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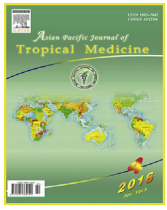

Original research http://dx.doi.org/10.1016/j.apjtm.2016.03.005

Molecular survey on zoonotic tick-borne bacteria and chlamydiae in feral pigeons (*Columba livia domestica*)

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ARTICLE INFO

Article history:
Received 15 Jan 2016
Received in revised form 20 Feb 2016
Available online 5 Mar 2016

Keywords:
Pigeon (*Columba livia domestica*)
Tick-borne bacteria
Chlamydophila psittaci
PCR

ABSTRACT

Objective: To determine the presence of zoonotic tick-borne bacteria in feral pigeons (*Columba livia domestica*) from urban areas.
Methods: Spleen samples from 84 feral pigeons, found dead with traumatic injuries in urban areas, were examined by PCR to detect DNA of *Anaplasma phagocytophilum*, *Bartonella* spp., *Borrelia burgdorferi* sensu lato, *Coxiella burnetii*, *Rickettsia* spp., and *Chlamydophila* spp.
Results: Twenty (23.8%) pigeons were infected by tick-borne agents, in particular 2 (2.38%) animals resulted positive for *Bartonella* spp., 5 (5.95%) for *C. burnetii*, 5 (5.95%) for *Rickettsia* spp., 13 (15.47%) for *B. burgdorferi* sensu lato. All birds scored negative for *A. phagocytophilum*. Moreover, 17 (20.23%) pigeons were positive for *Chlamydophila* spp. and among them 10 (11.9%) for *Chlamydophila psittaci*. Mixed infections by two or three agents were detected in 8 (9.52%) animals.
Conclusions: Feral pigeons living in urban and periurban areas are a hazard for the human health as source of several pathogens. The obtained results confirm pigeons as reservoirs of chlamydial agents and suggest that they may be involved in the epidemiology of zoonotic tick-borne infections too.

1. Introduction

Urban and periurban areas are frequently home to wild birds, particularly feral pigeons (*Columba livia domestica*), which can be present at high density. These animals are known as reservoirs of zoonotic viruses, bacteria, fungi and protozoa [1].

In particular, columbiform birds, including pigeons, have been ranked as the second major reservoir, after psittaciformes, of *Chlamydophila psittaci* (*C. psittaci*) [2]. This is a highly infectious, obligate intracellular bacterium which affects a wide range of avian hosts inducing asymptomatic forms or pneumonia, poor growth, diarrhea and central nervous system disorders. *C. psittaci* is transmissible to humans causing severe zoonotic infections [3].

Wild birds, including feral pigeons, are often carriers of infected ticks. In particular, the most common hard tick species associated with mammals and avian hosts in Europe is *Ixodes ricinus*, which is known as a competent vector of viral, bacterial and protozoan agents of medical and veterinary importance [4].

Some studies were carried out on vector-borne pathogens in arthropods collected from birds, whereas very little information is available about the presence of tick-borne bacteria in these animals.

Previous surveys found *Coxiella burnetii* (*C. burnetii*), the causative agent of Q fever, in feral pigeons and other birds [5,6].

Bartonella henselae DNA was detected in two northern mockingbirds (*Mimus polyglottus*) and one red-winged blackbird (*Agelaius phoeniceus*), and *Bartonella koehlerae* in a red-bellied woodpecker (*Melanerpes carolinus*) and a common loon (*Gavia immer*) in North Carolina, USA [7].

Rickettsia helvetica (*R. helvetica*), frequently involved in severe cases of human disease, was recently found in passerine birds, in particular five robins (*Erithacus rubecula*) and one dunnock (*Prunella modularis*) [8].

In Europe, wild birds belonging to different species scored positive to *Anaplasma phagocytophilum* (*A. phagocytophilum*) by PCR [8,9]. *Borrelia burgdorferi* (*B. burgdorferi*) sensu lato (s.l.), agent of the Lyme disease, was frequently detected in bird-associated ticks and in some studies it was directly found in avian specimens worldwide [10].

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Peer review under responsibility of Hainan Medical College.

At the best of our knowledge, no data were reported about the spreading of arthropod-borne agents among avian populations in Italy. The aim of this study was to evaluate the presence of some zoonotic tick-borne bacteria, in particular *A. phagocytophilum, Bartonella* spp., *B. burgdorferi* s.l., *C. burnetii*, and *Rickettsia* spp., in feral pigeons found dead in urban areas of Tuscany, Central Italy. The same specimens were also tested for *C. psittaci* DNA to monitor the spreading of this zoonotic agent among feral pigeons.

2. Material and methods

2.1. Sampling

During 2011–2013, 84 feral pigeons (*C. livia domestica*) were examined in the Avian Pathology Division of the Department of Veterinary Science, University of Pisa. All animals had been found dead and collected by private citizens in different urban areas of Tuscany.

The animals were submitted to necropsies, and spleen samples were collected. Animals' gender and age were also established: 55 pigeons were males and 29 females; 33 were considered young, 51 adult. It was generally accepted that complete development of the left ovary in females and testicles in males was achieved at the end of the fourth month of life. Therefore, pigeons under 4 months of age were considered young and those above 4 months adult [11].

All pigeons showed evidence of traumatic injuries with wing or/and leg trauma probably due to collisions. No ticks were detected during external examinations.

2.2. Molecular analysis

Spleen samples were submitted to DNA extraction using the DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) and according to the manufacturer's instructions. DNA samples were stored at 4 °C until employment as templates for PCR assays.

2.2.1. A. phagocytophilum

A primary amplification was carried out to amplify a 932 bp fragment of the 16S rRNA gene of *A. phagocytophilum*, using the primers GE 3a and GE 10r. A nested PCR, with the primers GE 9f and GE 2, amplified a 546 bp fragment of the same gene. Primary and secondary amplifications were performed with the same cycling conditions [12].

2.2.2. Bartonella spp.

DNA samples were employed in a PCR assay to identify the *Bartonella* genus. The primers p24E and p12B were used to amplify a 296 bp fragment of the *Bartonella* 16S rRNA gene as previously described by Relman *et al* [13].

2.2.3. B. burgdorferi s.l

Primers JS1 and JS2 were used to amplify a 261 bp fragment of the 23S rRNA gene of *B. burgdorferi* s.l. [14].

2.2.4. C. psittaci

A genus-specific amplification was carried out using primers 201CHOMP and CHOMP336s to amplify a 450 bp fragment of the variable domains III and IV of the *ompA* gene. One μL of the genus-specific PCR product was used as template in a second amplification with primers 218PSITT and CHOMP336s specific for a 389–404 bp fragment of *C. psittaci* [15].

2.2.5. C. burnetii

C. burnetii was identified by amplifying a 687 bp fragment of the IS1111a gene using primers Trans-1 and Trans-2, as described by Berri *et al* [16].

2.2.6. Rickettsia spp.

PCR with the primers Rr190.70p and Rr190.701 were carried out to amplify a 632 bp fragment of the gene encoding the outer surface protein ompA of *Rickettsia* spp., as described by Roux *et al* [17]. Since this protocol did not allow to detect *R. helvetica*, *R. akari*, *R. australis*, and *R. bellii*, a second PCR assay was performed using the primers RpCS.877p and RpCS.1258n which amplified a 381 bp fragment of the *gltA* gene [18].

HotStarTaq (Qiagen) was used for all PCR assays. Standard precautions were taken to avoid contamination of samples and reaction mixture, including strict separation of the areas for reagent preparation, DNA extraction and amplification. A negative control without template DNA was included to ensure the absence of contamination in the reaction mixture. No DNA positive controls were added to avoid possible cross-contamination.

All the amplification products were analyzed by electrophoresis on 1.5% agarose gel at 100 V for 45 min; gel was stained with ethidium bromide and observed. GelPilot 100 bp Plus Ladder (Qiagen) was used as DNA marker.

2.3. Statistical analysis

Statistical evaluation was carried out by the χ^2 test to analyze the results in relationship to age and gender of examined pigeons. Differences were considered significant when $P < 0.05$.

3. Results

Tick-borne infections were observed in 20 pigeons, with a 23.8% prevalence. More in details, 2 (2.38%) animals showed positive for *Bartonella* spp., 5 (5.95%) for *C. burnetii*, 5 (5.95%) for *Rickettsia* spp., 13 (15.47%) for *B. burgdorferi* s.l. All birds scored negative for *A. phagocytophilum*.

A total of 17 (20.23%) pigeons were positive for *Chlamydophila* spp. and among them 10 (11.9%) were positive for *C. psittaci*.

Statistical analysis did not find significant differences between the age and gender groups (Table 1).

Mixed infections were detected in 8 (9.52%) pigeons: 3 animals were positive for *Rickettsia* spp. and *B. burgdorferi* s.l., 2 for

Table 1
Number of examined pigeons PCR-positive for tick-borne agents and *Chlamydophila* spp. according to age and gender.

Groups	Number of examined pigeons	Tick-borne infected pigeons (%)	*Chlamydophila* spp. infected pigeons (%)
Adult	51	14 (27.45)	11 (21.56)
Young	33	6 (18.18)	6 (18.18)
Female	29	5 (17.24)	3 (10.34)
Male	55	15 (27.27)	14 (25.45)
Total	84	20 (23.80)	17 (20.23)

C. psittaci and *B. burgdorferi* s.l., 1 for *C. psittaci* and *C. burnetii*, 2 for *C. psittaci, Rickettsia* spp. and *B. burgdorferi* s.l.

4. Discussion

Contamination of urban environments caused by feral pigeons and the resultant health risks for humans have been known for a long time. Direct and indirect contact between feral pigeons and humans commonly occurs in squares, public gardens, parks, markets, and railway stations. In addition, the behavioral habit of pigeons in assembling and resting on roofs, balconies, window sills and shutters brings them even closer to humans. Epidemiological studies in feral pigeon populations have detected at least 110 organisms that are pathogenic to humans, including 8 viruses, 41 bacteria, 55 fungi and 6 protozoa. Among them, the most important pathogen transmissible from feral pigeons to humans is considered *C. psittaci* [19].

Our results, with 11.9% of *C. psittaci*-positive feral pigeons, confirm these animals as reservoirs of these bacteria. *C. psittaci* is usually excreted in feces and ocular and respiratory secretions of infected birds; infection in other animals and humans occurs through fecal dust, feather particles and dried excreta from infected birds that contaminate water, food and aerosols. Moreover, *C. psittaci* could be considered an arthropod-borne agent, because it may be transmitted by red mites *Dermanyssus gallinae* [20]. About 8% of the tested pigeons showed positive to *Chlamydophila* genus. In the literature, studies about avian infections by chlamydiae have usually been confined to the search of *C. psittaci*, thus data about the presence of other chlamydiae are scant. A previous survey detected *Chlamydia trachomatis*, *Chlamydophila abortus* and *Chlamydophila pecorum* in urban pigeons; moreover 19.5% of all *Chlamydiaceae*-positive cases turned out to be infected by non-classified organisms [21].

The 23.8% of the pigeons examined during the present investigation showed infected by tick-borne bacterial pathogens. All pigeons had no ectoparasites at the moment of examination and sampling. This finding was probably due to the fact that ectoparasites, in particular ticks, quite promptly leave dead animals; thus it cannot be excluded that the examined pigeons had been previously infested by hematophagous arthropods.

The 15.47% prevalence found for *B. burgdorferi* s.l. shows that, among birds, also *C. livia* is susceptible to this pathogen. Previous investigation found that the bird host competency for maintaining and transmitting *Borrelia* spirochetes varies in different bird species. Pheasants in the United Kingdom [22,23], blackbirds and song thrushes in Central Europe have been shown to be important reservoirs of *Borrelia garinii* and *B. valaisiana* [24–26]. *Borrelia turdi* DNA was recently detected in *Turdus merula* and their ticks in Portugal [27].

Two pigeons resulted infected by *Bartonella* spp. Various *Bartonella* spp. have been associated to human infections causing mild or severe diseases; these agents have been identified in domestic and wild animals, including canids, deer, cattle, rodents and marine mammals. *B. henselae* and *B. koehlerae* have been found in a few birds in USA [7], and *B. grahamii* DNA has been amplified from a bird tick in Korea [28]. However, attention to the spreading of these bacteria in avian population is very scant, thus data about the prevalence and the pathogenic role in birds are not available.

No pigeons showed positive to *A. phagocytophilum*. Information about infection by this pathogen in feral pigeons are not available, but studies carried out in Europe indicate that migrating birds may be important in the dispersal of *A. phagocytophilum* infected *I. ricinus* [29,30]. However, *A. phagocytophilum* DNA has been sometimes detected in ticks collected from birds at low prevalence, and it was questioned by some authors whether birds may really be involved in the spreading of the pathogen whereas other authors discussed their possible involvement [31]. The involvement of birds and their ticks in the lifecycle of *A. phagocytophilum* has been also tested in a transmission study in the US. For the two bird species involved, *Turdus migratorius* and *Dumetella carolinensis*, no significant role in the lifecycle was found [32].

C. burnetii DNA was amplified from spleen samples of five pigeons. *C. burnetii* is considered a tick-borne bacterium, even if infection is usually acquired by humans and animals through inhalation of contaminated aerosol or ingestion of contaminated food, mainly raw milk and dairy products [16]. Moreover, infected mammals shed the organism in placentas, and feces. It is not possible to determine the source of infection for the five positive pigeons; in fact they could have contracted coxiellae from arthropods, but also through direct or indirect contact with infected animals living in the neighboring rural areas.

The implication of birds in *C. burnetii* infection was described in Italy by Babudieri and Moscovici [5], who found that *C. livia* and *Anser anser domesticus* may be naturally infected with *C. burnetii*. Successively, it has been shown that infected domestic poultry can transmit coxiellosis to humans who consume raw eggs from infected hens or through aerosolized fomites. It has been also observed that experimentally infected hens shed *C. burnetii* in their feces for 7–14 d. The excretion of coxiellae in feces of infected birds has been demonstrated by Stein and Raoult [6] too, when they described a human Q fever outbreak resulted from exposure to contaminated pigeon feces and ticks.

Rickettsia spp. DNA was found in spleen samples collected from five pigeons. Infections by rickettsiae belonging to the Spotted Fever Group have been frequently reported in Europe, including Italy. However, literature data concern the detection of these pathogens in ticks and in wild and domestic animals; moreover cases of human rickettsiosis have been often described. Previous studies have found *Rickettsia* spp. DNA in ticks collected from birds, and *R. helvetica* bacteremia has been recently demonstrated in passerine birds. However, it has been supposed that the transmission of rickettsiae from birds to tick vectors may occur with low efficacy [8].

Significant differences were not detected between the gender and age groups; however, the higher values of prevalence observed in adult birds could be related to the time of exposure to the pathogens, which was longer in this group.

In conclusion, feral pigeons, which live in urban and peri-urban areas, are traditionally considered a hazard for the human health, as source of pathogens excreted with feces, such as salmonellae and chlamydiae. The results of the present survey, even if carried out on a small number of animals, suggest that these birds may be source of chlamydial agents, other than *C. psittaci*. Moreover, pigeons seem to be involved in the epidemiology of tick-borne zoonotic pathogens too.

Although it is well known that wild birds are vectors of infected ticks, further studies should be performed to better understand the role of pigeons as reservoirs of the tick-borne agents.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The present research was carried out with funds from the University of Pisa.

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