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Characterization, and comparison, of human clinical and black-headed gull (*Larus ridibundus*) extended-spectrum β-lactamase-producing bacterial isolates from Kalmar, on the southeast coast of Sweden

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Background: Antibiotic resistance is one of the great challenges for modern healthcare. In Gram-negative bacteria, CTX-M-type extended-spectrum β-lactamases (ESBLs) have been rapidly spreading through Europe since the early 2000s. In Sweden, ESBL-producing *Escherichia coli* are still rare, but a 3-fold increase has been seen from 2004 to 2007. Enterobacteria and normal flora of wild animals, with or without antibiotic resistance traits, constitute a potential source of human infection and colonization. We studied wild birds with the aim to understand the environmental dissemination of antibiotic resistance and, focusing on clinically relevant resistance types, we made comparisons with human clinical samples.

Methods: In this study, ESBL-producing human clinical isolates and isolates from juvenile black-headed gulls from Kalmar County hospital and the city of Kalmar, respectively, on the southeast coast of Sweden, were characterized and compared.

Results: Despite a low frequency of antibiotic resistance among the isolates from gulls, ESBL-producing *E. coli* isolates were found, two with $bla_{\text{CTX-M-14}}$ and one with $bla_{\text{CTX-M-15}}$. The same CTX-M types were dominant among human ESBL isolates. In addition, gull isolates were dispersed among the human samples in the PhenePlate™ clustering system, indicating that they neither differ from the human isolates nor form any separate clonal clustering.

Conclusions: The finding of CTX-M-type ESBLs in *E. coli* isolated from black-headed gulls in Sweden, where 'background resistance' is low, is consistent with an ongoing environmental spread of these plasmid-borne resistance genes. The results indicate that a potential for transfer between the human population and environment exists even in countries with a low level of antibiotic resistance.

Keywords: ESBL, CTX-M, clinical, environmental, wild birds

Introduction

Antibiotic resistance is becoming one of the big challenges to modern healthcare. It continues to spread and new resistance mechanisms are emerging. In Gram-negative bacteria, extended-spectrum β-lactamases (ESBLs) of the CTX-M type have been rapidly spreading through Europe since the early 2000s. We are now facing escalating problems with increasing numbers of ESBL-producing bacteria in healthcare-associated and community-acquired infections.

In Sweden, ESBL-producing *Escherichia coli* are still rare, but a 3-fold increase has been seen from 2004 to 2007. In 2007, the majority of the cefotaxime-resistant isolates (1.6%) were attributed to the presence of ESBLs of the CTX-M type.[1] The general resistance level in human *E. coli* isolates in Sweden is comparatively low and Kalmar County, in southeast Sweden, is no exception. Kalmar County Hospital has a clinical microbiology laboratory serving 234 000 inhabitants and the number of urine cultures positive for *E. coli* in Kalmar county in 2007 was 5136; out of these, only 27 (0.5%) produced ESBLs.

Gull populations, in general, have increased in numbers during recent decades. The population growth has forced the gulls to change both their feeding and breeding habits. Owing to their ability to adapt their feeding habits to environmental changes, some gull species have solved the population problem by moving into urban areas. Thus, they are found in close proximity to human activities where food is abundant, such as at refuse tips, sewage outlets and in newly sown fields.[2] Black-headed gulls (*Larus ridibundus*) are no exception and are a common domestic bird in Sweden, often seen together with feral pigeons in city squares etc.

The presence of enteropathogens in gulls has been known about for a long time. For example, *Salmonella* have been described in several of the >46 different gull species.[2] Gulls can also be carriers of ESBL-producing bacteria in their normal flora, as shown in our previous studies as well as in other recently published studies from Southern Europe.[3,4]

In this study, we screened a population of black-headed gulls breeding in the city of Kalmar for bacteria with reduced antibiotic susceptibility, with a focus on ESBL producers. ESBL-producing bacteria from gulls were compared with human clinical ESBL-producing isolates, collected during the same year and from the same geographical area.

Materials and methods

Bird samples

In June 2008 (first possible occasion after the entire human collection was made in 2007), we collected 100 faecal samples by cloacal swabs from juvenile non-fledged black-headed gulls (*L. ridibundus*). Sampling was performed in one colony of ∼500 nests, which was situated on a small island very close (∼50 m) to the shoreline in the city centre of Kalmar, southeast Sweden. The swabs were submerged in a bacterial storage medium [Luria broth (BD, Sparks, USA) in phosphate-buffered saline with the addition of 0.45% Na-citrate, 0.1% $MgSO_4$, 1% $(NH_4)_2SO_4$ and 4.4% glycerol]. After sampling in the field, the samples were transported to the laboratory and frozen at −70°C for later examination.

Isolation of *E. coli* for antibiotic susceptibility testing

Each sample was plated on Juhlin's 32 agar[5] for isolation of putative *E. coli* isolates. *E. coli* species identity was confirmed by biochemical testing. The susceptibility of one isolate (selected at random) per sample (in total, 83 isolates from 100 samples) was tested against a set of antibiotic agents, including tetracycline, ampicillin, streptomycin, chloramphenicol, nalidixic acid, cefadroxil, sulfamethoxazole, fosfomycin, tigecycline, trimethoprim, nitrofurantoin and mecillinam. These antibiotics were selected to represent commonly used agents against *E. coli* infections in human and veterinary medicine. Resistance was determined by antibiotic disc diffusion on Iso-Sensitest agar (Oxoid, Basingstoke, UK) in accordance with the recommendations of the Swedish Reference Group for Antibiotics,[6] using *E. coli* ATCC 25922 as a reference strain (as in all assessments).

Isolation of ESBL-producing bacteria from gulls

All faecal samples were also enriched in brain heart infusion broth (Becton Dickinson, Franklin Lakes, NJ, USA), supplemented with 16 mg/L vancomycin (ICN Biomedicals Inc., Aurora, OH, USA), for 18 h at 37°C. Samples were subsequently inoculated and cultured overnight at 37°C on chromID™ ESBL plates (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. Colonies were isolated and species identity was confirmed by biochemical testing. ESBL production was confirmed with the cefpodoxime/cefpodoxime+clavulanic acid double-disc test (MAST Diagnostics, Bootle, UK) and the Etest for cefotaxime, cefepime and ceftazidime, before genetic characterization (below).

Human clinical ESBL samples

Human samples comprised all ESBL-positive specimens isolated at the Kalmar County Hospital (clinical microbiology laboratory) during 2007. Isolates were tested for ESBL production and species identified previously by the laboratory, but were regarded as unknown and went through a similar procedure as avian samples. Thus, after receiving samples, they were plated on chromID™ ESBL plates (bioMérieux) and species identity was confirmed. A total of 27 isolates from first-time samplings were included.

Antibiotic susceptibility testing of ESBL-producing human and gull isolates

Antibiotic susceptibility was tested against the same antibiotics as previously mentioned for randomly selected *E. coli*. Newly adapted EUCAST methodology, similar to CLSI recommendations, using Mueller–Hinton agar was used.[7]

Genetic determination of ESBL variants in human and avian samples

The presence of the $bla_{CTX\text{-}M}$ genotype was detected using a previously described multiplex real-time PCR protocol,[8] displaying the group designation (CTX-M-1/-2/-8/-25 and -9) of $bla_{CTX\text{-}M}$-positive isolates. Positive isolates were sequenced using specific primers, as described previously (for CTX-M-1 and CTX-M-9),[9,10] using the GenScript BacReady Multiplex PCR system (GenScript Corporation, Piscataway, NJ, USA). Sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany). The presence of bla_{TEM} and bla_{SHV} was detected using previously described primers[11] and a SYBR® Green-based real-time PCR protocol as follows: a reaction tube of 25 μL was set up containing 1 μL of DNA extract solution, 0.05 μM (for bla_{TEM} detection) or 0–15 μM (for bla_{SHV} detection) each primer (forward and reverse), and 12.5 μL of SYBR® Green PCR Master Mix 2× (Applied Biosystems, Warrington, UK). Cycling conditions were (i) a 10 min incubation period at 95°C followed by (ii) 40 cycles of PCR (each cycle consisted of 30 s at 95°C, 40 s at 61°C (TEM) or 55°C (SHV) and 1 min at 72°C, with a fluorescence reading for the green channel at the end of the annealing and elongation stage) followed by (iii) a final elongation for 7 min at 72°C and (iv) a melt from 55°C to 99°C, with a 5 s hold on each step. Real-time data were analysed with Rotor-Gene software (version 6.0), and melt data analysis showed a peak at ∼84.7°C (TEM) and 91.0°C (SHV), respectively, for positive isolates. Control strains were *E. coli* ATCC 25922 (SHV- and TEM-negative), *Klebsiella pneumoniae* ATCC 700603 (SHV-positive, TEM-negative) and *E. coli* UKNE7630 (TEM-positive, SHV-negative). The specificity of the real-time PCR protocol was confirmed by using the previously described end-point PCR protocol and gel electrophoresis, which showed expected bands of 971 bp (TEM) and 885 bp (SHV) for positive samples.[11]

E. coli phylogenetic groups and PhenePlate™ system clustering

Phylogenetic groups were determined using the triplex PCR method developed by Clermont *et al.* and Gordon *et al.*,[12,13] but with modified

PCR conditions,[14] assigning isolates to phylogenetic groups A, A0, B1, B2 or D.

PhenePlate™ system (PhPlate Microplate Techniques AB, Stockholm, Sweden) clustering was performed according to product instructions for the *E. coli* system with 11 biochemical reactions (cellobiose, lactose, rhamnose, deoxyribose, sucrose, sorbose, tagatose, D-arabitol, raffinose, gal-lactone and ornithine) and as previously described.[15] From human isolates only *E. coli* were included. In short, a few colonies were suspended in PhP-suspension media, and then inoculated in the PhP-RE 96-well PhenePlates containing dehydrated reagents, and incubated at 37°C for 8, 24 and 48 h. Colour reactions were scored by use of a flatbed scanner (HP Scanjet 7400c) and analysis with PhenePlate™ system software (PhPWIN v4.24). The unweighted-pair group method using average linkages (UPGMA) was used and isolates with a similarity index of >0.975 were considered to be of the same PhP-subtype.

PCR-based replicon typing

Plasmid replicons were determined for the avian and a subset of the human ESBL-producing isolates using the PCR-based replicon typing described by Carattoli *et al.*[16] It detects the replication controls of plasmids, which differ between different plasmid types and directly relate to the compatibility groups of plasmids, i.e. which plasmid types can occur simultaneously in a bacterial cell. The method covers the major compatibility groups circulating in the Enterobacteriaceae family and, hence, most plasmid types will be detected.[16] As positive controls, PCR-positive strains with sequenced PCR products from the Department of Medical Microbiology, Uppsala University Hospital, were used.

Multilocus sequence typing (MLST)

The MLST scheme described by Wirth *et al*. is available online and was applied for analysing seven gene fragments, as previously described.[3,17,18] The MLST allele designations were determined via the online MLST database and novel sequence type (ST) designations were provided by the curator of the database. The MLST results of bird isolates were compared with the data fetched from the public database. For concatenated nucleotide datasets of the seven genes, the number of pair-wise differences among strains was determined using the Pearson coefficient for constructing a UPGMA tree with 47 strains of the ECOR collection as reference.[19]

Results

Antibiotic susceptibility of randomly selected E. coli from black-headed gulls

In order to determine a general picture of the antibiotic susceptibility of *E. coli* from black-headed gulls, we tested one *E. coli* isolate per sample against a panel of 12 antibiotics. Resistance levels were low and the majority (86.7%) of the isolates were fully susceptible to all tested agents. Of the 13.3% *E. coli* isolates with reduced susceptibility to at least one antibiotic, approximately half (7.2%) displayed resistance to two or more agents and one isolate displayed resistance to as many as four agents. The most common phenotypes were those with reduced susceptibility to tetracycline (five isolates) and ampicillin (five isolates), but additional resistance phenotypes were also found to trimethoprim ($n=2$), streptomycin ($n=2$), chloramphenicol ($n=1$), nitrofurantoin ($n=1$), cefadroxil ($n=1$) and fosfomycin ($n=1$). None of the isolates displayed reduced susceptibility to nalidixic acid (data not shown).

Identification of ESBL-producing bacteria and their replicon types

ESBL-producing bacteria from gulls were initially identified by growth on chromID™ ESBL plates. Three isolates (all *E. coli*) were positive in the disc diffusion synergy test and had a cefepime MIC concordant with ESBL production. All the human clinically isolated ESBL samples grew on chromID™ ESBL plates and were further confirmed to harbour ESBLs (23 *E. coli*, 3 *K. pneumoniae* and 1 *Providencia stuartii*). Two gull isolates were confirmed to harbour $bla_{\text{CTX-M-14}}$ and one isolate $bla_{\text{CTX-M-15}}$ (Table 1). In gulls, bla_{TEM} was found in combination with $bla_{\text{CTX-M-15}}$, but none harboured bla_{SHV}, regardless of other genotypes. Two of the isolates were positive for plasmids belonging to the IncF incompatibility group. In one of the isolates harbouring the $bla_{\text{CTX-M-14}}$ gene, FIB replicons were detected, whereas FIB and FIC replicons were found in the isolate carrying $bla_{\text{CTX-M-15}}$ and bla_{TEM}. The location of resistance genes on specific plasmid types was not investigated.

Human samples harboured mainly the $bla_{\text{CTX-M-15}}$ gene (17 isolates), but also $bla_{\text{CTX-M-14}}$ (5 isolates; one in combination with $bla_{\text{CTX-M-15}}$), $bla_{\text{CTX-M-14b}}$ (2 isolates; one in combination with $bla_{\text{CTX-M-15}}$) and $bla_{\text{CTX-M-65}}$ (1 isolate; a variant of $bla_{\text{CTX-M-14}}$). Thirteen isolates harboured bla_{TEM} and six bla_{SHV}. Several human isolates displayed combinations of the different *bla* genes (Table 1). Isolates harbouring any of the *bla* genes also exhibited ESBL-positive phenotypes. In human ESBL-positive isolates, plasmids belonging to the IncF incompatibility group were also the most common. Two $bla_{\text{CTX-M-15}}$-harbouring isolates carried FIB replicons only, while other isolates carried combinations of FIB with FIA or I1. In one $bla_{\text{CTX-M-15}}$-harbouring isolate, none of the replicons included in the method was detected.

E. coli phylogenetic groups, PhenePlate™ system clustering and MLST analysis

Phylogenetic group assessment, according to the triplex PCR method, assigned all three gull samples to the D group. Most human isolates belonged to the B2 group (14 isolates; 51.9%), followed by similar numbers of groups D and A (6 isolates; 22.2% and 7 isolates; 25.9%, respectively).

The PhenePlate™ system was used to determine diversity in the human and gull *E. coli* samples, and to detect any possible clonal relationship between the isolates. Gull isolates were dispersed among the human samples in the PhenePlate™ clustering, indicating that they are similar to the human isolates and do not form any separate clusters (Figure 1). Consequently, *E. coli* isolates 88:1 and 88:2, originating from the same bird sample, are indeed different despite displaying the same *bla* genotype. Further analysis of the three gull samples using MLST confirmed that these isolates belong to phylogroup D, as determined from phylogenetic analysis with ECOR strains using nucleotide sequences of the seven concatenated genes (data not shown). The three samples were assigned novel STs, named ST1340 (isolate 98), ST1646 (88:1) and ST1647 (88:2).

Antibiotic susceptibility of ESBL-producing gull and human isolates

Human ESBL isolates displayed a relatively high degree of resistance to a wide spectrum of antibiotics (e.g. 74.1% tetracycline, 70.4%

Table 1. Genotypic analysis of phenotypically positive ESBL-producing bacteria

Isolate[a]	Species	*bla* genotype			Phylogenetic group[d]	PhP type[e]	Replicon
		CTX-M[b]	TEM[c]	SHV[c]			
Human clinical isolates							
1	*Escherichia coli*	65			D	Si	FIB, I1
2		14			D	Si	*nt*
3		15			B2	Si	FIB
4		14			D	Si (CT1)[f]	*nt*
5		14	+		D	Si	FIB
6		15			B2	CT2	*nt*
7		14b	+		D	Si	FIA, FIB
8		15	+		B2	CT3	*nt*
9		15	+		B2	Si	*nt*
10		1			A	Si	*nt*
11		15			A	CT5	FIB
12				+	A	CT5	*nt*
13		15	+		B2	Si	FIB, I1
14		15	+		B2	CT6	*nt*
15			+	+	B2	Si	*nt*
16				+	B2	Si	*nt*
17		15			B2	CT2	*nt*
18		15	+		B2	CT6	*nt*
19		15/14	+		B2	Si	FIB, I1
20		15			D	CT3	—
21		15	+		B2	Si	*nt*
22		15	+		B2	CT6	*nt*
23		15			B2	Si	*nt*
24	*Klebsiella pneumoniae*	15	+	+	A	NA	*nt*
25		15	+	+	A	NA	*nt*
26		15/14b		+	A	NA	*nt*
27	*Providencia stuartii*				A	NA	*nt*
Bird isolates							
88:1	*Escherichia coli*	14			D	Si	FIB
88:2		14			D	Si	—
98		15	+		D	Si	FIB, FIC

NA, not applicable.
[a]Isolates indicated with laboratory identification numbers.
[b]$bla_{CTX\text{-}M}$-positive isolates were sequenced for the specific CTX-M genotype.
[c]+ indicates the presence of bla_{TEM} and bla_{SHV} genes.
[d]Phylogenetic group was determined by triplex PCR.
[e]PhenePlate™ type. 'Si' indicates no clustering above the identity level of 0.975. 'CT' indicates clustering above the identity level with one or several other isolates tested.
[f]Human isolate 4 clustered with *E. coli* reference strain ATCC 25922.

nalidixic acid, 66.7% sulfamethoxazole, 51.6% trimethoprim, 48.1% streptomycin, 25.9% chloramphenicol, 11.1% nitrofurantoin and 7.4% fosfomycin). Among gull ESBL isolates, CTX-M-14-producing strains displayed very low additional resistance (one had resistance to nalidixic acid and the other showed only resistance explained by the CTX-M genotype). The CTX-M-15-harbouring gull isolate had a similar resistance phenotype as human isolates (displaying resistance to nalidixic acid, streptomycin, tetracycline, sulfamethoxazole and trimethoprim).

None of the ESBL-producing isolates from humans or gulls displayed resistance to tigecycline.

Discussion

A comparison of the antibiotic susceptibility patterns of randomly selected gull *E. coli* with Swedish clinical isolates shows a difference in antibiotic resistance levels. For example, only 3% of the randomly selected *E. coli* isolates from gulls displayed reduced susceptibility to ampicillin and none was resistant to nalidixic acid; in comparison, the resistance level in Swedish human clinical isolates was 27% and 12%, respectively.[1] In our recent study of yellow-legged gulls in Southern France,[3] the general resistance levels (of randomly selected *E. coli* isolates)

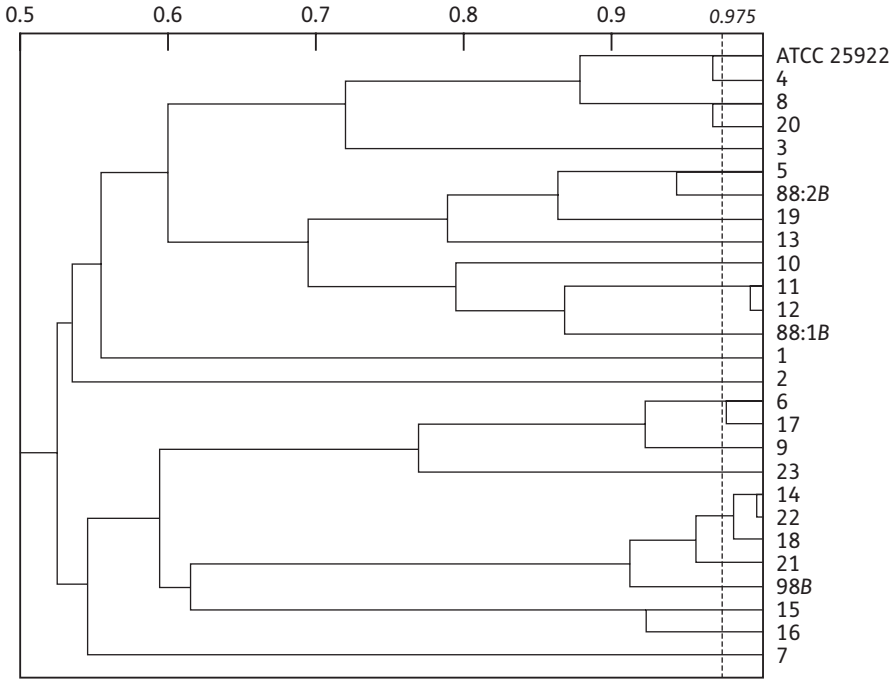

Figure 1. Dendrogram of UPGMA-clustered PhenePlate™ data for both human and gull *E. coli* samples isolated in Kalmar. The dotted line indicates the identity level of 0.975. Bird samples are denoted *B*.

were significantly higher than in the black-headed gulls in this study, probably reflecting a long-term difference in antibiotic pressure between Sweden and France.

Despite European dissemination beginning in the early 1990s, in Sweden the occurrence of ESBL-producing *E. coli* is a very recently emerged phenomenon. In 2007, they only contributed to 1.6% of invasive (obtained by blood culture) *E. coli* isolates in humans.[1] From this perspective, the finding of three different ESBL-producing *E. coli* in 2% of the gulls might seem surprising. However, as we discussed in our previous study of yellow-legged gulls,[3] and as found by others,[9] this illustrates the rapid dispersal potential of CTX-M genes in the environment. Similarly, the CTX-M genotypes (CTX-M-14 and CTX-M-15) were the same as those found among human isolates in the same region. Replicons found in both human and gull isolates were mainly of the IncF incompatibility group, a finding consistent with previous studies of CTX-M-14/-15-harbouring clinical human isolates and their replicon types.[20]

PhenePlate™ phenotyping did not in any way separate the gull *E. coli* isolates from the human *E. coli* isolates. Although this is a phenotypic clustering method, it indicates, similarly to the MLST performed on the isolates from yellow-legged gulls in France, that there is no host-species boundary for *E. coli*. This study showed that the bird isolate 88:1 with CTX-M-14 belongs to ST1646, a novel ST that is closely related to ST648, previously reported with ESBL, and isolated both from humans and poultry.[21,22] Our ST1646 differs at only one out of seven gene sequences (*recA*) from ST648, indicating a close genetic relationship between these STs. Interestingly, *E. coli* of ST648 is also present in urinary tract infections in humans living in southeastern Sweden (our own unpublished data). Assuming a close genetic relationship indicates the recent evolutionary origin of these STs, this finding is consistent with *E. coli* frequently crossing species barriers. Consequently, it may disseminate in a true zoonotic manner between humans and wildlife/the environment, and transfer resistance traits. Further studies on the population structure of *E. coli* harbouring CTX-M in humans and gulls will further clarify the dissemination of CTX-M in environmental settings.

Black-headed gulls are common in most parts of Sweden and often occur in close contact with humans, both on farms and in cities. The finding of CTX-M-type ESBLs in a species like the black-headed gull in Sweden, a country with low 'background resistance', indicates a rapid environmental spread of these resistance genes. It also indicates that chances for the back and forth transfer of antibiotic resistance genes between humans and the environment exist even in countries with a low level of antibiotic resistance. The dissemination of resistance into the environment is complex and most likely includes both clonal dissemination of successful pathogenic clones as well as horizontal gene transfer.[23]

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Transparency declarations

None to declare.

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