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Short communication

Characteristics of higher generation cephalosporin resistant Escherichia coli in feral birds in Switzerland

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Abstract

A worrisome phenomenon is the progressive global spread of Enterobacteriaceae harbouring plasmid-mediated production of enzymes which inactivate β-lactam-antibiotics into the environment and subsequent colonization of synanthropic and wild animal populations. The aim of this study was to investigate the presence of higher generation cephalosporin resistant Escherichia coli in faecal samples of feral pigeons (Columba livia) located in the city of Zurich and of great cormorants (Phalacrocorax carbo) located on the banks of the Rhine and to further characterize detected isolates. Six strains were isolated from 298 pigeons and 30 cormorants. Three (1%) of the pigeons and 2 (6.7%) of the cormorants were found to carry multidrug- resistant, predominantly pathogenicity-associated extra-intestinal E.coli. The worldwide frequently found bla\text{CTX-M-15} was detected in one pigeon and one cormorant isolate. Three pigeon strains harboured the plasmid-encoded AmpC-β-lactamase gene blacMY-2. One cormorant was found to carry the pandemic E.coli ST 131 clone containing blac\text{CTX-M-27}. Both urban pigeons and great cormorants in Switzerland are potential carriers of epidemiologically important ESBL-producing E. coli. Transmission of multiresistant strains into the urban environment and waterways via their faecal deposits constitute a potential hazard to public health.

Keywords:
ESBL; Escherichia coli; birds; faecal carriage; wildlife; reservoir
1. Introduction

One of the currently most important antibiotic resistance mechanisms in *Enterobacteriaceae* is based on plasmid-mediated production of enzymes which inactivate β-lactam-antibiotics including cephalosporins and monobactams by hydrolyzing their β-lactam ring. These so-called extended-spectrum β-lactamases (ESBLs) have been detected in human clinical isolates of *Enterobacteriaceae* since the early 1990s (Paterson and Bonomo, 2005) originally as derivates of the TEM- and SHV-β-lactamase families, then increasingly as CTX-M enzymes, or other less frequent ESBLs such as OXA- or PER-ESBLs (Bush and Jacoby, 2010; Coque et al., 2008). In addition, plasmid-mediated AmpC-type β-lactamases (pAmpCs) are increasingly reported world-wide, representing a new threat to successful antibiotic therapy because they are not, like ESBLs, susceptible to β-lactamase inhibitors such as clavulanic acid or sulbactam, and they possess a wider spectrum of enzymatic activity (Philippon et al., 2002).

As a further matter of concern, resistance caused by ESBLs or pAmpCs is often associated with resistance to other classes of antibiotics such as fluoroquinolones, aminoglycosides, and sulfamethoxazole/trimethoprim, resulting in multidrug resistant strains (Coque et al., 2008).

Since the first description of ESBL-producing *Enterobacteriaceae* from hospitalized humans, ESBL-producing *Escherichia coli* have been reported in numerous nosocomial and later also community-associated infections worldwide (Paterson and Bonomo, 2005) and have been detected in food-producing animals (Carattoli, 2008) and household pets (Ewers et al., 2011). In addition, ESBL-producing *E. coli* appear to be spreading into the natural environment, resulting in wildlife populations that act as reservoirs and disseminators of ESBL-producing *Enterobacteriaceae* (Guenther et al., 2011). Several studies have described antibiotic resistant *Enterobacteriaceae* in gulls (Bonnedahl et al., 2010; Poirel et al., 2012),
water birds (Tausova et al., 2012) and pigeons (Radimersky et al., 2010), suggesting that birds play an important role in the epidemiology of resistance genes.

The aim of the present study was therefore (i) to assess the prevalence of resistance to extended-spectrum β-lactam antibiotics in Enterobacteriaceae harboured by a population of urban pigeons (Columba livia forma domestica) in the city of Zurich, Switzerland as well as by great cormorants (Phalacrocorax carbo) from in the banks of the Rhine river in the northern district of the Canton of Zurich, Switzerland and (ii) to characterize such isolates by antibiotic susceptibility testing, identification of the blaESBL/blaAPmC genes, multi-locus sequence typing (MLST), determination of phylogenetic groups and detection of virulence genes.

2. Material and methods

2.1 Bacterial isolates

Pigeon swabs were obtained from the pigeon management programme in the city of Zurich. Between March 2012 and August 2012, a total of 298 postmortem cloacal swabs were taken from culled pigeons by an authorized gamekeeper using a swab tube containing Amies gel transport medium (Copan, Brescia, Italy). Tubes were numbered consecutively and sent to the laboratory for analysis.

Cormorants were shot between December 2011 and January 2012, by gamekeepers authorized by the Swiss Cormorant Action Plan, in collaboration with the Swiss Agency for the Environment, Forests and Landscape and the Institute for Veterinary Bacteriology, Vetsuisse Faculty, Zurich, Switzerland. Twelve pooled cloacal samples were obtained from a total of 30 dissected cormorants.

Each sample was incubated for 24 hours at 37 °C in 10 ml of EE Broth (BD, Franklin Lakes, USA) for enrichment. One loopful each of the enrichment cultures was inoculated onto
chromogenic Brilliance ESBL agar and Brilliance CRE agar (Oxoid, Hampshire, UK) to select for extended-spectrum β-lactamase and carbapenemase producers, respectively, and incubated at 37 °C for 24 hours under aerobic conditions. All colonies with different coloration and morphology were picked from the selective plates and subcultured on sheep blood agar (Difco laboratories; 5% sheep blood, SB055, Oxoid) at 37 °C for 24 hours. Oxidase-negative isolates were thereafter subjected to identification by API ID 32 E (bioMérieux, Marcy l’Etoile, France).

2.2 Antibiotic susceptibility testing and phenotypic ESBL detection

Susceptibility testing was performed by agar diffusion methods, using antibiotic disks (Becton Dickinson and Company, Maryland, USA) and Etest ESBL epsilometer strips (bioMérieux, Marcy l’Etoile, France), according to the manufacturers' protocols. Results were interpreted according to the criteria of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2008). Strains exhibiting resistance to 3 or more antibiotic classes were classified as multidrug resistant.

2.3 Characterization of β-lactamases

Bacterial strains confirmed for either production of ESBLs or expression of higher generation cephalosporin resistance were further analysed by screening for *bla* genes. DNA was extracted by a standard heat lysis protocol. Thereafter, specific primer sets (custom-synthesized by Microsynth, Balgach, Switzerland) were used to amplify β-lactamase-encoding genes belonging to *bla*TEM, *bla*SHV and *blaCTX-M* (Geser et al., 2012) and *bla*CMY families (Briñas et al., 2003; Endimiani et al., 2012), the latter supplemented with the following newly designed primer cmy-dn-3 from the *bla*CMY-2 downstream flanking region: 5’ATGCGCATGGGATTTTCCTTGC3’. Resulting amplicons were purified using the PCR Purification Kit (QIAGEN, Courtaboeuf, France) according to the manufacturer’s
recommendations. Custom-sequencing was performed by Microsynth (Balgach, Switzerland) and the nucleotide and protein sequences were analysed with Codon Code Aligner V. 3.7.1.1. For database searches the BLASTN program of NCBI (http://www.ncbi.nlm.nih.gov/blast/) was used.

2.4 Multi-locus sequence typing of ESBL-producers

Internal fragments of 7 housekeeping genes (adk, fumC, gyrB, icd, mdh, purA, and recA) were sequenced (Wirth et al., 2006) and alleles and sequence types (ST) were assigned in accordance with the E. coli MLST website (http://mlst.ucc.ie/).

2.5 Determination of E. coli phylogenetic groups

Phylogenetic analyses have shown that E. coli strains fall into four main groups (A, B1, B2, and D), in which groups A and B1 typically contain commensal isolates, and isolates of groups B2 and D are considered to be more likely to carry pathogenicity-associated genes (Clermont et al., 2000). After DNA extraction using a standard heat lysis protocol, ESBL-producing E. coli isolates were subjected to phylogenetic grouping by PCR as described previously (Clermont et al., 2000).

2.6 Virulence factors in ESBL-producers

Strains were examined for the presence of genes for putative virulence factors mediating adhesion (adhesion siderophore iha, long polar fimbriae lpfA, S fimbrial adhesin sfaS, and temperature sensitive haemagglutinin tsh), increasing iron-uptake (e.g. siderophore for iron iroN), and of genes encoding toxins (secreted autotransporter toxin sat, serine protease pic, vacuolating toxin vat, and cytotoxic necrotizing factor cnf1). An ArrayTube-based DNA microarray approach (Clondiag Chip Technologies, Jena, Germany) was used according to the manufacturer’s instructions.
3. Results and discussion

Using selective isolation on Brilliance ESBL agar plates, phenotypically positive ESBL-producing *Escherichia coli* were detected from 2 pigeon samples (W117E and W132) and 2 great cormorant samples (W34 and W43). Selective cultivation on Brilliance CRE agar plates gave rise to 2 isolates originating from pigeons (W117C and W265). One pigeon (W117) was thus found to have harboured 2 distinct strains; one selected from an ESBL- the other from a CRE- agar plate (W117E and W117C, respectively). In total, 6 resistant strains were collected for analysis.

The antibiotic susceptibility profiles of the *E. coli* isolates are summarized in Table 1. On account of their resistance patterns, all the isolates were classified multidrug resistant. All strains were resistant to ampicillin and the 1st-generation cephalosporin cephalothin. Three of 6 isolates (W117E, a pigeon- and W34 and W43, cormorant isolates, respectively) tested resistant to third-generation cephalosporin cefotaxime in the disk diffusion test and susceptible to ampicillin-clavulanic acid, thus suggesting an ESBL phenotype. Further 3 strains, W117C, W132 and W265 (all from pigeons), tested resistant to ampicillin-clavulanic acid in the disk diffusion test and showed reduced susceptibility to cephalosporin-clavulanic acid combinations in the E tests, suggesting an AmpC-phenotype. All isolates remained susceptible to the 4th generation cephalosporin cefepime and to the carbapenem antibiotic imipenem. The latter result indicated the absence of carbapenemases in both the pigeon population as well as in the cormorants, despite initial growth on Brilliance CRE agar of strains W117C and W265, suggesting reduced susceptibility of these strains to the selective agent in the CRE medium.

In addition, resistance to nalidixic acid was detected in 3 of 4 (75%) of the pigeon strains and in one cormorant strain (W43), the latter additionally testing resistant to ciprofloxacin. Resistance to aminoglycosides was restricted to streptomycin and identified in
2 pigeon and both cormorant strains. All isolates were resistant to tetracycline. One pigeon isolate (W117E) and both cormorant strains were resistant to sulfamethoxazole and trimethoprim. All strains tested fully susceptible to chloramphenicol.

The results of ESBL- and AmpC gene identification, MLST analysis, determination of phylogenetic groups, and detection of putative virulence genes are summarized in Table 2. Thereby, three (W117C, W132 and W265) of the 4 strains (75%) originating from pigeons carried a \( \text{bla}_{\text{CMY-2}} \) gene, whereas one strain (W117E) harboured a \( \text{bla}_{\text{CTX-M-15}} \) gene. The 2 strains (W34 and W43) isolated from cormorants carried \( \text{bla}_{\text{CTX-M-15}} \) and \( \text{bla}_{\text{CTX-M-27}} \), respectively.

MLST typing identified all CMY-2-producers as belonging to genotype ST 457. The CTX-M-15 -producing strain isolated from a pigeon swab (W177E) exhibited a yet undefined MLST profile, due to a point mutation A to G in the gene \( \text{mdh88} \) at nucleotide 69 (numbering according to \( \text{www.mlst.ucc.ie} \)). Strains isolated from cormorants were characterized as belonging to ST 120 in the case of the CTX-M-15-producer, and to ST 131 in the case of the strain expressing CTX-M-27, categorizing the latter as a member of the world-wide pandemic multiresistant clone strongly associated with potentially severe infections in humans and animals (Rogers et al., 2011).

Phylogenetic grouping showed that all CMY-2-producers belonged to pathogenicity-associated extra-intestinal \( \text{E. coli} \) group D. The CTM-X-15-producing strain from a pigeon (W117E) was classified as belonging to group B2, defining it also as a potentially virulent extra-intestinal type, with heterogeneous distribution of virulence factors, as demonstrated by analysis of their virulence genes. Of the cormorant-originating strains, the CTM-X-15-positive isolate (W34) belonged to the commensal phylogenetic group B1, whereas the CTX-M-27-producing strain was assigned to group B2, a phylogenetic group in which CTX-M ESBLs are found rarely, except in conjunction with clonal group ST 131 (Brisse et al., 2012).
Our study is the first investigation of the occurrence of β-lactam-resistant *E. coli* in urban pigeons and wild cormorants in Switzerland. The data presented show that 1% of the sampled pigeon population hosts multidrug resistant, virulent extra-intestinal *E. coli* that produce either CTX-M-15 or the plasmid-encoded AmpC-β-lactamase CMY-2. Furthermore, our results indicate that a proportion of these avian hosts carry more than one distinct strain of multidrug-resistant β-lactamase-producing *E. coli*, thus increasing the risk of horizontal transmission of mobile antibiotic resistance genes among the host's faecal flora. This is particularly alarming, because urban pigeons live in close contact to humans and animals and are known to contribute, via faeces, to the spread of pathogens, including antibiotic-resistant bacteria (Silva et al., 2009). While pigeons are known to host multidrug resistant *E. coli*, and CTX-M-15-producing *E. coli* have been detected in pigeons in isolated cases (Guenther et al., 2010), a study performed recently by Radimerski and collaborators (Radimersky et al., 2010) declares, in contrast to our findings, the absence of ESBL-producing *E. coli* in a population of urban pigeons analysed in the city of Brno, Czech Republic. This illustrates the complexity of the epidemiology of ESBL-*E. coli* in the environment and suggests a geographical and temporal heterogeneity which needs to be monitored carefully. In this context, it is of particular interest that both feral pigeons and wild cormorants in Switzerland carry producers of CTX-M-15 - the ESBL type found most frequently (41%) among human carriers of ESBL producers in this country (Geser et al., 2012).

As a further example of complex β-lactam resistance distribution in urban birds, we report an OXA-1-producing *E. coli* isolated from a crow (*Corvus corone ssp. corone*), located in the city of Zurich (data not shown).

The detection of CTX-M ESBLs in cormorants, especially of CTX-M-27 in the potentially highly virulent and pandemic *E. coli* clone ST 131 is worrisome. Together with the very recent first report of a CTX-M-27-positive isolate in cormorants by Tausova and collaborators (Tausova et al., 2012), we provide evidence for the emergence of CTX-M-27-
producing, multidrug-resistant epidemiologically important *E. coli* ST131 in water birds in Europe. A further indication that clonal group ST 131 producing CTM-X-27 is emerging continuously is its recent detection in companion animals in Japan (Harada et al., 2012).

Our study provides further evidence that synanthropic as well as water-associated birds should be considered environmental reservoirs of ESBL-producing *E. coli*, constituting a potential threat to human and animal health. Further studies are necessary to gain insight to the factors that contribute to the dissemination of antibiotic resistant bacteria to and from wildlife. There is urgent need for surveillance of the prevalence of ESBL- and pAmpC-producing *E. coli* in humans, animals and the environment.

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