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Molecular characterisation of carbapenemases in urban pigeon droppings in France and Algeria

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ABSTRACT

Objectives: The main objective of this study was to detect the presence of carbapenemase-encoding genes in stool samples of urban pigeons.
Methods: Stool samples were collected from 73 pigeons in two Mediterranean cities, namely Marseille (France) and Annaba (Algeria). Faecal samples were screened by real-time PCR and standard PCR for the presence of carbapenemase-encoding genes.
Results: Carbapenem resistance genes were detected in 16 (21.9%) of the samples, with 8 positive for bla_{OXA-23}, 12 positive for $bla_{OXA-51-like}$ and 13 positive for bla_{OXA-58}. No samples were positive for bla_{NDM-1}, bla_{OXA-24}, bla_{OXA-48}, bla_{VIM} or bla_{KPC}. All positive samples were screened for the presence of *Acinetobacter* spp. by partial *rpoB* gene sequence amplification, and the results showed the presence of five *Acinetobacter* spp., with percentage similarities to related species in GenBank ranging between 96% and 100%. The dominant species was *Acinetobacter guillouiae*, followed by *Acinetobacter baumannii*, *Acinetobacter haemolyticus*, *Acinetobacter pittii* and *Acinetobacter nosocomialis*. One DNA sequence showed a very low degree of homology (92%) with *Acinetobacter gerneri*, suggesting a new *Acinetobacter* spp.
Conclusions: Here we report the first detection of carbapenemase-encoding genes from urban pigeon stools. These results question the potential of birds as a reservoir for the spread of these resistance determinants both in animals and humans.

1. Introduction

Carbapenems are widely used to treat human infections caused by multidrug-resistant Gram-negative bacteria. However, the increasing use of carbapenems over the last decade has led to the emergence of carbapenem-resistant isolates, particularly Enterobacteriaceae and *Acinetobacter* spp. isolates [1] Resistance to carbapenems may be due several mechanisms, mainly the presence of extended-spectrum β-lactamase or AmpC enzymes in combination with porin mutations [2,3].

The majority of these enzymes have been identified in *Acinetobacter* spp., notably in *Acinetobacter baumannii*. Resistance is largely associated with acquired carbapenem-hydrolysing class D β-lactamases, with bla_{OXA-23} being the most commonly identified worldwide [4]. This class of β-lactamases confers resistance to carbapenems, such as imipenem, meropenem and doripenem, with a low hydrolytic activity towards these antibiotics [5], causing problems in the treatment of life-threatening infections. Furthermore, the emergence of resistance is not confined to nosocomial infections.

Possible reservoirs of carbapenemases can also be found in the natural environment, including animals. Indeed, several authors have reported the emergence of carbapenemase-producing isolates such as *Escherichia coli*, *Salmonella* spp. and *Acinetobacter* spp. from farm animals (cattle, pig, poultry) [6–12] and companion animals (dogs and cats) [13,14] as well as food-producing animals [1,9]. Kempf et al. [10] even detected a $bla_{OXA-like}$ gene in a human ectoparasite (head lice) in Senegal. Carbapenemase-encoding genes have also been detected in wild animals such as birds [15]. In 2013, Fischer et al. [15] isolated an

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NDM-1 carbapenemase-producing *Salmonella enterica* subsp. *enterica* serovar Corvallis from a wild bird (black kite, *Milvus migrans*) in Germany. However, the prevalence of resistance of zoonotic or commensal bacteria from animal sources is still unknown and underestimated.

Among wild birds, urban pigeons have a close proximity to human activities. Pigeons colonise our cities and proliferate in abundance in the absence of predators [16–18]. This relationship is so strong that urban pigeons have settled and have changed their diet to become omnivorous and opportunistic, feeding mainly on trash, landfills and occasional supply provided by some residents. These exchanges promote the possible horizontal transfer of bacteria between humans and pigeons. Human pathogenic bacteria carrying multidrug resistance determinants may reach the pigeons' gut via food, and humans can be infected by contaminated pigeon droppings. None the less, only a few studies have investigated the possible health hazard from urban pigeons [16]. Recently, da Silva et al. [16] demonstrated the presence of multidrug-resistant enterococci in the faeces of urban pigeons, whereas no data are available on the possible presence of carbapenemases in urban pigeons.

The main aim of this study was to investigate the presence of carbapenemase-encoding genes in pigeon stools collected from two Mediterranean cities, namely Annaba (Algeria) and Marseille (France). Pigeons were chosen as an indicator of our close environment and the Mediterranean region, which appears to have a high level of antibiotic consumption both in human and veterinary medicine [19,20].

2. Materials and methods

2.1. Sample collection

A total of 73 fresh stool samples from free-living feral pigeons were collected from different locations in two Mediterranean cities, namely Annaba ($n = 33$) and Marseille ($n = 40$). Samplings were carried out between April–October 2013 in public gardens and markets places, known to be the most relevant feeding sites for urban pigeons. Each dropping sample was collected using a sterile clamp, was placed into 2 mL of storage medium and was stored at −80 °C until analysis.

2.2. DNA extraction from stool samples

Whole DNA was extracted using a QIAGEN® BioRobot® EZ1® automated system (QIAGEN, Courtaboeuf, France). Briefly, DNA was extracted from 1 g of stool sample with the EZ1 DNA Tissue Kit (QIAGEN) as recommended by the manufacturer. The extracted DNA was eluted in 200 μL of elution buffer and was stored at −20 °C.

2.3. Real-time and conventional PCR assays for carbapenemase-encoding genes detection

Carbapenemase-encoding genes were screened by real-time PCR using specific primers for $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-58}}$, $bla_{\text{NDM-1}}$, bla_{VIM} and bla_{KPC}. The oligonucleotide primers used in this study are listed in Table 1. A QuantiTect® Probe PCR Kit (QIAGEN) was used for PCR, and the concentrations of primers and probes were 20 μM and 2.5 μM, respectively. PCR was performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Marnes-la-Coquette, France). Samples with a cycle threshold (Ct) of >35.0 were considered negative for carbapenemase-encoding genes. Three negative controls were used for each assay. To confirm and validate the results, conventional PCR was performed for all real-time PCR-positive samples. The primers used for standard PCR are listed in Table 1.

2.4. Acinetobacter *species detection in whole DNA from stool samples*

Acinetobacter spp. detection was performed by targeting the RNA polymerase subunit β gene (*rpoB*) of *Acinetobacter* using standard PCR [26]; the primers are listed in Table 1. Amplification was performed with an initial denaturation step for 15 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 50 °C and elongation for 1 min at 72 °C, followed by a final extension step for 7 min at 72 °C. Negative and positive controls were included in each assay.

2.5. Sequencing

Analyses of the PCR products of carbapenemase-encoding genes and the *Acinetobacter rpoB* gene were performed using a BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an automated ABI 3130 Sequencer (Applied Biosystems). Data were analysed with CodonCode Aligner (http://www.codoncode.com/aligner/) and MEGA5 software (http://www.megasoftware.net/). The obtained sequences were compared with those deposited in GenBank using the Blastn and Blastx programs (http://www.ncbi.nlm.nih.gov/blast) and with those of the ARG-ANNOT database [27].

3. Results

3.1. Real-time and standard PCR detection of carbapenemase-encoding genes

Of the 73 faecal samples collected from different sites in Marseille and Annaba, 16 samples (21.9%) (12 from Marseille and 4 from Annaba) were positive for carbapenemase genes. Among them, 5 samples harboured $bla_{\text{OXA-23}}$, $bla_{\text{OXA-58}}$ and $bla_{\text{OXA-51-like}}$ genes, 4 harboured both $bla_{\text{OXA-58}}$ and $bla_{\text{OXA-51-like}}$ genes, 3 harboured both $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-51-like}}$ genes, and 4 samples harboured only the $bla_{\text{OXA-58}}$ gene (Table 2). All carbapenemase-positive samples were detected with Ct values ranging from 23 to 34. None of the tested samples were positive for $bla_{\text{NDM-1}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-48}}$, bla_{VIM} or bla_{KPC}. The real-time PCR results were confirmed by conventional PCR.

3.2. Acinetobacter *spp. detection in whole DNA from stool samples*

PCR amplification targeting a partial sequence of the *Acinetobacter rpoB* gene revealed that 14 of the 73 DNA stool samples analysed were positive, confirming the presence of *Acinetobacter* strains among the stool microbiota of urban pigeons.

3.3. Sequencing

Blast results for the obtained sequences of $bla_{\text{OXA-23-like}}$ (Fig. 1) and $bla_{\text{OXA-51-like}}$ (Fig. 2) against the ARG-ANNOT database showed a high degree of similarity of 98–100% for a $bla_{\text{OXA23-like}}$ gene and 99–100% for a $bla_{\text{OXA-51-like}}$ gene. Standard PCR of the $bla_{\text{OXA-58}}$ gene did not generate any product.

rpoB partial sequencing revealed the presence of various species (Table 2). The amplicons sequences exhibited a concordance ranging between 96% and 100% compared with GenBank reference sequences. *A. baumannii* was detected in four samples, with a percentage similarity ranging within 99–100% with related species. Five samples were positive for *Acinetobacter guillouiae* (99% similarity), two showed the presence of *Acinetobacter haemolyticus* (96% and 97% similarity) and single samples shown