Original article

Microbiological and parasitological survey of zoonotic agents in apparently healthy feral pigeons

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Abstract

Microbiological and parasitological investigation was carried out on a colony of feral pigeons, located in a green area near the main hospital of a Central Italy city. One hundred pigeons were submitted to clinical examination. Cloacal swabs, grouped in pool of 4 samples, were analyzed to detect the presence of *Coxiella burnetii*, *Chlamydia psittaci*, *Chlamydophila* spp. using a biomolecular procedure, while individual cloacal samples were examined for *Salmonella* spp., *Campylobacter* spp., and yeasts by means of a specific culture media. An ELISA test was used to determine the presence of *Giardia* spp., and *Cryptosporidium* spp. coproantigens. Individual serological samples were also tested with the modified agglutination test (MAT) in order to detect antibodies against *Toxoplasma gondii*. The pigeons did not show any clinical signs. The cloacal pools proved to be negative for *C. burnetii* DNA while three pools were positive for *C. psittaci* or *Chlamydophila* spp. DNAs. *Salmonella* spp. was not detected. *C. jejuni* and *C. coli* were found in 13% and 4% of the samples, respectively. No *Giardia* spp. and *Cryptosporidium* spp. were detected. Thirty-three out of 100 samples (33%) were positive for yeast colonies. The seroprevalence for *T. gondii* was 8%. Although with moderate incidence, potentially zoonotic agents were present thus highlighting the need for sanitary surveillance on feral pigeon colonies.

Key words: feral pigeon, zoonotic agent, hospital, public health

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Introduction

Nowadays feral pigeons can be found all over the world interacting with human beings and their activities in several ways. Pigeons appear to be harmless yet they become a threat for Public Health (Jerolmack 2008). At present, approximately several hundred million feral pigeons are estimated to live in cities worldwide and the size of their colonies mainly depends on the amount of food provided by humans and on natural seasonal foodstuffs to a lesser extent (Haag-Wackernagel 2011). In some cases, the increase in number of these populations can cause concern as they frequently colonize squares, public parks or areas, where people at risk, such as children, the elderly or people with depressed immune systems, may inadvertently come into contact with their excreta in the form of aerosolized particles (Haag-Wackernagel and Moch 2004). In this context, feral pigeons have a significant epidemiological role, since they can be reservoirs and potential vectors of a large number of zoonotic pathogens as well as responsible for allergic diseases (Haag-Wackernagel and Bircher 2009, Magnino et al. 2009). Alexander et al. (1985) described approximately 110 zoonotic agents isolated from pigeons up to now. *Salmonella* spp., *Campylobacter* spp., Shiga toxin-producing, *E. coli*, chlamydial agents and *Cryptococcus* spp. are carried by clinically healthy pigeons (Haag-Wackernagel and Moch 2004, Pedersen et al. 2006, Kobayashi et al. 2007, Sachse et al. 2012). However, the association between human practice and illnesses contracted from feral pigeons has seldom been reported (Haag-Wackernagel and Moch 2004*). Cryptococcus neoformans, Candida albicans,* and *Chlamydophila psittaci* seem to be the most widespread zoonotic agents in the pigeon populations that reside in cities worldwide (Heddema et al. 2006, Magnino et al. 2009, Tomić et al. 2013). Epidemiological surveys for *C. psittaci* carried out on pigeons across Europe during the last thirty years detected high seropositivity and high prevalence in feces (Heddema et al. 2006, Magnino et al. 2009, Vazquez et al. 2010), although differences were observed among the diagnostic procedures used and the epidemiological conditions found in various studies. However, the presence of zoonotic organisms in feral pigeons does not necessarily cause disease in man, since several other factors such as the transmission route, the infectious dose, the immune status of the people involved and the closeness of contact strongly influence the possibility of infection (Haag-Wackernagel and Moch 2004). The aim of this study was to carry out a microbiological and parasitological survey on a colony of feral pigeons located near the main hospital situated in the suburbs of Perugia, a city of Central Italy, in order to assess the potential pathogen risk of transmission to both healthy and immunocompromised populations.

Materials and Methods

One hundred pigeons were captured with nets from a colony of approximately 200 feral pigeons, located near the main hospital in the suburbs of Perugia. After clinical examination, cloacal swabs and blood samples were collected in order to determine the presence of the following zoonotic agents: *Coxiella burnetii*, *Chlamydophila psittaci*, *Chlamydophila* spp., *Salmonella* spp., *Campylobacter* spp., *Giardia* spp., *Cryptosporidium* spp., *T. gondii* and yeasts.

Biomolecular investigation was performed to detect *C. burnetii*, *C. psittaci* and *Chlamydophila* spp.. Twenty-five cloacal pools (no. 1 to 25) were obtained by mixing 4 individual samples and their DNAs were extracted from 200 fl of each pool with a commercial kit (QIAamp DNA Stool mini kit, Qiagen, Italy) according to the manufacturer's instructions. A PCR protocol described by Berri et al. (2000), which involves amplifying a fragment of 687 bp of the IS1111 insertion sequence, was used with modifications. Two different nested PCR assays were used to identify the *Chlamydophila* spp. genus and *C. psittaci* species. Briefly, the *Chlamydophila* genus was recognized in the first step of the protocol described by Messmer et al. (1997) which targets the 16S rRNA gene, while the *C. psittaci* species was identified by both the nested step of the protocol by Messmer et al. (1997) and the amplification of the ompA gene (Van Look 2005). The DNA of each sample was tested in duplicate. The primers and cycling conditions are described in Table 1. Positive and negative DNA controls were used for each set of reactions. The PCR products of the expected size were purified by means of a gel extraction kit (Qiaquick PCR purification kit, Qiagen, Italy) and directly sequenced on both strands with the same primers using DNA analyzer (ABI 3730, Applied Biosystems) capillary sequencer (Primm Srl) as previously described. The sequences were assembled and aligned using BioEdit (Biological sequence alignment editor software, version 7.0.8). The sequence similarity was checked against sequences deposited in GenBank using BLAST (Basic local alignment search) software to confirm the species specificity of the PCR.

Table 1. PCR test protocols used for the detection of *Coxiella burnetii*, *Chlamydia psittaci*, and *Chlamydophila* spp.

PCR assay	Target gene	Primer sequence (5'-3')	Amplification profile					Product size (bp)	PCR type	References
			initial denaturation	denaturation	annealing	extension	final extension			
Coxiella burnetii	IS1111 insertion sequence	Trans1 (F): TAT GTA TCC ACC GTA GCC AGT C	94°C, 2 min	94°C, 30 sec	40 cycles	72°C, 1 min	72°C, 5 min	687	conventional	Berri et al., 2000
		Trans2 (R): CCC AAC AAC ACC TCC TTA TTC			59°C, 30 sec					
Chlamydophila spp. and *Chlamydia psittaci*	16S rRNA (genus level)	F: ACG GAA TAA TGA CTT CGG	94°C, 5 min	94°C, 30 sec	35 cycles	72°C, 45 sec	72°C, 5 min	436	conventional	Messmer et al. 1997
		R:TAC CTG GTA CGC TCA ATT			55°C, 30 sec					
	16S rRNA (species-specific level)	FN: ATA ATG ACT TCG GTT GTT ATT			35 cycles			127	nested	Messmer et al. 1997
		RN:TGT TTT AGA TGC CTA AAC AT	94°C, 5 min	94°C, 30 sec	55°C, 30 sec	72°C, 45 sec	72°C, 5 min			
Chlamydia psittaci	ompA	F CCT GTA GGG AAC CCA GCT GAA			35 cycles			872	conventional	Van Loock et al. 2005
		R:GGT TGA GCA ATG CGG ATA GTA T	94°C, 5 min	94°C, 30 sec	60°C, 30 sec	72°C, 45 sec	72°C, 5 min			
		FN: GCA GGA TAC TAC GGA GA			35 cycles			472	nested	Van Loock et al. 2005
		RN: GGA ACT CAG CTC CTA AAG	94 °C, 5 min	94°C, 30 sec	47°C, 30 sec	72°C, 45 sec	72°C, 5 min			

F = forward primer of the first round of PCR; R = reverse primer of the first round of PCR; FN = forward primer of the second round of PCR; RN=reverse primer of the second round of PCR.

Table 2. Yeast identification by biochemical test and by MALDI TOF MS.

Biochemical identification		Maldi-tof ms identification	
Yeast species	No. isolates	Yeast species	No. isolates
Candida zeylanoides	2	*C. zeylanoides*	2
Candida famata	5	*C. guillermondi*	5
Candida incospicua	1	*C. incospicua*	1
Candida lusitaniae	3	*C. lusitaniae*	1
Cryptococcus albidus	4	*C. albidus*	1
Rhodotorula mucilaginosa	3	*R. mucilaginosa*	3
Rhodotorula glutinis	1	*R. mucilaginosa*	1
Zygosaccharomyces spp.	14	*C. krusei*	4
Total	33	Total	18

The enrichment medium, Rappaport-Vassiliadis broth, was used for detecting *Salmonella* spp. The samples were subcultured at 37ºC on xylose lysine desoxycholate agar (XLD, Oxoid, Italy) and brilliant green agar (Oxoid, Italy) solid selective media. The results were read after 24 and 48 h of incubation. Suspected colonies were identified by means of composite biochemical media and commercial biochemical tests such as API RAPID 20E (BioMerieux, Italy). Cloacal specimens were analyzed to detect *Campylobacter* spp. diluted in Maximum Recovery Diluent (MRD, Oxoid, Italy) and vortexed until dissolution. The samples were surface spread-plated in duplicates onto dried, modified Cefaperazone Charcoal Desoxycholate Agar (mCCDA, Oxoid, Italy) plates, that were incubated micro aerobically for 48 h at 41.5ºC. The presence of *Campylobacter* spp. was confirmed by the oxidase activity, absence of growth in O_2 and agglutination test (Microgen® Campy Latex Agglutination Kit, Microgen Bioproduct, United Kingdom).

Giardia spp. and *Cryptosporidium* spp. coproantigens were investigated using individual cloacal samples which were then submitted to ELISA test (ProSpecT, Oxoid, Italy), according to the manufacturer's instructions. Individual cloacal swabs were cultured in Sabouraud Dextrose Agar, incubated at 24±1ºC in aerobiosis and observed on a daily basis in order to monitor the macro and microscopic characteristics of the yeast growth. The yeasts were subsequently identified with biochemical tests (API ID32C, BioMérieux, Italy); contemporarily a more feasible and specific method based on MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) was used in order to confirm the biochemical identification of the isolates. In the event of discordant results, MALDI-TOF MS was used to obtain definitive identification.

Serum samples were tested for *T. gondii* antibodies by means of the MAT (modified agglutination test), as described previously (Yan et al. 2011). In accordance with a recent study (Dubey 2010), MAT serum titers of 1:5 or higher were considered positive.

Results

The pigeons were well fed and did not show any clinical signs.

All cloacal pools were negative for *C. burnetii* DNAs, while one (no. 3) proved to be positive for *C. psittaci* DNA with both nested PCR protocols. Pool numbers 8 and 12 were only positive for the presence of gene 16S rRNA at the nested step. Sequencing of the ompA amplicon of pool no. 3 (GenBank accession no. KP704660) confirmed it as being *C. psittaci* at 99-100% homology with the sequences reported in GenBank. The sequencing of the amplicons, obtained from the three pools positive to the 16S rRNA gene (nos. 3, 8, and 12), identified various chlamydial species such as *C. psittaci*, *C. abortus*, *C. avium*, *C. felis*, *C. muridarum*, *C. pecorum*, *C. trachomatis*, although the nested step was considered species-specific for *C. psittaci*. For this reason, only pool no. 3 was identified as *C. psittaci* DNA and proved to be positive with both nested PCR protocols which was confirmed through sequencing, while the other two (nos. 8 and 12) probably contained DNA of various chlamydial species.

Salmonella spp. was not detected. *C. jejuni* and *C. coli* were found in 13% and 4% (positives/total subjects) of cases, respectively.

No *Giardia* spp. and *Cryptosporidium* spp. coproantigens were detected.

Thirty-three out of 100 samples were positive for yeast colonies at macro and micro features. Isolated yeast species with different tools are reported in Table 2.

The overall seroprevalence of *T. gondii* was 8% with the 1:10 end-point titre.

Discussion

In this study the positivity for *C. psittaci* DNA proved that pigeons are possible carriers of the infection in the investigated area, although it is impossible to estimate to what extent since the results were obtained from pool samples. However, the positive results were relevant as the pigeons did not show any clinical signs, in accordance with data reported by Vazquez et al. (2010), thus supporting the hypothesis that they may act as asymptomatic reservoirs of *C. psittaci* or other chlamydial species. Moreover, in the absence of clinical signs, the shedding of chlamydia is generally intermittent making it difficult to detect (Tomić et al. 2013). Nevertheless, there is a remarkable discrepancy between the widespread distribution of this pathogen in pigeon populations and the low number of human cases reported (Haag-Wackernagel and Moch 2004, Magnino et al. 2009), although it is possible that they may be underestimated due to diagnostic difficulties and the variability of clinical signs. Moreover, several authors have suggested that pigeon-derived *C. psittaci* strains have a low pathogenicity for man (Magnino et al. 2009). Lastly, pigeons could be also vectors of other chlamydial species that are relevant for their zoonotic role as previously reported (Sachse et al. 2012), which was confirmed in this study due to the detection of DNAs of various chlamydial species. However, further characterization of these sequences and an in-depth study of their zoonotic and epidemiological role are required. In this study *C. burnetii* DNA was not detected. Pigeons had previously been reported as being responsible for Q fever outbreaks in humans (Stein and Raoult 1999). In our samples *C. jejuni* and *C. coli* were found in 13% and 4% of the cases, respectively. Casanovas et al. (1995) demonstrated that *C. jejuni* was the most frequently isolated bacterium in 105 pigeons (26.2%) with a greater incidence in the districts of the city of Barcelona regardless of seasonal variation. Vazquez et al. (2010) also detected a high prevalence of *C. jejuni* and low prevalence of *C. coli*, as in our study. However, despite the frequent isolation of *Campylobacter* spp. from pigeons, direct transmission to humans has not yet been reported (Haag-Wackernagel and Moch 2004). On the basis of their studies regarding the adhesion and invasion models, Teske et al. (2013) suggested that pigeon-derived *C. jejuni* isolates were capable of infecting human enterocytes and producing the virulence factor cytolethal-distending toxin.

In this study *Salmonella* spp. was not detected thus confirming the observations of previous studies in which the isolation of *Salmonella* in feral pigeons was generally low (Pedersen et al. 2006). Similar results were obtained in a previous study, which only reported a single isolate of *S. typhimurium* in 202 pigeons monitored in the center of Perugia (Maresca et al. 2011). A review of health hazards caused by feral pigeons reported one single case of *S. enterica* serotype Kiambu infection in man (Haag Wackemagel and Moch 2004). In a two-year study carried out on racing pigeons, Teske et al. (2013) reported a range from a minimum of 0.9 to 3.7% of the positive lots investigated. In older studies (Vindevogel and Duchatel, 1979) the incidence of *Salmonella* spp. reached 26% in diseased pigeons submitted for diagnostic purposes. However, only clinically healthy pigeons were included in the present study. In this type of scenario, the incidence of *Salmonella* spp. is generally low and it is seldom transmitted from feral pigeon to man.

Concerning parasitological investigations, our samples proved to be negative for enteric protozoa while several studies have detected *C. parvum* and *C. hominis,* and the zoonotic assemblages of *G. duodenalis* (A and B) in faeces of various avian species indicating that birds might serve as mechanical vectors of cysts and oocysts and as possible sources of these infections in animals and humans (Graczyk et al. 2008, Abreu-Acosta et al. 2009, Majewska et al. 2009, Papini et al. 2012, Reboredo-Fernandez et al. 2015). At present, little information is available concerning the role played by pigeons in the zoonotic transmission of these pathogens. Probably, giardiasis and cryptosporidiosis spread more easily in overcrowded environments or stressful conditions, i.e. animals kept in zoos (Papini et al. 2012), than in small or medium-sized colonies like these examined in our study.

In our investigation, the overall seroprevalence of *T. gondii* (8%) was in accordance with data previously obtained in Italy (Grandini and Sarti 1995) even if a more recent report carried out in colonies of pigeons in Central Italy showed a seroprevalence of 83% (Maresca et al. 2011). Moreover, contrasting percentages of prevalence ranging from 4% to 100% can also be found in international literature due to differences in geographical conditions, diet and lifestyle, number of cats and rodents, as well as the serological test used (Mushi et al. 2001, Salant et al. 2009, Karatepe et al. 2011, Cong et al. 2014). Considering that pigeons share the environment with humans, the serological results suggest that they could be a good model and an indicator of the extent of oocyst contamination of the ground. Moreover, they can serve as sources of tissue cysts for domestic felids thus contributing to the epidemiological circuit of *T. gondii* (Karatepe et al. 2011).

In this study the pigeons frequently harbored a mixed potentially pathogenic fungi flora in the gas-

trointestinal tract. Although yeast has been considered a commensal organism and part of the normal biota of pigeons, over the last decade, *Cryptococcus albidus* and *non-Candida albicans* species, which were also isolated in this study, have emerged in immunocompromised and hospitalized patients (Dorneanu et al. 2008, Beyda et al. 2013). Recent studies on outbreaks of *Candida* species showed an increased incidence of bloodstream infections caused by *C. parapsilosis* in neonatal intensive care units (Clarck et al. 2013). Other yeasts, such as *Rhodotorula* and *Trichosporon,* can cause blood infections in patients with indwelling central venous catheters and cancer (Cafarchia et al. 2006). Several studies have confirmed the role of pigeon in the epidemiology of these opportunistic pathogen agents causing human infection due to inhalation or contact with their dried feces (Andrade-Silva et al. 2010, Rosario et al. 2010). In the present work, not all isolates of yeasts evidenced by biochemical test were confirmed and/or detected by MALDI TOF MS. Despite the specificity of the tool, Clark et al. (2013) evidenced that data-bases of MALDI TOF MS are not developed for several yeast species and this could justify the lower number of isolates identified. However, the detection of several zoonotic yeasts from pigeons located in urban areas can provide useful preliminary information for epidemiological and risk assessment studies.

In conclusion, proper control of the feral pigeon population and careful monitoring for zoonotic agents should be always carried out in areas, such as parks, hospitals, and schools, where subjects with impaired or not completely developed immune systems could be present (Dolbeer et al. 2000).

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