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High colonization rate of a novel carbapenem-resistant *Klebsiella* lineage among migratory birds at Qinghai Lake, China

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Objectives: The emergence of carbapenemase-positive Enterobacteriaceae poses a serious threat to public health worldwide. Here we conducted a molecular surveillance study on carbapenem-resistant Enterobacteriaceae (CRE) colonization among migratory birds at Qinghai Lake in China.

Methods: A total of 420 samples from migratory birds and their surrounding environment were collected at three sites along the Qinghai Lake bird island. Carbapenem-non-susceptible isolates were identified by 16S rDNA sequencing and MALDI-TOF MS. Carbapenemase producers were determined by Carba NP testing. Antimicrobial susceptibility testing, transfer ability and PFGE were also performed, and 46 isolates from different pulsotypes were analysed by WGS.

Results: Three hundred and fifty isolates were carbapenemase producers based on Carba NP testing, while 233 *Klebsiella* spp. and 2 *Escherichia coli* isolates were NDM-5-carriers. PFGE was performed and showed that the isolates were grouped into five pulsotypes; among these, type A was predominant (86.7%, n=202) and belonged to a novel *Klebsiella* lineage, ST1697. WGS analysis indicated that ST1697 strains may be a hybrid of the recombination of *Klebsiella quasipneumoniae* subsp. *similipneumoniae* and *Klebsiella pneumoniae* genomes.

Conclusions: This high frequency of carbapenemase producers in migratory birds is unexpected. These results provide new insight into the spread of antibiotic resistance, and highlight that continued vigilance for MDR carbapenemase-producing Enterobacteriaceae in migratory birds is urgently needed.

Introduction

The prevalence of antimicrobial resistance continues to increase globally, becoming one of the most serious threats to human and animal health. The concerns are highlighted by the global spread of carbapenem-resistant Enterobacteriaceae (CRE) among different ecological niches, including humans, livestock, wildlife, pets, insects and the environment.[1,2] Owing to their great mobility and heterogeneous environmental exposure, wild birds, especially migratory birds, may play an important role in the emergence and transmission of antimicrobial resistance and infectious diseases.[3] Thus, wild migratory birds have been postulated to be sentinels, reservoirs and potential vectors for the spread of antimicrobial resistance;[4,5] however, the spread of CRE in migratory birds is less frequently described, and the prevalence, species and genotypes associated with CRE colonization among migratory birds remain largely undetermined. Here we conducted a microbiological and molecular surveillance study of CRE colonization among migratory birds at Qinghai Lake in China, and surprisingly uncovered a high frequency of a novel *Klebsiella* lineage carrying NDM-5 MBL genes.

Materials and methods

Sample collection, bacterial isolation and carbapenemase-producing detection

A total of 420 samples from migratory birds and their surrounding environment (faeces n=406, fallen feathers n=4, water n=5, soil n=5) were collected at three sites along the Qinghai Lake bird island (Figure 1, Table S1, available as Supplementary data at *JAC* Online), China, in August 2016.

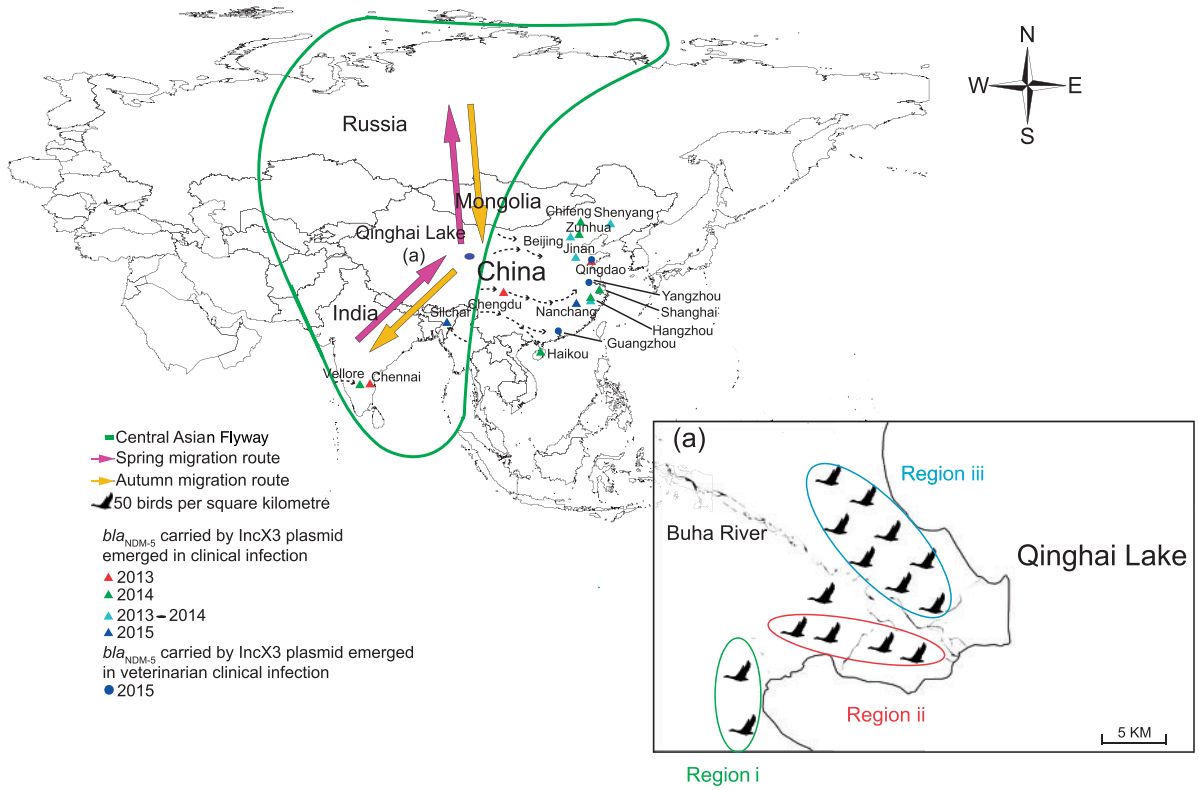


Figure 1. Map showing the location of Qinghai Lake. The map also shows the range of the Central Asian Flyway and specific information on sample sites. Dotted arrows indicate the tendency of the bla_{NDM-4}-like gene carried by IncX3 plasmid strains to spread around the Central Asian Flyway. (a) Schematic representation of migratory bird distribution density in Qinghai Lake bird island. Sampling regions (i), (ii) and (iii) are marked. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

During the autumn migration period, this island has the highest density and variety of migratory birds around Qinghai Lake;[6] therefore, we chose this time to conduct the study. As *Anser indicus* is one of the dominant species at Qinghai Lake[7] and its distribution density was higher than that of others, more *A. indicus* faeces was collected. Unlike other species of birds (e.g. *Phalacrocorax* and *Larus ichthyaetus*), *A. indicus* mainly feeds on grass; the colour of its faeces is mostly green, which is easy to distinguish from the faeces of other birds. Based on the morphological characteristics of bird faecal samples, 406 non-duplicate fresh faeces samples were collected in three regions of the island; 294 were from *A. indicus* and 112 were from other birds.

To mostly avoid repeated sampling from the same bird group, all samples in the three regions were collected on the same day before noon. In order to prevent cross-contamination, only wet, fresh and separate faeces were collected. Individual faecal samples were collected by swirling a sterile swab in the fresh faeces and placing it in sterile physiological saline solution. Individual fallen feathers were rinsed with sterile physiological saline solution. Feather flushing fluid, water samples and soil samples were incubated with LB broth without antibiotic selection. Then the diluted faeces samples and environment cultures were directly plated on MacConkey agar containing 1 mg/L meropenem and incubated for 18 h at 37°C. Carbapenem-non-susceptible isolates were identified using 16S rDNA sequencing[8] and MALDI-TOF MS (Shimadzu-Biotech).[9] The production of carbapenemase by all isolates was detected with the Carba NP (Carbapenemase Nordmann-Poirel) test as described previously.[10] All the carbapenemase-producing isolates were screened by PCR for the presence of carbapenemases, including NDM, IMP, VIM, KPC and OXA-48-like.[11] In addition, the entire bla_{NDM} gene coding region was amplified with primers reported previously,[12] followed by Sanger sequencing, and the 813 bp ORF of bla_{NDM} was translated and typed by protein BLAST.

Transfer of carbapenemase genes

The transferability of carbapenem resistance was determined by filter mating using streptomycin-resistant *Escherichia coli* C600 as a recipient strain.[13] The transferability and conjugation frequencies of bla_{NDM-5}-carrying plasmid pQH1207 to relevant species and genera were assessed between *E. coli* C600, *Enterobacter cloacae* ATCC 13047, *Salmonella* ATCC 14028 and *Acinetobacter baumannii* ATCC 19606. Transconjugants were selected on MacConkey agar plates containing streptomycin (1500 mg/L) and meropenem (1 mg/L). The replicon plasmid type of the transconjugants was classified using a PCR-based replicon typing (PBRT) scheme.[14,15]

Antimicrobial susceptibility testing

The MICs of 19 antibiotics (meropenem, imipenem, ertapenem, aztreonam, cefoxitin, ceftiofur, cefotaxime, ceftazidime, gentamicin, amikacin, tobramycin, ciprofloxacin, enrofloxacin, tetracycline, tigecycline, colistin, fosfomycin, florfenicol and trimethoprim/sulfamethoxazole) for all carbapenemase-producing isolates and their transconjugants were determined by agar dilution and interpreted according to the CLSI guidelines (CLSI, 2015: M100-S25). The breakpoints of colistin and tigecycline for Enterobacteriaceae were interpreted according to the EUCAST criteria (EUCAST 2015, version 5.0). Ceftiofur, enrofloxacin and florfenicol were interpreted in accordance with the veterinary CLSI (VET01-A4/VET01-S2). *E. coli* ATCC 25922 served as a quality control strain for susceptibility testing.

PFGE and WGS

The genetic relatedness of MBL-producing isolates was investigated by XbaI-PFGE for *E. coli* and *Klebsiella pneumoniae*. PFGE patterns were

analysed with the Dice coefficient and the unweighted pair group with arithmetic mean (UPGMA) clustering method using BioNumerics (Applied Maths, Ghent, Belgium), resulting in PFGE patterns with >90% similarity between clusters. MLST was performed according to published protocols (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search for *E. coli* and http://bigsdb.web.pasteur.fr/klebsiella for *K. pneumoniae*).

Based on the different resistance phenotypes and genetic relationships, 46 *K. pneumoniae* strains were selected for WGS. The complete DNA sequence of the selected isolates was extracted using the TianGen DNA Extraction kit (TianGen), and 250 bp paired-end reads were obtained using an Illumina MiSeq system (Illumina, San Diego, CA, USA). For each WGS, at least 100-fold coverage of raw reads was collected. A draft assembly of the sequences was generated using SPAdes,[16] and the putative coding sequences of the flanking region of bla_{NDM} were obtained using ORF Finder programs (www.ncbi.nlm.nih.gov/gorf/orfig.cgi). Among the 46 *K. pneumoniae* strains, a strain named QH1207, the PFGE profile of which occurred in 202 isolates, was chosen for further sequencing by the MinION platform (Oxford Nanopore).[17] The complete genome of isolate QH1207 was assembled using Illumina MiSeq short reads and nanopore long reads using Unicycler.[18]

Molecular characterization and phylogenetic analysis of K. pneumoniae isolates

Species identification was also performed for each isolate based on the genomic data. Average nucleotide identity based on BLAST (ANIb) was determined using JSpeciesWS.[19] The core genomes of strains were used to determine within-species/genus phylogenetic trees using Parsnp in the Harvest package,[20] and the *K. pneumoniae* population structure was estimated using BAPS v6.0 software.[21] SNP analysis was carried out with the Mauve programme,[22] *K. pneumoniae* subsp. *pneumoniae* MGH78578 and *Klebsiella quasipneumoniae* subsp. *similipneumoniae* ATCC 700603 were used as reference strains for comparison with QH1207. SNP density for each comparison was plotted using ggplot2 in R 3.5.0.

Plasmid analysis

The bla_{NDM-5}-harbouring plasmids extracted from transconjugants were subjected to restriction enzyme digestion (EcoRI) to determine the RFLP profile, and plasmids that differed in EcoRI RFLP profiles were further determined by S1-nuclease PFGE and Southern blotting.[5] Furthermore, five pairs of primers were designed to amplify five overlapping regions covering the complete sequence of IncX3 plasmid pNDM_MGR194, and PCR products were further digested with restriction enzyme BglII to determine the RFLP profile.

Accession number

All genome assemblies of 46 *K. pneumoniae* isolates were deposited in GenBank and are registered with BioProject number PRJNA514908.

Results and discussion

Sample collection, bacteria isolation and detection of carbapenemase genes

Qinghai Lake is the largest inland saltwater lake in China, located in the north-east of a basin in Qinghai Province (Figure 1). Every year, ~70000 waterfowl (>20 species) rest in this area during their migration from Mongolia in autumn and from South-East Asia in spring.[23] These birds usually stay at Qinghai Lake for nearly half a year. Qinghai Lake is thus one of the most important breeding and stopover sites for migratory birds along the Central Asian Flyway (Figure 1).[6,24,25] There are no domestic poultry and food animal farms in the vicinity of Qinghai Lake, making it an ideal study site to examine CRE colonization among migratory birds.

At Qinghai Lake, migratory birds are mainly concentrated on an island in the north-west of the lake (Figure 1). In this study, a total of 420 samples of bird stools and their surrounding environment (faeces n=406, fallen feathers n=4, water n=5, soil n=5) were collected at three sites along Qinghai Lake in August 2016 (Table S1). A total of 431 isolates were recovered from MacConkey agar containing 1 mg/L meropenem, 350 of which were carbapenemase producers based on the Carba NP test, including 233 *Klebsiella* spp., 2 *E. coli*, 88 *Stenotrophomonas maltophilia* and 27 *Pseudomonas* spp. isolates. The *Klebsiella* spp. isolates were mainly obtained from stools in region (iii) (84.5%), which had the highest residential density (Figure 1, Table S1), and included 166 isolates from *A. indicus*, 64 isolates from other birds, 2 isolates from soil and 1 isolate from a feather. The two *E. coli* isolates were collected from *A. indicus*. The 233 *Klebsiella* spp. and 2 *E. coli* isolates were subjected to PCR detection and Sanger sequencing for common carbapenemase genes in CRE. Interestingly, bla_{NDM-5} was the only carbapenemase gene identified, and it was detected in all Carba NP-positive *Klebsiella* spp. and *E. coli* isolates. Consequently, a very high carriage rate of NDM-5-producing *Klebsiella* spp. (233/420, 55.5%) and *E. coli* was identified in the migratory birds at Qinghai Lake.

Antimicrobial susceptibility testing

Besides carbapenems, these NDM-producing isolates also showed high-frequency (100% or approximately 100%) resistance to cephalosporins (cefoxitin, ceftiofur, cefotaxime and ceftazidime), tetracycline, enrofloxacin, fosfomycin, florfenicol, trimethoprim/sulfamethoxazole and gentamicin. The resistance rates to aztreonam and ciprofloxacin were 32.8% and 11.5%, respectively, but most isolates remained susceptible to amikacin, colistin, tobramycin and tigecycline (resistance rates <2.7%).

Molecular typing and phylogenetic characteristics

PFGE was successfully performed in 230 NDM-5-positive *Klebsiella* spp. (3 tests failed), and the results showed that they were grouped into five pulsotypes (A–E), with type A predominant (86.7%, n=202) (Figure S1, Table S2). A subset of 46 *Klebsiella* spp. strains, including isolates from each pulsotype, were selected for further WGS. *In silico* MLST analysis revealed five STs: ST35 (n=4), ST1697 (n=35), ST2582 (n=3), ST2583 (n=3) and ST2584 (n=1). Among them, ST2582, ST2583 and ST2584 were first described in this study; ST2582 showed a different *phoE* gene from ST2584 and showed a different *tonB* gene from ST2583. The *K. pneumoniae* MLST scheme is not limited to *K. pneumoniae* but is also applicable to other closely related *Klebsiella* species (namely, the *K. pneumoniae* complex), such as *K. quasipneumoniae* and *Klebsiella variicola*. Some STs may correlate to species backgrounds other than *K. pneumoniae*. Recent phylogenomic studies have divided the *K. pneumoniae* complex into three major phylogroups: KpI (*K. pneumoniae*), KpII (two subgroups: KpII-A, *K. quasipneumoniae* subsp. *quasipneumoniae*; and KpII-B, *K. quasipneumoniae* subsp. *similipneumoniae*) and KpIII (*K. variicola*),[26,27] and two additional novel groups (Kp5 and Kp6); Kp6 was recently assigned as *Klebsiella*

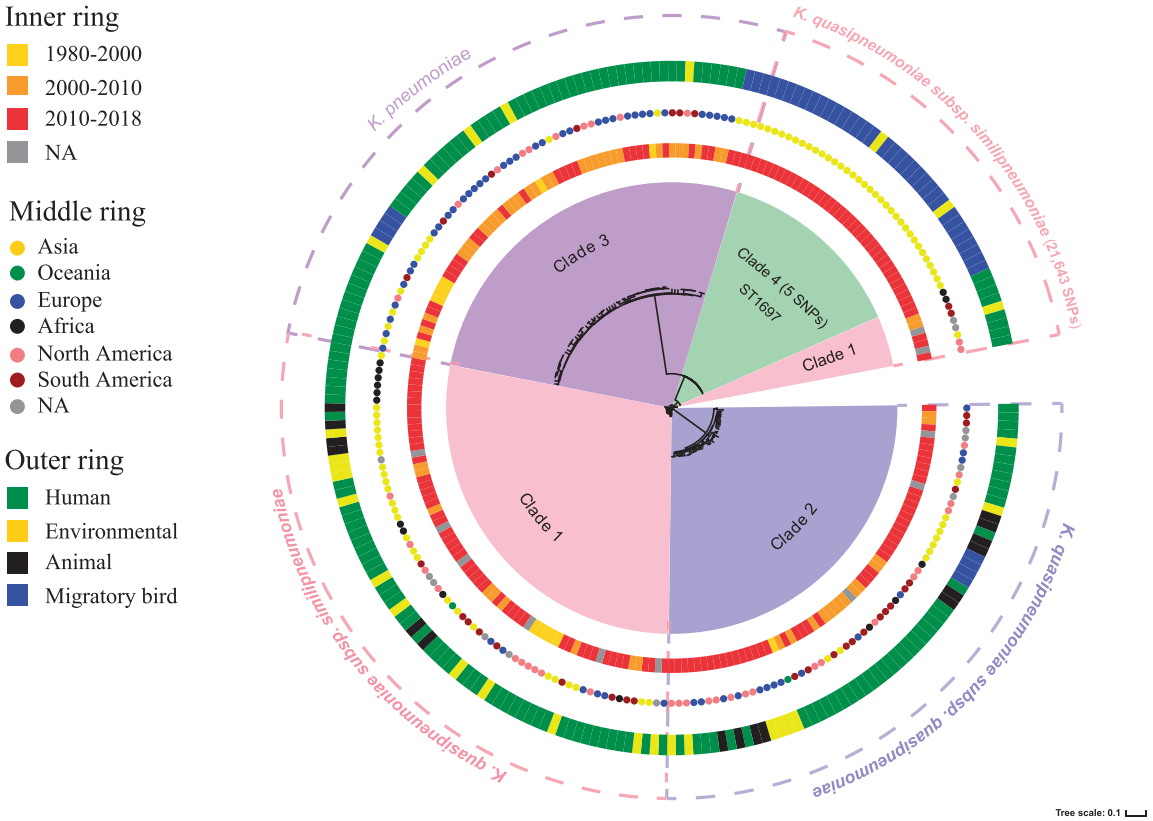


Figure 2. Clonal relationship and corresponding information on the *Klebsiella* spp. isolates. The midpoint-rooted maximum-likelihood phylogenetic tree of 241 *Klebsiella* spp. bacterial genomes was constructed using core-genome SNPs. Isolate collection date, isolate location and hosts from which isolates were obtained are marked on the inner, middle and outer rings, respectively. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

quasivariicola. In order to identify the phylogroups associated with NDM-5 carriage among the migratory birds, we then calculated the ANI of these genomes against *K. pneumoniae* complex reference genomes At-22 (*K. variicola*), MGH78578 (*K. pneumoniae*), 700603 (*K. quasipneumoniae* subsp. *similipneumoniae*), 18A069 (*K. quasipneumoniae* subsp. *quasipneumoniae*) and KPN1705 (*K. quasivariicola*). The results showed that genomes of ST35 and ST2582 had an ANIb value of >99% with respect to *K. pneumoniae* MGH78578, while ST2583 strains had an ANIb value of ~98.4% with respect to *K. quasipneumoniae* subsp. *quasipneumoniae* 18A069, and thus strains with these STs were designated *K. pneumoniae* (ST35 and ST2582) and *K. quasipneumoniae* subsp. *quasipneumoniae* (ST2583), respectively. However, the ST1697 genomes only showed the highest ANI values of ~97.8% with respect to *K. quasipneumoniae* subsp. *similipneumoniae*, and 93.2%–95.8% with respect to other species of *K. pneumoniae* complex. Pairwise ANI analysis of genomes from previously published *K. pneumoniae* complex isolates (n=17) showed that genomes from the same phylogroup usually shared >98.5% ANI, while 96%–98% was regarded as the boundary between different *K. pneumoniae* complex phylogroups.[28] If ANI values of 96% and 98% are used as the cut-offs for species differentiation and phylogroup designation, respectively, the ST1697 strains are more likely to be a novel subspecies or phylogroup that is closely related to *K. quasipneumoniae* subsp. *similipneumoniae*.

To confirm whether the ST1697 strains formed a new phylogroup, we performed a phylogenetic analysis with a total of 241 isolates, including 46 isolates in this study and 195 isolates downloaded from GenBank (Table S3). Among the 195 isolates downloaded, 136 belonged to KpII phylogroups *K. pneumoniae* and the remaining 59 *K. pneumoniae* isolates had detailed time and geospatial information. Consistent with some previous studies, the genomes from *Klebsiella* sp. were divided into four major clades sharing a total of 104 783 SNPs. All of the 34 ST1697 strains are clustered into clade 4, sharing only five SNPs among their core genome, and phylogenetically close to clade 1 (KpII-B, *K. quasipneumoniae* subsp. *similipneumoniae*) but formed a separate lineage, which supported the hypothesis that the ST1697 isolates could be a new subspecies in the *K. pneumoniae* complex (Figure 2).

We then used a BLAST-based *Klebsiella* species assignment program (https://github.com/rrwick/Klebsiella-assembly-species) to examine whether ST1697 is a hybrid strain resulting from cross-species recombination. Interestingly, the results showed that ~25.3% and 74.7% of the ST1697 genome (named QH1207, whose complete genome sequence was obtained from Illumina MiSeq and nanopore sequencing) showed high homology to the reference genome *K. pneumoniae* MGH78578 and *K. quasipneumoniae* subsp. *similipneumoniae* ATCC700603 respectively, suggesting that the ST1697 strain may be a hybrid of the

recombination of *K. quasipneumoniae* subsp. *similipneumoniae* and *K. pneumoniae* genomes. A further SNP frequency analysis was used to map the recombination sites (Tables S4 and S5). The analysis identified a ~1.3 Mbp contiguous recombination region, representing 25.3% the total chromosome. The above results suggested that the ST1697 strain is a hybrid strain, containing 74.7% (~3.8 Mbp) of the chromosome from *K. quasipneumoniae* subsp. *similipneumoniae* and 25.3% (~1.3 Mbp) from *K. pneumoniae* (Figure 3). Together, the ANI, phylogenetic and SNP analysis findings revealed that the ST1697 strain can be considered a novel subspecies of *K. quasipneumoniae*.

Plasmid transferability

In this study, different *K. pneumoniae* complex subspecies and *E. coli* have been found to carry the same bla_{NDM-5} variant. As bla_{NDM-5} is commonly found on conjugative plasmids, conjugation experiments were carried out among the 235 bla_{NDM-5}-positive strains (233 *Klebsiella* and 2 *E. coli*); plasmids carrying the bla_{NDM-5} gene from the 201 isolates (199 *Klebsiella* spp. presenting PFGE profiles A–D and 2 *E. coli*) were successfully transferred to *E. coli* C600 recipient strains. Interestingly, all 201 transconjugants had the carbapenem-resistance phenotype, and some transconjugants also showed resistance to cephalosporins (cefoxitin, cefotaxime and ceftazidime, >96%), florfenicol (12.4%), fosfomycin (7.5%), tetracycline and enrofloxacin (both 1.5%).

One bla_{NDM-5}-harbouring plasmid from a *K. quasipneumoniae* subsp. *similipneumoniae* ST1697 strain, QH1207, designated pQH1207, was completely sequenced. pQH1207 is 46 161 bp in size and belongs to the IncX3 incompatibility group, nearly identical to several plasmids reported previously in China and other counties (GenBank accession numbers NC_022740, KY435936, CP019444, KU167609, KU167608, KU647721, KU761328 and CP014006). These plasmids shared highly conserved plasmid backbone genes and contained minor difference in the bla_{NDM-5} neighbouring and other regions (Figure 4). The first bla_{NDM}-harbouring IncX3 plasmid (pNDM-HN380, bla_{NDM-1}) was reported from a *K. pneumoniae* isolate discovered in China in 2012.[29] Since then, IncX3 plasmids containing different bla_{NDM} alleles, such as bla_{NDM-1}, bla_{NDM-4}, bla_{NDM-7} and especially bla_{NDM-5}, have been reported from different geographical regions and in different Enterobacteriaceae species from clinical samples, meat and the environment.

Plasmid characterization

The plasmids extracted from all 201 transconjugants were digested with EcoRI and the fragment polymorphism patterns were compared. The results showed that 201 bla_{NDM-5}-bearing plasmids were divided into three pattern types (A–C), and each of the pattern types had an indistinguishable RFLP pattern (Figure 4a). Therefore, one transconjugant from each pattern type was selected to determine the plasmid location of the bla_{NDM-5} and bla_{NDM-5}-harbouring IncX3 plasmid. S1-PFGE in combination with Southern blot analysis showed that three transconjugants carry the same ~50 kb bla_{NDM-5}-harbouring IncX3 plasmid, similar in size to pQH1207. Subsequently, five pairs of primers were designed to screen pQH1207-like IncX3 plasmids (Table S6, Figure 4d). The PCR RFLP results showed that three plasmids were positive in the PCR screening, and yielded indistinguishable restriction fragment patterns (Figure 4b and c), suggesting the presence of nearly identical IncX3 plasmids in the transconjugants of *K. pneumoniae*, *K. quasipneumoniae* subsp. *quasipneumoniae*, *K. quasipneumoniae* subsp. *similipneumoniae* and *E. coli* (Figure 4a–c), in support of the horizontal transfer of the bla_{NDM-5}-harbouring IncX3 plasmids among bacteria in these migratory birds.

We then examined the transferability and conjugation frequencies of pQH1207 to other Enterobacteriaceae strains, including *E. coli* C600, *E. cloacae* ATCC 13047 and *Salmonella* ATCC 14028, as well as *A. baumannii* ATCC 19606. The results showed that pQH1207 could be successfully transferred to *E. coli* C600, *E. cloacae* ATCC 13047 and *Salmonella* ATCC 14028 with different conjugation frequencies ($3.50\pm2.14\times10^{-5}$, $4.80\pm2.51\times10^{-6}$ and $1.07\pm0.86\times10^{-10}$, respectively), but not to *A. baumannii* ATCC 19606.

The above plasmid screening results were consistent with several previous reports that the IncX3 plasmid serves as a key trafficker for the spread of bla_{NDM} genes in various ecological vectors globally.[30] The molecular mechanisms underlying its epidemiological and ecological success largely remain elusive. A previous study showed that IncX3 plasmids are genetically conserved, and have high conjugative compatibility at different temperatures (25, 30 and 37°C), and exert no fitness cost on their bacterial hosts.[31] Our study showed that the IncX3 plasmid could co-transfer with other resistance phenotypes to recipient strains. Since IncX3 plasmids contain a complete machinery of conjugative transfer, it may serve as a helper plasmid to facilitate the co-transfer of other non-conjugative plasmids or mobile elements, and consequently increase the ability to withstand different environmental stresses and confer antimicrobial resistance.

In this study, PFGE and MLST analysis demonstrated that *K. quasipneumoniae*, especially *K. quasipneumoniae* subsp. *similipneumoniae* ST1697, was the main clone carried by the migratory birds (202/233). The high detection rate of *K. quasipneumoniae* subsp. *similipneumoniae* among migratory birds suggested these bacteria may commonly colonize the intestine of these birds, probably through food or drinking water. *Klebsiella* spp. are common in humans, mammals and the environment; several previous studies demonstrated that *Klebsiella* spp. occurred in the intestines of wild birds, including pathogenic and resistant strains.[32–34] However, the original source of the NDM-5 in migratory birds remains unclear. The migration routes of migratory birds from Qinghai Lake were in the Central Asian Flyway and mainly linked three natural habitats, including Siberia, the west of China and the south-west of Asia (Figure 1).[23] The birds may acquire NDM-5-producing Enterobacteriaceae through contaminated water or food, from their habitats or in certain stopover sites, and transfer them to Qinghai Lake. The major concern is that the birds will carry these MDR bacteria throughout their migration routes and thus transmit the resistance to various geographical regions, consequently causing human infections. We suspected that these NDM-5-producing strains may be spread through the migratory flyway to north and south Asia, and further molecular studies along this migratory flyway should be investigated. Of note, besides the three novel STs first reported in this study, both *K. pneumoniae* ST35 and *K. quasipneumoniae* subsp. *similipneumoniae* ST1697 showed the ability to cause infection in humans. For example, the ST1697 strain was found in clinical isolates from drainage in Beijing and

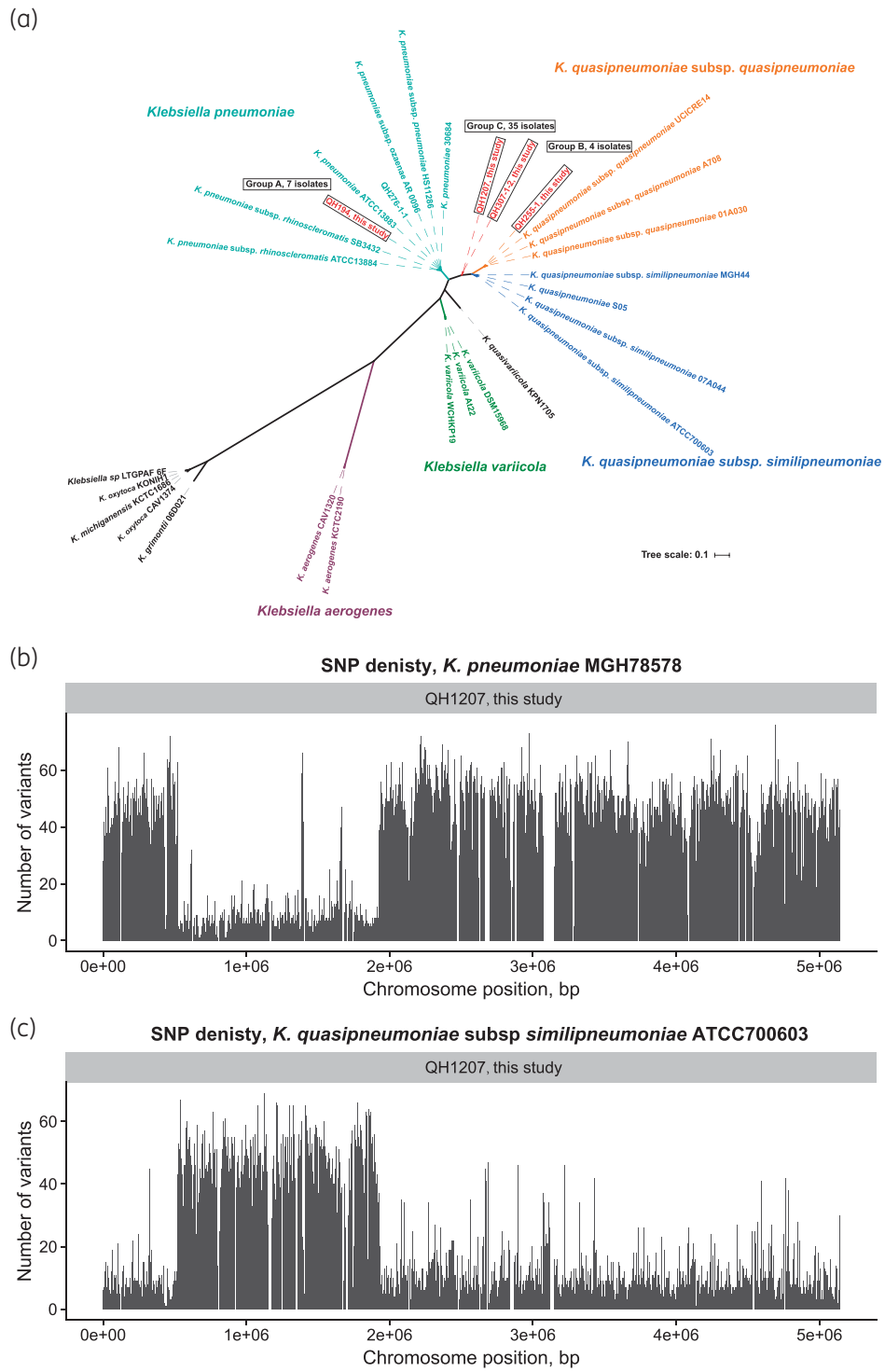
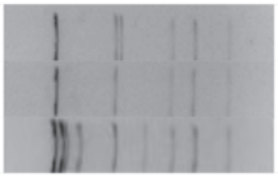


Figure 3. (a) Phylogenetic tree of *Klebsiella* isolates from this study and reference sequences from GenBank. Isolates from this study are represented in boxes. *K. pneumoniae*, *K. quasipneumoniae* subsp. *quasipneumoniae*, *K. quasipneumoniae* subsp. *similipneumoniae*, *K. variicola* and *Klebsiella aerogenes* are labelled. (b) Graphical distribution of variants of SNPs between *K. pneumoniae* subsp. *pneumoniae* MGH78578 and QH1207. (c) Graphical distribution of variants of SNPs between *K. quasipneumoniae* subsp. *similipneumoniae* ATCC70060 and QH1207. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

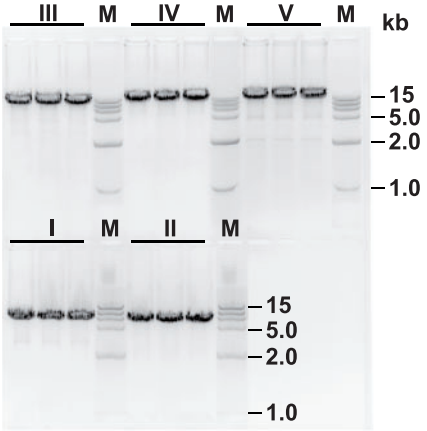
(a)

RFLP-EcoRI

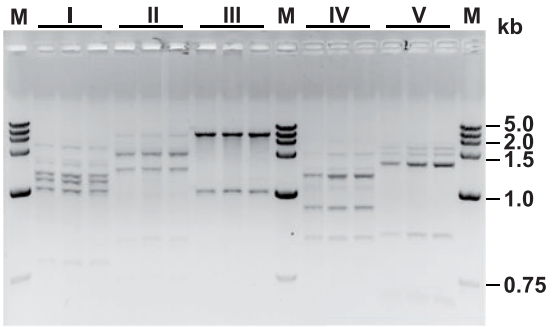


Pattern types	Number	Replicon types
A	2	IncX3
B	195	IncX3
C	4	IncX3

(b)



(c)



(d)

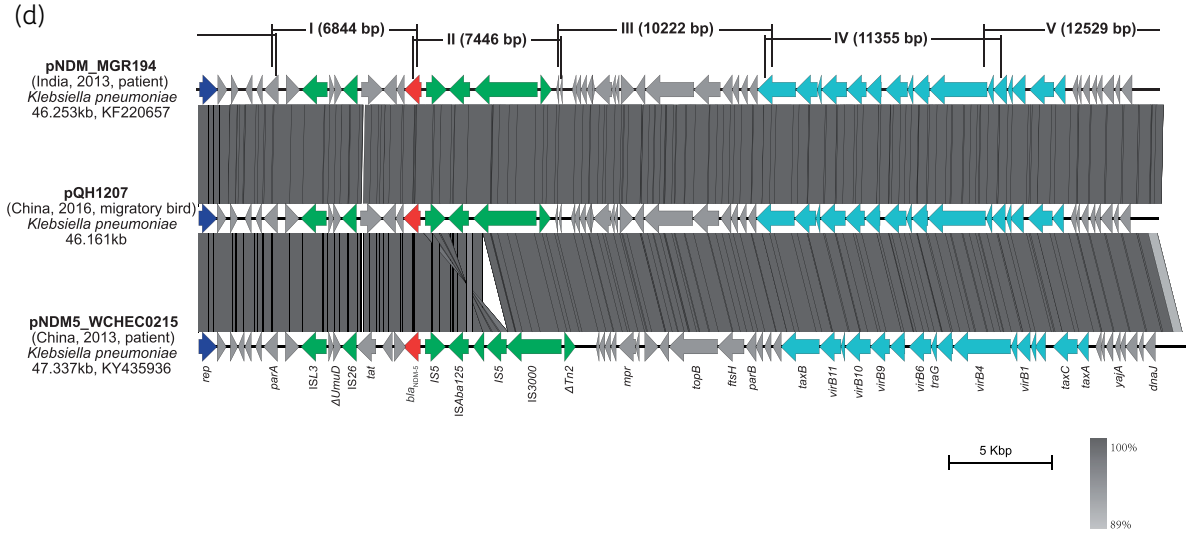


Figure 4. Plasmid analysis of bla_{NDM-5}-positive *Klebsiella* isolates. (a) EcoRI RFLP profiles and corresponding information on 201 transconjugants. (b) PCR results obtained using five primers. Lane M, DL 15000 marker. (c) BglII restriction digestion profiles of PCR products of the three transconjugants. Lane M, DL 5000 marker. (d) Linear sequence comparison of pQH1207 with other plasmids, including pNDM_MGR194 (accession number KU761327) and pNDM5_WCHEC0215 (accession number KY435936), both of which were derived from patients. The positions of five primers located on bla_{NDM-5}-IncX3 plasmid correspond to five conserved and variable regions. Boxed arrows represent the position and transcriptional direction of ORFs. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Taiwan as early as 2011, while ST35 appears to be an international clone, having been reported in several countries.[35] The data from our current study provided a novel route of bla_{NDM} gene transmission. Furthermore, because of the complicated migrational abilities of the migratory birds,[23,36] these NDM-5-positive *Klebsiella* spp. may accelerate the worldwide spread of the bla_{NDM-5} gene.

Taken together, these results uncover a high frequency of NDM-5-producing *K. quasipneumoniae* and an epidemic IncX3

resistance plasmid vector among migratory birds in the largest inland lake in China. Previous studies indicated that migratory birds may play an important role in the transmission of life-threatening bird flu,[36] and our results raise concern that these birds may serve as a reservoir to spread hard-to-treat MDR bacteria, posing a serious threat to public health. Continued vigilance for MDR carbapenemase-producing Enterobacteriaceae in migratory birds is urgently needed.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 and Tables S1–S6 are available as Supplementary data at *JAC* Online.

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