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Poultry in Poland as *Chlamydiaceae* carrier

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Abstract

Introduction: The study was conducted to investigate the prevalence and genetic diversity of *Chlamydia* spp. in poultry in Poland and estimate possible transmission to humans. **Material and Methods:** Molecular diagnostic methods followed by sequencing and strain isolation were used on cloacal/faecal swabs collected from 182 apparently healthy poultry flocks including chickens, turkeys, ducks, and geese. Serum samples obtained from people exposed (study group) and non-exposed (control group) to birds were tested by complement fixation test to acquire data on *Chlamydia* spp. antibody level. **Results:** Overall, 15.9% of the tested flocks were *Chlamydiaceae*-positive and three *Chlamydia* spp. were identified. Predominant chlamydial agent found was *C. gallinacea* occurring in 65.5% of all positive poultry flocks and in 73.0% of positive chicken flocks. The sequences from four chicken flocks were assigned to *C. abortus*, whereas *C. psittaci* was confirmed in one duck and one goose flock. The analysis of *omp*A variable domains revealed at least nine genetic variants of *C. gallinacea.* Chlamydial antibodies were detected in 19.2% of human serum samples in the study group in comparison with 10.8% in the controls. **Conclusion:** The obtained results confirm that chlamydiae are common among chicken flocks in Poland with *C. gallinacea* as a dominant species. Moreover, the presence of *C. abortus* in chickens is reported here for the first time. Further investigation should focus on possible zoonotic transmission of *C. gallinacea* and *C. abortus* as well as potential pathogenic effects on birds' health and poultry production.

Keywords: poultry, *Chlamydia gallinacea*, *Chlamydia abortus*, chlamydiosis, Poland.

Introduction

Chlamydiae are bacteria belonging to the family *Chlamydiaceae* that cause chlamydiosis in wild and domestic birds, mammals, and humans. The genus *Chlamydia* includes 11 recognised species (27). Three of them, namely *C. psittaci, C. avium*, and *C. gallinacea*, with the latter two brought into this classification recently, occur commonly in birds. Transient colonisations by *C. abortus*, *C. pecorum*, *C. trachomatis*, *C. suis*, and *C. muridarum* were also noted occasionally in Aves (8, 20, 25, 26). Additionally, an individual case of *C. ibidis*, with Candidatus status in the *Chlamydia* genus, was recorded in an African sacred ibis (34). It is commonly known that *C. psittaci* is widespread throughout the world and can infect more than 450 bird species, including chickens, turkeys, ducks, and geese. *C. psittaci* infection in birds can persist for months to years, often without causing obvious illness (11). Several chlamydia-specific proteins, which are the source of diversity among chlamydial genomes, have now been identified on the inclusion membrane: the group of polymorphic membrane proteins (pmps), inclusion member protein A (*inc*A), and outer membrane protein (*omp*A) (23, 33). Until recently, there were nine *omp*A genotypes described in *C. psittaci* (A–F, E/B, M56, and WC), along with a number of provisional genotypes (YP84, R54, 6N, CPX0308, I, and J) representing strains which are untypable so far (15, 24). Before the new emerging chlamydial agent – *C. gallinacea* – was described, *C. psittaci* was considered to be the dominant *Chlamydia* species in poultry. According to recent data, *C. gallinacea* was mostly found in asymptomatic poultry (8). However, a decrease in the rate of weight gain was reported in infected chickens (8). It should be highlighted that relatively little is known

regarding chlamydial agents in poultry in many countries, including Poland, and screening surveys of *Chlamydiaceae* presence in poultry have not been performed so far. Moreover, data about the genetic diversity and pathogenicity of recently classified *C. gallinacea* strains are scarce. Taking into account the fact that *C. gallinacea* found in poultry in a slaughterhouse in France coincided with atypical pneumonia cases reported in employees, its zoonotic potential cannot be ruled out (14). Therefore, the aim of the study was not only the evaluation of *Chlamydiaceae* prevalence and genetic diversity in poultry but also the estimation of the possibility of chlamydia transmission to humans, based on serological tests.

Material and Methods

Sampling. Sampling was performed between March 2014 and June 2016, in different regions of Poland (Fig. 1). In total, 1,830 cloacal/faecal swabs were collected from 182 apparently healthy poultry flocks (details in Table 1 and Supplementary file S1). On average, ten random samples were taken from each flock. Dry swabs were stored at −20°C for DNA extraction. Swabs in *Chlamydia* stabilising medium stored at −80°C were used for culture.

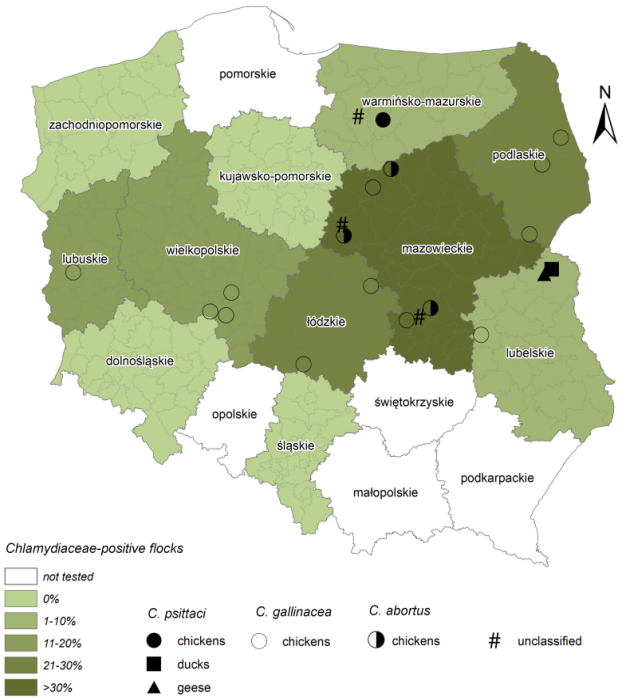

Fig. 1. Area of bird sampling (ArcMap 10.4 software)

Blood samples (n = 500) were taken from humans in two sampling groups: control and study (see Supplementary file S2). The first group consisted of randomly selected blood donors (n = 250) and the second group consisted of individuals who declared contact with birds (n = 250), including persons with occupational exposure on poultry farms (n = 215), as well as veterinarians (n = 20) and ornithologists (n = 15) involved in handling wild birds (30). None of the persons tested reported any clinical symptoms at the time of interview. Whole blood and serum samples were collected into Vacutainers® containing no additives or only a preservative. Sampling was performed by qualified medical staff.

DNA extraction. A QIAamp DNA Mini Kit (Qiagen, France) was used for the DNA extraction from cloacal/faecal swabs according to the manufacturer's instructions, but with one modification. DNA extracts were eluted in 100 µl of elution buffer instead of 200 µl. DNA extracts were stored at −20°C before analysis.

***Chlamydiaceae* identification.** All DNA extracts from birds (n = 1,830) were screened using a *Chlamydiaceae*-specific real-time PCR targeting a 23S rRNA gene fragment (111 bp) (4), which is conserved in all *Chlamydiaceae*. All analyses in the study were conducted on a 7500 Real-Time PCR System (Applied Biosystems, USA). Positive control using *C. trachomatis* (Genekam, Germany), and negative equivalents using DNase-RNase free water (Qiagen, Germany) were run with each assay. All sets of primers and probes used in this study are summarised in Supplementary file S3.

Microarrays. In total, 39 *Chlamydiaceae*-positive DNA samples (Ct < 36) from 16 poultry flocks were subjected to microarray tests performed by Alere Technologies GmbH, Germany.

Real-time PCR. All *Chlamydiaceae*-positive DNA extracts were included in further real-time PCR tests. Identification of *C. psittaci* based on the *inc*A gene was conducted according to the protocol by Menard *et al.* (17), whereas *C. gallinacea* was detected by the amplification of an *eno*A gene fragment according to Laroucau *et al.* (14). Furthermore, specific real-time PCR assays were performed on selected samples to identify other *Chlamydia* species including *C. abortus*, *C. pecorum*, *C. suis*, and *C. caviae* (20), *C. avium* (39), and *C. pneumoniae* (12). In order to distinguish true target negatives from negatives due to PCR inhibition, an internal positive control (TaqMan Exogenous Internal Positive Control, Applied Biosystems, USA) was added to reaction mixtures according to the manufacturer's instructions. Also, positive controls using *C. abortus*, *C. pecorum*, *C. suis*, and *C. caviae*, *C. avium*, and *C. pneumoniae* DNA, and negative equivalents using DNase-RNase free water (Qiagen, Germany) were run with each assay. All samples with a Ct value above 38.5 were considered negative.

Sequencing. Selected *Chlamydiaceae*-positive samples were used for DNA sequencing to confirm the identity of *Chlamydia* spp. Specific fragments of *omp*A, 16S rRNA, and 16S rRNA-23S rRNA intergenic spacer together with 23S rRNA domain I (IGS-23S rRNA) were amplified with previously published primer sets (5, 15, 22, 32) which are summarised in Supplementary file S3.

Table 1. Prevalence of chlamydiae in different poultry flocks and host species in Poland

Host species	Number of tested flocks	Number of samples	Number of *Chlamydiaceae*-positive flocks (%)	Species identification Number of positive flocks (%)			
				C. psittaci	*C. gallinacea*	*C. abortus*	Unclassified
chicken	113	1195	26 (23.0%)	1 (3.8)	19 (73.1)	4 (15.4)	2 (7.7)
duck	23	225	1 (4.3%)	1	-	-	-
turkey	28	230	1 (3.6%)	-	-	-	1
goose	18	180	1 (5.6%)	1	-	-	-
Total	182	1830	29 (15.9%)	3 (10.3)	19 (65.5)	4 (13.8)	3 (10.3)

To investigate the diversity of the *C. gallinacea* *omp*A gene the amplification of variable domains (VD): VD 1–2 (435 bp) and VD 3–4 (421 bp) was performed according to the procedure proposed by Guo *et al.* (8). All PCR assays were performed on a Biometra thermocycler (Biometra, Germany). Amplified products were detected on ethidium-bromide-stained agarose gels with ultraviolet illumination and sent to Genomed (Poland) for sequencing.

Phylogenetic and *omp*A variability analysis. All dendrograms were constructed using neighbour joining (NJ) with the robustness of the clusters assessed by bootstrapping 1,000 replicates. One representative sequence from each individual flock was used (if available). PCR products of the *omp*A gene obtained for 13 *Chlamydiaceae*-positive poultry flocks were sequenced and the data were analysed using Geneious Pro 8.0 software (Biomatters, New Zealand). Amplicons were subjected to BLAST analysis against the GenBank database (NCBI) to identify related entries and aligned with a panel of *Chlamydia* reference strains including avian *C. abortus* genotypes G1 and G2. Phylogenetic trees were constructed based on alignments of 936 bp and 1,006 bp for 16S rRNA and IGS-23S rRNA, respectively.

*Omp*A (830 bp) as well as VD 1–2 (343 bp) and VD 3–4 (338 bp) fragments were aligned with sequences of *C. gallinacea* to build separate dendrograms, and the sequences included European and Chinese strains constituting different genetic variants.

Sequencing data from the present study were deposited in the GenBank database with the following accession numbers: MF140888–MF140897 (16S rRNA), MF140898–MF140900 (IGS-23S rRNA), MF140901–MF140919 (*omp*A), MF140920–MF140946 (VD 1–2 of *omp*A), and MF140872–MF140887 (VD 3–4 of *omp*A).

Isolation and propagation in cell culture. Buffalo green monkey (BGM) cells in minimal essential medium (MEM) (Lonza, Germany) with 5% serum were seeded into Trac bottles containing glass coverslips (Bibby Sterilin Ltd., UK) and incubated at 37°C with 5% CO_2 in a fully humidified cabinet for four days. Swabs (with Ct value in *Chlamydiaceae* 23S real-time PCR <32) in 1–2 mL *Chlamydia* stabilising medium were ultrasonicated with a Branson 450D sonifier (ten 0.8 s pulses with 0.2 s pause between each pulse at an amplitude of 80%) (Branson Ultrasonics, USA) and 30–300 µL of medium were inoculated into six Trac bottles with confluent-grown BGM monolayers. After inoculation, the bottles were centrifuged at 3,000×*g* and at 37°C for 60 min and subsequently incubated for 2 h. The MEM was then replaced with serum-free medium UltraMDCK (Lonza, Germany) containing amphotericin (2.5 µg/mL), gentamicin (10 µg/mL), and vancomycin (25 µg/ mL). The medium was renewed after 18 h. Three days after inoculation, a single coverslip was fixed with methanol, and the monolayer was stained with IMAGEN Chlamydia (Oxoid Ltd., UK). A sample was considered positive when inclusions of typical chlamydial morphology appeared as bright apple-green spots after two passages.

Serological testing of human sera. In total, 500 human sera were tested by complement fixation test (CFT) in order to detect *Chlamydia* spp. antibodies. The CFT was performed according to the manufacturer's protocol. The chlamydial antigen was obtained from Serion Immundiagnostica (Germany). A titre of 64 or higher was considered diagnostically significant and reported as a positive result.

Statistical analysis. All analyses were conducted using the programme STATISTICA ver. 10 (StatSoft, part of Dell Software, USA). The chi-squared test was carried out to calculate correlation among dependent variables of seropositivity level in study and control groups. The odds ratio (OR) was also calculated for assessment of the chance of chlamydial antibodies in both sampled populations.

Results

Bird survey. The *Chlamydiaceae*-specific real-time PCR (summary in Table 1 and Supplementary file S1) showed that 15.9% (29/182) of apparently healthy poultry flocks from different areas of Poland were positive (Fig. 1). Among 182 tested farms, the presence of *Chlamydiaceae* was molecularly confirmed mainly in chickens (26/113 farms; 23.0%). Only one turkey (1/28 farms; 3.6%), goose (1/18 farms; 5.6%), and duck (1/23 farms; 4.3%) flock was *Chlamydiaceae*-positive. In seven chicken flocks, the number of *Chlamydiaceae*-positive samples ranged between eight and ten (out of ten), whereas in the remaining 19 flocks, only one to five (out of ten) positive samples were detected. In the other tested hosts, no more than three positive samples were noted per flock.

Re-analysis of positive samples with a microarray assay revealed that *C. gallinacea* was present in most of

the tested flocks. This result was confirmed by further molecular analysis. The majority of the *Chlamydiaceae*-positive chicken flocks (19/26, 73.1%) were positive in *C. gallinacea* qPCR with Ct values ranging from 21.8 to 38.3. Weak signals (average Ct value of 35.9 to 38.2) in *C. abortus* qPCR were obtained in four chicken flocks. Out of three *C. psittaci*-positive flocks, only in the duck flock (flock 15-63) was the shedding level high (Ct 23.8), while in the chicken (flock 15-2) and goose flocks (flock 15-41) average Ct values were 37.7 and 33.1, respectively. In a few flocks, microarrays and qPCR detected *C. abortus* and *C. psittaci* beside *C. gallinacea.* A co-infection of *C. psittaci* and *C. gallinacea* in chickens (flock 14-156) and geese (flock 15-41) was noted in qPCR tests. In chicken flock 14-67 species-specific qPCRs identified the three *Chlamydia* species: *C. psittaci*, *C. gallinacea*, and *C. abortus*, but further phylogenetic analysis confirmed only the presence of *C. gallinacea*. In two chicken and one turkey flocks (flocks 15-12, 14-157, and 14-200), species identification was unsuccessful.

Swabs sampled from chicken of flock 15-56 (samples 1 and 2) were inoculated onto BGM cell culture and two strains of *C. gallinacea* were successfully isolated from these samples. Partial *omp*A sequences were obtained from isolates and found to be 100% identical to those amplified directly from corresponding dry swabs.

Sequencing of 16S rRNA and IGS-23S rRNA was carried out to characterise studied DNA isolates. Successful amplification of the 16S rRNA gene was achieved for samples from eight poultry flocks, while IGS-23S rRNA amplicons were sequenced only for three flocks. Dendrograms constructed on the basis of these gene fragments and aligned with reference sequences of known *Chlamydia* species (Fig. 2 and 3) showed similar topology. All sequences from our study were positioned within the *Chlamydiaceae* family and were assigned to three *Chlamydia* species: *C. psittaci*, *C. abortus*, and *C. gallinacea*.

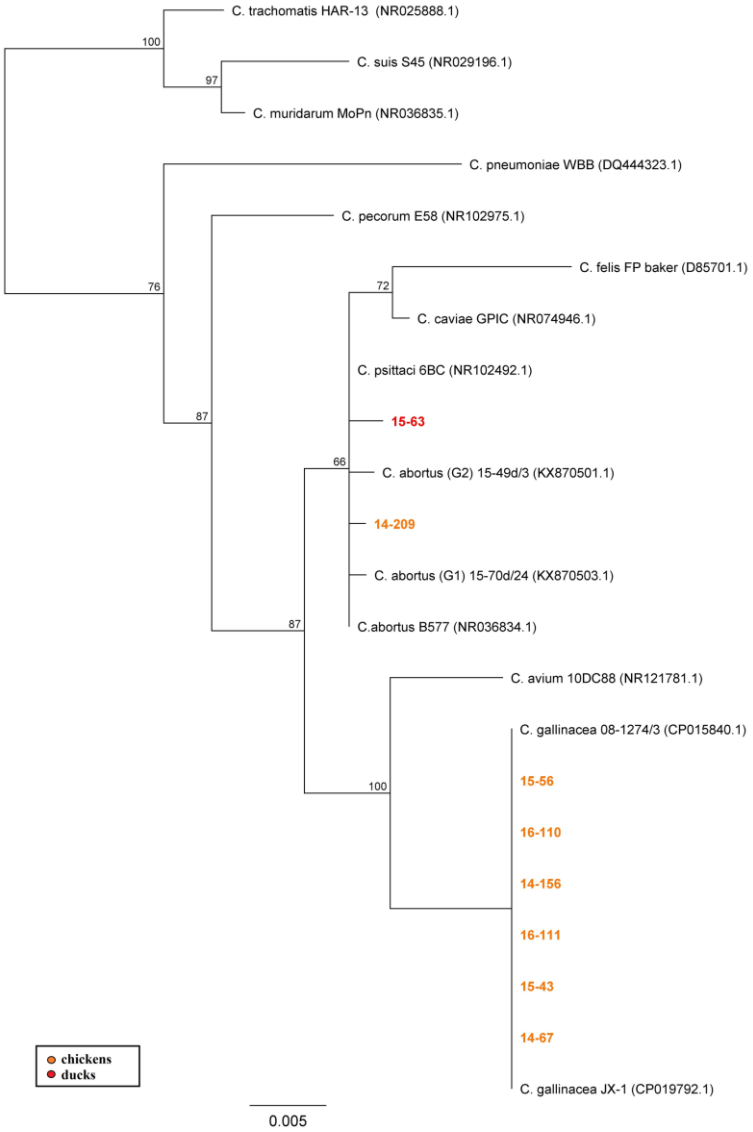

Fig. 2. NJ dendrogram based on 16S rRNA gene fragment. Representative sequences of established *Chlamydiaceae* species were used; bar corresponds to the number of substitutions per site

Phylogenetic analysis revealed that 2 out of 12 obtained *omp*A sequences (Fig. 4) from chicken (15-63) and goose flocks (15-41) were grouped with *C. psittaci* GD (AF26926.1) and *C. psittaci* (KX062086.1) with maximum 100% bootstrap support. A further three amplicons (14-204, 14-206, and 14-209) from chicken flocks were assigned to the *C. abortus* cluster with high bootstrap support and *C. abortus* B577 (M73036.1) as the closest relative. The remaining seven *omp*A sequences (14-67, 16-110, 14-205, 14-154, 14-156, 15-43, and 15-56) were grouped together with the *C. gallinacea* strains. The variability of this gene can be noted in the five subclades formed within the *C. gallinacea* group. The sequence 16-110 forms a subclade with *Chlamydia* spp. (HE660097.1) from a Slovenian isolate with 100% bootstrap support. Indicative of the heterogeneity, the sequence from flock 14-205 nevertheless was grouped together with Croatian *Chlamydia* sp. (HE660095.1) and a sister clade was formed by the 14-154 and 14-156 sequences. Both were supported by the highest bootstrap value. Sequence HE660099.1 obtained from Greek chickens was most similar to amplicons 15-43 and 15-56, while 14-67 from the present study did not group closely with any of the described strains. In-depth analysis of *omp*A variable domains was also performed. Sequences of VD 1–2 from 17 flocks were obtained but only 10 VD 3–4 fragments were sequenced successfully. Phylogenetic comparison of the resulting products identified nine genetic variants that were clearly separated, six of which were new (see Supplementary file S4).

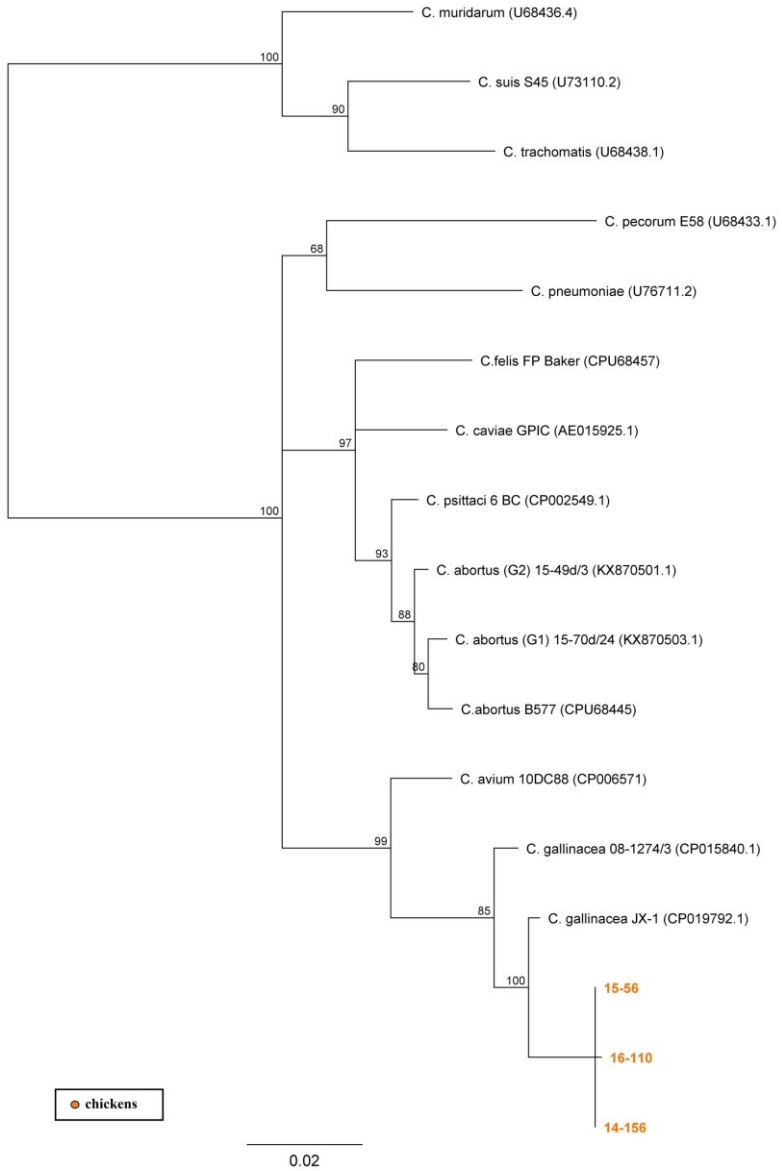

Fig. 3. NJ dendrogram based on IGS-23S rRNA gene fragment. Representative sequences of established *Chlamydiaceae* species were used; bar corresponds to the number of substitutions per site

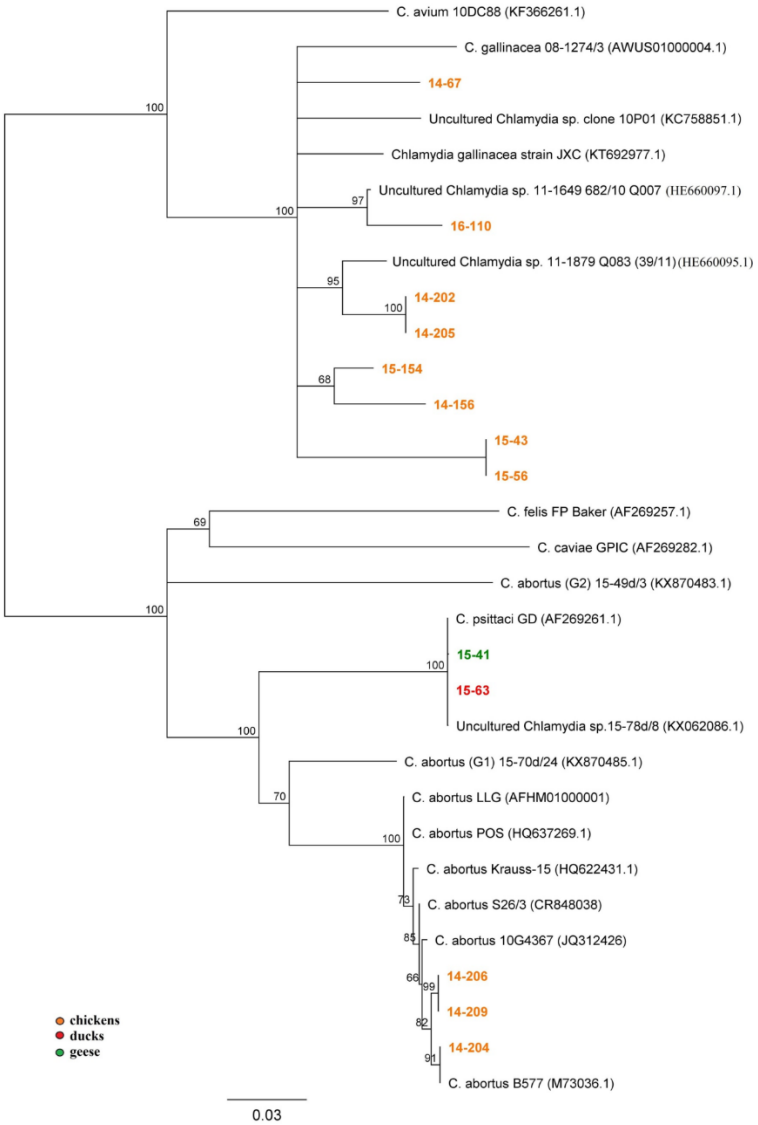

Fig. 4. *omp*A-based NJ dendrogram (822 bp); bar corresponds to the number of substitutions per site

Human survey. Out of 250 human serum samples obtained from people declaring considerable exposure to birds (study group), 48 were positive in CFT for chlamydial antibodies (19.2%), whereas in the control group *Chlamydia* spp. antibodies were confirmed in 27 (10.8%) serum samples (see Supplementary file S2). Statistical analysis confirmed significant difference in seropositivity level between tested groups and odds ratio was calculated as 1.96. Specimens taken from workers employed on six *C. gallinacea*-positive farms (n = 50) were included in the study group, but in these samples the presence of antibodies specific to chlamydiae was not detected.

Discussion

A variety of *Chlamydia* species occurring in poultry hosts were detected in different European countries and China (8, 13, 38). *C. psittaci* has long been considered the main *Chlamydia* species in poultry, while recent scientific reports show that *C. gallinacea,* a newly emerging agent, is predominantly found in asymptomatic chickens (8). A monitoring survey of *Chlamydia* spp. dissemination in poultry in Poland has not been performed so far, and literature data are limited to description of the first case of *C. gallinacea* in hens (29). The present study was carried out on cloacal/faecal swabs sampled from poultry flocks in 11 out of 16 provinces to explore the prevalence of *Chlamydiaceae* shedders. Our results showed that 15.9% of tested flocks were *Chlamydiaceae*-positive. At the same time, it was revealed that Polish poultry, excluding chickens, are almost free from chlamydiae. However, within the chicken population the percentage of *Chlamydiaceae*-positive flocks was 23.0%. *Chlamydia gallinacea* was noted in 73.0% of *Chlamydiaceae*-positive chicken flocks, indicating this species to be the dominant chlamydial agent in this host. Available data show that *C. gallinacea* occurs in poultry flocks in at least five European countries: France, Greece, Slovenia, Croatia, and Poland, as well as in China (29, 38). In contrast, *C. gallinacea* was not found in a Belgian study (13). An extensive comparison of prevalence levels of *C. gallinacea* within Europe is impossible due to the lack of monitoring studies from other countries, but

prevalence is probably lower than in China where *C. gallinacea* is endemic in chickens (8). Guo *et al.* (8) suggested that *C. gallinacea* is not commensal but a pathogen of moderate pathogenicity, and persistent infection can lead to reduced weight gain in poultry production. No clinical signs of chlamydia infection were observed in poultry included in our study.

In the presented study, *C. psittaci* and *C. abortus* were recorded less often in chicken flocks, with prevalence of 3.8% and 15.4% in *Chlamydiaceae*-positive flocks, respectively. The low rate of *C. psittaci* detection is not surprising as chickens are not a typical host of this chlamydial species.

The analysis of the *omp*A gene included here revealed that *C. gallinacea* strains encountered in Poland are diverse and different from previously known European and Chinese strains (8), but still share high sequence similarity with them (Fig. 4). Furthermore, none of the amplicons from the current study grouped closely with the *C. gallinacea* sequence (KC758851.1) obtained from strain 10P01 described in the first case report in Poland (29).

The two *C. psittaci* sequences from our study (15-41 and 15-63) represented genotype C that is typical for poultry. Interestingly, these sequences grouped together with a sequence obtained earlier by our research group from a swan in Poland (KX062086.1) (30). This is a clear indication of transmission of *C. psittaci* between wild and domestic birds.

It should be noted that three sequences from chickens (14-204, 14-206, and 14-209) were placed in the *C. abortus* clade together with typical mammalian strains. They share 100% sequence homology with *C. abortus* B577 (M73036.1) and *C. abortus* S26/3 (CR848038.1), both isolated from sheep, whereas up to seven single-nucleotide polymorphisms (SNPs) were present between them and the remaining *C. abortus* sequences. It is worth noting that this is the first report of natural infection with *C. abortus* in chickens. Previous data showed the presence of this pathogen in a few bird species, *e.g*. turkey, budgerigar, oriental white stork, and pigeon (3, 25, 28). *C. abortus* has attracted increasing scientific attention due to its pathogenicity and several events of systemic infection in humans (2, 9, 16, 21, 35, 36), as well as the isolation of the agent from new hosts including birds (30). Therefore, Pannekoek *et al*. (18) and ourselves, in our recent report on *Chlamydiaceae* prevalence in wild birds (30), propose a modification of the initial *C. abortus* species definition published by Everett *et al.* (5) and an expansion of this species to include not only the classical strains obtained from mammals but also avian isolates. Taking into account that the tested poultry was apparently healthy, it can be assumed that these birds were asymptomatic carriers of *C. abortus*. Crossing of the species barriers by chlamydiae is well known, with literature describing cases of *C. trachomatis* and *C. pecorum* as examples of a possible host change from mammals to birds (6, 19, 25) and *C. psittaci* as a *vice versa* example (10).

Avian chlamydiosis poses also a potential risk to humans. Four *Chlamydia* species, namely *C. trachomatis, C. pneumoniae, C. abortus*, and *C. psittaci*, are known to be able to infect humans. Outbreaks of the disease have been reported through direct contact with birds, both wild and breeding (31), though inadvertent exposure particularly in endemic areas also occurs (1). Human-to-human transmission has also been reported, raising the spectre of uncontrolled outbreaks (37). Description of *C. gallinacea* in poultry and *C. avium* in wild fowl by Sachse *et al.* (26) has raised the question of its aetiological role and possible zoonotic potential. Our survey in humans exposed to birds was based on available serological tools for detection of antibodies against *Chlamydia* spp. It should be noted that the lack of specific serological methods precluded the identification of any humoral immune response specific to *C. gallinacea*. Our results showed that the percentage of *Chlamydia* spp. seropositive samples is significantly higher in the study group than in the control. However, because CFT lacks specificity, it is unclear whether this discordance is due to different exposure to chlamydia-infected birds or to infections by the typical human pathogens, namely *C. trachomatis* and *C. pneumoniae.* Especially antibodies against *C. pneumoniae* are very common among the adult human population with seropositivity rising to 80% with age (7). Interestingly, 19.2% of tested individuals exposed to birds included in our survey were seropositive. All of them were bird breeders or poultry farm workers; however, no positive serological reactions were recorded among workers on some of *C. gallinacea*-positive farms, nor veterinarians and ornithologists who had had contact with wild birds. Taking into account the results of this study and the current state of knowledge, the zoonotic potential of the new *Chlamydia* players (*C. gallinacea* and *C. avium*) can be neither acknowledged nor excluded.

In conclusion, we have demonstrated that *Chlamydiaceae* are common in chicken flocks in Poland. The dominant species noted in this host is *C. gallinacea*, which exhibits different genetic variants. Moreover, this is the first report showing the occurrence of *C. abortus* in chickens. Further studies should focus on possible zoonotic transmission of *C. gallinacea* and *C. abortus* as well as potential pathogenic effects on poultry health and productivity. Moreover, a thorough molecular investigation of *C. abortus* and *C. gallinacea* strains isolated from production birds using next generation sequencing should be performed.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: Samples from birds were collected during clinical studies or routine activities following standard procedures. According to the Local Ethical Committee on Animal Testing at University of Life Sciences in Lublin (Poland) formal ethical approval is not required for this kind of study. Guidelines published by this ethics committee (Resolution No. 22/2006 of the National Ethic Committee for Experimentation of November 7 (2006) Poland. 2006) were used, which confirm that this work is acceptable without specific ethical approval. Moreover, consent of bird owners was obtained for sampling.

Human Rights Statement: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration. Sampling and laboratory testing of obtained specimens were approved by the Bioethics Committee at the Regional Chamber of Physicians Decision No. 224/2014/KB/V.

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The online version of this article (DOI:10.1515/jvetres-2017-0072) offers the following supplementary material: 1) Supplementary file S1. Identity, origin and results obtained for *Chlamydiaceae*-positives samples; 2) Supplementary file S2. Summary of human survey results; 3) Supplementary file S3. Summary of primers used in the study; 4) Supplementary file S4. NJ dendrograms displaying variability of VD 1–2 (A) and VD 3–4 (B) *omp*A domains.

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