlamydia transport medium or per ml ChlamyTrap air collection medium (Mayr *et al.*, 1974).

C. psittaci **genotyping.**

For all samples, DNA extraction was performed as previously described. Outer membrane protein A (*ompA*) genotyping was performed by a *C. psittaci* genotype-specific real-time PCR (Geens *et al.*, 2005). The test is based on using genotype-specific primers and genotype-specific TaqMan probes. Real-time PCR allowed molecular characterization of the *C. psittaci* strains involved, as well as quantification of chlamydial DNA using the human beta-actin housekeeping gene for normalization (Van Droogenbroeck *et al.*, 2009).

Results and discussion

The results on bioaerosol monitoring and on the examination of human swabs are presented in Tables 1 and 2, respectively. *C. psittaci* was cultured from the air of both the turkey/guinea fowl and chicken hatching chambers and in both cases high titers of live organisms (up to $10^{10.75}$ per ml ChlamyTrap for the turkey hatching chamber; up to $10^{6.25}$ per ml ChlamyTrap for the chicken hatching chamber) were present in the air. Viable *C. psittaci* was present in hatching chambers from start to finish of the hatching process (Table 1). Moreover, the microorganism was even there before the hatching process started, in cleaned, disinfected hatching rooms. *C. psittaci* titers

in air increased 100 to 10000 times during the hatching process of chickens or turkeys, respectively.

The hatching chamber for chickens contained *C. psittaci ompA* genotype D on all examined time points. Recently, Dickx *et al.*, (2010), also found genotype D in chickens being processed in the abattoir as well as in the air of the abattoir. Genotype D is considered highly virulent and is excreted extensively (Vanrompay *et al.*, 1995b). The empty hatching chamber for turkey/guinea fowl eggs contained genotype C and during hatching of turkeys genotype A was found (Table 1). Thus, cleaning and disinfection (formaldehyde fumigation) of both hatching chambers after the previous egg incubation period was not sufficient to remove *C. psittaci*, as viable *C. psittaci* was still present in air samples taken at T0. Interestingly, guinea-fowl eggs from France were incubated during the previous incubation period. *C. psittaci* has recently been detected by PCR in commercially raised guinea fowl in France. However, *ompA* genotyping failed (Laroucau *et al.*, 2009). Genotype C is mostly found in ducks and geese and has been isolated from poultry workers in relation with respiratory disease (Harkinezhad *et al.*, 2009).

Fumigation of eggs upon arrival in the hatching chamber seemed to have an effect on the amount of live organisms in the air, as genotype C was no longer detected at T1. Instead, genotype A was present, originating from turkey eggs. Genotype A was isolated before from turkeys (Van Loock *et al.*, 2005a;Verminnen *et al.*, 2008) and is highly virulent.

Eggshells were negative by both PCR and culture. Thus, contamination of eggs by secretions/excretions of the breeders was undetectable. Perhaps, since only a small percentage of eggs was sampled. However, it could also be due to the egg washing procedure performed in a hatchery upon arrival of the eggs and/or to fumigation of the eggs after being placed in the hatching chamber. On the other hand, *C. psittaci*, which is an extremely small bacterium, might rapidly penetrate the eggshell during cooling of fresh laid eggs in the breeder farm. Thus, in the

future, eggshell contamination should be examined in the breeding facility instead of in a hatchery, using fresh laid eggs.

C. psittaci was not detected by either PCR or culture on eggshell membranes. Again, this could be due to: i) examining only a small percentage of the eggs or ii) because the organism is present in the animal itself, in the amnion or allantois fluid and/or in the yolk, the yolk-sac membrane and/or the egg white. Maybe, egg white, yolk sac and/or yolk sac membrane, become infected during artificial insemination with *C. psittaci* contaminated sperm or during formation of the egg in the ovarium/oviduct. This could lead to the transfer of *C. psittaci* from the egg white and/or yolk to the embryo where the organisms might stay as aberrant temporary non-reproductive bodies, otherwise the embryo would die, in cells of the intestine and/or liver. Moreover, transovarian transmission through hematogenous spread of *C. psittaci* might also occur (Vanrompay *et al.*, 1995b). Sampling egg contents and internal organs of embryos to monitor *C. psittaci* dissemination during the embryonic period could provide answers.

Nevertheless, vertical or horizontal egg contamination might be reduced by vaccinating hens and roosters on the breeder farms. However, *C. psittaci* vaccines are not available. Thus, prophylactic measures like monitoring *C. psittaci* infections in breeders, optimal hygiene and disinfection of eggs soon after laying (Cox *et al.*, 2000) are currently the main weapons against egg contamination.

All employees (N = 4) were *C. psittaci* positive by culture and mixed infections with up to three different *ompA* genotypes (A, C and D) were discovered. Previously, Van Droogenbroeck et al., (2009), described a mixed *C. psittaci* genotype D, F and E/B asymptomatic infection in a veterinarian. The infection originated from diseased industrial turkeys. Interestingly, the currently examined employees were also healthy. All employees were working in the hatching facility for more than 20 years. In the past, they all had to seek medical attention because of respiratory

disease and were treated with tetracylines without an etiological diagnosis being performed. Thus, we cannot discuss on a possible link with *C. psittaci* infections. However, as suggested by Dickx et al., (2010), poultry workers are almost continuously exposed to *C. psittaci* and therefore could have natural immunity against disease.

Genotype A was the most prevalent genotype in the employees, as shown by quantitative real-time PCR (Table 2). This is in accordance with the high prevalence of this genotype in air samples (Table 1). Men were less infected than women. The men spend most of their daytime in the administrative office and only assisted in the hatching chambers during 'peak moments'. The women spend most of their daytime handling both eggs and hatchlings.

Psittacosis is recognized as an occupational disease in the USA, Belgium, France, the Netherlands, Germany, Slovakia and the UK. In those countries, the occupational physician is obliged to report each case of psittacosis. In Belgium, there is a Fund for Occupational Diseases to assess *C. psittaci* cases and possibly accept them as an occupational disease, and take care of financial compensations for the employee if necessarily.

Each employer is responsible for the health and safety of his employees and should focus on prevention of infections. An adequate prevention starts with a risk assessment. The employer, assisted by the occupational physician and occupational hygienist, evaluates the exposure to biological agents, taking into account the nature (contact with people or animals, or the workplace itself), intensity (the amount of infectious material handled) and duration of the worker's exposure (HSE, 2010). The risk assessment must also identify workers and other people who may not be in the workplace all the time (cleaners, maintenance and repair workers, contractors, students on placements) and members of the public who might be present (visitors) (HSE, 2010). Based on this information, adequate preventive measures can be designed. The second phase is the implementation of the preventive measures. In this stage, education and

training of the employees is very important to ensure that the measures are well understood and executed. When present, a company doctor might play a crucial role in both prevention and recognition of *C. psittaci* infections.

On a personal level, prevention includes a good hand hygiene protocol, protective clothing that does not retain dust and a mouth and eye (full face) mask. It is necessarily to have a transition room where protective clothing may be kept. Employees should only stay in the hatching chambers for as short as possible. Good environmental hygiene is also important, such as daily cleaning and disinfection of work areas and equipment, hereby preventing the creation of infectious aerosols. Some safe cleaning techniques include wet mopping of the floor with disinfectants or spraying the floor with a disinfectant or water before sweeping it. For larger areas, such as industrial hatching chambers, low pressure washers instead of high pressure cleaners are strongly recommended.

CONCLUSIONS

Until now, *in ovo* transmission of *C. psittaci* was considered of minimal impact. At present, we found increasing amounts of *C. psittaci* in the air during hatching of turkeys or chickens. Thus, hatchlings could already become infected before arriving on the farm. Diagnostic monitoring and reporting of *C. psittaci* infections in poultry workers should be promoted. Additionally, an efficient veterinary vaccine and information campaigns on zoonotic risk and preventive measures against *C. psittaci* transmission would be beneficial to public health.

Acknowledgements

This study was funded by the Federal Public Service of Health, Food Chain Safety and Environment (convention RF-6177).

227 Table 1. Diagnostic results on air samples from the turkey/guinea fowl or chicken hatching
228 chambers.

	Presence of *C. psittaci* in the air of hatching facilities		
	Turkey/guinea fowl hatching chamber		
Time point	Culture score	Titer (log_{10} $TCID_{50}$ per ml)	*ompA* genotype
T0	4	6.75	C
T1	4	6	A
T2	4	9	A
T3	4	10.75	A
	Chicken hatching chamber		
T0	4	4.25	D
T1	4	5.75	D
T2	4	5.75	D
T3	4	6.25	D

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Table 2. Normalized number of *ompA* copies per 5 µl DNA extract determined by genotype-specific real-time PCR

Pharyngeal sample	Normalized number* of *C. psittaci ompA* copies		
	Genotype A	Genotype D	Genotype C
M1	3.12×10^4	1.66×10^2	Neg
M2	1.55×10^3	1.34×10^2	Neg
F1	3.60×10^6	3.60×10^5	1.23×10^2
F2	4.12×10^6	4.60×10^5	2.69×10^5

*The number of human beta-actin copies in the reaction was determined in order to correct for inter-sample variability due to differences in sample taking and efficiency of DNA extraction. Absolute *C. psittaci* copy numbers were then normalized to the amount of human beta-actin gene copies in each sample.

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