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Chlamydiosis in backyard chickens (*Gallus gallus*) in Italy

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

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10

11 **Keywords:** *Chlamydia gallinacea*, *Chlamydia psittaci*, chicken, epidemiology, PCR

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13 Running title

14 Chlamydiosis in chickens

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21 **Abstract**

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

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

35  
36

## 37 **Introduction**

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

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

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

59

## 60 **Materials and Methods**

61 From March to June 2016, cloacal swabs were collected from 160 asymptomatic backyard  
62 chickens (*Gallus gallus*), reared in 16 farms located in six Italian regions (Table 1). The main races  
63 were Siciliana, Romagnola, Moroseta and Livorno, reared for ornamental purposes and/or self-  
64 consumption of meat. In July 2016, 13 chickens from the two farms where the highest number of  
65 chickens tested positive were sampled to attempt chlamydial isolation, according to Donati et al.  
66 (2010).

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

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

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

85 Then the *C. gallinacea ompA* sequences obtained in this study were edited, obtaining a gene  
86 fragment of 337-370 bp in length corresponding to nucleotides 210 to 582 of the *ompA* gene of *C.*  
87 *gallinacea* type strain 08-1274/3 (GenBank accession number NZ AWUS01000004) including the  
88 *ompA* VDs I-II. The gene fragments were compared with other *C. gallinacea* corresponding  
89 sequences and phylogenetic relationships were evaluated.

90

## 91 **Results**

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

96 specific rt-PCRs. The 32 *C. gallinacea ompA* nucleotide sequences from field samples and isolates  
97 showed 83-100% similarity between them and 86-96% to the corresponding sequence of the 08-  
98 1274/3 *C. gallinacea* type strain. Nucleotide alignment distinguished the 32 *C. gallinacea ompA*  
99 sequences in 12 different *ompA* types (01-12). Phylogenetic comparison showed that some *ompA*  
100 types were closely related to some *C. gallinacea ompA* sequences from France or Asia (Figure 1).  
101 Some *ompA* types were highlighted in geographically distant backyard farms and several *ompA*  
102 types were highlighted in the same farm (Table 1).

103

## 104 **Discussion**

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

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

135

#### 136 **Conflict of interest statement**

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

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

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

204

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

206

Farms	Regions	Total numbers of chickens per farm	rt-PCRs		Cell culture isolation			<i>C. gallinacea</i> ompA types
			No. pos./ No. sampled	No. <i>C. gallinacea</i> +ve samples	No. chickens sampled	No. isolates	<i>Chlamydia</i> 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	<i>C. gallinacea</i> (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	<i>C. gallinacea</i> (n=3) <i>C. psittaci</i> (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

207