Journal of Global Antimicrobial Resistance 35 (2023) 257–261

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Carbapenem-resistant *Escherichia coli* in Black-headed gulls, the Danube, and human clinical samples: A One Health comparison of contemporary isolates

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ARTICLE INFO

Article history:
Received 23 July 2023
Revised 10 September 2023
Accepted 2 October 2023
Available online 11 October 2023

Editor: Dr Jon Hobman

Keywords:
Antibiotic resistance
ST410
Urbanised birds
Human-animal interaction
blaNDM-1
carbapenemase

ABSTRACT

Objectives: Our aim was to characterize and compare contemporary carbapenem-resistant Enterobacterales (CRE) isolates from gulls, the River Danube, and humans in Hungary, Budapest.
Methods: Multiresistant Enterobacterales were sought for in 227 gull faecal and 24 Danube water samples from 2019 to 2020. Eosin-methylene blue agar containing 2 mg/L cefotaxime and Colilert-test containing 10 mg/L cefotaxime were used for gull and water samples, respectively. Isolates were characterized by polymerase chain reactions (PCRs); acquired carbapenemase producers were further analysed by whole-genome sequencing, together with 21 Hungarian human CR *Escherichia coli* (CREc) isolates.
Results: Gull and water samples exhibited a CRE prevalence of 7.4% (9/122) and 6.7% (7/105), none and 5/12 water samples yielded CRE from 2019 and 2020, respectively; CRE were found only in samples taken downstream of Budapest. The dominant species was *Escherichia coli* and the most prevalent carbapenemase was *blaNDM-1*. High-risk CREc clones were found both in gulls (ST224, ST372, ST744) and the Danube (ST10, ST354, ST410); the closest associations were between ST410 from humans and the Danube, among ST1437 among gulls, and between ST1437 in gulls and the Danube (46, 0, and 22–24 allelic distances, respectively). Direct links between human and gull isolates were not demonstrated.
Conclusion: The study demonstrates potential epidemiological links among humans, a river crossing a city, and urbanised birds, suggesting a local transmission network. Water bodies receiving influent wastewater, together with animals using such habitats, may serve as a local reservoir system for CRE, highlighting the importance of One Health in CRE transmission, even in a country with a low CRE prevalence in humans.

1. Introduction

The emergence and spread of carbapenem-resistant Enterobacterales (CRE) is of great concern to public health. Though CRE are mostly associated with nosocomial infections, growing evidence indicates that CRE have spread outside of hospitals [1]. Hospitals and wastewater treatment facilities release high amounts of anthropogenic resistant bacteria into surface waters [2], which can persist there and colonize wild animals creating secondary reservoirs [1,3]. Landfills are another anthropogenic factor in the transmission of resistance into wildlife, especially to those foraging on urban waste, such as omnivorous gulls and rooks [3,4]. These birds are highly mobile and wander in high numbers, which makes them perfect vectors of antibiotic-resistant bacteria over long distances

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https://doi.org/10.1016/j.jgar.2023.10.002

[3,5], exemplifying the importance of the One Health concept in the dissemination of antibiotic resistance. One such possible vector species can be the Black-headed gull (*Chroicocephalus ridibundus*). This species frequently visits landfills and lives near rivers, such as the Danube, and during winter, they flock in large numbers at the docks of Budapest. The aim of this study was to characterize and compare CRE isolates from gulls, the Danube River, and humans, as few studies have investigated wildlife, their habitats, and local epidemiology in humans as a complex system.

2. Materials and methods

Between January and March of 2019 and 2020, 122 and 105 faecal samples were collected from Black-headed gulls captured for ringing at the docks of Budapest, Hungary in order to research multiresistant Gram-negative bacteria. Isolates were recovered using eosin-methylene blue media containing 2 mg/L cefotaxime. In parallel, a total of 24 water samples were taken from the Danube River; 12 to 12 water samples were collected in 2019 and 2020, six-six upstream and six-six downstream of Budapest. Water samples were processed using Colilert-18/Quanti-Tray (IDEXX Laboratories, Westbrook, ME) supplemented with 10 mg/L ceftriaxone. Each positive well was opened and 30 µL was plated onto eosin-methylene blue media. Each different morphology was processed further and identified by MALDI-TOF-MS (Bruker, Bremen, Germany). Susceptibility testing was performed by disk diffusion method to ertapenem, meropenem, imipenem, cefotaxime, ceftazidime, cefepime, amoxicillin-clavulanic acid, ciprofloxacin, amikacin, gentamicin, tobramycin, fosfomycin, and trimethoprim-sulfamethoxazole according to European Committee on Antimicrobial Susceptibility Testing guidelines; MIC Test Strip (Liofilchem, Italy) was used for ertapenem, imipenem, and meropenem. Colistin susceptibility was determined by broth microdilution (MERLIN Diagnostika GmbH, Germany). Carbapenem non-susceptible isolates were further investigated by MASTDISCS Combi Carba test (Mast Group Ltd, United Kingdom) and multiplex polymerase chain reactions (PCRs) [6]; acquired carbapenemase-producers were selected for characterization by whole-genome sequencing (WGS). In addition, 21 human-derived CR *Escherichia coli* (CREc) sent to or collected at the National Public Health Centre between 2013 and 2020 were included in this study and sequenced.

Total DNA isolation and library preparation was performed as described previously [4] and WGS was carried out in Illumina MiSeq or NextSeq instruments. Raw reads generated in this study are available under BioProject ID PRJNA807502. Species identification was confirmed by KmerFinder v3.2 [7]. Core genome multilocus sequence typing (cgMLST) and resistance gene, point mutation (ResFinder v4.1), and replicon identification (PlasmidFinder v2.1) were performed as described elsewhere [4]. Based on this cgMLST scheme, we considered isolates closely related if <10 allelic distances were found. The identity of contigs harbouring a carbapenemase gene was confirmed by running Nucleotide Basic Local Alignment Search Tool [8] at the NCBI webserver (https://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed: 14.03.2022.). The contigs containing carbapenemase genes were annotated using Prokka [9] and visualized using clinker [10] to identify the immediate genetic context of the carbapenemase genes.

3. Results

3.1. Prevalence and characteristics of CRE carried by gulls in 2019 and 2020

The overall prevalence of CRE carriage in 2019 was 7.4% (9/122); six *E. coli, E. fergusonii*, and two *Enterobacter cloacae* complex (ECC) were recovered; in 2020, of 105 samples, seven (6.7%) were positive for CRE yielding eight *E. coli*, one *Klebsiella pneumoniae*, one *Citrobacter braakii*, and one ECC; two birds carried multiple CRE isolates (Supplementary Table S1). All *Escherichia* isolates were metallo-beta-lactamase (MBL)-producers except for one *E. coli*, which carried *blaOXA-181*. The most prevalent gene was *blaNDM-1* (14/15), followed by *blaVIM-4* (5/15), which always occurred together with *blaNDM-1*. Except for the OXA-181-producer, *Escherichia* isolates were co-resistant to all tested beta-lactams, fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole (Supplementary Table S2). Four *E. coli* were also resistant to fosfomycin and carried the *fosA3* or *fosL1* gene. One isolate had an amino acid substitution in the *pmrB* region (p.V161G) with the minimum inhibitory concentration (MIC) value for colistin at 2 mg/L. The *E. coli* isolates belonged to nine sequence types (STs): ST224 (2), ST226, ST372, ST744 (2), ST1437 (4), ST2772, ST8890, ST13079, and a novel ST. (Supplementary Table S1, Fig. 1). The ST1437 isolates recovered in 2019 shared the same resistome pattern and no allelic differences were found in their cgMLST profiles, while those collected in 2020 had similar resistance patterns with 22–24 allelic distances between them. ST744 isolates were not related based on their resistance and cgMLST profiles (allelic distance of 124). ST224 strains were recovered from 2019 and 2020 with similar resistance patterns and 24 allelic distances between them (Fig. 1). Two ECC isolates were non-carbapenemase producers; the other belonged to ST1001 (*Enterobacter kobei*), carried *blaVIM-1*, and was resistant to colistin (MIC >64 mg/L), but we did not find any resistance gene (*mcr-1–10*) or known *PmrAB/PhoPQ* mutation associated with this phenotype. The *K. pneumoniae* was assigned to ST273 and was resistant to fosfomycin, harbouring *fosA5*. The *C. braakii* belonged to a new ST; it carried *mcr-9* but the MIC value for colistin was 1 mg/L.

3.2. Prevalence and characteristics of CRE in the Danube River

All water samples obtained in 2019, as well as samples taken upstream of Budapest in 2020, were negative for CRE; five of six downstream samples in 2020 were positive. Two samples from the bank of the Danube contained an ECC and an *E. coli*, while six *E. coli* were found in three stream-channel specimens. The last downstream sample taken furthest from Budapest contained four different CREc (Supplementary Table S1). The ECC was porin deficient. All *E. coli* harboured MBL-type carbapenemases; three *blaNDM-1*, three *blaNDM-1*+*blaVIM-4*, and one *blaNDM-5* carriers were found (Supplementary Table S1). All isolates were also resistant to tested beta-lactams, fluoroquinolones, and aminoglycosides (Supplementary Table S2). Two isolates were resistant to fosfomycin and harboured *fosA3* or *fosA4*. Each isolate belonged to a different ST (ST10, ST354, ST410, ST624, ST746, ST1303, ST1437) (Supplementary Table S1, Fig. 1).

3.3. Characteristics of CREc isolates from humans

MBL-producers were predominant and the following carbapenemases were found: *blaNDM-1* (5/21), *blaVIM-4* (5/21), *blaOXA-48* (4/21), *blaNDM-5* (3/21), *blaKPC-3* (2/21), *blaOXA-244* (2/21), *blaNDM-7* (1/21), and *blaOXA-181* (1/21). Two isolates co-harboured *blaVIM-4* and *blaOXA-48*. Co-resistances are shown in Supplementary Table S2. One isolate carried *mcr-9*, yet the MIC of colistin was 0.125 mg/L. The isolates belonged to the following STs: ST38 (2), ST69 (2), ST73 (2), ST88, ST131 (2), ST405 (5), ST410 (3), ST448, ST617, ST648, and ST1011. Isolates of the same STs (ST38, ST69, ST73, ST405) were highly uniform and related (allelic distances of 15, 8, 1, and 1, respectively; Supplementary Table S1, Fig. 1), except for ST131 isolates (allelic distance of 122). The *blaNDM-5*-harbouring ST410 strains shared the same resistome and the allelic distances between them were 0 (Supplementary Table S1, Fig. 1).

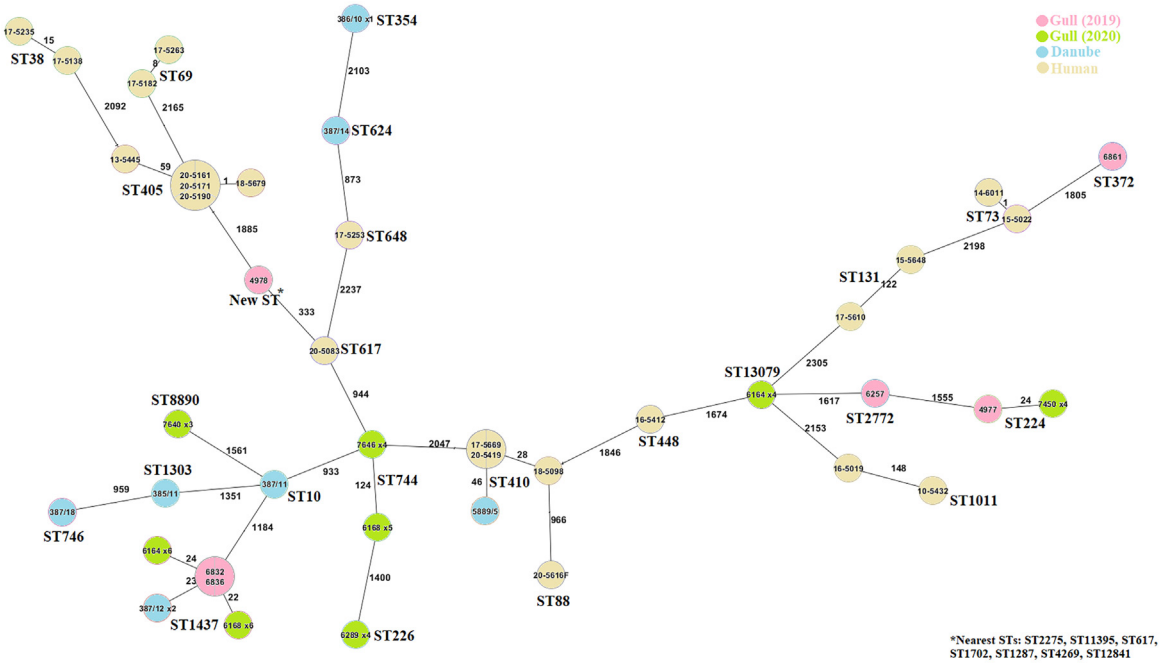

Fig. 1. Minimum spanning tree based on cgMLST allelic profiles of the whole-genome sequenced *Escherichia coli* isolates. Each circle represents an allelic profile based on sequence analysis of 2513 cgMLST target genes. The numbers on the connecting lines illustrate the numbers of target genes with different alleles.

3.4. Comparing the CREc isolates from gulls, the Danube River, and humans

The most prevalent gene in all isolate collections was *blaNDM-1; blaVIM-4* occurred only with *blaNDM-1* in gull and water isolates, while it was co-harboured with *blaOXA-48* or found alone in human isolates. All *blaKPC* and *blaOXA-48*-like (excepting one gull isolate) genes were found in human isolates. Fosfomycin-resistant isolates were present only in river and gull isolates, *fosA3* being the most frequent resistance gene. The 42 *E. coli* isolates belonged to 25 different STs; in general, different STs were found in human, river, and gull isolates. Nevertheless, STs shared between sample types were also found. ST1437 was present in a Danube sample and in gulls from both periods; gull isolates collected in 2019 were highly similar (allelic distance of 0), while those collected in 2020 and the river isolate differed by 22 to 24 alleles with minor differences in their resistomes (Fig. 1, Supplementary Table S1). NDM-5-producing ST410 isolates were recovered from humans and the Danube; the river isolate showed an allelic distance of 46 compared with the human isolates and similar resistance patterns. *blaNDM* genes were located within the same immediate genetic context (dsbD-trpF-bleMBL-bla*NDM1/5/7* or bla*NDM1*-IS*Aba125*-aph(3')-VIa) (Supplementary Table S3). *blaKPC-3, blaVIM*-like, and *blaOXA-48*-like genes were found on shorter (~1500–2100 bp) contigs; therefore, the genetic content of these genes could not be mapped. Consistent co-carriage of resistance genes found on the same contig was demonstrated in the case of *blaNDM-1* and *aph(3')-VI* in river and gull isolates, *blaOXA-181* and *qnrS1* (on a IncX3 replicon) in human and gull isolates, and blaVIM-4 and *aac(6')-Ib-cr* in all *blaVIM-4* carriers.

In most countries, carbapenems are restricted only to human clinical use; however, carbapenem residuals and resistant strains may remain in treated hospital wastewater released into surface waters, representing an important selection pressure for the persistence and dissemination of CRE in the environment [11]. Carbapenem-resistant Enterobacterales were described from wastewater treatment plants, rivers, streams, and lakes in European countries [11,12]. Carbapenem-resistant Enterobacterales were reported from the Danube in Serbia and Romania in 2013 (but not in Hungary), and later in Austria in 2016, though the species distribution (*K. pneumoniae* and ECC) and carbapenemase genes (*blaKPC-2, blaVIM-4*, and *blaOXA-48*) differed from our results [12,13]. In previous European studies, mainly *blaKPC-2, blaVIM-1*, and/or *blaOXA-48*-like genes were found; *blaNDM-1* was rare, while *blaVIM-4* and its combinations, prevalent in our study, were not reported to our knowledge [11,12]. Interestingly, in this work, the dominant carbapenemase genes in water were *blaNDM-1* and *blaVIM-4*, frequently in the same isolate, which may be explained by the dominance of these genes in CREs of human origin in Hungary [14]. Of note, simultaneous carriage of *blaNDM-1* and *blaVIM-4* was never found in human isolates. However, some OXA-48-like-producing strains could have been lost because of the methodology.

The prevalence of CRE found in Black-headed gulls in this work was similar to that found in other European studies (1.5%–16%) conducted on different gull species in countries with higher CRE rates in humans [15–18]. Although the prevalence of CRE found in gulls and the Danube River seems to be low, because the incidence of CRE (especially of *E. coli* in humans) is still rare in Hungary [14], this prevalence is surprising. Gene distribution in the gull samples reported here was similar to that found in the Danube River; likewise, in other studies, gene distributions in gulls were similar to those reported from their respective water bodies [2,16,19]. It was observed that human, water, and gull samples from certain geographical regions show similarities in gene distribution points towards a regional/local gene circulation between One Health compartments. This is supported by shared co-carriage of carbapenemase and other resistance genes.

Important high-risk *E. coli* clones were found in the Danube River; *E. coli* ST10 and ST354 are pandemic multiresistant lineages causing severe infections in humans [20] and appearing as colonizers or pathogens in various production [21] and companion [2] animals. ST410 is a major human-associated high-risk pandemic extraintestinal pathogenic *E. coli* (ExpEc) clone associated mainly with *blaCTX-M-15, blaOXA-181*, and *blaNDM-5* production and is increasingly reported worldwide [22].

Similarly, STs of human or veterinary importance were found in gulls. *Escherichia coli* ST224 and ST744 are high-risk ExpEc clones causing various diseases in humans and animals and are associated with production of extended spectrum beta-lactamases and/or carbapenemases [2,4]. ST372 is an international ExPEc clone causing mainly urinary tract infection in dogs [23], but it is also associated with human infection [24]. As dog faeces is a common contaminant of the urban environment and gulls consume faecal material, presence of ST372 in gulls suggests a One Health link between wild and companion animals as well as humans.

ST1437 isolates shared by the Danube River and gulls in different years were related, suggesting that *E. coli* ST1437 is maintained in the gull population or is a frequent and recent acquisition from a common source visited by gulls. ST1437 *E. coli* is a rarely reported commensal strain, probably of porcine origin, associated with *fosA3, blaCTX-M*, and *blaNDM* [25–27]. Its presence in gulls may derive from a source of porcine origin (e.g., arable land fertilised by manure), and its carbapenemase production may represent a novel threat.

The only *K. pneumoniae* isolate, which carried blaNDM-1, belonged to ST273, which is a single locus variant of ST147 associated with *blaCTX-M-15* in Hungary, Italy, Spain, and Tunisia; it has also been reported as a carbapenemase producer [27]. This ST147-ST273 complex is regarded as a strain with high epidemic potential [28,29]. The presence of CRECC in gulls, and that some were co-resistant to colistin and fosfomycin, is worrisome, as these are important globally emerging nosocomial pathogens in humans [30].

Close similarity of carbapenemases and their combinations between human, gull, and water isolates with a diversity of carrier STs indicates that epidemiology of CRE in this setting is shaped predominantly by horizontal gene transfer (HGT). This presumption is supported by the close similarity of the genetic environment of *blaVIM-4* from various sources as well as of *blaNDM-1* from water and gull samples. Another example is *blaOXA-181*, which is usually associated with IncX3 plasmids. Our contigs containing both *blaOXA-181* and the IncX3 replicon were highly similar to corresponding regions of IncX3 plasmids harbouring *blaOXA-181* found in different *E. coli* STs in river, canine, and human samples in Switzerland [31]. Furthermore, *blaOXA-181* carried on IncX3 plasmids was previously found dominant in gulls in the coastline of Portugal [15].

Aquatic environments are excellent sites for HGT [11]; resistance genes may accumulate in the sediment [12] and wild animals in proximity to water bodies can acquire those resistant strains from hospital waste reaching the surface waters. In Hungary, VIM-4-producing *K. pneumoniae* is the dominant CRE in humans [14]. Considering the high prevalence of *blaNDM-1* among the clinical *E. coli* isolates and that ST410 was common among our human strains, it seems probable that a proportion of carbapenemases found in the Danube River are of hospital origin (samples taken upstream of Budapest were negative). Thus, even treated hospital wastewater of Budapest may play an important role in shaping the epidemiology of carbapenemases in the environment and, consequently, in wildlife on a local scale. Furthermore, the increasing incidence of NDM-producers in humans in many parts of Europe [32], the rapid spread of *blaNDM-1* via HGT, and the vagrant behaviour of gulls may have contributed to the dominance of these genes in gull isolates [18]. Gulls then may serve as long-distance vectors for the carried strains linking geographical regions by their vagrancy. Black-headed gulls wintering in Budapest typically range from alpine-subalpine lakes in Switzerland and Italy and the Adriatic coast to the German and Polish seacoast, linking Szeged (Hungary), Zagreb (Croatia), and Vienna (Austria) to Budapest; however, they may roam as far as the Netherlands and the Atlantic coast based on 37786 mapped recovery data of ringed birds [33].

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. ÁLK was supported by János Bolyai Research Scholarship of the Hungarian Academy of Sciences, New National Excellence Programme of the Ministry of Innovation and Technology ÚNKP-21-5, NKFIH OTKA FK-138698 grants. The Danube samples provided were obtained through the project 'Drinking water: multidisciplinary assessment of secure supply from the source to the consumers', a program financed by the NRDI fund (2018–1.2.1-NKP-2018–00011). Additional support was provided by the National Research, Development and Innovation Fund, financed under the TKP2021-EGA-01 funding scheme.

Competing interests: The authors declare no conflict of interests. Ethical approval was not required for the investigation.

Acknowledgments: The help from the workers of the Centre for Ecological Research, Institute of Aquatic Ecology is gratefully acknowledged.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2023.10.002.

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