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Genomically diverse carbapenem resistant *Enterobacteriaceae* from wild birds provide insight into global patterns of spatiotemporal dissemination

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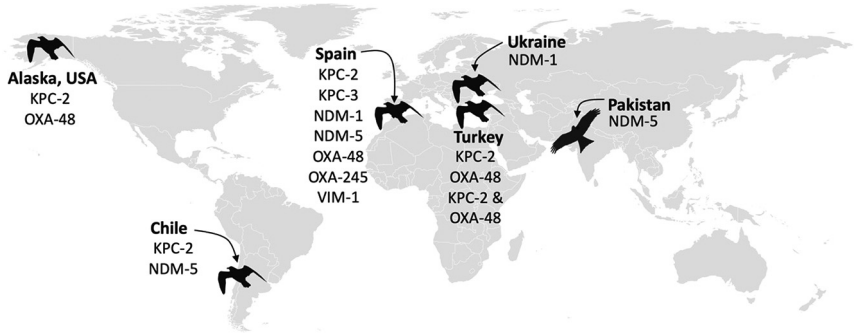
HIGHLIGHTS

- High diversity of carbapenem resistance genes found in wild birds sampled globally.
- Carbapenem resistant hypervirulent *K. pneumoniae* isolated from wild birds in Europe.
- Highly similar OXA-48-producing *E. coli* found in gulls from Alaska and Turkey.
- Limited evidence to support spatial and temporal dissemination of CRE clones
- Some CRE isolates from Chile and Spain also harbored colistin resistance genes.

GRAPHICAL ABSTRACT



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ABSTRACT

Carbapenem resistant *Enterobacteriaceae* (CRE) are a threat to public health globally, yet the role of the environment in the epidemiology of CRE remains elusive. Given that wild birds can acquire CRE, likely from foraging in anthropogenically impacted areas, and may aid in the maintenance and dissemination of CRE in the environment, a spatiotemporal comparison of isolates from different regions and timepoints may be useful for elucidating epidemiological information. Thus, we characterized the genomic diversity of CRE from fecal samples opportunistically collected from gulls (*Larus* spp.) inhabiting Alaska (USA), Chile, Spain, Turkey, and Ukraine and from black kites (*Milvus migrans*) sampled in Pakistan and assessed evidence for spatiotemporal patterns of dissemination. Within and among sampling locations, a high diversity of carbapenemases was found, including *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-beta-lactamase (NDM), oxacillinase (OXA), and Verona integron Metallo beta-lactamase (VIM). Although the majority of genomic comparisons among samples did not provide evidence for spatial dissemination, we did

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Hypervirulent
Gull
Kite

find strong evidence for dissemination among Alaska, Spain, and Turkey. We also found strong evidence for temporal dissemination among samples collected in Alaska and Pakistan, though the majority of CRE clones were transitory and were not repeatedly detected among locations where samples were collected longitudinally. Carbapenemase-producing hypervirulent *K. pneumoniae* was isolated from gulls in Spain and Ukraine and some isolates harbored antimicrobial resistance genes conferring resistance to up to 10 different antibiotic classes, including colistin. Our results are consistent with local acquisition of CRE by wild birds with spatial dissemination influenced by intermediary transmission routes, likely involving humans. Furthermore, our results support the premise that anthropogenically-associated wild birds may be good sentinels for understanding the burden of clinically-relevant antimicrobial resistance in the local human population.

1. Introduction

Carbapenems are considered antibiotics of last resort that are reserved for treating multidrug resistant (MDR) infections in humans. Until recently, carbapenem resistant infections were generally limited to healthcare settings and nosocomial spread; however, community onset carbapenem resistant *Enterobacteriaceae* (CRE) infections may now be more prevalent than hospital onset (Jernigan et al., 2020; Kelly et al., 2017). Carbapenem resistance has also recently been reported in environmental sources globally (Köck et al., 2018; Mills and Lee, 2019), including in wild birds inhabiting all continents except Antarctica, sometimes at high prevalence (Ahlstrom et al., 2019; Bouaziz et al., 2018; Bueno et al., 2020; Dolejska et al., 2015; Fischer et al., 2013; Vittecoq et al., 2017; Wang et al., 2017).

The impact of CRE maintained in wildlife and the environment to human health is not well understood, though it is possible that these bacteria could lead to infections in humans, especially in lower-income countries (Dolejska and Literak, 2019; Hasan et al., 2016; Van Boeckel et al., 2015). For example, interspecies transmission of clinically important antimicrobial resistant (AMR) bacterial clones has been demonstrated among humans, domestic animals, and wildlife (Schaufler et al., 2016; Wang et al., 2017) through mechanisms such as backyard production of agricultural animals (Li et al., 2019). Similarly, wild birds appear to acquire clinically important bacteria through exposure to anthropogenic sources that they may subsequently maintain and disperse (Ramey and Ahlstrom, 2020), though the occurrence and frequency of zoonotic transmission remains enigmatic. More specifically, urbanization has affected feeding and breeding habits of certain birds, including some gull and raptor species, promoting associations between these birds and humans. Therefore, there may be increasing risk of interspecies transmission of antimicrobial resistance and certain pathogens (Duhem et al., 2008; Plaza and Lambertucci, 2017). Furthermore, despite evidence that birds are capable of dispersing clinically important bacteria long distances through their migratory movements (Ahlstrom et al., 2021), there is limited direct evidence to support that such long-distance dispersal events are common (Guenther et al., 2012). As such, there are considerable data gaps pertaining to the pathways through which wild birds may disseminate clinically important AMR bacteria, including CRE.

As CRE in wild birds has increasingly been reported, a spatiotemporal comparison of isolates from different regions and timepoints may be useful for elucidating potentially important epidemiological information such as the extent to which CRE in wild birds is limited to particular clones, geographic areas, and/or sampling periods. The objective of this study was therefore to characterize the diversity of CRE from opportunistically collected wild bird samples and to assess evidence for spatiotemporal patterns of dissemination. We focused on birds that have a propensity to utilize landfills and other anthropogenically modified habitats for forage (i.e. plausible environmental pathways for exposure to CRE), including gulls (*Larus* spp.) inhabiting Alaska (USA), Chile, Spain, Turkey, and Ukraine and from black kites (*Milvus migrans*) sampled in Pakistan.

2. Materials and methods

2.1. CRE isolates

CRE was isolated from the feces of gulls and black kites from six countries sampled as part of existing research programs (Fig. 1). In Pakistan, two sampling sites were visited once every two weeks and fresh feces from black kites were collected for a period of 9 months starting from May 2019 to January 2020. Six samples were collected at each site at each visit. Charcoal swabs were used for sample collection and after collection swabs were streaked directly on ChromAgar supplemented with 1 μg/mL meropenem. Pink *Escherichia coli* colonies were confirmed using API 20E biochemical strips (bioMérieux, Marcy l'Etoile, France) and subjected to DNA extraction using conventional Phenol-Chloroform method. PCR was used to confirm the presence of the bla_{NDM} gene using the primers NDM F and NDM R (Pfeifer et al., 2011).

In Turkey, a total of 200 fecal samples from yellow-legged gulls (*Larus michahellis*) were collected during January 2015 in the Kadiköy (n = 100) and Kumkapi (n = 100) district of Istanbul, Turkey. Fecal samples were collected by swirling a sterile cotton swab in freshly deposited fecal matter from the ground. The swabs were then inserted into tubes with freeze medium (Luria-Bertani broth, BD Sparks, USA, and phosphate buffered saline containing 0.45% sodium citrate, 0.1% MgSO4, 1% (NH4)2 SO4 and 4.4% glycerol). After collection in the field, samples were kept on ice and shipped to Kalmar, Sweden for analysis. At arrival, they were stored at −80 °C until analyzed. Each fecal sample was cultured in tryptic soy broth (Sigma-Aldrich, USA) supplemented with meropenem (0.125 mg/L) and vancomycin (6 mg/L) for 18 h at 37 °C. The broth was thereafter inoculated onto chromID®-CARBA SMART plates (bioMérieux, Marcy L'Etoile, France) and cultured overnight at 37 °C. Putative *Enterobacteriaceae* colonies were sub-cultured on chromID®CARBA SMART plates for confirmation, before the bacteria were identified to the species level with API20E biochemical strips (bioMérieux SA, Marcy-lEtoile, France) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltronics Scandinavia AB, Solna, Sweden). Phenotypic carbapenamase production and its type were determined, using MBL&KPC&OXA-48 discs kit (Liofilchem, Italy) according to the manufacturer's instruction.

In Chile, freshly deposited feces from 100 Franklin's gulls (*Larus pipixcan*) were sampled in February 2020 near the Aconcagua River Delta, Concon, Chile. All samples were inoculated in 2 mL brain heart infusion (BHI) broth (Becton Dickinson, Sparks,USA), supplemented with vancomycin (16 mg/L; ICN Biomedicals Inc., Santa Ana, USA) for selection of gram-negative bacteria, and incubated for 18–24 h at 36 °C. Following incubation, 10 μL of BHI broth was streaked onto Supercarba plates (CHROMagar, Paris,France), a selective medium that supports growth of bacteria with reduced susceptibility to carbapenems. Plates were incubated in aerobic conditions for 18–24 h at 36 °C. Putative *E. coli* and *Klebsiella* spp. isolates were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

In Spain, fresh feces were collected from yellow-legged gulls and lesser black-backed gulls (*Larus fuscus*) at a beach front in Estepona, Spain. Samples were collected in February 2018 (n = 100), November 2018 (n = 93), January 2019 (n = 100), May 2019 (n = 100), and September 2019 (n = 100). Samples were processed the same as in Chile.

In the Azov-Black Sea region of Ukraine, fresh fecal material from Caspian gulls (*Larus cachinnans*), were collected in, Kherson June 2019 (n = 52), August 2019 (n = 7), September 2019 (n = 10), January 2020 (n = 3) and Odesa June 2019 (n = 6). Samples were processed the same as in Chile.

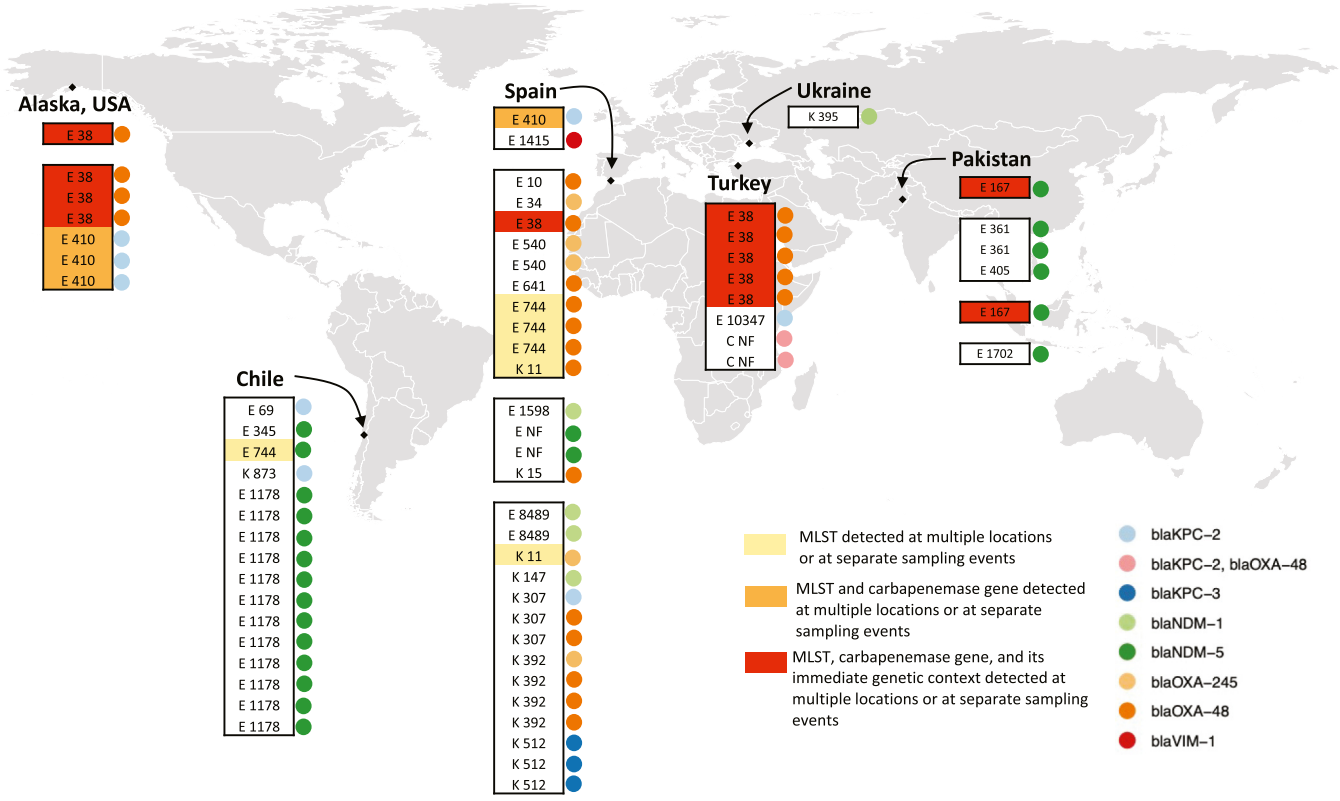

Fig. 1. Map displaying locations of CRE-positive samples collected from wild birds, displaying bacterial genera (*Citrobacter* spp. = C; *E. coli* = E; *K. pneumoniae* = K), multilocus sequence type (NF indicates not found), and carbapenemase genes identified at each location. Shaded colors represent strains with identical sequence type (yellow), carbapenemase gene (orange), and immediate genetic context of the carbapenemase gene (red) isolated from separate locations or sampling events.

In Alaska, fresh fecal material from glaucous gulls (*Larus hyperboreus*), glaucous-winged gulls (*Larus glaucescens*), herring gulls (*Larus argentatus*), and hybrids was collected from seven locations in Alaska, May–August 2016: Samples were collected by inserting a sterile swab into recently deposited gull fecal material and subsequently placing it into a vial with chilled Luria broth (LB) (Sigma-Aldrich, Stockholm, Sweden). Complete information and the initial characterization of isolates is reported in Ahlstrom et al. (2019).

Antimicrobial susceptibility testing (AST) was performed on all isolates using disk diffusion for ampicillin (10 μg), cefadroxil (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), mecillinam (10 μg), nalidixic acid (30 μg), nitrofurantoin (100 μg), piperacillin/tazobactam (30/6 μg), tetracycline (30 μg), trimethoprim (5 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg) and meropenem (10 μg) (Thermo Fisher Scientific Oxoid Ltd., UK). Minimum inhibitory concentration testing was performed using *E*-tests (0.002–32 mg/L) for doripenem, ertapenem, imipenem and meropenem (Biomerieux, France), and micro broth dilution for colistin (0.0625–64 mg/L) (Merlin Diagnostika GmbH, Germany). All AST was performed and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2013), except for tetracycline as this antimicrobial has no defined clinical breakpoint (Kronvall and Smith, 2016).

Conjugation experiments were performed for select isolates from Alaska and Turkey by growing donor (A1_180, A1_181, A1_136, E1ec20oxaOxa, E2ec2oxaOxa, E3ec26oxaOxa, E4ec35oxaOxa, E5ec36oxaOxa) and recipient (*E. coli* MG1655) cultures overnight in LB broth supplemented with ertapenem 1 mg/L (Sigma-Aldrich, Stockholm, Sweden) or chloramphenicol 12.5 mg/L (Sigma-Aldrich, Stockholm, Sweden) and rifampicin 100 mg/L (Sigma-Aldrich, Stockholm, Sweden), respectively. Donor and recipient cultures were washed in LB broth, mixed 1:1 (totally 200 μL), pelleted and resuspended in 20 μL LB broth, which was filtered on 0.45 μm filter 22 mm ϕ (Whatman, Sigma-Aldrich, Stockholm, Sweden). Filters were incubated on blood agar plates at 35 °C overnight and then transferred to 2 mL LB broth. Transconjugants were selected by plating mating mixture onto LB plates with ertapenem 1 mg/L, chloramphenicol 12.5 mg/L and rifampicin 100 mg/L. Transconjugants were verified by qPCR for bla_{KPC}, as previously described (Singh et al., 2016).

2.2. Whole genome sequencing and bioinformatic analysis

DNA was extracted from CRE isolated from the above sampling programs using the MagnaPure compact nucleic acid isolation kit (Roche, Stockholm, Sweden). Whole genome sequencing was performed using the Illumina HiSeq 4000 (Illumina, San Diego, USA). In the case of samples from Alaska (USA), whole genome sequencing information was previously collected and described as part of a prior investigation (Ahlstrom et al., 2019), though information was included for comparative analyses as part of this project. All other genomic information is presented here for the first time. All sequences are publicly available in the sequence read archive under accession number PRJNA800400.

Raw reads were trimmed and filtered using fastp (Chen et al., 2018) using default settings and then assembled de novo with Unicycler (Wick et al., 2017). Antimicrobial resistance genes, virulence genes, and plasmid replicons were detected from assemblies using Abricate (Seeman, https://github.com/tseemann/abricate) using the ResFinder (Zankari et al., 2012), virulence factor (Chen et al., 2016), and PlasmidFinder (Carattoli et al., 2014) databases, respectively. In silico multilocus sequence typing (MLST) was performed using SRST2 (Inouye et al., 2014) based on the seven gene *E. coli* #1 typing scheme retrieved from pubmlst.org. Kleborate (Lam et al., 2021) was used to determine virulence and K loci of *Klebsiella pneumoniae* isolates. The immediate genetic context of carbapenemase genes was identified using TETyper (Sheppard et al., 2018) and by aligning contigs containing carbpenemase genes in Geneious (Kearse et al., 2012).

Isolates within a particular sequence type were reference mapped to a completed genome using the Burrows-Wheeler Alignment tool (Li and Durbin, 2009) and variant sites were identified using SAMtools (Li et al., 2009). Variant sites were filtered based on depth of coverage (at least 2 high quality single nucleotide polymorphisms (SNPs) on both the forward and reverse strand), mapping quality (>40), and heterozygosity (<5%). An alignment of concatenated SNPs were used to create maximum likelihood phylogenetic trees using PhyML (Guindon et al., 2010).

Simpson's diversity index (1-D) was computed in the statistical program R (R Core Team, 2016) using the package vegan (Oksanen et al., 2018) and was based on 1) the number of unique sequence types, 2) the number of unique carbapenemase genes detected at each location, and 3) the combined sequence type/carbapenemase gene combination.

2.3. Assessment of spatial and temporal dissemination of CRE by wild birds

We assessed support for spatiotemporal patterns of dissemination of CRE by gulls and kites based on the bacterial sequence type, carbapenemase gene content, and immediate genetic context of the carbapenemase gene. We considered isolates with an identical sequence type but different carbapenemase genes to provide weak evidence for dissemination. We considered isolates sharing an identical sequence type and carbapenemase gene, but with different immediate genetic contexts to provide moderate support for dissemination. We considered isolates with an identical sequence type, carbapenemase gene, and identical genomic context of the carbapenemase gene to provide strong support for dissemination through space/time. All remaining isolate comparisons, including those without shared sequence types, were considered not to support dissemination through space/time. Additional comparisons of isolate diversity, dominant clones, and virulence genes among isolates from globally diverse sites was included to provide further information on potential dissemination patterns and implications to public/veterinary health; though, such information was not considered in a formal analytical framework.

3. Results

A total of 68 CRE isolates were successfully cultured and sequenced from wild bird fecal samples collected from globally diverse sites, including *Citrobacter* spp. from Turkey (n = 2); *E. coli* from Alaska (n = 7), Chile (n = 15), Spain (n = 16), Turkey (n = 6), and Pakistan (n = 6); and *K. pneumoniae* from Spain (n = 14), Chile (n = 1), and Ukraine (n = 1) (Fig. 1). Genes encoding for variants of four carbapenemases were identified, including *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-beta-lactamase (NDM), oxacillinase (OXA), and Verona integron Metallo beta-lactamase (VIM). All isolates, with the exception of one, a VIM-1 producer from Spain, phenotypically demonstrated reduced susceptibility to at least one carbapenem antibiotic (Supplementary Table 1). No isolates demonstrated phenotypic resistance to colistin. Given that our isolates were obtained opportunistically from different project objectives and sampling strategies, we do not compare prevalence or detection levels of CRE in wild birds among locations or host species.

3.1. Identity and diversity of multilocus sequence types and carbapenemase genes

Twenty-eight unique sequence types were identified, only three of which were found in multiple locations. *E. coli* ST38 was identified from samples collected from Alaska, Spain, and Turkey; *E. coli* ST410 was identified from samples from Alaska and Spain; and *E. coli* ST744 was identified from samples collected in Chile and Spain (Fig. 1). Three sequence types were identified from samples longitudinally collected at the same location including *E. coli* ST38 (Alaska), *E. coli* ST167 (Pakistan), and *K. pneumoniae* ST11 (Spain) (Fig. 1).

Seven different carbapenemase genes were detected, including $bla_{\text{KPC-2}}$, $bla_{\text{KPC-3}}$, $bla_{\text{NDM-1}}$, $bla_{\text{NDM-5}}$, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-245}}$, $bla_{\text{VIM-1}}$ (Fig. 2A). Common carbapenemase genes found in isolates from multiple locations included $bla_{\text{KPC-2}}$ (Alaska, Chile, Spain, and Turkey), $bla_{\text{NDM-1}}$ (Spain and Ukraine), $bla_{\text{NDM-5}}$ (Chile, Pakistan, and Spain), and $bla_{\text{OXA-48}}$ (Alaska, Spain, and Turkey). Two *Citrobacter* spp. isolates from Turkey were double carbapenemase producers, harboring both $bla_{\text{KPC-2}}$ and $bla_{\text{OXA-48}}$. A total of 32 unique MLST/carbapenemase gene combinations were detected (Fig. 1; Supplementary Table 1).

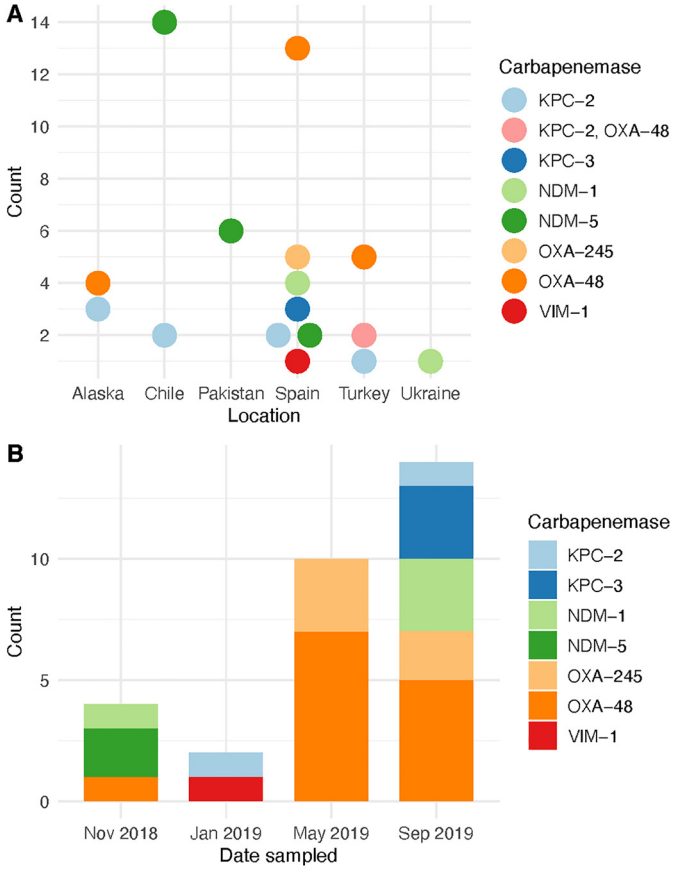

Fig. 2. Count and identity of carbapenemases found in *Enterobacteriaceae* isolated from (A) six locations and (B) in Spain during four sampling occasions.

Among six sampling sites from which bird feces were collected and cultured, Spain exhibited notably high diversity of both sequence types and carbapenemase genes (Table 1, Fig. 2B). In contrast, Chile and Pakistan had relatively low diversity of sequence types and carbapenemase genes, respectively (Table 1; Fig. 1). Generally, locations sampled longitudinally across seasons (Spain and Pakistan) exhibited relatively high diversity values for sequence types/carbapenemase genes as compared to locations sampled once or within a given season (Alaska, Chile, Turkey, and Ukraine) (Table 1). A single clone (i.e. identical sequence type and carbapenemase gene) also represented >50% of isolates per site for these latter sampling locations (Fig. 1).

Table 1
Number of CRE isolates genomically characterized from each location and diversity metrics based on MLST, carbapenemase genes, or the MLST/carbapenemase gene combination.

Location	Number of isolates	Diversity MLST	Diversity carbapenemase genes	Diversity MLST/carbapenemase gene
Alaska, USA	7	0.49	0.49	0.49
Chile	16	0.42	0.22	0.42
Pakistan	6	0.72	0	0.72
Spain	30	0.92	0.75	0.94
Turkey	8	0.53	0.53	0.53
Ukraine	1	0	0	0

3.2. Plasmid replicons

Diverse plasmid replicon types were identified among isolates from six sampling sites (Fig. 3). Full genetic context of carbapenemase genes was not possible to infer using only short read sequencing; however, the immediate genetic context of carbapenemase genes (i.e. genetic elements within the same contig) could be inferred for most isolates, except for those harboring $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-245}}$. KPC genes were located within three different variants of Tn4401 and a non-Tn4401 genetic element (Fig. 3; Supplementary Table 1). Three *E. coli* isolates from Alaska harbored KPC genes within Tn4401a-1, one *E. coli* and one *K. pneumoniae* isolate from Chile, both from the same gull, harbored $bla_{\text{KPC-2}}$ within a non-Tn4401 element, and three *K. pneumoniae* isolates from Spain harbored $bla_{\text{KPC-3}}$ within Tn4401a-2. Two isolates from Spain (one *E. coli* and one *K. pneumoniae*, each sampled at different time points) and three isolates from Turkey (one *K. pneumoniae* and two *Citrobacter* spp.) harbored $bla_{\text{KPC-2}}$ within a truncated Tn4401 element. NDM genes were located within the same immediate genetic context as previous reports of NDM-producing *E. coli* (*dsbD-trpF-bleMBL-*$bla_{\text{NDM1/5}}$), though longer contigs indicated some differences downstream of bla_{NDM} genes. OXA-48-like genes were found on short (~2300 bp) contigs; thus, the genetic context of these genes could not be conclusively resolved. However, an IncL/M pOXA-48 plasmid was detected in 20 of the 29 isolates harboring OXA-48-like genes (Fig. 3). No plasmid replicons were detected in five *E. coli* ST38 isolates from Turkey, suggesting chromosomal integration of $bla_{\text{OXA-48}}$. Carbapenem resistance failed to be transferred from these five isolates through conjugation experiments, further supporting chromosomal integration of $bla_{\text{OXA-48}}$. Chromosomal integration of $bla_{\text{OXA-48}}$ was confirmed in the four isolates from Alaska via long read whole genome sequencing (Ahlstrom et al., 2019).

3.3. Temporal dissemination

We found variable evidence for temporal dissemination of CRE at three locations (Alaska, Spain, and Pakistan) with longitudinal sampling (Fig. 1) as inferred through genomic comparisons among 27 clones. In Pakistan, one clone was detected at multiple sampling time points, an *E. coli* ST167 clone that harbored $bla_{\text{NDM-5}}$ within the same genetic context was isolated in May and then again in December of the same year. We previously reported the repeated detection of an *E. coli* ST38 clone harboring chromosomal $bla_{\text{OXA-48}}$ from gulls in Alaska, USA in June and then again in August (Ahlstrom et al., 2019). Both of these examples provide strong support for temporal dissemination. In Spain, two isolates with the same *K. pneumoniae* sequence type (ST11) were identified at multiple sampling events, though they harbored two different bla_{OXA} carbapenemase genes, providing only weak evidence for dissemination.

3.4. Spatial dissemination

We also found evidence for spatial dissemination of CRE among sample locations (Fig. 1). A total of 32 clones were detected among six sites, though only two clones were found in multiple locations. *E. coli* ST38 OXA-48-producing isolates were found in Alaska, Turkey, and Spain and comparisons among isolates provided strong evidence of dissemination. Reference mapping to the completed genome of the previously reported Alaska strain and comparison among all ST38 isolates identified in this study for the first time revealed only 1 to 5 SNPs (divergence = 0.0000002–0.0000009%) between the isolates from Turkey and Alaska. In contrast, the ST38 isolate from Spain differed by 388–392 SNPs (divergence = 0.00007%) (Fig. 4). The second clone, *E. coli* ST410 KPC-2-producing isolates, was found in Alaska and Spain and provided moderate support for dissemination, as the immediate genetic context of the $bla_{\text{KPC-2}}$ was different (Supplementary Table 1). Lastly, *E. coli* ST744 isolates were found in both Chile and Spain, though the carbapenemase genes differed providing only weak support for dissemination.

3.5. Hypervirulence and multidrug resistance

A total of 16 *K. pneumoniae* isolates were recovered from Chile, Spain, and Ukraine, all of which were MDR. Two isolates, one each from Spain (2507b) and Ukraine (3132), were identified as hypervirulent based on presence of yersiniabactin and aerobactin virulence genes. The single isolate from Ukraine was ST395, capsule type K2, harbored $bla_{\text{NDM-5}}$ and the mucoid phenotype regulator gene *rmpA*. The other hypervirulent strain was isolated from a gull in Spain and was ST11, capsule type K24, and harbored $bla_{\text{OXA-48}}$.

Genes conferring resistance to a total of 12 different antibiotic classes, including rifampicin, aminoglycoside, betalactam, phenicol, trimethoprim, macrolide, fosfomycin, lincosamide, colistin, fluoroquinolone, sulphonamide, and tetracycline, were identified in our collection of 68 CRE isolates (Fig. 5). Ninety-seven percent (66/68) of isolates were MDR, with some isolates harboring AMR genes conferring resistance to up to 10 different antibiotic classes. Colistin resistance genes were identified in gulls sampled in Chile and Spain. One *E. coli* isolate from Spain harbored the mobile colistin resistance gene *mcr-10* and three *K. pneumoniae* isolates from Spain harbored *K. pneumoniae* with mutations in *mgrB*, which may confer colistin resistance. Thirteen *E. coli* isolates from Chile harbored *mcr-9*, including the dominant ST1178 clone as well as a single ST744 isolate.

4. Discussion

4.1. High diversity and limited spatiotemporal dissemination

Our results reveal extensive diversity of CRE harbored by wild birds sampled in diverse global settings. In four out of the five locations where more than one isolate was found, two or more different classes of carbapenemases were identified. Very high diversity was found in Spain, where seven carbapenemase genes were identified over the course of four sampling events, two to five of which were identified during a single sampling event. This is in contrast to previous research in Australia and France, where a single carbapenemase gene was found when samples were collected at a single time point, despite the relatively high prevalence of CRE in gulls (Dolejska et al., 2015; Vittecoq et al., 2017). On the other hand, relatively high diversity of carbapenemases and bacterial sequence types was recently reported in gulls inhabiting the Lisbon coastline of Portugal (Aires-de-Sousa et al., 2020). We found lowest diversity in Pakistan, where a single carbapenemase gene was identified. Our results suggest recurrent local acquisition of CRE by wild birds, rather than international dispersal through migration or a distinct wildlife reservoir of CRE. A single clone was dominant at some locations, which could reflect transmission within the wild bird population or exposure to the same point source.

At some sampling events, particularly in Chile and Spain, a high proportion of CRE was recovered from wild bird samples (10–16 isolates out of 100 samples collected). During these sampling events, gulls harbored KPC, OXA, and NDM carbapenemases within *E. coli* and *K. pneumoniae* clones known to be pathogenic in humans, exemplifying potentially important public health implications of CRE in wild birds. The frequency at which CRE was detected at these locations/times suggests prevalence in wild birds may be equal or exceed levels found in many human populations, including in Latin America and Spain (European Centre for Disease Prevention and Control, 2019; Hansen, 2021). Although we do not directly estimate prevalence of CRE in wild birds in this study given methodological considerations (see Materials and Methods and Results), the high number of detections is noteworthy and emphasizes the need to further understand the role that wild birds may play in the maintenance and dissemination of CRE. For example, the well-visited beach in Estepona, Spain where the sampling took place is situated close to the densely populated city center, but not in the absolute vicinity of any farms. There are only small private hospitals in Estepona and the wastewater treatment plant and landfill are situated quite far from the city center. More detailed studies are clearly needed to identify the most relevant point sources for AMR acquisition by gulls.

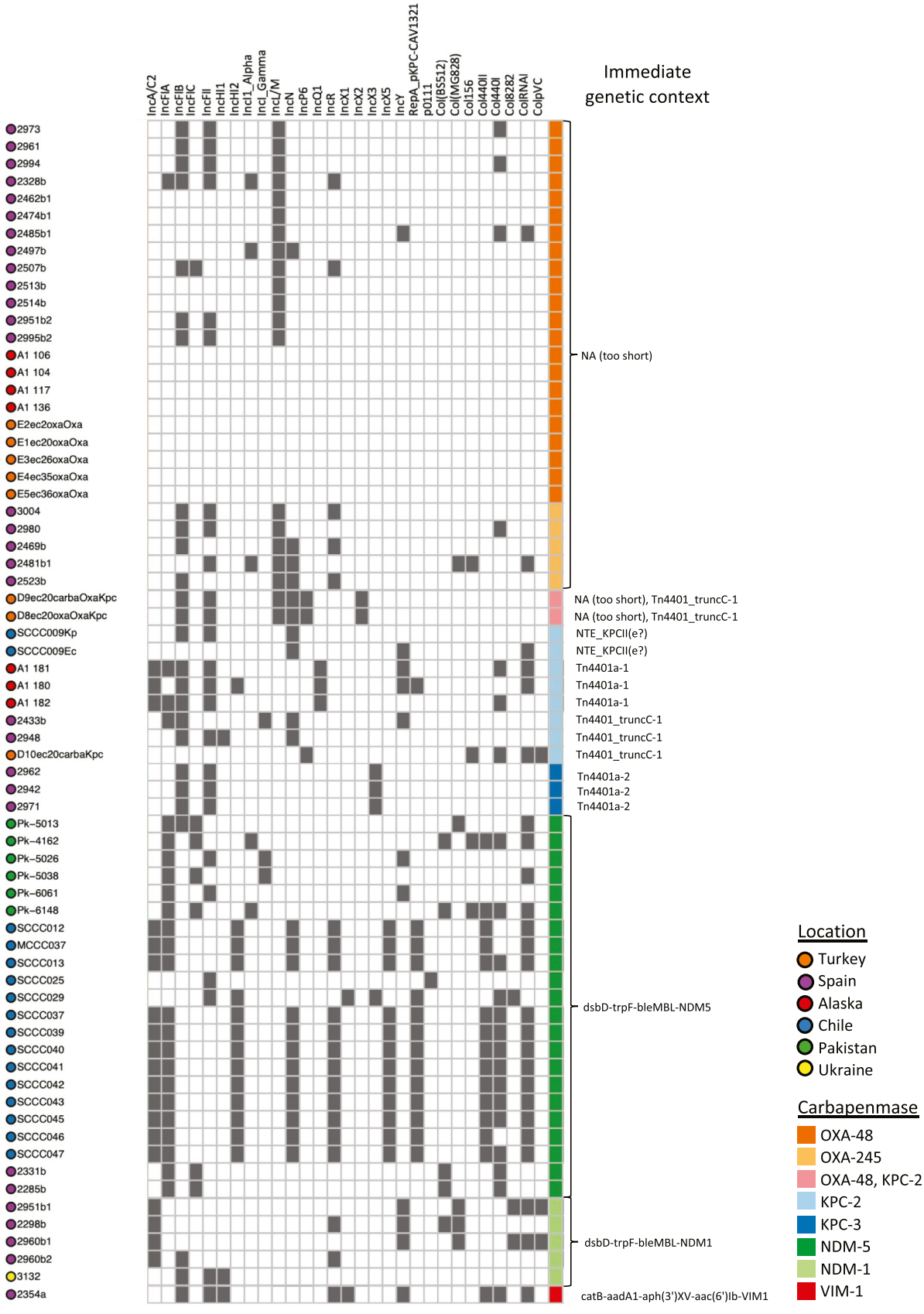

Fig. 3. Depiction of plasmid replicons identified among CRE isolates, grouped according to carbapenemase. Colored circles represent locations where samples were collected and the matrix indicates presence of specific plasmid replicons (grey shading). To the right, boxes are colored according to carbapenemase and the immediate genetic context of the carbapenemase gene is indicated. NA (too short) indicates contig sizes were of insufficient length to reliably infer genetic context.

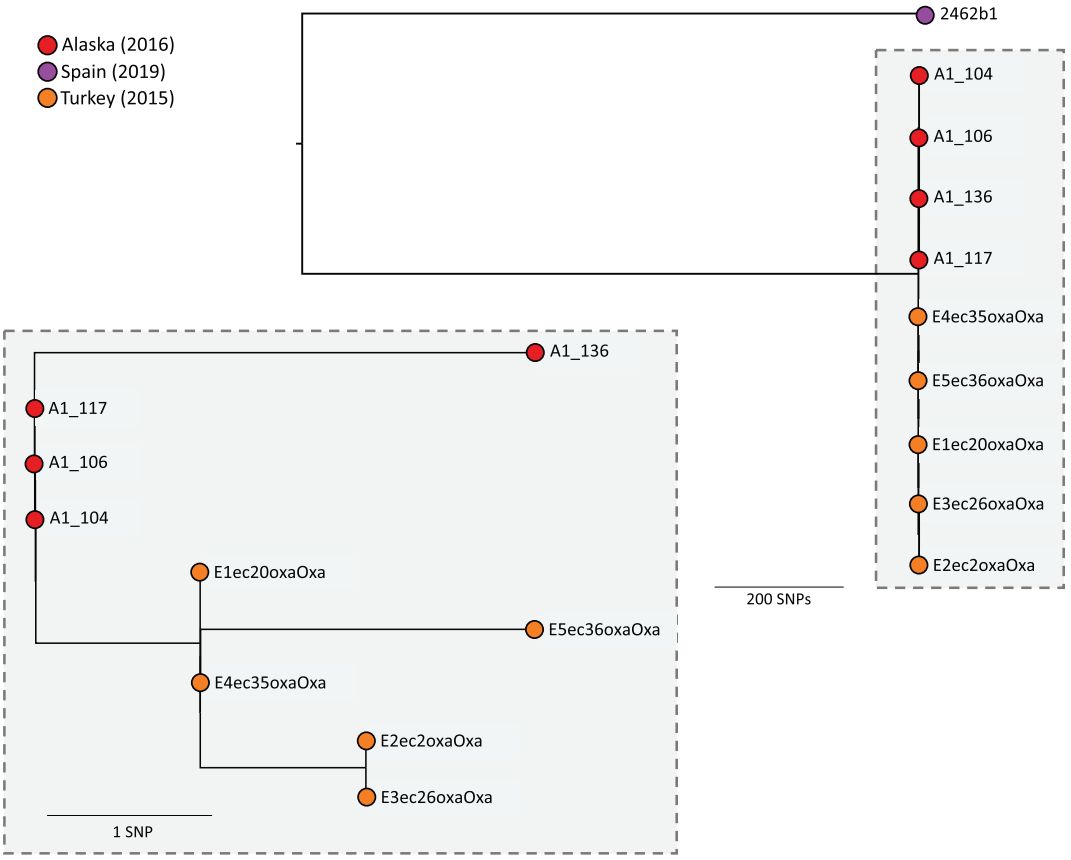

Fig. 4. Maximum likelihood phylogenetic tree of *E. coli* ST38 OXA-48-producing isolates isolated from feces of large-bodied gulls in three countries, based on an alignment of SNPs identified through reference mapping to Alaska strain A1_136 (CP040390). Tips are colored according to sampling location: Alaska, USA (red), Spain (purple), Turkey (orange). The shaded region of the phylogenetic tree is magnified (bottom left) to illustrate genetic similarity among isolates. Year of sampling at each location is provided in the upper left legend.

In Alaska, Pakistan, and Spain, where samples were collected longitudinally, we found some evidence for temporal dissemination of clones over time. A single ST167 clone in Pakistan appeared to persist over a period of several months (May – December) and we previously reported an *E. coli* ST38 clone detected in Alaska gulls sampled in June and August of the same year (Ahlstrom et al., 2019). This indicates that gulls and kites may be capable of facilitating the persistence of CRE or are repeatedly exposed to the same CRE in the environments they inhabit. The former is plausible given that gulls are capable of shedding clinically-important antibiotic resistance genes (e.g. *mcr-1* conferring colistin resistance) for at least 16 days following experimental challenge that can persist in the environment for at least 29 days (Franklin et al., 2020). Weak evidence for temporal dissemination, on the other hand, was found in Spain, where only one sequence type was isolated from multiple sampling events, though with different carbapenemase genes. Two sampling events in Spain (e.g. May and September) provided a high number of detections of CRE. Such temporal variation in detection of CRE among wild birds is consistent with previous research in which samples from mallard ducks had a higher prevalence of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in warmer months (Hessman et al., 2018). This weak evidence of temporal dissemination in Spain is somewhat unexpected given the findings of Franklin et al. (2020) and may indicate that a greater number of samples are required to detect temporal trends.

Strong evidence for spatial dissemination was found among samples collected in Alaska, Spain, and Turkey. We identified remarkable genomic similarity between *E. coli* ST38 $bla_{\text{OXA-48}}$-positive clones isolated from gulls in Turkey (sampled in 2015) and Alaska (sampled in 2016), with more diversity found within isolates from each site than between sites. These isolates harbored chromosomally-encoded $bla_{\text{OXA-48}}$, which has previously been reported in multiple countries and clinical settings (Beyrouthy et al., 2014; Hendrickx et al., 2021; Pitout et al., 2020). The other ST38 isolate recovered from Spain, as well as all other $bla_{\text{OXA-48}}$-positive isolates, harbored an IncL/M plasmid that is frequently associated with OXA-48. This isolate from Spain also had a larger number of SNPs compared to ST38 isolates from Alaska and Turkey, suggesting a more distant epidemiological connection. There is no migratory flyway that connects Alaska and Turkey; thus, intermediary transmission routes almost certainly played some role in the clonal dispersal of CRE between gulls from these two distant locations. OXA-48 was first identified in an isolate from Turkey in 2001 (Poirel et al., 2004) and has since spread widely, especially in the Middle East (Poirel et al., 2012). Human infections caused by OXA-48-producing CRE, on the other hand, are rare in the United States and are often associated with overseas travel (Lyman et al., 2015; Walters et al., 2018). It is therefore plausible that visitors to Alaska or residents travelling abroad became colonized with this clone and shed it into an environment where gulls were exposed.

We also identified moderate and weak evidence for spatial dissemination among samples from Alaska and Spain and samples from Chile and Spain, respectively. Although *E. coli* ST410 harboring $bla_{\text{KPC-2}}$ was found in both Alaska and Spain, the immediate genetic context of the $bla_{\text{KPC-2}}$ gene was different. These differences likely reflect different epidemiological histories and modes of carbapenemase gene acquisition (i.e. independent acquisition of $bla_{\text{KPC-2}}$ by two different ST410 clones) (David et al., 2020; Matlock et al., 2021) and thus provide only moderate evidence for spatial dissemination. *E. coli* ST744 isolates from Chile and Spain are unlikely to be epidemiologically linked, as they harbored different carbapenemase genes. MLST is a relatively course tool to determine genomic similarity and ST744 is geographically widespread, reported

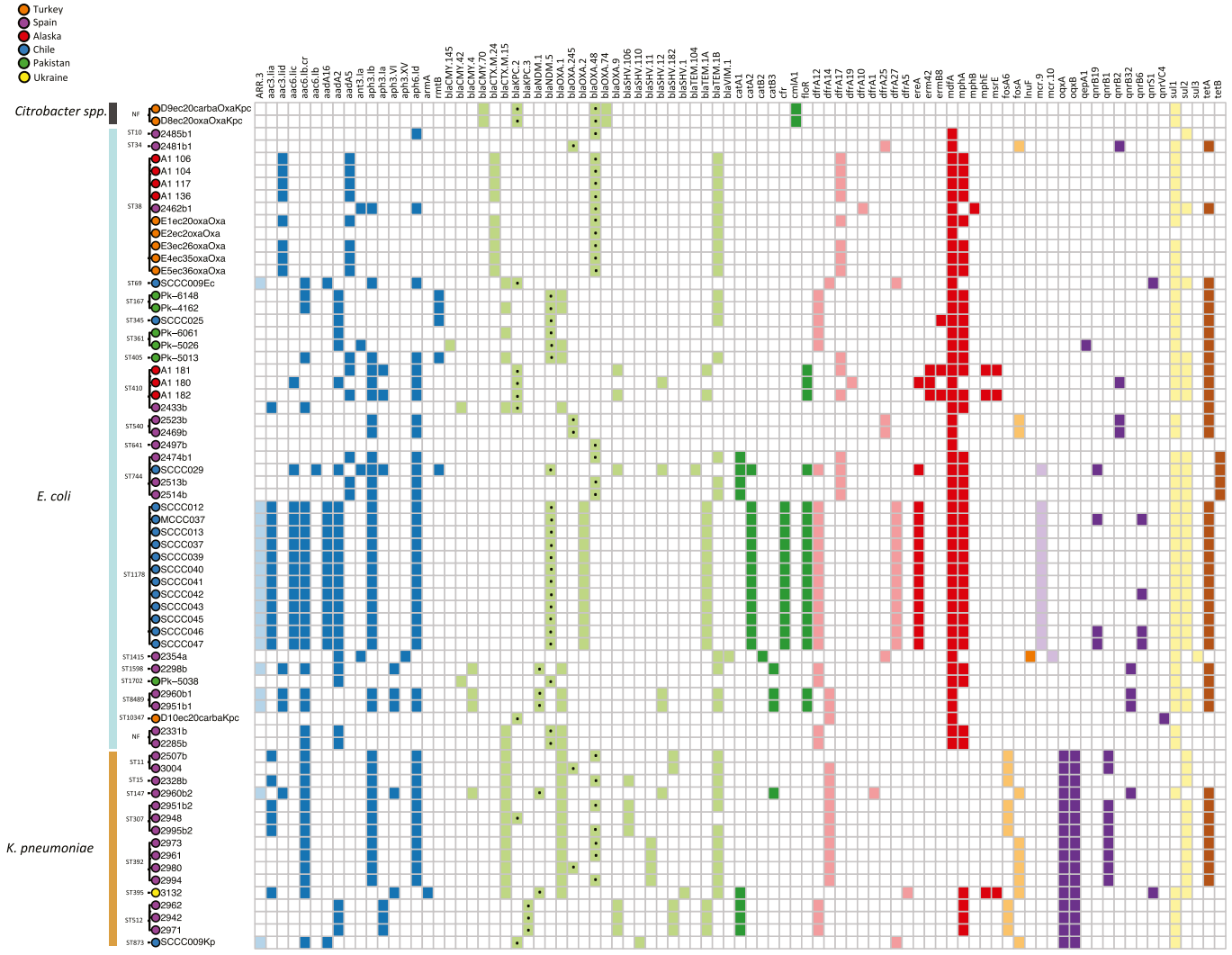

Fig. 5. Depiction of AMR genes identified among CRE isolates grouped according to bacterial species and multilocus sequence type. Colored circles represent locations where CRE was isolated and the matrix to the right indicates presence of AMR genes, colored according to antimicrobial class. Green shading with a black dot indicates the carbapenemase gene(s) detected.

in Africa, Asia, Europe, North America, and South America (http://enterobase.warwick.ac.uk).

4.2. Clinically-relevant clones and virulence

Most CRE isolated in this study are well-known MDR human pathogens (Dunn et al., 2019; Wyres et al., 2020), often reflecting clones or carbapenemases reported in spatially proximate human populations. For example, NDM-5-producing *E. coli* was dominant in wild birds in Pakistan, which corresponds to the dominance of NDM carbapenemases on the Indian subcontinent (Hornsey et al., 2011; Logan and Weinstein, 2017; Qamar et al., 2019). Furthermore, carbapenemase producing *E. coli* ST167 and ST405 found in black kites in Pakistan have been previously reported in clinical settings in Pakistan (Hadjadj et al., 2021). Similarly, we identified a KPC-2 gene embedded in a non-classical transposon element in isolates originating from gulls in Chile, a genetic context which has also been reported in clinical isolates from a hospital outbreak in the same region of the country (Wozniak et al., 2021). In Spain, several carbapenemases were reported among clinical isolates in Andalucía between 2014 and 2018, including KPC-3-producing *K. pneumoniae* ST512, and OXA-48-like-producing *K. pneumoniae* ST11, ST15, ST392, and ST307 (López-Hernández et al., 2020), all of which we detected in gulls in southern Spain. Such similarity of CRE clones isolated from spatially proximate humans and gulls was also recently observed in gulls sampled in Portugal (Aires-de-Sousa et al., 2020).

Co-occurrence of colistin and carbapenem resistance genes was found in 25% of isolates (17/68) despite the fact that none of the isolates exhibited phenotypic resistance to colistin through laboratory culture. Given that colistin is one of the few remaining treatment options for carbapenem-resistant infections, further evaluations of the co-occurrence of resistance to these two classes of antimicrobials in diverse environmental settings may be warranted to inform the evaluation of risk to human and veterinary medicine. Co-resistance to colistin and carbapenems has been reported in *Enterobacteriaceae* isolated from humans, agricultural environments, and a wild bird (Macesic et al., 2021; Wang et al., 2017; Yang et al., 2016) and human-animal transmission of an *E. coli* ST744 clone co-producing NDM-5 and MCR-1 was evidenced from humans and their backyard animals in China (Li et al., 2019). Macesic et al. (2021) described silent spread of *mcr-9.1* on IncHI2 plasmids in Australia among isolates that did not demonstrate phenotypic resistance to colistin. We similarly isolated 13 phenotypically colistin sensitive *E. coli* isolates harboring *mcr-9* from Chile and one isolate harboring *mcr-10* from Spain. In Spain, we also isolated three KPC-3-producing *K. pneumoniae* ST512 isolates with a deletion in *mgrB*, a clone that has been previously reported in southern Spain (Oteo et al., 2016). Should CRE in environmental sources develop phenotypic resistance to colistin or become more common, our data suggest that environmental

pathways could facilitate the dissemination of bacteria that may be extremely difficult to treat in clinical settings.

The presence of carbapenem resistant hypervirulent *K. pneumoniae* (CR-hvKp) in gull feces sampled in two European countries may also have implications to veterinary and public health. MDR strains and hypervirulent strains of *K. pneumoniae* were once considered two distinct populations, though strains that possess both phenotypes have been increasingly reported (Lan et al., 2021). We detected a bla_{NDM-1}-positive ST395 hypervirulent isolate from a gull in Ukraine, which is the same clone that was isolated from hospitalized patients in Russia (Lazareva et al., 2020). We also detected a bla_{OXA-48}-positive hypervirulent ST11 isolate in Spain. Both hypervirulent isolates belong to the *K. pneumoniae* clonal group 258 (Wyres et al., 2015), which has a strong association with carbapenem resistance; thus, these carbapenem resistant isolates likely acquired virulence genes via horizontal gene transfer, as opposed to hypervirulent clones acquiring carbapenemase genes (Lan et al., 2021).

Our comparisons of isolates from gulls and kites to previously reported findings are consistent with local acquisition of clinically-relevant CRE by wild birds and support the premise that anthropogenically-associated wildlife may be good sentinels for understanding the burden of antimicrobial resistance in the local human population (Nieto-Claudin et al., 2021; Ramey and Ahlstrom, 2020). Long read sequencing could confirm similarity of clones and plasmids among isolates in this study and previously reported isolates from humans and the environment, which may help to clarify epidemiologic relationships among these sectors. For example, the clinical isolates from Chile harboring KPC genes in a non-classical transposon element within an IncN plasmid (Wozniak et al., 2021) could be compared to the gull isolates harboring KPC-2 genes in the same non-classical transposon element. Furthermore, the KPC-2 gene in this genetic context was identified in both an *E. coli* and *K. pneumoniae* isolate from the same gull fecal sample, both of which harbored IncN plasmid replicons. This suggests possible transfer of the same KPC-2-containing plasmid between genera, and perhaps between humans and gulls, though confirmation via long read sequencing is requisite to improve resolution of inference.

4.3. Future directions

Our results provide valuable data from which to inform future studies specifically designed to assess spatiotemporal patterns of CRE dissemination among wild birds globally. Carefully selected geographic locations throughout migratory pathways of anthropogenically associated wild birds could provide more robust inference on the spatial patterns of dissemination, while bird tagging and/or movement data (e.g. obtained through banding or satellite telemetry) could resolve whether longitudinally collected samples reflect a consistent population of birds and further refine migratory connectivity. Given the opportunistic nature of our samples, only bacterial isolates collected from locations where CRE was detected were included in analyses, which did not necessarily reflect the most spatiotemporally refined sampling locales for detecting epidemiological connections. Thus, future studies designed to systematically collect data over space and time would minimize such sampling bias and provide opportunities to compare prevalence estimates of CRE among locations and assess seasonal variations within discrete or interconnected populations of birds. Finally, genomic comparisons of CRE derived from human clinical cases and wild birds from the same geographic region and time period could help determine epidemiological relationships between these host species and clarify the zoonotic potential of CRE harbored by wild birds.

5. Conclusions

We found a high diversity of CRE among wild bird fecal samples collected from six countries, with relatively high proportions of birds at some locations harboring CRE. Strong evidence for temporal and spatial dissemination was found, though the majority of clones were not detected at multiple locations or repeated sampling events. CRE detected in wild birds often appears to reflect clones and/or carbapenemase genes circulating in the local human population and detection of CR-hvKp among birds in two European countries exemplifies the public health significance of CRE harbored by wild birds. Increased surveillance for clinically-relevant antimicrobial resistant bacteria in the environment, including wild birds, might elucidate the epidemiological role of these hosts in the maintenance and dissemination of CRE, not only through space and time, but also among humans, animals, and other wildlife.

Abbreviations

AMR	Antimicrobial resistant
CRE	Carbapenem-resistant Enterobacteriaceae
MLST	Multilocus sequence typing
MDR	Multidrug resistant
ST	Sequence type
CR-hvKp	Carbapenem resistant hypervirulent *K. pneumoniae*

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CRediT authorship contribution statement

Christina A. Ahlstrom: Conceptualization, Methodology, Software, Formal analysis, Data curation, Writing – original draft, Visualization. **Hanna Woksepp:** Methodology, Validation, Investigation, Resources, Formal analysis, Data curation, Writing – review & editing. **Linus Sandegren:** Methodology, Investigation, Resources, Data curation, Writing – review & editing. **Mashkoor Mohsin:** Investigation, Resources, Data curation, Writing – review & editing. **Badrul Hasan:** Investigation, Resources, Data curation, Writing – review & editing. **Denys Muzyka:** Investigation, Resources, Data curation, Writing – review & editing. **Jorge Hernandez:** Investigation, Resources, Data curation, Writing – review & editing. **Filip Aguirre:** Investigation, Resources, Data curation, Writing – review & editing. **Atalay Tok:** Investigation, Resources, Data curation, Writing – review & editing. **Jan Söderman:** Investigation, Resources, Data curation, Writing – review & editing. **Bjorn Olsen:** Conceptualization, Investigation, Resources, Data curation, Writing – review & editing. **Andrew M. Ramey:** Conceptualization, Methodology, Resources, Writing – original draft, Supervision, Funding acquisition. **Jonas Bonnedahl:** Conceptualization, Methodology, Resources, Writing – original draft, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare ththey have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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