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Prevalence of *Chlamydophila psittaci* in Fecal Droppings from Feral Pigeons in Amsterdam, The Netherlands

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In many cities, the feral rock dove is an abundant bird species that can harbor *Chlamydophila psittaci*. We determined the prevalence and genotype of *C. psittaci* in fresh fecal samples from feral pigeons in Amsterdam, The Netherlands. The prevalence was 7.9% overall (26/331; 95% confidence interval, 5 to 11). Ten genotyped PCR-positive samples were all genotype B.

In many European cities, the feral rock dove (*Columbia livia*) is an abundant bird species that often lives in close contact with humans. It is known that pigeons, like many other bird species, can harbor *Chlamydophila psittaci*. This bacterium is a pathogen of birds but can cause zoonotic disease (5). Birds can shed this bacterium in the environment when they are either overtly ill or without any symptoms. In birds, the bacterium can be isolated from feces, the cloacae, and respiratory and conjunctiva secretions. For this study, we determined the prevalence of *C. psittaci* shedding in feces from feral pigeons in Amsterdam, The Netherlands, and the genotype of the PCR-positive samples. *C. psittaci* in these specimens was detected with a recently developed real-time PCR (6).

Setting and sampling. The city of Amsterdam consists of 14 town councils. Pigeon samples were obtained from nine locations in eight town councils. These locations were geographically widely distributed in Amsterdam, and all were situated in public areas and chosen based on previous research of assembling locations for feral pigeons in Amsterdam (3). At these locations, pigeons were attracted with food, and their fresh fecal droppings were sampled with sterile cotton swabs (MW&E, United Kingdom). Since shedding occurs intermittently and can be activated by stress factors, such as breeding, samples were taken on 3 February and 8 March 2005, when breeding activity was low (low-breeding period), and on 2 May 2005, when breeding was frequent (12).

DNA extraction and PCR. The cotton swabs were placed in 1.5-ml tubes in 300 μl Baker water (Boom B.V. Meppel, The Netherlands) and vortexed thoroughly. Fifty microliters of each fecal suspension was used as input for the DNA extraction procedure (1). *C. psittaci* PCR was performed as previously described (6). Briefly, this real-time PCR targets an 82-bp fragment of the *ompA* gene of *C. psittaci* as well as an internal control plasmid, using primers CpsittF (5′-CGCTCT CTCCTTACAAGCC-3′) and CPsittR (5′-AGCACCTT CCCA CATAGTG-3′). The internal control, which was added to each sample, has the same primer sites, length, and nucleotide content as the *C. psittaci* amplicon but has a shuffled probe binding region. To prevent false-positive reactions due to amplicon carryover, we used the uracil *N*-glycosylase system and a unidirectional workflow combined with separation of PCR mix preparation and DNA extraction from all (post)amplification activities.

Genotyping. PCR-positive samples were genotyped by *ompA* sequence analysis. The gene was amplified with the primers CPsittGenoFor (5′-GCTACGGGTTCCGCTCT-3′; nucleotides 400 to 416) and CPsittGenoRev (5′-TTTGTTGATYTG AATCGAAGC-3′; nucleotides 1420 to 1441) (nucleotide positions are according to the *C. psittaci* 6BC *ompA* gene [GenBank accession no. X56980]), resulting in a 1,041-bp amplicon. These primers target the conserved regions of the *ompA* gene enclosing the four variable domains. Genotype PCR was performed in a LightCycler 2.0 instrument (Roche Diagnostics, Germany). The final reaction mixture (20 μl) included 8 μl eluate and was essentially the same as that described previously (6). The real-time PCR steps were as follows: 50°C for 10 min; 95°C for 10 min; and 45 cycles of 95°C for 10 s, 62°C for 5 s, 72°C for 50 s, and 30°C for 30 s. Ten microliters of the PCR product was analyzed by 1% agarose gel electrophoresis. The expected amplicon was excised from the gel, purified by a simplified guanidinium thiocyanate extraction procedure (2.5 μl silica; wash cycles with L2, ethanol, and acetone), and eluted in 15 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) (2). To obtain sufficient product for sequence analysis, reamplification for only 20 cycles was performed in a GeneAmp 9700 thermocycler (Perkin-Elmer). The reaction mixture for reamplification (50 μl) included 2 μl of eluate, 5 μl (10×) PCR II buffer, 5 μg bovine serum albumin, 0.25 U AmpliTaq Gold, a 0.16 μM concentration of each primer, and 4.5 mM $MgCl_2$. The PCR steps were as follows: 95°C for 10 min; 20 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and 72°C for 10 min. When a single band of approximately 1,041 bp was obtained by agarose gel electrophoresis, the PCR product was subjected to sequence analysis (BigDye Terminator sequencing kit; Applied Biosystems). Overlapping sequences were obtained with four sequencing

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TABLE 1. Number of *C. psittaci* PCR-positive fecal samples in feral pigeons by sampling location in Amsterdam, The Netherlands

Town council	No. of positive samples/total no. of samples	
	Low-breeding period	Breeding period
Oost Watergraafsmeer	0/15	6/15
Oud Zuid	3/15	0/20
Binnenstad (Dam)	0/20	2/27
Binnenstad (Leidse plein)	2/25	3/27
Zeeburg	0/15	3/15
Zuider Amstel	0/15	3/15
Geuzenveld	0/15	0/15
Bos en Lommer	0/20	0/15
Oud West	3/20	1/22
Total	8/160 (5%; 95% CI, 2% to 10%)	18/171 (10%; 95% CI, 6% to 16%)

primers, including the above-mentioned genotype primers and two inner primers, CPsittFinner (5′-CGCTCTCTCCTTACA AGCC-3′) and CPsittRinner (5′-GATCTGAATCGAAGCA ATTTG-3′). We used the *C. psittaci* ORNI (genotype A) strain and a *C. abortus* strain as positive controls. To prevent amplicon carryover, the same measures as those described for real-time PCR were taken. The resulting sequences were aligned, and a similarity index based on the resulting amino acids was calculated for an 894-bp fragment of the *ompA* gene. Similarity (1 − distance) was calculated by using the pairwise distance method generated by MEGA3 (8). Reference *ompA* genotype sequences A to F (available in the GenBank database under accession numbers AY762608 to -12 and AF269261) were included in this analysis (4).

Results. In total, 331 fecal samples were obtained, with 160 samples obtained before and 171 samples obtained during the breeding period (Table 1). At least 15 samples were collected at each location. During the low-breeding period, 5% (8/160; 95% confidence interval [CI], 2% to 10%) of all samples were PCR positive. Among samples obtained during the breeding period, 10% (18/171; 95% CI, 6% to 16%) were positive, and hence the prevalence of positive samples during the breeding period was twice that in the low-breeding period ($P = 0.07$ by Fisher's exact test; GraphPad Software, San Diego, CA). The overall prevalence was 7.9% (26/331; 95% CI, 5% to 11%). All negative samples were truly negative since all internal controls were amplified correctly, thus excluding the occurrence of PCR inhibition. It was possible to genotype 10 of the 26 PCR-positive samples. The obtained sequences were all 100% similar to reference genotype B. Similarities based on amino acid sequences were 98% (genotype A), 56% (C), 43% (D), 99% (E), and 51% (F). The positive controls (*C. psittaci* ORNI and *C. abortus*) were amplified as expected and could subsequently be sequenced.

Discussion. This study shows that between 5 and 10% of our sample of urban feral pigeons in Amsterdam shed *C. psittaci* in their feces. Only genotype B was found in these isolates. We were unable to genotype all PCR-positive samples. For genotyping, a 1,041-bp fragment had to be amplified; the genotyping PCR was less sensitive than the optimized diagnostic real-time PCR, which amplifies a fragment of only 82 bp. Therefore, samples with relatively low *C. psittaci* loads could not be amplified by genotyping PCR. The major advantage of this study was the use of an internally controlled real-time PCR assay. PCR is a sensitive and specific test compared to enzyme-linked immunosorbent assays and tissue culture assays available for *C. psittaci* detection in birds (7, 9). Salinas et al. reported one of the largest series on the prevalence of *C. psittaci* in feral pigeons (10). In their study, *C. psittaci* was found by culture in 18% (7/39; 95% CI, 9% to 33%) of fecal samples, a prevalence that is similar to our results obtained by PCR. Recently, Tanaka et al. found *C. psittaci* in 106 of 463 (22.9%; 95% CI, 19% to 27%) fecal samples obtained from feral pigeons. However, they did not use exclusively fresh fecal samples and applied a nested PCR protocol, which is known to be particularly prone to contamination (13).

A previous study indicated that in 2001, the pigeon population size in Amsterdam averaged approximately 30,000 (3). Combined with our results, the number of feral pigeons shedding *C. psittaci* in their feces would be, on average, about 2,400 (95% CI, 1,500 to 3,300). Our isolates were all identical to genotype B. Currently, at least nine genotypes are known. Each genotype is more or less associated with a specific group of birds from which it is most commonly isolated. Geens et al. and Vanrompay et al. also found genotype B to be particularly associated with the pigeon host (4, 14). However, this genotype has been recovered from many bird species, including turkeys, parakeets, and ducks (11, 15). Whether shedding of *C. psittaci* by feral pigeons in Amsterdam poses a substantial zoonotic risk for humans has to be determined. Besides the zoonotic potential, there is also the risk of infection of domesticated birds, such as pet birds and poultry, which live in closer contact with human beings. Diagnosing *C. psittaci* infections has been hampered by a lack of sensitive and specific methods. Culture is only performed in some select laboratories, serologic tests do not fully differentiate infections with the various *Chlamydia* spp., and PCR is not routinely performed. However, PCR can provide a definitive diagnosis of psittacosis. We recommend that psittacosis in humans be diagnosed by detection of the agent by PCR, with or without serologic testing, instead of by serologic testing alone. Subsequent *ompA* gene sequence analysis can identify the responsible genotype. This approach could lead to a better understanding of the epidemiology of the different genotypes of *C. psittaci* in infected bird populations and human psittacosis cases and determine the relationship between the two.

Nucleotide sequence accession numbers. The *ompA* sequences of the positive control strains and the genotype B sequence obtained from the fecal pigeon samples were submitted to GenBank under accession no. DQ267973, DQ435299, and DQ435300.

Y. Pannekoek, University of Amsterdam, Amsterdam, The Netherlands, provided the *C. psittaci* ORNI strain. D. Vanrompay, Ghent University, Ghent, Belgium, provided the *C. abortus* strain.

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