First Experimental Evidence for the Transmission of *Chlamydia psittaci* in Poultry through Eggshell Penetration

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**Summary**

Eggshell penetration by pathogens is considered a potential route for their transmission in poultry flocks. Additionally, in case of zoonotic pathogens, contact with infected eggs or their consumption can result in human infection. *Chlamydia psittaci* is a zoonotic bacterium that causes a respiratory disease in poultry and humans. In this study, we provide an experimental evidence for eggshell penetration by *C. psittaci*. Additionally, we show that after eggshell penetration, *C. psittaci* could eventually infect the growing embryo. Our findings portend the potential of horizontal trans-shell transmission as a possible route for the spread of *C. psittaci* infection in poultry flocks. Considering that horizontal transmission of pathogens via eggs mainly occurs in hatcheries and hatching cabinets, we suggest the latter as critical control points in the transmission of *C. psittaci* to hatching chicks and broilers, as well as to the hatchery workers and consumers of table eggs.

**Introduction**

*Chlamydia psittaci* is a Gram-negative obligate intracellular bacterium that causes respiratory disease in poultry (psittacosis or avian chlamydiosis). The infection is often systemic and can be manifested by nasal and ocular discharges, dyspnoea, diarrhoea, hyperthermia, lethargy and dullness. The disease greatly affects the welfare in poultry farms and results in severe economic losses due to body weight loss, reduced egg production, increased mortality and costly antibiotic treatment (Vanrompay et al., 1995). In addition, *C. psittaci* is a zoonotic pathogen that causes a respiratory disease with potentially fatal complications in human (psittacosis or parrot fever) (Vanrompay et al., 1995; Kovacova et al., 2007).

Infected birds shed the pathogen in large quantities in their faecal droppings and respiratory secretions. Hence, transmission among birds occurs mainly through the inhalation of aerosols and airborne faecal dust (Vanrompay et al., 1995). Additionally, previous studies have suggested the transmission of *C. psittaci* from infected chicken and turkey layers to their progenies via the egg (vertical or transovarian transmission) (Wittenbrink et al., 1993; Lublin et al., 1996). These suggestions were based on the isolation or the detection of live bacteria in tissues of growing embryos. However, it is well established that pathogens can gain access to freshly laid eggs via eggshell penetration (horizontal trans-shell transmission) (Berrang et al., 1999). Eggshell penetration by pathogens mainly occurs as a result of the negative pressure created inside the egg shortly after laying (suck-in effect). The drop in ambient temperature, from the body temperature of the hen (≈42°C) to the temperature of the barn (≈21°C), causes the egg contents to contract and thus creates a negative pressure. The latter acts as a vacuum that pulls the microbes present on the eggshell into the egg (Berrang et al., 1999). Eggshell penetration by *C. psittaci* can lead to the transmission of the pathogen to broiler flocks, farm personnel and consumers of table eggs.
commercial table eggs. In this study, we provide the first evidence for eggshell penetration by *C. psittaci*, under experimental conditions mimicking the suck-in phenomenon. Additionally, we prove that after eggshell penetration, *C. psittaci* can survive the passage through the egg albumen, which possesses anti-microbial properties, and infects the growing embryo.

**Materials and Methods**

*C. psittaci* and SPF eggs

*C. psittaci* genotype D strain 92/1293 (Vanrompay et al., 1993) was used in this study. The bacterium was grown on Buffalo green monkey (BGM) cells as previously described (Vanrompay et al., 1992). Bacterial titration was performed by the method of Spearman and Kaerber to determine the number of bacteria used in the experiments (Vanrompay et al., 1992). Specific pathogen-free (SPF) chicken eggs (Valo BioMedia GmbH, Osterholz-Scharmbeck, Germany) were used in our experiments. The donor flock of the SPF eggs supplier was seronegative to *C. psittaci* antigens, as evident by our MOMP-based ELISA (Verminnen et al., 2006). Also, sample SPF eggs, obtained from the same donor flock, were negative to *C. psittaci* DNA, as shown by our *C. psittaci*-specific nested PCR (Van Loock et al., 2005). The SPF eggs used in our experiments were screened for cracks or eggshell deformities by candling.

**Faecal contamination of eggshells by Chlamydia psittaci in chicken broilers**

Freshly laid eggs (*n* = 96) were randomly collected during 1 day from a chicken broiler farm (East Flanders, Belgium), with a history of *C. psittaci* infection. The eggs were collected from the egg collection line prior to any handling by workers. Eggshell sampling was performed according to the method described by De Reu et al. (2005), with slight modifications. Briefly, each egg was dipped in a sterile plastic bag filled with 15 ml of Chlamydia transport medium (CTM) (Vanrompay et al., 1992). Thereafter, the eggshell was washed by massaging the egg by hand from the outside of the plastic bag for 2 min. After sampling, the media were kept on ice for transportation to our laboratory. Media from each of four eggs were pooled (24 sample groups) and then subjected to ultracentrifugation (45,000 × g at 4°C) for 45 min to harvest *C. psittaci*. The obtained bacterial pellets were resuspended in 1 ml of sucrose–phosphate–glutamate (SPG) buffer for genomic DNA extraction by the G-spin kit (Intron Biotechnology, Daejoen, South Korea), according to the manufacturer’s instructions. The DNA was tested for the presence of *C. psittaci* genomic DNA using a nested PCR with an inhibition control, as previously described (Van Loock et al., 2005). The lowest detection limit of the nested PCR is one organism (Van Loock et al., 2005).

**Examination of eggshell penetration by Chlamydia psittaci**

Specific pathogen-free (SPF) eggs (*n* = 15) were processed using the protocol described by De Reu et al. (2006) with slight modifications. Briefly, the adapted method consisted of replacing the egg content with CTM (Vanrompay et al., 1992); thereafter, eggshell openings were sealed with silicon and stored at 4°C till use on the next day. Eggshell of each egg was fully smeared with 200 µl of SPG buffer (Robertson et al., 2010) containing 10⁶ TCID₅₀ (50% tissue culture infective dose) of *C. psittaci* using a cotton swab. Immediately after the inoculation of the bacteria on the eggshell, eggs were incubated at 37°C for 30 min, followed by incubation at 20°C for 60 min to mimic the suck-in effect. At the end of the incubation time, the silicon seals were enucleated and the contained CTM was retrieved from the eggs. The media were subjected to ultracentrifugation (45,000 × g at 4°C) for 45 min to harvest *C. psittaci*. The obtained bacterial pellets were resuspended in 1 ml of SPG for genomic DNA extraction by the G-spin kit (Intron Biotechnology), according to the manufacturer’s instructions. The DNA was tested for the presence of *C. psittaci* genomic DNA using a nested PCR with a control for internal inhibition, as previously described (Van Loock et al., 2005).

**Infection of growing chicken embryos with Chlamydia psittaci via eggshell penetration**

The conditions to allow the eggshell penetration of the bacteria were performed as described above. Afterwards, SPF eggs (*n* = 17) were incubated in an automatic hatching chamber at 37.6°C and 47–52 % relative humidity, with frequent turning. At Day 13, the eggs were ripped, under sterile conditions, and the growing embryos were collected. Each embryo was mechanically homogenized using a 70-µm cell strainer (Falcon: BD Labware, NJ, USA) to a cell suspension in total volume of 10 ml PBS. Genomic DNA extraction was performed on 400 µl cell suspension using the G-spin kit, according to the manufacturer’s instructions. The yielded DNA was examined for the presence of *C. psittaci* genomic DNA using nested PCR, as described above. Additionally, BGM cell monolayers, in *Chlamydia* Trac bottles, were inoculated by 100 µl of the embryo cell suspension for further propagation of the bacteria. After three or 6 days of incubation, a second passage of *C. psittaci* was performed on BGM cells for 6 days. Thereafter, BGM monolayers were stained using the commercially available direct immunofluorescence (DIF) kit (IMAGEN™: Oxoid, Basingstoke, UK) for the detection of live Chlamydia according to the manufacturer’s instructions.
Results

PCR analysis of the pooled eggshell washings that were obtained from a farm with *C. Psittaci* infection history showed the presence of *C. psittaci* on eggshells of freshly laid eggs (7/24, 29% for pools or 7.3–29% for individual eggs). Accordingly, we next determined whether *C. psittaci* can penetrate the eggshell by means of the suck-in effect. Specific pathogen-free (SPF) eggs filled with CTM were challenged by the application of *C. psittaci* on their shells and then exposed to a drop in ambient temperature. Nested PCR showed that the media collected from all the challenged eggs (15/15, 100%) were positive for *C. psittaci* genomic DNA. This finding indicates the penetration of the eggshell by *C. psittaci* as a result of the suck-in effect. After demonstrating the penetration of *C. psittaci* through the eggshell, we next examined whether the penetrating bacteria could survive the passage through the egg albumen and infect the growing embryo. Challenged eggs were incubated for 13 days to allow the embryos to grow. Thereafter, the embryos were collected and homogenized to obtain a single-cell suspension. Nested PCR showed that the DNA extracted from the embryos was positive *C. psittaci* genomic DNA (11/17, 65%). Internal inhibition controls were all positive, indicating the absence of circumvent polymerase inhibitors in samples of the template genomic DNA. Cell suspensions from PCR-positive embryos were further examined for the presence of live bacteria using DIF, after a passage on BGM cell monolayers. Indeed, DIF staining of inoculated BGM cells, either after 3 or 6 days of incubation, showed the presence of live bacteria in all PCR-positive embryos (11/11, 100%) (Fig. 1).

Discussion

Eggshell penetration is recognized as a possible route for the transmission of avian pathogens to broilers (Berrang et al., 1999). Moreover, in case of zoonotic pathogens, infected eggs represent a potential source of infection for farm labourers and egg consumers. Pathogens penetrate the eggshell by means of the negative pressure created inside the egg due to the drop in ambient temperature as the egg leaves the hen’s cloaca (suck-in effect) (Berrang et al., 1999). Birds infected with *C. psittaci* shed the pathogen in large quantities in their faecal droppings (Vanrompay et al., 1995). Consistently, our study illustrates the common presence of *C. psittaci* on the eggshell on a broiler farm with a history of infection. Thus, freshly laid eggs are readily predisposed to penetration by *C. psittaci*. Indeed, challenging the eggshell of chicken SPF eggs with *C. psittaci* has resulted in the penetration of the eggshell by the bacteria. While eggshell and eggshell membrane can be penetrated by microbes, the egg albumen represents the main line of defence against microbial invasion. The egg albumen comprises a number of microbiocidal factors, such as the highly alkaline pH, the presence of iron-binding factors (e.g. ovotransferrin) and bactericidal enzymes (e.g. lysozyme) (Berrang et al., 1999). Therefore, one of the typical characteristics of eggborne pathogens is their ability to survive the passage through egg albumen and infect the growing embryo. Our findings show that *C. psittaci* is an egg pathogen, as it could survive the passage through egg albumin and eventually infect the growing embryo.

Our findings have implications for the transmission and control of *C. psittaci* infection in poultry farms as well as to humans. Infected eggs could represent a potential source for the horizontal transmission of *C. psittaci* to hatching chicks in the hatcheries and hatching cabinets. Also, these eggs represent a source for the transmission of pathogen to farm workers and consumers of table eggs. Therefore, hatcheries should be regarded as control points in the transmission of *C. psittaci* within poultry farms, where dipping or spraying disinfection procedures could be applied to prevent horizontal egg transmission. Such early intervention strategy could improve the status of animal welfare and the economic profit in poultry farms and minimize the risk of human infection.

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**Fig. 1.** Detection of *Chlamydia psittaci* in Buffalo green monkey (BGM) cells by immunofluorescence staining after inoculation with embryo homogenates obtained from Specific pathogen-free (SPF) eggs that were infected by trans-shell penetration. Image shows the *C. psittaci* intracellular inclusion (green) due to the propagation of the bacteria in BGM cells. Bar = 50 μM.
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Author Contribution Statement
All authors have substantially contributed to the study design, acquisition, analysis and interpretation of the data, manuscript drafting and revision of its intellectual contents. All authors have approved the submitted manuscript.

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