Chlamydiosis in backyard chickens (Gallus gallus) in Italy

Chlamydiosis in Backyard Chickens (*Gallus gallus*) in Italy

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Running title

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Abstract

Until recently, *Chlamydia psittaci* was considered to be the only etiological agent of avian chlamydiosis, but two new avian species, *Chlamydia gallinacea* and *Chlamydia avium*, have

recently been described in poultry and pigeons or psittacine birds, respectively. The aim of this study was to explore the occurrence of *C. psittaci* and *C. gallinacea* in backyard chickens in Italy. Cloacal swabs were taken from 160 asymptomatic chickens reared in 16 backyard farms. Samples were tested for *C. psittaci* and *C. gallinacea* by specific real-time polymerase chain reaction assays, with 24 (15%) of the 160 chickens resulting positive for *C. gallinacea*. In order to attempt chlamydial isolation, new samples were obtained from two farms harboring a high prevalence (60% and 70%, respectively) of *C. gallinacea*-positive chickens. In total, eight *C. gallinacea* and one *C. psittaci* isolates were successfully recovered from 13 chickens. *C. gallinacea* was confirmed to be the endemic chlamydial species in chickens, with a high *omp*A intraspecies diversity.

The presence of viable *C. psittaci* and *C. gallinacea* demonstrated by isolation from chickens in backyard farms poses a potential public health problem.

Introduction

Avian chlamydiosis is a bacterial disease of birds caused by members of the genus *Chlamydia*. *Chlamydia psittaci* has been the primary pathogenic chlamydial species identified in clinical infection, known as psittacosis, but at least two additional species, *Chlamydia avium* and *Chlamydia gallinacea*, have now been recognized (Sachse et al. 2014). Wild birds, pet birds and poultry are major reservoirs of *C. psittaci*. Depending on the virulence of the infecting strain and the species and age of the bird, *C. psittaci* infection can be subclinical or characterized by mild to severe respiratory, enteric and ocular symptoms (Andersen 1997). Zoonotic transmission mainly occurs through inhalation of an infectious aerosol or direct contact with contaminated feces or feathers, particularly in high-risk individuals, such as bird owners, veterinarians, bird breeders, pet shop staff, poultry, slaughterhouse and laboratory workers (Deschuyffeleer et al. 2012). In humans, clinical signs vary from a mild flu-like illness to severe atypical pneumonia.

C. avium has been found in asymptomatic or sick pigeons but also in psittacines (Hölzer et al. 2016). *C. gallinacea* was first identified in poultry flocks that had been linked to human chlamydiosis and it is suspected to be a zoonotic pathogen (Laroucau et al. 2009). Its virulence for birds is still unclear, although persistent infection has been linked to reduced body weight gain in broiler chickens (Guo et al. 2016).

In Italy, epidemiological studies on avian chlamydiosis have mostly been performed on wild birds such as pigeons (Magnino et al. 2009), collared doves (Donati et al. 2015) and corvids (Di Francesco et al. 2015). To our knowledge, no systematic investigations on chlamydiosis using highly specific diagnostic assays have been performed in poultry. The aim of this study was to explore the occurrence of *C. psittaci* and *C. gallinacea* in backyard chickens in Italy.

Materials and Methods

From March to June 2016, cloacal swabs were collected from 160 asymptomatic backyard chickens (*Gallus gallus*), reared in 16 farms located in six Italian regions (Table 1). The main races were Siciliana, Romagnola, Moroseta and Livorno, reared for ornamental purposes and/or self-consumption of meat. In July 2016, 13 chickens from the two farms where the highest number of chickens tested positive were sampled to attempt chlamydial isolation, according to Donati et al. (2010).

Genomic DNA was extracted from the cloacal swabs and the chlamydia-positive cell cultures using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the supplier's recommendations. DNA was screened by a *Chlamydiaceae*-specific real-time polymerase chain reaction (rt-PCR) targeting a region of the 23S rRNA gene conserved among all *Chlamydiaceae* (Ehricht et al. 2006). Samples with Ct values <40 were considered positive and reanalyzed by a *C.*

psittaci-specific rt-PCR targeting the *incA* gene (Ménard et al. 2006) and *C. gallinacea*-specific rt-PCR targeting the *eno*A gene (Laroucau et al. 2015).

The polymorphism of *C. gallinacea* in the backyard poultry studied was investigated by amplifying the *omp*A gene of *C. gallinacea* positive samples using the new primers CG3 (5'-GGAGATTATGTTTTCGA-3') and CG4 (5'-CTTGCCATTCATGGTATT-3'). The primers targeted a fragment of approximately 600 base pairs, that included *omp*A variable domains (VDs) I-II-III (Kaltenboeck et al. 1993). *C. gallinacea* 08-1274/3 type strain was used as positive control. The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and both DNA strands were sequenced (Bio-Fab Research, Rome, Italy). The sequences were compared to each other and to the *omp*A sequence of the *C. gallinacea* 08-1274/3 type strain using the BLAST server from the National Centre for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences found in the current study were submitted to the GenBank under accession numbers KY363892 to KY363923.

Then the *C. gallinacea omp*A sequences obtained in this study were edited, obtaining a gene fragment of 337-370 bp in length corresponding to nucleotides 210 to582 of the *omp*A gene of *C. gallinacea* type strain 08-1274/3 (GenBank accession number NZ AWUS01000004) including the *omp*A VDs I-II. The gene fragments were compared with other *C. gallinacea* corresponding sequences and phylogenetic relationships were evaluated.

Results

The results are reported in Table 1. Twenty-four of the 160 (15%) samples collected in the first sampling were chlamydia-positive by 23S rt-PCR. All the chlamydia-positive samples reacted positively to *eno*A-based rt-PCR for *C. gallinacea*. In the second sampling, nine chlamydial isolates were successfully obtained and identified as *C. gallinacea* (n = 8) and *C. psittaci* (n = 1) by the

specific rt-PCRs. The 32 *C. gallinacea ompA* nucleotide sequences from field samples and isolates showed 83-100% similarity between them and 86-96% to the corresponding sequence of the 08-1274/3 *C. gallinacea* type strain. Nucleotide alignment distinguished the 32 *C. gallinacea ompA* sequences in 12 different *ompA* types (01-12). Phylogenetic comparison showed that some *ompA* types were closely related to some *C. gallinacea ompA* sequences from France or Asia (Figure 1). Some *ompA* types were highlighted in geographically distant backyard farms and several *ompA* types were highlighted in the same farm (Table 1).

Discussion

The present study detected *C. gallinacea* in 100% of the PCR chlamydia-positive chickens and in 89% of the chlamydial isolates. These results are consistent with those of previous reports suggesting that *C. gallinacea* is the endemic chlamydial species in chickens (Zocevic et al. 2012, Hulin et al. 2015, Guo et al. 2016, Li et al. 2017). The flocks examined were very similar in terms of zootechnical characteristics (free-range farms) and chicken races (Mediterranean light chicken breeds). The high chlamydia prevalence (60% and 70%) observed in two flocks, compared with others, could be explained by the higher chicken turnover in these two farms. Sequence analysis of the *C. gallinacea ompA* gene fragments confirmed the high intraspecies diversity previously reported (Guo et al. 2016, Li et al. 2017), although the value of phylogenetic comparison based on partial gene sequences of *ompA* is limited (Li et al. 2017). The presence of the same ompA type in different farms, as well as several *ompA* types in the same farm and the close relationship of some *C. gallinacea* strains with European or Asiatic strains are not surprising considering the features of the farms tested, which commonly introduce animals from Italian farms and from European or extra-European countries, and participate in Italian or foreign exhibitions.

Nine chlamydial isolates were obtained, eight of which identified as *C. gallinacean* and one as *C. psittaci*. To our knowledge, this is the first isolation of *C. gallinacea* in Italy. The detection of viable bacteria confirmed the results of the PCR assays on chlamydia circulation in the tested farms, raising a potential public health problem. Whereas the principles and practices of on-farm biosecurity may be familiar to commercial farmers, hobbyists and backyard farmers may not be aware of the steps required to keep infectious diseases out of their flock and prevent their spread. Unlike *C. psittaci*, the zoonotic potential of *C. gallinacea* has yet to be investigated. In a previous study (Laroucau et al. 2009), three workers at a French slaughterhouse who had handled *C. gallinacea*-infected chickens showed signs of atypical pneumonia, even though previous exposure of these individuals to *C. psittaci* cannot be ruled out. Until recently, *C. psittaci* was considered the only agent of avian chlamydiosis. In the past, mainly before the common use of molecular assays, the diagnosis of some human cases of chlamydiosis could have stopped at genus level, disregarding other potential etiological agents. Taking into account the complex etiology of avian chlamydiosis and the endemic circulation of *C. gallinacea* in poultry, highly specific diagnostic methods should be systematically used both in birds and in humans to explore the potential zoonotic role of this new chlamydial species.

Conflict of interest statement

The authors of this paper do not have personal or financial relationships with people or organizations that could inappropriately influence the content of the paper.

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Figure 1. Phylogenetic comparison of *Chlamydia gallinacea* from backyard poultry in the present study.

Phylogenetic relationships among the *Chlamydia gallinacea ompA* variable domains I-II obtained in this study and other *C. gallinacea* corresponding sequences from poultry in France and China were evaluated using MEGA 6.0. A pylogenetic tree was constructed by the neighbor-joining method using the Tamura 3-parameter model with gamma distribution. A reference sequence of *Chlamydia psittaci* (GenBank accession number CP003790) was used as outgroup. Bootstrap values were determined by 1000 replicates to assess the confidence level of each branch pattern and values ≥ 60% were reported. *Omp*A sequences obtained in this study are shown in bold type.

205 **Table 1**. Information and results on investigated backyard chicken farms.

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			rt-PCRs		Cell culture isolation			*C. gallinacea* *omp*A types
Farms	Regions	Total numbers of chickens per farm	No. pos./ No. sampled	No. *C. gallinacea* +ve samples	No. chickens sampled	No. isolates	*Chlamydia* spp.	
1	Piedmont	100	1/10	1/1	–	–	–	01
2	Piedmont	180	1/10	1/1	–	–	–	02
3	Emilia-Romagna	90	0/10	–	–	–	–	
4	Emilia-Romagna	60	0/10	–	–	–	–	
5	Tuscany	120	6/10	6/6	7	5	*C. gallinacea* (n=5)	03, 10
6	Tuscany	120	0/10	–	–	–	–	
7	Sardinia	350	2/10	2/2	–	–	–	04, 05
8	Sardinia	120	2/10	2/2	–	–	–	01
9	Piedmont	120	2/10	2/2	–	–	–	01, 06
10	Sardinia	250	0/10	–	–	–	–	
11	Tuscany	100	2/10	2/2	–	–	–	07, 08
12	Lombardy	100	0/10	–	–	–	–	
13	Lazio	100	0/10	–	–	–	–	
14	Lazio	100	7/10	7/7	6	4	*C. gallinacea* (n=3) *C. psittaci* (n=1)	09, 11, 12
15	Lazio	100	1/10	1/1	–	–	–	10
16	Lazio	160	0/10	–		–	–	
Total=16	Total=6	Total=2170	Total=24	Total=24	Total=13	Total=9	Total=9	Total=12

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