GLOBALLY OR REGIONALLY SPREAD OF EPIDEMIC PLASMIDS CARRYING CLINICALLY IMPORTANT RESISTANCE GENES: EPIDEMIOLOGY, MOLECULAR MECHANISM, AND DRIVERS

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Editorial: Globally or Regionally Spread of Epidemic Plasmids Carrying Clinically Important Resistance Genes: Epidemiology, Molecular Mechanism, and Drivers

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Keywords: plasmid, resistance gene, epidemiology, transfer mechanism, horizontal spread

Editorial on the Research Topic

Globally or Regionally Spread of Epidemic Plasmids Carrying Clinically Important Resistance Genes: Epidemiology, Molecular Mechanism, and Drivers

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Plasmids play a key role in the evolution of bacterial antibiotic resistance by acting as vehicles for horizontal gene transfer. Crucially, some clinically relevant resistance genes, like $bla_{\mathrm{CTX-M}}$, *mcr-1*, and bla_{NDM}, are commonly carried by epidemic plasmids (e.g., IncI2, IncX4, IncX3). These resistance plasmids have been disseminated globally to bacterial strains of diverse sources (e.g., animal, human being, foods, and environment). In addition, some epidemic plasmids, such as IncI1 plasmids harboring the $bla_{\mathrm{CTX-M-1}}$ gene, and the IncF33 plasmids carrying the $bla_{\mathrm{CTX-M-55/65}}$, *fosA3*, and/or $bla_{\mathrm{KPC-2}}$ genes, are known to have spread regionally among some European countries and China, respectively. The emergence of bacteria carrying resistance plasmids in various environmental niches is evidence of rapid propagation of antibiotic resistance among bacterial populations of diverse genetic backgrounds. Nevertheless, our knowledge regarding how and to what extent epidemic plasmids carrying clinically relevant resistance genes propels the evolution of antibiotic resistance remains inadequate. Within this topic, 11 articles published recently complement our knowledge of epidemiology, molecular mechanism, and drivers for the dissemination of the epidemic plasmids carrying clinically relevant resistance genes among bacteria from various environmental sources and geographical regions.

The sources of plasmids carrying antibiotic resistance genes as reported in these 11 articles were diverse, comprising human (Fan et al.; Prussing et al.; Wang et al.; Hirabayashi et al.; Huang et al.; Toledano-Tableros et al.), animal (Dor et al.) and food samples (Kurittu et al.). Resistance genes investigated in these works include not only the *bla* variants that encode β-lactamases, which degrade β-lactam antibiotics (Prussing et al.; Wang et al.; Hirabayashi et al.; Huang et al.; Toledano-Tableros et al.), and the *mcr-1* gene that confers resistance to polymyxins (Fan et al.), but also the *lsa*(E) gene that mediates resistance to pleuromutilin, lincosamide, and streptogramin A (PLSA phenotype) (Yan et al.). The studies described in these publications reveal that resistance plasmids of different origins are often structurally related to each other.

Strains of Enterobacteriaceae producing extended-spectrum beta-lactamase (ESBL), plasmid-mediated AmpC-type cephalosporinase (pAmpC) and carbapenemase pose a serious threat to human health. The potential zoonotic origin of ESBL/AmpC-producing bacteria was

investigated by two studies (Dor et al.; Kurittu et al.). Kurittu et al. observed ESBL/pAmpC carriage in *Escherichia coli* and/or *Klebsiella pneumoniae* in 14 (7%) of 200 food products collected from 35 countries, as well as 36 (90%) of 40 broiler meat samples. The finding that the dissemination of ESBL/pAmpC genes is mediated by highly transmissible epidemic plasmids (e.g., IncFII and IncI1) highlights the risks of spreading resistant organisms through international trade (Kurittu et al.). An additional article by Dor et al. reported the emergence of ESBL-producing *Salmonella enterica* (ESBL-S) in hospitalized horses in Israel and attributed the surge of CTX-M-3 ESBL-encoding strains mainly to the dissemination of a broad host range plasmid (IncM2) among animal-borne *Salmonella* strains (Dor et al.).

Five studies reported the presence and genetic features of carbapenemase-producing Enterobacteriaceae strains isolated from patients. Prussing et al. investigated the prevalence of KPC-producing bacteria in two healthcare facilities in the United States and reported identical plasmids carrying the bla_{KPC-2} gene in four isolates belonging to three bacterial genera (*Citrobacter freundii*, *K. pneumoniae*, and *E. coli*) in the United States (Prussing et al.). Huang et al. provide key insights into the diverse genetic structures carrying the bla_{KPC-2} gene in clinical carbapenem-resistant hypervirulent *K. pneumoniae* (CR-hvKP) strains in Southern China. In their study, the IncF plasmids with a bla_{KPC}-bearing non-Tn4401 element and the pLVPK-like virulence plasmid were found to be responsible for the propagation of CR-hvKP, especially those of the ST11 type (Huang et al.). Also, horizontal transfer of the bla_{NDM-1} gene through IncF-like plasmids among *K. pneumoniae* strains of different sequence types in a tertiary referral hospital in Mexico was reported (Toledano-Tableros et al.). On the other hand, carbapenemase-resistance genes (e.g., bla_{NDM-5}, bla_{OXA-48}, and bla_{OXA-23}) harbored by clinical isolates were reported by Hirabayashi et al.. Based on on-site genomic and epidemiological analyses of carbapenemase-producing Gram-negative bacteria (CPGNB) using portable laboratory equipment, this work provided the first glimpse into the epidemiology of CPGNB in Cambodia and found that two *E. coli* isolates and one *Acinetobacter baumannii* isolate harbored the carbapenemase genes bla_{NDM-5} (carried by IncF), bla_{OXA-48} (carried by IncX3), and bla_{OXA-23} (located in chromosome), respectively (Hirabayashi et al.). In another study from China, Wang et al. reported the recovery of an extensively drug-resistant (XDR) *E. coli* strain from a patient. This XDR *E. coli* strain was found to carry two plasmids: an IncFIB plasmid carrying the bla_{NDM-5} gene and an IncFII plasmid carrying a truncated bla_{TEM} gene (Wang et al.). These two plasmids were found to be responsible for the resistance phenotypes expressed by this strain, including resistance to all commonly used β-lactam/BLI combinations (Wang et al.).

Rapid dissemination of the plasmid-borne colistin resistance determinant *mcr-1* threatens clinical usage of polymyxins, one of the last-resort antibiotics, to treat multidrug-resistant infections. Fan et al. investigated the origin and genetic characteristics of the *mcr-1* gene in clinical *Salmonella* strains collected from patients in China between 2009 and 2018 (Fan et al.). They reported that the *mcr-1* gene was detectable in six of the 689 *Salmonella* strains (0.87%) collected during this period and that *mcr-1* might be carried by two types of plasmids (IncHI2, $n = 4$; IncI2, $n = 1$) or located in the chromosome ($n = 1$). Besides, recovery of plasmids harboring the *lsa* genes also raised much concern as dissemination of these genes to *Enterococcus faecium* and *Staphylococcus aureus* may further increase the clinical burden due to drug-resistant bacterial infections. Yan et al. collected 96 *E. faecium* strains from one hospital in Beijing in 2013 and found that 46 (47.92%) and two isolates (2.08%) were positive to *lsa*(E) and *lsa*(A), respectively (Yan et al.). In this work, evaluation of the transferability of the *lsa*(E) gene provided the first evidence that *lsa*(E) can be transferred via plasmid conjugation from *E. faecium* to *S. aureus in vitro*.

To better understand how plasmids evolve, Zhang et al. characterized the diversification and evolution history of plasmids belonging to the incompatibility group C (IncC) (Zhang et al.). These researchers identified 20 IncC-positive strains from 870 Enterobacteriaceae strains isolated from food-producing animals in China. Based on four key structural differences in the IncC backbone, 20 IncC plasmids were classified into type 1 ($n = 4$), type 1/2 hybrid ($n = 15$), and type 2 ($n = 1$), respectively. Further analysis of the accessory resistance modules in these plasmids revealed that the various phenotypes expressed by the host strains carrying such plasmids were driven by loss and gain of various genetic modules within the bla_{CMY}-bearing region and two antibiotic resistance islands (ARI-A and ARI-B), as well as *via* IS*26* or IS*1294*-mediated genetic rearrangements (Zhang et al.).

Plasmids are generally transferred through either conjugation or natural transformation. The transfer of the resistance genes *via* a genetic vehicle (plasmids) has been widely investigated under laboratory conditions; however, research on bacterial plasmid conjugation in the intestinal microbiota remains scarce. A mini-review by Neil et al. describes the molecular mechanisms underlying plasmid conjugation in the gut microbiota. This article summarized, based on currently available evidence, that known conjugative plasmids were not efficiently transmissible among members of the intestinal microbiota. Moreover, these authors discussed the mating-pair stabilization (MPS) mechanisms and factors that influence bacterial conjugation, such as T4SS compatibility, replication compatibility and MPS specificity. Knowledge of bacterial plasmid conjugation *in situ* could facilitate the use of microbiome editing technologies to control antibiotic resistance (Neil et al.).

In summary, articles presented in this Research Topic demonstrated that plasmids are one of the main driving forces in the evolution of bacterial antibiotic resistance and highlighted the importance of collaboration among experts in different fields to tackle antibiotic resistance-related problems. More effort is needed to decipher the molecular mechanisms contributing to the formation and extensive transmission of epidemic plasmids among bacterial pathogens.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Nanopore MinION Sequencing Reveals Possible Transfer of *bla*$_{KPC-2}$ Plasmid Across Bacterial Species in Two Healthcare Facilities

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Carbapenemase-producing *Enterobacteriaceae* are a major threat to global public health. *Klebsiella pneumoniae* carbapenemase (KPC) is the most commonly identified carbapenemase in the United States and is frequently found on mobile genetic elements including plasmids, which can be horizontally transmitted between bacteria of the same or different species. Here we describe the results of an epidemiological investigation of KPC-producing bacteria at two healthcare facilities. Using a combination of short-read and long-read whole-genome sequencing, we identified an identical 44 kilobase plasmid carrying the *bla*$_{KPC-2}$ gene in four bacterial isolates belonging to three different species (*Citrobacter freundii*, *Klebsiella pneumoniae*, and *Escherichia coli*). The isolates in this investigation were collected from patients who were epidemiologically linked in a region in which KPC was uncommon, suggesting that the antibiotic resistance plasmid was transmitted between these bacterial species. This investigation highlights the importance of long-read sequencing in investigating the relatedness of bacterial plasmids, and in elucidating potential plasmid-mediated outbreaks caused by antibiotic resistant bacteria.

Keywords: carbapenem-resistant enterobacteriaceae, *klebsiella* pneumoniae carbapenemase, horizontal gene transfer, plasmids, long-read sequencing, hybrid genome assembly, molecular epidemiology

INTRODUCTION

Carbapenem-resistant *Enterobacteriaceae* (CRE) are an urgent global health threat, and have been categorized by the World Health Organization (World Health Organization [WHO], 2017) and the United States Centers for Disease Control and Prevention (CDC) (Centers for Disease Control and Prevention [CDC], 2019a) as top priorities for research, drug discovery, surveillance, and control. CRE that produce carbapenemases are particularly concerning epidemiologically because carbapenemase genes can be transferred among bacteria via mobile genetic elements, including plasmids (Bonomo et al., 2017). In the United States, the most commonly identified carbapenemase is *Klebsiella pneumoniae* carbapenemase (KPC), which has become endemic in parts of the country since it was first described in 1996 (Woodworth et al., 2018; Castanheira et al., 2019).

Though first identified in *K. pneumoniae*, the gene encoding KPC has been detected across multiple genera of gram-negative bacteria (Woodworth et al., 2018; Brandt et al., 2019). Over twenty variants of bla_{KPC} have been described, of which bla_{KPC-2} and bla_{KPC-3} are the most frequently detected (Castanheira et al., 2019). bla_{KPC} genes are commonly carried inside transposons, in particular Tn*4401*, a 10kb self-mobilizing transposon in the Tn*3* family (Cuzon et al., 2011; Partridge et al., 2018). Tn*4401* and bla_{KPC} are often encoded on plasmids (Brandt et al., 2019), but can also be found integrated into the bacterial chromosome (Mathers et al., 2017).

Though dissemination of KPC-producing bacteria can result from epidemic spread of clonal lineages (Kitchel et al., 2009; Hargreaves et al., 2015), horizontal transfer of KPC-encoding plasmids across unrelated bacteria of the same or different species has also been described (Conlan et al., 2014, 2019; Sheppard et al., 2016; Li et al., 2018; Evans et al., 2020). Detecting such instances of plasmid transfer requires the use of whole-genome sequencing (WGS) to characterize the genomic context of antibiotic resistance genes (Boolchandani et al., 2019). As short-read sequencing has been shown to be limited in its ability to resolve the highly repetitive regions common in plasmids (Arredondo-Alonso et al., 2017), combined short-read and long-read WGS of antibiotic resistant bacteria has become an increasingly common method to better elucidate plasmid structures, and to detect plasmid-mediated outbreaks (Lemon et al., 2017; Conlan et al., 2019; Decano et al., 2019; Van Dorp et al., 2019; Wyres et al., 2019). In this study, we describe a possible case of plasmid transfer detected by hybrid analysis of short read Illumina MiSeq and long-read Oxford Nanopore Technologies (ONT) MinION sequencing, in which an identical plasmid carrying bla_{KPC-2} was identified across three bacterial species isolated from epidemiologically linked patients in two healthcare facilities.

MATERIALS AND METHODS

Epidemiological Investigation

The Maine Center for Disease Control and Prevention utilizes a combination of required and voluntary reporting, along with isolate submission to the state public health laboratory (SPHL), to identify CRE that produce carbapenemases. In 2018, the SPHL identified KPC-producing bacteria in two unique clinical specimens in the same month. In response, and in accordance with guidance from the Centers for Disease Control and Prevention [CDC], 2019b, investigators arranged for colonization screens of epidemiologically linked patients who had been admitted at two associated healthcare facilities by rectal swab. Specimens from patients who had been discharged home were collected by walk-in clinics at one associated facility and by patients' primary care providers.

The Wadsworth Center, New York State Department of Health's public health laboratory and the Northeast Regional Laboratory for the CDC funded Antimicrobial Resistance Laboratory Network (ARLN), analyzed all rectal swab specimens from colonization screens with the Cepheid Xpert® Carba-R test. This test is a real-time PCR assay for rapid detection and differentiation of five genes (bla_{KPC}, bla_{VIM}, bla_{OXA-48}, bla_{IMP-1}, and bla_{NDM}) responsible for carbapenem resistance.

This screening identified three additional patients with KPC-producing bacteria representing three species: *Citrobacter freundii*, *Klebsiella pneumoniae*, and *Escherichia coli*. Traditionally, outbreak definitions include a requirement for matching organisms; however, as carbapenemase genes are transferrable among bacterial species and epidemiological links were present, further investigation of possible plasmid transfer was undertaken.

Microbiological Methods

Isolates were recovered from rectal swab specimens that were positive for bla_{KPC} by the Cepheid Xpert® Carba-R test by streaking on MacConkey agar. For clinical and colonization isolates, identification to species was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Antibiotic susceptibility testing was performed by broth microdilution with the Thermo Scientific Sensititre Aris 2X using the GNX2F panel, and by ETEST® (bioMérieux) for ceftazidime-avibactam (all specimens) and ertapenem, meropenem, and imipenem (Isolate 5 only). In addition, disk diffusion was used to test the susceptibility of Isolate 5 to doripenem, ertapenem, imipenem, and meropenem. Antibiotic susceptibilities were interpreted using CLSI M100-ED29 breakpoints for Enterobacteriales (Clinical and Laboratory Standards Institute [CLSI], 2019). The modified carbapenem inactivation method (mCIM) (Clinical and Laboratory Standards Institute [CLSI], 2019) was used to detect carbapenemase production. Molecular characterization of resistance mechanisms was performed using New York State Clinical Laboratory Evaluation Program (CLEP)-approved multiplex real-time PCR assays to detect bla_{KPC} and bla_{NDM} (developed at the Wadsworth Center), as well as bla_{VIM}, bla_{IMP} (all variants), and $bla_{OXA-48-like}$ genes (developed at the CDC).

Illumina WGS and Analysis

Genomic DNA was extracted from isolates using the DNeasy Blood & Tissue Kit on a QIAcube (QIAGEN). DNA was quantified using the Qubit dsDNA BR assay system. Sequence libraries were prepared using the Nextera XT DNA Sample Preparation Kit and sequenced on the Illumina MiSeq system at the Wadsworth Center Applied Genomic Technologies Core.

Raw Illumina reads were processed with *Trimmomatic* v0.38 (Bolger et al., 2014) and bacterial species identification was confirmed *in-silico* using *Kraken* v1.0 (Wood and Salzberg, 2014) with the MiniKraken 8GB database; paired, 250 bp reads were then de novo assembled into contigs with *SPAdes* v3.12.0 (Bankevich et al., 2012). Assembly quality was assessed using quantitative measurements, including *BUSCO* v3.1.0 (Simão et al., 2015; Waterhouse et al., 2017), prior to multilocus sequence typing analysis (MLST) with *mlst* v2.16.2[1] and AR gene identification with *ABRicate* v0.8.13.[2] Final analysis of the

[1]https://github.com/tseemann/mlst

[2]https://github.com/tseemann/abricate

antibiotic resistance genes in the genome assembly compared gene identification between the NCBI Bacterial Antimicrobial Resistance Reference Gene Database (NCBI) (Feldgarden et al., 2019), ResFinder (Zankari et al., 2012), and Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2016) databases to determine the best matches.

For mutation event analysis, *Mash* v1.1 (Ondov et al., 2016) was used to select the best possible reference genome from a number of candidates prior to mapping with *BWA/Samtools* [v0.7.17 and v1.9, respectively (Li and Durbin, 2009, 2010; Li et al., 2009)]. Mutation events (ME), defined as the number of single nucleotide polymorphisms (SNPs) and insertion/deletion events, were called using *FreeBayes* v1.0.2 (Garrison and Marth, 2012). A ME matrix was constructed from all isolates within a cluster by pairwise comparison of all reference-aligned sequences to count MEs while ignoring ambiguous or missing bases.

ONT MinION WGS

For genomic DNA extraction, all isolates were sub-cultured from frozen stocks twice on blood agar plates. Colonies were resuspended in 2 ml sterile water to 4 McFarland concentration and harvested as pellets. High molecular weight genomic DNA was extracted from Isolates 1, 3, 4, and 5 using Genomic-tip 20/G (QIAGEN) and Genomic DNA buffers (QIAGEN). Genomic DNA from Isolate 2 was extracted using the Nanobind CBB Big DNA kit (Circulomics). The protocol for genomic DNA isolation for gram negative bacteria was followed for both methods as suggested by the respective manufacturer. The genomic DNA was quantified using a Qubit fluorometer (ThermoFisher Scientific). Quality of the genomic DNA was assessed using the TapeStation (Agilent).

MinION sequencing libraries were prepared from 1.5 μg of input DNA. Genomic DNA was sheared in 50 μl total volume in Covaris G tubes using an Eppendorf 5425 centrifuge at 6000 rpm. The sequencing library was prepared according to manufacturer's instructions (Oxford Nanopore) and multiplexed using 1D Native barcoding kits (EXP-NBD104, EXP-NBD114) followed by ligation and sequencing kit (SQK-LSK109). The library was loaded on a SpotON flowcell R9.4.1 FLO-MIN106 and sequenced for 72 h on the MinION device. The fast5 data from MinKNOW was converted to fastq format using the Guppy basecaller in fast mode on a MinIT device (Oxford Nanopore, United Kingdom). The fastq reads were demultiplexed using qcat v1.0.1.[3]

Hybrid Genome Assembly and Annotation

MinION reads were quality filtered using *filtlong* v0.2.0[4] with a minimum read length of 1000 and the target number of bases set to 500,000,000 (to provide approximately 100X coverage of the target species' genomes). Paired-end MiSeq reads were trimmed to remove adapters and low-quality ends (<q10) using *trim_galore* v0.6.4.[5]

[3]https://github.com/nanoporetech/qcat

[4]https://github.com/rrwick/Filtlong

[5]https://github.com/FelixKrueger/TrimGalore

Genomes were assembled using two methods: *Unicycler* v0.4.8 (Wick et al., 2017) hybrid assembly using the filtered MinION reads and trimmed Illumina reads, with default settings; and *Flye* v2.6 (Kolmogorov et al., 2019) using the filtered MinION reads, with plasmids and meta options enabled. *Flye* assemblies were polished with *racon* v1.4.7 (Vaser et al., 2017) (-m 8 -x -6 -g -8 -w 500 –no-trimming) using MinION reads mapped to the assembly with *minimap2* v2.11 (Li, 2018), followed by *medaka* v0.8.1[6] and two rounds of *pilon* v1.23 (Walker et al., 2014) using Illumina reads mapped to the assembly with *bwa-mem* (Li, 2013). Discrepancies between the assemblies were assessed by comparing the percentage of Illumina reads that aligned to the assembly using *bwa-mem*, and the percentage of filtered MinION reads that aligned to the assembly using *minimap2*. In addition, Assembly Likelihood Evaluation (ALE) scores (Clark et al., 2013) were calculated using both the Illumina and MinION alignment files, and *Nanovar* v0.1.2 (Tham et al., 2019) was used to identify structural discrepancies between MinION reads and the assemblies. All Illumina and MinION sequences were deposited in the NCBI sequence read archive (SRA), and final assemblies in GenBank (BioProject ID PRJNA636827).

Plasflow v1.1 (Krawczyk et al., 2018) was used to classify contigs as plasmids or chromosomes. Final genomes were annotated with *prokka* v1.14.0 (Seemann, 2014). *Abricate* v0.9.8 (see text footnote 2) detected antimicrobial resistance genes using the CARD (Jia et al., 2016), ResFinder (Zankari et al., 2012), and NCBI (Feldgarden et al., 2019) databases, and plasmid replicon genes using the PlasmidFinder (Carattoli et al., 2014) database. Identified plasmids were queried against the PLSDB database v2019_10_07 (Galata et al., 2018) using mash (-S 42 -k 21 -s 1000) (Ondov et al., 2016) to identify related plasmids.

RESULTS

Following the identification of two patients with KPC-producing bacteria associated with Facility A (Patient 1, Isolate 1: *C. freundii*; Patient 2, Isolate 2: *K. pneumoniae*), a retrospective investigation including colonization screening of patients who had been roommates with or who had overlapped on the same unit for three or more days with either of the two source patients was performed. Rectal swabs from two additional patients who had been on the same unit at Facility A tested positive for KPC-producing bacteria after these patients had been discharged. Isolate recovery from one rectal swab specimen was unsuccessful (Patient X), but a KPC-producing *K. pneumoniae* (Isolate 3 from Patient 3) was recovered from the second specimen. Following the initial investigation, routine point prevalence surveys were done at Facility A on the affected unit to identify any additional KPC-producing bacteria. 3 months after the initial investigation, Isolate 4 (*C. freundii*) was recovered from a rectal swab collected from Patient 4, who had recently been admitted to Facility A (**Figure 1**).

Prior to the initiation of this investigation, Patients 2, X, and 3 had been transferred from Facility A to Facility B. Patients 2 and X

[6]https://github.com/nanoporetech/medaka

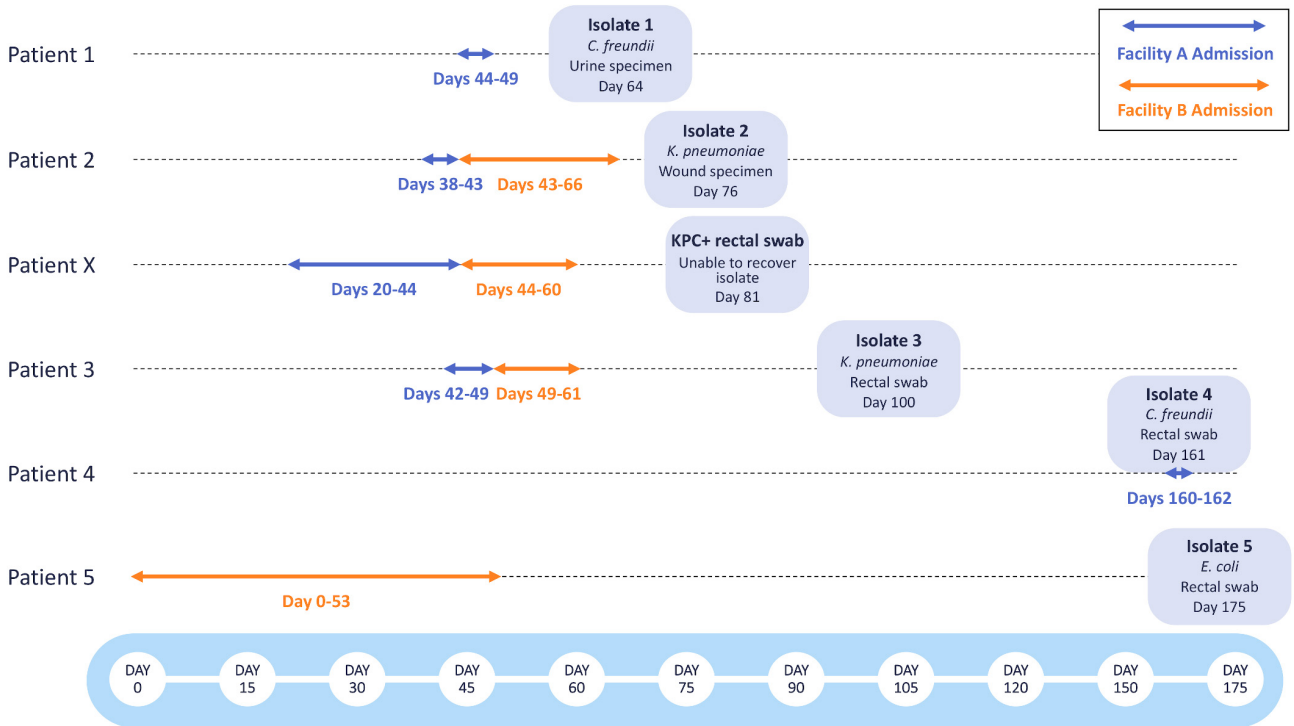

FIGURE 1 | Timeline of KPC-producing bacterial isolates collected during epidemiological investigations at Facilities A and B. The admission dates of Patients 1-5 and Patient X at Facility A (blue) and Facility B (orange) are shown as arrows. The bacterial species, specimen type, and date of specimen collection are shown for each bacterial isolate.

were placed in isolation at the time of admission to Facility B, but Patient 3 was assigned to a double occupancy room with a shared bathroom, sharing the room with Patient 5 for 5 days. After discharge from Facility B, a rectal swab obtained from Patient 5 yielded Isolate 5 (*E. coli*). All other rectal swabs collected from patients overlapping on the same unit for three or more days at Facility B tested negative for KPC-producing bacteria.

Isolates 1–5 differed in their antibiotic susceptibility profiles (**Table 1**), but all isolates were resistant to aztreonam and ticarcillin/clavulanic acid; Isolates 3 and 4 were non-susceptible to all beta-lactam antibiotics tested. Isolate 5 was susceptible to all carbapenems by broth microdilution. By disk diffusion, Isolate 5 had intermediate susceptibility to ertapenem (21 mm) and imipenem (22.5 mm), and was susceptible to doripenem (23 mm) and meropenem (23 mm). By gradient diffusion, Isolate 5 was susceptible to ertapenem (MIC 0.25 μg/ml), meropenem (MIC 0.19 μg/ml), and imipenem (MIC 1.0 μg/ml). All isolates tested positive for carbapenemase production by mCIM except for Isolate 4, which was not tested. Real-time PCR detected only bla_{KPC} in all isolates.

By Illumina WGS, the two *C. freundii* isolates (Isolates 1 and 4) were not able to be assigned to a sequence type, but had distinct alleles at six of seven MLST loci, and therefore were determined to be unrelated. The two *K. pneumoniae* isolates (Isolates 2 and 3) were both ST37 and were closely related, differing by only seven mutation events. Between five and twenty antibiotic resistance genes were identified from each of the five isolates (**Supplementary Table 1**). Two of these genes, both encoding beta-lactamases (bla_{KPC-2} and bla_{TEM-1}) were identified at >90% coverage and identity from all isolates.

Hybrid genome assembly methods combining ONT MinION and Illumina reads were used to determine the location of bla_{KPC-2} within the genomes of the five isolates. Complete genome assemblies consisting of only circular contigs were generated for all isolates with the exception of Isolate 2, which consisted of four circular contigs and a single small (8.5 kilobase) linear contig (**Supplementary Methods**). Assemblies for all five isolates consisted of closed chromosomes and between two and six additional small contigs. Details on assembly evaluation and rationales for the final choice of assembly method for each isolate are provided (**Supplementary Methods**). Each isolate harbored at least one plasmid that carried one or more antibiotic resistance gene (**Table 2**).

Isolate 1 (*C. freundii*) carried copies of the bla_{KPC-2} gene on two unique plasmids: a 43,621 bp plasmid that also carried bla_{TEM-1} (p1C44), and a 73,366 bp plasmid that did not carry other known resistance genes (p1C73). p1C44 is 100% identical across its entire length to the plasmid pKPC_UVA01 (Genbank accession no. CP017937.1), first described in a *K. pneumoniae* isolated from an abdominal abscess at the University of Virginia Health System in 2007 (Mathers et al., 2015).

Isolate 2 (*K. pneumoniae*), Isolate 3 (*K. pneumoniae*), and Isolate 5 (*E. coli*) also carried the bla_{KPC-2} gene on a plasmid identical (p5E44) or nearly identical (p2K44

TABLE 1 | Antibiotic susceptibility testing results.

Antibiotic Class/Antibiotic	Isolate 1: *C. freundii* MIC (μg/ml)/ Interpretation	Isolate 2: *K. pneumoniae* MIC (μg/ml)/ Interpretation	Isolate 3: *K. pneumoniae* MIC (μg/ml)/ Interpretation	Isolate 4: *C. freundii* MIC (μg/ml)/ Interpretation	Isolate 5: *E. coli* MIC (μg/ml)/ Interpretation
Aminoglycosides					
Amikacin	≤4/S	≤4/S	≤4/S	≤4/S	≤4/S
Gentamicin	>8/R	>8/R	>8/R	>8/R	≤1/S
Tobramycin	>8/R	>8/R	>8/R	2/S	≤1/S
Beta-lactams					
Aztreonam	16/R	>16/R	>16/R	>16/R	16/R
Cefepime	≤2/S	8/SDD	16/R	16/R	≤2/S
Cefotaxime	8/R	>32/R	>32/R	32/R	≤1/S
Ceftazidime	2/S	>16/R	>16/R	>16/R	2/S
Ceftazidime/ Avibactam	0.38/S	0.38/S	1.0/S	2/S	0.25/S
Doripenem	1/S	1/S	2/I	>2/R	0.5/S
Ertapenem	2/R	2/R	4/R	>4/R	≤0.25/S
Imipenem	2/I	4/R	4/R	8/R	≤1/S
Meropenem	4/R	2/I	4/R	8/R	≤1/S
Piperacillin/ tazobactam	>64/R	>64/R	>64/R	>64/R	32/I
Ticarcillin/ clavulanic acid	>128/R	>128/R	>128/R	>128/R	>128/R
Fluoroquinolones					
Ciprofloxacin	>2/R	2/R	>2/R	>2/R	≤0.25/S
Levofloxacin	>8/R	≤1/S	4/R	>8/R	≤1/S
Lipopeptides					
Colistin	0.5/NI	≤0.25/NI	≤0.25/NI	0.5/NI	≤0.25/NI
Polymyxin B	0.5/NI	0.5/NI	≤0.25/NI	0.5/NI	≤0.25/NI
Tetracyclines					
Doxycycline	8/I	16/R	>16/R	4/S	≤2/S
Minocycline	4/S	4/S	>16/R	4/S	≤2/S
Glycycyclines					
Tigecycline	0.5/NI	0.5/NI	2/NI	0.5/NI	≤0.25/NI

Susceptibility testing was performed by gradient diffusion (Ceftazidime/Avibactam) or broth microdilution (all other antibiotics), and results were interpreted using CLSI M100-ED29 breakpoints. MIC = minimum inhibitory concentration, S = susceptible, SDD = susceptible-dose dependent, I = intermediate, R = resistant, NI = not interpretable.

and p3K44) to pKPC_UVA01 (**Figure 2**). Compared with pKPC_UVA01, there were two single base indels in p2K44 (a deleted thymine corresponding to position 12,990 of pKPC_UVA01 and an inserted guanine corresponding to position 30,639 of pKPC_UVA01, both located in five-nucleotide homopolymers). Similarly, there was a deleted cytosine in p3K44 in a five nucleotide homopolymer corresponding to position 13,910 of pKPC_UVA01. As the five plasmids were otherwise identical across their entire lengths, and as these indels all occurred in homopolymers, the length of which is known to be commonly mis-identified by MinION sequencers (Wick et al., 2019), they likely represent sequencing, assembly, or polishing errors and not true differences in the plasmid sequences. This is supported by mapping of Illumina reads from Isolates 2 and 3 to pKPC_UVA01, which shows that the Illumina reads do not support the deletions in p2K44 or p3K44, though they do support the insertion in p2K44 (**Supplementary Methods**).

Unlike the other isolates, Isolate 4 (*C. freundii*) carried bla_{KPC-2} on a 12,158bp Col440I plasmid (p4C12). Like pKPC_UVA01, p1C73 and p4C12 carried bla_{KPC-2} within the transposon Tn4401b, but the plasmids did not otherwise share sequence identity (**Figure 3**). A PLSDB query found that p4C12 was most similar (mash distance 0.006, 781 of 1000 shared hashes) to a 9,803bp bla_{KPC-3}-containing plasmid isolated from *K. pneumoniae* in Spain (GenBank accession no. NC_019151.1). No plasmids similar to p1C73 were identified (lowest mash distance 0.04, 291 of 1000 shared hashes for GenBank accession no. NZ_CP039300.1).

In addition to sharing high chromosomal genomic similarity and near-identical bla_{KPC-2} plasmids, Isolates 2 and 3 also shared similar 157kb IncFIA(HI1) plasmids carrying eleven antibiotic resistance genes (**Table 2**). Despite carrying identical replicon and resistance genes, these two plasmids were not identical across their entire sequence (mash distance 0.004, 836 of 1000 shared hashes).

TABLE 2 | Characterization of plasmids carrying antibiotic resistance genes.

Sample ID	Final Assembly Method	Plasmid ID and length (bp)	Plasmid replicon genes	Resistance genes
Isolate 1: *C. freundii*	Unicycler hybrid	p1C157: 156,725	IncFIB(pB171), IncFII(S)	*sul1*, *arr-3*, *catB3*, bla_{OXA-1}, *aac(6′)-Ib-cr*, bla_{TEM-1}, *aac(3)-IId*, *mphA*, *sul2*
		p1C73: 73,366	None identified	bla_{KPC-2}
		p1C44: 43,621	repA_1_pKPC-2	bla_{KPC-2}, bla_{TEM-1}
Isolate 2: *K. pneumoniae*	Flye	p2K157: 156,883	IncFIA(HI1)	*aac(3)-IIa*, bla_{OXA-1}, *aac(6′)-Ib-cr*, $bla_{CTX-M-15}$, bla_{TEM-1}, *aph(6)-Id*, *aph(3″)-Ib*, *sul2*, *dfrA14*, *qnrB1*, *tet(A)*
		p2K44: 43,621	repA_1_pKPC-2	bla_{KPC-2}, bla_{TEM-1}
Isolate 3: *K. pneumoniae*	Flye	p3K157: 156,980	IncFIA(HI1)	*aac(3)-IIa*, bla_{OXA-1}, *aac(6′)-Ib-cr*, $bla_{CTX-M-15}$, bla_{TEM-1}, *aph(6)-Id*, *aph(3″)-Ib*, *sul2*, *dfrA14*, *qnrB1*, *tet(A)*
		p3K44: 43,620	repA_1_pKPC-2	bla_{KPC-2}, bla_{TEM-1}
Isolate 4: *C. freundii*	Flye	p4C141: 140,774	IncFII(Yp), IncFIB(pB171)	*mphA*, *dfrA12*, *aadA2*, *sul1*, *sul2*
		p4C12: 12,158	Col440I_1	bla_{KPC-2}
Isolate 5: *E. coli*	Unicycler hybrid, no depth filter	p5E44: 43,621	repA_1_pKPC-2	bla_{KPC-2}, bla_{TEM-1}

Resistance genes and plasmid replicon genes with at least 90% coverage and 90% identity are shown. All contigs were circular and identified as plasmids by PlasFlow.

DISCUSSION

Using a combination of short and long-read WGS, we identified identical plasmids carrying bla_{KPC-2} in four isolates of three bacterial genera recovered from epidemiologically linked patients associated with two healthcare facilities. The combination of these epidemiological and genetic findings strongly supports the transfer of this antibiotic resistance plasmid among different bacterial species in these facilities. Additionally, *K. pneumoniae* Isolates 2 and 3 shared high chromosomal genetic similarity, differing by only seven mutation events across their genomes. Thus, it is likely that in this case, the entire bacterial organism, including the bla_{KPC-2}-carrying plasmid, was transferred between patients.

Isolate 4 (*C. freundii*) was identified during a routine point prevalence study at Facility A, 3 months after the outbreak. Sequencing analysis determined that this isolate was genetically unrelated to the other *C. freundii* isolate from this investigation (Isolate 1), and that the plasmid harboring bla_{KPC-2} in Isolate 4 was unrelated to the plasmids harboring the gene in Isolates 1, 2, 3, and 5. Further investigation of Isolate 4 identified that the patient was a resident of Massachusetts, a state that had previously identified cases of KPC-producing bacteria (Centers for Disease Control and Prevention [CDC], 2019c), and that this colonization was likely present on admission.

Upon identification of KPC-producing bacteria through colonization screening, Facility A initiated weekly rectal swabs for all patients on the affected unit. No additional KPC-producing bacteria were identified. One year later, there has been no other healthcare-onset KPC-producing bacteria identified at either Facility A or Facility B.

Prevalence of carbapenemase-producing CRE was low in Maine in 2018. At that time, most healthcare facilities in the state did not routinely conduct active surveillance cultures to identify colonized patients upon admission (Centers for Disease Control and Prevention [CDC], 2015). Facility A did note a housekeeping staffing shortage during 2018. Unrecognized colonization and missed opportunities in environmental cleaning may have played a role in the transmission of gastrointestinal flora from the source patient to other patients on the same unit in Facility A. Unrecognized colonization and a shared bathroom may have led to transmission from the source patient to the roommate at Facility B. It has been shown that toilet flushing generates aerosolized bacteria that can land on nearby surfaces or drift in air currents to land on surfaces further away, which can contribute to the direct and/or indirect transmission of gastrointestinal flora (Barker and Jones, 2005; Johnson D. L. et al., 2013; Johnson D. et al., 2013). As environmental screening was not done in the course of this investigation, it is impossible to determine if horizontal transfer of the plasmid harboring bla_{KPC-2} may have occurred in the environment at Facility A and/or B, or within a patient, or both.

While the epidemiological and genetic data from this investigation support the transmission of the bla_{KPC-2}-carrying plasmid among three species of bacteria, it is also possible that the bacteria independently acquired this resistance plasmid. The identified plasmid is 100% identical to a plasmid first sequenced from *K. pneumoniae* more than 10 years earlier in Virginia (Mathers et al., 2015). This plasmid (pKPC_UVA01) has also been subsequently identified at 100% identity in other bacterial species (*Kluyvera intermedia*, *C. freundii*) from the same hospital in which it was first isolated (Sheppard et al., 2016; Barry et al., 2019), and highly similar plasmids have been identified in *Enterobacter* species from New York, Michigan, Maryland, Illinois, and Florida (Chavda et al., 2016). This suggests that, despite the fact that pKPC_UVA01 has been shown to have a relatively low conjugation efficiency into *K. pneumoniae* and *E. coli in vitro* (Hardiman et al., 2016), it may be widespread in bacterial populations. More long-read sequencing of KPC-producing bacterial populations globally is necessary to gain more insight into the phylogenetic diversity of pKPC_UVA01 and related plasmids.

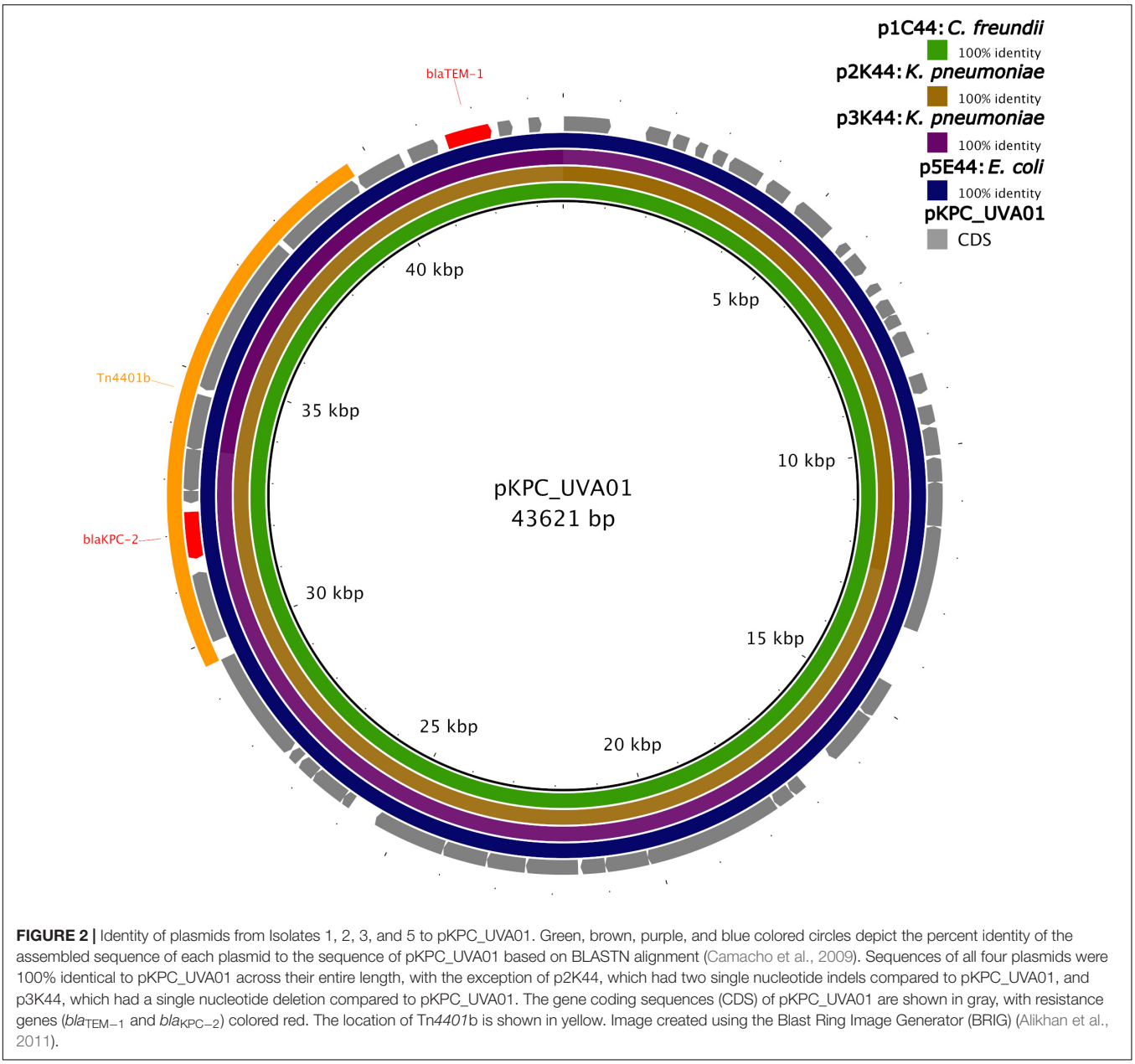

FIGURE 2 | Identity of plasmids from Isolates 1, 2, 3, and 5 to pKPC_UVA01. Green, brown, purple, and blue colored circles depict the percent identity of the assembled sequence of each plasmid to the sequence of pKPC_UVA01 based on BLASTN alignment (Camacho et al., 2009). Sequences of all four plasmids were 100% identical to pKPC_UVA01 across their entire length, with the exception of p2K44, which had two single nucleotide indels compared to pKPC_UVA01, and p3K44, which had a single nucleotide deletion compared to pKPC_UVA01. The gene coding sequences (CDS) of pKPC_UVA01 are shown in gray, with resistance genes (bla_{TEM-1} and bla_{KPC-2}) colored red. The location of Tn*4401*b is shown in yellow. Image created using the Blast Ring Image Generator (BRIG) (Alikhan et al., 2011).

The identification of three unique plasmids carrying bla_{KPC-2} across a small sample of KPC-producing bacteria in patients associated with two healthcare facilities highlights the diversity of the plasmid contexts of this resistance gene. The location of bla_{KPC} genes in transposons such as Tn*4401*b, which are themselves located on plasmids, allows for a high amount of mobility of these genes among bacterial species, patients, and healthcare facilities (Sheppard et al., 2016; Martin et al., 2017; Stoesser et al., 2017; Brandt et al., 2019; Mathers et al., 2019; Hendrickx et al., 2020). The location of bla_{KPC} genes in highly conserved transposons such as Tn*4401*b also highlights the necessity of using long-read sequencing to differentiate between these plasmids. Genome assembly methods based on short reads alone may not be able to assemble plasmids to the extent necessary to differentiate between plasmids that contain identical transposons on very different backbones (**Figure 3**). Similarly, the identification of two unique plasmids carrying bla_{KPC-2} within Tn*4401*b in Isolate 1 would have been impossible without the use of long-read sequencing.

Long-read and hybrid assembly of bacterial genomes is still an area of active development (De Maio et al., 2019; Wick and Holt, 2019). The discrepancies between assemblies produced by different methods in the current study, including inconsistencies in the genome assembly of Isolate 1 using Flye and in the genome assemblies of Isolates 3 and 5 using Unicycler (**Supplementary Methods**), support the use of more than one assembly method as well as thorough inspection and evaluation of genome assemblies.

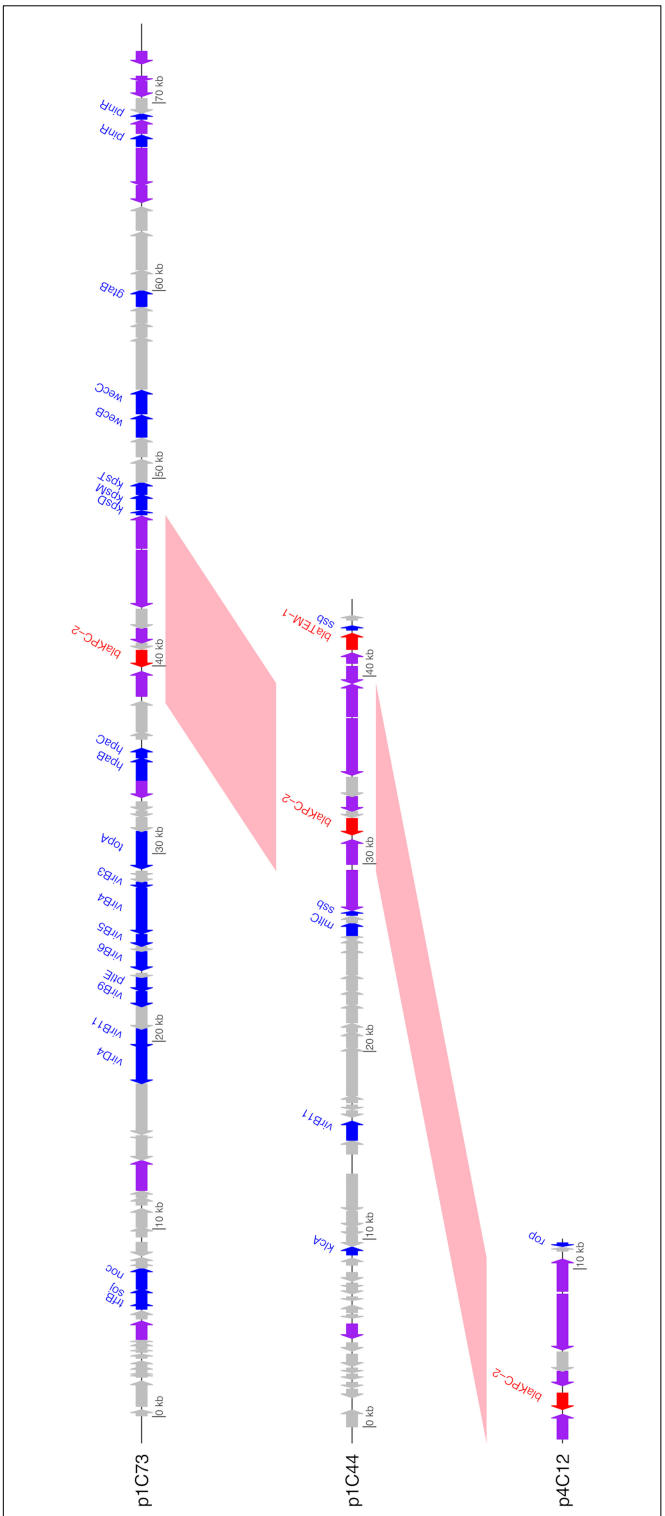

FIGURE 3 | Annotated gene maps of unique identified KPC-2 plasmids. BLASTN hits from pairwise comparisons of the plasmids are shown in pink; all plasmids contain the 10 kb transposon Tn*4401b* (>99% sequence identity for both comparisons). Coding sequences (CDS) encoding resistance genes are colored in red, transposases/integrases/recombinases in purple, other identified genes in blue, and hypothetical proteins in gray. Image generated using the genoplotR package (Guy et al., 2010).

Despite carrying a plasmid encoding bla_{KPC-2}, and testing positive for carbapenemase production by mCIM, Isolate 5 was not carbapenem resistant by broth microdilution (**Table 1**) or gradient diffusion, though it showed intermediate susceptibility to ertapenem and imipenem by disk diffusion. Carbapenemase producing bacteria with low carbapenem MICs have been previously reported (Fattouh et al., 2016; Tamma et al., 2016), highlighting the importance of utilizing multiple molecular and phenotypic methods to screen clinical isolates for carbapenemase production.

The ability to identify identical plasmids across bacterial species was instrumental in defining this event as an outbreak. Genomic evidence to support the epidemiological suspicion of an outbreak was of great benefit in acceptance by the facility that an outbreak occurred and for the promotion of infection control and prevention activities needed to respond to these novel organisms statewide.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA636827.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CP wrote the manuscript with contributions from RO, ES, NS, and WH. RO conducted epidemiological investigation and coordinated sample collection. KM, EN, and KM supervised microbiological laboratory work. NS conducted long-read sequencing and base-calling. CP, ES, and WH conducted data analysis with guidance from PL and EL-N. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.02007/full#supplementary-material

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Characterization of IncC Plasmids in Enterobacterales of Food-Producing Animals Originating From China

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Incompatibility group C (IncC) plasmids have received attention due to their broad host range and because they harbor key antibiotic resistance genes. Because these resistance genes can spread from food-producing animals to human, the proliferation of these plasmids represents a public health risk. In this study, a total of 20 IncC plasmids were collected from food-producing animals in China, and characterized by Oxford Nanopore Technologies long-read sequencing. Based on four key differences of the IncC backbone, 4 IncC plasmids were classified as type 1, 15 were classified as type 1/2 hybrid, and one was classified as type 2. The 15 type 1/2 hybrids were further divided into 13 type 1/2a and 2 type 1/2b, based on sequence differences arising from different homologous recombination events between type 1 and type 2 IncC backbones. Genome comparison of accessory resistance modules showed that different IncC plasmids exhibited various phenotypes via loss and gain of diverse modules, mainly within the bla_{CMY}-carrying region, and two antibiotic resistance islands designated ARI-A and ARI-B. Interestingly, in addition to insertion and deletion events, IS*26* or IS*1294*-mediated large sequence inversions were found in the IncC genome of the 4 type1/2a plasmids, suggesting that insertion sequence-mediated rearrangements also promote the diversity of the IncC genome. This study provides insight into the structural diversification and multidrug resistance of IncC plasmids identified from food-producing animals in China.

Keywords: antibiotic resistance, food-producing animal, insertion sequence, inversion, IncC plasmids

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INTRODUCTION

The emergence and spread of antimicrobial resistance in bacteria pose a serious threat to global health and food security (Laxminarayan et al., 2016; Liu et al., 2016; He et al., 2019). Consequently, the WHO officially recognized antimicrobial resistance as a significant threat to global health in 2019.[1] Mobile genetic elements (MGEs) are responsible for the capture, accumulation, and dissemination of resistance genes. Plasmids are important vehicles for other MGEs and acquired antimicrobial resistance genes associated with these elements in both Gram-negative and Gram-positive genera of bacteria (Partridge et al., 2018). Accordingly, plasmids play a significant role

[1]https://www.who.int/news-room/feature-stories/ten-threats-to-global-health-in-2019

in the worldwide dissemination of multidrug resistance (MDR; Carattoli, 2009; Chen et al., 2018; Partridge et al., 2018; Pinilla-Redondo et al., 2018) and the evolution of antimicrobial resistance in bacteria (San Millan et al., 2016; San Millan, 2018). Incompatibility group C (IncC) plasmids were first reported in the 1960s, and were grouped with the related IncA plasmid RA1 as the A–C complex in 1974, with the term "IncA/C" subsequently coined in the late 1980s (Datta and Hedges, 1973; Hedges, 1974; Couturier et al., 1988; Harmer and Hall, 2015). More recently, the original names of IncA and IncC are becoming more widely used to more accurately describe compatibility and backbone divergence (Harmer and Hall, 2015; Ambrose et al., 2018a,b). IncC plasmids occur widely in Gram-negative bacteria that are resistant to multiple antibiotics, indicating a broad host range (Partridge et al., 2018). These plasmids have received most attention due to their association with the dissemination of the bla_{CMY} cephalosporinase and bla_{NDM} carbapenemase genes (Mulvey et al., 2009; Mataseje et al., 2010; Guo et al., 2014; Harmer and Hall, 2014), and these plasmids can harbor genes conferring resistance to several different antibiotics, such as aminoglycoside and fluoroquinolone resistance genes, allowing resistance to many clinically useful aminoglycosides and fluoroquinolone (Shoma et al., 2014; Wasyl et al., 2015).

An approximately 1% nucleotide divergence of the IncC plasmids backbones separate a group including type 1, and a group including type2, which are represented by reference plasmids pR148 (Del Castillo et al., 2013), and pR55 (Doublet et al., 2012), respectively. These two lineages are also distinguished by two variable regions (R1 and R2), *orf1832* in type 1 or *orf1847* in type 2, *rhs1* in type 1 or *rhs2* in type 2, and two additional sequences (i1 and i2) in the type 2 IncC backbone (Harmer and Hall, 2014). Type 1 can be further divided into two sub-groups, type 1a and 1b, based on the presence (1a) or absence (1b) of a diverged segment that contains Single-nucleotide polymorphisms (SNPs) concentrated in a 14.5 kb part of the IncC genome (Harmer and Hall, 2017). More recently, a small number of novel type 1/2 hybrid IncC plasmids were reported (Harmer and Hall, 2015; Lei et al., 2017; Papagiannitsis et al., 2017). These plasmids share backbone features with both type 1 and type 2 plasmids, indicating that despite strong entry exclusion and incompatibility, homologous recombination can occur between type 1 and type 2 IncC plasmids (Ambrose et al., 2018b). One study found that IS*26* can act to generate an the IncC-IncX3-cointegrated plasmid (Li et al., 2019), with expanded resistance profile, effectively broadening the host spectrum of the resistance-encoding mobile elements.

For a long time, due to the importance of bla_{CMY-2}, bla_{NDM}, or other carbapenemase resistance genes, the reports and sequencing had a clear preference for type1a IncC plasmids (Harmer and Hall, 2015; Ambrose et al., 2018b). Ever-increasing studies of IncC plasmids have provided a fascinating insight into their evolutionary history (Ambrose et al., 2018b; Cheng et al., 2019), but the reports about animal derived IncC plasmids remain sporadic and limited. To further characterize plasmid strategies to disseminate antibiotic genes, we characterized 20 IncC plasmids identified from food-producing animals in China. Our sequencing results allow a comprehensive genomic comparison, providing insight into the structural diversification and MDR capacity of IncC plasmids found from food-producing animals in China.

MATERIALS AND METHODS

Bacterial Strains and the detection of IncC plasmids

A total of non-duplicate 870 Enterobacterales strains (369 *Escherichia coli* strains, 212 *Klebsiella pneumoniae* strains, 125 *Salmonella enterica* strains, 56 *Proteus mirabilis* strains, 43 *Proteus vulgaris* strains, 38 *Citrobacter* strains, and 27 *Enterobacter cloacae* strains) were used for IncC plasmid identification in this study. The strains were recovered from samples of feces, diseased tissues, or cloacal or anal swabs of animals from 58 livestock farms (27 poultry and 31 swine farms) located in 16 provinces in China, with samples collected between 2015 and 2019 (**Supplementary Table S1**). All isolates were identified by BD Phoenix 100 diagnostic systems (Sparks, MD, United States). IncC plasmids were identified by PCR amplification with primer pair C-F (5′–3′ GAGAACCAAAGACAAAGACCTGGA)/C-R (5′–3′ ACGACAAACCTGAATTGCCTCCTT) that targets *repA* gene (Carattoli et al., 2005). And amplification was carried out with the following thermal cycling conditions: 3 min at 95°C and 35 cycles of amplification consisting of 25 s at 94°C, 25 s at 55°C, and 10 s at 72°C, with 5 min at 72°C for the final extension. The positive isolates identified by PCR were sequenced by whole genome sequencing (WGS, see Section "DNA Extraction, Purification and Library Preparation") combined PlasmidFinder 2.1 analysis (Carattoli et al., 2014) to further determine the presence of the IncC plasmid.

Antimicrobial Susceptibility Testing

Bacterial antimicrobial susceptibility was determined by the broth dilution or agar dilution method (for fosfomycin, using agar media supplemented with 25 μg/mL of glucose-6-phosphate) according to CLSI (Clsi, 2018b) and Veterinary CLSI (Clsi, 2018a) guidelines. The tested antimicrobial agents included ampicillin (AMP), cefoxitin (FOX), cefotaxime (CTX), ceftriaxone (CRO), imipenem (IPM), amoxicillin-clavulanic acid (AMC), florfenicol (FFC), ciprofloxacin (CIP), gentamicin (GEN), doxycycline (DOX), sulfamethoxazole (SUL), trimethoprim (TMP), fosfomycin (FOS), and polymyxin B (POL). *E. coli* ATCC25922 was used as a quality control strain.

DNA Extraction, Purification and Library Preparation

Genomic DNAs of IncC-positive strains were extracted by using a MiniBEST Bacteria Genomic DNA Extraction Kit (TaKaRa, Dalian, China), and automatically recovered using the BluePippin (Sage science, United States). The quality and quantity of genomic DNA were confirmed using a Qubit 2.0 fluorometer (Life Technologies). PromethION and

Illumina sequencing library preparation were performed using SQK-LSK109 ligation genomic DNA kit (Oxford Nanopore Technologies, United Kingdom) and NEBNext®Ultra™ DNA Library Prep Kitfor Illumina (NEB, United States), respectively.

Whole Genome Sequencing, Assembly, MLST, and SNP Analysis

Whole genome sequencing was performed using the Illumina PE150 platform (350-bp paired-end reads) combined with the Nanopore PromethION 48 platform (Novogene, China), and the read length and depth for each sample was shown in **Supplementary Table S2**. The quality check for sequencing reads were performed by NanoPlot 1.3.1 soft (Q > 7). The reads were assembled using the software SPAdes_3.12.0 and Unicycler GPLv3 (Wick et al., 2017),[2] and the complete nucleotide sequences of all IncC plasmids were verified by PCR. The assembled sequences were analyzed to identify multi-locus sequence typing (MLST) by the MLST 2.0 (Larsen et al., 2012).[3] Single-nucleotide polymorphism alignments were computed using the CSI phylogeny 1.4 pipeline (Kaas et al., 2014) according to the default parameters.[4]

Sequence Annotation and Comparison

The complete plasmid sequences were annotated with the Rapid Annotation using Subsystem Technology (RAST) tool (Brettin et al., 2015) and the NCBI BLAST algorithm.[5] For analysis and annotation of resistance genes, mobile elements, and other features, BLAST, ResFinder 4.0 (Zankari et al., 2012), INTEGRALL (Moura et al., 2009), IntegronFinder Galaxy v1.5.1 (Cury et al., 2016)[6] and ISfinder (Siguier et al., 2006)[7] programs were utilized. Easyfig 2.2.3 was used for comparative genome alignments and generation of physical maps.

Conjugation Experiment

Conjugation was performed using the 20 IncC-containing isolates identified in this study as the donor strain respectively and rifampin-resistant *E. coli* EC600 as the recipient strain with selection on nutrient agar plates containing 20 mg/L rifampin and 8 mg/L florfenicol. Positive transconjugants were characterized by assessing mobilization of *repA* gene by PCR with the C-F/R primer pairs described above and determination of the antimicrobial resistance profile.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of 20 IncC plasmids and isolates in this study were submitted to GenBank and the assigned accession numbers are listed in **Table 1** and **Supplementary Table S1**, respectively.

[2]https://github.com/rrwick/Unicycler

[3]https://cge.cbs.dtu.dk/services/MLST/

[4]https://cge.cbs.dtu.dk/services/CSIPhylogeny/

[5]http://www.ncbi.nlm.nih.gov/BLAST/

[6]https://galaxy.pasteur.fr/root?tool_id=toolshed.pasteur.fr%2Frepos%2Fkhillion%2Fintegron_finder%2Fintegron_finder%2F1.5.1

[7]https://www-is.biotoul.fr/

RESULTS AND DISCUSSION

The Strains Carrying IncC

A total of 20 strains (20/870 total, 2.3%) were determined for carrying IncC plasmids. The positive strains included 14 *E. coli* strains (14/369 total, 3.8%), 3 *K. pneumoniae* strains (3/212 total, 1.4%), 1 *S. enterica* strain (1/125 total, 0.8%), 1 *P. mirabilis* strain (1/56 total, 1.8%), and 1 *C. braakii* strain (1/38 total, 2.6%). 14 *E. coli* isolates were identified as 13 different sequence types (STs) in this study, and on minimum, 11 and maximum, 49,317 SNPs were identified when compared with each other. The two ST4663 *E. coli* strains (EC2 and EC8) were isolated from the same poultry farms in different years, and there were 11 SNPs between them, revealing that they were almost identical. Similarly, the two ST423 *K. pneumoniae* isolates (KC1 and KC2) seemed to spread by clonal expansion (8 SNPs) in another poultry farms. As shown in **Figures 1**, **3**, due to the insertion of IS-mediated unit in backbone or resistance islands (the IS*26*-unit in ARI-B of pEC2-1/2b, and the IS*Kpn25*-unit in backbone of pKC2-1/2a), the IncC plasmids carried by the clonal isolates are structurally different. The minimal inhibitory concentrations (MICs) of the individual strain were listed in **Supplementary Table S1**, and all the 20 isolates were MDR (defined as resistant to three or more classes of antibiotics).

Sequence Overview of IncC

The 20 plasmids varied in size from 69.6 to 247.4 kb, with G + C content that ranged from 51.8% to 53.5%, and 100 to 314 ORFs predicted by RAST. Based on four key differences of the IncC backbone (i1, i2, R1 and R2), 15 of the identified IncC plasmids were recognized as type 1/2 hybrid (**Table 1**). Based on the presence of i1 or i2, the plasmids were further divided into different subtypes (see section "Backbone Features of IncC"). The remaining four IncC plasmids were assigned as type 1, and one was identified as a type 2 IncC plasmid (**Table 1**). Using the BLAST program, the plasmid sequences were analyzed together with the reference type 1a pR148, type 1b pYDC637 (Lee et al., 2015), type1/2a pPm14C18 (Lei et al., 2017), and type 2 pR55. The pairwise comparison of backbone sequences showed that these 24 plasmids displayed >99% nucleotide identity with ≥42% coverage among the same plasmid type, and had >97% nucleotide identity with ≥38% coverage between different plasmid types (**Supplementary Table S3**). The 20 plasmids possessed the relatively conserved backbone organization, corresponding to mobilization, replication, maintenance, metabolism, regulation, and some other functional genes. It is in agreement with the findings of previous studies (Fricke et al., 2009), indicating that the core backbones of IncC plasmids are highly syntenic. However, the insertion of accessory modules always led to loss or disruption of functional genes (show in the **Figure 1** and **Supplementary Table S4**). All 20 plasmids identified in this study contained accessory modules, with collections of resistance genes, but the insertions with different

TABLE 1 | IncC plasmids characterized in this study.

Type	Plasmid	Accession number	orf1832/ orf1847	*rhs1*/*rhs2*	i1[a]	i2	Organism	Total length (bp)	Length of backbone (bp)	Mean G + C content (%)	Total number of ORFs
Type 1/2a	pEC1-1/2a	MT551208	orf1832	*rhs1*	NP	i2	*E. coli*	139,489	113,808	52.1%	184
Type 1/2a	pEC2-1/2a	MT559985	orf1832	*rhs1*	NP	i2	*E. coli*	247,388	117,312	52.2%	314
Type 1/2a	pEC3-1/2a	MT559986	orf1832	*rhs1*	NP	i2	*E. coli*	160,789	117,312	52.6%	204
Type 1/2a	pEC5-1/2a	MT559988	orf1832	*rhs1*	NP	i2	*E. coli*	146,625	112,022	52.4%	191
Type 1/2a	pEC6-1/2a	MT559989	orf1832	*rhs1*	NP	i2	*E. coli*	214,513	117,312	52.5%	302
Type 1/2a	pEC7-1/2a	MT559990	Δorf1832	Δ*rhs1*	NP	i2	*E. coli*	93,442	74,054	52.0%	129
Type 1/2a	pEC8-1/2a	MT559991	orf1832	*rhs1*	NP	i2	*E. coli*	157,847	117,312	52.2%	204
Type 1/2a	pEC10-1/2a	MT559993	Δorf1832	*rhs1*	NP	i2	*E. coli*	110,589	74,541	52.7%	153
Type 1/2a	pEC13-1/2a	MT559996	Δorf1832	Δ*rhs1*	NP	i2	*E. coli*	88,958	70,058	51.8%	128
Type 1/2a	pKC1-1/2a	MT559999	orf1832	*rhs1*	NP	i2	*K. pneumoniae*	116,633	79,954	52.6%	153
Type 1/2a	pKC2-1/2a	MT560000	orf1832	*rhs1*	NP	i2	*K. pneumoniae*	124,795	79,962	52.6%	156
Type 1/2a	pSC1-1/2a	MT560003	orf1832	*rhs1*	NP	i2	*S. enterica*	147,359	88,201	53.5%	189
Type 1/2a	pPC1-1/2a	MT560002	orf1832	*rhs1*	NP	i2	*P. mirabilis*	153,373	117,312	52.3%	197
Type 1/2b	pKC3-1/2b	MT560001	Δorf1832	*rhs1*	+	−	*K. pneumoniae*	193,802	123,809	52.6%	252
Type 1/2b	pCC1-1/2b	MT559998	−	*rhs1*	+	−	*C. braakii*	180,389	110,009	52.6%	242
Type 1b	pEC4-1b	MT559987	orf1832	*rhs1*	−	−	*E. coli*	89,953	62,552	52.1%	124
Type 1b	pEC11-1b	MT559994	orf1832	*rhs1*	−	−	*E. coli*	166,437	116,870	52.5%	217
Type 1b	pEC12-1b	MT559995	−	*rhs1*	−	−	*E. coli*	69,554	50,286	53.1%	100
Type 1b	pEC14-1b	MT559997	orf1832	*rhs1*	−	−	*E. coli*	167,577	70,058	52.6%	220
Type 2	pEC9-2	MT559992	orf1847	*rhs2*	i1	i2	*E. coli*	137,087	97,100	52.9%	186

[a]*NP = region surrounding i1 location was not present.*

profiles ranging from the simple to the very complex (see section "Functional Genes of IncC Plasmids").

Backbone Features of IncC

In this study, 15 IncC plasmids were identified as type 1/2 hybrid. Thirteen of these plasmids (**Figures 1A,B**) contain the backbone features similar to the backbone of plasmid pPm14C18 (Lei et al., 2017), so were designated type 1/2a plasmids. These plasmids harbor the type 2 version of the additional sequence i2 and the type 1 versions of R1 (*orf1832*) and R2 (*rhs1*). In four type 1/2a plasmids, pEC5-1/2a, pEC6-1/2a, pEC7-1/2a and pSC1-1/2a, IS-mediated (IS*26* or IS*1294*) recombination resulted in different sequence inversions of the genome, which were similar to the inversion event in previously described study (such as those in p427113-Ct1/2 and pA1763-Ct2) (Cheng et al., 2019). But more complicated, in pEC6-1/2a and pSC1-1/2a, there were the larger-scale IncC genome inversion mediated by IS*26* between the ARI-B and ARI-A. The large genetic fragments of the backbone region with accessory modules (including multiple inversions arising from within ARI-A, ARI-B and the IS*Ecp1*-bla_{CMY-2} island) were positioned in inverse orientation relative to the other plasmids in this group (**Figure 1A**). Another subtype was comprised of plasmids pKC3-1/2b and pCC1-1/2b, type 1/2b, which contained *orf1832*, *rhs1*, and additional type 2 sequence i1 (**Figure 1C**). Analysis of the genome of pKC3-1/2b (GenBank accession number MT560001) by Blast analysis revealed that the backbone sequence from 0-bp to 50,800-bp and from 175,375-bp to 193,802-bp showed 99.97% nucleotide identity to the corresponding backbone region of type 2 IncC plasmid pR55 (GenBank accession no. JQ010984). The remaining backbone, from 50,801-bp to 175,374-bp, showed 99.99% nucleotide identity to the corresponding backbone region of type 1 IncC plasmid pR148 (GenBank accession no. JX141473). There were different backbone features in the 15 type 1/2 hybrid IncC plasmids, suggesting various homologous recombination between type 1 and type 2 IncC, with IS-mediated rearrangements increasing the diversity of the IncC plamids.

Like type 1b IncC plasmid pYDC637 (GenBank accession no. KP056256), the four type 1b IncC plasmids identified in this study (pEC4-1b, pEC11-1b, pEC12-1b, pEC14-1b) (**Figure 1D**) lacked the SNPs, which are typically concentrated in a 14.5-kb part of the type 1a IncC genome (Ambrose et al., 2018b). In addition, compared to pR55, the only type 2 IncC plasmid, pEC9-2, contained an extremely simple backbone due to the deletion of a large section of the backbone regions, but still retained all the characteristics of type 2 plasmids in the sequences of i1, i2, *orf1847*, and *rhs2*. This plasmid had a deletion of up to 27.6-kb between the *traC* and *nuc* genes relative to pR55, but no related mobile elements were detected (**Figure 1C**).

Structures of ARI-B and ARI-A are sometimes associated with backbone deletion (**Table 2**), and this process is mostly mediated by IS*26*. Of the IncC plasmids identified here, the most commonly seen event was a 10,984-bp IS*26*-mediated deletion arising within the ARI-B, which was in agreement with previously described ARI-B (Harmer and Hall, 2015). This deletion was identified in all 13 of the type 1/2a and all four of the type 1b plasmids identified here, but the configuration of ARI-B was slightly different in some plasmids (**Table 2**). Another event was a 4,477 bp backbone deletion upstream from the *parA* gene, found in the two type 1/2b plasmids, pKC3-1/2b and pCC1-1/2b, and the one type 2 plasmid,

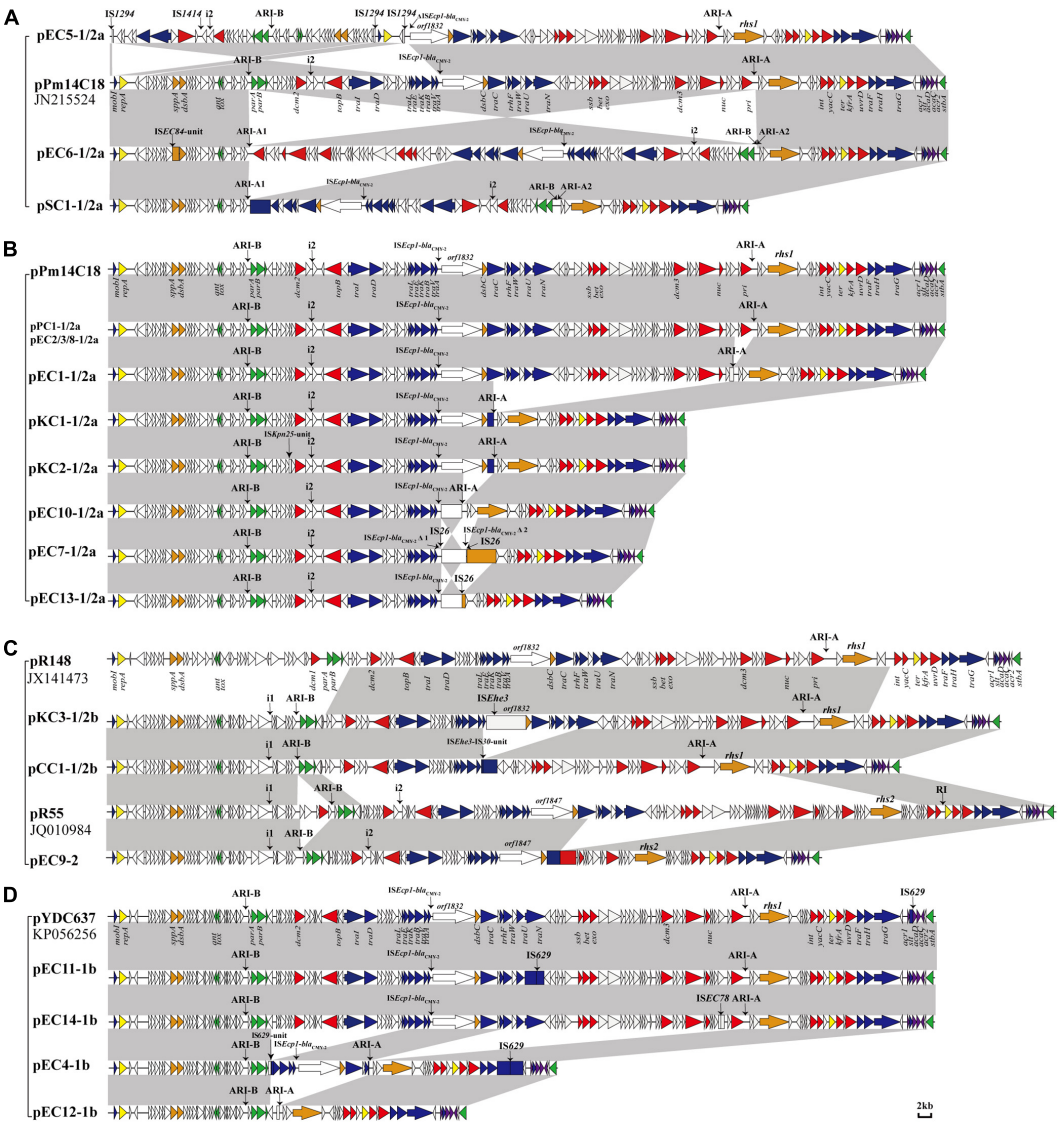

FIGURE 1 | Comparison of linear maps of the IncC plasmids. **(A)** The large sequence inversions mediated by IS-mediated found in the 3 type 1/2a IncC plasmids. **(B)** The remaining type 1/2a IncC plasmids. **(C)** The type 1/2b and type 2 IncC plasmids. **(D)** The type 1b plasmids. The GenBank accession numbers of the reference type 1a pR148, type 1b pYDC637, type1/2a pPm14C18 and type 2 reference pR55 are JX141473, KP056256, KU605240 and JQ010984. Genes and ORFs are shown as arrows, and their orientations of transcription are indicated by the arrowheads. The truncated genes are shown as rectangle. The positions of accessory modules, and the i1 or i2 insertions are indicated by vertical arrows. Genes coding for proteins are colored according to the following key: blue, conjugative transfer; yellow, replication; green, plasmid maintenance; red, DNA metabolism; purple, regulation; orange, other functional genes; white, no known function. Shared regions with above 99.9% identity are indicated by shading.

pEC9-2, which was associated with the ARI-B forms containing the IncN-related fragment previously characterized (Harmer and Hall, 2015; **Figures 3E,F**). Similarly, IS*26*-mediated backbone deletions with various sizes can often be found at the upstream of the ARI-A (**Table 2**). Additionally, other acquired regions (IS elements or transposons) were identified that interrupt or delete the backbone of IncC genome (**Table 2**).

Functional Genes of IncC Plasmids

As shown in **Figure 1**, the insertion mediated by accessory modules always resulted in the loss or disruption of the functional genes (some *tra* genes and metabolism genes) in varying degrees. The mobilization modules contained type IV secretion system (T4SS, *traLEKBVACWUFHG*) genes (Guglielmini et al., 2013), other *tra* genes (*traIDN* and *trhF*), *mobI* and *slt* genes, and only 8 IncC plasmids (pEC1-1/2a, pEC2-1/2a, pEC3-1/2a, pEC6-1/2a, pEC8-1/2a, pPC1-1/2a, pKC3-1/2b, and pEC14-1b) of this study maintained all these genes intact, but only 7 plasmids the ability to transfer (see section "Transferability and Antimicrobial Susceptibility"). In this study, the genes required for IncC plasmid replication (*repA* and *ter*) and maintenance (*ant*, *tox*, *parAB*, *sta*, and a putative gene, *053*) of all 20 plasmids were

TABLE 2 | Accessory modules in IncC plasmids.

Plasmid	ARI-A		ARI-B		IS*Ecp1*-bla_{CMY-2}	Others	
	Resistance	Deletion(bp)	Variant	Deletion (bp)		IS	Deletion(bp)
pEC1-1/2a	pDU_{mer}	3,499	A	10,984	+	–	–
pEC2-1/2a	*tmrB*, *aac(3)-IIa*, *erm*(B), *mph*(A)	–	D	10,984	interrupted	IS*1*	–
pEC3-1/2a	*aacA7, sul1, lnu(F), aadA22,* pDU_{mer}	–	A	10,984	+	–	–
pEC5-1/2a	pDU_{mer}	–	A	10,984[b]	interrupted	IS*1294*, IS*1414*	5,303
pEC6-1/2a	*sul3*, *aadA2*, *dfrA12*, bla_{TEM-1B}, *mph*(A), $\Delta Tn21_{mer}$	–	C	10,984[c]	+	IS*Ec84*-unit	–
pEC7-1/2a	–	–	A	10,984	interrupted	IS*26*	43,217
pEC8-1/2a	*tmrB*, *aac(3)-IIa*, *erm*(B), *mph*(A)	–	A	10,984	interrupted	IS*1*	–
pEC10-1/2a	*erm*(B), *mph*(A), pDU_{mer}	41,765	A	10,984	+	–	–
pEC13-1/2a	–	–	B	10,984	+	IS*26*	47,248
pKC1-1/2a	*aadB*, *cmlA1*, bla_{TEM-1B}, $\Delta Tn21_{mer}$, ΔpDU_{mer}	37,361	A	10,984	+	–	–
pKC2-1/2a	*aadB*, *cmlA1*, bla_{TEM-1B}, $\Delta Tn21_{mer}$, ΔpDU_{mer}	37,361	A	10,984	+	IS*Kpn25*-unit	–
pPC1-1/2a	*aacA7*, *sul1*, ΔpDU_{mer}	–	A	10,984	+	–	–
pSC1-1/2a	*mph*(A), *qepA*, *dfrA12*, *aadA2, sul1, qnrS1*, bla_{TEM-1B}, $\Delta Tn21_{mer}$, pDU_{mer}	29,106	C	10,984[c]	+	–	–
pKC3-1/2b	*dfrA14*, *arr-2*, *cmlA1*, bla_{OXA-10}, *aadA1*, *sul1*, *qnrB4*, bla_{DHA-1}, *mph*(A), $Tn21_{mer}$	–	F	4,477	–	IS*3*	–
pCC1-1/2b	*dfrA14*, *arr-2*, *cmlA1*, bla_{OXA-10}, *aadA1*, *sul1*, *qnrB4*, bla_{DHA-1}, *mph*(A), $Tn21_{mer}$	–	F	4.477	–	IS*3*, IS30	13,790
pEC4-1b	–[a]	34,760	A	10,984	+	IS*629-unit*	19,544
pEC11-1b	*qnrVC4*, *aac(6')Ib-cr*, *cmlA1*,*aadA1*, bla_{OXA-10}, *aadA1*, *dfrA14*, *mph*(A), *aph(3')-Ia*, pDU_{mer}	–	A	10,984	+	IS*3*	–
pEC12-1b	*mph*(A)	66,835	A	10,984	–	–	–
pEC14-1b	*qnrVC4*, *aac(6')Ib-cr*, *cmlA1*,*aadA1*, bla_{OXA-10}, *aadA1*, *dfrA14*, *mph*(A), *aph(3')-Ia*, pDU_{mer}	–	A	10,984	+	IS*66*	–
pEC9-2	–	–	E	4,477	–	–	27,636

[a] *Due to the IS26- mediated recombination, in ARI-A, only ΔIS4321 was retained.*
[b] *The ARI-B was reversed with the adjacent backbone region.*
[c] *The IS26 transposition event occurred between the ARI-B and ARI-A.*

complete and uninterrupted at their genome. The *repA* gene, a toxin-antitoxin (TA) system (*ant* and *tox*) and a set of 3 partitioning-related genes (*parA*, *parB* and *053*) have been identified roles for the stability (Hancock et al., 2017), so the integrity of these genes is critical for plasmid maintenance in host.

Resistance Islands of IncC Plasmids

Except for the type 2 plasmid, pEC9-2, and the two type 1/2a plasmids, pEC7-1/2a and pEC13-1/2a, ARI-A islands were identified in the remaining 17 plasmids (**Table 2** and **Figure 2**). The structure of ARI-A in the IncC plasmid pRMH760 (GenBank accession no. KF976462) was the first to be described in detail (Partridge and Hall, 2003; Harmer and Hall, 2017). Similar to pRMH760, there were 10 plasmids in this study have both ends of ARI-A intact, with this sequence flanked by a 5-bp duplication (TTGTA) of the target (**Figure 2A**, the ARI-A of pEC5-1/2a is not shown). The 10 ARI-A islands were all identified as a complex mosaic structure derived from Tn*1696* and composed of a different class 1 integron and multiple resistance units or transposons (**Figure 2**), of which the two

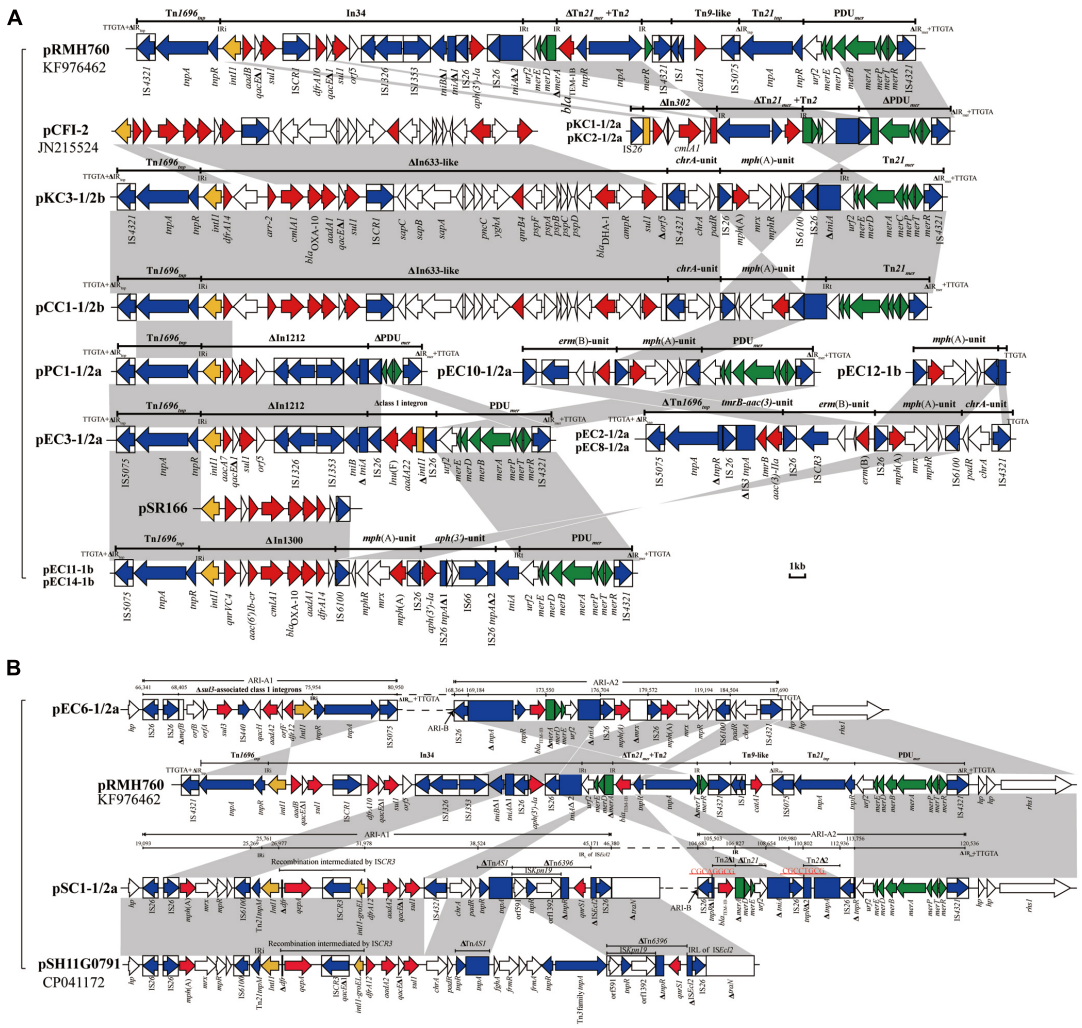

FIGURE 2 | Comparison of ARI-A containing resistance genes. **(A)** The common ARI-A forms. **(B)** The ARI-A forms inverted by IS*26*. The GenBank accession numbers of pRMH760, pCFI-2 (Yim et al., 2013), pSR166, and pSH11G0791 for reference are KF976462, JN215524, KU886277, and CP041172, respectively. Genes and ORFs are shown as arrows, and their orientations of transcription are indicated by the arrowheads. The truncated genes are shown as rectangle, which are indicated with a Δ. The IS elements are indicated by boxes around the blue arrows. Genes coding for proteins are colored according to the following key: blue, transposase genes; yellow, integrase genes; red, antimicrobial resistance genes; green, mercury resistance genes; white, other genes. Shared regions with above 99.9% identity are indicated by shading.

outermost inverted repeats are interrupted by either IS*4321* or IS*5075* elements. In agreement with the conclusions of a previous study about this form of ARI-A (Harmer and Hall, 2015), these islands include varying resistance island (RI) sequences, and this interruption of the IR effectively "locks" this island in place, making all insertions had the same boundaries in the plasmid backbone and evolution *in situ*. As a result of IS*26*-mediated recombination, the other 7 plasmids contained deletions originating within the island, which removed part of ARI-A and different portions of the backbone adjacent to the upstream of ARI-A (**Table 2**), resulting in a relatively ARI-A structure. It is also notable that, in pEC6-1/2 and pSC1-1/2, the IS*26* transposition event occurred between ARI-B and ARI-A, resulting in the interruption of ARI-A into two parts (ARI-A1 and ARI-A2), and an inversion of the adjacent genetic fragments of the backbone, with all of ARI-B and parts of ARI-A (ARI-A1) oriented in the inverse orientation (**Figures 1A,B**). The lack of 8-bp target inverted repeats (IRs) flanking the inversion, suggests that this complex reorganization was mediated by IS*26*. Also due to IS*26*-mediated rearrangements, compared to pRMH760, part of Tn*21*$_{mer}$ and the Tn*2* region in ARI-A2 of pSC1-1/2a and pEC6-1/2a (corresponding to bases 105,503-109,980 in accession no. MT560003, and bases 169,184-176,704 in accession no. MT559989) were in inverse orientation, and the inverted part of pSC1-1/2a was flanked by 8-bp IRs (CGCAGGCG).

In all 20 IncC plasmids, ARI-B was located upstream of the *parA* gene, and all contained the *sul2*-carrying remnants of GI*sul2* (Nigro and Hall, 2011; Harmer et al., 2017). Except for pKC3-1/2b and pCC1-1/2b, the 18 other plasmids all contain another four resistance genes (*floR*, *tet*(A), *strA*, and *strB*). Thirteen of the

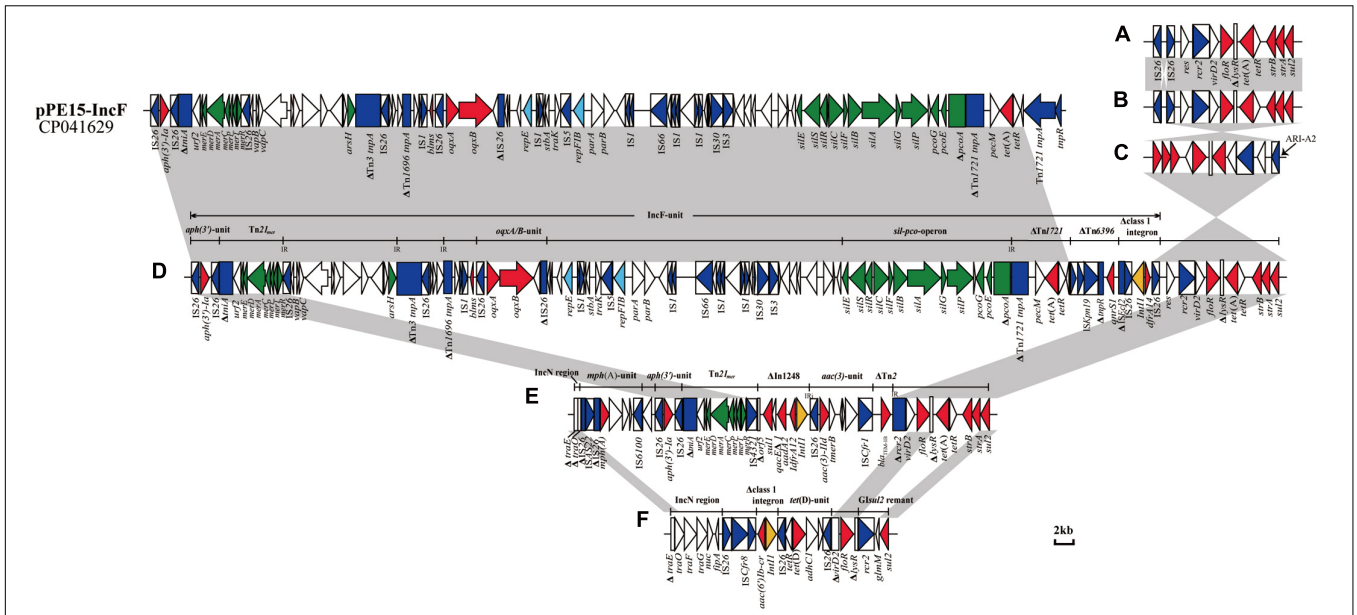

FIGURE 3 | Configurations of ARI-B in IncC. **(A)** The most common ARI-B configuration in this study. **(B)** Configuration present in pEC13-1/2a.**(C)** Configuration present in pEC6-1/2a and pSC1-1/2a. **(D)** Configuration present in pEC2-1/2a. **(E)** Configuration present in pEC9-2. **(F)** Configuration present in pKC3-1/2b and pCC1-1/2b. The GenBank accession number of pPE15 is CP041629. Genes and ORFs are shown as arrows, and their orientations of transcription are indicated by the arrowheads. The truncated genes are shown as rectangle, which are indicated with a Δ. The IS elements are indicated by boxes around the blue arrows. Genes coding for proteins are colored according to the following key: blue, transposase genes; yellow, integrase genes; red, antimicrobial resistance genes; green, mercury resistance genes; light blue, *rep* genes; white, other genes. Shared regions with above 99.9% identity are indicated by shading.

plasmids (**Table 2** and **Figure 3A**) contain the most commonly seen configuration of ARI-B (Fig. 7A in the review by Harmer and Hall, 2015), which is associated with a 10,984 bp, IS*26*-mediated deletion of the backbone. The ARI-B of pEC13-1/2 (**Figure 3B**) was different from variant A (**Figure 3A**) only by inversion of the short segment between the two IS*26*. In three plasmids with a large inversion (pEC5-1/2a, pEC6-1/2a, and pSC1-1/2a), their ARI-B sequence and the adjacent backbone region were reversed, and the right hand of ARI-B in pEC6-1/2a and pSC1-1/2a was linked to ARI-A2 by one IS*26* (**Figures 2A**, **3C**). There was also a deletion event that removed 4,477 bp of the backbone in pKC3-1/2b, pCC1-1/2b, and pEC9-2, generating a junction between the backbone and a segment derived from the *tra* region of an IncN plasmid, and these plasmids have a dramatically different set of resistance genes, as shown in **Figures 3E,F**. In pEC2-1/2a (**Figure 3D**), a distinct ARI-B form contains a 90.0-kb segment surrounded by two IS*26* elements sharing 99.99% nucleotide identity with the IncF plasmid pPE15 (GenBank accession no. CP041629), explaining acquisition of newer metal (*sil-pco*-operon and Tn*21*$_{mer}$) and antibiotic (*aph(3')-Ia*, *oqxA/B*, *tet*(A), *qnrS1*, *dfrA14*) resistance modules with a highly mosaic nature, and suggesting that IS*26* promotes diversity of ARI-B in IncC genome.

In 16 plasmids of this study, as previously reported, the bla_{CMY-2} gene was associated with the mobile element IS*Ecp1* in an island that was positioned downstream of the *traA* gene. In four plasmids, the IS*Ecp1* sequence was interrupted by IS*1* or IS*91*. Additionally, pEC7-1/2a contained an inversion of the IS*Ecp1*-bla_{CMY-2} island and *orf1832* mediated by two IS*26* elements in opposite orientation (**Figure 1B**), resulting in a 43.2-kb deletion of the backbone.

Transferability and Antimicrobial Susceptibility

We successfully obtained seven transconjugants harboring IncC, indicating successful transfer from wild-type isolates into EC600 through conjugation, as listed in **Supplementary Table S5**. The antimicrobial susceptibility testing of the transconjugants showed that the bla_{CMY-2} gene could be successfully transferred to EC600, and the antimicrobial resistance profiles of the resulting strains are shown in **Supplementary Table S5**. The seven IncC plasmids are all have an intact mobilization module, but the plasmid pEC6-1/2a, which failed to transfer, carried the unbroken but inverted mobilization modules (tra*IDLEKBVACWUN* and *trhF*). The failure to transfer in the remaining IncC plasmids may reflect the deletion or inversion of the related transfer genes, and the underlying mechanism will be explored in future studies.

CONCLUSION

In this study, we characterized 20 IncC plasmids identified from livestock farms in China and determined the antibiotic resistance. The accessory modules insertions always resulted in the loss or disruption of the functional genes (some *tra* genes and metabolism genes) to varying degrees, which might account for conjugation failure of some IncC plasmids. Meanwhile, the evolution of resistance islands via loss and gain of diverse

modules within the large resistance regions of ARI-A and ARI-B has driven leading to different resistance phenotypes. Variation existed in the 15 type 1/2 hybrids suggests various homologous recombination between type 1 and type 2 IncC plasmids. In addition to insertion of accessory modules and deletion of backbone regions, IS-mediated rearrangements resulted in large sequence inversions of the MDR regions extending outside the IncC backbone. The identification of the four IncC plasmids with IS-mediated inversion event suggested that IS-mediated rearrangements increase the diversity of IncC genome, especially the IS*26*, and we will pay attention to its role in promoting diversity in future studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MT551208; https://www.ncbi.nlm.nih.gov/genbank/, MT559985; https://www.ncbi.nlm.nih.gov/genbank/, MT559986; https://www.ncbi.nlm.nih.gov/genbank/, MT559988; https://www.ncbi.nlm.nih.gov/genbank/, MT559989; https://www.ncbi.nlm.nih.gov/genbank/, MT559990; https://www.ncbi.nlm.nih.gov/genbank/, MT559991; https://www.ncbi.nlm.nih.gov/genbank/, MT559993; https://www.ncbi.nlm.nih.gov/genbank/, MT559996; https://www.ncbi.nlm.nih.gov/genbank/, MT559999; https://www.ncbi.nlm.nih.gov/genbank/, MT560000; https://www.ncbi.nlm.nih.gov/genbank/, MT560003; https://www.ncbi.nlm.nih.gov/genbank/, MT560002; https://www.ncbi.nlm.nih.gov/genbank/, MT560001; https://www.ncbi.nlm.nih.gov/genbank/, MT559998; https://www.ncbi.nlm.nih.gov/genbank/, MT559987; https://www.ncbi.nlm.nih.gov/genbank/, MT559994; https://www.ncbi.nlm.nih.gov/genbank/, MT559995; https://www.ncbi.nlm.nih.gov/genbank/, MT559997; https://www.ncbi.nlm.nih.gov/genbank/, MT559992; https://www.ncbi.nlm.nih.gov/genbank/, JACXRF000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRG000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRH000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRI000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRJ000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRK000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRL000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRM000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRN000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRO000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRP000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRQ000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRR000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRS000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRT000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRU000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRV000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRW000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXRE000000000; https://www.ncbi.nlm.nih.gov/genbank/, JACXSK000000000.

ETHICS STATEMENT

This study was carried out in accordance with the recommendation of ethical guidelines of Sichuan University. The protocol was approved by the Sichuan University Animal Ethics Committee. Individual informed consent for the use of samples was obtained from all the animal owners.

AUTHOR CONTRIBUTIONS

YZ, C-WL, and XC performed the experiments. YZ, T-GY, J-WY, W-LH, and XM analyzed the data. YZ and C-WL wrote the manuscript. YZ and C-WL conceived of the study. All authors contributed to manuscript revision and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.580960/full#supplementary-material

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A Clinical Extensively-Drug Resistant (XDR) *Escherichia coli* and Role of Its β-Lactamase Genes

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An extensively-drug resistant (XDR) *Escherichia coli* W60 was isolated from the urine sample of a patient. The genetic basis for its XDR phenotype was investigated, particularly the basis for its resistance toward β-lactam/BLI (β-Lactamase Inhibitor) combinations. Following determination of the XDR phenotype, third generation genomic sequencing was performed to identify genetic structures in *E. coli* W60. Further cloning analysis was performed to identify determinants of β-lactam/BLI combination resistance. It was found that *E. coli* W60 is resistant to nearly all of the tested antibiotics including all commonly used β-lactam/BLI combinations. Analysis of the genomic structures in *E. coli* W60 showed two novel transferable plasmids are responsible for the resistance phenotypes. Further genetic analysis showed bla_{NDM-5} leads to high resistance to β-lactam/BLI combinations, which was enhanced by co-expressing ble_{MBL}. pECW602 harbors a truncated bla_{TEM} that is not functional due to the loss of the N-terminal signal peptide coding region. Research performed in this work leads to several significant conclusions: the XDR phenotype of *E. coli* W60 can be attributed to the presence of transferable multidrug resistance plasmids; NDM-5 confers high resistance to β-lactam/BLI combinations; co-expression of ble_{MBL} enhances resistance caused by NDM-5; the signal peptides of TEM type β-lactamases are essential for their secretion and function. Findings of this work show the danger of transferable multidrug resistance plasmids and metallo-β-lactamases, both of which should be given more attention in the analysis and treatment of multidrug resistant pathogens.

Keywords: antimicrobial resistance, extensively drug resistance, *Escherichia coli*, β-lactamase, β-lactamase inhibitor, multidrug resistant plasmid

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INTRODUCTION

Escherichia coli is one of the most common clinical bacteria, of which many isolates are pathogenic. *E. coli* can cause enteritis, urinary tract infection and many other diseases, leading to significant morbidity and mortality (Russo, 2003). In the past few decades, following the increased use of antibiotics, the resistance of clinical *E. coli* to antibiotics rises, making it difficult for treatment. In particular, many *E. coli* strains developed multi-, extensively- or pan-drug resistance (MDR, XDR, or PDR) phenotypes, posing a great challenge to infection treatment (Magiorakos et al., 2012;

Du et al., 2017; Jeong et al., 2018; Lv et al., 2018). Therapeutic options to these antibiotic resistant *E. coli* strains include last-resort antibiotics such as carbapenems and tigecycline, along with those still under development (Karaiskos and Giamarellou, 2014).

β-lactam antibiotics are the most widely used antibiotics in the treatment of bacterial infection. However, antibiotic resistant bacteria often produce β-lactamase, inactivating β-lactams. To address this, β-lactamase inhibitors (BLI) were developed to reenable the use of β-lactam antibiotics. Today, the most commonly used BLIs include tazobactam, clavulanate, sulbactam, and avibactam (Ehmann et al., 2012). Effective β-lactam/BLI combinations include piperacillin–tazobactam, amoxicillin–clavulanate, ticarcillin-clavulanate, ampicillin–sulbactam, and ceftazidime–avibactam (Tooke et al., 2019). The use of these combinations has replaced other last-resort antibiotics to become the most popular option in treating β-lactam resistant bacteria infections.

Based on sequence homology, β-lactamases are divided into four classes A, B, C, and D (Ambler, 1980). Despite differing by their mechanisms, all β-lactamases deactivate β-lactams by hydrolytic opening of the β-lactam ring. TEM is one of the most prevalent and typical class A β-lactamases. It was discovered in as early as 1965 when a plasmid harboring $bla_{\mathrm{TEM-1}}$ was found (Datta and Kontomichalou, 1965). A large number of TEM variants have been identified to date that mediate resistance to most β-lactams (Paterson and Bonomo, 2005). Among β-lactamases, metallo-β-lactamases (MBL) such as New Delhi Metallo-β-Lactamases (NDMs) rank among the most detrimental for their ability to lead to resistance against not only β-lactams but also carbapenems, and unlike other serine β-lactamases that exploit a serine active site for hydrolysis, MBLs rely on zinc ions in their active site to facilitate hydrolytic reaction (Bebrone, 2007). This different mechanism of MBLs on β-lactam hydrolysis leads to the consensus that BLIs are ineffective against MBLs. However, experimental evidence for whether all common β-lactam/BLI combinations are ineffective against MBLs is still needed. Statistics in recent years show that the prevalence of NDMs is increasing worldwide (Bush, 2018). Since the discovery of NDM-1, a total of 24 different NDM variants have been identified, the coding genes of which (bla_{NDM}) are hosted by a variety of bacteria, predominantly *Enterobacteriaceae* followed by other pathogenic bacteria such as *Acinetobacter spp*. (Wu et al., 2019). Transferable plasmids play an important role in the dissemination of bla_{NDM} by hosting and spreading of the gene through horizontal gene transfer (HGT) (Adamczuk et al., 2015; Wailan et al., 2015; Sugawara et al., 2017; Liu et al., 2019). This has led to the wide distribution of NDM worldwide, posing a severe threat to public health (Dortet et al., 2014; Dadashi et al., 2019; Wu et al., 2019).

In this study, an extensively-drug resistant (XDR) *E. coli* W60 was isolated from the urine sample of a patient following his bladder tumor surgery. This strain was found resistant to all tested antibiotics except tigecycline. In particular, *E. coli* W60 was found resistant to all commonly available β-lactam/BLI combinations. Whole-genome sequencing revealed that W60 hosts two novel transferable plasmids, the IncFIB-type plasmid pECW601 and the IncFII-type plasmid pECW602, and showed that the two multidrug resistance plasmids carry the main genetic determinants of antimicrobial resistance for *E. coli* W60. pECW601 contains the $bla_{\mathrm{NDM-5}}$ gene, which encodes the metallo-β-lactamase NDM-5. pECW602 contains a truncated bla_{TEM} gene. Further genetic analysis provides experimental evidence that NDM5 leads to resistance to β-lactam/BLI combinations and that the N-terminal 28 amino acids containing signal peptide appear essential for the functionality of TEM. This work provides a detailed insight into the resistance mechanisms of a clinical XDR *E. coli* strain, and provides evidence on the role of β-lactamase genes. In particular, this work demonstrates MBLs indeed renders BLIs ineffective, further stressing the danger of these now widespread β-lactamase genes.

MATERIALS AND METHODS

Bacterial Strains

The strain *E. coli* W60 used in this study was isolated from a urine sample of a patient from the Second Hospital of Shandong University who had an infection after bladder tumor resection. The preliminary identification results of the hospital showed that the bacterium was resistant to multiple antibiotics, so further research was needed to develop a treatment plan for the patient. The handling and experiments of the studied bacteria followed security and safety guidelines of Shandong University and the Second Hospital of Shandong University. All procedures were approved by the Scientific Ethics Committee of the Second Hospital of Shandong University with Approval No. KYLL-2020(LW)-044.

Susceptibility Tests

Drug susceptibility testing was carried out by the disk diffusion method, and the standard for inhibition zones followed the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2018b). Minimum Inhibition Concentrations (MICs) for all antibiotics (ampicillin, amoxicillin-clavulanate, ceftazidime-avibactam, piperacillin-tazobactam, ampicillin-sulbactam, ticarcillin-clavulanate, cefoperazone, cefotaxime, ceftazidime, cefoxitin, cefepime, cefazolin, imipenem, meropenem, kanamycin, ciprofloxacin, gatifloxacin, nalidixic acid, chloramphenicol, trimethoprim, and tetracycline) but tigecycline was determined with the agar dilution method following CLSI guidelines (Clinical and Laboratory Standards Institute, 2019). For tigecycline, MIC was determined with the broth microdilution method following European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (Marchaim et al., 2014). *E. coli* ATCC 25922 was used as the control strain for most antibiotics. *E. coli* ATCC 27853 was used as the control strain for carbapenems. For resistance against β-lactam/BLI combinations, *E. coli* ATCC 35218 was used as the control strain as instructed by the CLSI guidelines (Clinical and Laboratory Standards Institute, 2018a,b, 2019).

Whole Genome Sequencing and Sequence Analyses

The genomic DNA of *E. coli* W60 was extracted with the SDS method (Natarajan et al., 2016). Libraries for single-molecule real-time (SMRT) sequencing was constructed with an insert size of 10 kb using the SMRTbell™ Template kit, version 1.0. Sequencing libraries were generated using NEBNext™ Ultra® DNA Library Prep Kit for Illumina (NEB, United States) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. The whole genome of *E. coli* W60 was sequenced using PacBio Sequel platform and Illumina NovaSeq PE150 at the Beijing Novogene Bioinformatics Technology Co., Ltd., SMRT Link v5.1.0 software was used[1] for read assembly (Ardui et al., 2018; Reiner et al., 2018), which was further optimized by the Arrow software (part of SMRT Link v5.1.0). General function annotation databases GO (Gene Ontology, 2015), KEGG (Kanehisa and Goto, 2000), COG (Natale et al., 2000), NR (Li et al., 2002), Pfam (El-Gebali et al., 2019), TCDB (Saier et al., 2006), and Swiss-Prot (Boeckmann et al., 2003) were used for functional annotation of genes, and the Comprehensive Antibiotic Resistance Database (CARD) was used to manually annotate antimicrobial resistance genes (ARGs) (Jia et al., 2017). PlasmidFinder 2.1[2] was used to analyze plasmid types (Carattoli et al., 2014).

Mating Experiment

The ability of plasmids to transfer was determined by mating experiment, and the *E. coli* J53 was used as recipient strain. Transconjugants were selected on LB agar supplemented with different antibiotic agents: pECW601 was selected by LB agar containing NaN_3 (100 μg/ml) and trimethoprim (30 μg/ml), pECW602 was selected by LB agar containing NaN_3 (100 μg/ml), fosfomycin (50 μg/ml), and glucose 6-phosphate (25 μg/ml) according to CLSI standards (Clinical Laboratory Standards Institute, 2018b). Different antibiotics were used for screening transconjugants because pECW601 and pECW602 are, respectively, the only genetic determinants in the donor *E. coli* W60 strain that encode resistance for trimethoprim and fosfomycin as predicted by genomic analysis. Transconjugants were confirmed by amplifying the drug resistance genes and plasmid specific *rep* genes, followed by sequencing for final confirmation (**Supplementary Figure S1**). The sequences of primers for transconjugant confirmation are shown in **Supplementary Table S1**.

Cloning of bla_{NDM-5} and $bla_{TEM-W60}$

The bla_{NDM-5} and $bla_{TEM-W60}$ genes were amplified and cloned into pBCKS(+). Primers used are shown in **Supplementary Table S2**.

Accession Numbers

The nucleotide sequences of *E. coli* W60 genome and its plasmids can be found on NCBI under accession numbers CP058342, CP058343 and CP058344.

[1]https://www.pacb.com/support/software-downloads/

[2]https://cge.cbs.dtu.dk/services/PlasmidFinder/

Bioinformatics

SerotypeFinder 2.0[3] was used for serotype prediction (Joensen et al., 2015), followed by serum aggregation reaction experiment for confirmation. Prediction of signal peptides was performed using SignalP 5.0[4] (Armenteros et al., 2019). Sequence alignment was performed using ESPript 3.0[5] with the Maximum Likelihood method and Poisson correction model (Robert and Gouet, 2014). Phylogenetic analysis was done by MEGA-X (Kumar et al., 2018). Protein structure analysis was performed using PyMol (Seeliger and de Groot, 2010).

Ethics

All experiments in this work were performed adhering to the Declaration of Helsinki and were approved by the Scientific Ethics Committee of the Second Hospital of Shandong University with Approval No. KYLL-2020(LW)-044.

RESULTS

Isolation and Resistance Properties of a Clinical XDR *E. coli* W60 Strain

Escherichia coli strain W60 was isolated from the urine sample of a bladder tumor patient from the Second Hospital of Shandong University. *In silico* prediction of the serotype was performed for *E. coli* W60, suggesting it was either serotype O101, O8, or H9. Further serum aggregation reaction assay was performed, showing that *E. coli* W60 belongs to serotype O101 (**Supplementary Figure S2**). O101 is a common enterotoxigenic *E. coli* (ETEC) serotype originated from pigs and cattle (Staaf et al., 1997). The antibiotic resistance profiles were determined for this strain by testing its resistance against major classes of antibiotics including β-lactams, quinolones, carbapenems, aminoglycosides, chloramphenicol, trimethoprim, tigecycline, macrolides, and polymyxins. *E. coli* W60 was found resistant to all antibiotics tested except for tigecycline (**Table 1**). Of particular interest, *E. coli* W60 is highly resistant to all commonly available β-lactam/BLI combinations with MIC values much higher than the resistance breakpoint.

Genome Characteristics and Genotypic Basis for Antibiotic Resistance of *E. coli* W60

Whole genome sequencing was performed with PacBio and Illumina sequencing on *E. coli* W60. *E. coli* W60 has a chromosome at the size of 4,808,792 bp and GC content of 50.8% (**Figure 1**). Two circular plasmids were identified from *E. coli* W60, respectively, named pECW601 and pECW602. BLAST analysis of both plasmids found no known plasmids that share both high sequence identity and coverage.

pECW601 has a size of 140,410 bp. Its closest known relative is a *E. coli*-harboring unnamed plasmid from that has a size of

[3]https://cge.cbs.dtu.dk/services/SerotypeFinder/

[4]http://www.cbs.dtu.dk/services/SignalP/

[5]http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi

TABLE 1 | Antimicrobial resistance of *Escherichia coli* W60.

Antibiotic class	Antibiotics	Antimicrobial resistance[1]	
		Inhibition zone (mm)	MIC (mg/L)
β-lactam	Ampicillin (AMP)	*R*(0)	*R*(>512)
	Amoxicillin-clavulanate (AMC)	*R*(0)	*R*(64/32)
	Ceftazidime-avibactam (CAZ-AVI)	*R*(13)	*R*(>512/4)
	Piperacillin-tazobactam (TZP)	*R*(0)	*R*(>512/4)
	Ampicillin-sulbactam (SAM)	*R*(0)	*R*(>256/128)
	Ticarcillin-clavulanate (TIM)	*R*(7)	*R*(>256/2)
	Cefoperazone (CFP)	*R*(0)	*R*(>512)
	Cefotaxime (CTX)	*R*(0)	*R*(>512)
	Ceftazidime (CAZ)	*R*(0)	*R*(>512)
	Cefoxitin (FOX)	*R*(0)	*R*(>512)
	Cefepime (FEP)	*R*(0)	*R*(512)
	Cefazolin (CFZ)	*R*(0)	*R*(>512)
Carbapenem	Imipenem (IPM)	*R*(14)	*I*(2)
	Meropenem (MEM)	*R*(0)	*R*(4)
Aminoglycoside	Kanamycin (KAN)	*R*(12)	*R*(64)
Quinolone	Ciprofloxacin (CIP)	*R*(0)	*R*(32)
	Gatifloxacin (GAT)	*R*(9)	*R*(8)
	Nalidixic acid (NAL)	*R*(0)	*R*(512)
Phenicol	Chloramphenicol (CHL)	*R*(8)	*R*(256)
Diaminopyrimidine	Trimethoprim (TMP)	*R*(0)	*R*(>512)
Glycylcycline	Tigecycline (TGC)	*S*(18)	*S*(0.5)
Tetracycline	Tetracycline (TET)	*R*(10)	*R*(128)

[1]*R, resistant; I, intermediate; S, sensitive.*

286,854 bp (GenBank accession number CP025329.1). Plasmid replication analysis showed that pECW601 is an IncFIB type plasmid. Comparison of pECW601 and CP025329.1 show that both plasmids share similar conjugation genes, iron oxidase related genes and IS*26* transposase genes but their MDR regions are fundamentally different (**Figure 2A**). In addition, CP025329.1 has two copies of the conjugation gene clusters but pECW601 has only one. Therefore, a conclusion can be made that pECW601 is a new multidrug resistance plasmid.

pECW602 has a size of 94,780 bp. Its closest known relative is pECO-fce from *E. coli* Eco889 (GenBank: CP015160.1). Plasmid replication analysis showed that pECW602 is an IncFII type plasmid. Both plasmids share the backbone conjugation gene cluster although CP015160.1 has 2 such clusters while pECW602 has only one. The MDR regions of the two plasmids have little in common, along with other minor different features between the two plasmids (**Figure 2B**). This comparison suggests that pECW602 is also a new plasmid.

Analysis of the genomic sequence of *E. coli* W60 reveals resistance determinants putatively responsible for the antimicrobial resistance phenotype of this strain. Two AmpC-type and one AmpH-type β-lactamases are encoded by the chromosome that are potentially responsible for resistance against β-lactams (**Supplementary Table S3**). The chromosome harbors a *gyrA* gene that encodes a D87N/S83L variant and a *pacC* gene that encodes a S80I variant (**Supplementary Table S3**). Both variants are responsible for resistance to quinolones (Yoshida et al., 1990). Respectively, 6 and 8 antimicrobial resistance genes (ARGs) were found on pECW601 and pECW602 (**Table 2** and **Supplementary Tables S4, S5**). ARGs responsible for the resistance to aminoglycosides, β-lactams and sulfonamides are found on both plasmids. pECW601 harbors ARGs responsible for the resistance to trimethoprim, tetracycline and glycopeptides, while pECW602 harbors ARGs responsible for the resistance to chloramphenicol and fosfomycin. Therefore, genetic features were found for all the major resistant antibiotic classes investigated in the antimicrobial resistance phenotype analysis (**Table 1**), and the two multidrug resistance plasmids account for the resistance to most of these antibiotics.

Genetic analysis of the *E. coli* W60 genome leads to two interesting observations: *E. coli* W60 is resistant to all the β-lactam/BLI combinations tested, while the genetic basis for this observation remains unclear; pECW602 harbors a truncated version of $bla_{\mathrm{TEM-1}}$, leading us to wonder its role in mediating β-lactam resistance. Both these observations were further investigated in this work.

Transferability of pECW601, pECW602, and β-Lactam/BLI Combination Resistance Phenotypes

Conjugation assays between *E. coli* W60 and the recipient *E. coli* J53 strain show that both pECW601 and pECW602 are transferable plasmids. Analysis of the antimicrobial resistance phenotypes of both transconjugants leads to the finding that the pECW601-harboring transconjugant showed nearly the same high level of resistance to β-lactam/BLI combination as *E. coli* W60 (**Table 3**). Considering the only β-lactamase-coding gene on pECW601 is $bla_{\mathrm{NDM-5}}$, a hypothesis is raised that $bla_{\mathrm{NDM-5}}$ can lead to high level resistance to β-lactam/BLI combinations in *E. coli*.

β-Lactam/BLI Combination Resistance of $bla_{\mathrm{NDM-5}}$-Harboring *E. coli* Strain

To further explore the role of $bla_{\mathrm{NDM-5}}$ in the resistance of β-lactam related antibiotics, we cloned $bla_{\mathrm{NDM-5}}$ into pBCKS(+) plasmid and transformed the resulting construct to *E. coli* DH5α. An empty pBCKS(+) vector does not increase the resistance of *E. coli* DH5α to β-lactams or β-lactam/BLI combinations. However, $bla_{\mathrm{NDM-5}}$-containing pBCKS(+) increased the resistance of *E. coli* DH5α to β-lactams by 4–256-fold, and increased the resistance of *E. coli* DH5α to β-lactam/BLI combinations by 8–1,024-fold (**Table 4**). The finding that $bla_{\mathrm{NDM-5}}$ leads to high resistance to β-lactam/BLI combinations in *E. coli* DH5α, together with the observation that pECW601-containing *E. coli* J53 transconjugant is highly resistant to β-lactam/BLI combinations, experimentally confirm that $bla_{\mathrm{NDM-5}}$ leads to resistance to β-lactam/BLI combinations, and is the reason for the high level of resistance to β-lactam/BLI combinations of *E. coli* W60.

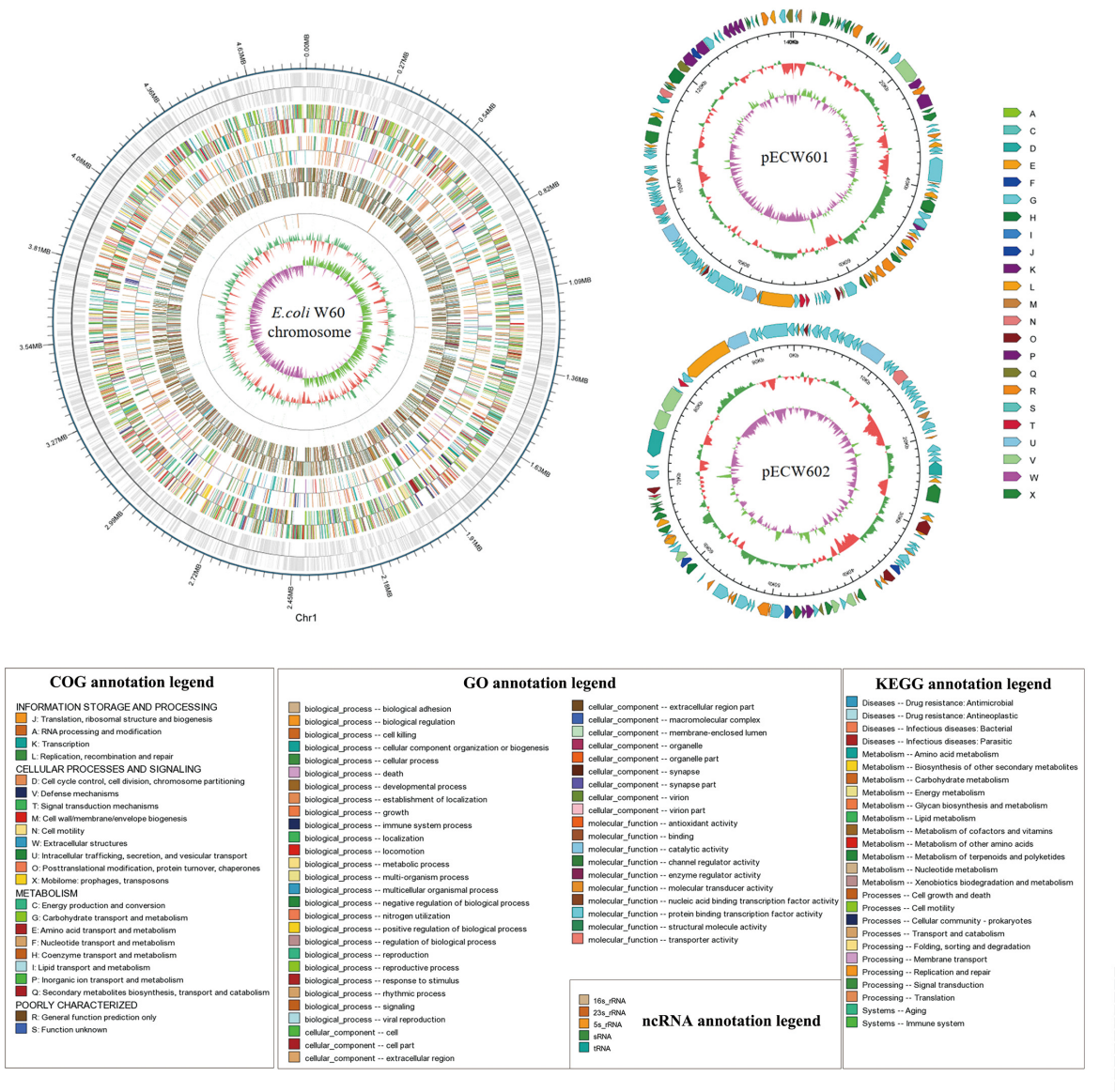

FIGURE 1 | Whole genome map of pECW601, pECW602 and chromosome of *E. coli* W60. The outermost circle is the coordinates of the genomic feature. From outside to inside the circles indicates coding genes, gene function annotation results, ncRNAs, genome GC contents, genome GC skew values. For plasmids, from the outside to the inside the circles indicate COG functional annotation classification genes (clockwise arrow indicates positive strand coding), genome sequence position coordinates, genome GC content, genome GC skew values distribution. COG (KOG), KEGG, GO databases were used for gene annotation. Different colors represent different functions of genes. For genome GC content, the inward red part indicates that the GC content of the region is lower than the average GC content of the whole genome, and the outward green part indicates the opposite; for the genome GC skew value, the inward pink part indicates that in the region the G content is lower than the C content, and the outward light green part indicates the opposite.

A bleomycin resistance-conferring ble_{MBL} gene is located downstream of bla_{NDM-5} under the control of the same promoter on pECW601. The potential impact of this gene on the function of bla_{NDM-5} was probed by cloning both bla_{NDM-5} and ble_{MBL} to pBCKS(+), transforming the construct to *E. coli* DH5α, and comparing the resistance of the transformant to β-lactams and β-lactam/BLI combinations (**Table 4**). Increased resistance, although by only two–fourfold, was found for ampcilin, ceftazidime, cefoxitin, and ampicillin-sulbactam. This finding suggests a potential function of ble_{MBL} in enhancing the role of bla_{NDM-5} in β-lactam resistance.

Presence and Function of a Truncated bla_{TEM} Gene on pECW602

A truncated bla_{TEM} gene that encodes a TEM β-lactamase missing the N-terminal 28 amino acids, termed $bla_{TEM-W60}$, was found on pECW602. Sequence comparison of TEM-W60

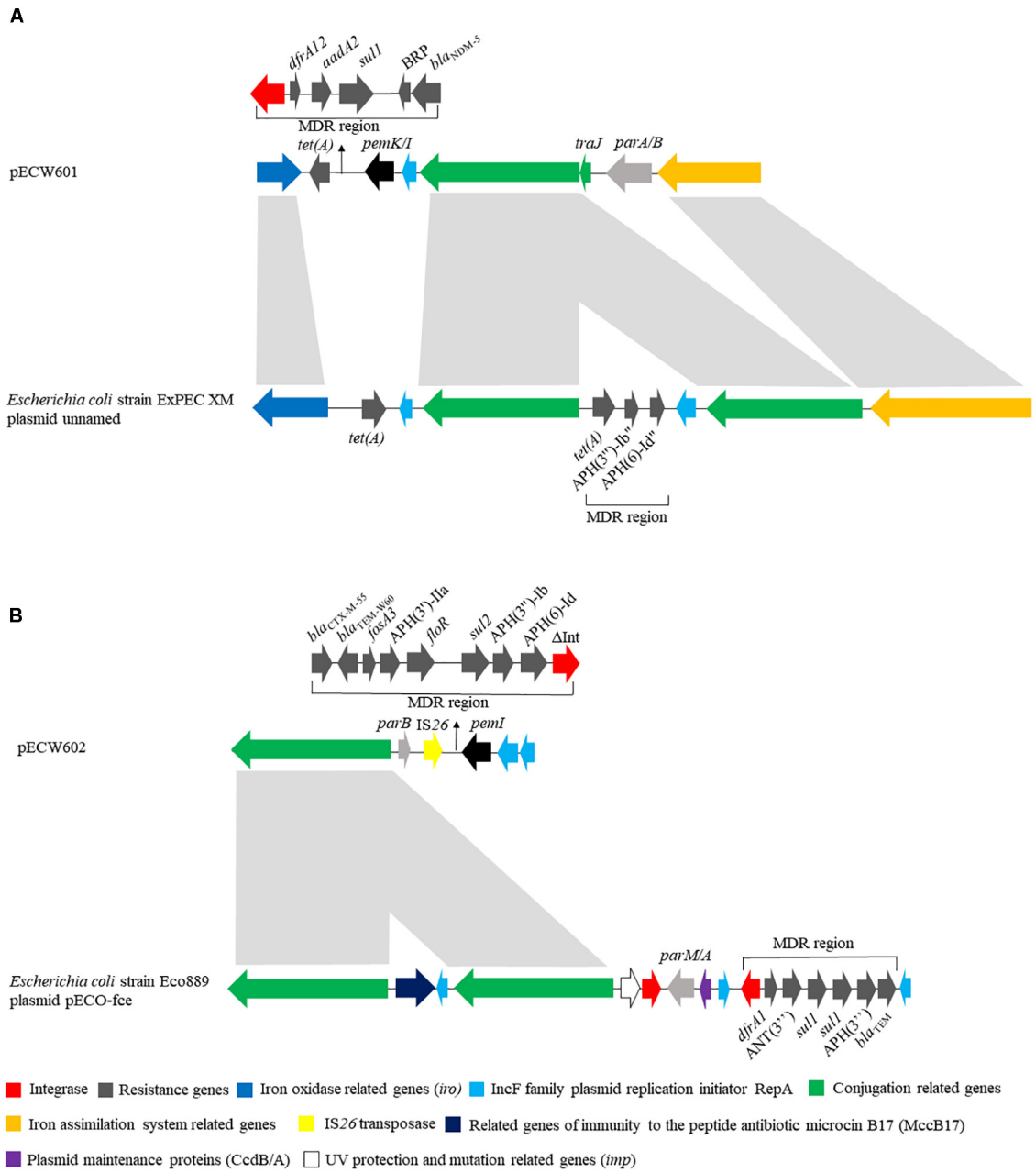

FIGURE 2 | Linear schematic of sequence comparison between plasmids found in this work and their closest relatives. **(A)** Comparison between pECW601 and *Escherichia coli* strain ExPEC XM plasmid unnamed; **(B)** comparison between pECW602 and *E. coli* strain Eco889 plasmid pECO-fce. Different colors represent gene clusters with different functions. Arrows indicate the direction of genes. The light gray indicates high similarity between sequences.

with TEM-1 and TEM-2 showed that other than missing the N-terminal 28 amino acids, TEM-W60 also has two mutations V29L and L38A (**Figure 3**). Phylogenetic analysis suggest that TEM-W60 does not cluster with known TEM β-lactamases (**Supplementary Figure S3**). The function of $bla_{\mathrm{TEM-W60}}$ was analyzed by cloning it into pBCKS(+), transforming the construct into *E. coli* DH5α, and analyzing the resistance of the transformant to β-lactams and β-lactam/BLI combinations (**Table 5**). It was found that $bla_{\mathrm{TEM-W60}}$ has little impact on resistance to β-lactams and β-lactam/BLI combinations. Structural analysis showed the two mutated amino acids reside on the N-terminal α-helix of TEM β-lactamase, far away from the active site and all other sites important for the activity of β-lactamase (**Figure 4**) (Jelsch et al., 1993; Minasov et al., 2002). It is therefore unlikely that these two substitutions significantly impact β-lactamase activity. Further sequence analysis predicted that the first 23 amino acids of TEM-1 form the signal peptide that is critical for secretion (**Supplementary Figure S4**). As

TABLE 2 | Presence of ARGs on plasmids.

Targeted antibiotic class	pECW601	pECW602
Aminoglycosides	*aadA2*	*APH(3′)-IIa* *APH(3″)-Ib* *APH(6)-Id*
β-lactam	bla_{NDM-5}	$bla_{CTX-M-55}$ $bla_{TEM-W60}$
Diaminopyrimidine	*dfrA12*	
Sulfonamides	*sul1*	*sul2*
Phenicols		*floR*
Tetracycline	*tetA*	
Fosfomycin		*fosA3*
Glycopeptide	ble_{MBL}	

TABLE 3 | Antibiotic sensitivity of pECW601 and pECW602-containing transconjugants.

Antibiotics[1]	W60[2] (mg/L)	J53[3] (mg/L)	J53/pECW601[4] (mg/L)	J53/pECW602[5] (mg/L)
AMP	>512	2	>512	>512
CFP	>512	<0.125	>512	128
CTX	>512	<0.125	>512	128
CAZ	>512	0.25	>512	16
FOX	>512	16	>512	32
FEP	512	<0.125	512	16
CFZ	>512	1	>512	>512
AMC	64/32	4/2	64/32	4/2
CAZ-AVI	>512/4	0.25/4	>256/4	0.125/4
TZP	>512/4	4/4	>256/4	4/4
SAM	>256/128	4/2	>256/128	16/8
TIM	>256/2	2/2	>256/2	16/2

[1]*AMP, ampicillin; AMC, amoxicillin-clavulanate; CAZ-AVI, ceftazidime-avibactam; TZP, piperacillin-tazobactam; SAM, ampicillin-sulbactam; TIM, ticarcillin-clavulanate; CFP, cefoperazone; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; FEP, cefepime; CFZ, cefazolin.*
[2]*W60, E. coli W60.*
[3]*J53, E. coli J53.*
[4]*J53/pECW601, E. coli J53 transconjugant harboring pECW601.*
[5]*J53/pECW602, E. coli J53 transconjugant harboring pECW602.*

β-lactamases are extracellular or periplasmic proteins and need to be secreted for their function (Livermore, 1995), it strongly suggests that the loss of function for TEM-W60 is due to the loss of a signal peptide and subsequent inability for secretion.

DISCUSSION

MDR and XDR pathogenic bacteria pose a significant threat to human health (Karaiskos and Giamarellou, 2014). Due to their resistance to multiple antibiotic agents, the treatment of postoperative infections becomes difficult, thus increasing the morbidity and mortality of patients. Understanding the underlying drug resistance mechanisms of these pathogens helps us finding solutions for the long-standing antibiotic resistance problem. For instance, β-lactamase inhibitors were developed to inhibit β-lactamases that caused β-lactam resistance.

In this study, we identified an XDR *E. coli* W60 strain in the urine sample of a patient with postoperative infection. *E. coli* W60 belongs to serotype O101 after analysis by agglutination reaction assay. Unlike the enterohaemorrhagic *E. coli* O157, which is highly pathogenic and virulent, although serotype O101 has been shown to be related to diarrhea and urinary tract infection (Mandal et al., 2001; Sun et al., 2019), it is only a risk factor and has no direct connection with human disease. This strain shows resistance to almost all common antibiotic agents, including β-lactams, aminoglycosides, carbapenems, quinolones and etc. Whole genome sequencing shows that *E. coli* W60 contains two new multidrug resistance plasmids, pECW601 and pECW602. Analysis of the ARGs harbored by these two plasmids suggested that these plasmids are the primary reason for the extensively-drug resistance phenotype, while resistance genes located into the chromosomes are presumably responsible for only β-lactam and quinolone resistance. Further conjugation assays show both plasmids are transferable. These findings again confirm the danger of multidrug resistance plasmids: the concentration of different multidrug resistance plasmids into one bacterium can lead to the generation of highly resistant pathogens as demonstrated in this work and the work of others (Zhao et al., 2010; Guo et al., 2017; Li et al., 2019). Because transfer of plasmids is way more efficient than the evolution of new antibiotic resistance genotypes, we suspect this is the primary route for the generation of extensively- or pan-drug resistant pathogens. The danger of multidrug resistance plasmids should therefore be given high attention. The fact that both multidrug resistance plasmids found in this work are new rings a bell for us: there could be many more such plasmids out there waiting to be found. We therefore would like to call upon scientists and doctors in the field of antimicrobial resistance to perform more surveillance studies on clinical multidrug resistance plasmids and have a better understanding on the types and structures of these mobile genetic elements.

A particularly interesting and troubling feature of *E. coli* W60 is that it is resistant to all the β-lactam/BLI combinations tested. Further genetic analysis shows that the bla_{NDM-5} gene harbored by pECW601 is the reason for the resistance of β-lactam/BLI combinations. β-lactams are by far the most important antibiotics for their high efficiency to both Gram-positive and Gram-negative bacteria, and for their relatively better safety to human in comparison with other more recently introduced last-resort antibiotics (Bush and Bradford, 2016). Therefore, reusing β-lactams that already develop widespread resistance by combining BLIs is a great strategy and the first choice when treating infections of β-lactam resistant pathogens. It has been long suspected that this strategy does not work well with MBLs for their different resistance mechanism (Bebrone, 2007). This work provides solid microbiological and genetic evidence that NDM-5 can lead to high β-lactam/BLI resistance against all commonly available β-lactam/BLI combinations, and it is already causing strong resistance in a clinical pathogen. The mechanism behind this phenotype is likely that these commonly used BLIs (tazobactam, clavulanate, sulbactam, and avibactam) are serine-β-lactamases inhibitors that inhibit the serine active site of

TABLE 4 | Antibiotic sensitivity of bla_{NDM-5} and bla_{NDM-5}/ble_{MBL}-harboring strains.

Antibiotics[1]	DH5α[2] (mg/L)	DH5α/pBCKS(+)[3] (mg/L)	DH5α/pBCKS(+)-NDM5[4] (mg/L)	DH5α/pBCKS(+)-NDM5+BLE[5] (mg/L)
AMP	1	2	64	128
CFP	<0.125	<0.125	8	8
CTX	<0.125	<0.125	32	32
CAZ	<0.125	<0.125	64	128
FOX	16	16	64	256
FEP	<0.125	<0.125	1	1
CFZ	2	1	256	256
AMC	2/1	4/2	64/32	64/32
CAZ-AVI	<0.125/4	<0.125/4	128/4	128/4
TZP	2/4	4/4	16/4	16/4
SAM	2/1	2/1	64/32	128/64
TIM	1/2	2/2	256/2	256/2

[1]AMP, ampicillin; AMC, amoxicillin-clavulanate; CAZ-AVI, ceftazidime-avibactam; TZP, piperacillin-tazobactam; SAM, ampicillin-sulbactam; TIM, ticarcillin-clavulanate; CFP, cefoperazone; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; FEP, cefepime; CFZ, cefazolin.
[2]DH5α, E. coli DH5α.
[3]DH5α/pBCKS(+), E. coli DH5α containing empty pBCKS(+) vector.
[4]DH5α/pBCKS(+)-NDM5, bla_{NDM-5}-harboring E. coli DH5α strain.
[5]DH5α/pBCKS(+)-NDM5 + BLE, bla_{NDM-5}/ble_{MBL}-harboring E. coli DH5α strain.

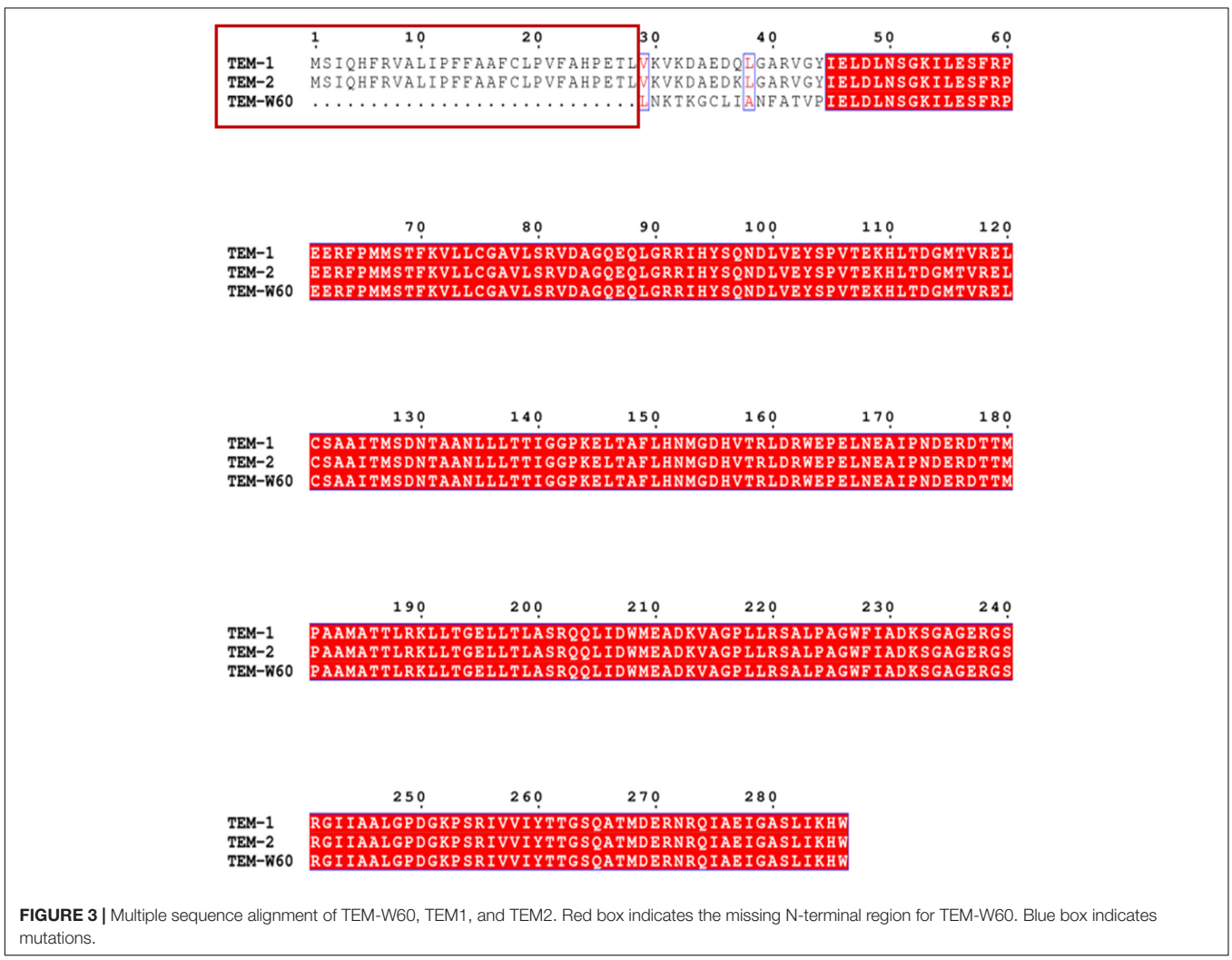

FIGURE 3 | Multiple sequence alignment of TEM-W60, TEM1, and TEM2. Red box indicates the missing N-terminal region for TEM-W60. Blue box indicates mutations.

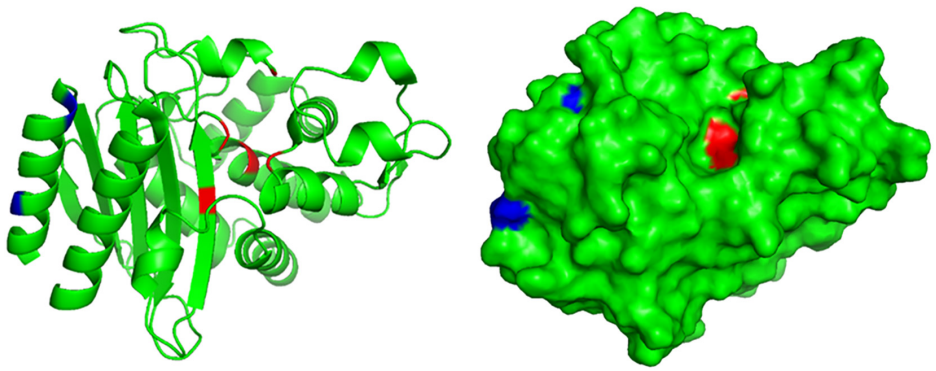

FIGURE 4 | Structural analysis of TEM-W60. The structure of TEM was previously determined (PDB ID 1M40). Blue color indicates mutated amino acids in TEM-W60. Red color indicates key residues for the activity of TEM.

β-lactamase, but are ineffective against the zinc ion-containing active sites for MBLs (Docquier and Mangani, 2018). It needs to be pointed out that the β-lactamases besides bla_{NDM-5} encoded by *E. coli* W60 also contribute to this β-lactam/BLI combination resistance phenotype, as *E. coli* DH5α harboring only bla_{NDM-5} showed a weaker resistance in comparison with *E. coli* W60 or pECW601-containing *E. coli* J53. The finding that bla_{NDM-5} confers widespread resistance to β-lactam/BLI combinations again confirms the danger of MBLs, as they render β-lactams, β-lactam/BLIs, and carbapenems (in other words all β-lactam related antibiotics) ineffective. Susceptible testing showed that *E. coli* DH5α containing both bla_{NDM-5} and ble_{MBL} is slightly more resistant to ampicillin, ceftazidime, cefoxitin, and ampicillin-sulbactam than *E. coli* DH5α containing only bla_{NDM-5} (**Table 4**). ble_{MBL} encodes a BRP protein that exerts resistance to bleomycin by specifically binding to bleomycin family antibiotics, but does not appear to interact with β-lactams or β-lactamases. Previous research reported that ble_{MBL} and bla_{NDM} genes are often co-transcribed, and suggested that BRP influences *E. coli* mutation rates to stabilize NDM resistance traits (Dortet et al., 2012). An earlier report suggested that the existence of bleomycin resistance phenotype protects bacteria from external DNA damage through the DNA repair system, thereby conferring better fitness of bacteria and facilitating the inheritance of genetic characteristics (Blot et al., 1991). The enhancement of β-lactam resistance by ble_{MBL} found in this work could be for the same reason, and this new role of ble_{MBL} in β-lactam resistance makes more sense for the frequently observed co-transcription of ble_{MBL} and bla_{NDM}.

TABLE 5 | Antibiotic sensitivity of $bla_{TEM-W60}$-harboring strains.

Antibiotics[1]	DH5α[2] (mg/L)	DH5α/pBCKS(+)[3] (mg/L)	DH5α/pBCKS(+)-TEM-W60[4] (mg/L)
AMP	1	2	2
CFP	<0.125	<0.125	<0.125
CTX	<0.125	<0.125	<0.125
CAZ	<0.125	<0.125	<0.125
FOX	16	16	32
FEP	<0.125	<0.125	<0.125
CFZ	2	1	1
AMC	2/1	4/2	4/2
CAZ-AVI	<0.125/4	<0.125/4	<0.125/4
TZP	2/4	4/4	4/4
SAM	2/1	2/1	2/1
TIM	1/2	2/2	2/2

[1]*AMP, ampicillin; AMC, amoxicillin-clavulanate; CAZ-AVI, ceftazidime-avibactam; TZP, piperacillin-tazobactam; SAM, ampicillin-sulbactam; TIM, ticarcillin-clavulanate; CFP, cefoperazone; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; FEP, cefepime; CFZ, cefazolin.*
[2]*DH5α, E. coli DH5α.*
[3]*DH5α/pBCKS(+), E. coli DH5α containing empty pBCKS(+) vector.*
[4]*DH5α/PBCKS(+)-TEM-W60, $bla_{TEM-W60}$-harboring E. coli DH5α strain.*

A survey of other β-lactamase genes leads to the finding of a truncated bla_{TEM} gene on pECW602 that encode a TEM β-lactamase with 28 amino acids deleted at the N-terminus. This gene was found unfunctional presumably due to the loss of the signal peptide coding region in comparison with other bla_{TEM} genes. This finding confirms the importance of the signal peptide for TEM β-lactamase, understandably for its critical role in β-lactamase secretion.

CONCLUSION

Combining genomic, microbiological and genetic approaches, we identified the genetic basis for the extensively-drug resistance phenotype of the clinical *E. coli* W60 strain. Two new conjugative multi-resistance plasmids pECW601 and pECW602 were found in *E. coli* W60, and were confirmed to be the primary determinants of the extensively drug resistance phenotype. Resistance phenotype analysis showed that *E. coli* W60 is resistant to all commonly available β-lactam/BLI combinations. Further genetic analysis showed that the NDM-5 β-lactamase coded on pECW601 is responsible for this phenotype, which is further enhanced by co-expressing BRP. A new unfunctional

truncated TEM β-lactamase that lacks the signal peptide-containing N-terminus is encoded by pECW602, suggesting the critical role of the signal peptide on the function of β-lactamases. Findings in this work shows the danger of transferable multidrug resistance plasmids and metallo-β-lactamases. We hope with this work these dangers are given enough attention in further developing methods for containing antimicrobial resistance.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject (accession: PRJNA642190).

AUTHOR CONTRIBUTIONS

WW, LL, MZ, ZL, WS, and FL performed microbial and genetic experiments. YN, TL, and XZ isolated bacteria. MW and WW performed bioinformatic analysis. MW, WW, XZ, and HX analyzed the data. MW, WW, XZ, and HX wrote the manuscript. MW, XZ, and HX conceived of the study and oversaw the project. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.590357/full#supplementary-material

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Emergence and Spread of Different ESBL-Producing *Salmonella enterica* Serovars in Hospitalized Horses Sharing a Highly Transferable IncM2 CTX-M-3-Encoding Plasmid

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Salmonella enterica is a major causative pathogen of human and animal gastroenteritis. Antibiotic resistant strains have emerged due to the production of extended-spectrum β-lactamases (ESBLs) posing a major health concern. With the increasing reports on ESBL-producing Enterobacterales that colonize companion animals, we aimed to investigate ESBL dissemination among ESBL-producing *Salmonella enterica* (ESBL-S) in hospitalized horses. We prospectively collected ESBL-S isolates from hospitalized horses in a Veterinary-Teaching Hospital during Dec 2015–Dec 2017. Selection criteria for ESBL-S were white colonies on CHROMagarESBL plates and an ESBL phenotypic confirmation. *Salmonella enterica* serovars were determined using the Kaufmann-White-Le-Minor serological scheme. ESBL-encoding plasmids were purified, transformed and compared using restriction fragment length polymorphism (RFLP). Whole genome sequencing (Illumina and MinION platforms) were performed for detailed phylogenetic and plasmid analyses. Twelve ESBL-S were included in this study. Molecular investigation and Sequence Read Archive (SRA) meta-analysis revealed the presence of three unique *Salmonella enterica* serovars, Cerro, Havana and Liverpool, all reported for the first time in horses. PFGE revealed the clonal spread of *S.* Cerro between seven horses. All twelve isolates carried $bla_{CTX-M-3}$ and showed an identical multidrug resistance profile with co-resistance to trimethoprim/sulfamethoxazole and to aminoglycosides. Plasmid RFLP proved the inter-serovar horizontal spread of a single $bla_{CTX-M-3}$-encoding plasmid. Complete sequence of a representative plasmid (*S.* Havana strain 373.3.1), designated pSEIL-3 was a ~86.4 Kb IncM2 plasmid, that encoded nine antibiotic resistance genes. pSEIL-3 was virtually identical to pCTX-M3 from *Citrobacter freundii*, and showed high identity (>95%) to six other $bla_{CTX-M-3}$ or bla_{NDM-1} IncM2 broad host range plasmids from various Enterobacterales of human origin. Using a specific six gene-based multiplex PCR, we detected pSEIL-3 in various Enterobacterales species that co-colonized the horses' gut. Together, our findings show

the alarming emergence of ESBL-S in hospitalized horses associated with gut shedding and foal morbidity and mortality. We demonstrated the dissemination of CTX-M-3 ESBL among different *Salmonella enterica* serovars due to transmission of a broad host range plasmid. This report highlights horses as a zoonotic reservoir for ESBL-S, including highly transmissible plasmids that may represent a 'One-Health' hazard. This risk calls for the implementation of infection control measures to monitor and control the spread of ESBL-S in hospitalized horses.

Keywords: *Salmonella enterica*, WGS, ESBL, serovars, IncM2, $bla_{CTX-M-3}$, MDR plasmid, horizontal transfer

INTRODUCTION

Salmonella enterica is the major causative pathogen of human and animal Salmonellosis (Scallan et al., 2011; Omer et al., 2018). Human Salmonellosis recently poses a major health concern due to the dissemination of multidrug resistant (MDR) strains that produce extended-spectrum β-lactamases (ESBLs) that limit the appropriate treatment options (Antonelli et al., 2019; Jajere, 2019). ESBL-producing *Salmonella enterica* (ESBL-S) are increasingly reported from livestock animals (European Centre for Disease Prevention and Control., 2019). Shared ESBL-S serovars among livestock and humans suggest that food animals are possible zoonotic reservoir for this human-associated pathogen (Sjölund-Karlsson et al., 2013).

In the last decade, along with food animals, ESBL-producing Enterobacterales colonization in companion animals is steadily increasing (Doi et al., 2017). Although the zoonotic potential of these bacteria is still enigmatic, there is a consensus regarding their role as being a reservoir for antibiotic resistance, and as a possible hazard to human health due to the close physical contact between companion animals and humans (Madec et al., 2017).

Horses in specific are in close interaction with humans and children in various interfaces including private use, sport events and as therapeutic animals. As such, they may serve as a zoonotic source for antibiotic resistant pathogens. Horses have been shown previously to be colonized and infected with various clinically important pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA) (Tirosh-Levy et al., 2015), *Acinetobacter baumannii*, and various ESBL-producing enteric pathogens (Walther et al., 2018; Shnaiderman-Torban et al., 2019). As for the genus *Salmonella*, horses may be sub-clinically infected with the bacterium or suffer from clinical signs which may vary from mild disease as fever and dehydration, to diarrhea, colic and manifestations of septicemia (Hernandez et al., 2014; Cummings et al., 2016). However, reports on ESBL-S strains in horses are still rare. A report from Germany described an SHV-12-producing *S.* Newport causing an outbreak in an equine hospital, which led to a three-month facility closure (Rankin et al., 2005). Another report from the United States described 11 ESBL-S clinical isolates from an equine referral hospital that belonged to various serovars including Braenderup, Anatum, Agona, Rubislaw, and Newport (Leon et al., 2018).

In a previous study, we investigated the shedding rate of ESBL-producing Enterobacterales in farm horses versus hospitalized horses and observed a significant increase in ESBL shedding rate among hospitalized horses together with first isolation of three colonizing isolates that we identified as ESBL-S isolates (Shnaiderman-Torban et al., 2020). The present study investigated and characterized the molecular epidemiology of ESBL-S isolates that were isolated during our surveys, together with ESBL-S isolates recovered from clinical infections from hospitalized horses during the study period. We aimed to describe the emergence of ESBL-S in hospitalized horses and to explore the dissemination of ESBL in this important pathogen.

MATERIALS AND METHODS

Isolation of ESBL-Producing Enterobacterales From Hospitalized Horses

During a prospective surveillance study of ESBL-producing Enterobacterales (ESBL-E) gut colonization in hospitalized horses that we performed in the Koret School of Veterinary Medicine-Veterinary Teaching Hospital (KSVM-VTH) in Israel (Dec 2015–Dec 2017), rectal swabs were collected from horses on admission and after 72 h of hospitalization. The study protocol was approved by the Internal Research Review Institution Committee (Protocol number: KSVM-VTH/15_2015). Isolation of ESBL-E from swabs was performed after swab enrichment in Tryptic Soy Broth supplemented with Ampicillin (100 mg/L), and an overnight incubation at 37°C to increase sensitivity of detection (Jazmati et al., 2016). After incubation, samples were plated onto CHROMagarESBL plates (HyLabs, Rehovot, Israel). In addition, *Salmonella enterica* clinical isolates recovered from horses during the study period that were processed at the Clinical Microbiology Lab at the Meir Medical Center, Kfar Saba, Israel, were collected and stored for retrospective molecular characterization.

Isolation of ESBL-Producing *Salmonella enterica* and the Identification of ESBL Genes

Following the former described procedure, all the white colonies that were obtained on the CHROMagarESBL plates, suspected as ESBL-producing *Salmonella enterica* (ESBL-S)

were further isolated onto selective *Salmonella/Shigella*-agar plates (HyLabs) following verification using the slide agglutination polyvalent serum assay (Remel Inc., United States). All ESBL-S isolates (both fecal and clinical isolates) were identified by the VITEK 2 automated system (Biomerieux, United States) together with antibiotic susceptibility testing using AST-N270 and AST-GN65 cards. Susceptibility results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. All isolates were confirmed for a positive ESBL production phenotype using the cephalosporin/clavulanic-acid combination disk assay (Oxoid, United Kingdom). The bla_{CTX-M} genes were identified by multiplex-PCR (Woodford et al., 2006) and Sanger sequencing (Macrogen, Netherlands). Sequences were analyzed (Snap-Gene) and compared with NCBI database to identify the specific ESBL gene allele.

Salmonella enterica Serovar Identification and Pulsed Field Gel Electrophoresis (PFGE)

Salmonella enterica serotyping was performed using the Kauffmann-White-Le Minor scheme (Le Minor et al., 1982). *Xba*I-restricted (New England BioLabs) PFGE was performed according to the PulseNet International Standard Protocol (Ribot et al., 2006) with *S.* Braenderup H9812 as a reference strain. The PFGE fingerprinting patterns were analyzed with BioNumerics software (version 7.6.3, Applied Maths, Sint-Martens-Latem, Belgium). The unweighted-pair group method using average linkages (UPGMA) clustering method and Dice similarity coefficients were used (1% optimization and 1% tolerance). Isolates were defined as genetically related if they presented $\geq$ 98% PFGE similarity.

Meta Data of the NCBI Sequence Read Archive (SRA) for Statistical Analysis

We explored the global occurrences and the isolation sources of the *Salmonella enterica* serovars using the NCBI SRA data. In order to perform the mata-analysis, we retrieved the SRA accession numbers and meta-data for all the publically available isolates using the NCBI E-Utilities. Statistical correlations between the serovar type and the isolation source were calculated using Phi coefficients with p-values < 0.01. P-values were corrected for multiple tests in step-down method using Bonferroni adjustments (alpha = 0.01). All statistical analyses were performed using Python statistics modules.

Salmonella enterica Whole Genome Sequencing (WGS) and Data Analysis

Total DNA was isolated using Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. WGS was performed by Illumina MiSeq platform using 2 × 250 paired-end libraries prepared with the NEBNext Ultra II FS DNA Library Prep Kit. Assembly was performed using SPAdes-3.11.1. Plasmid replicon types and antibiotic resistance genes (ARGs) were identified using the Center for Genomic Epidemiology (CGE) pipeline.

Whole Genome Multi Locus Sequence Typing (wgMLST) Phylogenetic Analysis

The EnteroBase database was searched for sequences predicted as *S.* Cerro, *S.* Havana, or *S.* Liverpool, according to the SISTR1 and SeqSero2 algorithms. Strains with source country metadata were selected for a phylogenetic analysis and for comparison with the Israeli sequences. A GrapeTree depiction of a NINJA NJ tree based on the wgMLST allelic distances was generated for each serovar population.

Purification and Characterization of *Salmonella enterica* ESBL-Encoding Plasmids

ESBL-encoding plasmid DNA was extracted using the Plasmid Midi Kit (Qiagen) following the manufacturer's instructions. Plasmids were transformed into electro-competent *Escherichia coli* DH10B and transformants were selected on ampicillin containing LB plates (100 mg/L), followed by bla_{CTX-M} PCR screening (Woodford et al., 2006). A second transformation and plasmid purification was performed to ensure plasmid purity. ESBL-encoding plasmids purified from all the 12 ESBL-S isolates were compared using RFLP analysis following restriction with *Sac*I, *Eco*RI and *Hin*dIII (New England BioLabs) and electrophoresis.

Complete Sequencing and Annotation of $bla_{CTX-M-3}$-Encoding Plasmid pSEIL-3

Since ten of 12 isolates harbored CTX-M-3-encoding plasmids with identical RFLP patterns, one representative plasmid (pSEIL-3 from *S.* Havana strain 373.3.1) was sequenced using MinION device (Oxford Nanopore Technologies, ONT, Oxford, United Kingdom) following hybrid assembly, resulting in a complete plasmid sequence (Wick et al., 2017). Plasmid DNA (200 ng) fragment library was prepared (SQK-RBK004 ONT Rapid barcoding sequencing kit) according to the manufacturer's instructions, and loaded onto the MinION flow cell FLO-MIN106. The hybrid read set (WGS Illumina and Nanopore reads) was assembled using Unicycler (v0.4.0) to yield a single circular plasmid designated pSEIL-3, annotated by RAST (Aziz et al., 2008). Replicon type assignment, ARG content, virulence genes and IS elements identification were performed using the CGE pipeline, and by ISfinder (Siguier et al., 2012). Homologous plasmids were identified from the NCBI Nucleotide (nt/nr) database using BLASTn search. Linear plasmid maps were generated using Easyfig-2.2.3.

GenBank Submission

WGS Illumina reads of the three *S.* serovars were deposited in the NCBI Sequence Read Archive database under project number PRJNA559324 (**Table 2**). The complete pSEIL-3 sequence isolated from *S.* Havana strain 373.3.1 (BioSample SAMN12532154) was

submitted to the NCBI Nucleotide database under the accession number MN380440.

Conjugation Experiments of pSEIL-3

Conjugation experiments were performed with *S.* Cerro strain 339.3.3 and *S.* Havana strain 373.3.1 as the donor strains and *Klebsiella pneumoniae* B199 (resistant to nalidixic-acid) and *E. coli* J53 (rifampicin resistant) as the recipient strains. Filter-mating was performed (donor and recipient, 1:1 ratio) on LB plates followed by selection of transconjugant colonies on LB agar plates containing ceftriaxone (2 mg/L) and either nalidixic acid (64 mg/L) or rifampicin (300 mg/L). Transconjugants were verified by the colony color obtained on CHROMagarESBL plates and by PCR detection of $bla_{CTX-M-3}$, and then were subjected to the VITEK 2 for antibiotic susceptibility testing.

Molecular Screening for the Presence of pSEIL-3

A novel six-gene multiplex PCR scheme for the molecular screening of pSEIL-3 was developed. The primers were designed (**Table 3**) based on the sequences of six genes whose combination was unique according to the NCBI Nucleotide database search. The multiplex PCR was performed with PCRBIO HS Taq Mix Red (PCRBIO-systems, United Kingdom) at the following conditions: denaturation at 95°C for one minute, 29 cycles of denaturation (95°C, 15 s), annealing (61.1°C, 15 s) and elongation (72°C, 90 s).

RESULTS

ESBL-Producing *Salmonella enterica* Isolates Recovered From Hospitalized Horses

Overall, 12 ESBL-S isolates were recovered from 12 hospitalized horses during the study period. All these strains were isolated > 72 h after admission and therefore were defined as nosocomial (**Table 1**). Ten out of the 12 horses were also sampled on admission, as part of an ESBL surveillance study, and two of them were found to be colonized with an ESBL-producing Enterobacterales (ESBL-E) strain. Nine out of the 12 horses (75%) that were colonized with an ESBL-S were also found to be colonized with different ESBL-E species. Diverse ESBL-E colonizing species were found: *E. coli* (n = 7), *K. pneumoniae* (n = 4), *Klebsiella oxytoca* (n = 2), and *Citrobacter freundii* (n = 1) (**Table 1**). Ten of the ESBL-S isolates originated from rectal swabs, of which, four were sampled from asymptomatic horses, and six from horses with clinical signs of gastroenteritis. Two additional ESBL-S isolates were clinical isolates that caused joint and umbilicus infections. The majority of the horses from which ESBL-S was recovered were neonates (8/12,

TABLE 1 | Molecular and epidemiological characteristics of the 12 ESBL-producing *Salmonella enterica* (ESBL-S) isolates included in this study and their equine host.

Isolate	ESBL-producing Enterobacterales carriage status on admission	ESBL-S Isolation date	Equine host	Colonization or infection (outcome)[a]	*Salmonella* serovar	$bla_{CTX-M-3}$-plasmid[c]	Co-colonizing ESBL-E[d]
72.2.3	Negative	20 Dec 2015	Mare	Gut colonization (S)	Havana (group G)	pSEIL-3-like IncM2	*Citrobacter freundii*
124.2.3	Negative	28 Jan 2016	Gelding	Gut colonization (S)	Havana (group G)	pSEIL-3-like IncM2	*E. coli*
229.2.2	Negative	12 Mar 2016	Foal	Gastroenteritis[b] (S)	Cerro (group K)	pSEIL-3-variant[e]	*E. coli*[e]
302.2.1	Negative	18 Apr 2016	Mare	Gut colonization (S)	Cerro (group K)	pSEIL-3-like IncM2	*E. coli K. oxytoca*[f]
320.2.3	Positive (ESBL-K. *pneumoniae*)	30 Apr 2016	Foal	Gastroenteritis[b] (D)	Cerro (group K)	pSEIL-3-like IncM2	*E. coli*[f] *K. pneumoniae*
322.2.2	Negative	5 May 2016	Foal	Gastroenteritis[b] (D)	Cerro (group K)	pSEIL-3-like IncM2	*E. coli*[f] *K. pneumoniae*[f]
303.4.3	Negative	9 May 2016	Foal	Gastroenteritis (D)	Cerro (group K)	pSEIL-3-like IncM2	*E. coli*[f] *K. oxytoca*[f]
339.3.3*	Negative	May 2016	Foal	Umbilical infection (D)	Cerro (group K)	pSEIL-3	*E. coli*[f] *K. pneumoniae*[f]
347.2.2	Positive (ESBL-*E. coli*)	30 May 2018	Mare	Gut colonization (S)	Cerro (group K)	pSEIL-3-varient[d]	*K. pneumoniae*[f]
373.3.1*	Negative	Nov 2016	Foal	Infected joint (S)	Havana (group G)	pSEIL-3	Unknown
667220	Unknown	21 Dec 2017	Foal	Gastroenteritis[b] (S)	Liverpool (group E4)	pSEIL-3-like IncM2	Unknown
667275*	Unknown	31 Dec 2017	Foal	Gastroenteritis[b] (D)	Liverpool (group E4)	pSEIL-3	Unknown

[a] *Outcome status 'S' - survival; 'D' - death.*

[b] *ESBL-S was recovered from diarrhea specimen.*

[c] *The non-sequenced $bla_{CTX-M-3}$-encoding plasmids that possessed the same RFLP pattern were designated pSEIL-3-like IncM2 plasmids.*

[d] *ESBL-producing Enterobacterales isolates that co-colonized the same horse and were recovered at the same sampling time together with the ESBL-S. All were PCR-positive for $bla_{CTX-M-1}$ –group (**Figure 1**).*

[e] *pSIEL-3-like plasmids are $bla_{CTX-M-3}$-encoding plasmids that showed a different RFLP pattern compared to pSEIL-3 but were positive in the pSIEL-3-specific multiplex PCR.*

[f] *Non-Salmonella ESBL-producing Enterobacterales isolates that co-colonized the horses gut together with ESBL-S and were found to carry pSEIL-3 by the pSEIL-3-specific multiplex PCR.*

**Salmonella enterica isolates sent to WGS; Unknown – The horse was not screened for ESBL-E carriage during hospitalization due to a positive ESBL-S clinical culture.*

67%, **Table 1**), which were all diagnosed with sepsis (Wong et al., 2018), presenting various clinical signs. These foals were all treated with ampicillin and amikacin, and if they suffered from diarrhea, metronidazole therapy was added. Five out of eight (62.5%) died or were euthanized during hospitalization.

Serovars, Genotyping and Antibiotic Susceptibility Profiles

ESBL-S isolates belonged to three different serovars – Cerro (n = 7), Havana (n = 3), and Liverpool (n = 2), with Cerro being the major serovar, representing more than 50% of the isolates (**Table 1**). All the seven ESBL-producing *S.* Cerro isolates clustered in time (a two-month period) and PFGE genotyping suggested the clonal expansion of this serovar (87.8–100% isolate identity, **Supplementary Figure 1**).

All the 12 ESBL-S isolates carried $bla_{CTX-M-3}$ and showed an identical MDR profile independent with their serovar antibiotic susceptibility testing showed resistance to ceftriaxone, aminoglycosides, amikacin, tobramycin, gentamicin, and trimethoprim-sulfamethoxazole. Isolates were susceptible to carbapenems, quinolones and fosfomycin (**Supplementary Table 1**).

WGS of ESBL-S Serovars and Identification of Plasmid Replicons and Resistome

To further explore the three ESBL-S serovars identified in the equine population, we performed WGS of three representative isolates, one of each serovar (data was deposited under project number PRJNA559324 in the GenBank). The WGS data is summarized in **Table 2**. Sequence types were identified *in silico*, and plasmid replicon analysis revealed that all three serovars harbored common IncM2 and ColRNAI plasmids, accompanied by other plasmids, that were unique for each isolate. Alongside with $bla_{CTX-M-3}$, they all encoded a wide resistome encompassing nine to 12 ARGs that correlated with their susceptibility profiles (**Table 2**).

Local and Global Occurrences and Comparative Genomics of *S. enterica* Serovars Cerro, Havana, and Liverpool

In order to assess the origin of the *Salmonella enterica* serovars identified in this study, we analyzed the *Salmonella* national database that consists data on all human and non-human *Salmonella* isolates recovered in Israel (the reference *Salmonella* laboratory, the Ministry of Health, Israel). The data indicated

TABLE 2 | Description of WGS data of three equine ESBL-producing *Salmonella enterica* serovars.

Salmonella enterica strain (Bio Sample No).	Serovar/ST[a]	Genome size/GC%	N50 bp/L50	No. of ORFs/RNA /ARGs	Plasmid replicon[b]	Plasmids resistome[c]	Resistance pattern[d]
339.3.3 (SAMN12532153)	Cerro ST1593	4.76 Mb/52.22	17575/8	4820/ 88/12	IncM2	*aac(3)-lid*-like, *aadA2*, *armA*, *blaCTX-M-3*, *blaTEM-1B*, *dfrA12*, *mph(E)*-like, *msr(E)*, *sul1*, *sul2*	CTX, AMC(I), AMK; GEN; TOB, TMS
					IncI1	*aadA1*, *dfrA1*	
					ColRNAI	None	
373.3.1 (SAMN12532154)	Havana ST5248	4.77 Mb/52.16	407943/4	4836/ 96/9	IncM2	*aac(3)-lid*-like, *aadA2*, *armA*, *blaCTX-M-3*, *blaTEM-1B*, *dfrA12*, *mph(E)*-like, *msr(E)*, *sul1*, *sul2*	CTX, AMC(I), AMK; GEN; TOB, TMS
					Col156	None	
					ColRNAI	None	
667275 (SAMN12532152)	Liverpool ST1959	4.9 Mb/52.15	762498/3	5007/ 100/12	IncM2	*aac(3)-lid*-like, *aadA2*, *armA*, *blaCTX-M-3*, *blaTEM-1B*, *dfrA12*, *mph(E)*-like, *msr(E)*, *sul1*, *sul2*	CTX, AMC(I), AMK; GEN; TOB, TMS; CIP(I)
					IncX2	*qnrS1*, *tet(A)*-like	
					ColRNAI	None	

[a] *PubMLST (https://pubmlst.org/salmonella).*
[b] *PlasmidFinder 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/).*
[c] *ResFinder 3.2 (https://cge.cbs.dtu.dk/services/ResFinder/).*
[b] *and* [c] *ARGs and a replicon type that were identified on the same scaffold were defined as a plasmid that carried these ARGs. This was verified using BLAST-match of the ARGs-carrying scaffold with IncM2 pSEIL-3.*
[d] *[I] represents intermediate resistance phenotype. Antibiotics abbreviations: CTX-cefotaxime; AMC-amoxicillin/clavulanate; AMK-amikacin; GEN-gentamicin; TOB-tobramycin; TMS-trimethoprim-sulfamethoxazole; CIP- ciprofloxacin.*

that during the study period (2015–2017), the annual prevalence of Cerro, Havana and Liverpool serovars was relatively low; In human infections it ranged from 0.1–0.2% (out of an average of 3,952 *Salmonella* isolates/year). In non-human sources, the prevalence increased throughout these years but was also low (0, 0.2% and 1% for Cerro, Havana and Liverpool in 2015, to - 0.6%, 0.4%, and 3.3%, in 2017, respectively).

In order to evaluate the global abundance of these *Salmonella* serovars and to hypothesize about their main reservoirs we performed a meta-analysis on a global dataset of SRA *Salmonella enterica* isolates belonging to the respected serovars (**Supplementary Table 2**, $n = 1394$). The meta-analysis indicated that these serovars were recovered previously from various human, animal and food sources, with Cerro being the most prevalent serovar. This meta-analysis confirmed that these three serovars are reported herein for the first time in horses.

In order to study the relations with globally reported lineages and clusters, we compared our Israeli genomic sequences with all global genomes of *S.* Cerro, *S.* Havana, and *S.* Liverpool with geographical source, available in Enterobase database. A minimum spanning tree representing all wgMLST profiles for each serovar is shown in **Figure 2**. The analysis of the global population highlights closely clustered genotypes that originate from specific geographical locations. The Israeli genotypes did not significantly cluster with strains from other countries, and the minimum allelic distances from the nearest neighbors were 40 for *S.* Cerro, 779 for *S.* Havana, and 39 for *S.* Liverpool (**Figure 2**). The analysis indicated that the antibiotic resistance phenotypes of the Israeli strains are linked to genomic profiles unique to Israel.

Characterization of the ESBL-Encoding *Salmonella* Plasmids

The WGS data revealed similar plasmid content between the serovars with a common IncM $bla_{CTX-M-3}$-encoding plasmid (**Table 2**). The $bla_{CTX-M-3}$-encoding plasmids of all twelve isolates were successfully transformed into *E. coli* DH10B The $bla_{CTX-M-3}$–positive transformants possessed exactly the same antibiotic susceptibility profile showing resistance to all cephalosporins except for ceftazidime and co-resistance to trimethoprim/sulfamethoxazole and aminoglycosides (**Supplementary Table 1**).

To examine and support the possible inter-serovar plasmid transmission we compared all 12 $bla_{CTX-M-3}$-encoding plasmids

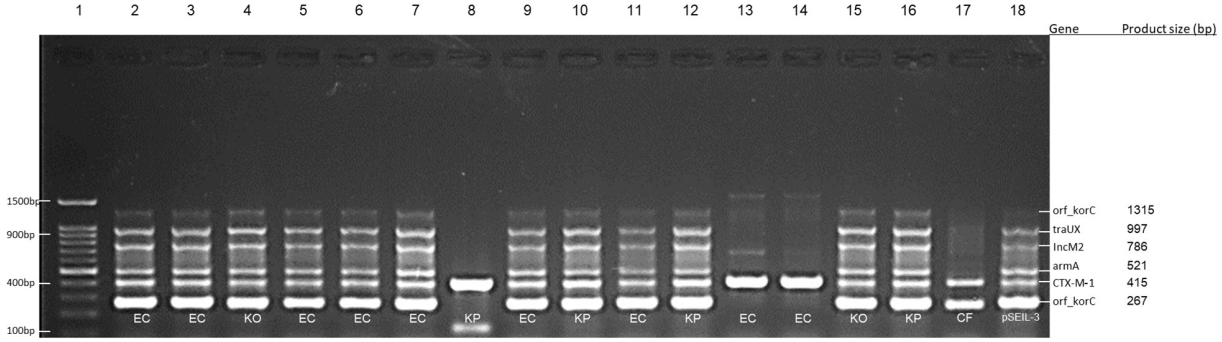

FIGURE 1 | Multiplex PCR for screening of pSEIL-3 in non-Salmonella CTX-M-1-producing Enterobacteriales species co-colonizing eight horses. Six-gene-multiplex PCR amplification for the detection of pSIEL-3 was performed on 16 CTX-M-1 positive ESBL-E isolates co-colonizing (together with ESBL-S) eight horses. foal 229, lane 2; foal 303, lanes 3–6; foal 320, lanes 7–8; foal 322, lanes 9–10; foal 339, lanes 11–12; gelding 124, lane 13; mare 302, lanes 14–15; mare 347, lane 16; mare 72, lane 17; pSIEL-3, lane 18; DNA 100 bp ladder, lane 1. EC - E. coli; KP - K. pneumoniae; KO - K. oxytoca; CF - Citrobacter freundii.

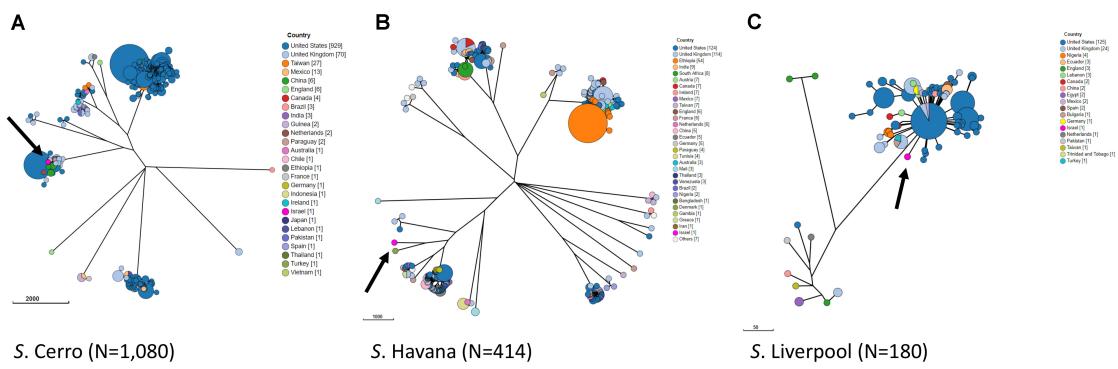

FIGURE 2 | Phylogenetic analysis by wgMLST of global populations of *S.* Cerro **(A)**, *S.* Havana **(B),** and *S.* Liverpool **(C)**. GrapeTree visualization of a NINJA NJ tree based on wgMLST allelic distances. Strains with allelic distances of ≤10 alleles were clustered into a single node. The three Israeli strains of each serovar are shown in pink and are indicated by an arrow.

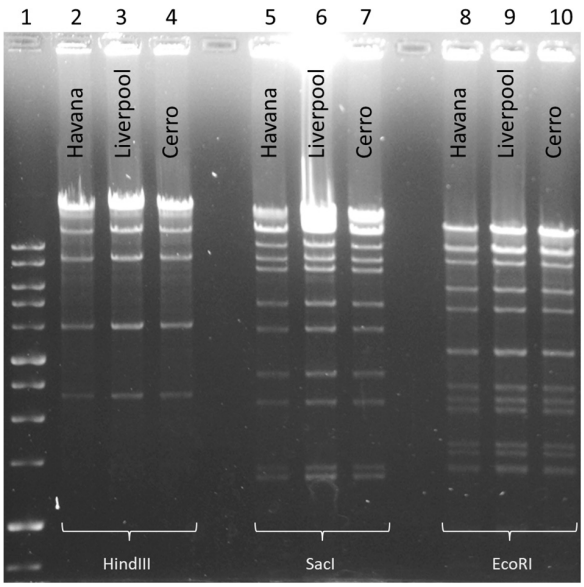

FIGURE 3 | Plasmid RFLP mapping of the CTX-M-3-encoding plasmid DNA from *Salmonella* serovars. RFLP analysis of plasmid DNA (1 μg) isolated from three *Salmonella* isolates representing each of the serovars (*S.* Liverpool 667220; *S.* Havana 373.3.1; and *S.* Cerro 303.4.3) was restricted with three restriction enzymes, *Sac*I (Lanes 2–4), *Eco*RI (Lanes 5–7), and *Hin*dIII (Lanes 8–10). 1 kb DNA size ladder (Lane 1). Plasmid RFLP analysis revealed identical restriction patterns demonstrating the presence of an identical plasmid common to all the three serovars.

by plasmid-RFLP. Ten out of 12 plasmids (83%) showed an identical RFLP pattern, suggesting an inter-serovar horizontal plasmid transfer. We designated this plasmid as pSEIL-3 (plasmid of *S. enterica* from Israel encoding $bla_{CTX-M-3}$). *In silico* analysis of the WGS data confirmed the presence of pSEIL-3 in all three serovars. A representative RFLP analysis of pSEIL-3 is presented in **Figure 3**. Two out of the 12 $bla_{CTX-M-3}$-carrying ESBL-S isolates (229.2.2 and 347.2.2, **Table 1**) were IncM2 plasmids with a different RFLP pattern (Results are not presented) suggesting the presence of a variant of this plasmid (**Table 1**).

Complete Sequence of pSEIL-3

To deepen our understanding on the transferability of pSEIL-3 we sequenced the purified plasmid (Havana 373.3.1) and performed long-read MinION sequencing. Using a hybrid assembly, we generated the complete sequence of the circular 86207-bp plasmid. pSEIL-3 was an IncM2 plasmid (Carattoli et al., 2015) and encoded 118 ORFs, 24 conjugation genes and a single toxin-antitoxin pair *pemIK*. The pSEIL-3 resistome encompassed nine ARGs conferring broad resistance to cephalosporins (bla_{TEM-1B} and $bla_{CTX-M-3}$), aminoglycosides (the modifying enzymes, *aac2* and *aadA2* and the 16S rRNA methyl transferase, *armA*), trimethoprim (*dfrA12*), sulfonamide (*sul1*) and to macrolides (*msrE* and *mphE*) (**Figure 4**).

Blast-based search of plasmids related to pSEIL-3 revealed that our sequenced plasmid was virtually identical to pCTX-M-3 from *Citrobacter freundii* except for a 3902 bp region that encodes *mucAB* of the *umuDC*-like gene family that is involved in UV-resistance, and four additional ORFs that encode hypothetical proteins in pCTX-M-3. The conjugation genes in both plasmids were identical except for a truncation in *orf36* that was shown previously to be involved in plasmid mobilization efficiency (Dmowski et al., 2018). In addition, pSEIL-3 resembled six other $bla_{CTX-M-3}$ and bla_{NDM}-encoding plasmids aligning to >90% of its sequence (**Figure 4**). These plasmids were isolated from various human Enterobacterales strains (*E. coli* - 4; *C. freundii* - 1; *K. pneumoniae* - 1; *S. enterica* - 1) isolated from different countries and years, demonstrating the broad-host-range and high stability nature of these plasmids. Plasmid alignment revealed several DNA rearrangements that seemed to be host-dependent and presumably were linked to the presence of IS*26* (**Figure 4**).

Transferability of pSEIL-3

Plasmid pSEIL-3 proved to be self-conjugable and was transferrable into both *E. coli* and *K. pneumoniae*. Acquisition of pSEIL-3 resulted in the same antibiogram as the donor ESBL-S strains (**Supplementary Table 1**).

In order to examine the *in situ* transferability of pSEIL-3 and pSEIL-3-like IncM2 plasmids in the horses' gut, we screened 16 non-*Salmonella* isolates that co-colonized the horses, and that were PCR positive for $bla_{CTX-M-1}$ –group, for the presence of pSEIL-3 using a specific six-gene multiplex PCR we have developed (**Table 3**). We identified pSEIL-3 and pSEIL-3-like plasmids in 12/16 (75%) of the ESBL-E isolates tested. These isolates belonged to various Enterobacterales species, including *E. coli*, *K. pneumoniae, K. oxytoca,* and *C. freundii*. Therefore, we defined it as a broad host range plasmid (**Figure 1**).

DISCUSSION

In this study, we report for the first time the emergence of three MDR CTX-M-3-producing *S. enterica* serovars - Cerro, Havana and Liverpool, which colonize and cause severe infections in hospitalized horses. Based on WGS and molecular studies we elucidated the route of ESBL spread in *S. enterica* and discovered an inter-serovar horizontal transfer of an IncM2 broad host range plasmid, pSEIL-3. Furthermore, we identified the clonal expansion of $bla_{CTX-M-3}$-producing *S.* Cerro that was responsible for more than half of the cases.

Global phylogenetic serovar analysis indicated the genetic uniqueness of our strains, and the metadata analysis revealed that these three serovars have not been described before in horses. Previously, *S.* Cerro was mainly reported in cattle in the United States (Tewari et al., 2012; Webb et al., 2017), and as the main causative Salmonellosis pathogen in dairy farms (Van Kessel et al., 2007; Kovac et al., 2017). In the United States and the Far East, *S.* Cerro has also been reported in poultry (Roy et al., 2002; Murase et al., 2004). The second serovar we found, *S.* Havana was reported both in humans (Backer, 2000; Bekal et al., 2013) and in poultry (Clemente et al., 2013), and less frequently in wild birds (Reche et al., 2003) and in environmental setting

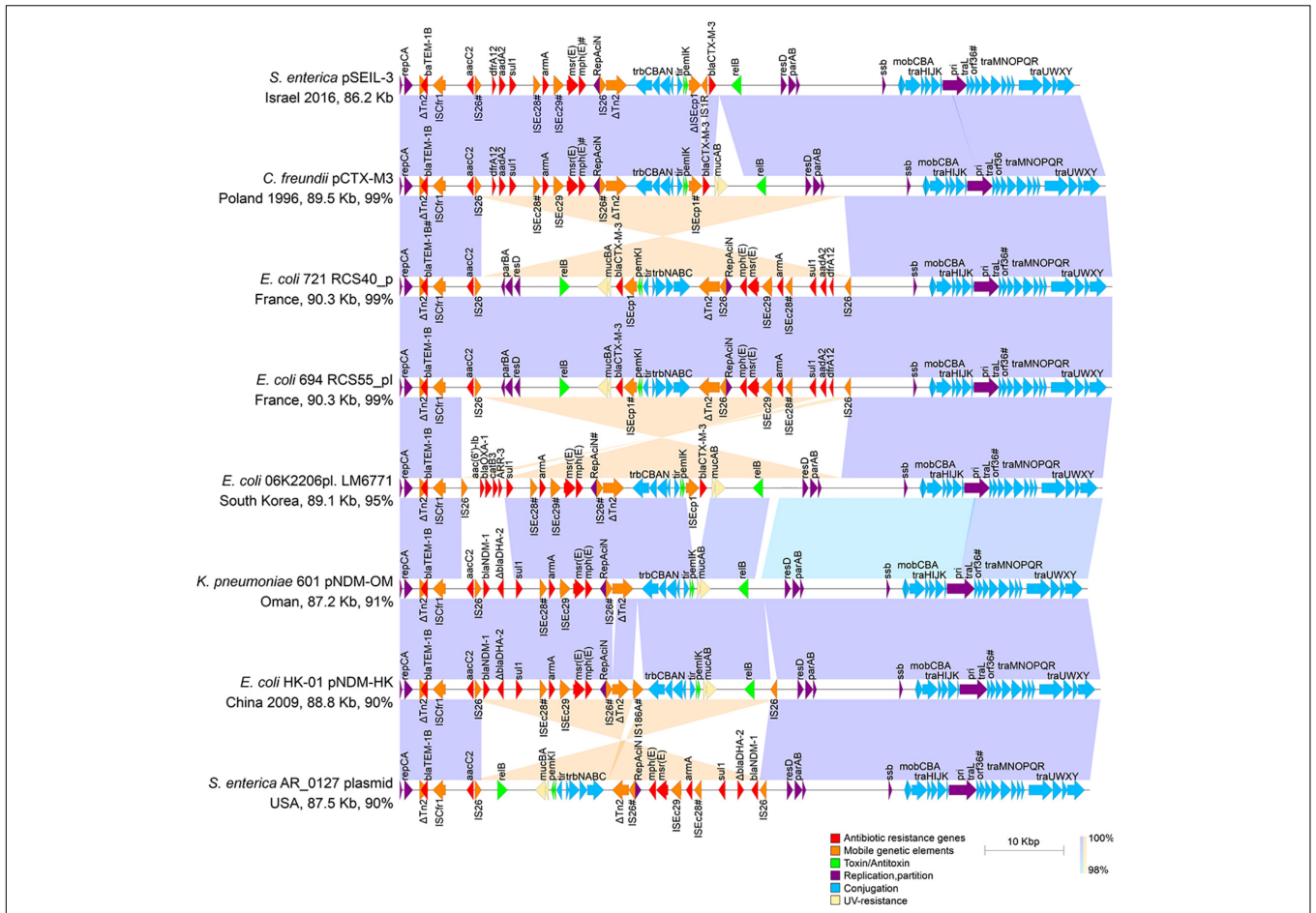

FIGURE 4 | Linear plasmid maps of pSEIL-3 and seven highly related IncM2 related plasmids. Linear schematic maps of pSEIL-3 and IncM2-related plasmids retrieved from the GenBank (aligning for >90%). Plasmids' GenBank accession numbers: pCTX-M3 - AF550415; RCS40_p - LT985241; RCS55_pI - LT985387; LM6771 - KX009507; pNDM-OM - JX988621; pNDM-HK - HQ451074; AR_0127 - CP032193. Arrows (colored by function) represent the major annotated gene groups and IS elements. The alignment between related plasmids is shown (purple, same direction alignment; orange, and opposite direction). The year of plasmid isolation is indicated when available. Truncated genes are labeled with 'Δ' and mutated genes (identity > 99%) are labeled with #.

TABLE 3 | Description of the genes and primers used for the screening of pSEIL-3 multiplex PCR.

Gene	Primer ID	Sequence	Product size	Primers coordinates on pSEIL-3 (5′-3′)	
replicon	IncM2_FW	GGATGAAAACTATCAGCATCTGAAG	786	86138	86162
	IncM2_RV	CTGCAGGGGCGATTCTTTAGG		716	696
aminoglycoside resistance	armA_F	GGGGTCTTACTATTCTGCCTAT	521	18264	18285
	armA_R	GCTGGTAATTCTCTTCCATTCC		18784	18763
*bla*CTX-M-1 ESBL	CTX-M1-F	AAAAATCACTGCGCCAGTTC	415	39216	39235
	CTX-M1-R	AGCTTATTCATCGCCACGTT		39630	39611
pSEIL-3 backbone region	pSEIL3_orf_korC_F	CTGGGACCGGATGCGTGAT	1315	53404	53422
	pSEIL3_orf_korC_R	TCGTTTTGATGTTGCGCCGG		54718	54699
Tra	traJ_F	CGGACTGATATGCGGCGAGA	267	67834	67853
	traJ_R	AGGCGGTTAAGGAGCTCACC		68100	68081
Tra	pSEIL3_traUX_F	TGCGATCCTGGACATGCAAAAC	997	80710	80731
	pSEIL3_traUX_R	TGTTAATCAGCGTGGCCTGGAT		81706	81685

The multiplex PCR was performed with PCRBIO HS Taq Mix Red (PCRBIO-systems, United Kingdom) at the following conditions: denaturation at 95°C for one minute, 29 cycles of denaturation (95°C, 15 s), annealing (61.1°C, 15 s), and elongation (72°C, 90 s).

(Magwedere et al., 2015). It was also identified previously as an ESBL-producer, carrying various bla_{CTX-M} alleles (Bekal et al., 2013; Clemente et al., 2013). *S.* Liverpool is a more rarely reported serovar, with a single report that describes its origin from cattle feces in an EU registered slaughterhouse (Madden et al., 2007).

The source of the *S. enterica* serovars that we identified is unknown. In spite a large nation-wide survey of poultry-associated *Salmonella enterica* was recently reported from Israel (Cohen et al., 2020), data on the serovars that are circulating in the community or in hospital equine populations is still lacking. The large animal department in the KSVM-VTH serves equine patients from diverse farms that occasionally may be housed together with different farm animals. In addition, various animals, often rescued from rural areas, are sporadically admitted for intensive care to the same department. These farm animals may be the source for these *Salmonella* serovars however, a solid support for this is lacking.

Interestingly, the majority of the horses included in our study were not detected as positive ESBL carriers on admission to the hospital, suggesting the nosocomial acquisition of the ESBL-producing strains or the ESBL genetic elements (the $bla_{CTX-M-3}$ gene or its encoding plasmid). In the United States, studies that describe asymptomatic community carriage of *Salmonella* in horses report the prevalence of 0.8% without information on the existing serovars (Traub-Dargatz et al., 2000). Other studies in horses that describe the prevalence of clinical *Salmonella* isolates indicate that the main serovars are Typhimurium, Newport, Agona, Javiana, Anatum, Infantis, and Braenderup (Hernandez et al., 2014; Martelli et al., 2019). Nevertheless, the serovars that we describe herein are unique and are mentioned for the first time in the context of equine population.

Dissemination of ESBL among the hospitalized horses showed a complex epidemiology that included the clonal expansion of *S.* Cerro between seven horses alongside with an in-hospital spread of pSEIL-3 that horizontally transferred to all three *Salmonella* serovars. Acquisition of this single plasmid with its wide resistome was responsible for the dissemination of multidrug resistance. Complete plasmid sequencing of pSEIL-3 indicated that it is merely identical to the previously reported wide-host-range pCTX-M-3 plasmid from *Citrobacter freundii* (Golebiewski et al., 2007) and to other MDR plasmids, that encode various carbapenemases, all from human origin. The findings of pSEIL-3-like IncM2 plasmids in other non-*Salmonella* ESBL-E species that colonized the horses' gut is alarming, and proves their high inter-species transmissibility. The presence of pSEIL-3 in horses, and previously in humans, highlights the risk of horizontal transmission of MDR plasmids between human, animals and environmental pools.

The potential transmission of pSEIL-3-like plasmids is disturbing not only due to their broad host range, but also due to their wide resistome, which confers resistance to all aminoglycosides and to trimethoprim/sulfamethoxazole. Considering the massive use of aminoglycosides antibiotics, often combined with β-lactamase inhibitors, for treating ESBL-producing pathogens in humans, food and companion animals, emphasizes the risk of this plasmid as it may lead to limited treatment options. Additional reports regarding this clinically important ARGs combination in *S. enterica* are infrequent, with one recent study that described a similar MDR pattern of *S. Virchow* from food animals in South Korea (Na et al., 2020), and another study describing shedding of quinolone resistant and ESBL-producing *S. enterica* serovars in swine population in the United States (Elnekave et al., 2019).

The clinical impact of ESBL-S and specifically pSEIL-3-like plasmids in a 'One-Health' perspective is vast. The clonal expansion of the *S.* Cerro underlines the lack of current infection-control measures for detecting and controlling *Salmonella* infections in the veterinary hospital, and calls for the implementation of control measures to prevent further spread. The existence of highly transmissible plasmids such as pSEIL-3 and its spread into three uncommon *S. enterica* serovars highlights the importance of detailed molecular analyses for elucidation of these transmission paths. The developed multiplex PCR in this study enables the tracking of pSEIL-3 in future studies and in active surveillance actions.

This study describes horse-to-horse spread of a zoonotic pathogen harboring a wide-host-range MDR plasmid, which was reported previously in human pathogens, representing a major public health concern. Although the source of this highly transferable plasmid in the veterinary hospital and its circulating routes remains unclear, its disseminative nature is alarming.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, SAMN12532153; https://www.ncbi.nlm.nih.gov/genbank/, SAMN12532154; https://www.ncbi.nlm.nih.gov/genbank/, SAMN12532152.

AUTHOR CONTRIBUTIONS

ZD and AS-T collected the specimens. ZD performed all the microbiological and molecular analyses. KK assisted in the bioinformatics analysis. MD-C and AR performed the serovar typing, the PFGE and the wgMLST analyses. AS was involved in the study design. SN-V was responsible for the design of the study and data analyses. ZD and SN-V wrote the manuscript. All authors read and approved the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.616032/full#supplementary-material

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Plasmid Dynamics of *mcr-1*-Positive *Salmonella* spp. in a General Hospital in China

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Salmonella is an important food pathogen that can cause severe gastroenteritis with more than 600,000 deaths globally every year. Colistin (COL), a last-resort antibiotic, is ineffective in bacteria that carry a functional *mcr-1* gene, which is often spread by conjugative plasmids. Our work aimed to understand the prevalence of the *mcr-1* gene in clinical isolates of *Salmonella*, as the frequency of occurrence of the *mcr-1* gene is increasing globally. Therefore, we analyzed 689 clinical strains, that were isolated between 2009 and late 2018. The *mcr-1* gene was found in six strains, which we analyzed in detail by whole genome sequencing and antibiotic susceptibility testing, while we also provide the clinical information on the patients suffering from an infection. The genomic analysis revealed that five strains had plasmid-encoded *mcr-1* gene located in four IncHI2 plasmids and one IncI2 plasmid, while one strain had the chromosomal *mcr-1* gene originated from plasmid. Surprisingly, in two strains the *mcr-1* genes were inactive due to disruption by insertion sequences (ISs): IS*Apl1* and IS*Vsa5*. A detailed analysis of the plasmids revealed a multitude of ISs, most commonly IS*26*. The IS contained genes that meditate broad resistance toward most antibiotics underlining their importance of the mobile elements, also with respect to the spread of the *mcr-1* gene. Our study revealed potential reservoirs for the transmission of COL resistance and offers insights into the evolution of the *mcr-1* gene in *Salmonella*.

Keywords: *Salmonella*, *mcr-1*, IS, inactivation, IS*Apl1*, IS*Vsa5*

INTRODUCTION

Colistin (COL) is a polypeptide antibiotic that was first isolated from the supernatant of a *Bacillus polymyxa var. colistinus* culture (Tambadou et al., 2015). It has been used in both human and veterinary medicine for more than 50 years, although the parenteral use in humans is limited due to issues with nephrotoxicity and neurotoxicity (Landman et al., 2008; Biswas et al., 2012). Due to the increase of antimicrobial resistance and the lack of antibiotic compounds that are effective, COL can be deployed in the clinic in combination with other drugs that protect renal function. At present, COL is considered as the last resort option for the treatment of infections caused by

Gram-negative bacteria that are multidrug-resistant (MDR), extensively drug-resistant (XDR), or pandrug-resistant (PDR) (Nation and Li, 2009).

In 2015, Liu et al. first discovered plasmid-encoded COL resistance, mediated by the gene *mcr-1*, in *Escherichia coli* isolated from animals, and demonstrated that the gene, encoded on conjugative plasmids, can be used by different strains to mediate low levels of COL resistance (Liu et al., 2016). While the acquisition of the *mcr-1* gene does not result in a new bacterial strain, the recipient strain develops resistance to COL (Schwarz and Johnson, 2016). As the *mcr-1* gene is highly transmissible, it has been observed in more than 30 countries on six continents (Wang et al., 2019; Shen et al., 2020), and can be found in different genera (such as *E. coli*, *Klebsiella pneumoniae*, *Shigella sonnei,* and *Salmonella*) isolated from animals, food, or humans worldwide (Hu et al., 2016; Liu et al., 2016; Pham Thanh et al., 2016; Schwarz and Johnson, 2016; Al-Tawfiq et al., 2017; Zhang et al., 2019). Strains of *Salmonella* are important pathogens of concern in food safety, as they are frequently transmitted between agricultural animals, food, and humans (Foley and Lynne, 2008); the pathogens often cause gastroenteritis, in some cases severe, and are responsible for >600,000 deaths annually (Lokken et al., 2016). Increasing antimicrobial resistance in *Salmonella* species is considered an important public health concern of the 21st century (Lozano-Leon et al., 2019). *Salmonella* strains that acquire multidrug resistance genes would be difficult to treat and would result in an even higher number of cases with severe morbidity and high frequency of mortality. After *Salmonella* species carrying *mcr-1* were detected in the United Kingdom in 2016, the level of occurrence of *mcr-1* in *Salmonella* among livestock and humans, but also the environment, is increasing worldwide (Cui et al., 2017; Yi et al., 2017b). In addition, although another five *mcr* variants (*mcr-2*, *mcr-3*, *mcr-4*, *mcr-5,* and *mcr-9*) have been identified in *Salmonella* species (Carattoli et al., 2018; Garcia-Graells et al., 2018; Borowiak et al., 2019; Carroll et al., 2019; Sun et al., 2020), *mcr-1* is still the most common *mcr* gene found in COL-resistant *Salmonella* spp. (Borowiak et al., 2020).

Many studies have focused on the *mcr-1* gene-carrying *E. coli* and *K. pneumonia* strains, while the prevalence and molecular characteristics of the *mcr-1* gene in *Salmonella* spp. have been investigated in much less detail. The goal of our study is to understand the prevalence of the human-derived *mcr-1* gene in community-acquired *Salmonella* infections in clinical isolates. We conducted a retrospective study to determine the prevalence of *mcr-1* positive *Salmonella* in 689 clinical strains that were isolated between 2009 and 2018. To analyze the level of drug-resistance, we also tested the sensitivity of the isolates to different antibiotic classes, other than COL, and performed a detailed genomic analysis of all *mcr-1* positive strains.

MATERIALS AND METHODS

Strains Source

Salmonella clinical strains were collected from patients in the First People's Hospital of Hangzhou, Zhejiang Province, China between 2009 and 2018. Most specimens were collected from the departments of Pediatrics, Internal Medicine, Gastroenterology Department and Infectious Medicine while other departments were only a secondary source for the isolates. The specimen types of clinical isolates collected included blood, feces, and pus. The cultured bacteria were stored in glycerol broth at −80°C. The DNA from isolates were extracted and screened for the *mcr-1* gene by PCR. The *mcr-1*-positive isolates were verified by Sanger sequencing. This study was approved by the Ethics Committee of Hangzhou First People's Hospital (2020103-1) with a waiver of informed consent because of the retrospective nature of the study. All strains were identified using the automated Vitek 2 system (BioMérieux, Marcy-l'Étoile, France) and MALDI-TOF MS (Bruker, Bremen, Germany). *Salmonella* serotyping was conducted by slide agglutination with specific antisera (Tianrun Bio-Pharmaceutical Co., Ltd., Ningbo, China) according to the White-Kauffmann-Le Minor scheme (9th edition).

PCR Amplifications and Sequencing

All collected isolates were screened for the presence of *mcr-1* positive strains using PCR with the primers *mcr-1*-forward (5′-GCTCGGTCAGTCCGTTTG-3′) and *mcr-1*-reverse (5′-GAA TGCGGTGCGGTCTTT-3′). The amplicons were subsequently sequenced by Sanger sequencing (Quan et al., 2017).

Antimicrobial Susceptibility Test

Broth micro-dilution and *E*-test method were used to determine the minimal inhibitory concentrations (MICs) of COL and 13 other antibiotics: ampicillin (AMP), ceftriaxone (CRO), cefepime (FEP), tetracycline (TET), amoxicillin/clavulanic acid (AMC), cefotaxime (CTX), chloramphenicol (C), meropenem (MEM), imipenem (IPM), azithromycin (AZM), cefoxitin (FOX), amikacin (AK) and ciprofloxacin (CIP), according to the Clinical and Laboratory Standards Institute (CLSI) M100-S29 (CLSI, 2019). Most results were interpreted in accordance with CLSI, except COL and tigecycline, for which we used the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints v8.1. The quality control strain used in the antimicrobial sensitivity test was *E. coli* ATCC 25922.

Genomic DNA Sequencing and Bioinformatics Analysis

Genomic DNA was extracted from six *mcr-1*-positive strains and sequenced using the Illumina HiSeq and Nanopore MinION platforms. Long-read library preparation for Nanopore sequencing was performed with a 1D sequencing kit (SQK-LSK109; Nanopore) without fragmentation. The libraries were then sequenced on a MinION device with a 1D flow cell (FlO-MIN106; Nanopore) and base called with Guppy v2.3.5 (Nanopore). The long read and short read sequence data were used in a hybrid *de novo* assembly using Unicycler v0.4.8 (Wick et al., 2017), then polished with Pilon v1.23 (Walker et al., 2014). Antibiotic resistance genes were identified using the ResFinder database (Zankari et al., 2012) with Abricate 0.8[1] or BacAnt (Hua et al., 2020). Multi-locus sequence typing (MLST) was performed

[1]https://github.com/tseemann/abricate

using mlst[2]. Sequence comparisons were performed using BLAST and visualized with Easyfig 2.2.2 (Sullivan et al., 2011).

Plasmid Conjugation Experiments

Conjugation experiments were performed by broth and filter mating using the sodium azide-resistant *E. coli* J53 as the recipient strains. The mixture (ratio of 1:1) of donors and the recipient strain J53 were subjected to incubation on MH agar plates for 18 h at 37°C. The successful transconjugants were selected on MH agar plates supplemented with 250 μg/mL sodium azide and 2 μg/mL CTX (or 1 μg/mL COL). The carriage of such a plasmid in the parental strain and the corresponding transconjugants were confirmed by PCR and MALDI-TOF MS. The MIC profiles of the transconjugants were also determined with antibiotics (AK, CTX, COL, TET, and AMP) by the broth microdilution method (CLSI, 2019).

RESULTS

Screening for *mcr-1* Positive Strains

A total of 689 clinical *Salmonella* isolates were screened in this study, all of which were derived from patient specimen isolates from May 2009 to December 2018 in Hangzhou First People's Hospital, Zhejiang Province, China. The isolates analyzed included more than ten species of *Salmonella*, that cause a wide range of pathologies including typhoid fever, swine cholera, and enteritis and belong to various *Salmonella* spp. such as *S.* Typhimurium, *S.* Enteritidis, *S.* Choleraesuis, *S.* Thompson, *S.* Manhattan, *S.* Derby, *S.* London, *S.* Senftenberg, and *S.* Aberdeen (**Table 1**). When screening for the *mcr-1* gene by PCR, we detected six isolates (0.87%) of the 689 *Salmonella* strains, that were *mcr-1* positive. Interestingly, the isolate S520 showed a longer *mcr-1* PCR product than other strains (**Supplementary Figure 1**), indicating an insertion in the *mcr-1* gene, or possibly a duplication. To confirm that these six strains harbored the *mcr-1* gene, we performed whole genome sequencing. While five strains had a plasmid-encoded *mcr-1* gene, one strain contained the gene as part of the bacterial chromosome. The results are described below in more detail.

Clinical Information on Patients Suffering From a *Salmonella mcr-1* Positive Infection

The detailed clinical information about *mcr-1* positive patients is shown in **Table 2**. The oldest *mcr-1* positive strain was isolated in May 2015, from a 1-year-old male infant. The second oldest strain was isolated in 2016. Three strains have been obtained in 2017, and one strain in 2018. Four patients suffering from infections with a *mcr-1* positive strain were young children under 3 years, while the remaining two were isolated from elderly people (65 and 84 years old). All patients had symptoms of gastroenteritis, gastrointestinal dysfunction, or diarrhea. Among the six strains, five isolates (S304, S438, S441, S520, S585) belonged to *S.* Typhimurium/ST34 (O4:Hi), the remaining one (S530) to *S.* Indiana/ST17 (O4:Hz:H7).

Drug Susceptibility of the *mcr-1* Positive Strains

Clinical strains that show COL resistance are often resistant to several antibiotics. We therefore tested the susceptibility of the isolates that were *mcr-1* positive toward several antibiotics applying the MIC standard values of the 2019 CLSI standard. The drug susceptibility results are shown in **Table 2** and **Supplementary Data Sheet 1**. Surprisingly, only four of the six *mcr-1* positive strains were resistant to COL in various degrees (three up to 32 μg/mL, one 4 μg/mL), while the two isolates S520 and S530 were not resistant to COL, indicating that their *mcr-1* genes (or their regulation) might not be functional. All six strains displayed resistance to AMP, CRO, FEP, TET, AMC, CTX, and chloramphenicol, but were not resistant to MEM nor IPM. With the exception of strains S441 and S530, four strains were sensitive to AZM. Two isolates, S304 and S441, were resistant to FOX, while the others were sensitive to the compound. Four strains were sensitive to AK, while two (S520 and S530) were resistant to the antibiotic. Strains S304 and S438 were sensitive, two intermediate (S441 and S585) and two (S520 and S530) were resistant to CIP. The results of our antibiotic susceptibility testing show that most strains that carry a COL resistant gene are also resistant to many other antibiotics, and can thus be considered multidrug resistant, making treatment challenging.

Genomic Location of *mcr-1* in the Six Isolates

The genomic characteristics of the six isolates with regard to antimicrobial resistance are shown in **Table 3**. In four isolates (four *S.* Typhimurium), the *mcr-1* gene was located on derivatives of an IncHI2 plasmid (∼253–284 kb). All four IncHI2 plasmids belonged to sequence type 3 and were similar to a number of IncHI2 plasmids of *S.* Typhimurium from different origins including animals, food, and humans (**Supplementary Figure 2**). The genetic context of the *mcr-1* gene was relatively similar within these plasmids in three strains (S438, S441, S520), with the exception of strain S585. The *mcr-1* gene in S585 was located in variable region of IncHI2 plasmid without being embedded in an IS. In addition to the *mcr-1* gene, all four IncHI2 plasmids encoded other antibiotic resistance genes, including beta-lactam ($bla_{CTX-M-14}$), aminoglycoside [*aac(3)-IVa*, *aadA2,* and *aph(4)-Ia*], sulphonamide (*sul1*, *sul2,* and *sul3*), phenicol (*floR*), quinolone (*oqxAB*), and fosfomycin (*fosA3*). A comparison of the plasmid sequences indicated many ISs and transposon insertion events, including IS*Apl1*, IS*186B*, IS*103*, IS*2*, Tn*5403*. Furthermore, our analysis revealed that IS*26* was responsible for a majority of inversion events (**Figure 1**). Plasmid pS438 contains 21 ISs, of which nine were IS*26* copies, two IS*Vsa3* copies, two IS*2* copies, and one copy each of IS*Kpn8*, IS*Ec59*, IS*Apl1*, IS*Aba1*, IS*Aba1*, IS*103*, IS*150*, and IS*1006*, respectively. An inversion of ca 23 kb fragment of nine antibiotic resistance genes including *aadA1*, *cmlA1*, *aadA1,* and *sul3* was observed in pS438 when compared to the pSH16G4918 (GenBank accession

[2]https://github.com/tseemann/mlst

TABLE 1 | Serotype distribution of 679 *Salmonella* isolates.

Serogroup	Serotype	O antigens	H antigens	No. of isolates (%)
A group (2)	*S.* Paratyphi A	1, 2, 12	a: [1, 5]	2 (0.3)
B group (289)	*S.* Typhimurium	1, 4, [5], 12	i: 1, 2	227 (33.0)
	S. Derby	1, 4, [5], 12	f, g: [1, 2]	20 (2.9)
	S. Saintpaul	1, 4, [5], 12	e, h: 1, 2	9 (1.3)
	S. Agona	1, 4, [5], 12	f, g, s: [1, 2]	12 (1.7)
	S. Stanleyville	1, 4, [5], 12, [27]	d: 1, 2	7 (1.0)
	S. Indiana	1, 4, 12	z: 1, 7	4 (0.6)
	others	1, 4, 12	–	10 (1.5)
C1 group (118)	*S.* Choleraesuis	6, 7	c: 1, 5	12 (1.7)
	S. Infantis	6, 7, 14	r: 1, 5	18 (2.6)
	S. Irumu	6, 7	l, v: 1, 5	5 (0.7)
	S. Virchow	6, 7, 14	r: 1, 2	5 (0.7)
	S. Thompson	6, 7, 14	k: 1, 5	27 (3.9)
	S. Potsdam	6, 7, 14	l, v: e, n, z15	10 (1.5)
	S. Braenderup	6, 7, 14	e, h: e, n, z15	7 (1.0)
	S. Mbandaka	6, 7, 14	z10: e, n, z15	4 (0.6)
	S. Rissen	6, 7, 14	f, g: –	5 (0.7)
	S. Montevideo	6, 7, 14	g, m, [p], s: [1, 2, 7]	2 (0.3)
	others	6, 7, 14	–	23 (3.3)
C2 group (35)	*S.* Manhattan	6, 8	d: 1, 5	3 (0.4)
	S. Newport	6, 8, 20	e, h: 1, 2	7 (1.0)
	S. Bovismorbificans	6, 8, 20	r, [i]: 1, 5	10 (1.5)
	S. Litchfield	6, 8	l, v: 1, 2	9 (1.3)
	others	6, 8	–	6 (0.9)
D group (157)	*S.* Enteritidis	1, 9, 12	g, m: [1,7]	154 (22.4)
	S. Gallinarum-pullorum	1, 9, 12	–	2 (0.3)
	others	1, 9, 12	:H5	1 (0.1)
E1 group (51)	*S.* London	3, {10} {15}	l, v: 1, 6	37 (5.4)
	S. Weltevreden	3, {10} {15}	r: z6	5 (0.7)
	S. Ruzizi	3, 10	l, v: e, n, z15	1 (0.1)
	S. Vejle	3, {10} {15}	e, h: 1, 2	1 (0.1)
	S. Anatum	3, {10} {15} {15,34}	e, h: 1, 6	1 (0.1)
	others	10	–	6 (0.9)
E4 group (25)	*S.* Senftenberg	1, 3, 19	g, [s], t: –	21 (3.0)
	others	19	–	4 (0.6)
F group (5)	*S.* Aberdeen	11	i: 1, 2	5 (0.7)
Other groups (5)	*S. enterica* subsp. *diarizonae*			2 (0.3)
	others			3 (0.4)

no. MK477619). In pS438, two copies of IS*26* adjacent to the 23 kb fragment are in opposite orientation and are flanked by an identical 8-bp repeat inverted relative to each other. This inversion was caused by the intramolecular transposition *in trans* of IS*26* into a target site.

In the isolate S304 (a *S.* Typhimurium strain), the *mcr-1* gene was located on IncI2 (60 kb), which was similar to a number of plasmids from different hosts, origins, and regions (**Figure 2**). The pS304_2 had the simplest *mcr-1* transposon structure (*mcr-1-pap2*) without the assistance of the IS*Apl1* gene, as reported previously (Wang J. et al., 2017). These findings confirmed that IncI2-type plasmids have contributed to the successful spread of *mcr-1* gene among species of different diverse genetic environments. Interestingly, in one isolate (*S.* Indiana), the *mcr-1* gene was located in the chromosome. The COL-sensitive isolates S520 and S530 contained *mcr-1* genes that were both disrupted by insertion elements. IS*Apl1* disrupted the *mcr-1* gene in S520, while IS*Vsa5* inserted into the *mcr-1* gene in S530, resulting in non-functional gene products. The identified tandem site duplications (TSDs) with the sequences GA and GACCGAGCG indicate the occurrence of an IS insertion (**Figure 3**).

Genetic Environment of the Chromosomal *mcr-1*

The genetic arrangement of chromosome-borne *mcr-1* in S530 was investigated. The chromosomal *mcr-1* was adjacent to IS*Apl1* and the *pap2* gene, and they consisted of a "IS*Apl1-mcr-1-pap2*" structure, which is commonly presented in chromosome-borne *mcr-1* harbored strains (Peng et al., 2019). However, to our

TABLE 2 | Basic situation of *mcr-1* positive strains.

Strain	Inspection time	Specimen type	Patient age	Patient sex	Diagnosis of infection	Visiting department	Serovar	Serotype	AMR phenotype
S304	2015.5	Stool	1	Male	Acute enteritis	Pediatrics	*S.* Typhimurium	O4:Hi:	COL, AMP, CRO, FEP, TET, AMC, CTX, FOX, C,
S438	2016.12	Stool	3	Male	Enteritis	Pediatrics	*S.* Typhimurium	O4:Hi:	COL, AMP, CRO, FEP, TET, AMC, CTX, C
S441	2017.3	Stool	3	Female	Infectious diarrhea	Pediatrics	*S.* Typhimurium	O4:Hi:	COL, AMP, CRO, AZM, FEP, TET, AMC, CTX, FOX, C
S520	2017.9	Stool	1	Female	Diarrhea	Pediatrics	*S.* Typhimurium	O4:Hi:	AMP, CRO, FEP, TET, AMC, CTX, CIP, AK, C
S530	2017.1	Stool	84	Female	Fracture, diarrhea	Recovery unit	*S.* Indiana	O4:Hz:H7	AMP, CRO, AZM, FEP, TET, AMC, CTX, CIP, AK, C
S585	2018.12	Stool	65	Female	Gastrointestinal disorders	Recovery unit	*S.* Typhimurium	O4:Hi:	COL, AMP, CRO, FEP, TET, AMC, CTX, C

AMR, antimicrobial resistance; COL, colistin; AMP, ampicillin; CRO, ceftriaxone; AZM, aztreonam; FEP, cefepime; TET, tetracycline; AMC, amoxicillin/clavulanate; CTX, cefotaxime; FOX, cefoxitin; CIP, ciprofloxacin; AK, amikacin; IPM, imipenem; C, chloramphenicol; MEM, meropenem.

TABLE 3 | The summary of the features associated with the genome and plasmid identified in *Salmonella* genomes.

Sequence name	Size (bp)	ST type	Replicon Type (s)	Antibiotic resistance genes
S304	4,937,766	ST34		bla_{TEM-1B}, bla_{TEM-1B}, *aph(6)-Id*, *aph(3″)-Ib*, *sul2*, *aac(6′)-Iaa*, *mdf(A)*
pS304-1	254,873		IncHI2 IncHI2A	*aadA2*, *cmlA1*, *ant(3″)-Ia*, *sul3*, *aac(3)-IVa*, *aph(4)-Ia*, *aph(3′)-Ia*, *aph(6)-Id*, *aph(3″)-Ib*, bla_{TEM-1B}, *tet(A)*, *qnrS1*, *ARR-3*, *cmlA1*, bla_{OXA-10}, *ant(3″)-Ia*, *dfrA14*, *tet(A)*, *floR*, $bla_{CTX-M-65}$
pS304_2	60.870		IncI2	*mcr-1.1*
pS304_3	7395		ColRNAI	
S441	4,944,769	ST34		*tet(B)*, *sul2*, *aph(3″)-Ib*, *aph(6)-Id*, bla_{TEM-1B}, *qnrS1*, $bla_{CTX-M-55}$, *aac(6′)-Iaa*, *mdf(A)*
pS441	260,707		IncHI2 IncHI2A	*mcr-1.1*, *fosA3*, $bla_{CTX-M-14}$, *aac(3)-IVa*, *aph(4)-Ia*, *sul2*, *floR*, *aac(3)-IId*, *sul3*, *ant(3″)-Ia*, *cmlA1*, *aadA2*, *sul1*, *oqxA*, *oqxB*
S438	4,971,941	ST34		bla_{TEM-1B}, *aph(6)-Id*, *aph(3″)-Ib*, *sul2*, *tet(B)*, *aac(6′)-Iaa*, *floR*, *sul2*, *aph(4)-Ia*, *aac(3)-IVa*, $bla_{CTX-M-14}$, *fosA3*, *mdf(A)*
pS438	253,817		IncHI2 IncHI2A	*mcr-1.1*, *fosA3*, $bla_{CTX-M-14}$, *aac(3)-IVa*, *aph(4)-Ia*, *sul2*, *floR*, *sul1*, *aadA2*, *dfrA12*, *aph(3′)-Ia*, *sul3*, *ant(3″)-Ia*, *cmlA1*, *aadA2*, *oqxA*, *oqxB*
S520	5,038,289	ST34		*aac(6′)-Iaa*, *mdf(A)*, *tet(B)*, *sul2*, *aph(3″)-Ib*, *aph(6)-Id*, bla_{TEM-1B}
pS520	253,797		IncHI2 IncHI2A	*mcr-1.1*, *fosA3*, $bla_{CTX-M-14}$, *aac(3)-IVa*, *aph(4)-Ia*, *sul2*, *floR*, *aadA2*, *cmlA1*, *ant(3″)-Ia*, *sul3*, *aph(3′)-Ia*, *dfrA12*, *aadA2*, *sul1*, *oqxA*, *oqxB*
S530	5,059,260	ST17	IncN IncQ1	*dfrA17*, *aadA5*, *aac(6′)-Ib-cr*, bla_{OXA-1}, *catB3*, *ARR-3*, *sul1*, *fosA3*, $bla_{CTX-M-14}$, *aac(3)-IVa*, *aph(4)-Ia*, *sul2*, *floR*, *mcr-1.1*, *mph(A)*, *sul3*, *tet(A)*, bla_{TEM-1B}, *sul2*, *aph(3″)-Ib*, *aph(6)-Id*, *oqxA*, *oqxB*, *mph(E)*, *msr(E)*, *armA*, *sul1*, *ARR-3*, *catB3*, bla_{OXA-1}, *aac(6′)-Ib-cr*, *aac(6′)-Iaa*, *mdf(A)*
S585	5,024,322	ST34		*mdf(A)*, *aac(6′)-Iaa*, *tet(B)*, *aph(6)-Id*, *aph(3″)-Ib*, *sul2*
pS585_1	284,303		IncHI2 IncHI2A	*sul1*, *aadA2*, *dfrA12*, *aph(3′)-Ia*, *sul3*, *floR*, *sul2*, *aph(4)-Ia*, *aac(3)-IVa*, *aac(6′)-Ib-cr*, bla_{OXA-1}, *catB3*, *ARR-3*, *qnrS2*, *mcr-1.1*, $bla_{CTX-M-14}$, *oqxA*, *oqxB*
pS585_2	6,079		ColRNAI	

surprise, the IncN/IncQ1 replicon and *repA* gene were identified as a sequence surrounding the *mcr-1* gene, indicating a plasmid origin. Bioinformatic analysis showed that a 129.85 kb *mcr-1*-carrying region inserted into the HTH-type transcriptional regulator protein encoding gene *aaeR*, compared with the genome of another *S.* Indiana/ST17 strain D90 (GenBank accession no. CP022450) (**Figure 4**). This region has a similar genetic environment compared with an IncN/IncHI2 plasmid pMCR_WCHEC050613 (GenBank accession no. CP019214), with 99.99% nucleotide identity at 86% coverage. In addition to the disrupted *mcr-1* gene, this putative plasmid region also harbored multiple resistance genes including *dfrA17*, *aadA5*, *aac(6′)-Ib-cr*, bla_{OXA-1}, *catB3*, *ARR-3*, *sul1*, *fosA3*, $bla_{CTX-M-14}$, *aac(3)-IVa*, *aph(4)-Ia*, *sul2*, *floR*, *mph(A)*, *sul3*, *tet(A)*, bla_{TEM-1B}, *sul2*, *aph(3″)-Ib*, *aph(6)-Id*, *oqxA,* and *oqxB* (**Table 3**). Further sequence analysis showed that two copies of IS*26* adjacent to the putative plasmid region in same orientation, were flanked by identical 8-bp TSDs (ACCTGAAG), indicating that IS*26* is

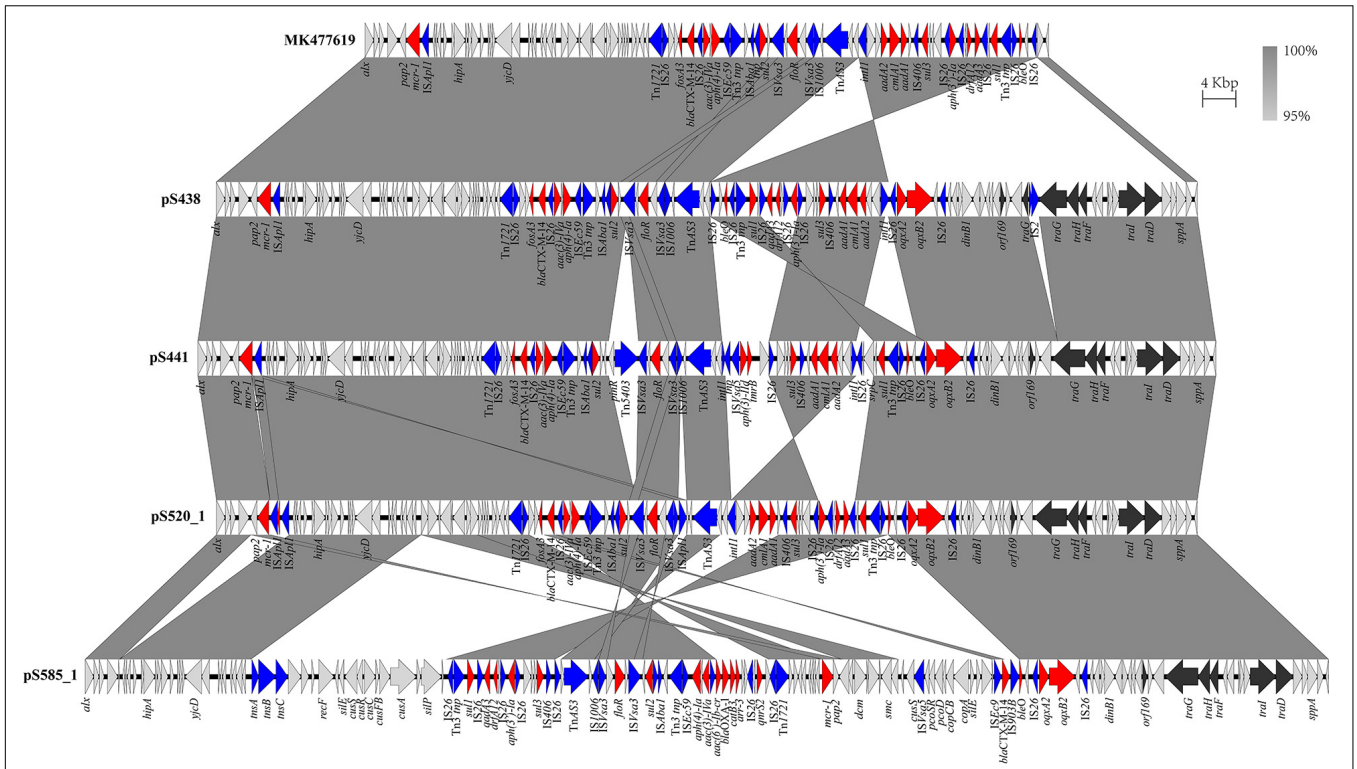

FIGURE 1 | Scaled, linear diagrams comparing the sequences of plasmid pSH16G4918 (GenBank no. MK477619), pS438, pS441, pS520, and pS585_1. Antibiotic resistance genes are indicated in red arrows. The individual conjugation-related genes are indicated with black arrows. Blue arrows denote transposon- and integron-associated genes. Other genes are shown as gray arrows.

involved in the insertion of a *mcr-1*-carrying MDR plasmid into the S530 chromosome.

Transfer Ability of the *mcr-1*-Harboring Plasmids

To evaluate the transferability of the *mcr-1*-harboring plasmids obtained in this study, conjugation experiments were performed. Transconjugants were obtained from each of the mixed cultures of the recipient *E. coli* J53 with one of the five *mcr-1*-positive strains. Four *mcr-1*-positive plasmids were successfully transferred from their hosts via conjugation. The result of antimicrobial susceptibility tests showed that all the positive transconjugants displayed elevated MICs to COL, CTX, and AMP, compared with that of *E. coli* J53 (**Supplementary Table 1**). It is important to state that since the IncI2 plasmid pS304_2 carried the only antimicrobial resistance *mcr-1*, the co-transferred resistance genes via the IncHI2 plasmid pS304_1 was able to confer the respective antibiotic resistance to the recipients. However, the *mcr-1*-harboring IncHI2 plasmid pS438 failed to transfer to the recipient *E. coli* J53, despite repeating the experiment several times. Further sequence analysis showed that pS438 possessed all the essential genes for mobility, but the transfer-related gene *traG* was disrupted by the insertion element IS*2* (**Figure 1**), suggesting that the non-transferability of pS438 may be caused by the IS*2* insertion into the *traG* gene.

DISCUSSION

In 2016, the Chinese Ministry of Agriculture decreed to completely stop the use of COL in animal husbandry for promoting livestock growth and preventing disease (Walsh and Wu, 2016). However, the widespread use of COL in animal farming worldwide over the past decades has resulted in an increase of COL resistance and the spread of *mcr-1*. From 2013 to 2014, the detection rate of pathogenic *E. coli mcr-1* in Japanese pigs was as high as 50% (Kusumoto et al., 2016) and up 21% in French cow dung samples from 2004 to 2014 (Haenni et al., 2016). These findings indicate that the misuse of antibiotics in the livestock industry will greatly increase the selection pressure of bacteria and provide evolutionary advantages for *mcr-1* positive bacteria. As the clinical use of COL has been restricted in the past, the current rate of *mcr-1* positive strains in clinical isolates and in healthy humans is much lower than that in samples that are obtained from farmed animals.

In our study, a total of 689 clinical *Salmonella* strains were screened which were isolated over the past 10 years. Only six of all strains (0.87%) were positive for the *mcr-1* gene. The rate of occurrence of human-derived *Enterobacteriaceae mcr-1* genes has been reported to be less than 2% (Yi et al., 2017a). Lu et al. (2019) collected 12,053 *Salmonella* isolates from a surveillance on diarrhoeal outpatients in Shanghai, China, 2006–2016, and 37 *mcr-1*-harboring strains were detected among them, in which

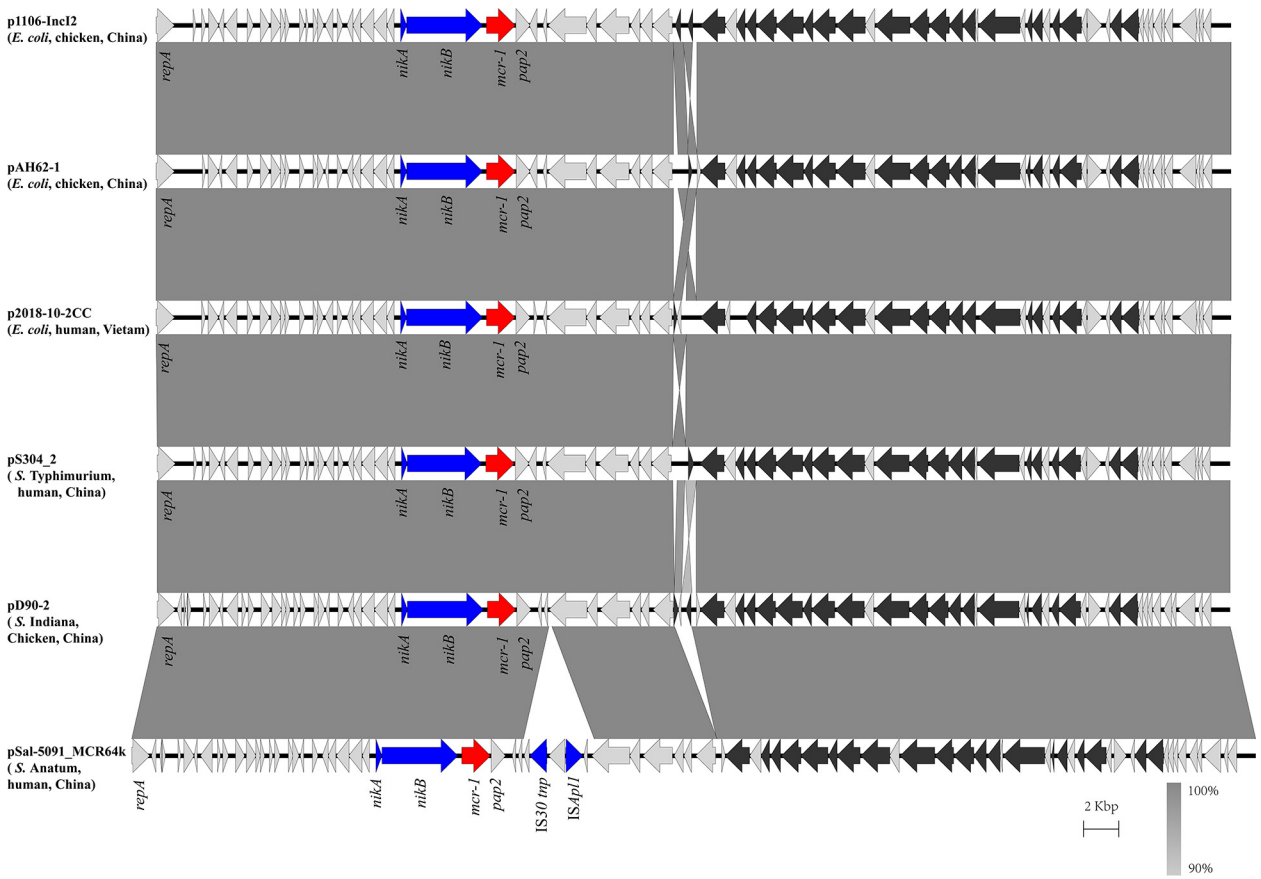

FIGURE 2 | Scaled, linear diagrams comparing the sequences between plasmid pS304_2, p1106-IncI2 (GenBank no. MG825374.1), pAH62-1 (GenBank no. CP055260.1), p2018-10-2CC (GenBank no. LC511622.1), pD90_2 (GenBank no. CP022452.1), and pSal-5091_MCR64k (GenBank no. CP045521.1). Antibiotic resistance genes are indicated in red arrows. The individual conjugation-related genes are indicated with black arrows. Blue arrows denote transposon- and integron-associated genes. Other genes are shown as gray arrows.

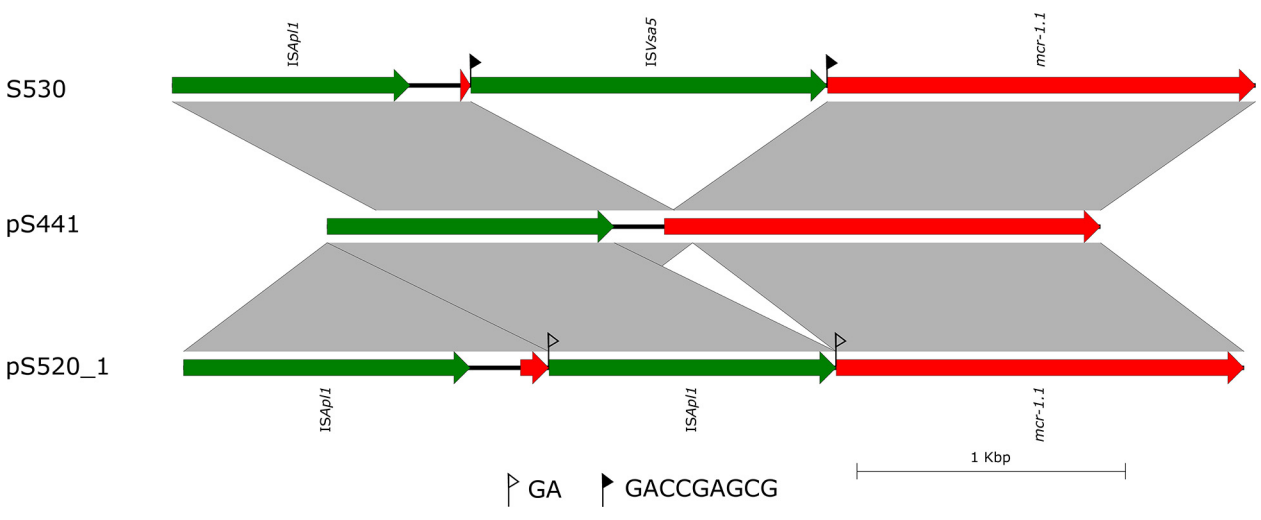

FIGURE 3 | Scaled, linear diagrams comparing the sequences of S530, plasmid pS441, and pS520_1. Insertion elements are shown as green arrows. Genes, including antibiotic resistance genes, are shown as labeled red arrows. Flags indicate the 8 bp target site duplications (TSDs).

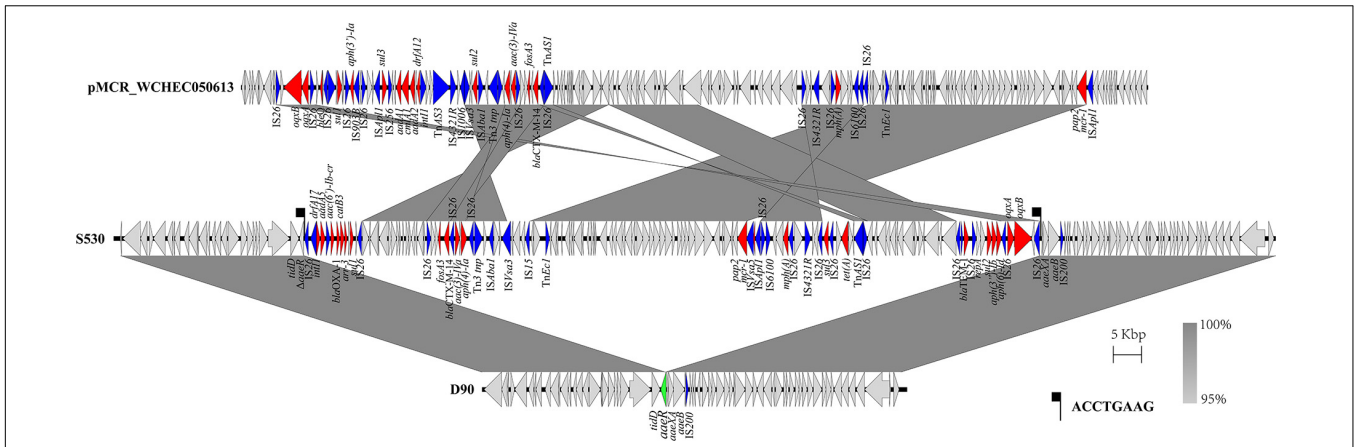

FIGURE 4 | Scaled, linear diagrams comparing the sequences between S530, D90 (GenBank no. CP022450), and pMCR_WCHEC050613 (GenBank no. CP019214). Antibiotic resistance genes are indicated in red arrows. The inserted gene *aaeR* is marked as the green arrow. Blue arrows denote transposon- and integron-associated genes. Other genes are shown as gray arrows.

35 were serovar Typhimurium. In China, the most common ST of *S.* Typhimurium, especially MDR *S.* Typhimurium, is ST34, which is also prevalent in Europe (Antunes et al., 2011; Wong et al., 2013). In this study, all the five plasmid-mediated *mcr-1*-positive *Salmonella* strains belong to *S.* Typhimurium/ST34, suggesting that *mcr-1*-bearing plasmids might have a strong association with specific serotypes of *Salmonella*. Most of the *S.* Typhimurium/ST34 strains carrying *mcr-1* gene were isolated from animals (Yi et al., 2017b), which suggests that the ST34 clone poses a great threat as it is able to disseminate COL resistance from food-producing animals to humans. While the antibiotic is still being used in agriculture (legally or illegally), the last-resort clinical deployment of COL for the treatment of MDR Gram-negative bacteria will lead to an increase in frequency of the *mcr-1* gene, which can be easily transmitted by conjugation. This will aggravate the global antibiotic resistance crisis even further.

Wang Y. et al. (2017) conducted a retrospective analysis of risk factors such as infection, frequency of occurrence and fatality rates of infections by *mcr-1* positive *E. coli* and *K. pneumoniae* in hospitals in Zhejiang and Guangdong, China. The results showed that the status of the immune system as well as the history of antibiotic use (especially carbapenem and fluoroquinolone) are risk factors for infection with *mcr-1* positive strains. In our study, we found that four patients that were infected with *mcr-1* positive strains were young children under 3 years, while the remaining two were 65 and 84 years old, being admitted to the hospital with symptoms of gastroenteritis, gastrointestinal disorders, or diarrhea. This indicated that age and low immune function may have certain effects on *mcr-1* infection. However, due to the small number of patients and *mcr-1* positive strains no statistically significant conclusion can be drawn.

The antimicrobial susceptibility test of the six *mcr-1* positive strains showed that all strains are multidrug resistant, with resistance to many antibiotics, including AK, CRO, FEP, TET, AMC, CTX, and chloramphenicol. Interestingly, only four of the six *mcr-1* positive strains displayed COL resistance. For the two COL-sensitive strains we identified inactive forms of *mcr-1* due to insertion. It has previously been reported that the *mcr-1* gene was inactivated by insertions of either IS*10R* or IS*12984b* (Terveer et al., 2017; Zhou et al., 2018). An inactivation of *mcr-1* by an intragenic 22-bp duplication was described in an isolate of *Shigella sonnei* from Vietnam (Pham Thanh et al., 2016). In our study, we observed two inactive forms of the *mcr-1* gene in *Salmonella*. The *mcr-1* gene was disrupted by IS*Apl1* and IS*Vsa5*, respectively. The strain S520 which harbored the *mcr-1* gene with the insertion of IS*Apl1* was plasmid-encoded in the IncHI2 plasmid pS520_1. As the plasmid also encoded genes mediating resistance to several other antibiotics, the inactive *mcr-1* gene might have been "carried along" on epidemic resistance plasmids. Interestingly, we found one chromosome-encoded *mcr-1* in strain S530, which was, however, disrupted by IS*Vsa5*. So far, a chromosome-embedded *mcr-1* in *Enterobacteriaceae* has been rarely reported (Falgenhauer et al., 2016; Peng et al., 2019). Our bioinformatic analysis showed that the *mcr-1* gene on the chromosome in strain S530 was flanked by IS*Apl1*. Our finding corroborates the hypothesis that the insertion of the *mcr-1* gene into the bacterial chromosome is mediated by IS*Apl1* (Peng et al., 2019). However, in our case, the chromosomal *mcr-1* gene was found to be of plasmid origin. The putative *mcr-1*-carrying IncN/IncQ1plasmid was flanked by two copies of IS*26* that mediated the integration into the *aaeR* gene of the S530. To the best of our knowledge, this study includes the first description of the mobilization of the *mcr-1* gene into a chromosome mediated by a plasmid.

Our study showed that the *mcr-1* gene was encoded by a variety of plasmids, among them IncI2, IncX4 and IncHI2 being the most predominant ones (Sun et al., 2018). Previously, the IncHI2/IncN replicon was reported to carry the *mcr-1* gene, which indicates that the *mcr-1* gene and its surrounding sequence might be derived from the IncHI2/IncN plasmid (Lu et al., 2020). In IncI2 plasmids, *mcr-1-pap2* was the most common, whereas *mcr-1* genes were usually flanked by IS*Apl1* (IS*Apl1*-*mcr-1-pap2* or Tn*6330*) in IncHI2 plasmids (Cao et al., 2020). This finding is also consistent with the results of our study,

with the exception of pS585 that is not embedded in an IS. Although the total positive rate of *mcr-1*-harboring *Salmonella* strains in this study was revealed to be rather low, our conjugation experiments demonstrated that all the *mcr-1*-harboring plasmids are capable of transferring to *E. coli*, except the one plasmid in which *traG* was disrupted by IS*2*. This clearly illustrates the ability of *Salmonella* to spread these plasmids to diverse genera of the *Enterobacteriaceae*. The same, or highly similar, plasmids were also found in various host strains from different sources (animal, food, and humans) isolated in different regions, demonstrating the general applicability of our findings regarding the transfer abilities of the studied *mcr-1*-harboring plasmids among a pool of various microbes, including those that are animal and human pathogens.

Mobile genetic elements, particularly insertion sequence elements (ISs) are able to reorganize the sequence in plasmids. In this study, we observed several IS and transposon insertion events, including those mediated by IS*Apl1*, IS*186B*, IS*103*, IS*2*, and Tn*5403*. Among all the ISs, IS*26* seems to play a major role in the rapid dissemination of antibiotic resistance gene in Gram-negative bacteria (Harmer et al., 2014; He et al., 2015). Partridge et al. (2018) had previously shown that additional IS*26* are easily acquired if the plasmid already possessed a copy of IS*26*. In our study, the insertion of one or more IS*26* mediated several inversion events which involved a plethora of antibiotic resistance genes. IS*26* copies lead to DNA sequence inversion via intramolecular replicative transposition *in trans* (He et al., 2015). This work, as well as our study, demonstrates the importance of replicative transposition in the reorganization of multiple antibiotic resistance plasmids.

In conclusion, we collected 689 clinical *Salmonella* strains and six of them (0.87%) were *mcr-1*-positive. Five strains harbored plasmid-encoded *mcr-1* gene and the other one carried chromosomal *mcr-1* gene originated from plasmid. Five plasmid-mediated *mcr-1*-positive *Salmonella* strains belong to *S.* Typhimurium/ST34 and carried *mcr-1* via two types of plasmids (four IncHI2 plasmids and one IncI2 plasmid). The same or highly similar plasmids were also found in different sources (animal, food, and humans), suggesting that *S.* Typhimurium/ST34 is the potential reservoir for transmission of COL resistance and presents a potential public health threat. Active surveillance of *mcr-1*-harboring *Salmonella*, especially *S.* Typhimurium/ST34, should be further conducted due to the potential high risk of COL resistance development.

DATA AVAILABILITY STATEMENT

The complete genome sequence of six *mcr-1*-positive *Salmonella* has been deposited in GenBank under accession number CP061115-CP061130.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Hangzhou First People's Hospital (2020103-1). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JF, YY, and XH designed the study. JF, LZ, JH, and MZ performed the experiments. XH, JH, SL, BL, and LZ analyzed the bioinformatics data. JF, JH, MZ, and XH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.604710/full#supplementary-material

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Plasmid-Borne and Chromosomal ESBL/AmpC Genes in *Escherichia coli* and *Klebsiella pneumoniae* in Global Food Products

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Plasmid-mediated extended-spectrum beta-lactamase (ESBL), AmpC, and carbapenemase producing Enterobacteriaceae, in particular *Escherichia coli* and *Klebsiella pneumoniae*, with potential zoonotic transmission routes, are one of the greatest threats to global health. The aim of this study was to investigate global food products as potential vehicles for ESBL/AmpC-producing bacteria and identify plasmids harboring resistance genes. We sampled 200 food products purchased from Finland capital region during fall 2018. Products originated from 35 countries from six continents and represented four food categories: vegetables (n = 60), fruits and berries (n = 50), meat (n = 60), and seafood (n = 30). Additionally, subsamples (n = 40) were taken from broiler meat. Samples were screened for ESBL/AmpC-producing Enterobacteriaceae and whole genome sequenced to identify resistance and virulence genes and sequence types (STs). To accurately identify plasmids harboring resistance and virulence genes, a hybrid sequence analysis combining long- and short-read sequencing was employed. Sequences were compared to previously published plasmids to identify potential epidemic plasmid types. Altogether, 14 out of 200 samples were positive for ESBL/AmpC-producing *E. coli* and/or *K. pneumoniae*. Positive samples were recovered from meat (18%; 11/60) and vegetables (5%; 3/60) but were not found from seafood or fruit. ESBL/AmpC-producing *E. coli* and/or *K. pneumoniae* was found in 90% (36/40) of broiler meat subsamples. Whole genome sequencing of selected isolates (n = 21) revealed a wide collection of STs, plasmid replicons, and genes conferring multidrug resistance. $bla_{\text{CTX-M-15}}$-producing *K. pneumoniae* ST307 was identified in vegetable (n = 1) and meat (n = 1) samples. Successful IncFII plasmid type was recovered from vegetable and both IncFII and IncI1-Iγ types from meat samples. Hybrid sequence analysis also revealed chromosomally located beta-lactamase genes in two of the isolates and indicated similarity of food-derived plasmids to other livestock-associated sources and also to plasmids obtained from human clinical samples from various countries, such as IncI type plasmid harboring $bla_{\text{TEM-52C}}$ from a human urine sample

obtained in the Netherlands which was highly similar to a plasmid obtained from broiler meat in this study. Results indicate certain foods contain bacteria with multidrug resistance and pose a possible risk to public health, emphasizing the importance of surveillance and the need for further studies on epidemiology of epidemic plasmids.

Keywords: antimicrobial resistance, whole genome sequencing, extended-spectrum beta-lactamases, multidrug resistance, imported food, hybrid sequencing, one health

INTRODUCTION

The increasing prevalence of bacteria producing extended-spectrum beta-lactamases (ESBL) and plasmid-encoded AmpC (pAmpC) enzymes mediating resistance to many commonly used antibiotics has led to global health problems. ESBL/pAmpC are commonly found in Gram-negative enterobacteria, such as *Escherichia coli* and *Klebsiella pneumoniae*, which belong to the normal human and animal intestinal microbiota. These bacteria may spread during food production, for example during the slaughter process, and thus contaminate food products, imposing a threat to consumers.

In addition to bacterial clonal spread, certain plasmids have been shown to be successful in transferring resistance genes between bacterial populations through horizontal gene transfer, thus increasing the spread of antimicrobial resistance (AMR) (Accogli et al., 2013; Carattoli, 2013).

Typically, ESBL/AmpC-producing bacteria are not more virulent than susceptible bacteria but problems may arise when bacteria cause infections requiring treatment with antimicrobials, such as urinary tract, bloodstream, intra-abdominal, and respiratory tract infections (Pitout and Laupland, 2008). In addition to healthcare associated infections, ESBL-producing *E. coli* have been recognized as a major cause of community-onset disease (Laupland et al., 2008). The World Health Organization (WHO) has issued guidelines on the use of medically important antimicrobials in food-producing animals to preserve the effectiveness of antimicrobials important for human medicine (WHO, 2017a). Cephalosporins (third, fourth, and fifth generation) and carbapenems have been classified as critically important antimicrobials for human medicine by the WHO (WHO, 2019). The WHO has additionally published a global priority list of antibiotic-resistant bacteria to guide research, which includes specific carbapenem-resistant and third-generation cephalosporin-resistant species of the Enterobacteriaceae family, including *E. coli* and *K. pneumoniae* (WHO, 2017b).

Antimicrobial resistance can disseminate in many environments, i.e., communities, hospitals, food production plants, and farms. International travel as well as import and export of goods work as mediators of the spread of AMR across country borders. The use of antimicrobials, both quantitatively and qualitatively, varies between countries, leading to differences in AMR levels (WHO et al., 2018; EFSA and ECDC, 2020). In Europe, antimicrobial sales for food-producing animals range from 3.1 to 423.1 mg/PCU (population correction unit) (European Medicines Agency [EMA], 2019). The median for all countries participating in the report was 61.9 mg/PCU. Northern European countries use less antimicrobials in animals in general compared with Southern European countries (European Medicines Agency [EMA], 2019). A recent study by Van Boeckel et al. (2019) identified the largest hot spots of AMR in animals in China and India, with predicted hot spots emerging in Kenya and Brazil. The use of antimicrobials affects the resistance levels in food-producing animals (Hoelzer et al., 2017) and the use of certain antimicrobials in livestock has been shown to correlate with the level of AMR in *E. coli* in pigs, poultry, and cattle (Chantziaras et al., 2014). Antimicrobial use in animals can even be seen in the levels of AMR in human populations (European Centre for Disease Prevention and Control [ECDC] et al., 2017; Tang et al., 2017). As the demand for animal-source nutrition is rising with the human population increase, more antimicrobials will be used in food-producing animals, especially in low- and middle-income countries (Van Boeckel et al., 2015; Schar et al., 2018). Regarding meat products, broiler meat has been recognized as having a high prevalence of ESBL/AmpC-producing *E. coli* (Ewers et al., 2012; EFSA and ECDC, 2020).

In addition to antimicrobial use, other factors such as lack of sanitation may also affect global AMR gene diversity and abundance (Hendriksen et al., 2019). In particular, fresh food products may be susceptible to bacterial contamination from environmental sources such as poor-quality irrigation water or by cross contamination in food-producing facilities (FAO and WHO, 2019). Application of manure of animal origin is another source of AMR dissemination in agriculture and food production (Hartmann et al., 2012). In addition, seafood is often grown in developing countries in unsanitary conditions, with an increased risk of AMR (Boss et al., 2016). Subsequently, fruits and vegetables may also acquire resistant bacteria through irrigation water contaminated by aquaculture production (Done et al., 2015). Animal crops fertilized with manure and contaminated by soil bacteria may be a source of resistant bacteria for food-producing animals and lead to amplification of AMR in animal gut microbiota with concurrent antimicrobial administration (Witte, 2000; Marshall and Levy, 2011; FAO and WHO, 2019). Also, human-derived pathogenic bacteria together with AMR may enter the food chain via wastewater or sewage sludge used for irrigation or fertilization in agriculture (Reinthaler et al., 2010).

Our objective was to study the occurrence of ESBL/AmpC-producing Enterobacteriaceae in food products from a wide selection of countries and multiple food categories to assess the risk of AMR. Furthermore, to study the epidemiology of bacterial isolates and their plasmids, a subset of ESBL/AmpC-producing Enterobacteriaceae isolates were subjected to whole genome sequencing (WGS) to determine sequence types (STs),

resistance and virulence genes, plasmid incompatibility (Inc) groups and subtypes, and to verify bacterial species identification. In addition, short-read sequences were combined with long-read sequences to verify beta-lactamase harboring plasmids to gain insight to plasmid epidemiology and to compare plasmid sequences to previously published plasmids to identify similarities to plasmids identified from different sources. To the best of our knowledge, this study is the first of its kind using WGS for studying AMR and beta-lactamase-harboring plasmids from a large set of import countries, with 35 countries included.

MATERIALS AND METHODS

Sampling

Food products were collected from nine grocery stores in the Helsinki region during November 2018.

Altogether 200 individual products were collected from four different food categories, including vegetables (n = 60), fruits and berries (n = 50), meat (n = 60), and seafood products (n = 30). A detailed list of sampled products including information on country of origin, store of purchase and whether the product was fresh or frozen, and whether it originated from a same batch number with another sampled product is provided in **Supplementary Table 1**. Briefly, 11 batch numbers were identical for 32 sampled products. These 32 products included 10 raw broiler meat samples originating from the same batch and purchased from the same store. The other same-batch products consisted each of two or three products. Food products were divided into different categories according to how they are traditionally perceived; for example, herbs were categorized into vegetables. Products varied in size and consisted of raw, ready-to-eat, frozen, and cooked products. Country or region of origin for samples from different food categories in presented in **Table 1**.

Subsamples

The meat category included raw broiler meat products (n = 10/60) from the same batch number purchased from the same store. One package of broiler meat consisted of two kilograms of raw chicken wings, and 10 of these packages were included in the study. Additional subsamples were taken from each package for further characterization of bacterial isolates to study the diversity of Enterobacteriaceae, STs, plasmid replicons, and resistance genes in raw broiler meat, which has been identified as a rich reservoir of ESBL/AmpC-producing bacteria (EFSA Panel on Biological Hazards, 2011). Four subsamples were taken from each broiler meat package, totaling 40 subsamples. The aim of subsampling was to further study the diversity of ESBL/AmpC-producing Enterobacteriaceae found in a single product.

Country and Region of Origin of Food Samples

The country and region of origin of the samples per food category is shown in **Table 1**.

TABLE 1 | Country/region of origin for samples per food category.

		Sample category			
Country of origin	**Number of samples (total)**	**Vegetables**	**Fruit and berries**	**Meat**	**Seafood**
Belgium	4	3		1	
Brazil	19		12	7	
Canada	1				1
Chile	2		2		
China	3	3			
Colombia	1		1		
Costa Rica	3		3		
Denmark	4			4	
Egypt	1	1			
Estonia	1				1
European Union	1			1	
France	4	2		1	1
Germany	22	2	2	18	
Hungary	2			2	
India	1		1		
Indian Ocean	1				1
Iran	1		1		
Israel	1		1		
Italy	6	3		2	1
Kenya	2	2			
Laos	6	6			
Lithuania*	11	1		10	
Malaysia*	4	3	1		
Morocco	1		1		
Mexico	3		3		
New Zealand	3		1	2	
Norway	3				3
Pacific Ocean	9				9
Peru	5	2	3		
Poland*	4			4	
Portugal	4		4		
South Africa	5		5		
Spain	12	4	3	4	1
Thailand	6	6			
The Netherlands	3	1		2	
Turkey	1	1			
United Kingdom	1			1	
Vietnam	8		1		7
Unknown	31	20	5	1	5
Total:	200	60	50	60	30

**Countries of origin for products in which ESBL/AmpC-producing E. coli and/or K. pneumonia were found.*

Microbiological Analysis of the Samples

Samples were transported to the laboratory and stored at 4°C, and analysis was started within 24 h. Using sterile scissors and forceps, 25 g of each sample was dissected at various sites of the product and placed into a sterile Stomacher strainer bag (Seward Stomacher 400 Classic Strainer bag, Worthing, United Kingdom) with 225 ml of sterile buffered peptone

water (Oxoid, Basingstoke, Hampshire, United Kingdom) and homogenized for 60 s (Stomacher 400 laboratory blender, Seward, United Kingdom). Samples were incubated at 37°C for 18–22 h. After incubation, a loopful (10 μl) of each enrichment was streaked onto two parallel MacConkey agar plates (Lab M, Lancashire, United Kingdom; Scharlau Chemie s.a, Sentmenat, Spain) with 1 mg/l cefotaxime. To improve the isolation of Enterobacteriaceae listed as the highest priority by the WHO (2017b), one of the plates was incubated at 44°C and the other at 37°C for 18–22 h. One colony from each morphologically different bacterial growth from each plate was re-streaked onto individual MacConkey agar plates with 1 mg/l cefotaxime and incubated overnight at 37°C. Bacterial colonies were re-streaked onto individual MacConkey agar plates with cefotaxime supplement until a pure culture was achieved.

Bacterial Species Identification

Isolates were streaked onto a bovine blood agar plate and incubated at 37°C overnight for bacterial species determination with a matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) based Bruker Biotyper (Bruker Daltonics). A score value of 2.0–3.0 was considered high and thus a confident match and was set as the criteria. All isolates identified as *E. coli* and *K. pneumoniae* were stored at −70°C for further characterization.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) was performed on *E. coli* and *K. pneumoniae* isolates to confirm production of ESBL, AmpC, and/or carbapenemase. AST was performed with a disk diffusion method; susceptibility to third-generation cephalosporins was tested with ceftazidime (10 μg) (Neo-Sensitabs, Rosco Diagnostica, Taastrup, Denmark) and cefotaxime (5 μg) (Oxoid, Basingstoke, Hampshire, United Kingdom), to fourth-generation cephalosporins with cefepime (30 μg), to cephamycins with cefoxitin (30 μg), and to carbapenems with meropenem (10 μg) (Neo-Sensitabs, Rosco Diagnostica, Taastrup, Denmark). Epidemiological cut-off values were used as a reference (EUCAST, 2017). Synergism between third-generation cephalosporins and clavulanic acid was tested with a combination disk diffusion test with cefotaxime + clavulanic acid (30 μg + 10 μg) and ceftazidime + clavulanic acid (30 μg + 10 μg) (Neo-Sensitabs, Rosco Diagnostica, Taastrup, Denmark). *E. coli* ATCC 25922 was included as a quality control. In addition to resistance to third-generation cephalosporins, resistance to cephamycin and <5 mm difference in inhibition zones in the combination disc diffusion test were used as criteria for AmpC production, whereas ESBL production was evidenced by resistance to third-generation cephalosporins and ≥5 mm difference in the combination disk diffusion test.

DNA Extraction and Sequencing

Short-Read Sequencing

From all food samples positive for ESBL/AmpC-producing *E. coli* or *K. pneumoniae*, a collection of isolates was chosen for WGS analysis in order to study the presence of AMR, virulence genes, and plasmid replicons, as well as to assess the multilocus sequence type (MLST). If applicable, a representative from each ESBL/AmpC enzyme type category (ESBL, AmpC, or ESBL together with AmpC) and bacterial species (*E. coli* or *K. pneumoniae*) was chosen from each positive food sample, excluding subsamples. Consequently, from one to three isolates were chosen for whole genome sequencing from each positive sample.

Bacterial DNA was extracted and purified with a PureLink Genomic DNA Mini Kit (Invitrogen by Thermo Fischer Scientific, Carlsbad, CA, United States) according to the manufacturer's instructions. The assessment of DNA quality was carried out using a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, United States) and DNA quantity was measured using a Qubit 2.0 fluorometer (Invitrogen, Life Technologies, Carlsbad, CA, United States). Library preparation was performed with an Illumina Nextera XT and sequencing with an Illumina Novaseq 6000 (Center for Genomics and Transcriptomics, Tübingen, Germany) with paired-end reads. Samples were sequenced with 100× coverage and 2× 100 bp read length.

Long-Read Sequencing

Subsequently, seven short-read sequenced *E. coli* isolates were chosen for long-read sequencing in order to study beta-lactamase harboring plasmids in more depth. Isolates were chosen from short-read sequenced isolates to represent a wide selection of different beta-lactamases, MLST types, and plasmid replicons. DNA extraction and purification were performed as described in Section "Short-Read Sequencing."

DNA extracts from three or two isolates at a time were multiplexed using a SQK-LSK109 ligation sequence kit (Oxford Nanopore Technologies, United Kingdom) according to the manufacturer's protocol. Libraries were loaded onto FLO-FLG001 R9.4.1 Flongle flow cells (Oxford Nanopore Technologies, United Kingdom) used with the MinION Mk1B sequencing device and sequenced with MinKNOW software v19.06.8 for 20–24 h.

All raw sequences have been deposited at European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB37779[1]. Accession numbers for each sequenced isolate are provided in **Supplementary Table 2**.

Bioinformatic Analyses

Short-Read Sequences

Bioinformatic analyses of bacterial DNA sequences were run on a web-based service (Center for Genomic Epidemiology, DTU, Denmark). Raw reads were assembled with SPAdes 3.9 (Nurk et al., 2013) and the rest of the analyses were carried out with assembled contigs according to the service's recommendations with default values for identity and coverage. Acquired AMR genes were determined using ResFinder 3.1 (Zankari et al., 2012), bacterial species identification was confirmed with KmerFinder 3.1 (Hasman et al., 2014; Larsen et al., 2014; Clausen et al., 2018), MLST was determined with MLST 2.0 (Larsen et al., 2012) using

[1]https://www.ebi.ac.uk/ena/data/view/PRJEB37779

E. coli scheme 1 (Wirth et al., 2006), virulence genes for *E. coli* isolates were determined with VirulenceFinder 2.0 (Joensen et al., 2014), plasmid replicons were determined with PlasmidFinder 2.1 (Carattoli et al., 2014), and pMLST 2.0 (Carattoli et al., 2014) was used for typing plasmid replicons, where applicable.

Long-Read Sequences and Plasmid Analysis

Nanopore FAST5 read files were basecalled using Guppy v3.4.1 (Oxford Nanopore Technologies, United Kingdom) with FASTQ output and demultiplexed with Qcat v1.1.0 (Oxford Nanopore Technologies, United Kingdom). Quality trimming was performed with BBDuk (BBTools v38.71, Joint Genome Institute, United States) using a QTRIM value of seven. Hybrid assembly of Illumina and nanopore sequences was performed with Unicycler v0.4.8 (Wick et al., 2017) set at default values. Hybrid assembled FASTA files were uploaded to ResFinder 3.2 (Zankari et al., 2012) to determine acquired beta-lactamase resistance genes with default values for identity and coverage. PlasmidFinder 2.1 (Carattoli et al., 2014) was used to determine plasmid replicons located in the same contigs as beta-lactamase genes. Plasmid STs were determined for beta-lactamase harboring plasmid replicons with pMLST 2.0 (Carattoli et al., 2014). VirulenceFinder 2.0 (Joensen et al., 2014) was used to confirm virulence genes present on plasmid contigs. The plasmid sequences were annotated with Prokka v1.13 (Seemann, 2014) and manually curated with BLASTn/BLASTp. The newly developed tool MobileElementFinder v1.0.3 (Johansson et al., 2021) was utilized to search for mobile elements together with BLASTn/BLASTp. Plasmid structures were visualized with Easyfig v2.2.2 (Sullivan et al., 2011) for each different plasmid Inc group identified in the study, and comparisons to previously published plasmids with available metadata were visualized with BRIG v0.95 (Alikhan et al., 2011).

RESULTS

Bacterial Species Identification

Altogether, 14 out of 200 food samples were positive for ESBL/AmpC-producing *E. coli* and/or *K. pneumoniae* (**Table 2**). ESBL/AmpC-producing *E. coli* was found in 3% (2/60) of vegetable samples. The positive samples were obtained from two coriander samples originating from Malaysia and were from the same batch and purchased from the same store. ESBL/AmpC-producing *K. pneumoniae* was found in 1% (1/60) of vegetable samples. The positive sample was obtained from chili pepper originating from Malaysia purchased from the same store as the coriander samples.

ESBL/AmpC-producing *E. coli* was found in 17% (10/60) of meat samples, all originating from raw broiler meat from the same batch originating from Lithuania and purchased from the same store. In addition, ESBL/AmpC-producing *K. pneumoniae* was recovered from 3% (2/60) of meat samples, originating from the aforementioned raw broiler meat (n = 1) and frozen turkey meat (n = 1) originating from Poland. Positive samples originating from food products are presented in **Figure 1**.

Altogether, 152 out of 200 food samples yielded a total of 313 isolates, which were subjected to bacterial species identification with MALDI-TOF MS. Isolates originated from samples incubated at both 44 and 37°C. Samples with bacterial growth (n = 152) yielded from one to four isolates per parallel agar plate. Information on samples and isolates positive for bacterial growth and ESBL/AmpC-producing *E. coli* and *K. pneumoniae* is provided in **Figure 2**. Altogether, 21 isolates were identified as *E. coli* and five as *K. pneumoniae*. Isolates positive for ESBL/AmpC-producing *E. coli* and/or *K. pneumoniae* and the selection process for WGS are presented in **Figure 2**.

Bacterial Species Identification of Subsamples

Altogether, ESBL/AmpC-producing *E. coli* and/or *K. pneumoniae* were found in 90% (36/40) of subsamples. The subsamples were taken from 10 raw broiler meat samples that were included in the main samples. These raw broiler meat samples originated all from the same batch from the same store. Altogether, 135 isolates were recovered from 40 raw broiler meat subsamples. From these subsample isolates, 109 were identified with MALDI-TOF MS with a score value of 2.0–3.0. From these isolates, 86 were identified as *E. coli* and four as *K. pneumoniae*. For 23 isolates, species identification was not possible.

TABLE 2 | Occurrence of ESBL/AmpC-producing Enterobacteriaceae in food samples.

Food category	Number of samples analyzed	Number of samples positive for ESBL/AmpC Enterobacteriaceae (%)[a]	Number of samples positive for ESBL/AmpC *E. coli* (%)	Number of samples positive for ESBL/AmpC *K. pneumoniae* (%)	Number of isolates obtained (samples positive for bacterial growth/total no. of samples)	Number of *E. coli* isolates/total no. of isolates (%)	Number of *K. pneumoniae* isolates/total no. of isolates (%)
Vegetables	60	3 (5)	2 (3)	1 (2)	127 (57/60)	2/127 (2)	2/127 (2)
Fruits and berries	50	0 (0)	0 (0)	0 (0)	40 (25/50)	0/40 (0)	0/40 (0)
Meat	60	11 (18)	10 (17)	2 (3)	103 (49/60)	19/103 (18)	3/103 (3)
Seafood	30	0 (0)	0 (0)	0 (0)	43 (21/30)	0/43 (0)	0/43 (0)
Total	200	14 (7)	12 (6)	3 (2)	313 (152/200)	21/313 (7)	5/313 (2)
Subsamples	40	36 (90)	35 (89)	4 (10)	135 (40/40)	86/135 (64)	4/135 (3)

[a]*Based on phenotypic tests (EFSA Panel on Biological Hazards, 2011; EUCAST, 2017) and species identification with matrix-assisted laser desorption ionization–time of flight mass spectrometry, including results with a score value of 2.0–3.0.*

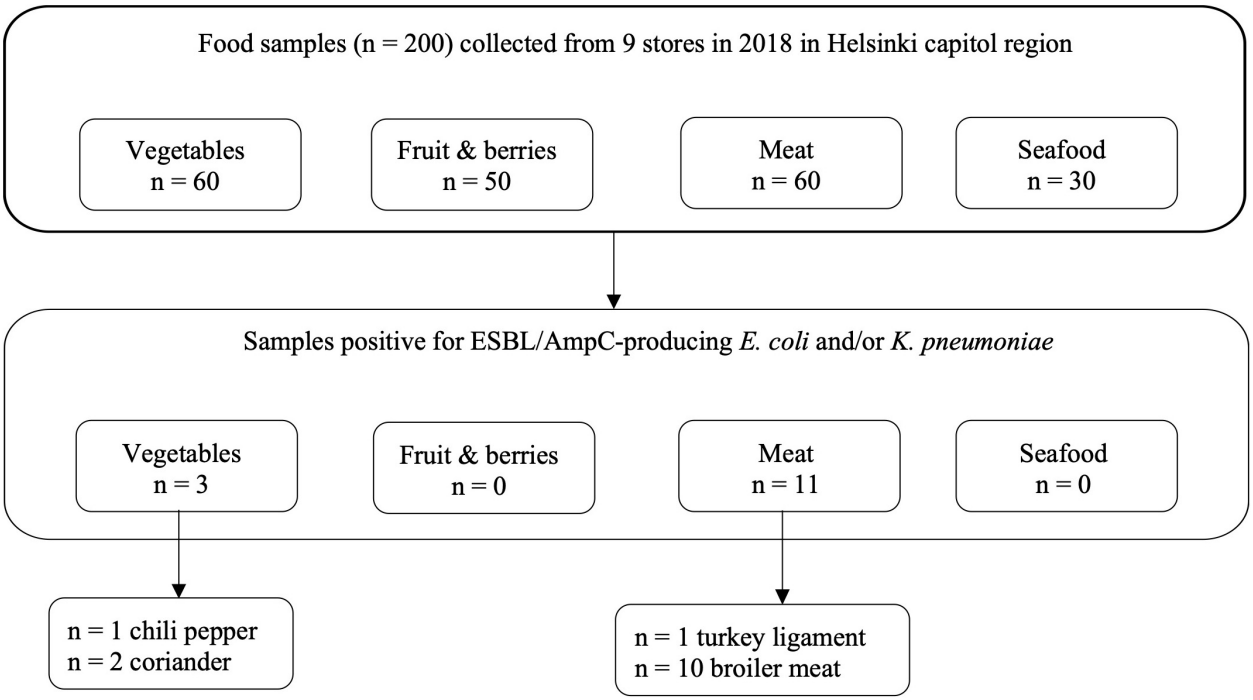

FIGURE 1 | Food samples positive for ESBL/AmpC-producing *Escherichia coli* and/or *Klebsiella pneumoniae* based on phenotypic tests (EFSA Panel on Biological Hazards, 2011; EUCAST, 2017) and species identification with matrix- assisted laser desorption ionization–time of flight mass spectrometry, including results with a score value of 2.0–3.0.

Antimicrobial Susceptibility Testing of *Escherichia coli* and *Klebsiella pneumoniae* Isolated From Food Samples

Altogether, 21 isolates from the main samples were identified as *E. coli*. From these, 13 (62%) were phenotypically ESBL producers, three (14%) AmpC producers, and five (24%) produced both AmpC and ESBL. From the five isolates from the main samples identified as *K. pneumoniae*, four were phenotypically ESBL producers and one an AmpC producer. All isolates were resistant to third-generation cephalosporin (cefotaxime and ceftazidime), except one *E. coli* isolate that was susceptible to ceftazidime but resistant to cefotaxime. None of the isolates were resistant to carbapenem (meropenem).

Antimicrobial Susceptibility Testing of *Escherichia coli* and *Klebsiella pneumoniae* Isolated From Raw Broiler Meat Subsamples

According to AST with disk diffusion, from the 86 isolates identified as *E. coli*, 43 (50%) were ESBL producers, 20 (23%) AmpC producers, and 23 (27%) produced both AmpC and ESBL. All four *K. pneumoniae* isolates were ESBL producers. All isolates from the subsamples were resistant to third-generation cephalosporin (cefotaxime and ceftazidime), except one *K. pneumoniae* isolate that was susceptible to ceftazidime but resistant to cefotaxime. None of the subsample isolates were resistant to carbapenem (meropenem).

Bioinformatic Analyses

Short-Read Sequences

Altogether, 21 isolates were subjected to short-read WGS with Illumina, consisting of 17 *E. coli* and four *K. pneumoniae* isolates, originating from one chili pepper, two coriander, one turkey, and 10 broiler meat samples. Genotypic results are presented in **Table 3**.

The sequenced isolates were confirmed to be either *E. coli* or *K. pneumoniae*. Altogether 18 MLST types were identified, 15 STs from the 17 sequenced *E. coli* isolates and three STs from the four sequenced *K. pneumoniae* isolates. Four different STs were identified from the four vegetable samples: ST155 and ST479 *E. coli* isolates from coriander, and ST307 and ST101 *K. pneumoniae* isolates from chili. The *K. pneumoniae* isolate from turkey meat was identified as ST307. The 16 isolates originating from broiler meat consisted of 14 different STs, including ST189, ST8330, ST4994, ST1011, ST423, ST1485, ST201, ST83, ST38, ST1638, ST641, ST117, and ST88 *E. coli* isolates and ST37 *K. pneumoniae* isolate.

Multiple AMR genes were observed in all of the sequenced isolates. All isolates harbored beta-lactamase gene(s) ($bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M-65}}$, $bla_{\text{SHV-12}}$, $bla_{\text{SHV-81}}$, $bla_{\text{SHV-28}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{TEM-52C}}$, $bla_{\text{CARB-2}}$, $bla_{\text{OXA-1}}$, $bla_{\text{DHA-1}}$, and/or $bla_{\text{CMY-2}}$) and almost all isolates harbored genes conferring resistance to aminoglycoside, macrolide, lincosamide, streptogramin B, phenicol, sulfonamide, tetracycline, and trimethoprim. Additionally, a few isolates

Samples positive for ESBL/AmpC-producing *E. coli* and/or *K. pneumoniae*

Sample	Food product
A35	chili pepper
A40	coriander
A41	coriander
C32	turkey ligament
C51	broiler meat
C56	broiler meat
C61	broiler meat
C66	broiler meat
C71	broiler meat
C76	broiler meat
C81	broiler meat
C86	broiler meat
C91	broiler meat
C96	broiler meat

Isolates obtained from positive samples

Isolate	Species	Phenotype	Short-read WGS	Long-read WGS
A35-1	*K. pneumoniae*	ESBL	X	
A35.2-2	*K. pneumoniae*	AmpC	X	
A40.2-1	*E. coli*	ESBL	X	X
A41.2-1	*E. coli*	ESBL	X	X
C32.1.2-1	*K. pneumoniae*	ESBL		
C32.1-2	*K. pneumoniae*	ESBL	X	
C51-1	*E. coli*	ESBL	X	X
C51.2-2	*E. coli*	ESBL+AmpC	X	
C56.1-1	*E. coli*	ESBL	X	X
C56.2-1	*E. coli*	ESBL		
C61-1	*E. coli*	ESBL+AmpC	X	X
C66-1	*E. coli*	ESBL	X	
C71.1-1	*E. coli*	ESBL	X	
C76.1-1	*E. coli*	ESBL+AmpC	X	
C76.2-1	*E. coli*	ESBL		
C76.1-2	*E. coli*	ESBL	X	
C76.3-2	*E. coli*	ESBL		
C81.1-1	*E. coli*	ESBL	X	X
C81.3-1	*E. coli*	AmpC	X	
C81.1-2	*E. coli*	ESBL+AmpC	X	
C81.2-2	*E. coli*	ESBL		
C86.1-1	*E. coli*	ESBL+AmpC	X	X
C86.1-2	*E. coli*	ESBL	X	
C86.3-1	*K. pneumoniae*	ESBL	X	
C91-2	*E. coli*	AmpC	X	
C96-1	*E. coli*	AmpC	X	

Isolates were chosen for short-read sequencing from each positive sample by selecting one isolate representing each resistance phenotype (ESBL, AmpC or ESBL+AmpC) and species (*E. coli* / *K. pneumoniae*)

Selected *E. coli* isolates were chosen for long-read sequencing to study resistance plasmids → isolates chosen to represent a diversity of bacterial sequence types and beta-lactamase genes

FIGURE 2 | Isolates obtained from food samples positive for ESBL/AmpC-producing *Escherichia coli* and/or *Klebsiella pneumoniae* and subsequent isolate selection for short-read and long-read whole genome sequencing (WGS).

TABLE 3 | Genomic characteristics of *Escherichia coli* and *Klebsiella pneumoniae* strains isolated from globally produced food.

								Resistance genes								
Isolate[a]	**Product**	**Origin**	**Species[b]**	**MLST[c]**	**Plasmid replicons**	**Phenotype[d]**	**Virulence genes**	**Aminoglycoside**	**Beta-lactam**	**Fluoroquinolone**	**Fosfomycin**	**Macrolide, Lincosamide, Streptogramin B**	**Phenicol**	**Rifampicin**	**Sulfonamide**	**Tetracycline**	**Trimethoprim**
A35-1	Chili pepper	Malaysia	*K. pneumoniae*	ST307	IncFIB(K), IncFII(K)	ESBL	*N/A*	*aac(3)-IIa, aac(6′)-Ib-cr, aph(3′′)-Ib, aph(6)-Id*	$bla_{CTX-M-15}$, bla_{OXA-1}, bla_{SHV-28}, bla_{TEM-1B}	*aac(6′)-Ib-cr, oqxA, oqxB, qnrB1*	*fosA*		*catB3*		*sul2*	*tet*(A)	*dfrA14*
A35.2-2	Chili pepper	Malaysia	*K. pneumoniae*	ST101	IncFIB(K), IncFII(pK91)	AmpC	*N/A*		bla_{DHA-1}, bla_{SHV-28}	*oqxA, oqxB, qnrS1*	*fosA*				*sul1*	*tet*(A)	*dfrA1*
A40.2-1	Coriander	Malaysia	*E. coli*	ST155	IncFIB, IncFIC(FII)	ESBL	*cma, gad, lpfA*	*aph(3′)-Ia, aph(3′′)-Ib, aph(6)-Id*	$bla_{CTX-M-55}$, bla_{TEM-1B}			*mdf*(A)	*floR*	*ARR-2*	*sul2*	*tet*(A)	*dfrA14*
A41.2-1	Coriander	Malaysia	*E. coli*	ST479*	IncFIB, p0111	ESBL	*gad, lpfA*	*aac(3)-IV, aadA5, aph(4)-Ia*	$bla_{CTX-M-65}$	*oqxA, oqxB*		*mdf*(A)	*floR*		*sul1, sul2*	*tet*(A)	*dfrA17*
C32.1-2	Frozen turkey ligament	Poland	*K. pneumoniae*	ST307	IncFIB(K), IncFII(K)	ESBL	*N/A*	*aac(3)-IIa, aac(6′)-Ib-cr, aph(6)-Id, aph(3′′)-Ib*	$bla_{CTX-M-15}$, bla_{OXA-1}, bla_{SHV-28}, bla_{TEM-1B}	*aac(6′)-Ib-cr, oqxA, oqxB, qnrB1*	*fosA*		*catB3*		*sul2*	*tet*(A)	*dfrA14*
C51-1	Raw broiler meat	Lithuania	*E. coli*	ST189	IncI1-I(Gamma)	ESBL	*astA, cif, eae, espA, espB, espF, espJ, gad, nleA, nleB, tir*	*aadA1, aadA2*	bla_{SHV-12}			*mdf*(A)	*cmlA1*		*sul3*	*tet*(A)	
C51.2-2	Raw broiler meat	Lithuania	*Escherichia fergusonii***	ST8330	ColpVC, IncB/O/K/Z, IncFIB, IncFII, IncI2, IncX1	ESBL + AmpC	*cma, gad, mchF*	*aac(3)-IV, aadA1, ant(2′′)-Ia, aph(3′)-Ia, aph(3′′)-Ib, aph(4)-Ia, aph(6)-Id*	bla_{CMY-2}, bla_{TEM-1B}				*catA1, floR*		*sul1, sul2*	*tet*(B)	
C56.1-1	Raw broiler meat	Lithuania	*E. coli*	ST4994	Col156, Col8282, ColpVC, IncI1-I(Gamma), IncI2(Delta)	ESBL	*air, astA, celb, eilA, gad, iha, iss*		$bla_{TEM-52C}$			*mdf*(A)					
C61-1	Raw broiler meat	Lithuania	*E. coli*	ST1011	IncFIB, IncFII, IncI1-I(Gamma)	ESBL + AmpC	*cma, eilA, iroN, iss, mchF*	*aadA2, aph(3′′)-Ib, aph(6)-Id*	bla_{CMY-2}, bla_{TEM-1B}			*mdf*(A)			*sul2*	*tet*(A)	*dfrA12*
C66-1	Raw broiler meat	Lithuania	*E. coli*	ST423	IncFIB(pLF82), IncFII(pSE11), IncI1-I(Gamma)	ESBL	*gad, lpfA*	*aadA1, aadA2*	bla_{CARB-2}, bla_{SHV-12}			*mdf*(A)	*cmlA1*		*sul3*	*tet*(A)	*dfrA16*
C71.1-1	Raw broiler meat	Lithuania	*E. coli*	ST1485	IncB/O/K/Z, IncFIA, IncFIB, IncFIC(FII), IncHI2, IncHI2A, p0111	ESBL	*air, eilA, gad, iha, iroN, lpfA, iss, mchF, mcmA, tsh*	*aadA1, aadA2, aph(3′′)-Ib, aph(6)-Id*	bla_{CMY-2}, bla_{TEM-1B}			*mdf*(A)	*cmlA1*		*sul2, sul3*	*tet*(A)	*dfrA14*
C76.1-1	Raw broiler meat	Lithuania	*E. coli*	ST201	IncFIB, IncFII(pCoo), IncI1-I(Gamma)	ESBL + AmpC	*cma, gad, iroN, lpfA, iss*	*aadA1*	bla_{CMY-2}, bla_{TEM-1B}	*qnrS1*		*mdf*(A)	*cmlA1*		*sul3*	*tet*(A)	*dfrA15*
C76.1-2	Raw broiler meat	Lithuania	*E. coli*	ST83*	IncFIA, IncFIB, IncI1-I(Gamma)	ESBL	*astA, gad, iroN, iss, mchF, tsh*	*aadA1, aadA2*	bla_{SHV-12}			*mdf*(A)	*cmlA1*		*sul3*	*tet*(A)	
C81.1-1	Raw broiler meat	Lithuania	*E. coli*	ST38	Col156, IncFII(29), IncI1-I(Gamma)	ESBL	*air, eilA, gad*	*aadA5*	$bla_{CTX-M-1}$			*mdf*(A)			*sul2*		*dfrA17*
C81.3-1	Raw broiler meat	Lithuania	*E. coli*	ST1638	IncFIB, IncFIC(FII), IncFII(pHN7A8), IncI1-I(Gamma), IncX1, Col(pHAD28)	AmpC	*gad, iss*	*aadA1, aadA2, aph(3′′)-Ib, aph(6)-Id*	bla_{CMY-2}, bla_{TEM-1B}	*qnrB19*		*mdf*(A)	*cmlA1*		*sul3*	*tet*(B)	*dfrA8*
C81.1-2	Raw broiler meat	Lithuania	*E. coli*	ST641	IncFIB, IncFIB(pLF82), IncFII(pSE11), IncX1	ESBL + AmpC	*etpD, gad, lpfA, iss*	*aadA1, aadA2*	bla_{CARB-2}, bla_{CMY-2}, bla_{TEM-1B}	*qnrS1*		*mdf*(A)	*cmlA1*		*sul3*	*tet*(A)	*dfrA16*
C86.1-1	Raw broiler meat	Lithuania	*E. coli*	ST641	IncFIB, IncFIB(pLF82), IncFII(29), IncFII(pSE11), IncX1	ESBL + AmpC	*etpD, gad, iss, lpfA*	*aadA1, aadA3*	bla_{CARB-2}, bla_{CMY-2}, bla_{TEM-1B}	*qnrS1*		*mdf*(A)	*cmlA1*		*sul3*	*tet*(A)	*dfrA16*

(Continued)

TABLE 3 | Continued

Isolate[a]	Product	Origin	Species[b]	MLST[c]	Plasmid replicons	Phenotype[d]	Resistance genes								
							Virulence genes	Aminoglycoside	Beta-lactam	Fluoroquinolone	Fosfomycin	Macrolide, Lincosamide, Streptogramin B	Phenicol	Rifampicin	Sulfonamide	Tetracycline	Trimethoprim
C86.1-2	Raw broiler meat	Lithuania	*E. coli*	ST38	Col156, IncFII(29), IncI1-I(Gamma)	ESBL	*air, eilA, gad, ireA*	*aadA1, aadA5, aph(3'')-Ib, aph(6)-Id*	$bla_{CTX\text{-}M\text{-}1}$			*mdf*(A)	*floR*		*sul1, sul2*	*tet*(A)	*dfrA17*
C86.3-1	Raw broiler meat	Lithuania	*K. pneumoniae*	ST37	IncFIA(H1), IncR	ESBL	*N/A*	*aac(3)-IId, aph(3'')-Ib, aph(6)-Id*	$bla_{CTX\text{-}M\text{-}15}$, $bla_{SHV\text{-}81}$, $bla_{TEM\text{-}1B}$	*oqxA, oqxB*	*fosA*				*sul2*		*dfrA14*
C91-2	Raw broiler meat	Lithuania	*E. coli*	ST117	ColpVC, IncB/O/K/Z, IncFIB, IncFIC(FII), IncFII(29)	AmpC	*celb, gad, ireA, iroN, lpfA, iss, mchB, mchC, mchF, pic*	*aadA1, aph(3'')-Ib, aph(6)-Id*	$bla_{CMY\text{-}2}$, $bla_{TEM\text{-}1B}$			*mdf*(A)			*sul1, sul2*	*tet*(A)	*dfrA8*
C96-1	Raw broiler meat	Lithuania	*E. coli*	ST88	IncFII(29), IncI1-I(Gamma)	AmpC	*gad, iss, lpfA*	*aadA1, aph(3'')-Ib, aph(6)-Id*	$bla_{CMY\text{-}2}$, $bla_{TEM\text{-}1B}$			*mdf*(A), *mph*(B)	*catA1*		*sul1, sul2*		*dfrA1, dfrA8*

[a]*Suffix −1 indicates first incubation temperature of 44°C and −2 of 37 °C.* [b]*Analysis tools by Center for Genomic Epidemiology: SPAdes 3.9 for assembly, KmerFinder 3.1 for species determination (** identified as E. coli with SpeciesFinder 1.2), MLST 2.0 for multilocus sequence typing [* scheme Escherichia coli #1 used first, if sequence type (ST) unidentified then #2 (Jaureguy et al., 2008) used], PlasmidFinder 2.0 for plasmid replicons, VirulenceFinder 2.0 for E. coli virulence genes (N/A, not applicable for K. pneumoniae), ResFinder 3.1 for acquired resistance genes.* [c]*MLST, multilocus sequence typing.* [d]*Based on phenotypic tests; ESBL, extended-spectrum beta-lactamase.*

harbored plasmid-mediated quinolone resistance (PMQR), fosfomycin, and/or rifampicin resistance genes. Nine out of the 16 sequenced broiler meat samples harbored the AmpC beta-lactamase $bla_{CMY\text{-}2}$.

All isolates were carrying AMR genes conferring resistance toward critically important antimicrobials (WHO, 2019), including aminoglycosides, third- and fourth-generation cephalosporins, macrolides, and quinolones. All of the *E. coli* isolates harbored the macrolide resistance gene *mdf(A)*. All of the *K. pneumoniae* isolates harbored PMQR and fosfomycin resistance genes, whereas only five *E. coli* isolates harbored PMQR genes and none harbored fosfomycin resistance genes. Aminoglycoside resistance genes were found in all but one *E. coli* and one *K. pneumoniae* isolate. Two *K. pneumoniae* isolates harbored gene *aac(6′)-Ib-cr*, which confers resistance toward both aminoglycosides and fluoroquinolones (Frasson et al., 2011). Phenicol resistance was found in 16 isolates, rifampicin resistance in one isolate, sulfonamide resistance in all but one isolate, and tetracycline and trimethoprim resistance in 17 isolates.

ESBL/AmpC phenotypes correlated with the detected genotype in all but four (isolates A35.2-2, C51.2-2, C61-1, and C71.1-1) of the sequenced isolates. Isolate A35.2-2 was phenotypically an AmpC producer but harbored both $bla_{DHA\text{-}1}$ and $bla_{SHV\text{-}28}$. Isolates C51.2-2 and C61-1 were phenotypically both ESBL and AmpC producers, although they possessed only $bla_{TEM\text{-}1B}$ in addition to $bla_{CMY\text{-}2}$. Isolate C71.1-1 was phenotypically an ESBL producer but harbored $bla_{CMY\text{-}2}$ in addition to $bla_{TEM\text{-}1B}$. $bla_{TEM\text{-}1B}$ confers resistance toward penicillin and ampicillin but not significantly toward extended-spectrum cephalosporins (Paterson and Bonomo, 2005). Complete resistance gene profiles are shown in **Table 3**.

A total of 24 different plasmid replicons were identified from the sequenced isolates with PlasmidFinder 2.1 (Carattoli et al., 2014) with IncF subtype IncFIB and IncI1-Iγ being the most common and appearing in 11 and 10 isolates, respectively. Plasmid replicons belonging to IncF groups were overall detected in 19 isolates. Each isolate harbored from one to seven different plasmid replicons, with the average amount of replicons being three per isolate. Typing of plasmids with plasmid multilocus sequence typing (pMLST) revealed 14 different STs, with the most common ones being ST12 and ST95-CC9 (clonal complex) appearing each in three isolates. Three ST/clonal complex clusters were linked to the IncI1-Iγ plasmid group. IncI1-Iγ ST3-CC3 was found in two ST38 *E. coli* isolates, both harboring resistance gene $bla_{CTX\text{-}M\text{-}1}$. IncI1-Iγ ST95-CC9 was associated with resistance gene $bla_{SHV\text{-}12}$, which was found in three *E. coli* isolates, all with different STs: ST189, ST423, and ST83. IncI1-Iγ ST12 was identified in three *E. coli* isolates harboring $bla_{CMY\text{-}2}$. All of the IncI1-Iγ plasmid replicons originated from broiler meat samples originating from the same batch and collected from the same store. pMLST results are presented in **Supplementary Table 3**.

The isolates harbored multiple different virulence genes. Virulence gene results are presented in **Table 3**. One of the isolates, originating from broiler meat, was positive for

adhesin intimin coding *eae* gene, which is associated with enteropathogenic *E. coli* (Frankel et al., 1998; Müller et al., 2016).

Plasmid Analysis

Altogether, seven *E. coli* isolates were subjected to long-read sequencing with Oxford Nanopore Technologies, consisting of two coriander and five broiler meat isolates. Results of the hybrid assembled plasmid sequences are presented in **Table 4**. All long-read sequenced and hybrid assembled isolates were found to harbor resistance genes and plasmid replicons matching with short-read sequence analysis. In the two coriander isolates, beta-lactamase genes were located in plasmid replicons belonging to the IncF group, whereas in four out of five broiler isolates beta-lactamase genes were located on IncI1-Iγ plasmids. Isolate C86.1-1 included $bla_{\text{CARB-2}}$-carrying IncF type plasmid replicon and $bla_{\text{TEM-1}}$-carrying IncX1 plasmid replicon in addition to a chromosomal $bla_{\text{CMY-2}}$. A chromosomal $bla_{\text{TEM-1B}}$ gene was detected also in isolate C61-1. All detected plasmid replicons represented different plasmid STs. Additionally, all IncF plasmid replicons, the IncX1 replicon and one IncI1-Iγ replicon were found to harbor multiple resistance genes.

Comparison of IncF type plasmids

Three of the hybrid assembled food isolates were found to carry plasmids belonging to the IncF group. Two of the isolates were of coriander origin (A40.2-1 and A41.2-1) and one from raw broiler meat (C86.1-1). RST analysis with pMLST tool (Carattoli et al., 2014) indicated new FIB alleles for pZPK-A41.2-1 and pZPK-C86.1-1 which were submitted to PubMLST database[2] and assigned FIB76 and FIB77, respectively. IncFII/IncFIB replicon carrying narrow-spectrum beta-lactamase $bla_{\text{CARB-2}}$ with a FAB formula of F68:A-:B77 from isolate C86.1-1 additionally carried $bla_{\text{CMY-2}}$ in the chromosome and an IncX1 plasmid harboring $bla_{\text{TEM-1}}$. From this isolate, the IncX1 plasmid (pZPK-C86.1-1_X1) was selected for further comparison analysis to achieve diverse ESBL-plasmid comparisons (**Table 4**).

[2]https://pubmlst.org/

pZPK40.2-1 from isolate A40.2-1 carried a multireplicon IncFIB/IncFIC with a size of 120.7 kb and C + G content of 51.45% and 136 predicted coding sequences (CDSs). pZPK41.2-1 from isolate A41.2-1 carried IncFIB replicon together with p0111 and was 153.3 kb in size with G + C content of 49.93% and 165 CDSs. Comparison to a previously published and annotated IncF plasmid with FAB formula F18:A-:B1:C4 (GenBank accession: MK878890.1) indicated high variability between the plasmids with multiple insertions of mobile elements in both pZPK-A40.2-1 and pZPK-A41.2-1 (**Figure 3A**).

pZPK-A40.2-1 harbored multiple resistance genes in addition to its beta-lactamases $bla_{\text{CTX-M-55}}$ and $bla_{\text{TEM-1B}}$, including *ARR-2, floR, dfrA14, aph(3″)-Ib, aph(3′)-Ia, aph(6)-Id, tet(A)* and *sul2*. In addition, multiple virulence genes (*cma, cvaC, hlyF, iucC, iutA, ompT, sitA*) were detected. Top BLAST hits with available metadata in NCBI included an IncFII/IncFIC type plasmid with a FAB profile F18:A-B1 and $bla_{\text{CTX-M-55}}$, $bla_{\text{TEM-1B}}$ and *mcr-1* from a human urine sample from United States (GenBank accession: KX276657.1) with 89% coverage and 99.73% identity. Another plasmid with the FAB profile F18:A-B1 with $bla_{\text{CTX-M-55}}$ obtained from a turkey in Canada (GenBank accession: CP059932.1) was found to be similar with 88% coverage and 99.78% identity (**Figure 4A**).

pZPK-A41.2-1 also carried multiple resistance genes in addition to the beta-lactamase $bla_{\text{CTX-M-65}}$, including *dfrA17, oqxB, oqxA, sul2, sul1, aac(3)-IV, aph(4)-Ia, aadA5, tet(A)* and *floR*. The only virulence gene detected was the P fimbriae encoding *papC*. BLASTn search against NCBI database identified only partly similar plasmid sequences with lower coverage values than for the other plasmids in the study. pZPK-A41.2-1 aligned with 68% coverage and 99.97% identity with another IncFIB/p0111 type plasmid obtained from chicken stool in China (GenBank accession: CP033251.1). This plasmid carried a $bla_{\text{CTX-M-14}}$ instead of $bla_{\text{CTX-M-65}}$. Another IncFIB/p0111 plasmid with $bla_{\text{TEM-1B}}$ from China recovered from a goose sample (GenBank accession: CP034590.1) aligned with 64% coverage and 99.5% identity (**Figure 4B**).

TABLE 4 | Hybrid assembled *Escherichia coli* food isolates and corresponding beta-lactamase harboring plasmids for comparative analysis.

Isolate	ESBL-plasmid	Plasmid replicon[a]	pMLST/RST	Plasmid size (bp)	Beta-lactamase gene	Other resistance genes on same plasmid replicon(s)	Virulence genes on plasmid replicon(s)
A40.2-1	pZPK-A40.2-1	IncFIB, IncFIC(FII)	[F-:A-:B1] FIC-4	120698	$bla_{\text{CTX-M-55}}$, $bla_{\text{TEM-1B}}$	*ARR-2, floR, dfrA14, aph(3″)-Ib, aph(3′)-Ia, aph(6)-Id, tet(A), sul2*	*cma, cvaC, hlyF, iucC, iutA, ompT, sitA*
A41.2-1	pZPK-A41.2-1	IncFIB, p0111	[F-:A-:B76]	153291	$bla_{\text{CTX-M-65}}$	*floR, oqxA, oqxB, dfrA17, aac(3)-IV, aadA5, aph(4)-Ia, sul1, sul2*	*papC*
C51-1	pZPK-C51-1	IncI1-Iγ	95 (CC-9)	121837	$bla_{\text{SHV-12}}$	*aadA1, aadA2b, tet(A), sul3, cmlA1*	*cib*
C56.1-1	pZPK-C56.1-1	IncI1-Iγ	36 (CC-3)	89504	$bla_{\text{TEM-52C}}$	–	–
C61-1	pZPK-C61-1	IncI1-Iγ	2 (CC-2)	97275	$bla_{\text{CMY-2}}$	–	*cia*
C81.1-1	pZPK-C81.1-1	IncI1-Iγ	3 (CC-3)	112374	$bla_{\text{CTX-M-1}}$	–	*cib*
C86.1-1	pZPK-C86.1-1_X1	IncX1	–	47686	$bla_{\text{TEM-1B}}$	*qnrS1*	

[a]*Analysis tools by Center for Genomic Epidemiology: ResFinder 3.2 for acquired resistance genes; PlasmidFinder 2.1 for determining beta-lactamase harboring plasmid replicons; pMLST 2.0 for plasmid sequence type (ST) (CC, clonal complex; pMLST, plasmid multilocus sequence typing; RST, replicon sequence typing).*

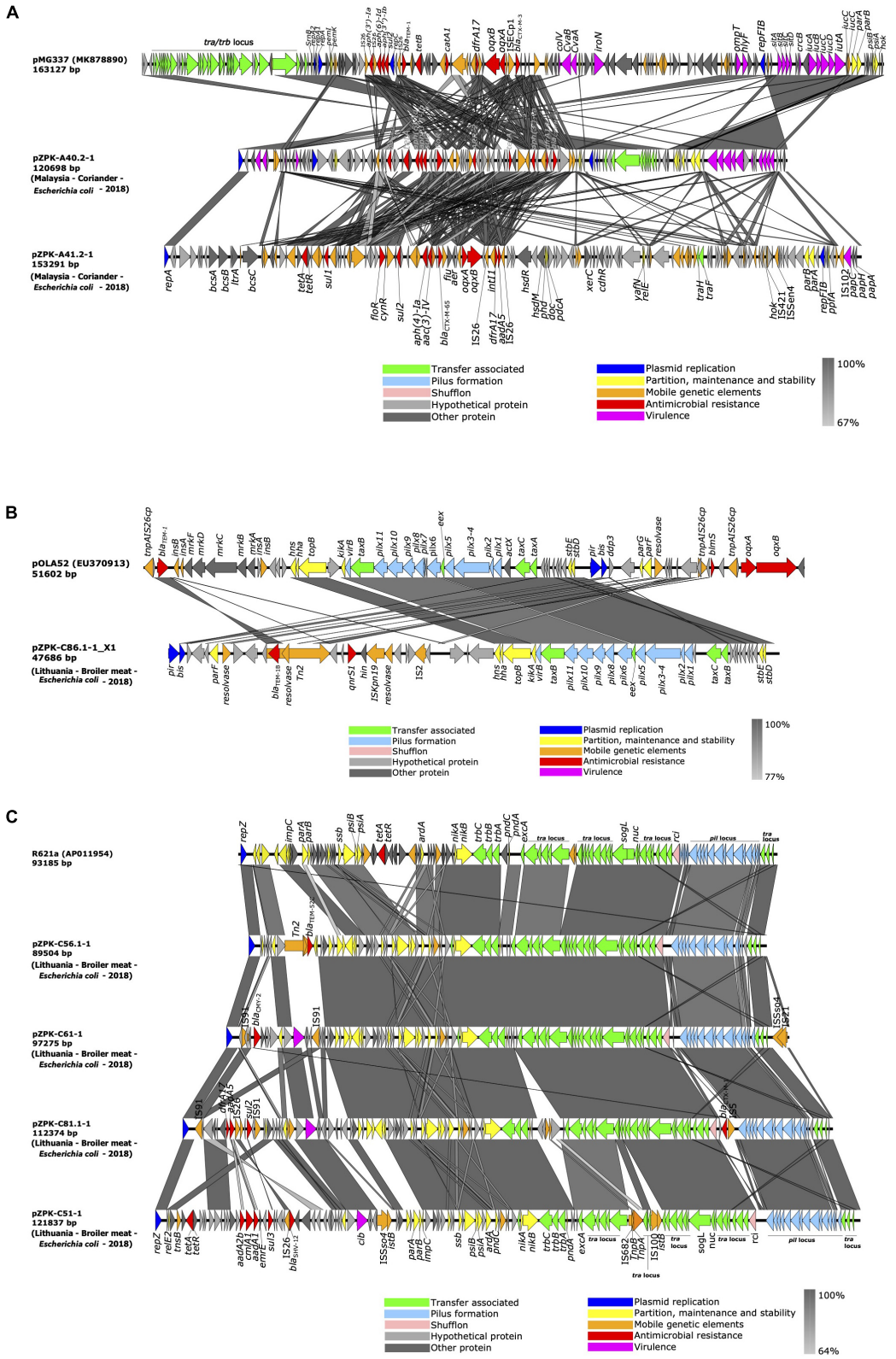

FIGURE 3 | Linear comparison of **(A)** IncFIB/IncFIC(FII) and IncFIB/p0111, **(B)** IncX1 and **(C)** IncI1-Iγ plasmids identified in this study with previously published plasmids (GenBank accession numbers in parentheses for reference plasmids; for plasmids identified in this study the country of origin, source, bacterial species and year of isolation is provided in parentheses). Gray areas between plasmid sequences indicate the percentage of nucleotide sequence identity. The arrows represent coding sequences and their orientation and are colored based on their predicted function.

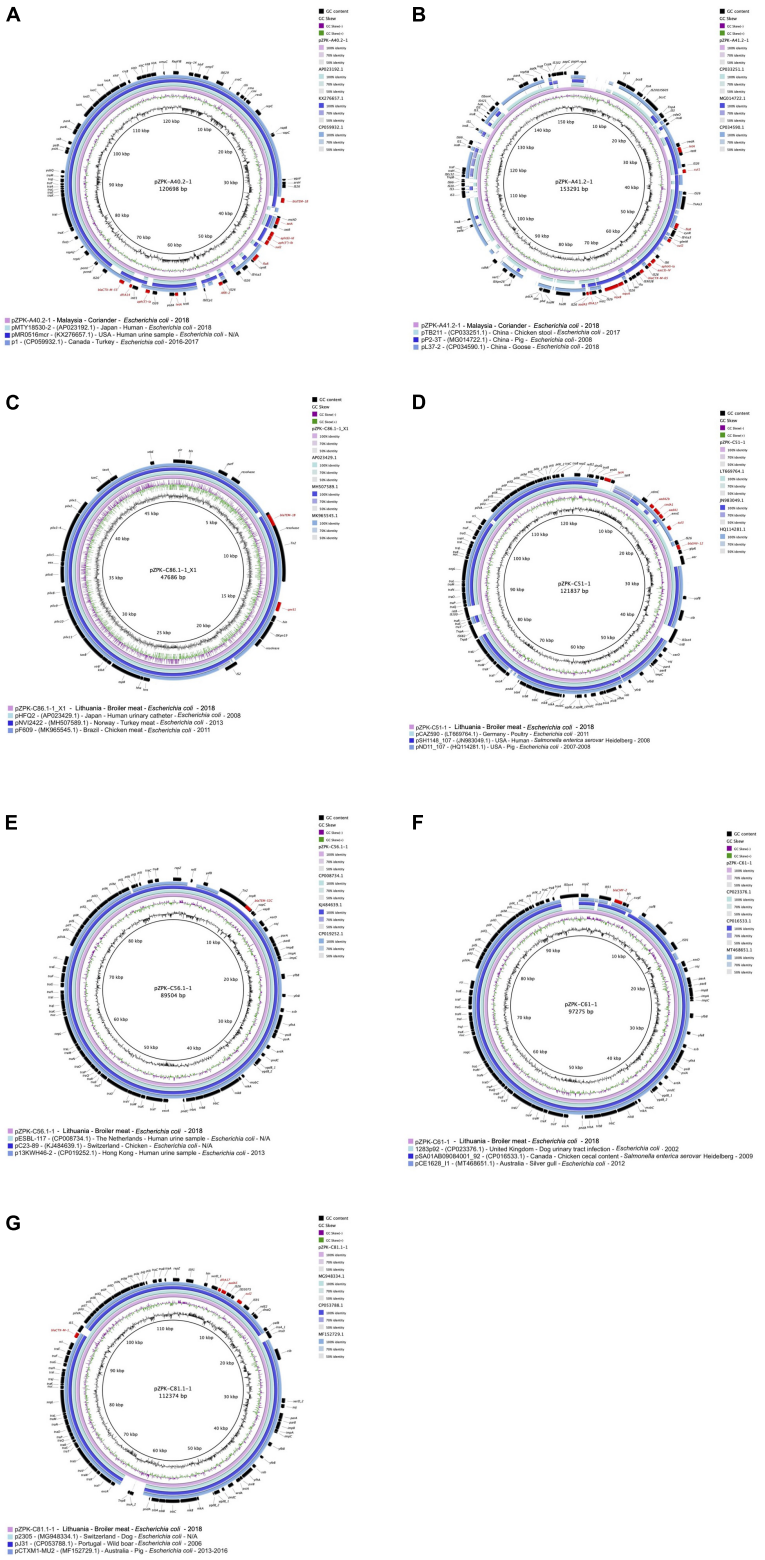

FIGURE 4 | Circular comparisons of the studied plasmids to previously published similar plasmids (GenBank accession numbers are provided in parentheses in the figure legends after plasmid name, followed by country, source, bacterial species and year of isolation; N/A, not available). GC content and GC skew of the studied plasmids are depicted in the inner map with distance scale and the outer ring represents predicted coding sequences with antimicrobial resistance genes highlighted in red. The plasmids in this study included **(A)** pZPK-A40.2-1, **(B)** pZPK-A41.2-1, **(C)** pZPK-C86.1-1_X1, **(D)** pZPK-C51-1, **(E)** pZPK-C56.1-1, **(F)** pZPK-C61-1 and **(G)** pZPK-C81.1-1.

Comparison of IncX1 type plasmid

IncX1 type plasmid (pZPK-C86.1-1_X1) was recovered from isolate C86.1-1 from raw broiler meat. The plasmid carried $bla_{\text{TEM}-1\text{B}}$ and PMQR gene *qnrS1* and no virulence genes were identified. The size of the plasmid was 47.7 kb with G + C content 43.13% and 56 CDSs predicted. Comparison of the plasmid structure to a IncX1 reference plasmid pOLA52 (GenBank accession: EU370913.1) identified the IncX plasmid backbone in the sequenced plasmid, including replication genes *pir* and *bis*, pilus associated genes *pilX* and genes involved in partition (*par*), stability (*stb*) and conjugation (t*ax*) (Johnson et al., 2012) (**Figure 3B**).

BLASTn search against NCBI database identified a highly similar plasmid obtained from *E. coli* from a human urinary catheter sample in Japan (GenBank accession: AP023429.1), aligning with 100% coverage and 99.98% identity and also carrying resistance genes $bla_{\text{TEM-1B}}$ and *qnrS1*. Another highly homologous plasmid with the same resistance profile was a plasmid obtained from turkey meat in Norway (GenBank accession: MH507589.1), aligning with 99% coverage and 99.99% identity. An IncX1 plasmid carrying $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$ and *qnrS1* recovered from chicken meat in Brazil (GenBank accession: MK965545.1) was also found to be similar, with 97% coverage and 100% identity to pZPK-C86.1-1_X1 (**Figure 4C**).

Comparison of IncI1-Iγ type plasmids

IncI1-Iγ type plasmid replicons were identified from four hybrid sequenced food isolates, all originating from broiler meat from the same batch and ranging in size from 89.5 to 121.8 kb. Overall G + C content ranged in size from 50.24 to 51.65% and between 97 and 136 CDSs were predicted per plasmid. Only one of the four IncI1-Iγ plasmids harbored additional resistance genes other than a beta-lactamase; this pZPK-C51-1 $bla_{\text{SHV-12}}$-harboring ST95(CC-9) plasmid with *aadA1, aadA2b, tet(A), sul3, cmlA1* was the largest of the four plasmids. The only virulence genes detected in IncI1-Iγ plasmids were the channel-forming colicin gene *cia* or *cib*. Plasmid pZPK-C56.1-1 (ST36 and CC-3) carried $bla_{\text{TEM-52C}}$ and no virulence genes. The *pndCA* plasmid addiction system was detected in all the IncI1-Iγ plasmid sequences.

Aligning the plasmid sequences with BLASTn with both IncI1 type reference plasmid R64 (GenBank accession: AP005147.1) and IncIγ type reference plasmid R621a (GenBank accession: AP011954.1) indicated three of the four of the plasmids were slightly more similar to the IncIγ type R621a, which was chosen for comparative genomic visualization (**Figure 3C**). The plasmids in our study aligned with a query coverage of 61–85% and with >98% identity with R621a. All studied plasmids had typical IncI type backbones including the conjugational, pilus formation and maintenance and stability regions, except for $bla_{\text{CTX-M-1}}$ and IS*5* located near the shufflon region in pZPK-C81.1-1. The accessory module was variable between plasmids with different inserted elements and resistance genes (**Figure 3C**). Alignment of *excA* and *traY* regions with both references (R64 and R621a) indicated more similarity with IncI1 type R64. The *parAB* region was also more similar to corresponding regions of R64 in all sequenced IncI1-Iγ plasmids except for pZPK-C51-1 which shared more similarity with *parAB* region of R621 plasmid. The results indicate the plasmids in our study share similarities with both I1 and Iγ replicon types for which reason plasmid results are here referred to as IncI1-Iγ.

BLASTn search against NCBI database indicated highly similar plasmids have been isolated from different locations and sources, mostly from different livestock but also human clinical samples and wild animal sources (**Figures 4D–G**). pZPK-C56.1-1 was found to be highly similar with a plasmid of the same ST type (ST36) also carrying $bla_{\text{TEM-52C}}$ isolated from a human urine sample in the Netherlands (GenBank accession: CP008734.1) with a coverage of 100% and identity of 99.99%. pZPK-C56.1-1 was found to align also with another plasmid from a human urine sample from Hong Kong (GenBank accession: CP019252.1) with 91% coverage and 98.98% identity. Also, pZPK-C51-1 carrying $bla_{\text{SHV-12}}$ matched with 99.76% identity and 80% coverage to a plasmid isolated from *Salmonella enterica* serovar Heidelberg from a human source in United States (GenBank accession: JN983049.1). $bla_{\text{CMY-2}}$-harboring pZPK-C61-1 was similar with a plasmid with the same sequence type ST2 CC-2 obtained from a dog urinary tract infection sample in the United Kingdom (GenBank accession: CP023376.1), aligning with 96% coverage and 99.99% identity. pZPK-C61-1 was also similar to a plasmid isolated from a silver gull sample from Australia (GenBank accession: MT468651.1) with 93% coverage and 98.65% identity. Also, pZPK-C81.1-1 with $bla_{\text{CTX-M-1}}$ matched with a plasmid obtained from a dog source in Switzerland (GenBank accession: MG948334.1) with 96% coverage and 99.92% identity and with a wild animal derived plasmid from a wild boar in Portugal (GenBank accession: CP053788.1) with 94% coverage and 99.98% identity. All IncI1-Iγ type plasmids in this study were found to match with similar plasmids from livestock-associated sources, such as a ST95 plasmid with $bla_{\text{SHV-12}}$ from poultry in Germany (GenBank accession: LT669764.1) with 94% coverage and 99.97% identity with pZPK-C51-1 and ST36 plasmid with $bla_{\text{TEM-52C}}$ from chicken in Switzerland (GenBank accession: KJ484639.1) with 100% coverage and 99.96% identity with pZPK-C56. 1-1.

DISCUSSION

Our results demonstrate that imported food products from various origins, acquired in Finland, possess wide genetic variety of ESBL/AmpC-producing Enterobacteriaceae. Hybrid sequence analysis combining long- and short-read sequencing proved beneficial in determining resistance gene locations on specific plasmid replicons. Hybrid assembly of plasmid sequences also resolved the chromosomal location of beta-lactamase genes in two studied isolates. The spread of ESBL/pAmpC genes is highly attributed to epidemic and highly transmissible plasmids, which emphasizes the importance of plasmid replicon determination in epidemiological studies. Although the sample size was limited, our study provides a global indicative insight into AMR prevalence in global food products from 35 countries. The diversity of AMR genes was high in the isolates from food samples positive for ESBL/AmpC-producing Enterobacteriaceae. In addition, resistance toward critically

important antimicrobials was found in all sequenced isolates. Raw meat, in particular broiler meat, was recognized as a common source of ESBL/AmpC-producing Enterobacteriaceae. Broiler meat samples originated from the same batch, which emphasizes the finding that one source can contain a wide variety of different resistance genes, STs, and plasmid replicons.

Compared with an earlier study investigating ESBL/AmpC-producing *E. coli* in Finnish poultry production, the isolates recovered from broiler meat in the current study showed broader resistance to multiple antimicrobial groups (Oikarainen et al., 2019). The isolates in the present study carried AMR genes conferring resistance against critically important antimicrobials (WHO, 2019), including aminoglycosides, third- and fourth-generation cephalosporins, macrolides, and quinolones, whereas no macrolide or PMQR was found in the isolates recovered from Finnish poultry production in the earlier study (Oikarainen et al., 2019). Another study of broilers at slaughterhouses found only limited sulfonamide resistance in addition to ESBL/AmpC with no other resistance genes in broiler meat and cecum samples (Päivärinta et al., 2020).

Our results show that vegetables, coriander and chili from Malaysia contained ESBL/AmpC-producing bacteria. Vegetables are often eaten without heating processes, leading to a greater risk of acquiring resistant bacteria. The three vegetable samples positive for ESBL/AmpC-producing Enterobacteriaceae in our study yielded a total of four isolates with varying STs, plasmid replicons, and a wide variety of beta-lactamase encoding genes, including $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-28}}$, $bla_{\text{CTX-M-55}}$, and $bla_{\text{CTX-M-65}}$. Samples from all food categories yielded many isolates, although most isolates were recovered from the vegetable samples. *Citrobacter* spp. and *Hafnia alvei* were also identified in the isolates, but these were left out of further analysis as our study focused on *E. coli* and *K. pneumoniae*, which are categorized as critical for research by the WHO (WHO, 2017b).

The finding of resistance genes $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-12}}$, and $bla_{\text{OXA-1}}$ commonly linked to human sources (Cantón et al., 2008; Livermore et al., 2019) highlights the potential transmission route of AMR via food products and emphasizes the importance of hygiene measures. In the current study, two of the three isolates carrying $bla_{\text{CTX-M-15}}$ were harboring the plasmid replicon IncFII(K), which has been previously identified from human infections with *K. pneumoniae* harboring $bla_{\text{CTX-M-15}}$ (Dolejska et al., 2012; Bi et al., 2018). Plasmids of the IncFII type have been identified as being epidemic (Carattoli, 2009; Mathers et al., 2015). The AmpC-type resistance gene $bla_{\text{CMY-2}}$ and ESBL types $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-55}}$, and $bla_{\text{CTX-M-65}}$ are associated with food-producing animals, especially poultry (EFSA Panel on Biological Hazards, 2011; Cormier et al., 2019; Day et al., 2019). Interestingly, $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-1}}$ were recovered from broiler meat samples and $bla_{\text{CTX-M-55}}$, and $bla_{\text{CTX-M-65}}$ from vegetable samples in our study. One explanation for the finding of AMR genes associated with food-producing animals in food products could be the possible use of contaminated irrigation water or animal manure in the farming process. As low-income countries have higher AMR abundance in their wastewater (Hendriksen et al., 2019), it can be speculated that the risk of ESBL/AmpC dissemination is greater, especially when water is contaminated by animal manure or wastewater from human sources is used for crop watering. However, no conclusions can be drawn from the current study regarding the possible differences in ESBL/AmpC-producing Enterobacteriaceae prevalence between different countries, because the sample size per country was limited. More attention should be paid to the comparison of different food categories from the results of the current study.

Food samples were found to harbor a variety of bacterial STs, some of which have been described from human infections, i.e., ST37 from Chinese strains (Zhu et al., 2015; Zhang et al., 2016; Xiao et al., 2017) and ST307 from Italian, Colombian, and United Kingdom strains (Villa et al., 2017) of *K. pneumoniae* and ST38 from Bangladeshi and United Kingdom strains of *E. coli* (Hasan et al., 2015; Day et al., 2019). Also, same ST types (ST88 and ST117) found in the Lithuanian broiler meat in the present study have been previously found also in Finnish broiler production (Heikinheimo et al., 2016; Oikarainen et al., 2019) and ST117 more widely in Nordic broiler production (Ronco et al., 2017). In a Canadian study (Vincent et al., 2010), *E. coli* ST117 isolates were identified from human infections and poultry sources with related pulsed-field gel electrophoresis profiles, pointing to a possible poultry source of human infections. With the large variety of different bacterial STs, it is difficult to draw any further conclusions about possible bacterial transmission routes from our study, but it seems that certain *E. coli* STs and their resistance genes are common in international food production and are found in food products produced in various countries. Noteworthy is the finding of clinically relevant bacterial STs.

Plasmid replicons linked to the spread of AMR, particularly of the IncI type (Carattoli, 2013), were detected in our study. IncI1-Iγ ST12 was recovered from three broiler isolates, and it has been previously linked to $bla_{\text{SHV-12}}$ from avian *E. coli* (Accogli et al., 2013). In addition, IncI plasmids with the $bla_{\text{CTX-M-1}}$ gene have been found in *E. coli* from poultry and humans in the Netherlands (Leverstein-van Hall et al., 2011). Also, plasmids of the IncF group were detected in 90% (19/21) of the sequenced isolates. Plasmids of the IncF type are one of the most common Inc types identified in humans and animals, especially in Asia, and most often carry AMR genes of the *bla* family (Rozwandowicz et al., 2018).

Long-read sequencing and hybrid assembly with Illumina short reads allowed more in-depth analysis of plasmids harboring beta-lactamases in our study. Hybrid assembly of the sequences resolved the correct plasmid replicons carrying *bla* genes, which would not have been possible with confidence from the fragmented short read data. As new assembly tools, such as Trycycler[3] have been developed to resolve genomes from WGS data it is important to bear in mind the requirements for high-quality assemblies with these new tools, such as high enough coverage of the long reads. With the advancement and improved availability of long-read sequencing technologies

[3]https://github.com/rrwick/Trycycler

plasmid analyses regarding AMR studies should prove to be even more affordable and accessible in the future.

Our analysis comparing plasmids carrying *bla* genes in this study with plasmid sequences deposited in open databases showed the plasmids in our study were similar with mostly livestock-derived plasmids obtained previously from various countries, but also with human clinical samples, especially from urinary tract infections. pZPK-A41.2-1 carried the P fimbriae encoding virulence gene papC which has been associated with uropathogenic *E. coli* infections (Yazdanpour et al., 2020). pZPK-A40.2-1 was also similar to a *mcr-1*-carrying plasmid isolated from a human urine sample from the United States (GenBank accession: KX276657.1), indicating a potential for IncF type plasmids being able to obtain new resistance genes. pZPK-C86.1-1_X1 was found the be a typical IncX1 plasmid in regards of carrying the PMQR gene *qnrS1*, which has been linked to *Salmonella* and *E. coli* from animal and human sources (Dobiasova and Dolejska, 2016). This plasmid was also found to be highly similar to a plasmid isolated from a human urinary catheter sample in Japan (GenBank accession: AP023429.1).

Five of the hybrid assembled plasmid sequences were obtained from raw broiler meat samples originating from the same batch number. Four of these carried the IncI-Iγ type plasmid, but all harbored a different *bla* gene, indicating diversity even among plasmids obtained from a homogenous origin. The similarity of all of the seven hybrid assembled plasmids, even from vegetable origin, to poultry and other livestock sources published previously also indicates certain plasmid-resistance gene combinations flourish in specific host environments.

Similarity to plasmids from small animal sources was also observed. pZPK-C61-1 of the IncI1-Iγ type with $bla_{\text{CMY-2}}$ was highly similar to a plasmid obtained from a canine urinary tract infection sample in the UK (GenBank accession: CP023376.1). pZPK-C81.1-1 with $bla_{\text{CTX-M-1}}$ also of IncI1-Iγ type was also similar to a $bla_{\text{CTX-M-1}}$-carrying plasmid obtained from *E. coli* from a dog in Switzerland (GenBank accession: MG948334.1). The finding of similar transmissible plasmids from food products and small animals indicates a possible transmission route between food, animals and potentially humans who are in close contact with pets. This highlights the need for plasmid studies implementing a One Health approach, since horizontal gene transmission via plasmids does not know country or species borders.

Interestingly, long-read sequencing and hybrid assembly revealed chromosomally located beta-lactamases in two of the sequenced isolates. Long-read sequencing provides reliable information on the true location of resistance genes, as plasmid replicons and genes can be matched together. As plasmids have been recognized as successful drivers of AMR, more studies combining short- and long-read sequencing are needed in order to sequence plasmids as a whole (Valcek et al., 2019) and to gain a deeper knowledge of the multifactorial epidemiology behind the spread of AMR.

Interestingly, no ESBL/AmpC-producing Enterobacteriaceae were obtained from fruit or seafood samples in our study. It is noteworthy, however, that sample sizes were limited. In another study conducted in Spain, raw fish products and sushi were found to have a prevalence of ESBL-producing Enterobacteriaceae of 10.6 and 19.4%, respectively (Vitas et al., 2018). Local fish products in Vietnam have been found to have an ESBL-producing *E. coli* prevalence of 62.5% with a high level of multidrug resistance (Le et al., 2015). Other AMR bacteria have also been recovered from seafood products in previous studies (Boss et al., 2016; Ellis-Iversen et al., 2019). In our study, seafood samples consisted largely of frozen or precooked products, which may, together with limited sample size, explain the absence of ESBL producers. Fortunately, no cross contamination with ESBL/AmpC-producing Enterobacteriaceae was found in cooked meat products, as these products are often eaten without additional heating, posing a greater risk of transmission of bacteria. The finding is in line with an earlier study conducted in Spain (Vitas et al., 2018) but not in line with a study by Jiang et al. (2014), which found a 6.7% prevalence of ESBL producers in *E. coli* isolates recovered from cooked meat products in China. $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-9}}$, and $bla_{\text{TEM-1}}$ were identified in the latter study, which may point to inadequate hygiene measures after heat processing or cross contamination, in which bacteria are introduced to products after heat processing. We did not recover any carbapenemase producing Enterobacteriaceae (CPE) in our study, although CPE have been recovered previously from meat, vegetable, and seafood products of Southeast Asian origin (Zurfluh et al., 2015a; Janecko et al., 2016; Sugawara et al., 2019). However, the selection with cefotaxime in the pre-enrichment of samples in our study might cause carbapenemase producing strains to be missed.

Earlier studies investigating the presence of ESBL/AmpC-producing bacteria in food products have partly differing results. A German study found only one out of 399 vegetable samples to be positive for cefotaxime-resistant *E. coli* (Kaesbohrer et al., 2019) but found cefotaxime-resistant *E. coli* in 74.9% of chicken meat and 40.1% of turkey meat. This is in line with our results, pointing to a higher prevalence of resistant Enterobacteriaceae in broiler meat and limited prevalence in vegetables. However, a Swiss study found 25.4% of vegetables imported from the Dominican Republic, India, Thailand, and Vietnam positive for ESBL-producing Enterobacteriaceae (Zurfluh et al., 2015b). This is remarkably higher than in our study with 5% of vegetable samples being positive for ESBL/AmpC-producing Enterobacteriaceae, although our study included samples from around the world, not only Asia and South America. In these continents, however, wastewater of human origin is commonly used for agriculture (Jiménez, 2006; Raschid-Sally and Jayakody, 2008). Using contaminated irrigation water may serve as a direct link to AMR contamination, especially if the water is applied to edible parts of fruits and vegetables (FAO and WHO, 2019). Antimicrobial-resistant bacteria have also been recovered from frozen products, vegetables, and ready-to-eat products from Chinese retail food (Ye et al., 2018). Ready-to-eat vegetables, in particular sprouts, have been previously recognized as a possible source of ESBL/AmpC-producing *E. coli* and *K. pneumoniae* (Kim et al., 2015).

Although food is not considered as the main source of human ESBL infections (Belmar Campos et al., 2014; Carmo et al., 2014; Börjesson et al., 2016; Day et al., 2019), similar ESBL strains

have been occasionally found from human infections and foods (Irrgang et al., 2017; Yamaji et al., 2018; Day et al., 2019; Roer et al., 2019; Valcek et al., 2019). In a recent population-based modeling study, 18.9% of human ESBL/AmpC-*E. coli* carriage was attributed to a food source (Mughini-Gras et al., 2019), highlighting the importance of AMR surveillance and proper hygiene measures for food products. Our results also show highly similar plasmids have been previously identified from human samples, indicating a possible transmission route of ESBL-harboring plasmids via food sources.

Antimicrobial resistance is a global problem with an uneven distribution around the globe and an accumulated burden especially in low-income countries (Alsan et al., 2015; Seale et al., 2017; Hendriksen et al., 2019). Although the burden of AMR is not equal in all countries, globalization, traffic of people, animals, and food enables resistant bacteria to disseminate around the world. To track the spread of AMR, it is of vital importance to execute AMR surveillance programs and to study different food products from various food categories in order to detect changes in the distribution of resistant bacteria, genes, and plasmids. As plasmids have been recognized as important drivers of AMR, efforts should be put into developing rapid WGS pipelines able to accurately detect plasmids carrying antimicrobial resistance genes of high importance.

CONCLUSION

Food products from different food categories around the world contain a wide variety of bacterial species resistant to third-generation cephalosporins among other critically important antimicrobials, with poultry meat serving as a rich reservoir of ESBL/AmpC-producing *E. coli* and *K. pneumoniae*. Although raw poultry meat was identified as the most common source of ESBL/AmpC-producing Enterobacteriaceae, the sporadic finding of resistant bacteria from fresh vegetable samples highlights the need for diverse One Health surveillance of AMR from multiple sources. Further studies should focus on identifying plasmids, resistance and virulence genes, and bacterial STs in food products relevant for human infections and food safety. The finding of high similarity between food-derived plasmids carrying *bla* genes with plasmids recovered previously from various sources globally highlights the risks posed by international trade, such as food products.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

PK, AH, MA, SN, EV, and A-LM contributed to the concept and design of the study. PK collected samples. PK and SN contributed to bacterial laboratory analysis. BK, MB, PK, and AH analyzed whole genome sequence data. MB and PK performed long-read sequencing. BK, PK, and MB performed subsequent analysis. BK and PK made visualizations for plasmid comparisons. PK and AH drafted the manuscript. MB, MA, and SN revised the manuscript. All authors have read and approved the final draft of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.592291/full#supplementary-material

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Dissemination of *bla*$_{NDM-1}$ Gene Among Several *Klebsiella pneumoniae* Sequence Types in Mexico Associated With Horizontal Transfer Mediated by IncF-Like Plasmids

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Nosocomial infections caused by multidrug-resistant (MDR) *Klebsiella pneumoniae* are a major health problem worldwide. The aim of this study was to describe NDM-1-producing *K. pneumoniae* strains causing bacteremia in a tertiary referral hospital in Mexico. MDR *K. pneumoniae* isolates were screened by polymerase chain reaction for the presence of resistance genes. In resistant isolates, plasmids were identified and conjugation assays were performed. Clonal diversity and the sequence types were determined by pulsed-field gel electrophoresis and multilocus sequence typing. A total of 80 *K. pneumoniae* isolates were collected from patients with bacteremia over a 1-year period. These isolates showed a level of resistance of 59% (47/80) to aztreonam, 56–60% (45–48/80) to cephalosporins, 54% (43/80) to colistin and 12.5% (10/80) to carbapenems. The carbapenem resistant isolates were *bla*$_{NDM-1}$ carriers and negative for *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$ and *bla*$_{OXA-48-like}$ carbapenemases genes. Conjugative plasmids IncFIIA and IncF group with sizes of 82–195 kbp were carriers of *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *aac(6′)-Ib* and/or *aac(3′)-IIa*. Clonal variability and nine different multilocus sequence types were detected (ST661, ST683, ST1395, ST2706, ST252, ST1198, ST690, ST1535, and ST3368) for the first time in the isolates carrying *bla*$_{NDM-1}$ in Mexico. This study demonstrates that *bla*$_{NDM-1}$ has remained within this hospital in recent years and suggests that it is currently the most prevalent carbapenemase among *K. pneumoniae* MDR strains causing bacteremia in Mexico. The horizontal transfer of *bla*$_{NDM-1}$ gene through IncF-like plasmids among different clones demonstrates the dissemination pathway of antimicrobial resistance

and underscore the need for strong and urgent joint measures to control the spread of NDM-1 carbapenemase in the hospital.

Keywords: *Klebsiella pneumoniae*, *bla*$_{NDM-1}$, IncF-like plasmids, carbapenems, MLST, Mexico

INTRODUCTION

Klebsiella pneumoniae (*K. pneumoniae*) is the causative agent of community and hospital acquired infections (Ramirez et al., 2019). In recent years, this bacterium has acquired high resistance to broad-spectrum antibiotics such as β-lactams, aminoglycosides, and quinolones (Ferreira et al., 2019). At the present time, the spread of carbapenemase-producing *K. pneumoniae* is a global public health concern (Villa et al., 2017). *Klebsiella pneumoniae* carbapenemase (KPC) was first reported in North Carolina in 2001. In the last decade it has disseminated globally due to the clonal spread of KPC-producing *K. pneumoniae* and in some countries its nosocomial dissemination has caused outbreaks (Martin et al., 2017). In Mexico, the first report of KPC-3-producing *K. pneumoniae* causing an outbreak was in 2013 (Rodríguez-Zulueta et al., 2013). Two subsequent works have reported the presence of a small number of strains of *K. pneumoniae* producing this carbapenemase (Bocanegra-Ibarias et al., 2017; Aquino-Andrade et al., 2018). One of the carbapenemases initially described in *K. pneumoniae* is New Delhi metallo-β-lactamase 1 (NDM-1), the dissemination of which is mostly hospital associated (Politi et al., 2019). The prevalence of carbapenemases in *K. pneumoniae* has been little studied in Mexico, however, recent research has demonstrated that NDM-1 carbapenemase is more frequent than that of KPC (Rodríguez-Zulueta et al., 2013; Bocanegra-Ibarias et al., 2017; Aquino-Andrade et al., 2018; Alcántar-Curiel et al., 2019b), which has been reported as endemic in the United States, Brazil, Argentina, Colombia and sporadically in Canada (Lee et al., 2016; Hammoudi Halat and Ayoub Moubareck, 2020).

Although *bla*$_{NDM-1}$ has been found on the bacterial chromosome, the vast majority is carried on plasmids (Wu et al., 2019). Currently there are 20 different incompatibility groups (Inc) of *bla*$_{NDM-1}$ carrying plasmids in *Enterobacteriaceae*, including IncA/C, IncFIA, IncFIB, IncFII and IncX3 (Wu et al., 2019), indicating the different possibilities of acquisition of *bla*$_{NDM-1}$ and the horizontal spread between bacteria of the same or different species.

In addition to this phenomenon, some *K. pneumoniae* carbapenemase producers are defined as high-risk clones because of their ability to colonize, spread and persist (Pitout et al., 2015). The multilocus sequence types (ST) ST258, and ST11, both belonging to the clonal complex (CC) 258, are prototypes of an epidemic clone which was identified as early 2000s and are currently spread around the world (Pitout et al., 2015; Lee et al., 2016).

The aims of this study were to investigate antimicrobial resistant genes, the plasmids associated with horizontal gene transfer and to determine the expansion of multilocus sequence types in *K. pneumoniae* causing bacteremia in a tertiary referral hospital in Mexico.

MATERIALS AND METHODS

Bacterial Isolation

Non-duplicate isolates of *K. pneumoniae* were consecutively collected from all blood cultures of patients with nosocomial bacteremia identified from January to December 2017 at Hospital Civil de Guadalajara, an 899-bed tertiary-care teaching hospital in Guadalajara, Jalisco, Mexico. The hospital infrastructure is made up of two buildings, the old, the architecture of the building is mixed, horizontal in its old area and a vertical tower of specialties with ten levels, also there is a building for the Care of Neonates and Women, an outpatient tower, the ophthalmology Unit and a Geriatric Care Unit.

Nosocomial infections were defined according to criteria published by the Centers for Disease Control and by Infectious Diseases Unit physicians (Horan et al., 2008). The isolates were stored in Luria Bertani (LB) broth (Difco, BD Biosciences, Franklin Lakes, NJ, United States) with 20% glycerol (Sigma-Aldrich, St. Louis, MO, United States) at −70°C.

Antimicrobial Susceptibility Testing

Identification and antimicrobial susceptibility against piperacillin–tazobactam, aztreonam, cefazolin, cefepime, ceftriaxone, ceftolozane–tazobactam, imipenem, meropenem, ciprofloxacin, amikacin, gentamycin, tobramycin, nitrofurantoin, tigecycline and trimethoprim-sulfamethoxazole were performed using the Vitek® 2 system (BioMérieux Durham, NC, United States). Minimal inhibitory concentrations (MIC) of colistin were determined by a microdilution method following the guidelines of the Clinical and Laboratory Standard Institute (CLSI, 2018). The production of extended spectrum β-lactamases (ESBLs) was confirmed phenotypically in all isolates resistant to penicillin/tazobactam and cephalosporins using the agar diffusion method (CLSI, 2018). Metallo-β-lactamases (MBLs) production in carbapenem-resistant isolates was determined by the diffusion test on agar using meropenem and imipenem sensidisks with or without 0.5 M EDTA and in combination with 400 μg/mL of phenylboronic acid for the presumptive identification of carbapenemase KPC (Alcántar-Curiel et al., 2019a).

Detection of Resistance Genes

Genes that encode antimicrobial resistance were detected by polymerase chain reaction (PCR) assay described previously (Alcántar-Curiel et al., 2019b). The presence of carbapenemase genes *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$ and *bla*$_{OXA-48-like}$ was determined by multiplex PCR (Poirel et al., 2011). Endpoint PCR was performed to detect *bla*$_{TEM}$, *bla*$_{CTX-M}$. Additional genes of aminoglycoside modifying enzymes (AMEs) genes *aac(3′)-Ia*, *aac(6′)-IIb*, the methyltransferases genes *rmtB* and *armA* and the colistin resistance *mcr-1* gene (Liu et al., 2016)

were included in order to further characterize the strains. The specific oligonucleotides used are described in **Supplementary Table 1**. The amplified fragments were purified using the Zymogen Purification Kit (Promega) and sequenced (Instituto de Biotecnología, Universidad Nacional Autónoma de México). The sequence analysis was performed with the BioEdit and Kaling bioinformatics tools to subsequently undergo a Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information database[1].

Plasmid Analysis and Conjugation Assays

Plasmids profile was obtained from isolates carrying ESBLs or MBLs according to Eckhardt technique (Eckhardt, 1978). Horizontal transfer of antibiotic resistant was confirmed by bacterial conjugation with *Escherichia coli* J53-2 as the recipient strain using Miller method (Miller, 1992). Transconjugants were selected on McConkey agar supplemented with rifampicin (200 μg/mL) plus ceftazidime (16 μg/mL) and plus meropenem (16 μg/mL) for isolates that were carrying carbapenemases genes and tested for antimicrobial susceptibility. Successful conjugation was confirmed by specific PCR amplification and the electrophoretic pattern of the conjugated plasmids was obtained. The bacterial artificial chromosomes (BACs) of 67, 86, 101, 122, 145, and 195 kb were used as a molecular weight markers (González et al., 2006). Plasmids of transconjugant strains were purified using the QIAGEN Plasmid Midi Kit (Qiagen, Hilden, Germany), following the manufacturer's specifications. Plasmids diversity was determined by restriction fragment length polymorphism (RFLP) (Ho et al., 2012) with *Eco*RI and *Hin*dIII (Invitrogen) restriction enzymes following the manufacturer's specifications. Finally, the groups Inc of conjugative plasmids were determined by PCR-based replicon typing (Carattoli et al., 2005).

Genotyping by Pulsed-Field Gel Electrophoresis

Clonality among all of the isolates was determined by pulsed-field gel electrophoresis (PFGE) (Alcántar-Curiel et al., 2019a). Chromosomal DNA of each isolate was prepared as described previously (Miranda et al., 1996) and macrorestricted with the restriction endonuclease *Xba*I (New England Biolabs, Beverly, MA, United States). Restriction fragments were resolved in a Gene Path System (BioRad®, Hercules, CA, United States). The classification of the isolates in clones was based on Tenover criteria (Tenover et al., 1995). The percentage of similarity profile was calculated using the Dice coefficient. Isolates with a Dice similarity coefficient >85% were considered as members of the same clone (Alcántar-Curiel et al., 2019a).

Multilocus Sequence Typing

To determine the sequence type (ST) of *K. pneumoniae* isolates harboring bla_{NDM-1}, MLST was performed according to the Pasteur scheme (Diancourt et al., 2005). The housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, *tonB*) were amplified, sequenced, and analyzed with the MLST database of the Pasteur Institute to identify allelic profile. In order to identify the clonal complex (CC) and visualize evolutionary relationships among isolates carrying bla_{NDM-1}, we used Phyloviz 2.0 program that generates eBURST and neighbor-joining diagram (Sepp et al., 2019).

[1]www.ncbi.nlm.nih.gov/blast/

RESULTS

Clinical Isolates and Antibiotic Susceptibility Pattern

A total of 80 isolates of *K. pneumoniae* causing bacteremia were collected over the course of 1 year at the Hospital Civil de Guadalajara. These isolates representing 8% of the total documented bacteremias. *K. pneumoniae* were more frequently derived in patients from surgical ward (32.5%) and medicine ward (31.2%) (**Table 1** and **Supplementary Data Sheet 1**). The isolates were resistant to penicillin/tazobactam 26% (21/80), aztreonam 59% (47/80), cefepime 56% (45/80), ceftriaxone 60% (48/80), imipenem and meropenem 12.5% (10/80), ciprofloxacin 18% (14/80), tobramycin 50% (40/80), gentamicin 55% (44/80), nitrofurantoin 16% (13/80), tigecycline 5% (4/80), trimethoprim-sulfamethoxazole 56% (45/80) and colistin 53% (42/80) (**Table 2**).

Antibiotic Resistance Enzymes

A total of 10 strains resistant to all β-lactams including the two carbapenems were detected. These strains were MBL producers and carried the bla_{NDM-1} carbapenemase gene (**Supplementary Data Sheet 1**). Furthermore, three of these strains carried the bla_{TEM-1} penicillinase gene and four strains bla_{TEM-1} and

TABLE 1 | Frequency of bla_{NDM-1}-producing *Klebsiella pneumoniae* isolates in the different hospital setting.

Hospital wards	Area	No. isolates (%)
Surgical		26 (32.5)
Neurology (NEU)	Old	13 (16)
General surgery (GES)	Old	10 (12.5)
Plastic surgery (PSU)	Old	1 (3.8)
Oral and maxillofacial surgery (OMS)	Old	1 (1.3)
Otorhinolaryngology (OTO)	Old	1 (3.8)
Medicine		25 (31.2)
Internal medicine (IME)	Old	7 (8.4)
Cardiology (CAR)	Old	5 (6.3)
Nephrology (NEP)	Tower 5 floor	5 (6.3)
Haematology (HEM)	Tower 9 floor	3 (3.8)
Infectious diseases unit (IDU)	Tower 7 floor	3 (3.8)
Gastroenterology (GAS)	Tower 6 floor	1 (1.3)
HIV/AIDS Unit (HIV)		1 (1.3)
Pediatric intensive care unit		20 (25)
Neonatal intensive care unit (NICU)	Newborn unit	13 (16.2)
Pediatric intensive care unit (PICU)	Tower 1 floor	7 (8.7)
Intensive Care Unit (ICU)	Tower 1 floor	9 (11.2)
Total		80 (100)

TABLE 2 | Minimum inhibitory concentration data and antimicrobial susceptibility of 80 *Klebsiella pneumoniae* isolates from January to December 2017 at Hospital Civil de Guadalajara.

Drug class	Antimicrobial agent	MIC* (μg/mL)		Antimicrobial susceptibility (%)		
		MIC_{90}	MIC_{50}	Susceptible	Intermediate	Resistant
β-lactam combination agents	Piperacillin/Tazobactam	128	16	65	9	26
Monobactam	Aztreonam	64	2	36	5	59
Cephems	Cefazolin	64	8	41	0	59
	Cefepime	64	2	44	0	56
	ceftriaxone	64	32	40	0	60
β-lactam combination agents	Ceftolozane/Tazobactam**	N/A	N/A	87	0	13
Carbapenems	Imipenem	8	0.06	87	0	13
	Meropenem	4	0.03	87	0	13
Fluoroquinolones	Ciprofloxacin	4	1	68	14	18
Aminoglycosides	Amikacin	128	8	56	0	24
	Gentamicin	128	32	45	0	55
	Tobramycin	32	1	50	0	50
Nitrofurans	Nitrofurantoin	128	64	26	58	16
Glycylcycline	Tigecycline***	2	1	91	4	5
Folate pathway antagonists	Trimethoprim/Sulfamethoxazole	16/304	16/304	43	1	56
Lipopeptides	Colistin***	32	4	47	0	54

**Susceptibility breakpoint categories were derived from CLSI (2018).*
***Susceptibility was determined by agar disk diffusion method.*
****Susceptibility categorization was determined according to the EUCAST criteria.*
N/A, Not applicable.

$bla_{CTX-M-15}$ (**Supplementary Figure 1** and **Supplementary Data Sheet 1**). Regarding to the 37 strains resistant to β-lactams but susceptible to carbapenems, 89% (33/37) were ESBL producers; 32 strains carried bla_{TEM-1} and $bla_{CTX-M-15}$ genes and one strain carried only $bla_{CTX-M-15}$ (**Supplementary Figure 1** and **Supplementary Data Sheet 1**).

In relation to the 55 isolates resistant to aminoglycosides tested, 56% (31/55) were carriers of *aac(3′)-IIa* and *aac(6′)-IIb* AME genes. AMEs genes were not detected in five isolates resistant only to amikacin and six isolates resistant to amikacin and gentamicin. *aac(6′)-Ib* was associated with resistance to tobramycin, while *aac(3′)-IIa* was associated with resistance to gentamicin (**Supplementary Table 2**). Regarding the *mcr-1* gene was not detected in 43 colistin-resistant isolates examined in this study.

Plasmid Analysis

Conjugation experiments in ten isolates bla_{NDM-1} carriers showed that five transconjugants acquired the bla_{NDM-1} gene (**Table 3**). Plasmid analysis indicated that one transconjugant harbored the bla_{NDM-1} gene on a ∼82 kbp plasmid. The other four transconjugants harbored the bla_{NDM-1} gene on a ∼195 kbp plasmid, only two of these plasmids were carriers of a single bla_{NDM-1} resistance gene, the other plasmid was a carrier of both bla_{NDM-1} and bla_{TEM-1} genes and the fourth plasmid was a carrier of bla_{NDM-1}, bla_{TEM-1}, $bla_{CTX-M-15}$ and *aac(3′)-IIa* and *aac(6′)-Ib*. All the five transconjugants were resistant to all β-lactams and aminoglycosides with the exception of one. Plasmid replicon typing showed that four of the conjugative plasmids belonged to the IncFIIA and one to the IncF group.

With respect to thirty-three bla_{TEM-1} and $bla_{CTX-M-15}$ carriers, 26 transconjugants were obtained (**Supplementary Table 3**), of which 13 harbored a >195 kbp plasmid, 12 carried a ∼195 kbp plasmid and only one acquired a ∼67 kbp plasmid. From the total 26 conjugative plasmids, 25 of them harbored the $bla_{CTX-M-15}$, bla_{TEM-1}, *aac*(3′)-*IIa* and *aac*(6′)-*Ib*, and only one of them harbored the $bla_{CTX-M-15}$, bla_{TEM-1} and *aac*(6′)-*IIa*. Twenty-five of these plasmids belonged to the IncF group and only one plasmid to the IncFIIA group.

Fragment length polymorphism (RFLP) analysis of the conjugative plasmids carrying both bla_{NDM-1} and $bla_{CTX-M-15}$ showed an average similarity of 88% (**Figure 1**). The five plasmids carriers bla_{NDM-1} revealed two different restriction profiles (P), four of them belonged to P8. The 26 plasmids carriers of bla_{TEM-1} and $bla_{CTX-M-15}$ belonged to eight different restriction profiles.

Clonality Analysis

Pulsed-field gel electrophoresis (PFGE) analysis was conducted with an average similarity of 66%. Sixty-nine different clones were detected among the 80 isolates, which showed clonal heterogeneity (data not shown). Clone 26 was the most prevalent with three isolates collected in May and June, all carriers of $bla_{CTX-M-15}$, bla_{TEM-1}, *aac(3′)-Ia* and *aac(6′)-IIb*. Clones 6, 15, 34, 35, 37, 39, 42, and 47 had two isolates each, while the rest of the isolates belonged to different clones (**Supplementary Data Sheet 1**). The 43 strains of *K. pneumoniae* carrying resistance genes belonged to 38 different clones, the 10 isolates carriers of bla_{NDM-1} belonged to different clones (**Supplementary Figure 1**).

TABLE 3 | Antimicrobial susceptibility of 5 *K. pneumoniae* carriers bla_{NDM-1} and their transconjugants.

Isolate	Multiresistant pattern	Resistant genes	Plasmids pattern		Transconjugant	Acquired multi-resistance profile	Acquired resistant genes	Conjugative plasmid size (kbp)
			No.	Size (kbp)				
12-Kpn	PTZ, AZT, CFZ, FEP, CRO, CAZ, C/T, IMI, MEM, CIP, TOB, NIT, SXT, COL	bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{TEM-1}	3	>195 195 82	12-Tc	PTZ, AZT, CFZ, FEP, CRO, CAZ, C/T, IMI, MEM	bla_{NDM-1} $bla_{CTX-M-15}$	82
18-Kpn	PTZ, AZT, CFZ, FEP, CRO, CAZ, C/T, IMI, MEM, AMK, GEN, TOB, NIT, COL	bla_{NDM-1}, bla_{TEM-1}	1	195	18-Tc	PTZ, AZT, CFZ, FEP, CRO, CAZ, C/T, IMI, MEM, AMK, GEN, TOB	bla_{NDM-1}, bla_{TEM-1}	195
19-Kpn	PTZ, AZT, CFZ, FEP, CRO, CAZ, C/T, IMI, MEM, GEN, TOB, NIT, CIP, COL	bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{TEM-1}, *aac(6')-Ib*, *aac(3')-IIa*	2	>195 195	19-Tc	PTZ, AZT, CFZ, FEP, CRO, CAZ, C/T, IMI, MEM, GEN, TOB, CIP	bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{TEM-1}, *aac(6')-Ib*, *aac(3')-IIa*	195
40-Kpn	PTZ, AZT, CFZ, FEP, CRO, CAZ, C/T, IMI, MEM, AMK, GEN, TOB, NIT, TGC, SXT	bla_{NDM-1}, bla_{TEM-1}	1	195	40-Tc	PTZ, AZT, CFZ, FEP, CRO, CAZ, C/T, IMI, MEM, AMK, GEN, TOB	bla_{NDM-1}	195
41-Kpn	PTZ, AZT, CFZ, FEP, CRO, CAZ, C/T, IMI, MEM, AMK, GEN, TOB, NIT, TGC, SXT, COL	bla_{NDM-1}, bla_{TEM-1}	1	195	41-Tc	PTZ, AZT, CFZ, FEP, CRO, CAZ, C/T, IMI, MEM, AMK, GEN, TOB	bla_{NDM-1}	195

PTZ, piperacillin-tazobactam; AZT, Aztreonam; CAZ, ceftazidime; CRO, ceftriaxone; C/T, ceftolozane-tazobactam; CFZ, cefazolin; FEP, cefepime; IMP, imipenem; MEM, meropenem; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; NIT, nitrofurantoin; TGC, tigecycline; SXT, trimethoprim-sulfamethoxazole; COL, colistin.

MLST Analysis

The analysis demonstrated nine different STs among ten isolates carriers of bla_{NDM-1} gene; ST661, ST683 belonged to CC258, ST1395, ST2706, ST252, ST1198, ST690, ST1535, and ST3368 (**Supplementary Figure 2**). Isolates 07-KP-17, and 11-KP-17 belonged to ST661 which corresponds to the founder member of CC661, these isolates were recovered in February and March 2017 respectively.

Phylogenetic analysis using the neighbor-joining method detected the genetic distance between the 10 isolates carriers of bla_{NDM-1} gene (**Figure 2**). Isolates recovered in February and March as well as the isolates recovered between April and June were significantly associated, while the October isolates including the 69-KP-17 isolate were not.

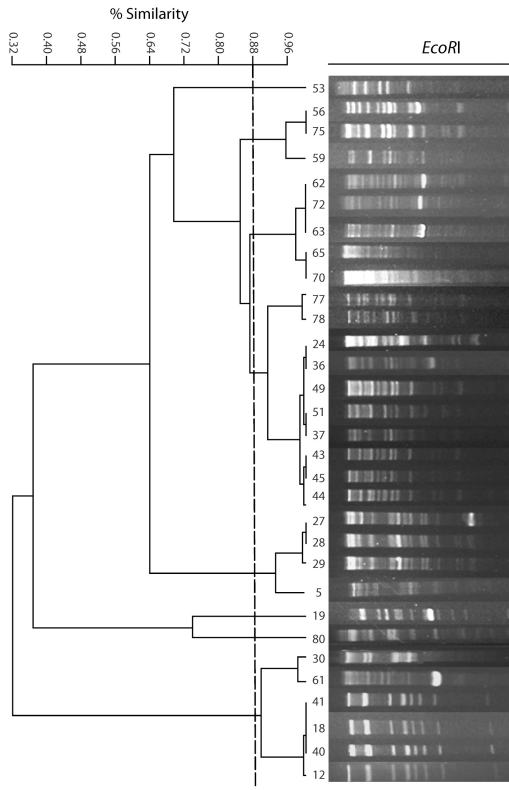
DISCUSSION

Klebsiella pneumoniae is included in the global priority list of antibiotic resistant bacteria, and there is a need for enhanced *K. pneumoniae* surveillance to rapidly identify and monitor convergent strains and/or plasmids (Wyres et al., 2020). The infections caused by *K. pneumoniae* during the present study period were 8%, lower than those reported for developing countries ranging from 16 to 28% (Khaertynov et al., 2018).

The rate of antimicrobial resistance observed in the isolates from our study was lower than the rate from the isolates that caused nosocomial outbreaks previously reported in the same hospital (Bocanegra-Ibarias et al., 2017). While this was a short study period, our findings are consistent with the high rate of resistance reported in Asia where they detected 60–75.8% to cephalosporins, 47–65.6% to imipenem and 40.8–76% to amikacin (Effah et al., 2020). Detection of 54% colistin resistance in this study is undoubtedly our biggest concern, a significant increase compared to the 4.7% reported 2 years earlier in this hospital (Bocanegra-Ibarias et al., 2017). This may be due to the increased use of colistin for the treatment of bacteremia caused by carbapenemase-producing isolates in hospital and because the isolates from the previous study were mostly derived from a nosocomial outbreak (Bocanegra-Ibarias et al., 2017).

The spread of NDM-producing bacteria and their association with nosocomial outbreaks is of concern worldwide (Dortet et al., 2014). The first report of NDM in *K. pneumoniae* was described in Mexico in 2014 (Barrios et al., 2014). The detection of bla_{NDM-1} in ten isolates included in this study, demonstrates the spread and persistence of this carbapenemase among *K. pneumoniae* isolates for at least two consecutive years at Hospital Civil de Guadalajara (Bocanegra-Ibarias et al., 2017). Additionally, the co-transfer of bla_{NDM-1} and AMEs genes together with fluoroquinolone resistance in 19-Kpn isolate (**Table 3**) demonstrates the concurrence of these genes, which represents a major challenge in the treatment of patients (Mitra et al., 2019).

It has been documented that in *K. pneumoniae* these resistance genes are encoded in small 25 kbp conjugative plasmids or smaller (Ramirez et al., 2019), which contrasts with the >195 kbp conjugative plasmids identified in this



Transconjugant	Size (kbp)	Restriction pattern	Inc group	N	C	T	A6	A3
53 Tc	195	P1	Inc F					
56 Tc	>195	P2	Inc F					
75 Tc	195	P2	Inc F					
59 Tc	>195	P2	Inc F					
62 Tc	>195	P3	Inc F					
72 Tc	>195	P3	Inc F					
63 Tc	>195	P3	Inc F					
65 Tc	>195	P3	Inc F IIA					
70 Tc	195	P3	Inc F					
77 Tc	195	P4	Inc F					
78 Tc	195	P4	Inc F					
24 Tc	195	P4	Inc F					
36 Tc	>195	P4	Inc F					
49 Tc	195	P4	Inc F					
51 Tc	>195	P4	Inc F					
37 Tc	>195	P4	Inc F					
43 Tc	195	P4	Inc F					
45 Tc	195	P4	Inc F					
44 Tc	195	P4	Inc F					
27 Tc	>195	P5	Inc F					
28 Tc	>195	P5	Inc F					
29 Tc	>195	P5	Inc F					
05 Tc	>195	P5	Inc F					
19 Tc	195	P6	Inc F					
80 Tc	195	P7	Inc F					
30 Tc	67	P8	Inc F					
61 Tc	195	P8	Inc F					
41 Tc	195	P8	Inc FIIA					
18 Tc	195	P8	Inc FIIA					
40 Tc	195	P8	Inc FIIA					
12 Tc	82	P8	Inc F					

FIGURE 1 | Restriction fragment length polymorphism analysis of conjugative plasmids carrying antimicrobial resistance genes. N: bla_{NDM-1}, C: $bla_{CTX-M-15}$, T: bla_{TEM-1}, A6: *aac*(6′)-*Ib*, A3: *aac*(3′)-*IIa*.

study (**Figure 1**). RFLP analysis showed that isolates in our study harbor different types of plasmids carrying bla_{NDM-1}, ESBLs and AMEs, suggesting that genetic rearrangements occurred at the plasmid level during this period of study. Furthermore, similar plasmids were detected among different clones, which indicates that the transfer of genes is common among bacteria allowing the spread of resistance genes in the hospital environment.

The bla_{NDM-1} gene has been identified in plasmids from different replicon types, in this study the plasmid carrying bla_{NDM-1} gene belongs to the IncF and IncFIIA subgroups; which are different from the IncFIIk and IncFIIy subgroups previously reported in this hospital (Bocanegra-Ibarias et al., 2017) and the IncFIA reported in isolates in Mexico City (Alcántar-Curiel et al., 2019a).

The prevalence of plasmid replicons is led by the IncX group, while the IncF group ranks third, worldwide (Partridge et al., 2018; Wu et al., 2019). However, in Latin America, particularly in Mexico, Brazil, and Colombia, the IncF group seems be the most prevalent (Torres-González et al., 2015; Bocanegra-Ibarias et al., 2017; Alcántar-Curiel et al., 2019b; Wu et al., 2019).

In this study, gentamicin and tobramycin resistance was associated with the production of AAC(3′)-IIa and AAC(6′)-Ib, but not for amikacin resistance since the resistant isolates did not carry these genes studied (**Supplementary Table 2**). The five transconjugants of the isolates that produced ESBLs and were resistant to the 3 aminoglycosides acquired both AMEs but only had resistance to gentamicin and tobramycin (**Table 2**), suggests that amikacin resistance may be due to another AME or another resistance mechanism not encoded in plasmids. Because none of the studied AMEs were detected in transconjugants carriers bla_{NDM-1} that acquired resistance to aminoglycosides, we consider that these isolates may carry others AMEs that were not investigated.

One of the most prevalent genes in *Enterobacteriaceae*, *Pseudomonadales* and *Vibrionaceace* worldwide is *aac(6′)-Ib* (Ramirez and Tolmasky, 2017; Fernández-Martínez et al., 2018; Galani et al., 2019), which is mostly associated with amikacin and gentamicin resistance (Ramirez and Tolmasky, 2017; Ramirez et al., 2019) and frequently encoded in plasmids and coexisting with ESBLs such as CTX-M. Our results are in agreement with these data with the exception that *aac(6′)-Ib* seems to be associated with tobramycin resistance since it was detected in isolates resistant only to this antibiotic. The observation that *aac(3′)-IIa* is more frequent in *Enterobacteriaceae* and is associated with gentamicin and tobramycin resistance (Fernández-Martínez et al., 2018) is in agreement with our results.

Colistin resistance was not transferred by conjugation and this was supported by the fact that none of the isolates carried the mcr-1 gene, suggesting that resistance may be due to be associated with chromosomal mutations that are directly involved in LPS

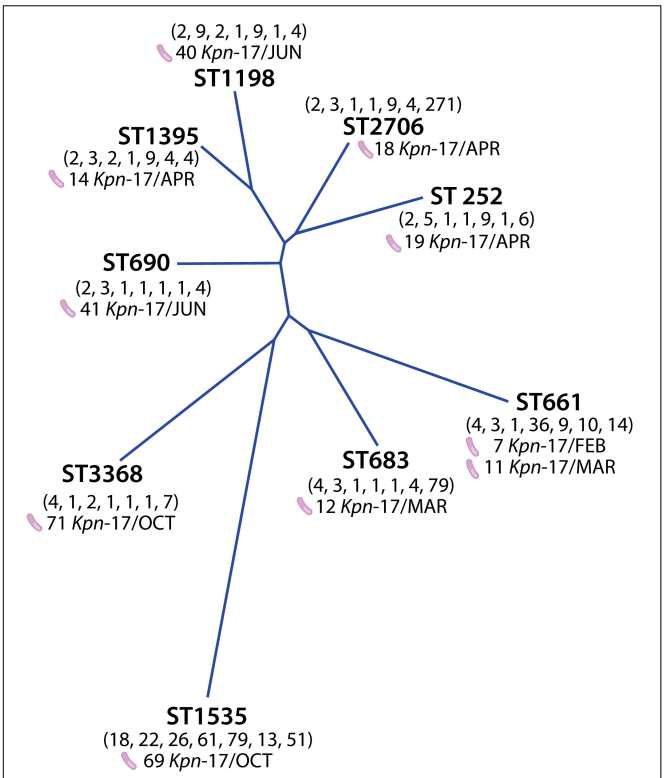


FIGURE 2 | Phylogenetic analysis of STs obtained for bla_{NDM-1}-producing *K. pneumoniae* strains using the neighbor-joining method.

modifications such alteration in the MgrB gene, a very common colistin resistance mechanism in *K. pneumoniae* from the clinical setting (Luo et al., 2017).

Throughout our study, it was interesting to find that isolates showed a wide clonal diversity, including the carbapenem-resistant isolates which carried the bla_{NDM-1} gene that has been frequently associated with outbreaks by *K. pneumoniae* (Dortet et al., 2014). However, the plasmids bla_{NDM-1} carriers and other resistance genes were similar, their detection in different clones partially explains their dissemination in different clones, coinciding with previously report of bla_{NDM-1} carriers *Enterobacteriaceae* in this hospital (Bocanegra-Ibarias et al., 2017).

The 9 STs detected in the ten *K. pneumoniae* carriers of bla_{NDM-1} gene have not been previously described in Mexico (Barrios et al., 2014; Torres-González et al., 2015; Bocanegra-Ibarias et al., 2017, 2019; Garza-Ramos et al., 2018). However, the isolate belonging to the ST683 is related to clonal complex 258, an epidemic clone with a global expansion. This clone is prevalent in Argentina and includes multi-drug resistant microorganisms that are KPC-producing and have been associated with high mortality rates (Cejas et al., 2019). Finally, three STs detected in this study (ST661, ST690, ST252) have been previously reported in other regions of the world although none of these *K. pneumoniae* strains carriers the bla_{NDM-1} gene (Coelho et al., 2012; Papagiannitsis et al., 2015; Martin et al., 2017; Fu et al., 2018; Marques et al., 2019; Piazza et al., 2019; Sghaier et al., 2019; Mori et al., 2020).

CONCLUSION

This study shows the prevalence of *K. pneumoniae* MDR isolates causing bacteremia in a tertiary referral hospital in Mexico. The carbapenem-resistant isolates were carriers of the bla_{NDM-1} gene harbored in similar IncF-like plasmids among clones with different STs, which supports their nosocomial dissemination and persistence in different plasmids which can be associated with genetic rearrangements that might be in favor the microevolution of this nosocomial pathogen. These results underscore the importance of maintaining microbiological and epidemiological surveillance actions to detect and prevent the spread of MDR bacteria.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

This study was evaluated and approved by the Institutional Research and Ethics Committee of the Hospital Civil de Guadalajara project number HCG/CEI-009316; 27 January 2016. The study does not involve humans it is an *in vitro* study. Written informed consent was not required for this study according to the institutional ethical, biosecurity and investigation committees because the Hospital Clinical Laboratory provided every bacterial isolates included in this study.

AUTHOR CONTRIBUTIONS

JET-T conceived and designed the study and performed the experiments, analyzed the data, and wrote and edited the manuscript. CG-V, MDJ-Q, JLF-V, and JDC performed the experiments, analyzed the data, and revised the manuscript. RM-O, ER-N, SG-C, GG, and JS-P analyzed the data and revised the manuscript. MA-C conceived, designed, and supervised the study, analyzed the data, and wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.611274/full#supplementary-material

Supplementary Figure 1 | Genetic relationship and molecular characteristics of the 43 strains of *K. pneumoniae* carrying bla_{NDM-1} and other resistance genes.

Supplementary Figure 2 | Diagram of the different clonal complexes (CC) and the multilocus sequence types (STs) identified by eBURST in bla_{NDM-1}-producing *K. pneumoniae* isolates. The ST (red circles) and the CC (green circles) obtained for each strain 2B.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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On-Site Genomic Epidemiological Analysis of Antimicrobial-Resistant Bacteria in Cambodia With Portable Laboratory Equipment

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The rapid emergence of carbapenemase-producing gram-negative bacteria (CPGNB) is a global threat due to the high mortality of infection and limited treatment options. Although there have been many reports of CPGNB isolated from Southeast Asian countries, to date there has been no genetic analysis of CPGNB isolated from Cambodia. Sequence-based molecular epidemiological analysis enables a better understanding of the genotypic characteristics and epidemiological significance of antimicrobial-resistant (AMR) bacteria in each country, and allows countries to enact measures related to AMR issues. In this study, we performed on-site genomic epidemiological analysis of CPGNB isolated in Cambodia using a portable laboratory equipment called Bento Lab, which combines a PCR thermal cycler, microcentrifuge, gel electrophoresis apparatus, and LED transilluminator, along with the MinION nanopore sequencer. PCR targeting of major carbapenemase genes using Bento Lab revealed that two *Escherichia coli* isolates and one *Acinetobacter baumannii* isolate harbored carbapenemase genes: bla_{NDM}, $bla_{\mathrm{OXA-48}}$, and $bla_{\mathrm{OXA-23}}$, respectively. The results of phenotypic diagnostic tests for CPGNB, such as the carbapenem inactivation method and double-disk diffusion test using a specific inhibitor of metallo-β-lactamases, were consistent with their AMR genotypes. Whole-genome sequencing analysis using MinION revealed that $bla_{\mathrm{NDM-5}}$ gene was carried on a 93.9-kb plasmid with IncFIA/IncFIB/IncFII/IncQ1 replicons, and $bla_{\mathrm{OXA-181}}$ gene was carried on a 51.5-kb plasmid with the IncX3 replicon in *E. coli* isolates. $bla_{\mathrm{OXA-23}}$ was encoded in two locations on the chromosome of *A. baumannii*. Plasmids carrying $bla_{\mathrm{NDM-5}}$ or $bla_{\mathrm{OXA-181}}$ in *E. coli* were highly structurally identical to plasmids prevalent in Enterobacterales in China and other countries, suggesting that they disseminated from a common evolutionary origin. Our findings demonstrate the potential impact of portable laboratory equipment on AMR bacteria research in hospitals and research centers with limited research facilities, and provide the first glimpse into the genomic epidemiology of CPGNB in Cambodia.

Keywords: AMR, carbapenemase, CPE, Acinetobacter, Cambodia

INTRODUCTION

Antimicrobial-resistant (AMR) bacteria have emerged and spread all over the world. Among antimicrobials, carbapenems are one of the most reliable last-resort antimicrobials for infections caused by AMR gram-negative bacteria. One of the mechanisms of AMR is drug inactivation mediated by acquired enzymes, such as β-lactamases (Santajit and Indrawattana, 2016). Among β-lactamases, extended-spectrum β-lactamases (ESBLs) and carbapenem-hydrolyzing β-lactamase (carbapenemases) are clinically important, as ESBLs hydrolyze a broad range of β-lactams, including cephalosporins, and carbapenemases hydrolyze most β-lactams, including carbapenems. β-lactamases are classified using the Ambler scheme as follows. Ambler class A includes ESBLs, such as CTX-M, as well as carbapenemases, such as KPC; class B includes metallo-β-lactamases (MBLs), such as NDM, IMP, and VIM; class C includes AmpC β-lactamases; and class D includes carbapenem-hydrolyzing oxacillinases, of which OXA-48 is prevalent in Enterobacterales, and OXA-23, OXA-24, and OXA-58 are prevalent in *Acinetobacter* spp. ESBL and carbapenemase genes are predominantly encoded on conjugative plasmids and have been transferred among Enterobacterales and other gram-negative bacteria (Tzouvelekis et al., 2012). Moreover, carbapenemase-producing gram-negative bacteria (CPGNB) harboring carbapenemase genes often co-harbor clinically relevant antimicrobial resistance genes, such as aminoglycoside and fluoroquinolone resistance genes (Wu et al., 2007; Chmelnitsky et al., 2008; Poirel et al., 2011a). There is great concern about the global spread of plasmids that carry multiple AMR genes that can be transferred between homogeneous and heterogeneous species.

Although CPGNB has been detected in large numbers in Southeast Asia, the publicly available information on CPGNB is limited to a small number of countries (Malchione et al., 2019). In 2015, the World Health Organization (WHO) adopted a global action plan on AMR and launched the Global Antimicrobial Resistance Surveillance System (GLASS), the first global collaborative report to standardize AMR surveillance (WHO, 2015; Tornimbene et al., 2018). Cambodia's Laboratory Information System (CamLIS) was developed by the Ministry of Health in Cambodia with the support of WHO starting in 2011 (WHO, 2019). As of 2018, 35 national, provincial, and referral laboratories contribute to CamLIS. Cambodia has started to prepare the GLASS report for 2020, and the actual status of AMR bacteria in the country will be revealed in the near future. To date, however, only a few studies have examined AMR bacteria clinically isolated in Cambodia, although other Southeast Asian countries are increasingly reporting cases of AMR bacteria (Suwantarat and Carroll, 2016; Gandra et al., 2020). To date, there has been no report on the genomic epidemiology of CPGNB in Cambodia.

In this study, we introduced portable laboratory equipment, Bento Lab and MinION, for on-site genomic epidemiological analysis of CPGNB in Cambodia. Bento Lab (Bento Bioworks Ltd., United Kingdom) is a DNA analysis device small enough to fit in a laptop-sized bag. It contains a PCR thermal cycler, microcentrifuge, and gel electrophoresis apparatus with LED transilluminator, and has sufficient functionality for laboratory work (Bento Lab, 2016). The MinION nanopore sequencer (Oxford Nanopore Technologies, United Kingdom) is a portable long-read sequencer with the size of a large USB memory stick. MinION was utilized for on-site genomic epidemiological analysis of the Ebola virus outbreak in West Africa in 2016 (Quick et al., 2016) and the Zika virus outbreak in the Americas in 2017 (Faria et al., 2017). Because carbapenemase genes are mostly carried on plasmids, long-read sequencing is useful for assembling whole plasmid sequences and tracking horizontal transfer of AMR plasmids in hospitals, as well as local and global communities (Conlan et al., 2014).

We organized an international collaborative research group with researchers from Japan, United Kingdom, and Cambodia, and successfully performed on-site genomic epidemiological analysis of CPGNB clinical isolates in Cambodia, where access to laboratory equipment is limited. Our findings demonstrate the potential impact of portable laboratory equipment on AMR bacteria research and provide the first glimpse into the genomic epidemiology of CPGNB in Cambodia.

MATERIALS AND METHODS

Ethics

Written informed consent was obtained from the individuals for the publication of any potentially identifiable images included in this article.

Subjects and Specimen Collection

The outpatient clinic of National Institute of Public Health (NIPH) in Phnom Penh, Cambodia has around 10 patients in a day and 456 bacterial strains were isolated from patient specimens, such as sputum, stool, urine, pus, body fluid, and cerebral spinal fluid, in 2017. Ethical approval of this study "Genomic epidemiological analysis of AMR bacterial isolates in Cambodia" was obtained from National Ethics Committee for Health Research (NECHR), Cambodia (approval no.: 178NECHR). Two carbapenemase-producing isolates NIPH17_0020 and NIPH17_0036 of *Escherichia coli* and one carbapenemase-producing isolate NIPH17_0019 of *Acinetobacter baumannii* analyzed in this study were obtained from abdominal pus, urine, and blood of patients, respectively, at NIPH, Cambodia in 2017.

Bacterial Isolates

Bacterial species identification was performed using conventional biochemical tests (e.g., citrate test, urease test, hydrogen sulfide test, oxidase test, indole test, lysine decarboxylase test, and carbohydrate fermentation test) and the API 20E/20NE systems (bioMérieux), and antimicrobial susceptibility testing with *E. coli* ATCC 25922 as quality control was performed using BBL Sensi-Disc Susceptibility Test Disks (BD) as part of routine diagnosis at NIPH, Cambodia. Minimum inhibitory concentrations (MICs) of selected antimicrobials, including imipenem (IPM), meropenem (MEM), ceftazidime (CAZ),

cefotaxime (CTX), aztreonam (AZT), amikacin (AMK), and ciprofloxacin (CPFX), against carbapenemase-producing isolates of *E. coli* (NIPH17_0020 and NIPH17_0036) and *A. baumannii* (NIPH17_0019) were further examined using the E-test strips (bioMérieux) in this study. The breakpoints for susceptible (S), intermediate (I), and resistance (R) to antimicrobials were adopted from the Clinical and Laboratory Standards Institute (CLSI) 2020 guidelines. Carbapenemase production was examined using the carbapenem inactivation method (CIM) according to the CLSI guidelines also as routine diagnosis at NIPH, Cambodia. The double-disk diffusion tests (DDDTs) with clavulanate (CVA) and sodium mercaptoacetic acid (SMA) as specific inhibitors for extended spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs), respectively, were performed as previously described (Arakawa et al., 2000; Hattori et al., 2013; CLSI, 2015). Briefly, the production of ESBLs was tested with the combination of CAZ, CTX, and clavulanate/amoxicillin (CVA/AMPC) disks (Eiken Chemical Co.), and production of MBLs was tested with the combination of IPM and SMA disks (Eiken Chemical Co.).

PCR, Whole-Genome Sequencing, and Bioinformatics Analysis

Draft genome analysis of carbapenemase-producing isolates of *E. coli* (NIPH17_0020 and NIPH17_0036) and *A. baumannii* (NIPH17_0019) using Bento Lab and MinION was performed in NIPH, Cambodia in July, 2017. Bacterial genomic DNAs (gDNAs) were extracted using the MagAttract HMW DNA Kit (Qiagen) and quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific). Library preparation for MinION sequencing using Rapid Sequencing Kits (SQK-RAD002 and SQK-RAD003) (Oxford Nanopore Technologies) were performed using the prototype model of Bento Lab (Bento Bioworks Ltd.) consisting of a thermal cycler, microcentrifuge, and gel electrophoresis apparatus with LED transilluminator (Bento Lab, 2016). The prototype is configured slightly differently from the current commercial versions, but there is no significant difference in performance (**Supplementary Figure 1**).

PCR for selected carbapenemase genes, bla_{NDM} (621-bp), bla_{KPC} (798-bp), bla_{IMP} (232-bp), bla_{VIM} (390-bp), $bla_{\mathrm{OXA-48}}$ (438-bp), $bla_{\mathrm{OXA-23}}$ (501-bp), $bla_{\mathrm{OXA-24}}$ (246-bp), $bla_{\mathrm{OXA-51}}$ (353-bp), and $bla_{\mathrm{OXA-58}}$ (599-bp)] was performed using primers as previously described (Woodford et al., 2006; Poirel et al., 2011b). PCR amplification products were subjected to agarose gel electrophoresis using electrophoresis apparatus supplied with Bento Lab, stained with SYBR Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific), detected using LED transilluminator built in Bento Lab, and photographed with iPhone 7 Plus (Apple).

Whole-genome sequencing was performed on the MinION nanopore sequencer (Oxford Nanopore Technologies) for 24 h with the offline-capable version of MinKNOW v1.7.3 and R9.4 flow cells according to the manufacturer's instructions. Nanopore reads were base called using Albacore v2.1.0 (Oxford Nanopore Technologies), corrected using Genome Finishing Module v1.7 plugged-in CLC Genomics Workbench v10.1.1 (Qiagen) with default parameters of Correct PacBio Reads (beta), and assembled *de novo* using Miniasm v0.2 (Li, 2016) with default parameters.

The extracted bacterial gDNAs were subsequently re-sequenced on a Illumina system in National Institute of Infectious Diseases, Japan for further error correction. Library for Illumina sequencing (insert size of 500–900 bp) was prepared using Nextera XT DNA Library Prep Kit (Illumina) and paired-end sequencing (2 bp× 150 bp) was performed using MiniSeq (Illumina). Illumina paired-end reads were mapped onto the on-site assembly sequences, and sequencing errors were corrected by extracting the consensus of the mapped reads five times using CLC Genomics Workbench v12.0 (Qiagen) with default parameters.

Genome sequences were annotated using the DFAST server[1]. Sequence type (ST), plasmid replicon type, AMR genes, and virulence genes were detected using MLST v2.0, PlasmidFinder v2.1, ResFinder v4.1, and VirulenceFinder v2.0, respectively, using the CGE server[2] with default parameters. Type IV secretion system (T4SS)-associated genes involved in conjugation were detected using TXSScan[3] with default parameters. Mobile gene elements (MGEs) were identified manually from CDS annotations and basically analyzed by comparing the sequences analyzed in previous studies. Linear comparisons of sequences carrying carbapenemase genes were performed using BLAST with default settings (the nucleotide collection database and the megablast program) and visualized using Easyfig v2.2.2[4]. The annotated bacterial circular chromosomes were visualized using the CGView Server[5].

Genome and plasmid sequences of carbapenemase-producing *E. coli* (NIPH17_0020 and NIPH17_0036) and *A. baumannii* (NIPH17_0019) isolated in Cambodia have been deposited at GenBank/EMBL/DDBJ under accession numbers AP024560 (NIPH17_0020), LC483178 (pNIPH17_0020_1), AP024561 (NIPH17_0036), LC483179 (pNIPH17_0036_1), LC603215 (pNIPH17_0036_2), and AP024415 (NIPH17_0019).

RESULTS

On-Site Genomic Epidemiological Analysis of Carbapenemase-Producing Gram-Negative Bacteria Isolated in Cambodia

In July 2017, we stayed for 5 days at the National Institute of Public Health (NIPH) in Phnom Penh, Cambodia. There, we set up potable laboratory equipment, including Bento Lab and MinION, in the Bacteriology laboratory, which has limited research facilities and no PCR machine (**Figure 1**). On the first and second days, we performed the carbapenem inactivation method (CIM) test, double-disk diffusion tests (DDDTs), minimum inhibitory concentrations (MICs) measurement,

[1]https://dfast.nig.ac.jp
[2]http://www.genomicepidemiology.org
[3]https://galaxy.pasteur.fr/
[4]http://mjsull.github.io/Easyfig/
[5]http://cgview.ca

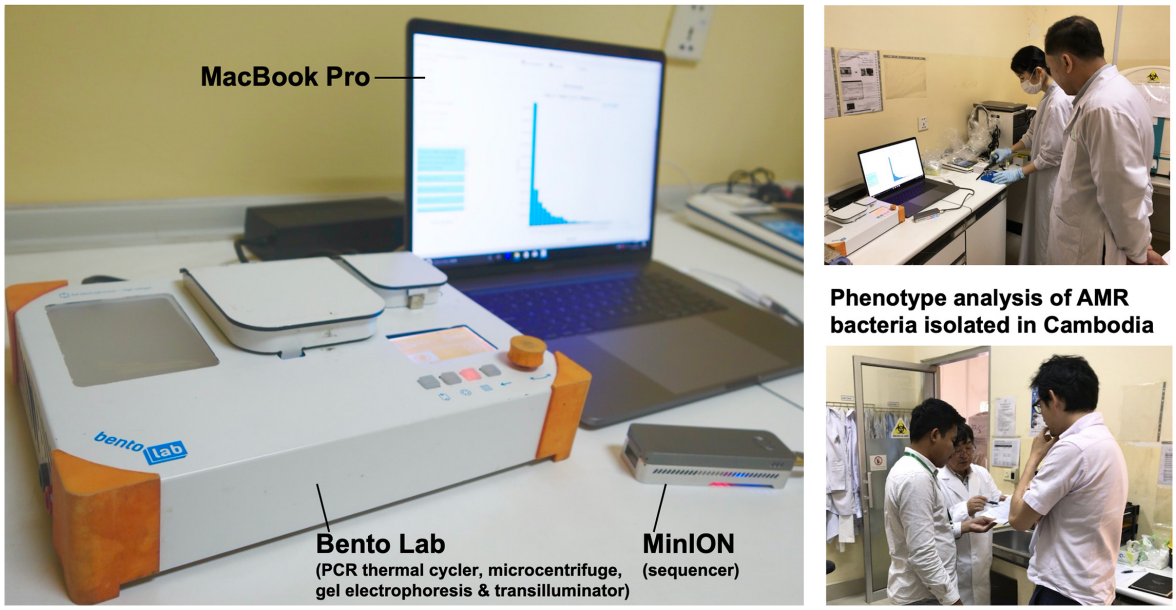


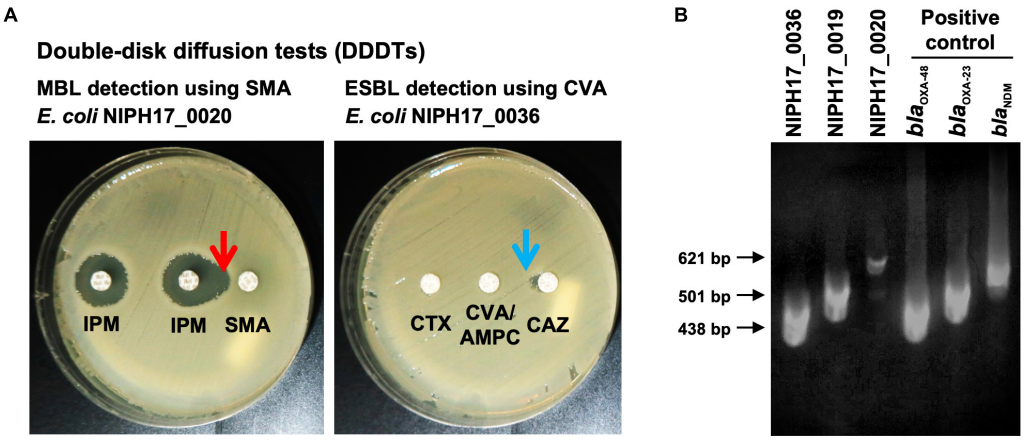
FIGURE 1 | On-site genomic epidemiological analysis of AMR bacteria in Cambodia. Bento Lab and MinION were used for genotype analysis and the carbapenem inactivation method (CIM) and double-disk diffusion tests (DDDTs) were used for phenotype analysis of AMR bacteria.

PCR, and MinION sequencing on two carbapenemase-producing isolates of *E. coli* (NIPH17_0020 and NIPH17_0036) and one carbapenemase-producing isolate of *A. baumannii* (NIPH17_0019) (**Figure 1**) stored in the laboratory prior to this study. On the third and fourth days, we examined diagnostic testing data (**Figure 2**) and analyzed sequencing data (**Figures 3–5**). On the final day, we discussed the results of genotype and phenotype analysis with researchers and technicians belonging to the Bacteriology laboratory at NIPH.

The CIM test is routinely performed at NIPH, and we confirmed that all three bacterial isolates were positive for carbapenemase production. DDDTs with sodium mercaptoacetic acid (SMA) and clavulanic acid (CVA) disks revealed that *E. coli* NIPH17_0020 and *E. coli* NIPH17_0036 were positive for MBL and ESBL production, respectively (**Figure 2A**). PCR targeting major carbapenemase genes revealed that *E. coli* NIPH17_0020, *E. coli* NIPH17_0036, and *A. baumannii* NIPH17_0019 were positive for bla_{NDM}, $bla_{\mathrm{OXA-48}}$, and $bla_{\mathrm{OXA-23}}$, respectively (**Figure 2B**). The MICs of imipenem (IPM) and meropenem (MEM) against *E. coli* NIPH17_0020, *E. coli* NIPH17_0036, and *A. baumannii* NIPH17_0019 were 2 (I) and 2 (I), 0.38 (S) and 0.25 (S), and > 32 (R) and > 32 μg/mL (R), respectively. Furthermore, the MICs of ceftazidime (CAZ), cefotaxime (CTX), aztreonam (AZT), amikacin (AMK), and ciprofloxacin (CPFX) against *E. coli* NIPH17_0020 were >256 (R), >32 (R), 4 (S), 1 (S), and >32 μg/mL (R), respectively; those against *E. coli* NIPH17_0036 were >256 (R), >32 (R), >256 (R), 2 (S), and >32 μg/mL (R), respectively; and those against *A. baumannii* NIPH17_0019 were >256 (R), >32 (R), >256, >256 (R), and >32 μg/mL (R), respectively (**Figure 2C**).

Based on the on-site *de novo* assembly sequences obtained from nanopore sequencing analysis, we determined the complete structures of chromosomes and plasmids of each bacterial isolate and detected AMR genes. As shown in **Supplementary Table 1**, *E. coli* NIPH17_0020 had two contigs (the 4.78-Mb chromosome and the 91.6-kb plasmid pNIPH17_0020_1); *E. coli* NIPH17_0036 had three contigs (the 4.68-Mb chromosome, the 50.2-kb plasmid pNIPH17_0036_1, and the 92.6-kb plasmid pNIPH17_0036_2); and *A. baumannii* NIPH17_0019 had only one contig (the 3.85-Mb chromosome). *E. coli* pNIPH17_0020_1 carried the $bla_{\mathrm{NDM-5}}$-like gene (96.3% identity and 3.0% gap relative to $bla_{\mathrm{NDM-5}}$: accession no. JN104597) (**Supplementary Figure 2A**); *E. coli* pNIPH17_0036_1 carried the $bla_{\mathrm{OXA-48}}$ family carbapenemase $bla_{\mathrm{OXA-181}}$-like gene (97.2% identity and 2.5% gap relative to $bla_{\mathrm{OXA-181}}$: accession no. CM004561) (**Supplementary Figure 2B**); and *A. baumannii* NIPH17_0019 harbored $bla_{\mathrm{OXA-23}}$-like genes in two separate locations of the chromosome (97.7% identity and 2.1% gap or 93.9 and 5.6% gap relative to $bla_{\mathrm{OXA-23}}$: accession no. AY795964) (**Supplementary Figure 2C**).

To summarize the results, the detected carbapenemase genes had a few-percent mismatch that caused frameshifts of genes relative to their putative reference sequences (**Supplementary Figure 2**); hence, we avoided performing CDS annotation for the on-site sequences. We detected AMR genes and plasmid replicons in the sequences analyzed on-site by sequence-based detection (**Figures 3–5** and **Supplementary Table 1**). AMR genes, such as $bla_{\mathrm{TEM-1B}}$, *aadA2*, *aph(3″)-lb*, *aac(3)-lld*, and *aph(6)-ld*, and plasmid replicons, including IncFIA, IncFIB, IncFII, and IncQ1, were detected in provisional



C

Isolate	CIM	DDDT		MIC (μg/ml)							PCR
		SMA	CVA	IPM	MEM	CAZ	CTX	AZT	AMK	CPFX	
E. coli NIPH17_0020	Posi.	Posi.	Neg.	2 (I)	2 (I)	>256 (R)	>32 (R)	4 (S)	1 (S)	>32 (R)	bla_{NDM}
E. coli NIPH17_0036	Posi.	Neg.	Posi.	0.38 (S)	0.25 (S)	>256 (R)	>32 (R)	>256 (R)	2 (S)	>32 (R)	bla_{OXA-48}
A. baumannii NIPH17_0019	Posi.	Neg.	Neg.	>32 (R)	>32 (R)	>256 (R)	>32 (R)	>256	>256 (R)	>32 (R)	bla_{OXA-23}

FIGURE 2 | Genotype and phenotype analysis of AMR bacteria isolated in Cambodia. **(A)** The double-disk diffusion test (DDDT) with imipenem (IPM) and sodium mercaptoacetic acid (SMA) disks against *E. coli* NIPH17_0020 for MBL detection and DDDT with ceftazidime (CAZ), cefotaxime (CTX), and clavulanate/amoxicillin (CVA/AMPC) disks against *E. coli* NIPH17_0036 for ESBL detection. Arrows indicate β-lactamase inhibition. **(B)** PCR amplifications for bla_{OXA-48} for *E. coli* NIPH17_0036 (438 bp), bla_{OXA-23} for *A. baumannii* NIPH17_0019 (501 bp), bla_{NDM} for *E. coli* NIPH17_0020 (621 bp) and their positive controls. Some of the images are unclear because they were acquired in a location with limited equipment. **(C)** Summary of genotype and phenotype analysis, including the carbapenem inactivation method (CIM), DDDTs with SMA or CVA, minimum inhibitory concentrations (MIC) measurement, and PCR targeting selected major carbapenemase genes. The breakpoints for susceptible (S), intermediate (I), and resistance (R) to antimicrobials were adopted from the CLSI 2020 guidelines.

bla_{NDM-5}-carrying pNIPH17_0020_1 in *E. coli* NIPH17_0020 (**Figure 3**), and *qnrS1* genes and the IncX3 replicon were detected in provisional $bla_{OXA-181}$-carrying pNIPH17_0036_1 in *E. coli* NIPH17_0036 (**Figure 4**). BLAST searches of *E. coli* pNIPH17_0020_1 and *E. coli* pNIPH17_0036_1 revealed that several plasmids from Asian and Western countries were highly identical to those plasmids (**Figures 3**, **4**).

Comparison of Plasmids and Genomic Regions in Carbapenemase-Producing Gram-Negative Bacteria Isolated in Cambodia With Those in Other Countries

After the on-site analysis in Cambodia, we further performed Illumina sequencing of carbapenemase-producing isolates of *E. coli* (NIPH17_0020 and NIPH17_0036) and *A. baumannii* (NIPH17_0019), corrected the on-site *de novo* assembly sequences using Illumina reads, and compared the on-site and error-corrected sequences (**Figures 3**, **4**, **5A**, and **Supplementary Figure 3**). The on-site sequences of *E. coli* pNIPH17_0020_1 (provisional 91.6-kb bla_{NDM-5}-carrying plasmid with IncFIA/FIB/FII/Q1 replicons) and *E. coli* pNIPH17_0036_1 (provisional 50.2-kb $bla_{OXA-181}$-carrying plasmid with IncX3 replicons) were highly identical to their error-corrected sequences (96.48% identity over 100% of the error-corrected 93.9-kb plasmid pNIPH17_0020_1: accession no. LC483178 and 96.70% identity over 100% of the error-corrected plasmid 51.5-kb pNIPH17_0036_1: accession no. LC483179, respectively) (**Figures 3**, **4**). Moreover, the on-site sequences of bla_{OXA-23}-containing chromosomal regions of *A. baumannii* NIPH17_0019 were also highly identical to their error-corrected sequences (96.95% identity over 100% of 216,633–233,441 nt and 97.36% identity over 100% of 3,924,674–3,941,483 nt in their error-corrected chromosome of NIPH17_0019: accession no. AP024415) (**Supplementary Figures 3A,B**). Although there were differences of a few percent between the on-site and error-corrected sequences, the best match types of AMR genes and plasmid replicons detected from the reference libraries were consistent (**Figures 3**, **4**, **5A**).

Escherichia coli NIPH17_0020 belonged to sequence type 410 (ST410) according to multilocus sequence typing (MLST) analysis and harbored putative virulence genes,

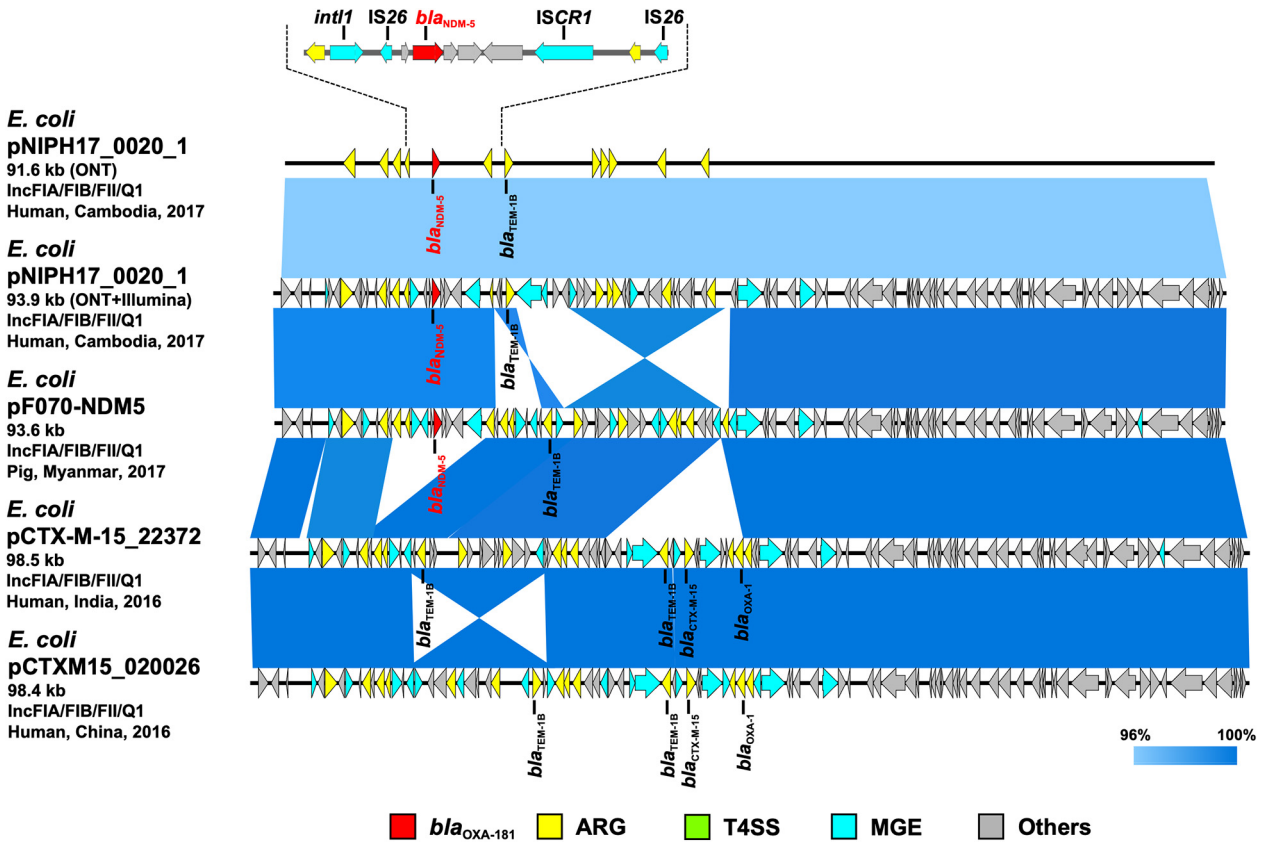


FIGURE 3 | Linear comparison of related plasmid sequences from Cambodia and other countries. *E. coli* pNIPH17_0020_1 with bla_{NDM-5} (this study, accession no. LC483178), *E. coli* pF070-NDM5 with bla_{NDM-5} (accession no. AP023238), *E. coli* pCTX-M-15_22372 with no bla_{NDM} (accession no. CP040398), and *E. coli* pCTXM15_020026 with no bla_{NDM} (accession no. CP034956) are shown. Regarding *E. coli* pNIPH17_0020_1, both the uncorrected sequence (from on-site ONT analysis) and corrected sequence (from subsequent ONT + Illumina analysis), and the detailed genetic structures around bla_{NDM-5} are shown (upper). Red, yellow, green, blue, and gray arrows indicate carbapenemase gene (bla_{NDM-5}), other AMR genes (ARG), type IV secretion system-associated genes involved in conjugation (T4SS), mobile gene elements (MGE), and other genes (Others), respectively. The colors in comparison of plasmids show percent identity.

including *fyuA*, *gad*, *irp2*, *lpfA*, and *terC* on its chromosome (**Supplementary Table 1**). NIPH17_0020 had one 93.9-kb plasmid, pNIPH17_0020_1, with a backbone consisting of IncFIA/FIB/FII/Q1 with multiple AMR genes, such as β-lactamase (bla_{NDM-5} and bla_{TEM-1B}) and aminoglycoside resistance genes [*aac(3)-lld*-like, *aadA2, aph(3")-lb, aph(6)-ld*] (**Supplementary Table 1**). *E. coli* pNIPH17_0020_1 was structurally highly identical to plasmid pPF070-NDM5 (accession no. AP023238) in *E. coli* isolated from a pig in Myanmar in 2017, plasmid pCTX-M-15_22372 (accession no. CP040398) in *E. coli* isolated from a human in India in 2016, and plasmid pCTXM15_020026 (accession no. CP034956) in *E. coli* isolated from a human in China in 2016 (99.8% identity over 92–94% of pNIPH17_0020_1) (**Figure 3**). pNIPH17_0020_1 contained several mobile gene elements (MGEs), including IS*26* and IS*CR1,* surrounding bla_{NDM-5} (**Figure 3** upper). pF070-NDM5 carried bla_{NDM-5} with the same MGEs, whereas pCTX-M-15_22372 and pCTXM15_020026 did not carry bla_{NDM-5} (**Figure 3**).

Escherichia coli NIPH17_0036 also belonged to ST410 according to MLST analysis and harbored putative virulence genes, including *gad*, *hra*, *lpfA*, and *terC* on its chromosome (**Supplementary Table 1**). NIPH17_0036 had two plasmids; one of them, 51.5-kb IncX3 plasmid pNIPH17_0036_1 carrying AMR genes, including $bla_{OXA-181}$ and *qnrS1* (**Supplementary Table 1**), was structurally nearly identical to plasmid pOXA-181-IHIT35346 (accession no. KX894452) in *E. coli* isolated from a pig in Italy in 2016 and plasmid pOXA181 (accession no. KP400525) in *E. coli* isolated from a human in China in 2014 (Liu et al., 2015), as well as plasmid pKS22 (accession no. KT005457) in *Klebsiella variicola* isolated from a fresh vegetable imported from Thailand or Vietnam (99.9% identity over 100% of pNIPH17_0036_1) (**Figure 4**). $bla_{OXA-181}$ in pNIPH17_0036_1 was surrounded by several MGEs, including IS*Kpn19*, IS*3000*, and IS*26* (**Figure 4** upper). *E. coli* NIPH17_0036 had another plasmid, pNIPH17_0036_2 (94.8-kb IncFIA/FIB/FII/Q1 plasmid, accession no. LC603215), carrying $bla_{CTX-M-15}$, bla_{OXA-1}, bla_{TEM-1B}, other β-lactamase genes, and multiple aminoglycoside resistance genes (**Supplementary Table 1**).

Acinetobacter baumannii NIPH17_0019 belonged to ST471 according to MLST analysis and harbored the bla_{OXA-23} genes in two separate regions on the chromosome (accession no.

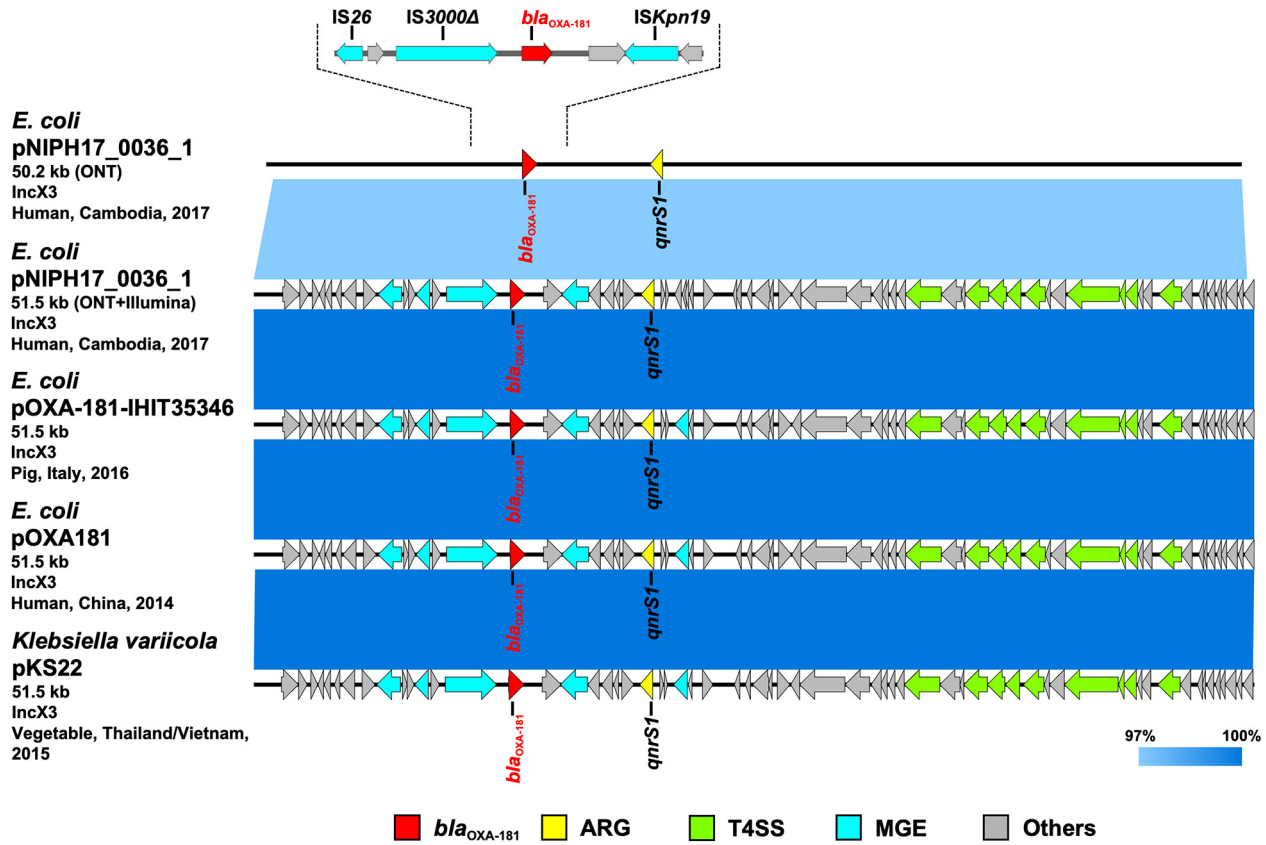


FIGURE 4 | Linear comparison of related plasmid sequences from Cambodia and other countries. *E. coli* pNIPH17_0036_1 with $bla_{OXA-181}$ (this study, accession no. LC483179), *E. coli* pOXA-181-IHIT35346 with $bla_{OXA-181}$ (accession no. KX894452), *E. coli* pOXA-181 with $bla_{OXA-181}$ (accession no. KP400525), and *Klebsiella variicola* pKS22 with $bla_{OXA-181}$ (accession no. KT005457) are shown. Regarding *E. coli* pNIPH17_0036_1, both the uncorrected sequence (from on-site ONT analysis) and corrected sequence (from subsequent ONT + Illumina analysis), and the detailed genetic structures around $bla_{OXA-181}$ are shown (upper). Red, yellow, green, blue, and gray arrows indicate carbapenemase gene ($bla_{OXA-181}$), other AMR genes (ARG), type IV secretion system-associated genes involved in conjugation (T4SS), mobile gene elements (MGE), and other genes (Others), respectively. The colors in comparison of plasmids show percent identity.

AP024415) (**Figure 5A**). Both copies of bla_{OXA-23} were located in Tn*2006* in AbaR4. AbaR is the resistance island containing AMR genes in *A. baumannii* (Bi et al., 2019). Comparison of the genetic environment around AbaR4 was performed between *A. baumannii* NIPH17_0019 and AbaR4-harboring *A. baumannii* D36 (accession no. JN107991), which was isolated from a human in 2008 in Australia (**Figure 5B**). The results revealed that two AbaR4 islands in *A. baumannii* NIPH17_0019 were highly identical to that of *A. baumannii* D36 (99.84–99.88% identity over 100% of the sequence) (**Figure 5B**). Interestingly, AbaR4 was integrated into the *comM* gene in *A. baumannii* D36, whereas AbaR4 was integrated into the *comM* gene (AbaR4 of the 216,633–233,441 nt region) and *mutY* genes (for AbaR4 of the 3,924,674–3,941,483 nt region) in *A. baumannii* NIPH17_0019.

DISCUSSION

In this study, we successfully conducted genomic analysis of carbapenemase-producing gram-negative bacteria (CPGNB) in Cambodia and provided the first glimpse into the genomic epidemiology of CPGNB in that country. The main analysis was performed on-site using portable laboratory equipment, namely, Bento Lab and MinION. The nearly complete genomes of carbapenemase-producing *E. coli* and *A. baumannii* isolates, including their plasmids, were determined using the MinION nanopore sequencing data; detection of AMR genes and plasmid replicons, as well as sequence similarity searches in public databases, were performed at a Cambodian laboratory with limited research facilities. Because the nanopore sequencing technology is still evolving, and the accuracy of sequencing is imperfect (the rates of indels and substitutions are a few percent each) (Jain et al., 2016), it was necessary to combine other sequencing technologies, such as Illumina sequencing by synthesis, for further molecular typing analysis of bacteria that require more accurate sequences at the single-nucleotide level.

The cost of commercial versions of Bento Lab is starting from $1,646.99[6], whereas MinION is free of charge because it is basically a rental from the company[7]. In this study, we

[6]https://us.vwr.com/store/product/27856428/bento-lab
[7]https://store.nanoporetech.com/configure-minion-basic

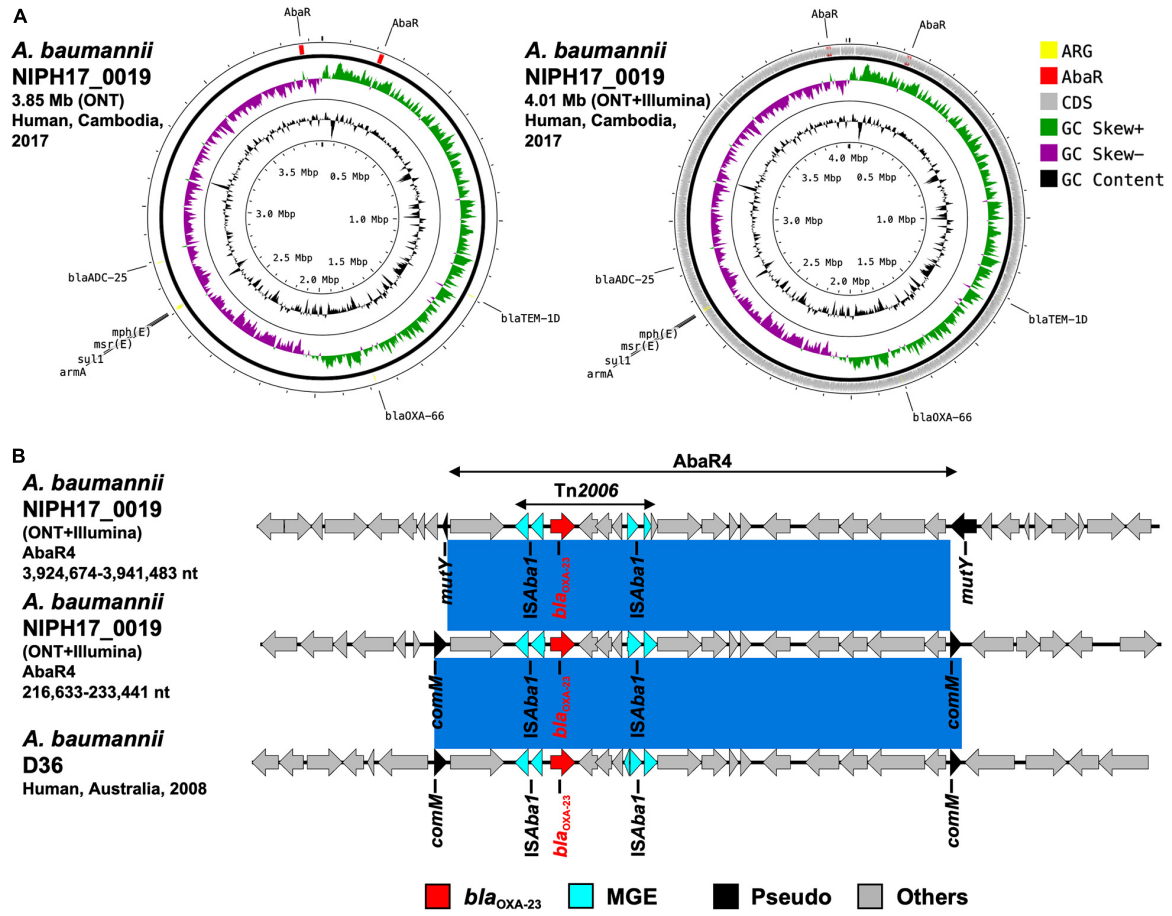


FIGURE 5 | (A) Circular chromosome representation of *A. baumannii* NIPH17_0019 harboring two copies of bla_{OXA-23}. Both the uncorrected sequence (from on-site ONT analysis) and corrected sequence (from subsequent ONT + Illumina analysis, accession no. AP024415) are shown. Yellow, red, gray, green, purple, and black indicate AMR genes (ARG), AbaR4 with bla_{OXA-23} (AbaR), other coding sequences (CDS), GC skew+, GC skew-, and GC content, respectively. **(B)** Linear comparison of AbaR4-containing genomic regions in *A. baumannii* from Cambodia and other countries. Two sets of AbaR4 with bla_{OXA-23} in *A. baumannii* NIPH17_0019 (3,924,674–3,941,483 nt region inserted within *mutY* and 216,633–233,441 nt region inserted within *comM* in accession no. AP024415) and AbaR4 with bla_{OXA-23} in *A. baumannii* D36 (41,312–58,123 nt region inserted within *comM* in accession no. JN107991) are shown. Red, blue, black, and gray arrows indicate carbapenemase gene (bla_{OXA-23}), mobile gene elements (MGE), pseudogenes disrupted by AbaR4 insertions (Pseudo), and other genes (Others), respectively. The blue color in comparison of sequences indicates nearly 100% identity.

did not use barcodes for MinION library preparation and performed MinION analysis using one flow cell per sample to ensure reliable data acquisition. The cost of the flow cell and library preparation reagent was $599 (for 6 reactions) and $900, respectively, resulting in MinION analysis cost of $999 per sample. If barcodes are used for library preparation, the unit cost of MinION analysis can be dramatically reduced. We usually analyze about six barcoded samples using one flow cell for bacterial species with genome sizes of around 5 Mb. If six samples are included in one analysis, the unit cost is going down to $171[8].

The carbapenem inactivation method (CIM) and double-disk diffusion test (DDDT) are simple, inexpensive, and useful, especially in developing countries where materials and facilities are limited. In this study, the results of both tests were reasonable, as validated by subsequent genetic analysis (**Figure 2**). The CIM test is routinely performed at NIPH, Cambodia, and NIPH had detected and stored carbapenemase-producing bacterial isolates prior to this study. The DDDTs with SMA and CVA disks clearly detected production of MBL in *E. coli* NIPH17_0020 and ESBL in *E. coli* NIPH17_0036 (**Figure 2A**). CIM and DDDT are important for screening for CPGNB because MICs of carbapenems against CPGNB are not always high, as is the case for bacteria with carbapenem-hydrolyzing oxacillinase genes, such as bla_{OXA-48} or its variants. The MICs of carbapenems against *E. coli* NIPH17_0036, which harbored $bla_{OXA-181}$ on its plasmid (**Figure 4**), were relatively low, whereas CIM yielded a positive result (**Figure 2C**). OXA-48 is capable of weakly hydrolyzing carbapenems while maintaining its activity against broad-spectrum cephalosporins. The $bla_{OXA-181}$ gene is a variant of bla_{OXA-48}, and the hydrolytic activity of OXA-181 toward β-lactams is similar to that of OXA-48 (Castanheira et al., 2011).

[8]https://store.nanoporetech.com/catalog/product/view/id/226/s/rapid-barcoding-kit/category/28/

We sequenced carbapenemase-producing *E. coli* and *A. baumannii* isolates on-site using MinION and Bento Lab, and revealed that *E. coli* NIPH17_0020 and *E. coli* NIPH17_0036 harbored bla_{NDM-5} and $bla_{OXA-181}$ on plasmids pNIPH17_0020_1 and pNIPH17_0036_1, respectively (**Figures 3**, **4**) and that *A. baumannii* NIPH17_0019 harbored two copies of bla_{OXA-23} on its chromosome (**Figure 5**). Although the MinION control software MinKNOW requires a constant internet connection, the company provided us with an offline-capable version of MinKNOW. For *de novo* assembly using nanopore long-read data, we used the Miniasm software, which is fast and computationally inexpensive (Li, 2016). Because Miniasm assembles without error correction, MinION reads were error-corrected using the CLC Genomics Workbench pipeline for long-read sequencers prior to *de novo* assembly. The resultant assembly sequences obtained from on-site analysis still contained a few percent of errors that are responsible for gene frameshifts. However, the on-site *de novo* assembly sequences were sufficient for subsequent molecular epidemiological analysis (e.g., detection of AMR genes and genomic locations where the genes are encoded) (**Figures 3–5** and **Supplementary Figures 2**, **3**), and the error-corrected sequences using Illumina sequencing were subsequently used to confirm the results of the on-site analysis (**Figures 3–5**).

Escherichia coli isolates NIPH17_0020 and NIPH17_0036 belonged to ST410 according to MLST analysis. ST410 is a high-risk clone associated with AMR and recently emerged in among humans and the environment in Southeast Asia (Nadimpalli et al., 2019). In this study, both *E. coli* isolates harbored carbapenemase genes, bla_{NDM-5} and $bla_{OXA-181}$, respectively, on their plasmids, and commonly harbored putative virulence genes, including *gad* (a glutamate decarboxylase gene), *lpfA* (a long polar fimbriae gene), and *terC* (a tellurium ion resistance gene) on their chromosomes (**Supplementary Table 1**). Furthermore, NIPH17_0020 harbored other virulence genes, *fyuA* (a siderophore receptor gene) and *irp2* (a siderophore gene), and NIPH17_0036 harbored other virulence gene *hra* (a heat-resistant agglutinin gene) on their chromosomes (**Supplementary Table 1**).

Escherichia coli pNIPH17_0020_1 [93.9-kb IncFIA/FIB/FII/Q1 plasmid with bla_{NDM-5}, accession no. LC483179] was structurally highly identical to *E. coli* pPF070-NDM5 in Myanmar (accession no. AP023238) (**Figure 3**) and harbored several mobile gene elements (MGEs), including IS*26* and IS*CR1* surrounding bla_{NDM-5} (**Figure 3** upper). The bla_{NDM-5}-containing regions between IS*26* and IS*CR1* in pNIPH17_0020_1 and pPF070-NDM5 were identical with those of IncFII plasmids, such as *E. coli* pM109_FII in Myanmar (accession no. AP018139), *E. coli* pGUE-NDM in France (accession no. JQ364967), and *K. pneumoniae* pCC1409-1 in South Korea (accession no. KT725789), implying that bla_{NDM-5} was disseminated via plasmids and MGEs, such IS*26*, among Enterobacterales around the world (Sugawara et al., 2017).

Escherichia coli pNIPH17_0036_1 [51.5-kb IncX3 plasmid with $bla_{OXA-181}$, accession no. LC483179] was structurally nearly identical to *E. coli* pOXA-181-IHIT35346 from Italy (accession no. KX894452), *E. coli* pOXA181 from China (accession no. KP400525), and *K. variicola* pKS22 from Thailand/Vietnam (accession no. KT005457) (**Figure 4**). Our analysis revealed that $bla_{OXA-181}$-carrying IncX3 plasmids widespread among Enterobacterales worldwide were also present in Cambodia. pKS22 was detected in coriander imported from Thailand or Vietnam, and the international fresh vegetable trade is suspected to be a route for the spread of AMR bacteria (Zurfluh et al., 2015). Because Cambodia is geographically and culturally close to Thailand and Vietnam, it is possible for AMR bacteria to be transmitted through foods. However, this study was very small, so further analysis with larger numbers of bacterial isolates in Cambodia according to One Health approaches will be necessary to characterize AMR bacteria in this country.

Acinetobacter baumannii NIPH17_0019 belonging to ST571 harbored two copies of bla_{OXA-23} in the chromosome (**Figure 5A**). According to a previous study of carbapenem-resistant *A. baumannii* (Hamidian and Nigro, 2019), ST2 is the most prevalent genotype and recognized as a high-risk clone associated with AMR, and bla_{OXA-23} is the most widespread carbapenem-resistance gene in the world. ST571 belongs to clonal complex 2, and ST571 strains harboring bla_{OXA-23} are widespread in medical settings in Vietnam (Tada et al., 2015). In general, carbapenem-hydrolyzing oxacillinases hydrolyze carbapenems weakly and do not contribute to strong carbapenem resistance on their own. However, elevated expression of oxacillinase genes by the upstream insertion of IS, such as IS*Aba1* in *Acinetobacter* spp., which serves as a promoter for the downstream genes, leads to strong resistance (Turton et al., 2006; Corvec et al., 2007). Two copies of bla_{OXA-23} in *A. baumannii* NIPH17_0019 were located downstream of IS*Aba1* (**Figure 5B**). Both bla_{oxa-23} were present in Tn*2006*, a common 4.8-kb transposon in *Acinetobacter* app., with a central segment of 2,445-bp flanked by two reverse-oriented copies of IS*Aba1* (Nigro and Hall, 2016). Tn*2006*-containing AbaR4 is frequently inserted within the *comM* gene in the chromosome of international *A. baumannii* clones (Hamidian and Hall, 2011; Seputiene et al., 2012; Hamidian et al., 2015), and the *comM* gene is the most-preferred hotspot for AbaR insertions (Bi et al., 2019). One of the insertion sites of AbaR4 (3,924,674–3,941,483 nt region) in *A. baumannii* NIPH17_0019 was not the *comM* gene but the *mutY* gene, encoding for an adenine DNA glycosylase (**Figure 5B**). According to a previous study (Bi et al., 2019), AbaR insertions at *acoA*, *pho,* and *uup* genes were occasionally observed; however, *mutY* has not been previously reported as an insertion site.

CONCLUSION

Based on our on-site genomic epidemiological analysis of carbapenemase-producing gram-negative bacteria in Cambodia, we revealed for the first time that plasmids and MGEs carrying clinically relevant carbapenemase genes reported in other countries have also been spreading in Cambodia. Bento Lab and MinION are useful for genomic analysis and surveillance of AMR bacteria in hospitals and research centers with limited facilities.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Ethics Committee for Health Research (NECHR), Cambodia. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individuals for the publication of any potentially identifiable images included in this article.

AUTHOR CONTRIBUTIONS

MS contributed to conceptualization. AH, HY, and MS contributed to methodology. MS contributed to software. AH, HY, HT, KY, and MS contributed to validation. AH and MS contributed to formal analysis, data curation, and visualization. AH, HY, HT, KY, KS, and MS contributed to investigation. PB, BW, VN, VL, MV, VA, and CD contributed to resources. AH, KS, and MS contributed to writing. CD and KS contributed to supervision. CD, KS, and MS contributed to project administration. KS and MS contributed to funding. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.675463/full#supplementary-material

Supplementary Figure 1 | Bento Lab for on-site genomic epidemiological analysis of AMR bacteria in Cambodia. **(A)** The prototype used in this study and **(B)** current commercial type are shown.

Supplementary Figure 2 | Multiple sequence alignment of carbapenemase genes analyzed by MAFFT v7.475. **(A)** Comparison between the bla_{NDM-5}-like gene in *E. coli* pNIPH17_0020_1 (from on-site ONT analysis) and the reference gene (bla_{NDM-5} in accession no. JN104597), **(B)** comparison between the $bla_{OXA-181}$-like gene in *E. coli* pNIPH17_0036_1 (from on-site ONT analysis) and the reference sequence ($bla_{OXA-181}$ in accession no. CM004561), and **(C)** comparison between the bla_{OXA-23}-like sequence in *A. baumannii* NIPH17_0019 (from on-site ONT analysis) and the reference sequence (bla_{OXA-23} in accession no. AY795964) are shown.

Supplementary Figure 3 | Linear comparison of AbaR4-containing genomic regions in *A. baumannii* NIPH17_0019 harboring two copies of bla_{OXA-23}. Two sets of AbaR4 with bla_{OXA-23} in *A. baumannii* NIPH17_0019: **(A)** 216,633–233,441 nt region inserted within *comM* and **(B)** 3,924,674–3,941,483 nt region inserted within *mutY* in accession no. AP024415, and **(A,B)** comparison of both the uncorrected sequences (from on-site ONT analysis) and corrected sequences (from subsequent ONT + Illumina analysis) are shown. Red, blue, black, and gray arrows indicate carbapenemase gene (bla_{OXA-23}), mobile gene elements (MGE), pseudogenes disrupted by AbaR4 insertion (Pseudo), and other genes (Others), respectively. The colors in comparison of sequences show percent identity.

Supplementary Table 1 | Summary of whole-genome sequencing and bioinformatics analysis of AMR bacteria isolated in Cambodia in this study. Bacterial isolates, nucleotides of raw reads of MinION sequencing and the corrected reads, genome size of the uncorrected sequence (from on-site ONT analysis) and corrected sequence (from subsequent ONT + Illumina analysis) are shown. Also, AMR genes (ARG) associated with resistance to β-lactams, aminoglycosides (AG), and fluoroquinolones (FQ), virulence genes, and accession nos. of sequences are shown.

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Molecular Mechanisms Influencing Bacterial Conjugation in the Intestinal Microbiota

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Bacterial conjugation is a widespread and particularly efficient strategy to horizontally disseminate genes in microbial populations. With a rich and dense population of microorganisms, the intestinal microbiota is often considered a fertile environment for conjugative transfer and a major reservoir of antibiotic resistance genes. In this mini-review, we summarize recent findings suggesting that few conjugative plasmid families present in *Enterobacteriaceae* transfer at high rates in the gut microbiota. We discuss the importance of mating pair stabilization as well as additional factors influencing DNA transfer efficiency and conjugative host range in this environment. Finally, we examine the potential repurposing of bacterial conjugation for microbiome editing.

Keywords: bacterial conjugation, microbiota, conjugative plasmids (CP), mating pair stabilization, antibiotic resistance

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INTRODUCTION

Antimicrobial resistance continues to rise worldwide, with alarming projections suggesting that antibiotic-resistant infections could become the second most common cause of death by 2050 (O' Neil, 2014). This led many research groups to study the global collection of antibiotic resistance genes, also called the resistome (Carattoli, 2013; Penders et al., 2013; van Schaik, 2015; Casals-Pascual et al., 2018), and to identify the intestinal microbiota as a major reservoir of antibiotic resistance genes (Ravi et al., 2014). The complex microbial communities found in the gut are dense and composed of diverse bacteria phyla (Turnbaugh et al., 2007; Qin et al., 2010), a context thought to be particularly favorable for horizontal gene transfer (Liu et al., 2012; Soucy et al., 2015) and antibiotic resistance gene dissemination (San Millan, 2018). Given that the intestinal microbiota also contains a variety of pathobionts (Palleja et al., 2018; Bakkeren et al., 2019), understanding the molecular mechanisms driving the spread of antibiotic resistance genes is particularly important to prevent infections that could become difficult or impossible to treat.

Horizontal gene transfer mechanisms include transformation, transduction, and bacterial conjugation. Bacterial conjugation is considered a major contributor to gene transfer and to the emergence of new antibiotic-resistant pathogens. Conjugative transfer is a well characterized phenomenon during which a donor bacterium assembles a type IV secretion system (T4SS) and transfers DNA to a recipient bacterium in close contact (Cascales and Christie, 2003; Alvarez-Martinez and Christie, 2009; Arutyunov and Frost, 2013; Virolle et al., 2020). Although thoroughly investigated in test tubes and Petri dishes, the study of bacterial conjugation in the intestinal microbiota remains far less characterized with most evidence being provided by epidemiologic studies (Norman et al., 2009; Chen et al., 2013; Soucy et al., 2015; Sun et al., 2016; San Millan, 2018). IncF, IncI, IncA, IncC, and IncH plasmids are the most frequently encountered in humans

and animals (Rozwandowicz et al., 2018) but few studies have quantified the transmission of mobile genetic elements *in situ* and described the underlying mechanisms. This mini-review summarizes recent findings on bacterial conjugation in the gut microbiome with a focus on enterobacteria.

THE MOBILITY OF GENES IN THE GUT MICROBIOTA

Many studies have reported conjugative transfer of plasmids in the intestinal microbiota (Licht and Wilcks, 2005). For instance, conjugation was found to occur with plasmids of different incompatibility groups (**Table 1**) harbored by Gram-negative (Kasuya, 1964; Reed et al., 1969; Jones and Curtiss, 1970; Duval-Iflah et al., 1980, 1994; Corpet et al., 1989; Garrigues-Jeanjean et al., 1999; Licht et al., 1999, 2003; García-Quintanilla et al., 2008; Stecher et al., 2012; Aviv et al., 2016; Gumpert et al., 2017; Bakkeren et al., 2019; Neil et al., 2020; Ott et al., 2020) or Gram-positive bacteria (Doucet-Populaire et al., 1991, 1992; McConnell et al., 1991; Schlundt et al., 1994; Igimi et al., 1996; Jacobsen et al., 1999; Moubareck et al., 2003; Lester et al., 2004). Most studies focused on *Escherichia coli* as the donor bacterium but lactic acid bacteria have also been investigated because of their abundance in fermented food products (Igimi et al., 1996). Despite major implications on microbial evolution and on the emergence of antibiotic-resistant pathogens, our knowledge of the molecular mechanisms facilitating bacterial conjugation in the gut microbiota remains sparse (Norman et al., 2009; Soucy et al., 2015; Casals-Pascual et al., 2018).

Several environmental conditions resembling those encountered in the intestinal tract were investigated *in vitro* and shown to influence conjugation (Rang et al., 1996). For example, the transfer rates of conjugative plasmids pES1 and pSLT were shown to be affected by lower oxygen levels and the presence of bile salt or by other factors such as NaCl concentration and temperature (García-Quintanilla et al., 2008; Aviv et al., 2016). Other plasmids were shown to be inhibited by the presence of mammalian cells in co-cultures, raising the possibility that human host secreted factors could affect plasmid transfer rates (Lim et al., 2008; Machado and Sommer, 2014). A pioneering study reported in 1999 that IncF plasmid R1drd19 can transfer between two *E. coli* strains within the mouse gut microbiome at rates similar to those obtained on agar plates (Licht et al., 1999). This led to the hypothesis that bacterial mating may occur in a stable matrix, most likely after the formation of biofilm in the gut. *In situ* transfer rates were also quantified directly in the mouse intestinal microbiota for other conjugative plasmids (**Table 1**). However, different models with several experimental variables were used. For example, different mice models ranging from germ-free to antibiotic-treated mice have been reported (Licht and Wilcks, 2005). Another important variable comes from the nature of the donor strain and recipient strains, which were shown to affect transfer rates in the gut (Ott et al., 2020). While some studies introduced and probed specific bacteria as recipient cells for conjugation, other investigations used endogenous residents of the microbiota. Furthermore, mixing donors and recipient strains before their introduction in the mice (Stecher et al., 2012) could also introduce differences since conjugation could occur between the two strains before or in the stomach immediately after their introduction in mice rather than in the intestinal microbiota. Taken together, these variations in experimental models make the comparison of transfer rates difficult between studies.

A recent study by our group adopted a standardized assay to evaluate and compare the mobility of conjugative plasmids in the mouse gut microbiota (Neil et al., 2020). Transfer rates were quantified for 13 conjugative plasmids representing 10 of the major conjugative plasmids incompatibility groups found in *Enterobacteriaceae* (**Table 1**). This work was performed in streptomycin-treated mice to deplete endogenous enterobacteria and facilitate the establishment of *E. coli* Nissle, 1917 derivatives as the donor and recipient bacteria. This work revealed that few conjugative plasmids were able to efficiently transfer *in situ* using this model, without any correlation with *in vitro* conjugation rates. A surprising finding was that incompatibility group I_2 ($IncI_2$) plasmid TP114 displayed only modest conjugation efficiencies *in vitro* but reached very high transfer rates in the intestinal microbiota, which prompted a more thorough investigation of this plasmid. A first observation was that hypoxic conditions increased the relatively modest TP114 *in vitro* transfer rates to very high frequencies of conjugation *in situ*. Transposon mutagenesis coupled to conjugation experiments also highlighted the crucial role of a group of genes encoding an accessory type IVb pilus (T4P) for TP114 conjugation in the intestinal tract (Neil et al., 2020). The T4P is a structure found in I-complex plasmids (IncB/O, IncI1, IncI2, IncK, and IncZ) that was previously proposed to stabilize the mating-pair in order to allow conjugation in unstable environments (Ishiwa and Komano, 2000; Praszkier and Pittard, 2005).

MATING-PAIR STABILIZATION MECHANISMS

The T4SS is a sophisticated nanomachine that plays an essential role in the transfer of DNA and/or protein macromolecules during bacterial conjugation. An important step during this process is mating-pair formation (MPF), which brings the donor and recipient bacteria in close contact (Chandran Darbari and Waksman, 2015; Christie, 2016; Virolle et al., 2020). In enterobacteria, two basic forms of conjugative pilus are associated with T4SS, either thin flexible or thick rigid, which influences the ability to support conjugation in liquid or solid environments (Arutyunov and Frost, 2013; Chandran Darbari and Waksman, 2015; Virolle et al., 2020). Besides MPF, a generally overlooked step called mating-pair stabilization (MPS) may be needed to keep the donor and recipient cells together long enough to allow successful DNA transfer. MPS is especially important in broth/*in vivo* conditions where bacterial mobility, flow forces, and other environmental factors could perturb the interaction between the donor and recipient cells (Clarke et al., 2008; **Figures 1A–C**). MPS relies on adhesins either displayed at the surface of the bacterium or on specialized

TABLE 1 | Transfer rates of various conjugative elements in the intestinal microbiota.

Name	Inc group	Resistance	Isolated in	Donor strain	Recipient strain	Transfer rates†*in vitro***	Transfer rates†*in situ*	MPS family	Genbank	References
pAMβ1	18	Er, Lc	*Enterococcus faecalis*	*Lactococcus lactis* IL1403	*Enterococcus faecalis* HS32	2.3×10^{-3}	$<1 \times 10^{-7}$ (a)	Not reported	NC_ 013514.1	Igimi et al., 1996
pAT191 (synthetic)*	18	Km	*Enterococcus faecalis*	*Enterococcus faecalis* BM4110	*Escherichia coli* K802N::Tn10	5×10^{-9}	3×10^{-9} (a)	Not reported	Not deposited	Doucet-Populaire et al., 1992
pAM714	Hly	Er	*Enterococcus faecalis*	*Enterococcus faecalis* FA2-2	*Enterococcus faecalis* JH2SS	$\sim 1 \times 10^{-2}$	1.4×10^{-1} (b)	Not reported	Not deposited	Huycke et al., 1992
pAM771	Hly	Er	*Enterococcus faecalis*	*Enterococcus faecalis* FA2-2	*Enterococcus faecalis* JH2SS	Not reported	2.9×10^{-2} (b)	Not reported	Not deposited	Huycke et al., 1992
pCAL1/pCAL2	Not found	Er	*Enterococcus faecium*	*Enterococcus faecium* 160/00	*Enterococcus faecium* 64/3-RFS	2×10^{-5}	$\sim 1 \times 10^{-6}$ (a)	Not reported	Not deposited	Lester et al., 2004
pCF10	Not found	Tc	*Enterococcus faecalis*	*Enterococcus faecalis* OG1RFS	*Enterococcus faecalis* OG1SS	Data not shown	$\sim 1 \times 10^{-3}$ (c)	Not reported	NC_ 006827.2	Licht et al., 2002
Tn1545	–****	Km, Er, Tc	*Streptococcus pneumoniae*	*Enterococcus faecalis* BM4110	*Listeria monocytogenes* LO17RF	2.5×10^{-7}	1.1×10^{-8} (a)	Not reported	AM903082.1	Doucet-Populaire et al., 1991
Tn916	–****	Tc	*Bacillus subtilis*	*Enterococcus faecalis* OG1SS	*Enterococcus faecalis* OG1RF	1.1×10^{-5}	$\sim 1 \times 10^{-9}$ (d)	Not reported	KM516885.1	Bahl et al., 2004
pYD1	Not found	14 antibiotic resistance markers	*Serratia liquefasciens*	*Serratia liquefasciens*	*Escherichia coli*	Not reported	$\sim 1 \times 10^{-6}$ (a)	Not reported	Not deposited	Duval-Iflah et al., 1980
ROR-1	Not found	Tc	Not found	*Escherichia coli* M7-18	*Escherichia coli* x820	$\sim 1 \times 10^{-5}$	$\sim 1 \times 10^{-4}$ (a)	Not reported	Not deposited	Jones and Curtiss, 1970
pIP72	B/O	Km	*Escherichia coli*	*Escherichia coli* Nissle1917	*Escherichia coli* Nissle1917	3.57×10^{-4}	3.56×10^{-5} (a)	PilV	MN612051.1	Neil et al., 2020
pVCR94ΔX3	C	Km	*Vibrio cholerae*	*Escherichia coli* Nissle1917	*Escherichia coli* Nissle1917	3.23×10^{-3}	Not detected (a)	TraN	KF551948.1	Neil et al., 2020
pSLTΔfinO	F	Km	*Salmonella enterica* subsp. *enterica* serovar Typhimurium	*Salmonella enterica* subsp. *enterica* serovar Typhimurium SV5535	*Salmonella enterica* subsp. *enterica* serovar Typhimurium SV5534	5×10^{-4}	5×10^{-5} (a)	TraN	AE006471.2	García-Quintanilla et al., 2008
pOX38	FI	Sp, Tc, Su	*Escherichia coli*	*Escherichia coli* Nissle1917	*Escherichia coli* Nissle1917	6.82×10^{-2}	4.89×10^{-5} (a)	TraN	MF370216.1	Neil et al., 2020
RIP71a	FII	Ap, Tc, Cm, Sm, Sp	*Escherichia coli*	*Escherichia coli* Nissle1917	*Escherichia coli* Nissle1917	2.64×10^{-3}	7.87×10^{-4} (a)	TraN	MN626601	Neil et al., 2020
R1	FII	Km, Cm, Su, Sp	*Salmonella enterica* subsp. *enterica* serovar Paratyphi B	*Escherichia coli* Nissle1917	*Escherichia coli* Nissle1917	2.97×10^{-3}	1.6×10^{-4} (a)	TraN	KY749247.1	Neil et al., 2020
R1drd19	FII	Km, Cm, Su, Sp, Ap	*Salmonella enterica* subsp. *enterica* serovar Paratyphi B	*Escherichia coli* BJ4	*Escherichia coli* BJ4	$\sim 1 \times 10^{-1}$	$\sim 1 \times 10^{-3}$ (a)	TraN	Not deposited	Licht et al., 1999

(Continued)

TABLE 1 | Continued

Name	Inc group	Resistance	Isolated in	Donor strain	Recipient strain	Transfer rates†*in vitro***	Transfer rates†*in situ*	MPS family	Genbank	References
pCVM29188_146	FIIA	Sm, Tc	*Salmonella enterica* subsp. *enterica* serovar Kentucky	*Salmonella enterica* subsp. *enterica* serovar Kentucky	*Escherichia coli* HS-4	$\sim 1 \times 10^{-4}$	$\sim 5 \times 10^{-4}$ (a)	TraN	CP001122.1	Ott et al., 2020
TP123	HI1	Sm, Cm, Su, Sp	*Salmonella enterica* subsp. *enterica* serovar Typhi	*Escherichia coli* Nissle1917	*Escherichia coli* Nissle1917	8.05×10^{-3}	Not detected (a)	TraN	MN626602.1	Neil et al., 2020
R64	I1α	Sm, Tc	*Salmonella enterica* subsp. *enterica* serovar Typhimurium	*Escherichia coli* Nissle1917	*Escherichia coli* Nissle1917	5.51×10^{-4}	1.54×10^{-6} (a)	PilV	NC_005014.1	Neil et al., 2020
p2kan	I1	Km	*Salmonella enterica* subsp. *enterica* serovar Typhimurium	*Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344	*Escherichia coli*	7.53×10^{-3} to 5.20×10^{-9}	$\sim 1 \times 10^{0}$ (a)	PilV	Not deposited	Stecher et al., 2012
pHUSEC41-1	I1	Su, Ap, Sm, Pip	*Escherichia coli* HUSEC41	*Escherichia coli*	*Escherichia coli*	Not reported	Not reported (a)	PilV	NC_018995.1	Gumpert et al., 2017
pES1	I1	Tc, Su, Tr	*Salmonella enterica* subsp. *enterica* serovar Infantis	*Escherichia coli*	*Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344	1.2×10^{-6}	2×10^{-7} (a)	PilV	NZ_CP047882.1	Aviv et al., 2016
TP114	I2	Km	*Escherichia coli*	*Escherichia coli* Nissle1917	*Escherichia coli* Nissle1917	7.05×10^{-3}	1.12×10^{-1} (a)	PilV	MF521836.1	Neil et al., 2020
pIP69	L/M	Ap, Km, Tc	*Salmonella enterica* subsp. *enterica* serovar Paratyphi	*Escherichia coli* Nissle1917	*Escherichia coli* Nissle1917	9.73×10^{-7}	Not detected (a)	Not reported	MN626603	Neil et al., 2020
RP1/RP4	P1α	Ap, Km, Tc	*Pseudomonas aeruginosa*	*Escherichia coli* HB101	*Escherichia coli* X7	2.05×10^{-1}	9.21×10^{-5} (a)	None	BN000925.1	Rang et al., 1996
				Escherichia coli BJ4	*Escherichia coli* BJ4	2.56×10^{-2}	Not detected*** (a)	None	BN000925.1	Licht et al., 2003
pRK24 (derived from RK2)		Ap, Tc	*Enterobacter aerogenes*	*Escherichia coli* Nissle1917	*Escherichia coli* Nissle1917	4.07×10^{-1}	Not detected (a)	None	Not deposited	Neil et al., 2020
pRts1	T	Km, Sp	*Proteus vulgaris*	*Escherichia coli* Nissle1917	*Escherichia coli* Nissle1917	2.63×10^{-4}	Not detected (a)	TraN	MN626604	Neil et al., 2020
R388	W	Su, Tm	*Escherichia coli*	*Escherichia coli* Nissle1917	*Escherichia coli* Nissle1917	3.09×10^{-4}	Not detected (a)	None	NC_028464	Neil et al., 2020
				Escherichia coli UB1832	*Escherichia coli* UB281	~ 1 (10^{0})	$\sim 1 \times 10^{-4}$ (a)	None	NC_028464	Duval-Iflah et al., 1994, 1998
R6K	X2	Ap, Sm	*Escherichia coli*	*Escherichia coli* Nissle1917	*Escherichia coli* Nissle1917	1.21×10^{-2}	2.5×10^{-4} (a)	Not reported	LT827129.1	Neil et al., 2020

**Derived from pAMβ1 conjugative plasmid, has pBR322 origin of replication.*
***Measure taken for conjugation on agar plate.*
****In conditions not selecting for transconjugants.*
*****Conjugative transposons integrate into the chromosome of their host, and hence, plasmid incompatibility groups do not apply.*
†Transconjugants/recipients.
(a) Mice model; (b) Hamster model; (c) Pig model; (d) rat model.
MPS family is indicated as "not reported" when the exact mechanism has not been described or with "none" when experimental evidence show that this function is absent.

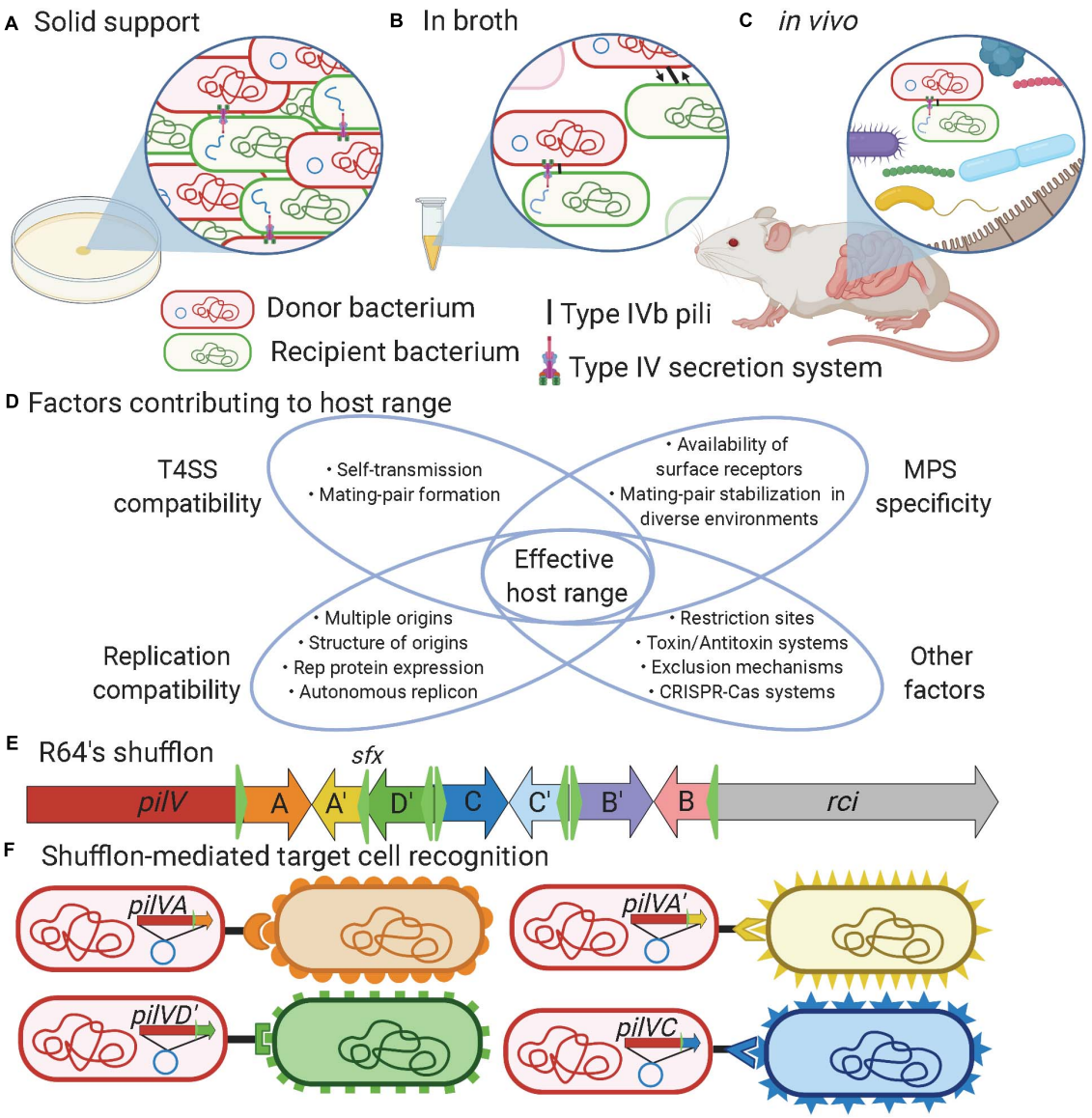

FIGURE 1 | Factors influencing bacterial conjugation. **(A)** Bacterial conjugation taking place on solid support provides high cell density and close proximity between donor and recipient cells that facilitate mating-pair formation to enable plasmid transfer. **(B)** Bacteria evolving in liquid environments or **(C)** *in vivo* benefit from mating-pair stabilization (MPS) provided either by F-pili or type IVb pili to bring cells together and keep them in close contact during plasmid transfer. **(D)** Venn diagram showing the factors contributing to the effective host range of a mobile genetic element. **(E)** Schematic representation of R64's shufflon where the C-terminus region of the *pilV* gene can undergo DNA rearrangement catalyzed by the shufflase (*rci*) to allow expression of seven variants of PilV. For example, DNA region A could be exchanged to express A′. **(F)** DNA rearrangement of the shufflon in the donor strain determines recipient specificity when mating occurred *in broth/in vivo* conditions. Created in BioRender.com.

pili (Hospenthal et al., 2017; González-Rivera et al., 2019). In enterobacteria, conjugative pili involved in MPS can be divided into two groups: conjugative pili and type IVb pili (T4Pb) that respectively comprise the *traN* or *pilV* adhesins (Neil et al., 2020). Additional MPS mechanisms might exist, as proposed for plasmid R6K (Neil et al., 2020), since this phenomenon remains poorly characterized in most mobile genetic elements.

Different types of conjugative pilus were reported in enterobacteria (Bradley, 1980), but the most studied is probably the F-pili (Smillie et al., 2010; Arutyunov and Frost, 2013; Koraimann, 2018). The establishment of contact between donor and recipient cells can be considered as the first rate-limiting step in conjugation as well as a key determinant for plasmid host range specificity (Virolle et al., 2020; **Figure 1D**). F-type pili elaborate long, thin and flexible pili that extend by polymerization of the TraA major pilin into a helical filament ranging from 1 to 20 μm in length (Christie, 2016; Koraimann, 2018). Upon contact, the F-pilus retracts, presumably by depolymerization, enabling donor cells to bring the recipient cell into close proximity for the formation of the mating pore (Clarke et al., 2008;

Hospenthal et al., 2017). TraN, also named *tivF6* (Thomas et al., 2017), is an essential component for DNA transfer machinery that promotes the formation of stable donor-recipient mating-pair by interacting with OmpA or lipopolysaccharides (Klimke et al., 2005). F-pili have also been shown to promote biofilm formation, which favors plasmid transfer (Ghigo, 2001).

Type IVb pili encoded on conjugative plasmids are required only for conjugation in broth (Kim and Komano, 1997) or in the gastrointestinal tract but not on solid support (Neil et al., 2020). T4Pb are thin, flexible, helical fibers distinct from the T4SS that are mainly composed of major pilin and PilV minor adhesins that are thought to be localized to the tip of the pilus. A single motor ATPase encoding gene is predicted in T4Pb, making the extension and retraction of the pilus uncertain since two ATPases are generally present in other types of T4P (Craig et al., 2019; Ellison et al., 2019). T4Pb structures can be found encoded in all plasmid families within the I-complex (IncB/O, IncI1, IncI2, IncK, and IncZ), which were grouped based on similar morphological and serological properties of their pili (Falkow et al., 1974; Bradley, 1984; Rozwandowicz et al., 2020). The adhesin gene in I-complex plasmids is generally the last gene of the T4Pb operon and its C-terminal portion comprises a shufflon (**Figure 1E**). The shufflon is a dynamic DNA locus that can be re-arranged by a shufflase, encoded by *rci* (recombinase for clustered inversion), thought to be constitutively active in IncI plasmids (Brouwer et al., 2019). The shufflase recognizes specific DNA sequences called *sfx* (green triangles in **Figure 1E**) and promotes the recombination by inversion between two head-to-head *sfx* sites (Gyohda et al., 2002). This results in variations of the C-terminal sequence of the minor pilin PilV (Komano, 1999), thus changing the specificity of these pili to recognize different structures in lipopolysaccharides (Ishiwa and Komano, 2000) or other cell surface appendages (**Figure 1F**).

FACTORS INFLUENCING CONJUGATION IN THE GUT MICROBIOTA

The gut microbiota is a complex assembly of microorganisms (Lloyd-Price et al., 2016). The high density of bacteria in this environment could thus be seen as a favorable context for conjugative elements to promote their dissemination (Norman et al., 2009). However, several factors that can act at different steps of conjugative transfer can limit the host spectrum or affect the transfer rates of conjugative elements (**Figure 1D**). The first barrier to bacterial conjugation in the gut is the regulation of mobile genetic element transfer genes by environmental conditions (Fernandez-Lopez et al., 2014; Getino and de la Cruz, 2018). For example, plasmid TP114 was found to be active by low oxygen concentrations (Neil et al., 2020). Many conjugative plasmids respond to specific conditions that may not be found in the gut and hence cannot reach high transfer rates in this environment (García-Quintanilla et al., 2008; Aviv et al., 2016; Neil et al., 2020). In some cases, MPS could be essential or significantly increase transfer rates by establishing and stabilizing the contact between the donor and recipient bacteria (Neil et al., 2020). For this purpose, conjugative elements may use adhesins that recognize receptors at the surface of recipient bacteria (Ishiwa and Komano, 2004). However, in certain environmental niches such as in a biofilm, the role of adhesins and MPS might not be as important, allowing the T4SS to enter in contact with potentially more diverse bacterial species (Król et al., 2013). The T4SS of the conjugative element also has to penetrate the recipient bacterium cell wall and membrane. The drastically different structures of Gram-negative and Gram-positive bacteria represent a physical barrier that is likely restraining the host range of some conjugative plasmids (Domaradskiĭ, 1985). Surface or entry exclusion represent more sophisticated mechanisms that impact conjugation (Garcillán-Barcia and De La Cruz, 2008; Arutyunov and Frost, 2013). In addition, DNA molecules that are successfully transferred must not be targeted by restriction enzymes or CRISPR-Cas systems (Wilkins, 2002; Garneau et al., 2010; Roy et al., 2020). Conjugative plasmids also have to interact with the cellular machinery of their new host to allow the expression of their genes and their maintenance. Establishing the host range of a particular conjugative element is thus a complex task that requires careful investigation of several factors (Jain and Srivastava, 2013) such as the environmental conditions, the nature of the host and recipient bacteria along with other key phenomena such as MPS, MPF, gene expression, and plasmid replication (**Figure 1D**).

THE RELATION BETWEEN CONJUGATIVE PLASMIDS IN THE GUT

Most *in situ* conjugation studies to date have used simplified models involving a single conjugative element present in the donor bacterium (Neil et al., 2020; Ott et al., 2020). This does not necessarily represent natural conditions as gut *Enterobacteriaceae* isolates often harbor multiple plasmids (Lyimo et al., 2016; Martino et al., 2019). Mobile genetic elements were shown to have complex relationships (Getino and de la Cruz, 2018). Some conjugative plasmids, such as IncI plasmids, encode transcription factors that inhibit IncF plasmid conjugation (Gasson and Willetts, 1975, 1976, 1977; Gaffney et al., 1983; Ham and Skurray, 1989). In other cases, the regulatory proteins from a conjugative plasmid or an integrative and conjugative element (ICE) can activate gene expression in other mobile genetic elements such as genomic islands. In an elegant study, it was also shown that some mobile genetic islands such as SGI1, encodes for T4SS subunits that can reshape the mating apparatus of IncC plasmid pVCR94 to promote SGI1 self-propagation over pVCR94 conjugation (Carraro et al., 2017). SGI1 was also found to destabilize pVCR94 maintenance mechanisms. Examples of these types of relationships are plentiful, illustrating how frequent the interaction between mobile genetic elements must be in natural environments (Harmer et al., 2016).

Some plasmids, such as the P-type systems (RP4, R388, and pKM101) lack MPS and display lower conjugation rates in unstable environments such as culture broth or the gut microbiota (Chandran Darbari and Waksman, 2015; Neil et al., 2020). For instance, IncP plasmid RP4 showed no transfer in the intestinal tract in absence of antibiotic selection for the

transconjugants (Licht et al., 2003). However, conjugation was shown to have implications in the stability of IncP plasmid pKJK5 in the intestinal microbiota of germ-free rats (Bahl et al., 2007). Other evidence suggests that these plasmids could highjack MPS mechanisms from other conjugative elements found in the same donor cells in a parasitic manner (Gama et al., 2017). This strategy could be beneficial to some plasmids, allowing their own transfer in a stable environment while taking advantage of other plasmids MPS systems in unstable environments. Therefore, plasmids that do not encode MPS systems should not be deemed strictly incapable of transferring in the gut microbiota. Additional work will be needed to evaluate, characterize and quantify this phenomenon and could bring new insights on the mobility of genes in the gut microbiota.

CONCLUSION AND APPLICATIONS OF THIS KNOWLEDGE

Bacterial conjugation can reach high transfer rates in the gut microbiota. Direct evidence suggests that MPS plays an important role in this environment but the genes that are involved in this mechanism are not encoded in all plasmid families (Neil et al., 2020). MPS has been overlooked by many groups since it is not required in classical bacterial conjugation assays on agar plates where cells are already in close contact. Plasmids encoding MPS genes could hence be seen as the most versatile conjugation machinery since they can promote DNA transfer under a wider diversity of conditions. Conjugative elements that do not encode MPS mechanisms could exploit plasmids that possess this feature to promote their dissemination. Understanding the interactions between plasmids in the gut microbiota could thus provide important insights on the dissemination of antibiotic resistance.

Alternatives to conventional antibiotics include, among other, vaccines (Scully et al., 2015), phage therapy (Ando et al., 2015; Nobrega et al., 2015), predatory bacteria (Dwidar et al., 2012), and anti-plasmid or anti-conjugation strategies (Thomas and Nielsen, 2005; Williams and Hergenrother, 2008; Oyedemi et al., 2016; Cabezón et al., 2017; Getino and de la Cruz, 2018). Inhibiting horizontal gene transfer in the intestinal microbiota will require the identification of potential drug targets. Given that MPS appears to be important for bacterial conjugation in the gut (Neil et al., 2020), strategies to limit or abolish this function could lower the spread of antibiotic resistance (Craig et al., 2019). This type of technology could be used in conjunction with antibiotic treatments or before medical procedures to limit the risk of resistance to treatment (Buelow et al., 2017).

Increased knowledge of bacterial conjugation *in situ* will also be instrumental to the development of microbiome editing technologies. Using a highly effective conjugative system, genes providing benefits to their host could be transferred and integrated into the chromosome of natural residents of the microbiota, avoiding probiotic colonization resistance (Ronda et al., 2019). This DNA mobilization technology could also be used as a CRISPR-Cas delivery vehicle (Bikard et al., 2014; Citorik et al., 2014; Yosef et al., 2015; Getino and de la Cruz, 2018; Neil et al., 2019). CRISPR could be programmed to eliminate specific bacteria causing dysbiosis, antibiotic-resistant bacteria, or pathogens, providing a precision tool for microbiome editing (Bikard and Barrangou, 2017). One could also imagine that MPS could be tuned to facilitate transfer to targeted bacterial populations while leaving other microorganisms untouched by the procedure. The study of bacterial conjugation could thus provide important knowledge that could be applicable in several aspects of the fight against antibiotic resistance.

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KN, NA, and SR contributed to the initial conceptualization of the review. KN and NA did initial literature reviews and manuscript drafting. SR contributed to the literature review and extensive manuscript editing. All authors contributed to the final proofs and approved the submitted version.

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Conflict of Interest: The authors have filed a patent application for the use of conjugative plasmids for microbiome editing. KN and SR are co-founders of TATUM bioscience.

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A Conjugative MDR pMG1-Like Plasmid Carrying the *lsa*(E) Gene of *Enterococcus faecium* With Potential Transmission to *Staphylococcus aureus*

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lsa(E) is a pleuromutilin, lincosamide, and streptogramin A (PLSA phenotype) resistance gene that was first described in *S. aureus* and was thought to have been transferred from *Enterococcus* sp. This study aimed to elucidate the prevalence of the *lsa*(E) gene among *E. faecium* isolates at a tertiary teaching hospital and to evaluate the transferability of the *lsa*(E) gene from *E. faecium* to *S. aureus in vitro*. A total of 96 *E. faecium* strains isolated from one hospital in Beijing in 2013 were analysed for quinupristin-dalfopristin (QDA) resistance genes, and multilocus sequence typing (MLST) was performed. The transferability of QDA resistance between ten *E. faecium* strains and four *S. aureus* strains was determined by filter mating. Genome sequencing of the transconjugant was performed. A total of 46 *E. faecium* isolates (46/96, 47.92%) tested positive for *lsa*(E), while two isolates (2/96, 2.08%) tested positive for *lsa*(A). Thirty-six *lsa*(E)-positive strains (36/46, 78.3%) belonged to ST78. Among 40 mating tests, *lsa*(E) was successfully transferred through one conjugation at a frequency of 1.125×10^{-7} transconjugants per donor. The QDA resistance of the transconjugant N7435-R3645 was expressed at a higher level (MIC = 16 mg/L) than that of the parent *S. aureus* strain (MIC = 0.38 mg/L). Next-generation sequencing (NGS) analysis of the transconjugant N7435-R3645 showed that the complete sequence of the *lsa*(E)-carrying plasmid pN7435-R3645 had a size of 92,396 bp and a G + C content of 33% (accession no. MT022086). The genetic map of pN7435-R3645 had high nucleotide similarity and shared the main open reading frame (ORF) features with two plasmids: *E. faecium* pMG1 (AB206333.1) and *E. faecium* LS170308 (CP025078.1). The *rep* gene of pN7435-R3645 showed 100% identity with that of pMG1, although it did not belong to the *rep*1-19 family but instead a unique *rep* family. Multiple antibiotic resistance

genes, including *lsa*(E), *aadE* and *lnu*(B), *erm*(B), *ant6*-Ia, and *lnu*(B), were present on the plasmid. In conclusion, an *lsa*(E)-carrying plasmid that can be transferred by conjugation from *E. faecium* to *S. aureus in vitro* was identified. This multidrug resistance (MDR) pMG1-like plasmid may act as a vector in the dissemination of antimicrobial resistance among species.

Keywords: *Enterococcus faecium*, conjugative plasmid, *lsa*(E), *Staphylococcus aureus*, quinupristin/dalfopristin

INTRODUCTION

Enterococci and *Staphylococcus aureus* are well-documented opportunistic pathogens. Due to the emergence of antimicrobial resistance as a result of antibiotic overuse, a great concern is infection by methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* species (VRE), which can lead to increased treatment failure and higher mortality rates (Blot et al., 2002; Yaw et al., 2014). Antibiotic resistance in *S. aureus* can emerge through point mutations or horizontal transfer of mobile genetic elements (MGEs). Genetic exchange of genes coding for antibiotic resistance between enterococci and *S. aureus* has been reported for genes such as the vancomycin resistance gene *vanA* (Weigel, 2003), the tetracycline resistance gene *tetM* (Leon-Sampedro et al., 2016), the trimethoprim resistance gene *dfrK* (Lopez et al., 2012), the multiresistance gene *cfr* (Liu et al., 2012) and the macrolide resistance gene *erm*(B) (Wan et al., 2016).

Quinupristin-dalfopristin (QDA) is a semisynthetic 70:30 mixture of streptogramin A and B and is used mainly for the treatment of glycopeptide-resistant *Enterococcus faecium* (GRE) and MRSA infections. The two mixture components act synergistically on the bacterial 50S ribosomal subunit, inhibiting protein synthesis. Resistance to streptogramin B does not confer resistance to QDA, while resistance to streptogramin A does (Hancock, 2005). Resistance to streptogramin A-type antibiotics can be caused by different mechanisms, such as the acetyltransferase Vat (Allignet et al., 1993), the ABC transporters Vga (Allignet et al., 1992, 1998; Kadlec and Schwarz, 2009; Schwendener and Perreten, 2011) and Lsa (Wendlandt et al., 2013b), and the methyltransferase Cfr (Long et al., 2006).

The *lsa*(E) gene was first described in three *S. aureus* strains of human origin, namely, one MRSA ST398-t011 strain and two methicillin-susceptible *S. aureus* (MSSA) ST9-t337 strains, and encodes an ABC transporter of unknown function (Wendlandt et al., 2013b). The *lsa*(E) gene was identified as a macrolide-lincosamide-streptogramin (MLS) resistance gene and was speculated to have been transferred from *Enterococcus* (Wendlandt et al., 2013b). The *lsa*(E) gene has been described not only in *S. aureus* but also in coagulase-negative staphylococci (CoNS) and other species, such as *Erysipelothrix rhusiopathiae*, *Streptococcus suis*, and *Streptococcus agalactiae* (Montilla et al., 2014; Wendlandt et al., 2015; Zhang et al., 2015; Huang et al., 2016). It is most often located in a multiresistance region in chromosomal DNA (Wendlandt et al., 2013b, 2014, 2015; Sarrou et al., 2016; Deng et al., 2017) and is sometimes detected on plasmids (Li et al., 2013; Wendlandt et al., 2013a).

We previously demonstrated that 98% (44/45) of QDA-resistant *S. aureus* isolates sampled from slaughter pigs in northeastern China harboured *lsa*(E) (Yan et al., 2014). Genome sequencing of the *lsa*(E)-positive strains revealed that the transposon with the *lsa*(E) gene cluster showed similarity to the plasmid pEF418 of *E. faecalis* and the plasmid pXD4 of *E. faecium* (Yan et al., 2016). However, limited information is known about the presence of the *lsa*(E) gene in *E. faecium* strains isolated from inpatients in China and the transferability of the *lsa*(E) gene between *E. faecium* and *S. aureus*.

The objective of this study was to elucidate the prevalence of the *lsa*(E) gene among *E. faecium* strains isolated at a tertiary teaching hospital and to evaluate the transferability of the *lsa*(E) gene from *E. faecium* to *S. aureus in vitro*.

MATERIALS AND METHODS

Bacterial Isolates

A total of 96 *E. faecium* strains isolated from one hospital in Beijing in 2013 were analysed in the present study (**Supplementary Table 1**). The isolates were identified as *E. faecium* using a Vitek-2 microbiology analyser (bioMérieux, Marcy l'Etoile, France).

Antimicrobial Susceptibility Testing and QDA Resistance Gene Detection

The susceptibility to 13 antimicrobial agents—ampicillin, penicillin, erythromycin, ciprofloxacin, levofloxacin, nitrofurantoin, tetracycline, vancomycin, linezolid, quinupristin/dalfopristin, tigecycline, high-level gentamicin and streptomycin—was tested with a Vitek-2 microbiology analyser according to the manufacturer's instructions. QDA resistance was reconfirmed by Etest (bioMérieux SA, Marcy l'Etoile, France). The minimum inhibitory concentrations (MICs) for all the antimicrobials were interpreted using Clinical and Laboratory Standards Institute (CLSI) criteria [Clinical and Laboratory Standards Institute (CLSI), 2021].

All the isolates were investigated for the QDA resistance genes *lsa*(A), *lsa*(C), *lsa*(E), *vat*D, *vat*E, *vatH*, and *vga*D by PCR, and *eat*(A) mutations, which are designated *eat*(A)v, were checked by sequencing (**Supplementary Table 2**).

Multilocus Sequence Typing (MLST)

MLST of *E. faecium* isolates was performed by amplifying seven housekeeping genes—*adk*, *atpA*, *ddl*, *gyd*, *gdh*, *purK* and *pstS*—as described previously (Homan et al., 2002). The sequences were

submitted to the MLST website for *E. faecium*[1], and sequence types (STs) were assigned according to the allelic profiles. The clonal complex (CC) was analysed with goeBURST v1.2.1.

Mating Experiments

The transferability of QDA resistance was determined by performing filter mating. Ten rifampin-susceptible *E. faecium* strains (9200, P9772, 5118, 6354, 6474, 3240, 4103, N7435, P2505 and P3814) harbouring *lsa*(E) were randomly selected as donors for the mating experiments. The recipients were four clinical *lsa*(E)-negative, rifampin- and methicillin-resistant *S. aureus* isolates (109, R3645, R3680, and 121) that were plasmid-free after plasmid extraction (**Table 1**). A donor:recipient ratio of 1:9 was used for the mating experiments (Tomita et al., 2002). Selection was performed on brain-heart infusion agar (BHI, OXOID LTD., Basingstoke, Hampshire, England) supplemented with 4 or 8 mg/L virginiamycin and 128 mg/L rifampicin. Rifampicin- and virginiamycin-resistant colonies of putative *S. aureus* transconjugants were isolated and identified by *lsa*(E) PCR. QDA was determined by Etest for the *lsa*(E)-positive transconjugant. The microdilution broth method was used to determine the MICs of 18 antimicrobial agents, namely, penicillin, cefoxitin, tetracycline, chloramphenicol, ciprofloxacin, gentamicin, rifampicin, vancomycin, nitrofurantoin, trimethoprim-sulphamethoxazole, erythromycin, teicoplanin, clindamycin, linezolid, tigecycline, mupirocin, fusidic acid, and daptomycin. The transfer frequency was expressed as the number of transconjugants per donor.

SmaI- and *S1* Nuclease (*S1*)-Pulsed-Field Gel Electrophoresis (PFGE), Southern Blotting and Hybridisation Assays

Transconjugants were further confirmed by Southern blotting. *SmaI*- and *S1*-PFGE analyses were performed as described previously (Tomita et al., 2002; Yan et al., 2011). Southern blotting was performed using a DIG High Prime DNA labelling and Detection Starter Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The digoxigenin-labelled *lsa*(E)-specific probe was prepared using primers (forward 5′-ACAGCGAGTTGTTTCCTGCT-3′; and reverse 5′-GCACGTTTCATCGCTTTTGC-3′) that amplified a 410-bp region of the *lsa*(E) gene. After *S1*-PFGE, the DNA was transferred to a nylon membrane (Hybond N, Amersham, United Kingdom) that was hybridised with the prepared *lsa*(E)-specific probe. Detection was performed using an NBT/BCIP colour detection kit (Roche, Switzerland).

Transconjugant Stability

The stability of the *lsa*(E)-carrying transconjugants was evaluated by daily serial passage on antibiotic-free blood agar. Colonies were tested daily for *lsa*(E) by PCR. The stability of the *lsa*(E)-carrying plasmid was also evaluated by growing on virginiamycin (4 and 8 mg/L) MH agar after storage at 4 and −80°C for 4 weeks.

[1]https://pubmlst.org/bigsdb?db=pubmlst_efaecium_seqdef

Plasmid Sequencing, Assembly and Annotation

The transconjugant N7435-R3645 genome (named with donor and recipient strains) was extracted using a commercial kit (Promega, Madison, United States). Genome sequencing was performed by using the Illumina HiSeq 4000 platform and PacBio RS II platform (10 kb insert library; Pacific Biosciences, Menlo Park, CA, United States) at the Beijing Genomics Institute (BGI, Shenzhen, China).

De novo assemblies and contig assembly for the plasmid pN7435-R3645 of transconjugant N7435-R3645 were performed using Soapdenovo 2.0. Open reading frames (ORFs) were predicted with GeneMarkS.[2] The overlapping regions were found by BLASTing the sequences of the beginning and the end of the final contig. The closed plasmid was confirmed by PCR (JH-F 5′-CTCTACCAGATGGTTGGAGCA-3′; JH-R 5′-CCTACGATCACGGCACCAAT-3′) and Sanger sequencing of the resulting amplicons. The plasmid nucleotide sequences were compared with sequences in the GenBank database using BLASTN.[3]

Nucleotide Sequence Accession Number

The sequence of the conjugated plasmid pN7435-R3645 was deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession number MT022086.

RESULTS

Antimicrobial Susceptibility

For the *E. faecium* isolates, the resistance rates to ampicillin, penicillin, erythromycin, ciprofloxacin, levofloxacin, nitrofurantoin, gentamicin, streptomycin, tetracycline and vancomycin were 91.67, 92.71, 94.79, 92.71, 92.71, 75.00, 66.67 56.25, 31.25 and 20.83%, respectively. A low resistance rate was observed for linezolid (1.04%) and tigecycline (1.04%).

Antimicrobial Resistance Genotype and Phenotype of QDA Resistance in *E. faecium* Strains

A total of 46 *E. faecium* isolates (46/96; 47.92%) tested positive for *lsa*(E), while two isolates (2/96; 2.08%) tested positive for *lsa*(A). The *eat*(A)v mutation (C1349T) was found in 41 of 96 *E. faecium* isolates. The *vatD, vatE, vatH, vgaD* and *lsa*(C) genes were not detected in any of the isolates. Four antibiotic resistance gene profiles were observed, namely, *lsa*(E) (n = 27), *eat*(A)*v* (n = 22), *lsa*(E)-*lsa*(A)-*eat*(A)*v* (n = 2), and *lsa*(E)-*eat*(A)*v* (n = 17) (**Table 2**).

QDA resistance was observed in 9 isolates (9.37%; 9/96), while 53 isolates (55.2%; 53/96) showed intermediate susceptibility. The majority of the *lsa*(E)-carrying strains (43/46; 93.48%) showed QDA resistance or an intermediate susceptible phenotype.

[2]http://topaz.gatech.edu/

[3]http://blast.ncbi.nlm.nih.gov/blast

TABLE 1 | Background of donor and recipient strains.

Donor/Recipient	Species	Strain name	MLST	*spa*	Antibiotic resistance profile[a]
Recipient	*S. aureus*	109	ST239	t1152	FOX-TC-GM-CI-EM-CM-RI
Recipient	*S. aureus*	R3645	ST239	t037	FOX-TC-GM-CI-EM-CM-RI
Recipient	*S. aureus*	R3680	ST239	t037	FOX-TC-GM-CI-EM-CM-RI
Recipient	*S. aureus*	121	ST239	t030	FOX-TC-GM-CI-EM-CM-RI
Donor	*E. faecium*	9200	ST747	–	
Donor	*E. faecium*	P9772	ST923	–	
Donor	*E. faecium*	5118	ST18	–	
Donor	*E. faecium*	6354	ST78	–	
Donor	*E. faecium*	6474	ST78	–	
Donor	*E. faecium*	3240	ST78	–	
Donor	*E. faecium*	4103	ST571	–	
Donor	*E. faecium*	N7435	ST18	–	
Donor	*E. faecium*	P2505	ST78	–	
Donor	*E. faecium*	P3814	ST78	–	

[a]*FOX, cefoxitin; TC, tetracycline; GM, gentamicin; CI, ciprofloxacin; EM, erythromycin; CM, clindamycin; RI, rifampin; QDA, quinupristin/dalfopristin.*

TABLE 2 | Quinupristin-dalfopristin (QDA) resistance gene profiles and ST types in *E. faecium* strains isolated from patients.

Quinupristin-dalfopristin (QDA) resistance gene profiles	Number of isolates	QDA phenotype			ST types (No. of isolates)	Clonal complex (No. of isolates)
		R	I	S		
lsa(E)	27	3	22	2	ST78 (23) ST18 (3) ST17 (1)	CC17 (27)
lsa(E)- eat(A)v	17	0	16	1	ST78 (13) ST571 (2) ST30 (1) ST414 (1)	CC17 (16) CC293 (1)
lsa(E)- lsa(A)- eat(A)v	2	2	0	0	ST747 (1) ST923 (1)	CC17 (1) Singleton (1)
eat(A)v	22	4	15	3	ST78 (14) ST812 (4) ST341 (2) ST94 (1) ST414 (1)	CC17 (17) CC39 (4) CC94 (1)
no QDA resistance gene	28	0	0	28	ST78 (14) ST18 (4) ST922 (3) ST812 (2) ST17 (1) ST389 (1) ST564 (1) ST921 (1) ST923 (1)	CC17 (26) CC39 (2)

Among the *lsa*(E)-positive strains, two strains carrying *lsa*(E)-*lsa*(A)-*eat*(A)*v* showed high QDA MIC values of 24 and 6 mg/L.

Molecular Characterisation of *E. faecium* Isolates

MLST for all the isolates revealed fifteen ST types that belonged to four clonal complexes and one singleton (**Table 2**). ST78 (CC17) was the most frequent ST type and was identified in 64 of 96 isolates (64/96, 66.7%), followed by ST18 (CC17) (7/96, 7.3%) and ST812 (CC39) (6/96, 6.3%). Moreover, forty-four *lsa*(E)-positive strains (44/46, 95.6%) belonged to CC17.

Conjugative Transfer of *lsa*(E) From *E. faecium* to *S. aureus*

Among the 40 mating tests performed, QDA resistance was successfully transferred in one conjugation at a frequency of 1.125×10^{-7} transconjugants per donor. Transfer occurred from *E. faecium* N7435 to *S. aureus* R3645. The match of the conjugated N7435-R3645 with the recipient was confirmed by comparing their *Sma*I-PFGE profiles (**Supplementary Figure 1**). One extra ∼100 kb band was observed in the N7435-R3645 *Sma*I-PFGE profile. The QDA resistance of conjugated N7435-R3645 was expressed at a higher level (MIC = 16 mg/L) than that of the parent *S. aureus* strain (MIC = 0.38 mg/L) (**Figure 1**). The erythromycin resistance of conjugated N7435-R3645 was also expressed at a higher level (MIC > 2048 mg/L) than that of the parent *S. aureus* strain (MIC = 512 mg/L). There was no difference in the MIC values of the other 17 antibiotics.

Location and Stability of the *lsa*(E) Gene in the Transconjugant

The location of the *lsa*(E) gene in the transconjugant N7435-R3645 was investigated by *S1*-PFGE followed by Southern blotting (**Figure 2**). *S1*-PFGE revealed that recipient R3645 did not harbour plasmids, while transconjugant N7435-R3645 carried a single ∼100 kb plasmid. Hybridisation assays showed that *lsa*(E) was located on the ∼100 kb plasmid in the transconjugant N7435-R3645.

lsa(E) was stable after ten overnight passages on antibiotic-free blood agar. The transconjugant continued to grow on MH agar supplemented with virginiamycin (4 and 8 mg/L) even after storage at 4 and −80°C for 4 weeks.

Characteristics of the Transconjugated Plasmid

Next-generation sequencing (NGS) analysis of the pN7435-R3645 transconjugant showed that the complete sequence of the *lsa*(E)-carrying plasmid pN7435-R3645 was 92,396 bp in size

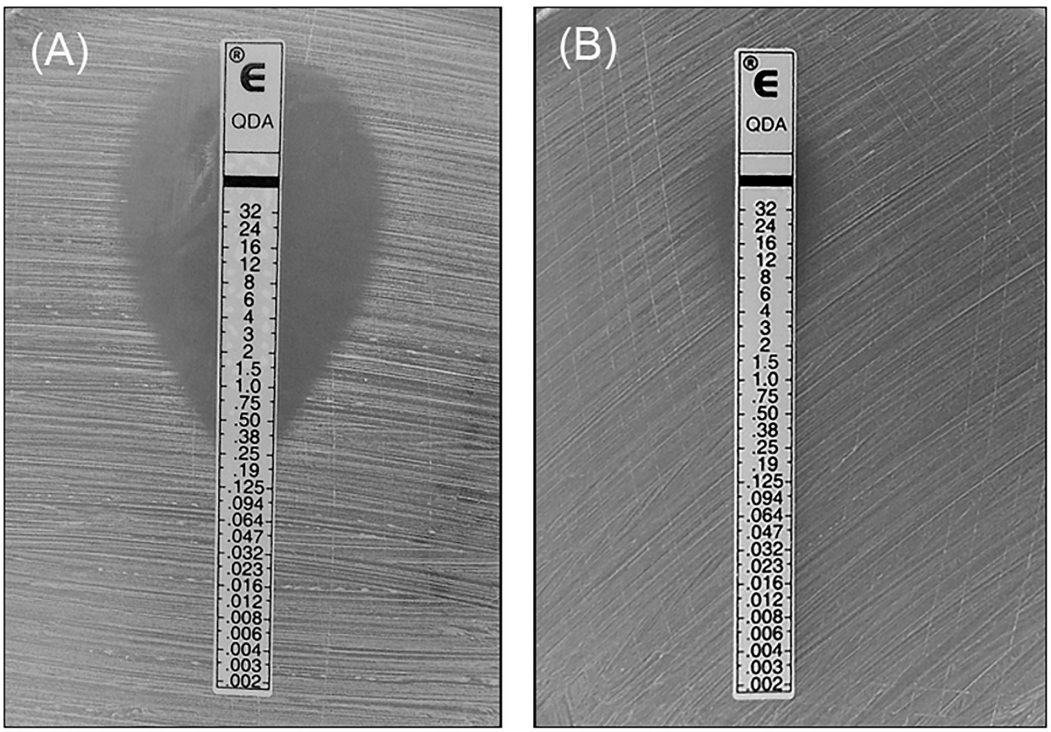

FIGURE 1 | The MIC value of QDA against the recipient strain and the transconjugant strain. **(A)** Recipient strain, R3645; **(B)** Transconjugant strain, N7435/R3645.

and had a G + C content of 33% (accession no. MT022086). Sequence analysis identified 119 ORFs. The genetic map of pN7435-R3645 is shown in **Figure 3** along with the maps of elements showing high nucleotide similarity and the main ORF features with two plasmids, *E. faecium* pMG1 (AB206333.1) and *E. faecium* LS170308 plasmid (CP025078.1).

pN7435-R3645 is highly equivalent to the 1–60,447 bp region of *E. faecium* pMG1, and this region of similarity is divided into two parts by the insertion of the *E. faecium* LS170308 plasmid. The insertion site was in the ORF region of the TraI topoisomerase-encoding gene. The pN7435-R3645 plasmid retained most of the genes from the *E. faecium* pMG1 plasmid and lost mainly the 1,822–6,469 bp and 60,448–64,920 bp regions, corresponding to the aminoglycoside resistance gene (*aac*/*aph*) and insertion sequence (IS) elements, respectively.

The conjugation region (43,513–77,131 bp; G + C content, 32.07%) of the pN7435-R3645 plasmid is approximately 33.6 kb. This region showed 99% identity with the conjugation region of pMG1 (13,600–45,300 bp). The *rep* gene of pN7435-R3645 showed 100% identity with that of pMG1, which did not belong to the *rep*1-19 family but belonged to a unique *rep* family. The IS elements in the pN7435-R3645 plasmid involved in possible recombination processes included mainly the *IS1216* transposase.

The resistance genes on the pN7435-R3645 plasmid were located mainly in the region of similarity with the *E. faecium* LS170308 plasmid. In addition, the plasmid structure was rearranged in these regions. Different AR elements were found in the following two pN7435-R3645 regions:

lsa(E) region (7,987–13,147 bp; G + C content, 35.69%). This region included three AR genes, *lsa*(E), *aadE* and *lnu*(B), which confer resistance to lincosamides/streptogramin A/pleuromutilins, aminoglycosides, and lincosamide, respectively. This region exhibited more than 99% nucleotide identity with multiple plasmids of *E. faecium* (plasmids of *E. faecium* strain LS170308, pEF37BA, pXD5, pY13, *etc.*), *E. faecalis* (pEF418, pE15, p11-27, *etc.*), *E. gallinarum* (pY15), and *S. aureus* (pV7037) as well as the chromosome region of *Streptococcus agalactiae*. This region was flanked by two identical IS1216 transposase genes with the same orientation.

erm(B), *ant6*-Ia, and *lnu*(B) regions (18,097–35,240 bp; G + C content, 35.72%). This segment contained three AR genes, *erm*(B), *ant6*-Ia, and *lnu*(B), which confer resistance to macrolides, aminoglycosides and lincosamide, respectively. This region exhibited 99% nucleotide identity with part of the *E. faecium* strain LS170308 plasmid and was flanked by two identical *IS1216* transposase genes.

DISCUSSION

Plasmids harbour a number of antibiotic genes and are widely found in enterococci, mainly *E. faecalis* and *E. faecium*, which are currently leading causes of multiresistant hospital-acquired infections. Conjugation is a primary means of intercellular DNA transfer in enterococci. Moreover, enterococci are reservoirs for antibiotic resistance genes, which can spread to other important pathogens, most notably *S. aureus*.

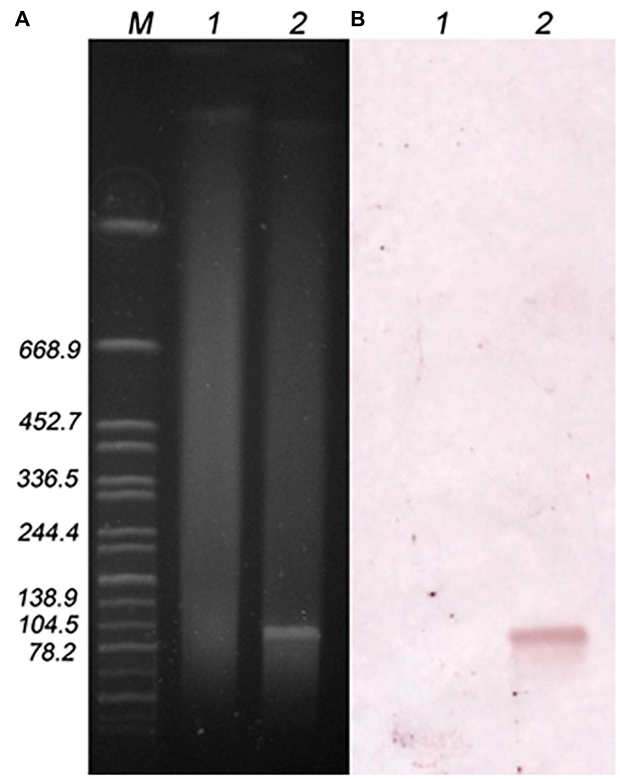

FIGURE 2 | *S1*-PFGE plasmid profile of the recipient and transconjugant strains **(A)** and hybridisation with the *lsa*(E) probe **(B)**. M, Global reference strain H9812 of the *Salmonella* Braenderup serotype digested with the *Xba*I enzyme; lane 1, recipient strain (R3645); lane 2, transconjugant strain (N7435/R3645).

The present study provided the first evidence of the ability of the *lsa*(E) gene to undergo plasmid-mediated transfer and of the ability of an *E. faecium* plasmid carrying a *lsa*(E) gene to replicate in a clinical MRSA strain. To date, only the *van*(A) gene has been shown to be transferred from *E. faecalis* to *S. aureus* by conjugation *in vitro* (de Niederhausern et al., 2011). Peptide sex pheromones secreted by *S. aureus* induce conjugation-related mating functions and may play an important role in Tn*1546*-containing pheromone-responding plasmid transfer in *E. faecalis* (Showsh et al., 2001). To our knowledge, *lsa* (E) is the first gene that has been confirmed to be transferred from *E. faecium* to *S. aureus in vitro* and probably has a different transfer mechanism than the *van*(A) gene. Plasmid pN7435-R3645 in this study retained most of the genes in the *E. faecium* pMG1 plasmid. pMG1, which has been completely sequenced, is a 65 kb conjugative plasmid from *E. faecium* containing a Tn*4001*-like element and is a non-pheromone-responding plasmid (Ike et al., 1998; Tanimoto and Ike, 2008). It can transfer relatively well to other *E. faecium* strains in broth as well as to *E. faecalis* and *E. hirae*. pMG1 family elements have significantly contributed to the spread of vancomycin and gentamicin resistance among enterococci, particularly within *E. faecium* (Tomita et al., 2003). Although insertion of the plasmid pLS170308 region resulted in partial deletion of the TraI topoisomerase-encoding gene, pN7435-R3645 still retained the complete conjugation region of pMG1 (13,600–45,300 bp) (Tanimoto and Ike, 2008). Therefore, we speculated that the horizontal transfer of *lsa*(E) between *E. faecium* and *S. aureus* was dependent mainly on a non-pheromone-responding pMG1-like plasmid through conjugation.

Another *lsa*(E)-carrying non-conjugative plasmid, pY13 (28,489 bp), from a porcine linezolid-resistant *E. faecium* isolate has been reported (Si et al., 2015). The conjugative plasmid pN7435-R3645, approximately 92,396 bp, in this study is much larger than pY13 and has a structure different from that of pY13. Rearrangement and inversion regions were observed on the plasmid pN7435-R3645. Since all of these segments were flanked by ISs, pN7435-R3645 may have derived from interplasmidic recombination events in which ISs, such as IS*1216* and IS*1252*, were involved.

In the present study, four clinical ST239 MRSA strains were selected as recipients, and only one strain (R3645) was successfully transferred. Mutations in genes of the SauI type I restriction-modification (RM) system and deficiency in the type

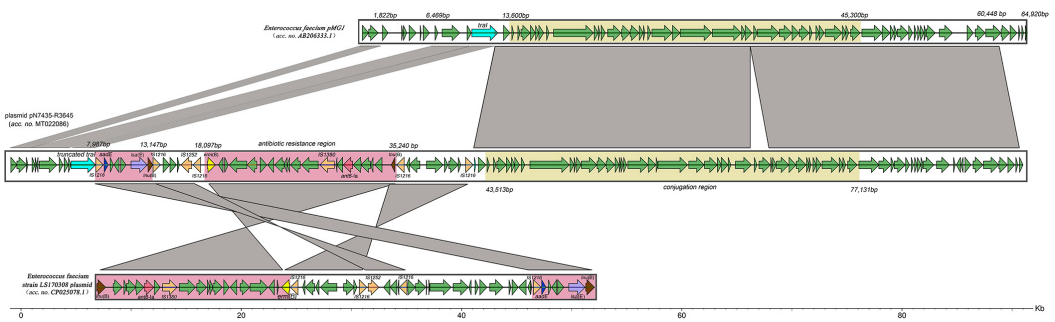

FIGURE 3 | Structure of the *lsa*(E)-carrying plasmid pN7435-R3645 and similar regions in plasmid pMG1 and plasmid LS170308 of *E. faecium*. The structure of the plasmid pN7435-R3645 is illustrated based on the nucleotide sequences deposited in the GenBank database (MT022086). ORFs are indicated by arrows coloured as follows: orange, insertion sequences; purple, *lsa*(E); blue, *aadE*; pink, *ant6*-Ia; brown, *lnu*(B); yellow, *erm*(B); light blue, *traI* or truncated *traI*; and green, other genes. The similarities in the structures are indicated by grey shading. Antibiotic resistance regions and conjugation regions are shown in pink and yellow, respectively.

IV RM system have been shown to increase a strain's ability to accept foreign DNA (Waldron and Lindsay, 2006; Corvaglia et al., 2010). Intact type I and IV RM systems were found in R3645 (data not shown). Other characteristics, such as mutations of CRISPR loci, that may contribute to the ability to acquire *lsa*(E)-carrying non-conjugative plasmids need to be further investigated.

E. faecalis is intrinsically resistant to QDA as a result of the presence of the *lsa* determinant, while *E. faecium* always acquires QDA resistance (Singh et al., 2002). To date, the prevalence of QDA resistance among *E. faecium* clinical isolates in many countries has been low, but relatively high resistance rates have occasionally been reported, such as 6.7% (9/135) in Poland (Sadowy et al., 2013), 10% (25/249) in Korea (Oh et al., 2005), and 60% in northwest Iran (6/10) (Haghi et al., 2019). The rate of intermediate resistance to QDA is relatively high in some countries, such as 17.6% (28/159) in Japan (Isogai et al., 2013), 26.7% (36/135) in Poland (Sadowy et al., 2013) and 28.9% (250/865) in Greece (Karanika et al., 2008). An investigation in a Chinese hospital in Wenzhou reported that 9 of 911 (1.0%) *E. faecium* isolates were resistant to QDA (Wang et al., 2016). In this study, QDA resistance was observed in 9 isolates (9.37%; 9/96), while 53 isolates (55.2%; 53/96) showed intermediate susceptibility. This finding indicated that QDA resistance differed among hospitals and regions in China. Although QDA has not been marketed in China, virginiamycin, which belongs to the same antibiotic class as QDA, has been widely used as an animal growth promoter in poultry, cattle and swine. The resistance of *E. faecium* strains isolated from animals to QDA ranged from 2.2 to 33.6%, and 38.5–83.2% of the strains were classified as not sensitive in European countries from 2004 to 2014 (Wang et al., 2016; de Jong et al., 2019). However, virginiamycin has been banned for use as a growth promoter in Europe since 1999. This may be explained by the possible co-selection of resistance genes by compounds currently approved to treat clinical diseases.

A high prevalence of *lsa*(E) (47.92%, 46/96) was found among clinical *E. faecium* isolates, and the majority of *lsa*(E)-carrying strains (43/46; 93.48%) showed QDA resistance or an intermediate susceptible phenotype in this study. Acetyltransferases encoded by *vat*D and *vat*E have been found in enterococci from various sources, including humans, animals and the environment in Europe, the United States and Asia (Soltani et al., 2000; Werner et al., 2000; Jackson et al., 2007; Hwang et al., 2010); however, the *vatD* and *vatE* genes were not detected in this study. To date, only two papers have reported the distribution of *lsa*(E) in enterococcus, and both papers are from China. The *lsa*(E) gene was found in 30.3% (10/33) of human enterococcal strains and 53.6% (37/69) of swine enterococcal strains in Henan Province, China. Most of them were clonally unrelated, with the exception of *E. faecium* ST29 ($n = 4$) and ST362 ($n = 4$) (Li et al., 2014). The *lsa*(E) gene was also detected in five *E. faecalis* strains, one *E. faecium* strain and one *E. gallinarum* strain among thirty-five enterococcal strains isolated from a pig farm in Guangxi Province, China (Si et al., 2015). In this study, thirty-six *lsa*(E)-positive strains (36/46, 78.3%) belonged to ST78, which is an epidemic clone in hospitals in China (Sun et al., 2019). This result suggested that the high *lsa*(E) detection rate in clinical strains may be due to the spread of *E. faecium*-resistant clones.

In conclusion, a high prevalence of *lsa*(E) was found in clinical *E. faecium* strains. An *lsa*(E)-carrying plasmid that can be transferred from *E. faecium* to *S. aureus in vitro* by conjugation was identified. This MDR pMG1-like plasmid may act as a vector in the dissemination of antimicrobial resistance among species.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MT022086.

AUTHOR CONTRIBUTIONS

X-MY, JW, and J-ZZ conceived the study. X-MY wrote the manuscript and performed the Southern blotting experiment. JW, H-BJ, HY, and Y-HY collected the strains and performed the antibiotic resistance experiments. X-XT, F-LM, BZ, and YH carried out the molecular typing, mating, and QDA resistance gene detection. X-MY and Y-HY analysed the genome sequencing data. J-ZZ revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.667415/full#supplementary-material

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Prevalence of the NTE$_{KPC}$-I on IncF Plasmids Among Hypervirulent *Klebsiella pneumoniae* Isolates in Jiangxi Province, South China

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Infection caused by carbapenem-resistant hypervirulent Klebsiella pneumoniae (CR-hvKP) has become a tricky health care threat in China and KPC-2 enzyme is a main factor mediating resistance to carbapenems of *K. pneumoniae*. Here, we report the characterization of the genetic environment of the blaKPC-2 gene in CR-hvKP clinical isolates from South China. Forty-five non-duplicated CR-hvKP isolates collected in Jiangxi Province from 2018 to 2019 were analyzed. Each of them were multidrug-resistant due to the presence not only of blaKPC-2 gene but also of other resistance determinants, including Metallo-β-lactamases (NDM-1), extended-spectrum β-lactamases (TEM-1, CTX-M-14, SHV-1), and plasmid-mediated quinolone resistance determinants (qnrS, aac(6′)-Ib-cr). After plasmid analyses of PCR-based replicon typing (PBRT), mapping PCR, amplicon sequencing, and whole-genome sequencing (WGS) were used to analyze the genetic environment of the blaKPC-2 gene. PCR analysis of pLVPK-like plasmids, Southern Blot, and mouse lethality assay were used to characterize the virulence phenotype of *K. pneumoniae*. Multilocus sequence typing (MLST) analysis showed ST11 CR-hvKP was the predominant clone. In conclusion, this is the first analysis of diverse genetic structures blaKPC-2 gene in CR-hvKP isolates from south China. Both the NTEKPC-I on the IncF plasmids and pLVPK-like virulence plasmids make contributions to the formation of CR-hvKP especially ST11 which need more attention.

Keywords: carbapenem resistance, hypervirulent *Klebsiella pneumoniae*, NTEKPC-2, IncFII-like plasmids, ST11

INTRODUCTION

Recently, carbapenem-resistant hypervirulent *Klebsiella pneumoniae* (CR-hvKP) have become important pathogens of morbidity and mortality among hospital-acquired and long-term care-associated infections (Gu et al., 2018; Zhang et al., 2020). Regardless which plasmids-associated mechanisms underlying the formation of CR-hvKPs clones, the acquisition of carbapenem resistance plasmids by the hypervirulent *K. pneumoniae* (hvKP) strains or the acquisition of virulence plasmids by the carbapenem-resistant *K. pneumoniae* (CRKP) strains, carbapenem resistance plasmids are the main actors (Wyres et al., 2019).

KPC-2, the most common variant of KPC carbapenemase enzymes, is a main factor mediating resistance to carbapenems of *K. pneumoniae* (Shen et al., 2016). Transmission of the KPC gene, *bla*$_{KPC}$, can be mediated by different molecular mechanisms such as the mobility of small genetic elements, horizontal transfer of plasmids, and the clonal spread (Munoz-Price and Quinn, 2009). In most countries and regions, such as Europe (Naas et al., 2008) and the United States (Chen et al., 2013), *bla*$_{KPC-2}$ is mainly located on *Tn4401* transposon. However, *bla*$_{KPC}$-bearing non-*Tn4401* elements (NTE$_{KPC}$) were first reported on the plasmid pKP048 from a Chinese clinical *K. pneumoniae* isolate in 2009 (Shen et al., 2009). Then, NTE$_{KPC}$ was not only reported in different provinces in China (Li et al., 2016; Wang et al., 2016; Fu et al., 2019), but also in other countries such as Brazil (Cerdeira et al., 2017, 2019) and Singapore (Octavia et al., 2019). In a review about molecular and genetic decoding in carbapenemase-producing *Klebsiella pneumoniae*, NTE$_{KPC}$ has been divided into three groups (NTE$_{KPC}$ I, NTE$_{KPC}$ II, and NTE$_{KPC}$ III) on the basis of the genes adjacent to *bla*$_{KPC}$ (Chen et al., 2014).

The characteristics of this genetic structure, which mobilizes *bla*$_{KPC}$ in CR-hvKP strains within and among different clones or plasmids, is unknown, and understanding it would provide insight into diffusion processes and their evolutionary history. This study aimed to present the genetic environment of *bla*$_{KPC-2}$ in CR-hvKP isolates in Jiangxi Province using a series of PCR assays and whole-genome sequencing.

METHODS AND MATERIALS

Bacterial Isolates and Antimicrobial Susceptibility Testing

A total of 45 non-duplicated CR-hvKP clinical isolates were collected from 11 prefecture-level cities in Jiangxi Province including Nanchang, Jingdezhen, Ganzhou, Jiujiang, Xinyu, Pingxiang, Yingtan, Ji'an, Yichun, Shangrao, and Fuzhou, from January 2018 to December 2019. Among them 38 CR-hvKP clinical isolates were collected from 513 non-duplicated CRKP clinical isolates in the First Affiliated Hospital of Nanchang University. *K. pneumoniae* isolates were identified by an automated Vitek II system (bioMerieux, Balmes-les-Grottes, France) and were further verified with 16S rRNA gene sequencing. According to the latest definition of hvKP (Xu et al., 2019; Zhang et al., 2020), we chose the CRKP strains carrying the pLVPK-like virulence plasmid as CR-hvKP strains in this study. Antibiotic susceptibilities were determined by the disk diffusion method on Mueller-Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Humphries et al., 2018). *K. pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 were used as quality control.

PCR Detection of Resistance Genes, Virulence Genes, and Plasmid Replicon Types

Single PCR was used to analyze quinolone resistance genes [*aac(6′)-Ib-cr*, *qnrA*, *qnrB*, *qnrS*, and *qepA*], ESBLs genes (*bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$), carbapenemase genes (*bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$, *bla*$_{IMP}$, *bla*$_{VIM}$), and pLVPK-related loci (*rmpA*, *iutA*, *rmpA2*), as previously reported (Liao et al., 2020). To confirm the existence of the pLVPK-like plasmid, specific primers of *repA*, *sopB*, *Lv049* were also designed based on a previous study (Zhao et al., 2019). Plasmid incompatibility was analyzed by using PCR-based replicon typing (PBRT)-KIT 2.0 (DIATHEVA, Italy) (Zhou et al., 2020). A PCR mapping approach was carried out to compare the genetic context of the *bla*$_{KPC-2}$ gene in all the CR-hvKP isolates with the *NTE*$_{KPC}$ in plasmid pKP048 (Shen et al., 2009). All the PCR products were purified and sequenced, and their sequences were compared with the reference sequences stored in the GenBank nucleotide database.

PFGE and MLST

S1 Nuclease Pulsed Field Gel Electrophoresis (S1-PFGE) and Southern blotting were used to determine the location of virulence genes. Hybridization were performed with the DIG-High Prime DNA Labeling and Detection Starter Kit II with of the probe *rmpA2* (Roche, Basel, Switzerland) (Li et al., 2020). All the CR-hvKP isolates were subjected to PFGE after digestion with XbaI. The molecular marker was *Salmonella* serotype *Braenderup* strain H9812. The cluster cutoff line at 80% similarity was used to analyze genetic relatedness. MLST of *Klebsiella pneumoniae* was performed online (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html), as previously described (Liao et al., 2020).

Whole-Genome Sequencing and Analysis

Given the PCR mapping analysis, we chose one isolate, respectively, from each different NTE$_{KPC}$ pattern for WGS. Genome sequences were obtained using a combination of Illumina Miseq (150 bp paired-end), and they were assembled with SPAdes version 3.9.1. An average sequencing depth of $\times$64 was achieved for the genomes. Genomics analysis was performed as described in a previous study (Octavia et al., 2019).

Nucleotide Sequence Accession Numbers

The genome sequences of 12 CR-hvKP strains were submitted to GenBank under the bioproject number PRJNA672246.

Mouse Lethality Assay

Determination of the virulence of *K. pneumoniae* in mouse lethality tests and the medium lethal dose (LD50, expressed as colony-forming units) was performed as previously described (Yu et al., 2008). In short, a graded dose of 10^1-10^7 CFU of each strain in 10-fold serial dilutions in 0.1 ml of normal saline was injected intraperitoneally into mice (four mice for each dose of inoculum). The survival rate of all the vaccinated mice was recorded daily in 2 weeks. The hvKP strain NTUH-K2044 and the classic *K. pneumoniae* strain ATCC700603 were used as controls of high and low virulence strains, respectively. The interpretation of virulence was referred to reference (Siu et al., 2012).

Ethics Statement

The study has been approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University. Patients participating in the study were anonymous, as a result of the retrospective study, so informed consent was not obtained.

RESULTS

Prevalence of ESBLs Genes and Quinolone Resistance Genes Among CR-hvKP Clinical Isolates

A total of 45 carbapenem-resistant hypervirulent *K. pneumoniae* isolates were selected from clinical specimens including 19 from sputum, 19 from blood, 4 from urine, 1 from pus, 1 from a deep vein catheter, and 1 from ascites. As shown in **Supplementary Table 1**, almost all the CR-hvKP isolates were resistant to 18 antibiotics commonly used in clinical treatment, except for some isolates that were sensitive to sulfamethoxazole, tobramycin, and amikacin. As shown in **Table 1**, all of the CR-hvKP isolates were found to carry carbapenemase gene bla_{KPC-2}, three of which were found to co-carry carbapenemase gene bla_{NDM-1}. Almost all of the CR-hvKP isolates were found to carry at least one ESBLs gene (bla_{CTX-M}, bla_{SHV}, bla_{TEM}) and most of them were found to carry quinolone resistance gene *qnrS* and *aac(6′)-Ib-cr*.

Virulence Assessment of CR-hvKP Clinical Isolates

In this scenario, PCR analysis of pLVPK-like plasmids revealed that all the CR-hvKP isolates were found to carry the pLVPK-like plasmid. These results were also verified by S1-PFGE and Southern blotting as shown in **Supplementary Figure 1**. However, PCR analysis of virulence genes revealed that not every isolate was found to carry the complete pLVPK-related loci (*rmpA, iutA, rmpA2*). The mouse lethality assay also proved that all the CR-hvKP isolates having the 50% lethal dose (LD50) of $< 10^5$ CFU were hypervirulent, and were a little lower virulent than the hvKP strain NTUH-K2044 having the LD50 of $< 10^2$ CFU, while classic *K. pneumoniae* strain ATCC700603 had the LD50 of more than 10^7 CFU.

Genetic Linkage of bla_{kpc-2} Among the CR-hvKP Isolates

The analysis of the genetic environment of bla_{KPC-2} genes showed five distinct NTE patterns compared with the classic *Tn3*-based structure in the plasmid pKP048. All the different NTE patterns were classified as NTE$_{KPC}$-I. In detail, the distribution of different NTE patterns among these clinical CR-hvKP strains was shown in the **Figure 1**.

NTE$_{kpc}$-I Plasmids Diversity

Almost all the different NTE patterns carrying *blaKPC-2* were shown to be carried on the IncF plasmids. Interestingly untyped plasmid replicons were detected in 11 CR-hvKP isolates (**Table 1**). As shown in **Figure 2**, these NTE$_{KPC}$ plasmids also had diversity of gene structure. Plasmid gene structure differences existed in the same ST (ST11) clinical *K. pneumoniae* strains. JX-CR-hvKP-9-p1 and JX-CR-hvKP-9-p4 from the same isolate had the totally different plasmid gene structure, the same goes for JX-CR-hvKP-10-p1 and JX-CRhvKP-10-p3. Interestingly JX-CR-hvKP-6-p2 and JX-CR-hvKP-5-p2 from ST23 CR-hvKP had the same plasmid gene structure, while JX-CR-hvKP-8-p2, JX-CR-hvKP-7-p2, JX-CR-hvKP-9-p1, JX-CR-hvKP-10-p1 from ST11 CR-hvKP also had highly similar plasmid gene structure.

Molecular Characteristics

The PFGE-based fingerprints of the CR-hvKP isolates displayed three different clusters (named A–C) using a similarity cutoff value of 80% (**Figure 3**), including cluster A (34/45, 75.6%), cluster B (7/45, 15.6%), and cluster C (4/45, 8.8%). The MLST analysis distinguished two different STs. The most prevalent ST in CR-hvKP isolates was ST11 (41/45, 91.1%), followed by ST23 (4/45, 8.9%).

DISCUSSION

Over the past few decades, hvKP has emerged worldwide, causing invasive infections since the first clinical hvKP report was published in 1986 (Russo and Marr, 2019). Although initially reported hvKP isolates were usually antimicrobial sensitive, clonal complexes of hypervirulent (hvKP) and carbapenem-resistant (CR) strains are non-overlapping (Liao et al., 2020). In this study, we collected 45 CR-hvKP strains causing nosocomial infections, including 41 ST11 CRKP acquiring the pLVPK like plasmid, 4 ST23 hvKP acquiring the carbapenemase plasmid. It was consistent with the evolution of CR-hvKP in a Chinese multicenter and molecular epidemiological analysis that the KPC-2-producing ST11 clone was the common type of CR-hvKP isolates (Zhang et al., 2020).

PCR analysis of *repA*, *sopB*, *Lv049* and Southern Blot revealed that all the CR-hvKP carrying pLVPK-like virulence plasmids were identified in this study. PCR analysis of pLVPK-related loci (*rmpA, iutA, rmpA2*) revealed that some virulence genes such as *rmpA*, *terW* were lost in a few CR-hvKP isolates. Maybe some virulence genes such as *rmpA*, *terW* were lost at the time of pLVPK-like plasmid transferring. It was consistent with the findings in a previous study about core genome allelic profiles of clinical *Klebsiella pneumoniae* strains based on multi-locus sequence typing scheme for hypervirulence analysis (Lan et al., 2020). Moreover, mouse lethality assay revealed that all the CR-hvKP isolates had a 50% lethal dose (LD50) of $< 10^5$ CFU, while classic *K. pneumoniae* (cKP) strain ATCC700603 had the LD50 of more than 10^7 CFU. To our best knowledge, at present, the mouse lethality assay is the most standard method to differentiate hvKP from cKP (Russo and MacDonald, 2020).

This study first provides key insights into the horizontal transfer of the NTE$_{KPC}$ and IncF plasmids, which appears to be a potential element driving the molecular diversification in ST11 CR-hvKP isolates. Transposon elements are believed to be responsible for the rapid spread of bla_{KPC} (Chen et al., 2011; Zhang et al., 2012). In China, a different genetic organization of the bla_{KPC} locus from the "traditional" Tn4401 was detected by Shen et al. (2009). The genetic locus located on the plasmid pKP048 contains a *Tn3*-based transposon and a partial Tn4401 segment, *ISKpn8*, and an *ISKpn6*-like element (Shen et al., 2009). Afterward, various mutations in the surrounding environment of the bla_{KPC-2} gene were detected, and most of which were mainly caused by the insertion of a truncated bla_{TEM} gene sequence

TABLE 1 | Main molecular features of all the CR-hvKP isolates.

Isolates	LD50(cfu)	Virulence genes	Carbapenemase genes	Other durg resistance genes	NTE$_{KPC}$ plasmid replicon type
Kp1	5.4×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, qnrS aac(6')-Ib-cr*	IncFII(k)
Kp2	1.2×10^5	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, qnrS aac(6')-Ib-cr*	IncFII(k)
Kp3	3.7×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1, qnrS aac(6')-Ib-cr*	–
Kp4	5.5×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1, qnrS aac(6')-Ib-cr*	–
Kp5	1.1×10^5	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1, qnrS aac(6')-Ib-cr*	IncFII(k)
Kp6	5.7×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1, qnrS aac(6')-Ib-cr*	–
Kp7	3.2×10^5	*rmpA, silS, iutA, rmpA2*	*KPC-2, NDM-1*	*CTX-M-14, SHV-1, TEM-1, qnrS*	IncFII(k)
Kp8	7.8×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1, qnrS aac(6')-Ib-cr*	IncFII(k)
Kp9	7.7×10^5	*rmpA, silS, iutA, rmpA2*	*NDM-1*	*CTX-M-14, SHV-1, TEM-1, qnrS aac(6')-Ib-cr*	–
Kp10	8.1×10^5	*rmpA, silS, iutA, rmpA2*	*KPC-2, NDM-1*	*CTX-M-14, SHV-1, TEM-1, qnrS aac(6')-Ib-cr*	IncFII(k)
Kp11	2.1×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, qnrS aac(6')-Ib-cr*	IncFII(k)
Kp12	3.3×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, TEM-1, qnrS aac(6')-Ib-cr*	IncFII(k)
Kp13	6.5×10^3	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1, qnrS aac(6')-Ib-cr*	IncFII(k)
Kp14	4.2×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, TEM-1, qnrS aac(6')-Ib-cr*	IncFII(k)
Kp15	7.9×10^5	*terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, TEM-1, qnrS aac(6')-Ib-cr*	IncFII(k)
Kp16	7.6×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, TEM-1, qnrS aac(6')-Ib-cr*	IncH1B/IncF$_{repB}$
Kp17	4.5×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2,VIM-2*	*CTX-M-14, SHV-1, TEM-1, qnrS aac(6')-Ib-cr*	IncFII(k)
Kp18	8.5×10^5	*terW, silS, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1*	IncFII(k)
Kp19	7.7×10^3	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*SHV-1, TEM-1, qnrS aac(6')-Ib-cr-*	–
Kp20	7.7×10^4	*terW, silS, iutA, rmpA2*	*KPC-2*	*SHV-1, TEM-1, qnrS aac(6')-Ib-cr-*	–
Kp21	6.1×10^3	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	–	IncFII(k)
Kp22	6.4×10^5	*terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1, qnrS*	IncFII(k)
Kp23	7.2×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1*	IncFII(k)
Kp24	8.8×10^5	*terW, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1*	IncFII(k)
Kp25	6.9×10^5	*terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1*	IncFII(k)
Kp26	8.9×10^5	*terW, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1, qnrS*	IncFII(k)
Kp27	6.2×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1, qnrS*	IncFII(k)
Kp28	6.4×10^5	*terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, TEM-1, qnrS*	IncH1B/IncF$_{repB}$
Kp29	5.7×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1, qnrS*	IncFII(k)
Kp30	6.2×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1, qnrS*	IncFII(k)
Kp31	3.8×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, qnrS*	–
Kp32	3.9×10^5	*terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1, qnrS*	–
Kp33	6.8×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, qnrS*	IncFII(k)
Kp34	3.9×10^3	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*TEM-1*	IncFII(k)
Kp35	6.3×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1, qnrS*	IncFII(k)

(Continued)

TABLE 1 | Continued

Isolates	LD50(cfu)	Virulence genes	Carbapenemase genes	Other durg resistance genes	NTE$_{KPC}$ plasmid replicon type
Kp36	8.3×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, qnrS*	IncFII(k)
Kp37	8.7×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1*	–
Kp38	3.4×10^5	*terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, TEM-1, qnrS*	IncFII(k)
Kp39	4.7×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, SHV-1, qnrS*	IncFII(k)
Kp40	5.6×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, qnrS*	IncFII(k)
Kp41	2.6×10^4	*rmpA, terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, TEM-1, qnrS*	–
Kp42	1.4×10^5	*terW, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, TEM-1, qnrS*	IncFII(k)
Kp43	2.7×10^5	*rmpA, terW, silS, rmpA2*	*KPC-2*	*SHV-1, qnrS*	–
Kp44	4.4×10^4	*terW, silS, iutA, rmpA2*	*KPC-2*	*SHV-1, qnrS*	IncFII(k)
Kp45	2.9×10^5	*rmpA, silS, iutA, rmpA2*	*KPC-2*	*CTX-M-14, TEM-1, qnrS*	IncFII(k)

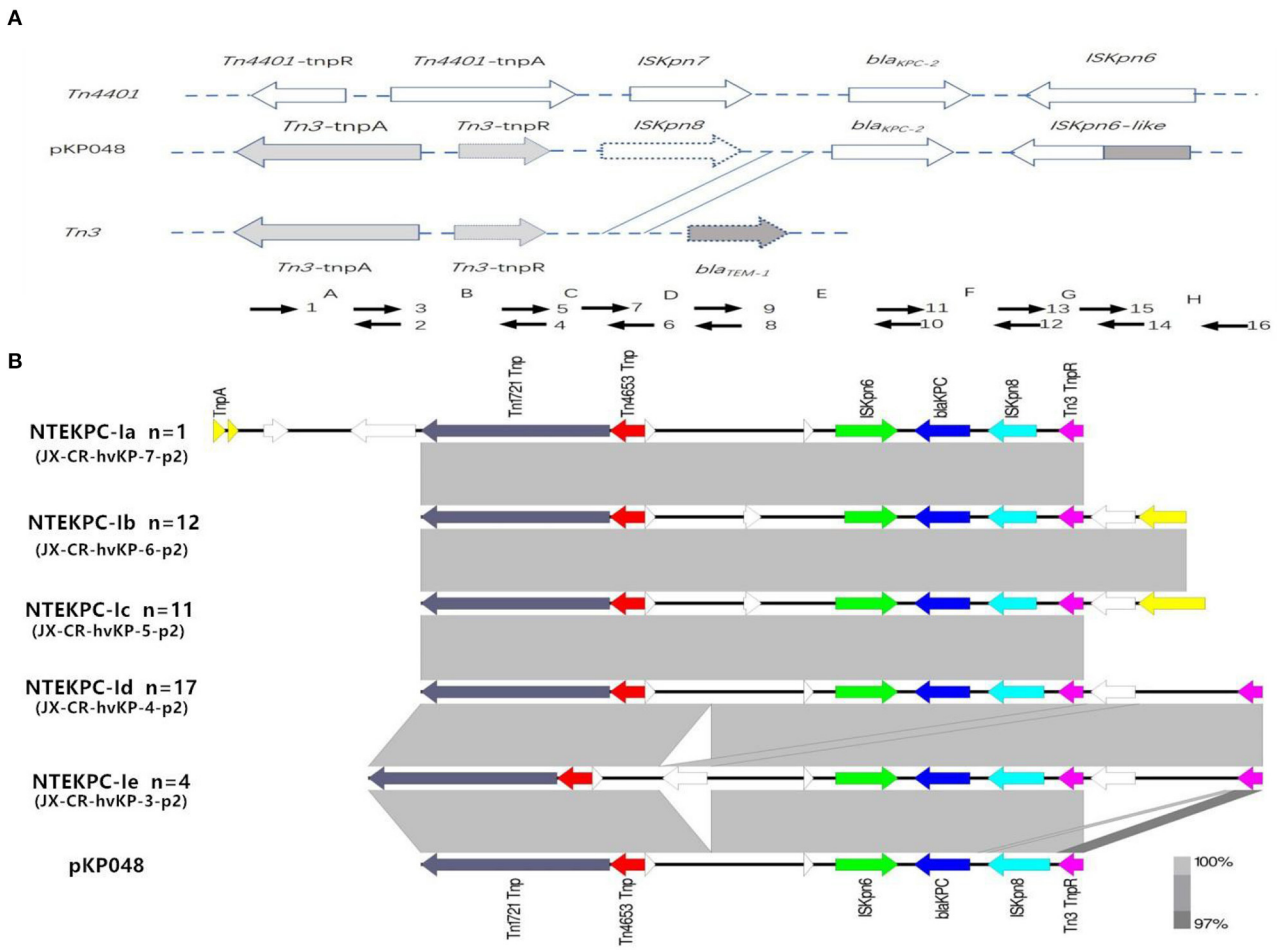

FIGURE 1 | Schematic representation of the genetic structures surrounding the blaKPC-2 gene in the CR-hvKP isolates. **(A)** Classic *Tn3*-based structure in plasmid pKP048; Short arrowheads with numbers were used for PCR mapping. **(B)** Five distinct NTE patterns. Gene bla_{KPC-2} is represented by a blue arrow, and the remaining genes are color coded. NTEKPC-Ib, NTEKPC-Ic, and NTEKPC-Id were the most common genetic structures in the CR-hvKP isolates.

between *ISKpn8* and bla_{KPC} gene with different sizes (Yang et al., 2013; Li et al., 2016). In this study, the KPC structures of all the CR-hvKP isolates should be separated into the NTE$_{KPC}$-I group because of the bla_{TEM} absence (NTE$_{KPC}$-I) (**Figure 1**) (Chen et al., 2014). PCR and WGS analysis showed that there were five different NTE$_{KPC}$-I patterns in these CR-hvKP strains. They had high similarity to the NTE$_{KPC}$ on the earliest plasmid pKP048. An increasing number of CR-hvKP in Jiangxi Province

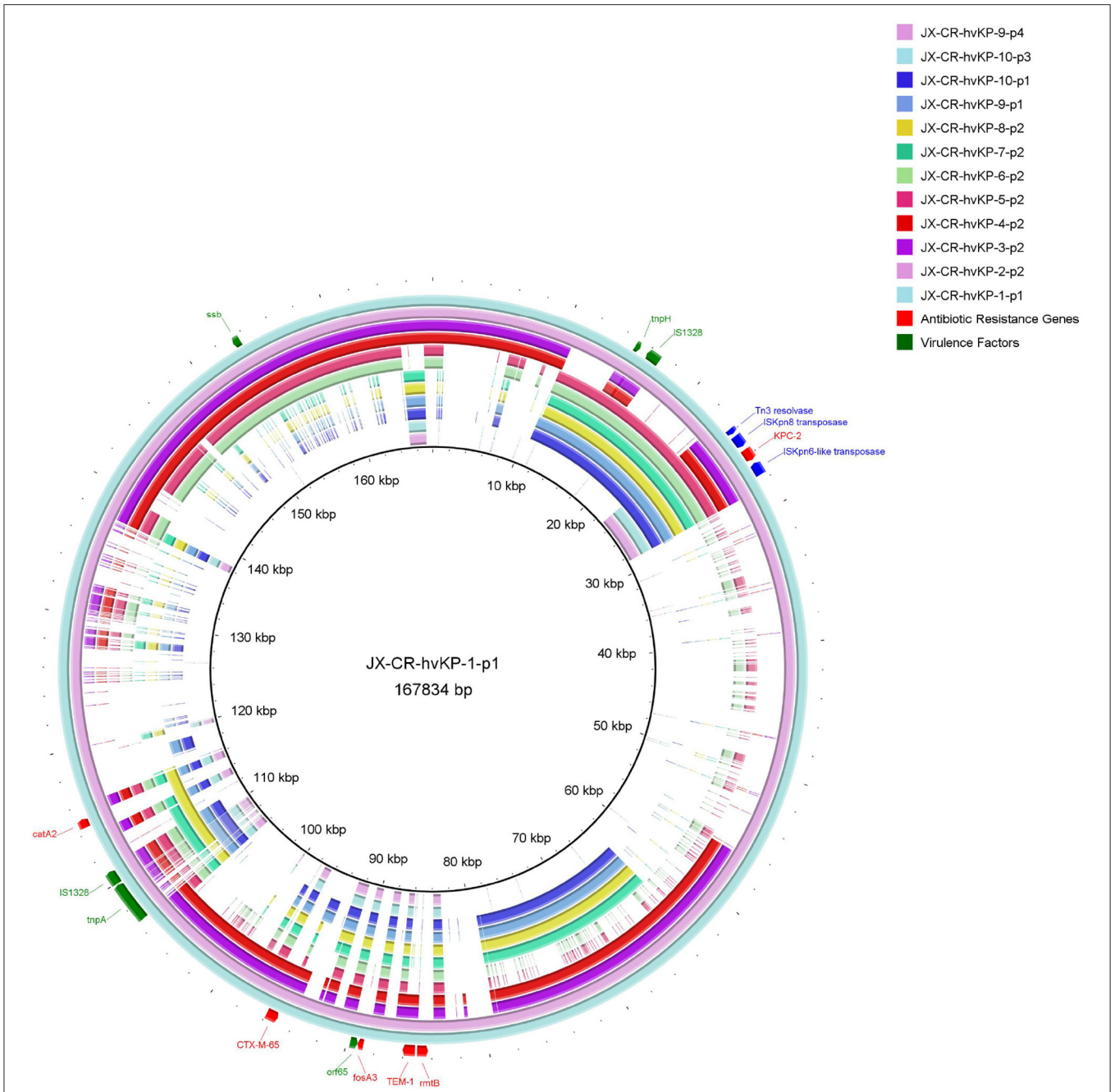

FIGURE 2 | Gene map of twelve NTE$_{KPC}$ plasmids harbored by CR-hvKP strains. JX-CR-hvKP-5-p2 and JX-CR-hvKP-6-p2 are from ST23 CR-hvKP, and the others are from ST11 CR-hvKP. The circular map was generated using the BLAST Ring Image Gnerator. A sequence comparison showed the polymorphism of *bla*$_{KPC}$ plasmids in CR-hvKP.

revealed that NTE$_{KPC}$-I had strong dissemination ability and good stability (Liao et al., 2020; Li et al., 2020). It is noteworthy that NTE$_{KPC}$s are primarily found in non-ST258 *K. pneumoniae* or other non-*K. pneumoniae* species (Chen et al., 2014).

The pandemic spread of *bla*$_{KPC-2}$ among *Klebsiella pneumoniae* ST11 in China is mainly related to the horizontal transfer mediated by incompatibility group F (IncF) plasmids (Chi et al., 2019; Fu et al., 2019). PCR-based replicon type and representative strain WGS analysis revealed that almost all the different NTE patterns carrying *bla*$_{KPC-2}$ in this study were also shown to be carried on the IncF plasmids. We suppose that there was also a close correlation between NTE$_{KPC}$-I and IncF plasmids in CR-hvKP, whereby ST11 CR-hvKP is a seemingly good colonizer to capture IncF plasmids. The gene map of

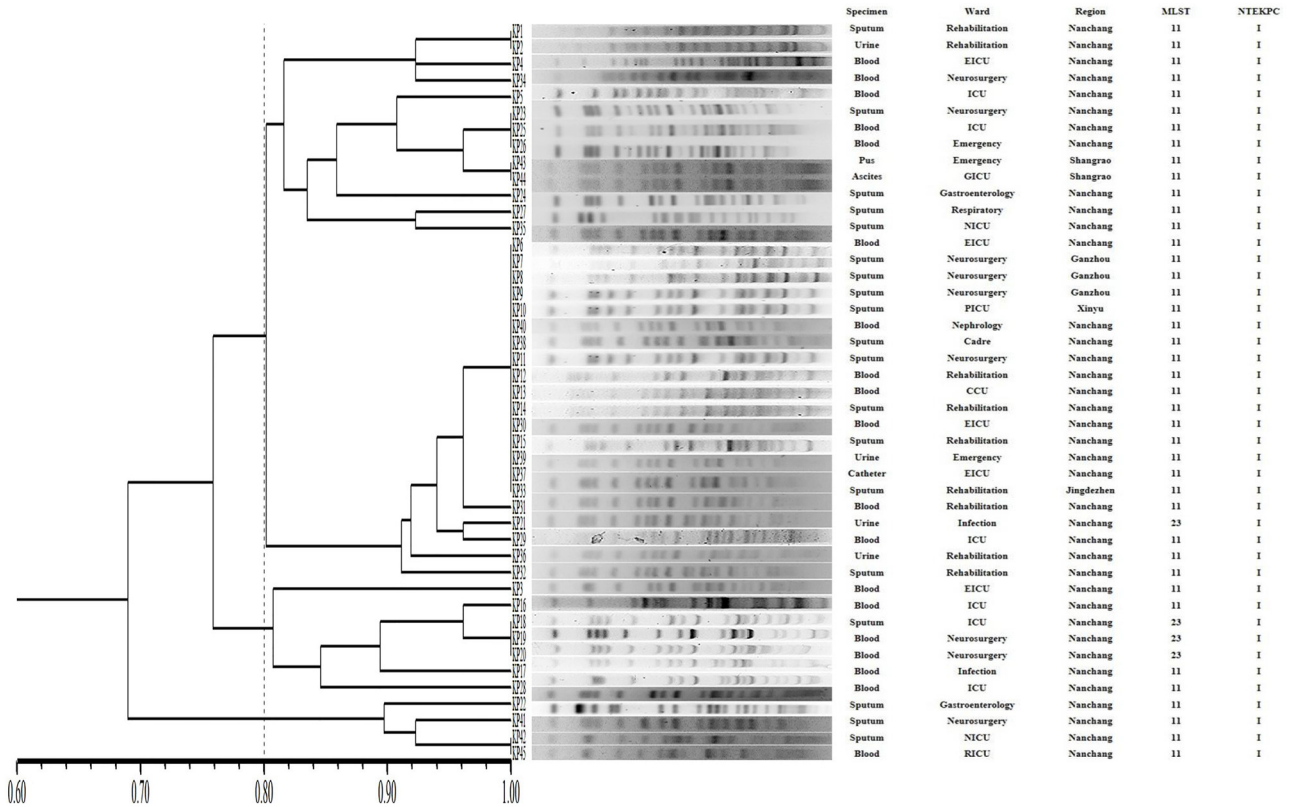

FIGURE 3 | Pulsed-field gel electrophoresis (PFGE) patterns, NTE$_{KPC}$ patterns, and STs among 45 clinical CR-hvKP isolates. NICU, neurosurgery ICU; GICU, gastroenterology ICU; RICU, respiratory ICU; PICU, Pediatric ICU; EICU, Emergency ICU.

twelve NTE$_{KPC}$ plasmids showed that there are gene structure differences among ST11 CR-hvKP strains in Jiangxi Province, which is consistent with the finding that the diversity of the plasmids of genetically related *K. pneumoniae* strains harboring the beta-lactamase gene *blaKPC-2* existed in the Netherlands from 2014 to 2019 (Hendrickx et al., 2020). Interestingly JX-CR-hvKP-6-p2 and JX-CR-hvKP-5-p2 from ST23 CR-hvKP had the same plasmid gene structure without any plasmid replicon type. It appeared to be that the NTE$_{KPC}$ plasmids lost their plasmid replicon after entering ST23 hvKP, while JX-CR-hvKP-8-p2, JX-CR-hvKP-7-p2, JX-CR-hvKP-9-p1, and JX-CR-hvKP-10-p1 from ST11 CR-hvKP had highly similar plasmid gene structure. This indicates that the resistance/virulence hybrid plasmids in JX-CR-hvKP-9 and JX-CR-hvKP-10 formed by the fusion of NTE$_{KPC}$ plasmids and pLVPK-like virulence plasmids. The hypothesis must be validated by further experiments.

The PFGE patterns show that all the CR-hvKP isolates were assigned to three clusters based on >80% pattern similarity. It is consistent with a previous study in China that the ST11 genomes were highly heterogeneous and clustered into at least three major lineages based on single nucleotide polymorphism (SNP) analysis (Dong et al., 2018).

The study has certain limitations, including its retrospective nature and a relatively small study population. Therefore, there may be selection bias, which limits the general application of study results to other areas. Consequently, a further study that includes more patients, especially for ST23 hvKp isolates, is needed.

CONCLUSION

In conclusion, this is the first analysis of the diverse genetic structures of the bla_{KPC-2} gene in CR-hvKP isolates from South China. Both the NTE$_{KPC}$-I on the IncF plasmids and pLVPK-like virulence plasmids make contributions to the formation of CR-hvKP, especially ST11, which need more attention.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: GenBank, PRJNA672246.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

L-GW and T-xX did strain characterization and participated in manuscript writing. ZX and DL conceived the study and performed data analysis. WL did the whole-genome sequencing and comparative genomics and participated in manuscript writing. YL and DW wrote the paper. Q-SH collected the clinical data and analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.622280/full#supplementary-material

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