Letter to the editor: Higher generation cephalosporin-resistant Escherichia coli in feral birds in Switzerland

Zurfluh, Katrin ; Nüesch-Inderbinen, Magdalena ; Stephan, Roger ; Hächler, Herbert

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Short communication

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

K. Zurfluh, M. T. Nüesch-Inderbinen, R. Stephan , H. Hächler *

Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Zurich, Switzerland

*Corresponding author at: Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Winterthurerstrasse 272, 8057 Zurich, Switzerland. Tel.: +41 44 635 8651; fax: +41 44 635 8908; *E-mail address:haechlerh@fsafety.uzh.ch*

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 worlwide 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 $bla_{\text{CMY-2}}$. One cormorant was found to carry the pandemic *E.coli* ST 131 clone containing $bla_{\text{CTX-M-27}}$. 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

48

49 1. Introduction

50 One of the currently most important antibiotic resistance mechanisms in
51 *Enterobacteriaceae* is based on plasmid-mediated production of enzymes which inactivate β-
52 lactam-antibiotics including cephalosporins and monobactams by hydrolyzing their β-lactam
53 ring. These so-called extended-spectrum β-lactamases (ESBLs) have been detected in human
54 clinical isolates of *Enterobacteriaceae* since the early 1990s (Paterson and Bonomo, 2005)
55 originally as derivates of the TEM- and SHV-β-lactamase families, then increasingly as CTX-
56 M enzymes, or other less frequent ESBLs such as OXA- or PER-ESBLs (Bush and Jacoby,
57 2010; Coque et al., 2008). In addition, plasmid-mediated AmpC-type ß-lactamases (pAmpCs)
58 are increasingly reported world-wide, representing a new threat to successful antibiotic
59 therapy because they are not, like ESBLs, susceptible to ß-lactamase inhibitors such as
60 clavulanic acid or sulbactam, and they possess a wider spectrum of enzymatic activity
61 (Philippon et al., 2002).

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

66 Since the first description of ESBL-producing *Enterobacteriaceae* from hospitalized
67 humans, ESBL-producing *Escherichia coli* have been reported in numerous nosocomial and
68 later also community-associated infections worldwide (Paterson and Bonomo, 2005) and have
69 been detected in food-producing animals (Carattoli, 2008) and household pets (Ewers et al.,
70 2011). In addition, ESBL-producing *E. coli* appear to be spreading into the natural
71 environment, resulting in wildlife populations that act as reservoirs and disseminators of
72 ESBL-producing *Enterobacteriaceae* (Guenther et al., 2011). Several studies have described
73 antibiotic resistant *Enterobacteriaceae* in gulls (Bonnedahl et al., 2010; Poirel et al., 2012),

74 water birds (Tausova et al., 2012) and pigeons (Radimersky et al., 2010), suggesting that
75 birds play an important role in the epidemiology of resistance genes.
76 The aim of the present study was therefore (i) to assess the prevalence of resistance to
77 extended-spectrum ß-lactam antibiotics in *Enterobacteriaceae* harboured by a population of
78 urban pigeons (*Columba livia* forma *domestica*) in the city of Zurich, Switzerland as well as
79 by great cormorants (*Phalacrocorax carbo*) from in the banks of the Rhine river in the
80 northern district of the Canton of Zurich, Switzerland and (ii) to characterize such isolates by
81 antibiotic susceptibility testing, identification of the bla_{ESBL}/bla_{pAmpC} genes, multi-locus
82 sequence typing (MLST), determination of phylogenetic groups and detection of virulence
83 genes.
84

85 2. Material and methods

86

87 2.1 Bacterial isolates

88 Pigeon swabs were obtained from the pigeon management programme in the city of
89 Zurich. Between March 2012 and August 2012, a total of 298 postmortem cloacal swabs were
90 taken from culled pigeons by an authorized gamekeeper using a swab tube containing Amies
91 gel transport medium (Copan, Brescia, Italy). Tubes were numbered consecutively and sent to
92 the laboratory for analysis.
93 Cormorants were shot between December 2011 and January 2012, by gamekeepers
94 authorized by the Swiss Cormorant Action Plan, in collaboration with the Swiss Agency for
95 the Environment, Forests and Landscape and the Institute for Veterinary Bacteriology,
96 Vetsuisse Faculty, Zurich, Switzerland. Twelve pooled cloacal samples were obtained from a
97 total of 30 dissected cormorants.
98 Each sample was incubated for 24 hours at 37 °C in 10 ml of EE Broth (BD, Franklin
99 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 ß-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 $bla_{\text{CTX-M}}$ (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 $bla_{\text{CMY-2}}$ downstream flanking region: 5'ATGCGCATGGGATTTTCCTTGC3'. Resulting amplicons were purified using the PCR Purification Kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's

126 recommendations. Custom-sequencing was performed by Microsynth (Balgach, Switzerland)
127 and the nucleotide and protein sequences were analysed with Codon Code Aligner V. 3.7.1.1.
128 For database searches the BLASTN program of NCBI (http://www.ncbi.nlm.nih.gov/blast/)
129 was used.
130
131 **2.4 Multi-locus sequence typing of ESBL-producers**
132 Internal fragments of 7 housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and
133 *recA*) were sequenced (Wirth et al., 2006) and alleles and sequence types (ST) were assigned
134 in accordance with the *E. coli* MLST website (http://mlst.ucc.ie/).
135
136 **2.5 Determination of *E. coli* phylogenetic groups**
137 Phylogenetic analyses have shown that *E. coli* strains fall into four main groups (A,
138 B1, B2, and D), in which groups A and B1 typically contain commensal isolates, and isolates
139 of groups B2 and D are considered to be more likely to carry pathogenicity-associated genes
140 (Clermont et al., 2000). After DNA extraction using a standard heat lysis protocol, ESBL-
141 producing *E. coli* isolates were subjected to phylogenetic grouping by PCR as described
142 previously (Clermont et al., 2000).
143
144 **2.6 Virulence factors in ESBL-producers**
145 Strains were examined for the presence of genes for putative virulence factors
146 mediating adhesion (adhesion siderophore *iha*, long polar fimbriae *lpf*A, S fimbrial adhesin
147 *sfa*S, and temperature sensitive haemagglutinin *tsh*), increasing iron-uptake (e.g. siderophore
148 for iron *iro*N), and of genes encoding toxins (secreted autotransporter toxin *sat*, serine
149 protease *pic*, vacuolating toxin *vat*, and cytotoxic necrotizing factor *cnf*1). An ArrayTube-
150 based DNA microarray approach (Clondiag Chip Technologies, Jena, Germany) was used
151 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

178 2 pigeon and both cormorant strains.All isolates were resistant to tetracycline. One pigeon
179 isolate (W117E) and both cormorant strains were resistant to sulfamethoxazole and
180 trimethoprim. All strains tested fully susceptible to chloramphenicol.
181
182 The results of ESBL-and AmpC gene identification, MLST analysis, determination of
183 phylogenetic groups, and detection of putative virulence genes are summarized in Table 2.
184 Thereby, three (W117C, W132 and W265) of the 4 strains (75%) originating from pigeons
185 carried a *bla*$_{\text{CMY-2}}$ gene, whereas one strain (W117E) harboured a *bla*$_{\text{CTX-M-15}}$ gene. The 2
186 strains (W34 and W43) isolated from cormorants carried *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-27}}$,
187 respectively.
188 MLST typing identified all CMY-2-producers as belonging to genotype ST 457. The
189 CTX-M-15 -producing strain isolated from a pigeon swab (W177E) exhibited a yet undefined
190 MLST profile, due to a point mutation A to G in the gene *mdh*88 at nucleotide 69 (numbering
191 according to www.mlst.ucc.ie). Strains isolated from cormorants were characterized as
192 belonging to ST 120 in the case of the CTX-M-15-producer, and to ST 131 in the case of the
193 strain expressing CTX-M-27, categorizing the latter as a member of the world-wide pandemic
194 multiresistant clone strongly associated with potentially severe infections in humans and
195 animals (Rogers et al., 2011).
196 Phylogenetic grouping showed that all CMY-2-producers belonged to pathogenicity-
197 associated extra-intestinal *E. coli* group D. The CTM-X-15-producing strain from a pigeon
198 (W117E) was classified as belonging to group B2, defining it also as a potentially virulent
199 extra-intestinal type, with heterogeneous distribution of virulence factors, as demonstrated by
200 analysis of their virulence genes. Of the cormorant-originating strains, the CTM-X-15-positve
201 isolate (W34) belonged to the commensal phylogenetic group B1, whereas the CTX-M-27-
202 producing strain was assigned to group B2, a phylogenetic group in which CTX-M ESBLs are
203 found rarely, except in conjunction with clonal group ST 131 (Brisse et al., 2012).

Our study is the first investigation of the occurrence of ß-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-ß-lactamase CMY-2. Furthermore, our results indicate that a proportion of these avian hosts carry more than one distinct strain of multidrug-resistant ß-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 ß-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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