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Authors: Zhang, Faming, Li, Shaowen, Yang, Jianming, Pang, Wanyong, Yang, Li, et al.

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Isolation and Characterization of *Chlamydophila psittaci* Isolated from Laying Hens with Cystic Oviducts

Faming Zhang,[A] Shaowen Li,[B] Jianming Yang,[A] Wanyong Pang,[A] Li Yang,[A] and Cheng He[AC]

[A]College of Veterinary Medicine, China Agricultural University, Beijing 100094, China
[B]College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China

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SUMMARY. The objective of this study was to isolate and identify a hypothetical *Chlamydiaceae* pathogen from laying hens with an oviduct cyst, and to characterize its potential causal relation with decreased egg production. Our clinical survey showed that cystic oviducts were prevalent at rates of 10% and 15.1% in breeder and commercial hen flocks, respectively. Chlamydial antigens were detected in 20 of 50 pharyngeal swabs (40%) and in 17 of 20 oviduct tissues (85%) using enzyme-linked immunosorbent assay (ELISA) antigen detection kits. The isolated pathogen was identified as *Chlamydophila psittaci* via complement fixation test, PCE-ELISA, and immunofluorescence assay. Avian influenza virus, Newcastle disease virus, and infectious bronchitis virus were excluded after oviduct tissues were inoculated onto the chorioallantoic membrane of embryonating eggs. The nucleotide sequence of the *omp1* gene (accession no. EF202608) from the isolate was similar to that of *C. psittaci* avian type C (accession no. L25436). Typical cystic oviducts were observed in specific-pathogen-free hens inoculated intraperitoneally with the isolate. The high presence of chlamydial antigen is consistent with the cystic oviducts and poor egg production. We conclude that the isolated *C. psittaci* is most likely associated with cystic oviducts in laying hens.

RESUMEN. Aislamiento y caracterización de *Chlamydophila psittaci* aislada de ponedoras comerciales con oviductitis quística. El objetivo de este estudio fue aislar e identificar una *Chlamydiaceae* hipotéticamente patógena de oviductos quísticos de gallinas ponedoras, y caracterizar su posible relación como potencial agente causal relacionado con baja en la postura. Nuestra encuesta epidemiológica mostró que la prevalencia de oviductos quísticos fue del 10% y 15.1% en lotes de reproductoras y ponedoras comerciales, respectivamente. Los antígenos clamidiales fueron detectados en 20 de 50 hisopos faríngeos (40%) y en 17 de 20 tejidos provenientes de oviducto (85%) usando kits para detección mediante el inmunoensayo con enzimas asociadas. El patógeno aislado fue identificado como *Chlamydophila psittaci* mediante la prueba de fijación de complemento, elemento fotorreductor conservado - ELISA y prueba de inmunofluorescencia. Los virus de influenza aviar, enfermedad de Newcastle y bronquitis infecciosa fueron excluidos después de que tejidos de oviducto fueron inoculados en la membrana corioalantoidea o en huevos embrionados. La secuencia de nucleótidos del gen *omp1* (No. de acceso EF202608) del aislamiento fue similar a la de *C. psittaci* aviar tipo C (No. de acceso no. L25436). Se observaron oviductos típicamente quísticos en gallinas libres de patógenos específicos inoculadas intraperitonealmente con el aislado. La alta presencia de antígenos clamidiales fue consistente con los oviductos quísticos y la pobre producción de huevos. Concluimos que el aislamiento de *C. psittaci* parece estar muy asociado con la presencia de oviductos quísticos en gallinas ponedoras.

Key words: isolation, characterization, *Chlamydophila psittaci*, cystic oviduct, laying hens

Abbreviations: AIV = avian influenza virus; CFT = complement fixation test; CPE = cytopathic effect; EID_{50} = 50% embryo infectious dose; ELISA = enzyme-linked immunosorbent assay; EMEM = Eagle's minimal essential medium; HA = hemagglutination; HI = hemagglutination inhibition; IBV = infectious bronchitis virus; IFT = immunofluorescence test; MOMP = major outer membrane protein; NDV = Newcastle disease virus; OMP-PCR = outer membrane protein polymerase chain reaction; PBS = phosphate buffered solution; PI = postinoculation; SPF = specific-pathogen-free

Chlamydiosis has spread worldwide and has been isolated in turkeys, chickens, ducks, and pigeons all over Europe and the United States (1). Moreover, infection in humans was also reported, especially in those closely connected to infected birds (2,3,4). Because of the severity of psittacosis, this bacterial species has been classified as a category B biowarfare agent in the United States (8). In the poultry industry, several economically important epidemics of chlamydiosis have been reported in turkeys (18) and ducks (15). Epidemics of avian chlamydiosis have also been reported in wild birds (10,11). The infections vary greatly in clinical severity, depending on the species and age of the infected bird and the *C. psittaci* strain. Cases of chlamydiosis in laying hens have been reported, but were often unapparent (4). Epidemiologic and laboratory examinations revealed that chickens are relatively resistant to chlamydiosis, especially older birds (1,3).

Since 2000 a very poor egg production (45.1%, instead of 96% during peak period) in laying hens became prevalent in China, particularly in Hy-line and Lohmann strains (7). This problem occurred in previously clinically healthy hen flocks, resulting in culling of large numbers of hens due to poor productive performance. Until recently, studies related to the isolation and characterizations of a potential pathogen remained unknown in China. Therefore, the purpose of this study was to isolate and characterize the pathogen(s) and its potential association with cystic oviducts.

MATERIALS AND METHODS

Clinical survey and detection of chlamydial antigens. The current study was approved by the Animal Care and Use Committee at China Agricultural University and was carried out in approved animal biosafety level 2 facilities. In the study, a total of 61,000 hens at the age of 25 wk were selected from different farms, including 10,000 affected breeder

[C]Corresponding author. E-mail: hecheng@cau.edu.cn

Table 1. Incidence of oviduct cyst in affected hens and unaffected hens.

	Breeder hens			Laying hens		
Species	No.[A]	Positive no. (%)	Negative no. (%)	No.[A]	Positive no. (%)	Negative no. (%)
Affected hens[B]	10.0	1.0 (10.0%)[**]	9.0 (90.0%)	25.0	3.79 (15.1%)[**]	21.2 (84.9%)
Unaffected hens[C]	15.0	0.15 (1.0%)	14.85 (99.0%)	11.0	0.28 (2.5%)	10.73 (97.5%)

[A]Bird numbers = no. in each column × 1000.
[B]Average egg production rate arranged from 45.5% to 52.1% in affected hens during peak period.
[C]Average egg production rate arranged from 88.5% to 91.0% in unaffected hens during peak period.
[**]Means $P < 0.01$ when compared to the unaffected breeder hens ($P = 0.0052$) or unaffected laying hens ($P < 0.0001$) in same column.

hens, 25,000 affected laying hens with egg drop, 15,000 unaffected breeder hens, and 11,000 unaffected laying hens as control groups. From their population, 50 pharyngeal swabs and 20 oviduct tissues from affected birds were subjected to testing to detect the possible of presence of antigens of *C. psittaci* by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (IMAGEN^{TM} CHLAMYDIA, DakoCytomation Ltd., Cambridgeshire, United Kingdom) following the manufacturer's procedure.

Pathogen isolation and characterization. Tissue samples, usually oviducts and spleens, were obtained aseptically from suspect hens during postmortem examination. Approximately 100 mg minced tissues were suspended with Eagle's minimal essential medium (EMEM). Gentamycin (200 μg/ml) and Vancomycin (1 mg/ml) were added to the suspension. The samples were vortexed with 3 mm–diameter glass beads, centrifuged at 500 × *g* for 5 min at 4 C, and the supernatants were collected for inoculation into chicken embryos and McCoy cells (2).

Tissue supernatants (0.4 ml) were injected into the yolk sac of specific-pathogen-free (SPF) 6-day-old chicken embryos. The embryonating eggs were then incubated in a humid atmosphere (55%) at 39 C. The yolk-sac membranes were harvested from the embryos that died between day 3 and 10 postinoculation (PI) (2). If no embryo death occurred, additional blind passages were performed before designating any samples as negative.

In another cultivation, 20 μl of the oviduct supernatant preparations were inoculated onto a McCoy cell monolayer. After incubation at 37 C for 2 hr in a 5% CO_2 atmosphere, inocula were removed and replaced with MEM. Cells were incubated for 72 hr and then subcultured twice at 48-hr intervals if earlier passages were negative. Positive cell cultures and harvested yolk sacs were stored at −80 C.

Antigen preparation. The yolk-sac harvests were ground to a smooth homogeneous paste with sterile sand, and diluted 1:3 (v/v) in phosphate buffered solution (PBS). After homogenization, the preparation was subjected to centrifugation at 1000 × *g* for 20 min at 4 C, and the middle layer was collected as undiluted antigen (2,5). The cell cultures were inactivated by the addition of phenol to a final concentration of 1.0% (v/v), and then antigens were collected as previously described (3).

Complement fixation test (CFT). The CFT Hemolytic System Set Vet was performed in 96-well round-bottom multiwell plates according to the manufacturer's procedures (Diesse Diagnostica Senese SPA, Siena, Italy).

Immunofluorescence test (IFT). Before staining, yolk-sac membranes and cell cultures were washed once with PBS and fixed with acetone for 10 min. An IFT was performed using *Chlamydia* test kits (IMAGEN^{TM} CHLAMYDIA, DakoCytomation Ltd.) following the manufacturer's instructions.

DNA extraction and PCR amplification. Amplicons were prepared from purified C*hlamydophila* using the DNeasy Tissue Kit (Qiagen GmbH, Gene Company Limited, Hilden, Germany) following the manufacturer's instructions. A confirmatory test for *Chlamydia* based on the major outer membrane protein (MOMP) gene can be used to distinguish *C. pecorum* from *C. psittaci*/*C. abortus* by absence of the ClaI restriction enzyme site. This nested polymerase chain reaction (PCR) should produce a product of 165 bp (13,14).

Reference genomic DNA prepared from *C. psittaci* strain ISN 1528 was a gift from Dr. David Longbottom (Moredun Research Institute, Edinburgh, United Kingdom). Another reference was *C. psittaci* 6BC (Chinese Institute of Veterinary Inspection, Beijing, China). An unaffected McCoy cell preparation was included as negative control. PCR products were separated by electrophoresis through a 2.5% agarose gel (Sigma) and visualized by ethidium bromide staining. DNA molecular weight markers (Boehringer, Mannheim, Germany) were used to estimate the size of the amplicons. Amplicons of isolates and reference strains were extracted to obtain the *omp1* gene sequence. The nucleotide sequence of *omp1* was then deposited in GenBank (accession no. EF202608).

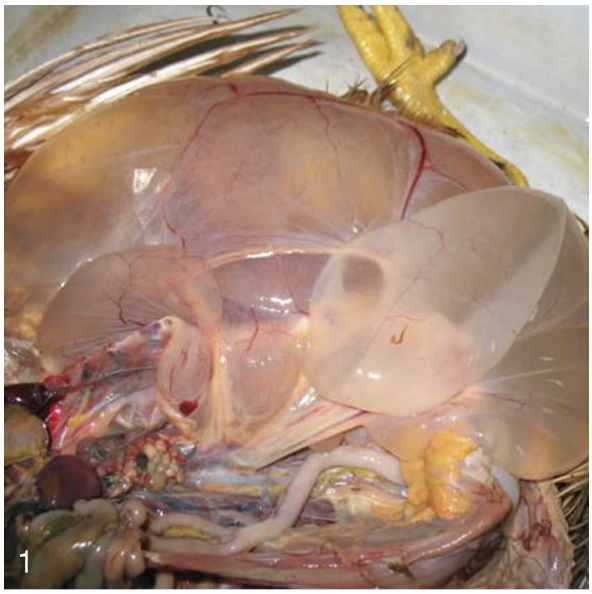
Virus isolation and differentiation diagnosis. Undiluted supernatant (0.1 ml) was inoculated into each of three 10-day-old embryonating chicken eggs. Inoculated eggs were incubated at 37 C for 4 days and candled daily. Virus-infected embryos were identified by hemagglutination (HA) activity in allantoic fluid harvested from chilled eggs. Confirmation of Newcastle disease virus (NDV) and avian influenza virus (AIV) in HA-positive samples was determined by hemagglutination inhibition (HI) tests with NDV and AIV antisera (1). Infectious bronchitis virus (IBV) was confirmed by observing typical lesions of embryos and by observing coronavirus-like particles in allantoic fluid under electron microscopy (6).

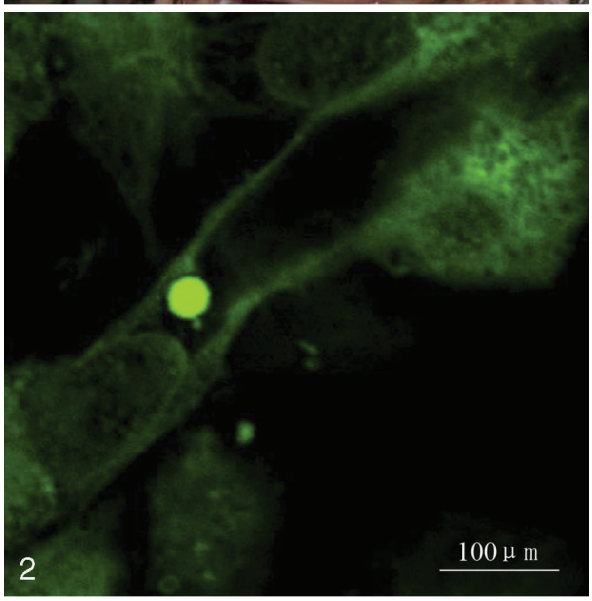
Birds challenge. Forty 10-week-old laying hens were randomly assigned to four groups with 10 hens per group and maintained in negative pressure isolators in biosafety level 2 facilities. Group 1 hens were infected orally with 0.5 ml containing 10^5 50% embryo infectious dose (EID_{50}) isolated pathogen. Group 2 hens received similar doses of the isolate intranasally. Group 3 hens were inoculated intraperitoneally with the same dosage. Group 4 hens were administered intraperitoneally with sterile physiological saline as a control group. Each group was observed daily. The hens were anesthetized by intraperitoneal injection with sodium pentobarbital and sacrificed on day 90 PI. The gross lesions were recorded and organ samples from spleens and uterus were examined by the IMAGEN direct immunofluorescense staining, as previously described (19).

Statistical analysis. Two samples test of equality of proportions was used to compare the mean positivity between affected group and unaffected group. To better understand the relationship between clinical lesion and antigen test, the linear regression analysis was used to evaluate the regression coefficient between the oviduct cyst and the PCE-ELISA test.

RESULTS

Clinical signs and egg production performance. In a total of 25,000 breeder hens, 10% affected birds and 1.0% unaffected birds were positive on having a cystic oviduct, respectively, which was significantly higher than that of unaffected birds ($P = 0.0052$). For the last four years, 36,000 commercial laying hens were tested, and the percentage of those having a cystic oviduct was 15.1% in the affected hens and 2.5% in the unaffected hens ($P < 0.0001$). Clinically the affected hens laid fading-shell eggs with lower fertility in breeder hens. Most infected hens walked like penguins and tended to lie down in cages. The symptoms slowly started to spread to all birds and was followed by decreased egg production. Although the mortality was not elevated, the infected hens had to be culled due to





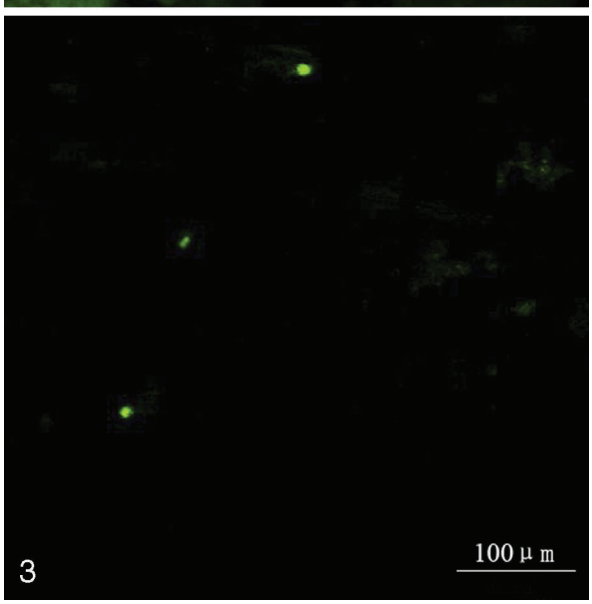


Fig. 1. Fluid-filled oviduct of a *Chlamydophila*-infected laying hen.
Fig. 2. *C. psittaci* microimmunofluorescence test of infected McCoy cell culture monolayers.
Fig. 3. Chlamydial inclusions observed in infected yolk-sac membranes revealed by immunofluorescence assay.

poor egg production. The average egg production rate ranged from 45.5% to 52.1% in affected hens during the peak period (Table 1).

At necropsy, the oviduct and uterus were dilated with yellow fluid, up to 1000 ml per hen (Fig. 1). The uterus became thin and slim due to a large volume of fluid. Meanwhile, the liver and other internal organs decreased in volume. No evident pathological changes were found in the ovary, ovarian follicle, and other organs of the diseased hens.

In PCE-ELISA antigen examination, 20 of 50 pharyngeal swabs (40%) in affected birds were significantly positive compared to 1of 20 (5%) positive in unaffected birds ($P = 0.0080$). Interestingly, 17 of 20 oviduct tissues (85%) were positive for chlamydial antigen, and no samples for control birds were positive ($P < 0.0001$) (Table 2). The coefficient between a cystic oviduct with poor egg performance and the PCE-ELISA antigen test was 0.889 by linear regression analysis.

Culture and isolation of *C. psittaci*. The inoculated embryos died between day 3 and day 10 PI, with a characteristic vascular congestion of the yolk sac in the embryo. A typical CPE occurred in McCoy cells after five passages, and chlamydial inclusions were observed from the second passage using the direct immunofluorescence test (IFT). The intracellular chlamydial inclusions appeared as bright apple-green sacs within the cytoplasm of the infected cells (Fig. 2) as well as in yolk-sac membranes (Fig. 3). Ten or more fluorescing inclusions were observed under one view both in cell cultures and in yolk-sac membranes.

In the complement fixation test (CFT) study the titration of purified antigen from the yolk sac ranged from 1:8 to 1:32, while titrations from cell cultures were 1:32 and 1:64 in the second and third passage, respectively. In a total of 10 tested cultures, the positivity of CFT, PCE-ELISA, and IFT was found to be 50.0%, 60.0%, and 80.0%, respectively, in cell cultures as compared to that of 27.3%, 54.5%, and 72.7%, respectively, in yolk sacs, showing a clear increasing trend with the IFT method (Table 3).

PCR characterization. The DNA extracted from the cell cultures and yolk sacs produced the expected PCR products using *omp1*-specific primers in both the first and the second steps (Fig. 4).

Virus identification. The allantoic fluid of inoculated embryos was found to be negative for NDV and AIV by HA. After the third passage in SPF embryonating eggs, typical viral pathologies, such as mortality of embryos, stunting, curling, and uric acid deposition in the kidneys, were not observed. Coronavirus-like particles were not detected in allantoic fluid by electron microscopic examination (not shown).

Bird challenge. The results of the challenge experiments can be seen in Table 4. No death occurred in any SPF hens inoculated with the isolated pathogen. Three of 10 hens inoculated intraperitoneally with the isolate were observed to lay fading-shell eggs, and two hens displayed a characteristically cystic oviduct in group 3. No clinical signs were found in other groups either by oral inoculation or by an intranasal route. Under microscopic examination, chlamydial inclusions could be observed in the uterus of 10 hens (100%) in group 3 as compared to 4 (40%) in uterus in group 2 by oral inoculation.

Table 2. Chlamydial antigen detection using PCE-ELISA in lower egg production flocks.

	Pharyngeal swabs			Oviduct tissues		
Samples	No.	Positive no. (%)	Negative no. (%)	No.	Positive no. (%)	Negative no. (%)
Affected hens[A]	50	20 (40.0%)[**]	30 (60.0%)	20	17 (85.0%)[**]	3 (15.0%)
Unaffected hens[B]	20	1 (5.0%)	19 (95.0%)	10	0 (0.0%)	10 (100.0%)

[A]Chlamydial antigens were detected in 20 of 50 (40.0%) pharyngeal swabs and in 17 of 20 (85.0%) oviduct tissues in affected hens.
[B]Chlamydial antigens were detected in 1 of 20 (5.0%) pharyngeal swabs and none positive in oviduct tissues of the unaffected hens.
[**]Indicates $P < 0.01$ when compared to the unaffected hens both in pharyngeal swabs ($P = 0.008$) and oviduct tissues ($P < 0.0001$) in the same column.

Table 3. Antigen detection using different methods both in yolk-sac membranes and cell cultures.

		CFT		PCE-ELISA		IFT	
Sample	No.	Positive no. (%)	Negative no. (%)	Positive no. (%)	Negative no. (%)	Positive no. (%)	Negative no. (%)
Yolk-sac cultures[A]	11	3 (27.3%)	8 (72.7%)	6 (54.5%)	5 (45.5%)	8 (72.7%)	3 (27.3%)
Cell cultures[B]	10	5 (50.0%)	5 (50.0%)	6 (60.0%)	4 (40.0%)	8 (80.0%)	2 (20.0%)

[A]The positivity of CFT, PCE-ELISA, and IFT was 27.3%, 54.5%, and 72.7%, respectively, in yolk-sac cultures.
[B]The positivity of CFT, PCE-ELISA, and IFT was 50%, 60%, and 80%, respectively, in cell cultures.

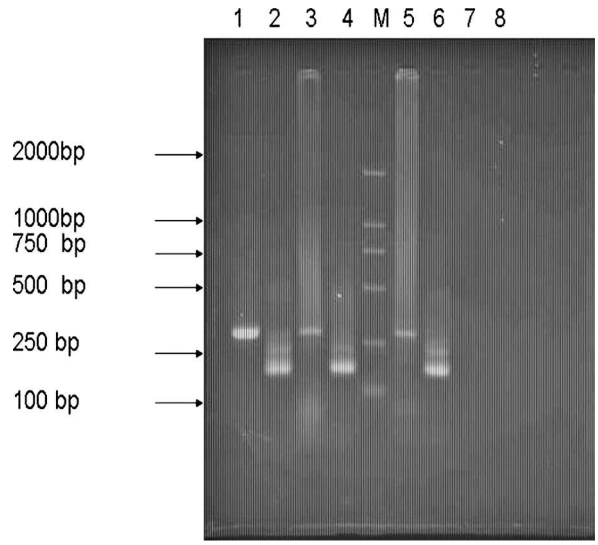


Fig. 4. Detection of *C. psittaci* with nested PCR. Bands of first-step PCR are 285 bp in Lanes 1, 3, 5, and 7. The bands of second-step PCR are 165 bp in Lanes 2, 4, 6, and 8. Lanes 1 and 2: *C. psittaci 6BC;* Lanes 3 and 4*: C. psittaci ISN* 158 strains; Lanes 5 and 6: isolated strains; Lanes 7 and 8: negative control; Lane M: 2000 bp ladder (Takara, Japan).

DISCUSSION

In this study 40% of pharyngeal swabs and 85% of oviduct tissues of the diseased laying hens were positive for chlamydial antigens using PCE-ELISA. The higher antigen positivity is consistent with the frequent lesion of a cystic oviduct with poor egg production in poultry flocks and is suggestive of the pathogenic role of *C. psittaci.* The pathogen isolated from oviduct samples could grow both in chicken embryos and in McCoy cells, resulting in embryo death and cytopathic effect (CPE) in a cultured monolayer. Using CFT, PCE-ELISA, and IFT, the pathogen was identified to be an avian chlamydial strain, while viral agents NDV, AIV, and IBV were excluded by differential diagnosis. The PCR product of the *omp*1 gene sequence of the isolate indicates that the new isolate is similar to two reference strains. In an effort to demonstrate Koch's postulates, SPF hens inoculated intraperitoneally with the isolated pathogen displayed cystic oviducts and typical chlamydial inclusions in the uterus. These results therefore fulfill Koch's postulates for confirming the role of a suspected bacterial pathogen in disease (9). The results of this study therefore strongly suggest that the isolated avian *C. psittaci* is responsible for cystic oviduct disease in laying hens with poor egg production in China.

These findings are different from previous conclusions that *C. psittaci* seldom spreads widely in poultry flocks and that chickens appear to be relatively resistant to *C. psittaci* (2,3). Although very little is known about the formation of oviduct cysts in laying hens, the clinical signs and gross lesions of the infected laying hens are different from those reported in hens (4), laying ducks (12), and

Table 4. Clinical signs and chlamydial inclusions PI with the isolated pathogens in SPF laying hens.

Group[A]	No.	Routes	Cystic oviduct positive no.[B] (%)	Inclusion body positive no. (%)
1	10	Oral	0 (0%)	0 (0%)
2	10	Intranasal[C]	0 (0%)	4 (40%)
3	10	Intraperitoneal[D]	2 (20%)	10 (100%)
Control	10	Intraperitoneal	0 (0%)	0 (0%)

[A]Group 1 hens were infected orally with 0.5 ml containing 10^5 EID_{50} isolated pathogen. Group 2 hens received similar doses of the isolate intranasally. Group 3 hens were inoculated intraperitoneally with the same dosage. Group 4 hens were administered intraperitoneally with sterile physiological saline as control group.
[B]Inclusion body in uterus.
[C]Chlamydial inclusion bodies were observed in the SPF laying hens administrated intranasally with the isolated *C. psittaci* strain.
[D]Cystic oviduct and inclusion bodies were observed in the SPF laying hens administrated intraperitoneally with the isolated *C. psittaci* strain.

laying turkeys (18). In laying turkey hens, egg production drops rapidly and remains low until complete recovery. Current knowledge of cystic oviduct in laying hens was characterized as liver atrophy and uterus exudation. A large volume of liquid might prevent follicles from entering into the oviduct, resulting in a lower egg production instead of a rapid drop in egg production. This also affects calcium deposition in egg shells, leading to a large production of shallow-color eggs and a lower fertility in breeder hens flocks. Transmission of the pathogen was also different from the infectious routes for turkeys and ducks. Laying hens were infected more frequently by intraperitoneal administration than by intranasal infection. In field observation, a cystic oviduct mostly occurs in laying hens originating from the same breeder flocks. This implies that *C. psittaci* may be transmitted from infected parent birds to their offspring. Identification and characterization of the isolated C*hlamydophila* strains will provide new insight into the pathogenesis of fluid accumulation in the oviduct of hens.

Many cases of severe infections in poultry with a potential zoonotic risk require reliable tests for specific detection of *Chlamydia* in clinical samples. The PCR approach based on *omp1* restriction mapping has enabled a precise differentiation of avian *C. psittaci* strains and has been proposed as a valuable taxonomical tool (13,14). Several PCR tests have been used for differential diagnosis of *C. psittaci.* Nested outer membrane protein polymerase chain reaction (OMP-PCR) tests are mainly performed for speciation of *C. psittaci* in poultry and occasionally for verification of real-time PCR results (16). In this study the nested OMP-PCR guaranteed specific diagnosis, in addition to simple handling and reduced contamination risk. The *omp1* sequence of isolated pathogens is close to *C. psittaci* avian type C, which was isolated from White Leghorn chickens in Belgium and classified as serovar B (17,19). For this purpose, serovar-specific analysis will be needed to reveal whether the isolate is more closely related to *C. psittaci* in turkeys or in White Leghorn chickens.

Although typical pathologies were observed in SPF hens infected via the intraperitoneal route, mechanisms of oviduct cysts had been unknown in laying hens. Up to now, mechanisms of oviduct cysts associated with *C. psittaci* in laying hens had been unknown. Further studies will be required to investigate the potential relationship between the isolate and its pathogenesis.

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