Detection of chlamydial infection in Iranian turkey flocks

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Abstract:

BACKGROUND: Avian chlamydiosis is a zoonotic disease of birds caused by the intracellular bacterium *Chlamydia psittaci*. Avian chlamydiosis leads to severe respiratory disease in young turkeys and egg production losses in layers. **OBJECTIVES:** Due to paucity of information about the prevalence of chlamydial infection in the turkey population in Iran, this study was conducted to detect chlamydial infection in some Iranian turkey flocks in different provinces. **METHODS:** A total of 177 samples were taken from turkeys and first verified as *Chlamydiaceae* by *Chlamydiaceae*-specific real-time polymerase chain reaction (real-time PCR) by detection of the 23S RNA gene of *Chlamydiaceae* (Ct values ranging from 34 to 38) and then positive samples were investigated for the presence of *C. psittaci* by a nested PCR. **RESULTS:** Seventeen of 177 samples (9.6%), corresponding to 13 farms of 48 examined farms were positive for *Chlamydiaceae* by real-time PCR. None of the positive samples were found to be *C. psittaci* in the nested PCR. **CONCLUSIONS:** This study showed no *C. psittaci* infection in the turkey population in Iran. We recommend investigation on other farm animals and wild populations for possible chlamydial infection and for better understanding of the source and epidemiology of this agent. Due to the challenges that exist for sampling and the relevant impact on reducing positive samples, investigation by parallel and complementary techniques may be useful in showing the true prevalence of infection in the target populations.

Introduction

Chlamydial infections leading to outbreaks of avian chlamydiosis in domestic, companion and wild birds are regularly reported from all parts of the world (Gade et al., 2008; Laroucau et al., 2009a; Sachse et al., 2012; Zocevic et al., 2012, Madani and Peighambari, 2013). The general importance of avian chlamydiosis includes both economic losses to the bird owners and potential zoonotic transmission to humans (Vanrompay et al., 2007; Laroucau et al., 2009a). The family *Chlamydiaceae* currently includes only the genus Chlamydia, from which nine species including *abortus*, *caviae*, *felis*, *muridarum*, *pecorum*, *pneumoniae*, *psittaci*, *suis* and *trachomatis* have been identified (Kuo et al., 2010).

Recent investigations have provided evidence of the occurrence of chlamydial species other than *Chlamydia psittaci* (*C. psittaci*) in birds (Herrmann et al., 2000; Chahota et al., 2006; Pantchev et al., 2009; Lemus et al., 2010; Sachse et al., 2012). Moreover, atypical strains of *Chlamydiaceae* were recently detected in chickens (Gaede et al., 2008; Laroucau et al., 2009a; Zocevic et al., 2012), pigeons (Gasparini et al., 2011; Sachse et al.,

2012), gulls (Christerson et al., 2010) and wild birds (Madani and Peighambari, 2013).

The clinical feature depends on the chlamydial strains and avian host (Andersen and Vanrompay, 2000). Transmission of *C. psittaci* predominantly occurs through inhalation of contaminated material and sometimes through ingestion, from an infected bird to a susceptible bird. Humans can become infected by inhaling an organism shed by infected birds, mouth-to-beak contact or by handling the plumage and tissues of infected birds. Human infections are common following handling or processing of infected turkeys or ducks (Laroucau et al., 2009b). The disease in humans contracted from turkeys is often more severe than that contracted from psittacine birds (Andersen and Vanrompay, 2000).

Diagnosis of chlamydial infection in birds is still a considerable challenge. Clinical changes are not pathognomonical and persistent infections can also occur. The infection can therefore only be confirmed by direct identification of the agent or indirectly by detection of specific antibodies. PCR is currently the method of choice for diagnosis of chlamydial infection. Specific and sensitive PCR methods targeting the 23S rRNA gene that allows the detection of *Chlamydiaceae* and identification of Chlamydia species have been developed (Everett et al., 1999; DeGraves et al., 2003; Ehricht et al., 2006). Analysis of the gene encoding outer membrane protein A (ompA) is most often used to study avian *C. psittaci* strains into genotypes (Sachse et al., 2008). Nine genotypes of *C. psittaci* are currently accepted (A-F, E/B, WC and M56) (Geens et al., 2005a; Geens et al., 2005b; Sachse et al., 2008).

Previous research in Iran avian/poultry population showed 12.6% *Chlamydia psittaci* infection in companion and wild birds (Madani et al., 2011) and 14.3% in Pigeons (Doosti and Arshi, 2011). At present, little is known about the prevalence of chlamydial infection in the turkey population, especially in Iran. This study was, therefore, conducted to detect chlamydial infection in Iranian turkey population by real-time PCR. Samples were first verified as *Chlamydiaceae* and then were investigated for the presence of *C. psittaci*.

Materials and Methods

Samples: During 2013-2014, a total of 177 samples were collected from turkey flocks located in the provinces of Ardabil, Ghazvin, Ghom, Gilan, Golestan, Hamedan, Isfahan, Lorestan, Mazandaran and Tehran. In total, 48 farms were sampled, from which 11 farms showed clinical signs including nasal discharge, sinusitis, conjunctivitis and diarrhea. Thirty samples were also collected from free range turkeys in Golestan province and cases referred to a private poultry clinic in Tehran (Table 1).

A single sterile cotton-tipped swab was used to take triple samples from the conjunctiva, choanal cleft and cloaca of each individual randomly selected turkey in the flock. Twenty triple sample swabs were collected from each flock, then each five swabs from the same flock were pooled to form a single sample. Tissue samples including air sac exudates, lungs, spleens and livers were collected in Najafabad abattoir (Isfahan province) and from cases referred to a poultry clinic in Tehran. Tissue samples from each bird were considered as a single sample. Swab samples were placed in plastic bags or in SPG (Sucrose Phosphate Glutamate) transport media (Madani and Peighambari, 2013) and transported to the laboratory in cold condition and preserved at -20° C until further use.

DNA extraction and Real-time PCR assay for Chlamydiceae: Template DNA was extracted and prepared using the High Pure PCR Preparation kit (Roche Applied Science, Mannheim, Germany) as instructed by the manufacturer.

A *Chlamydiaceae* family-specific real-time

PCR based on partial replication of the 23S gene and yielding a 111 base pairs (bp) fragment was used (Ehricht et al., 2006). The forward Ch23S-F (5′-CTGAAACCAGTAGCTTATAAGCGGT-3′) and reverse Ch23S-R (5′-ACCTCGCCGTTTAACTTAACTCC-3′) primers, and the probe Ch23S-p (FAMCTCATCATGCAAAAGGCACGCCG-TAMRA) used for the amplification process were synthetized by Metabion International AG (Martinsried, Germany). Real-time PCR reactions were run in a Rotor-Gene Q (QIAGEN Marseille S.A.) instrument. Each reaction mixture contained 2 µl sample DNA template, 10 µl of qPCR Probes MasterMix 2X (Jena Bioscience GmbH, Germany), 0.5 µl of each primer (25 mM), 2 µl of the probe (1 mM), and 5 µl deionized water. The cycling profile was included and initial denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 94°C for 15s and 60°C for 60s. A cycle threshold (Ct value) of <38.00 was considered as positive. The positive control DNA templates were provided from previous works in our laboratory (Madani and Peighambari, 2013). Sterile dH2O was also used as negative control. All samples were tested at least in duplicate.

OmpA nested PCR: All specimens that were positive in real-time PCR and positive control DNA were subjected to ompA nested PCR amplification for the detection of *C. psittaci* as described by Sachse and Hotzel (2003). The degenerate nested primers 191CHOMP (5′-GCIYTITGGGARTGYGGITGYGCI AC-3′) and CHOMP371 (5′-TTAGAAICKGAATTGIGCRTTIAYGTGIGCIGC-3′) for the identification of the genus *Chlamydia* spp. were synthesized by Metabion International AG (Martinsried, Germany). A 50 µl reaction mixture was prepared with 1 µl dNTP (10 mM), 1 µl of each primer (20 pmol/µl), 1.5 µl MgCl2 (50 mM), 5 µl 10X PCR Buffer, 0.2 µl SmarTaq™ DNA Polymerase, 5 µl DNA and 35.3 µl dH2O. Amplification was programmed in a thermocycler (Gradient Mastercycler, Eppendorff, Germany) as follows: an initial denaturation at 95°C for 30 s, followed by 35 cycles of denaturation (95°C for 30 s), alignment (50°C for 30 s), extension (72°C for 30 s), and a final extension at 72°C for 2 min. PCR products were visualized by agarose gel electrophoresis (1%) in TBE (Tris Base, boric acid, EDTA, pH8, 0.5 M), stained with RedSafe™ (iNtRON, South Korea). GeneRULER™ 100 bp Plus DNA Ladder (Fermentas, Germany) was used as marker on each gel running. This step provided amplicon with weights 576-597 bp. All amplification products were subjected to a second PCR to identify *C. psittaci* using the primers 218PSITT (5′-GTAATTTCIAGCCCAGCACAATTYGTG-3′) and CHOMP336s (5′-CCRCAAGMTTTTCTRGAYTTCAWYTTGTTRAT-3′) in a reaction with proportions of reactants as described above but varying the amounts of $MgCl_2$ (2 µl), distilled water (38.8 µl) and DNA (1 µl). Amplification conditions, programmed in Gradient Mastercycler (Eppendorff) were as follows: 95°C for 30 s followed by 20 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 2 min. The second PCR products were visualized by agarose gel electrophoresis as described above. Samples that showed bands with weights 389-404 bp were considered positive. DNA of *C. psittaci* which was extracted from the liver of a Chlamydia positive African grey parrot, taken at the Dutch Research Institute for Birds and Exotic Animals in the Netherlands (NOIVBD), was used as positive control; water was used as negative control. All PCR materials including SmarTaq™ DNA polymerase, $MgCl_2$, PCR buffer and dNTPs were purchased from Cinnagen (Tehran, Iran).

Results

Seventeen of 177 samples (9.6%), corresponding to 13 farms of 48 examined farms (Table 2) were positive for *Chlamydiaceae* by real-time PCR, i.e. the 23S RNA gene of

Table 1. The total number of examined birds and type of samples.

Sample type	No. of pooled samples/Farms	No. of samples used for pooling
Triple swab transported in dry condition	61/15	5
Triple swab in SPG transport medium	44/11	5
Tissue samples	72/22	1
Total	177/48	

Table 2. Detailed results obtained in real-time PCR assay.

Sample type (no.)	Age (Day)	Farm	Real-Time PCR		No. of positive samples in nested PCR total
			No. of positive samples in total tested	Ø Ct value	
Tissue samples (7)	56	A	7/72	36	0/7
	112	B			
	45	C			
	72	D			
	72	D			
	32	E			
	18	F			
Triple swab transported in dry condition (3)	100	G	3/61	37.44	0/3
	82	H			
	40	I			
Triple swab in SPG transport medium (7)	50	J	7/44	35.50	0/7
	50	J			
	60	K			
	160	L			
	160	L			
	65	M			
	160	Z			

Chlamydiaceae was detected (Ct values ranging from 34 to 38) (Fig. 1). None of the positive samples were found to be *C. psittaci* in the nested PCR (Fig. 2). Samples from free range turkeys in Golestan province were also negative.

Discussion

Chlamydia psittaci is a very important bacterial pathogen in veterinary and human medicine (Andersen and Vanrompay, 2000). In turkeys, *C. psittaci* causes infections of the respiratory tract followed by septicemia and localization in epithelial cells and macrophages in various organs (Vanrompay et al., 1995a). Nowadays, the increase in confinement-rearing of turkeys and the prevention of wild birds flying in and out the turkey houses seems to contribute to a decrease of severe outbreaks (van Loock et al., 2005). Since little is known about the prevalence of chlamydial infection in the turkey population of Iran, this study was designed to detect chlamydial infection in some Iranian turkey farms, for the first time in Iran, by molecular methods.

In this study, 177 sample were sampled in order to detect chlamydial infections by real-time PCR assay. Triple sample swabs were taken from each bird, as infected birds can

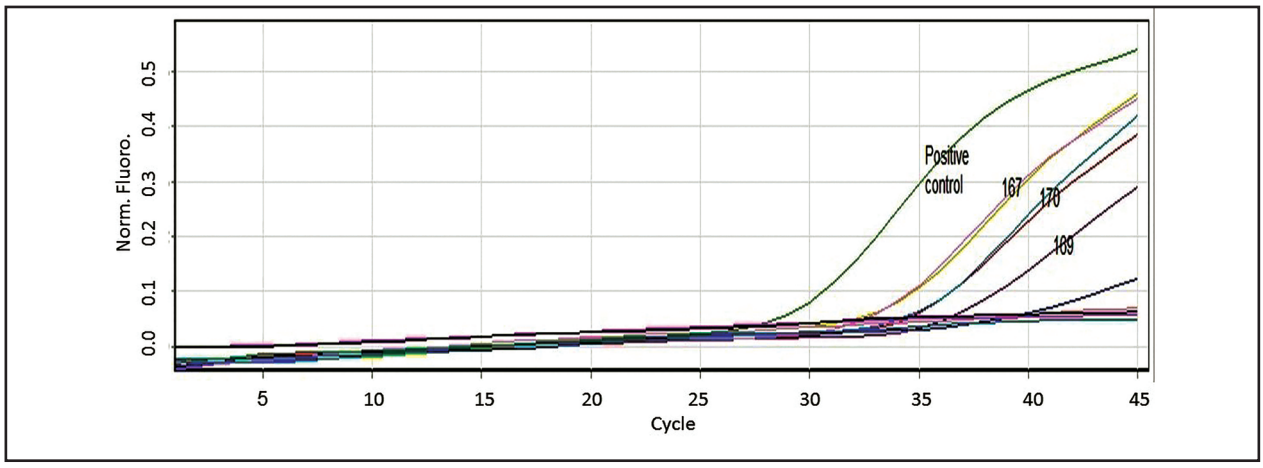

Figure 1. Curve of three positive samples in real-time PCR for 23S gene of *Chlamydiaceae*.

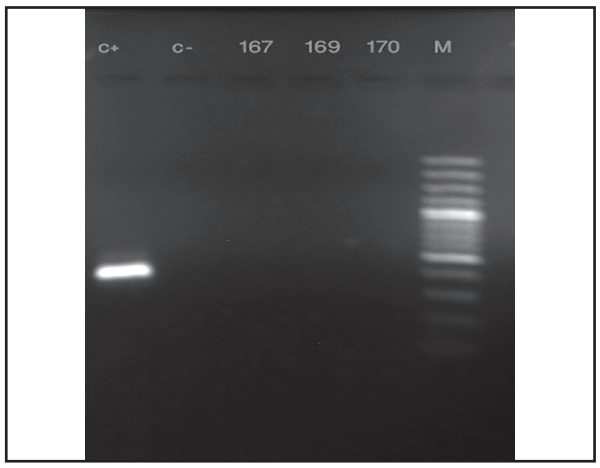

Figure 2. Gel electrophoresis of three nested-PCR amplified products previously. found as positive in real-time PCR for 23S gene of *Chlamydiaceae*. M = Molecular. weight marker (GeneRULERTM 100 bp Plus DNA Ladder, Fermentas, Germany), C+ = Positive control, C-= Negative control.

shed Chlamydiae through respiratory tract excretions and in feces (Andersen and Vanrompay, 2000; Harkinezhad et al., 2009). Seventeen out of 177 samples (9.6%), corresponding to 13 out of 48 sampled farms were positive for *Chlamydiaceae* by real-time PCR. Among the farms that were sampled in our study, 11 out of 48 (22%) farms showed clinical signs, while the others were apparently healthy.

There was considerable difference between our findings and those of others on prevalence of *C. psittaci* infection in turkey. There may be some reasons that have influenced our results. Sampling may have occurred at the times that birds had stopped shedding, as the shedding of *C. psittaci* occurs intermittently and healthy carrier birds might not excrete bacteria for extended periods (Vanrompay et al., 1995b). Most of the sampled turkeys were clinically healthy birds; therefore, the amount of chlamydial DNA in the swabs was probably lower than required for the test. Antibacterial agents can stop shedding of Chlamydiae and Chlamydia availability (Billington, 2005). Antibiotics such as tetracyclines, macrolides, fluoroquinolones and chloramphenicol may interfere with diagnostic tests by reducing antibody production, antigen shedding, and Chlamydia viability (Krieg et al., 2010). In Iran, because of imprudent use of antibacterial agents in farms, it is really difficult to find and sample turkey flocks without previous background of antibacterial usage.

Surprisingly, none of the positive samples in real-time PCR were positive in the nested PCR. One reason for this may be that the amount of investigated DNA of *C. psittaci* was under the detection limit of our nested PCR method. Real-time PCR is one of the favored methods for the detection of DNA of *Chlamydiaceae* (Geens et al., 2005a; Ehricht et al., 2006; Pantchev et al., 2009). Nested PCR is usually a bit more sensitive, but vulnerable to DNA cross-contamination (Sachse and Hotzel, 2003). The second reason may be due to failures in ompA gene fragment amplification. This may occur because this gene is represented by a single copy per genome, unlike

ribosomal RNA genes such as 23S gene that was amplified in real-time PCR (Arraiz et al., 2012). The third reason that can be speculated is the possible infection of our *Chlamydiaceae*-positive samples found in real-time PCR with a non-classified strain of *C. psittaci*. Non-classified strains that belong to another member of the Chlamydia genus are not amplified by the ompA nested PCR used in the present work. Based on systematic investigation on poultry samples submitted to laboratories for avian chlamydiosis diagnosis, Zocevic et al. (2012) confirmed that *C. psittaci* was not the predominant chlamydial species among chickens and suggested that the new chlamydial agents could putatively be widespread in poultry flocks in countries such as France, Greece, Croatia, Slovenia and China. However, Zocevic et al. (2013) suggested a limited dissemination of atypical strains compared to the usually higher prevalence of *C. psittaci* that is the main species associated with avian chlamydiosis in Parisian pigeon populations.

In conclusion, this study showed no *C. psittaci* infection in the turkey population in Iran. We recommend investigation on other farm animals and wild populations for possible chlamydial infection and for better understanding of the source and epidemiology of this agent. Due to the challenges that exist for sampling and the relevant impacts on reducing positive samples, investigation by parallel and complementary techniques such as cell culturing, serology and micro-array may be useful in showing the true prevalence of infection in the target population.

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