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Characterization of the population structure, drug resistance mechanisms and plasmids of the community-associated *Enterobacter cloacae* complex in China

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Objectives: To investigate the population structure, drug resistance mechanisms and plasmids of community-associated *Enterobacter cloacae* complex (CA-ECC) isolates in China.

Methods: Sixty-two CA-ECC isolates collected from 31 hospitals across China were typed by *hsp60* typing and MLST. ESBL and AmpC-overexpression phenotype was determined by double-disc synergy test. Replicon typing and conjugation were performed for plasmid analysis. All ESBL-positive isolates and representative conjugants were subjected to detailed characterization by WGS.

Results: *Enterobacter hormaechei* and *Enterobacter kobei* were predominant in our collections. MLST distinguished 46 STs with a polyclonal structure. ST591 was the most prevalent clone detected in northern China. Twenty-two isolates (35.5%) were ESBL positive and half of them were *E. kobei.* ESBL positivity was related to ESBL production (15/22) and to AmpC overexpression (18/22). Core-genome phylogenetic analysis identified intra- and inter-regional dissemination of ESBL-producing *E. kobei* clones. ESBL producers were exclusively classified as *E. hormaechei* and *E. kobei*, and $bla_{CTX\text{-}M\text{-}3}$ was the most prevalent ESBL genotype (10/15) detected in four different environments. In the ESBL-positive population, the ESBL producers encoded more drug resistance genes (8–24 genes) by carrying more plasmids (1–3 plasmids) than the non-ESBL-producing isolates, resulting in an inter-group difference in drug susceptibilities. IncHI-type plasmids were prevalent in the ESBL producers (12/15). All IncHI2-type plasmids ($n = 11$) carried ESBL genes and shared a similar backbone to p09-036813-1A_261 recovered from *Salmonella enterica* in Canada.

Conclusions: The species-specific distribution, species-dependent ESBL mechanism and endemic plasmids identified in our study highlight the necessity for tailored surveillance of CA-ECC in the future.

Introduction

Enterobacter spp. have emerged as important nosocomial pathogens in the last decade and are classified as one of the 'ESKAPE bugs'.[1] Among *Enterobacter* spp. the *Enterobacter cloacae* complex (ECC) is of major importance, accounting for 65%–75% of infections.[2] The ECC comprises at least five species (*E. cloacae*, *Enterobacter kobei*, *Enterobacter asburiae*, *Enterobacter hormaechei* and *Enterobacter ludwigii*) with different subspecies and the taxonomic status is consecutively updated. However, ECC species are often investigated at the complex level, which impedes our understanding of their clinical significance, epidemiology and drug resistance characterization at the species level. Species identification within the ECC is difficult and currently *hsp60* typing is the most widely used method. *hsp60* typing groups the ECC into 12 genetic clusters (*I–XII*) and one unstable sequence cluster (*XIII*);[3] some of these genetic clusters have been assigned to the named species and subspecies.[4] According to this typing method, *E. hormaechei* has been revealed as the predominant species both in the clinical setting and in the environment.[4–6]

Due to the prevalence of ESBLs and carbapenemases in the constituent species, the ECC has become the third major drug-

resistant Enterobacteriaceae species involved in nosocomial infections after *Escherichia coli* and *Klebsiella pneumoniae*.[7–8] Studies covering the years 1998–2013 from different parts of the world all reveal high ESBL carriage within the ECC (36%–66.7%).[9–13] Recently, large surveillance studies from North and South America, and China report increasing levels (7.4%–17.4%) of carbapenemase-producing ECC.[14–17] The ECC intrinsically carries *ampC* genes, resulting in a constitutive production of the AmpC β-lactamase and is thus resistant to ampicillin, amoxicillin, amoxicillin/clavulanate, first-generation cephalosporins and cefoxitin.[18] Moreover, the ECC overproduces AmpC β-lactamases by derepression of a chromosomal gene or by the acquisition of a transferable *ampC* gene on plasmids or other mobile elements, conferring resistance to third-generation cephalosporins. Of more concern, various ESBL and carbapenemase genes are widespread within the species. The most prevalent ESBL genotypes are $bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$ and $bla_{\text{SHV-12}}$.[11,13,19–21] KPC, NDM and IMP/VIM are the major types of carbapenemases.[14–17] It has been suggested that conjugative plasmids are primarily responsible for the spread of these resistance genes among enterobacterial isolates; mainly six plasmid families (IncF, IncA/C, IncL/M, IncN, IncI and IncHI2).[22]

MLST has revolutionized the clonal structure analyses of bacterial populations, allowing reliable large-scale epidemiological and evolutionary studies. Because of the relatively recent release of the MLST scheme for *Enterobacter* spp. (2013), only limited MLST data exist in the literature, largely impeding the understanding of the global epidemiology of ECC. Recently, multiple international clones (ST66, ST78, ST108 and ST114) that cause nosocomial infections were identified in Europe.[23–25] However, those clones have not been identified during our previous investigation in China,[19] suggesting that the epidemiology of nosocomially acquired ECC could depend on geography.

Of note, ECC strains have recently also become major pathogens involved in community-acquired infections, frequently causing pneumonia and urinary tract and bloodstream infections.[26–28] It has been suggested that the nosocomial isolates represent samplings from the diversity of commensal ECC in the community.[25] However, the epidemiological and drug resistance characterization of community-associated ECC (CA-ECC) is unclear, thus largely impeding verification of this hypothesis. This study was performed to investigate the population structure, drug-resistance mechanisms and plasmids of CA-ECC isolates collected in 31 hospitals in 12 provinces in China.

Materials and methods

Clinical isolates

Isolates were collected in the course of a national survey from 31 hospitals located in 12 provinces, representing seven geographical regions of China, between August 2010 and August 2011.[29] Patients with community-associated infections were included in the study in accordance with previously described criteria.[29] Species identification was performed using MALDI-TOF MS (Bruker Diagnostics, Bremen, Germany) and the ECC members were differentiated by *hsp60* typing.[3]

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined using the agar dilution method and the antibiotics are listed in Table 2. Results were interpreted according to the 2016 EUCAST clinical breakpoints (http://www.eucast.org/clinical_breakpoints/). Susceptibility to biapenem, cefoperazone/sulbactam and cefazolin was interpreted according to CLSI.[30] The breakpoints for biapenem and cefoperazone/sulbactam referred to those of imipenem and cefoperazone/sulbactam, respectively, as recommended by CLSI. The phenotypic detection of ESBL and AmpC expression was performed by the double-disc synergy test using cefotaxime and ceftazidime with clavulanate discs in the absence or presence of 250 mg/L cloxacillin.[23]

MLST and phylogenetic analysis

MLST was performed as described previously[31] and the STs were assigned using the MLST database (http://pubmlst.org/ecloacae). The relatedness between the STs was analysed using eBURST (http://eburst.mlst.net/). The seven DNA fragments were concatenated; the phylogenic tree was constructed by bootstrap phylogenetic inference using MEGA6 v. 6.0. The sequences of 14 concatenated reference sequences (ST2, ST3, ST9, ST12, ST78, ST108, ST114, ST119, ST122, ST130, ST131, ST144, ST162 and ST187) were included in the phylogenetic analysis, corresponding to the previously identified main ST clades.[24]

Plasmid analysis

The plasmid size was estimated by S1-PFGE. The conjugative transfer of plasmids was carried out with the recipient *E. coli* J53 and a 2:1 donor:recipient ratio at 30 °C. The transconjugants were selected on Mueller–Hinton medium with sodium azide (100 mg/L) and ceftriaxone (4 mg/L). The selected transconjugants were verified by replicon amplification using primers, as previously described;[32] the representative transconjugants were sent for WGS. Plasmid replicon typing was performed either as previously described[32] or by submitting the genomic sequences to PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/). Plasmid sequences were compared using BLASTn.

WGS and data analysis

The genomes were sequenced using an Illumina Hiseq2500 instrument (Illumina, San Diego, CA, USA) after constructing 2×125 bp paired-end libraries. The *de novo* assembly was performed using CLC Genomics Workbench v. 8.0 (QIAGEN, Hilden, Germany) after quality trimming (Qs $\geq$ 20); the annotation was performed using the RAST server (rast.nmpdr.org); the resistome analysis was carried out using the CGE server (https://cge.cbs.dtu.dk) and CARD (https://card.mcmaster.ca/); and the detection of SNPs and synteny and phylogenomic analyses were performed as described previously.[33]

Average nucleotide identity (ANI) analysis

The following type strains were used for the ANI analysis: *E. asburiae* ATCC 35953 (NZ_CP011863.1), *E. hormaechei* subsp. *steigerwaltii* DSM16691 (NZ_CP017179.1), *E. hormaechei* subsp. *oharae* DSM16687 (NZ_CP017180.1), *E. hormaechei* subsp. *hormaechei* ATCC 49162 (AFHR00000000), *Enterobacter xiangfangensis* LMG27195 (NZ_CP017183.1), *E. kobei* DSM13645 (NZ_CP017181.1) and *E. cloacae* subsp. *cloacae* ATCC 13047 (NC_014121.1). The ANI analysis of ECC genomes was performed using JSpecies v. 1.2.1 (http://imedea.uib-csic.es/jspecies). ANI value >95% was set as the threshold for species definition.

GenBank accession numbers

The Whole Genome Shotgun BioProject for the ECC isolates has been deposited at DDBJ/EMBL/GenBank under accession numbers NEEF00000000–NEEX00000000.

Results

E. hormaechei and E. kobei were prevalent among CA-ECC

In this study, 62 ECC isolates were detected (isolates ECC3018, ECC3026 and ECC3047 have been reported previously)[34] among 2946 community-associated clinical isolates; they constituted 6.7% of the detected Enterobacteriaceae ($n = 952$) and 78.5% of the detected *Enterobacter* spp. ($n = 79$). These isolates were detected in seven geographical regions (Figure S1, available as Supplementary data at *JAC* Online) and were obtained from sputum ($n = 23$), urine ($n = 17$), secretions ($n = 7$), abscesses ($n = 2$), bronchoalveolar lavage ($n = 1$) and blood ($n = 1$), or unknown sources ($n = 11$).

The 62 isolates were identified by MALDI-TOF MS as *E. cloacae* ($n = 35$), *E. asburiae* ($n = 14$) and *E. kobei* ($n = 13$). The *hsp*60 typing revealed that *E. hormaechei* was predominant ($n = 21$), followed by *E. kobei* ($n = 15$), *cluster IV* ($n = 7$), *cluster III* ($n = 6$), *cluster IX* ($n = 4$), *E. asburiae* ($n = 3$), *cluster XIII* ($n = 3$), *E. ludwigii* ($n = 2$) and *E. cloacae* ($n = 1$) (Table 1).

E. kobei is predominant in the ESBL-positive population

As summarized in Table 2, all isolates exhibited low susceptibility rates to ampicillin (0%), ampicillin/sulbactam (24.2%), cefazolin (12.9%) and cefuroxime (30.6%), and high susceptibility rates to piperacillin/tazobactam (65.2%), cefoperazone/sulbactam (90.5%), ceftazidime (67.8%), amikacin (80.7%) and fluoroquinolones (ciprofloxacin: 72.5%; levofloxacin: 77.4%). All isolates were susceptible to carbapenems.

Of the 62 isolates, 22 (35.5%) exhibited an ESBL-positive phenotype (Table 1). Half of the ESBL-positive strains were *E. kobei* ($n = 11$), followed by *E. hormaechei* ($n = 5$).

Detection of an emerging ESBL-positive E. kobei clone in northern China

MLST assigned the 62 isolates to 46 STs (including 33 novel STs as of February 2016), indicating a high genetic diversity of the CA-ECC isolates. The most prevalent ST was ST591 ($n = 8$), followed by ST32 ($n = 3$), ST564 ($n = 3$), ST634 ($n = 3$), ST145 ($n = 2$), ST618 ($n = 2$) and ST629 ($n = 2$). The remaining STs contained one isolate for each (Table 1). Clonality analysis suggested a polyclonal structure of the 46 STs (Figure S2). Eight STs (ST32, ST90, ST145, ST542, ST582, ST875, ST876 and ST878) were classified into eight clonal complexes. Thirteen STs were connected with one or two STs, and the others were singletons. The 22 ESBL-positive isolates represented 11 STs (Table 1). Little association was found between the ST prevalence and the specimen type or the geographical origin of the isolates. Notably, the eight ST591 *E. kobei* strains were obtained from two different regions (central north and northeast), suggesting that this ESBL-positive clone is circulating in northern China. ST32, ST145 and ST359 were also identified in different regions (Table 1 and Figure 1).

Phylogenetic analysis based on the concatenated sequences of seven MLST loci classified the 46 STs into 14 clusters (A–N), including 12 reported clusters (A–L)[24] and two novel clusters (M and N) (Figure 1). Clusters A, B and C constituted the largest clade of 24 STs. Of note, cluster A and B isolates belonged to *E. hormaechei* and cluster C isolates belonged to *cluster III*, implying a close relationship between *cluster III* and *E. hormaechei*. The clusters identified by MLST correlated well with the *hsp60* typing results (Figure 1).

Intra- and inter-regional dissemination of the ESBL-positive E. kobei clones

Core-genome phylogenetic analysis using 37113 SNPs classified the 22 ESBL-positive isolates into six clades (Figure 2). The 11 ESBL-producing *E. kobei* strains clustered with the type strain DSM13645 constitute the largest clade, with two subclades formed by ST591 and ST32. The ST591 isolates were clustered according to their origin, forming two different subclones differentiated by ~190 SNPs (Figure 2). Of note, the central north clone was isolated at two hospitals and showed 1–7 SNPs, indicating clonal dissemination in the region. The three ST32 isolates obtained from the middle south (ECC1752 and ECC1875) and south-west (ECC1097) differed by 1–19 SNPs. Surprisingly, ECC1752 was identical to ECC1097 (no SNPs), suggesting inter-regional transmission.

In another large clade composed of *E. hormaechei* strains, ECC904 and ECC1950 were clustered with *E. hormaechei* subsp. *steigerwalltii* DSM16691, while ECC3422 clustered with *E. xiangfangensis* LMG27195, a new subspecies of *E. hormaechei*.[4] These two subclades differed by >2960 SNPs and were separated from a subclade of two *cluster III* strains (ECC1840 and ECC3137) by >4760 SNPs. The *E. hormaechei* clade further grouped with the *cluster XIII* strain ECC1766, which suggests that they share a common ancestor.

The three *cluster IV* strains (ECC4202, ECC4240 and ECC4241) grouped with the type strain *E. asburiae* ATCC 35953. The three isolates were almost identical with ≤1 SNP, indicating clonal dissemination in the central south. The *E. cloacae* isolate ECC2288 clustered with *E. cloacae* subsp. *cloacae* ATCC 13047, and further clustered with *E. ludwigii* EN119, suggesting that they share a common ancestor. The *cluster IX* strain ECC2572 represented a singleton.

CTX-M-3 and SHV-12 are the prevalent genotypes

ESBL genes were detected in 15 of the 22 ESBL-positive isolates, with $bla_{\text{CTX-M-3}}$ the most prevalent ($n = 10$), followed by $bla_{\text{SHV-12}}$ ($n = 7$), $bla_{\text{CTX-M-9}}$ ($n = 3$), $bla_{\text{CTX-M-12}}$ ($n = 3$) and $bla_{\text{CTX-M-14}}$ ($n = 2$) (Figure 2). These ESBL genes were found in *E. kobei* (n = 11), *E. hormaechei* ($n = 2$) and *cluster III* ($n = 2$). Two types of AmpC β-lactamase genes, bla_{ATC} and bla_{MIR}, were detected in the majority of ESBL-positive isolates, with the exception of ST32 strains. The AmpC-overexpression phenotype was apparent in 18 ESBL-producing strains, including the seven strains lacking the ESBL genes (Figure 2). The AmpC-overexpression phenotype can be explained by multiple point mutations detected in *ampR* and *ampD* genes in the 18 strains (data not shown).

Differences in the resistome and drug susceptibility between the ESBL-producing and non-ESBL-producing isolates in the ESBL-positive population

We next analysed the resistomes of the ESBL-positive isolates. The 15 ESBL-producing isolates harboured many more drug-resistance

Table 1. ECC isolates used in this study

Isolate ID[a]	Specimen	Origin[b]	Isolation date (year/month/day)	Patient age[c]	*hsp60* typing	ST[d]	MLST cluster
873	sp	SW2	2011/01/19	34 days	EH-O	614-NEW	B
1248	sp	SW3	2011/03/12	32	EH-O	564	B
1249	ur	SW3	2011/03/13	23	EH-O	564	B
1499	sp	NC4	2011/03/08	45	EH-O	620-NEW	B
1568	se	NC5	2010/11/05	45	EH-O	621-NEW	B
1620	ur	NC2	2011/01/16	71	EH-O	622-NEW	B
1635	ur	NC2	2011/02/12	63	EH-O	623-NEW	B
1783	unknown	MS3	unknown	unknown	EH-O	564	B
2250	ur	SC3	2010/10/02	25	EH-O	878-NEW	B
2599	se	NW2	2010/11/22	28 days	EH-O	586-NEW	B
3422	ur	NC4	2011/04/18	33	EH-O	592-NEW	B
3595	ur	EC5	unknown	54	EH-O	488	B
898	sp	SW2	2011/02/15	6 months	EH-S	874-NEW	A
904	ur	SW3	2010/12/09	5	EH-S	617-NEW	A
1009	ur	SW5	2010/12/12	75	EH-S	582-NEW	A
1259	se	SW1	2011/02/23	11	EH-S	517	A
1375	ur	SC2	2011/02/10	26	EH-S	542	A
1767	unknown	MS3	unknown	unknown	EH-S	625-NEW	A
1950	sp	MS5	2011/03/10	60	EH-S	90	A
2229	sp	SC3	2010/11/08	88	EH-S	626-NEW	A
2589	ur	NW2	2010/11/16	22	EH-S	150	A
1097	ur	SW5	2010/12/17	55	EK	32	F
1752	unknown	MS3	unknown	unknown	EK	32	F
1790	unknown	MS3	unknown	unknown	EK	583-NEW	F
1802	unknown	MS3	unknown	unknown	EK	876-NEW	G
1875	unknown	MS3	unknown	unknown	EK	32	F
1892	unknown	MS3	unknown	unknown	EK	584-NEW	F
3018[e]	bal	NE3	unknown	unknown	EK	591	F
3026[e]	bl	NE3	unknown	unknown	EK	591	F
3047[e]	ab	NE3	unknown	unknown	EK	591	F
3380	sp	NC3	2011/05/29	42	EK	591	F
3382	sp	NC4	2011/03/16	39	EK	591	F
3393	sp	NC4	2011/03/22	57	EK	591	F
3402	sp	NC4	2011/04/01	57	EK	591	F
3408	sp	NC4	2011/04/05	67	EK	591	F
3587	sp	EC5	unknown	85	EK	280	F
1265	sp	SW2	2011/01/10	4 months	EA	618-NEW	N
1277	sp	SW2	2011/01/24	6 months	EA	618-NEW	N
3345	ur	NC2	2011/05/24	61	EA	587-NEW	J
1488	se	NC4	2010/10/26	30	EL	875-NEW	L
1831	unknown	MS3	unknown	unknown	EL	877-NEW	L
2288	ur	SC5	2010/11/24	3	EC	627-NEW	H
881	sp	SW2	2010/12/22	69	*cluster III*	616-NEW	C
897	sp	SW2	2011/02/21	75	*cluster III*	145	C
1491	se	NC4	2010/12/09	47	*cluster III*	619-NEW	O
1840	unknown	MS3	unknown	unknown	*cluster III*	97	C
3137	unknown	NE4	unknown	unknown	*cluster III*	102	C
3991	ur	EC2	2011/05/13	69	*cluster III*	145	C
2390	ur	NW4	2011/05/21	71	*cluster IV*	585-NEW	I
2926	sp	SW3	2011/04/17	81	*cluster IV*	629-NEW	I
2929	se	SW3	2011/04/16	63	*cluster IV*	629-NEW	I
3037	sp	NE3	unknown	unknown	*cluster IV*	41	I
4204	sp	SC4	2011/04/15	61	*cluster IV*	634-NEW	I

Continued

Table 1. *Continued*

Isolate ID[a]	Specimen	Origin[b]	Isolation date (year/month/day)	Patient age[c]	*hsp60* typing	ST[d]	MLST cluster
4240	sp	SC4	2011/04/04	52	*cluster IV*	634-NEW	I
4241	sp	SC4	2011/04/20	27	*cluster IV*	634-NEW	I
877	sp	SW2	2011/01/17	12 days	*cluster IX*	615-NEW	E
2572	ur	NW2	2010/11/08	50	*cluster IX*	628-NEW	E
3588	se	EC5	unknown	25	*cluster IX*	631-NEW	E
3593	ab	EC5	2011/01/18	48	*cluster IX*	632-NEW	E
1766	unknown	MS3	unknown	unknown	*cluster XIII*	624-NEW	K
3349	ur	NC2	2011/05/28	61	*cluster XIII*	630-NEW	K
3597	sp	EC5	unknown	77	*cluster XIII*	633-NEW	M

ab, abscess; bal, bronchoalveolar lavage; bl, blood; se, secretion; sp, sputum; ur, urine; EC, east central; MS, middle south; NC, north central; NE, north-east; NW, north-west; SC, south central; SW, south-west; EA, *E. asburiae*; EC, *E. cloacae*; EH-O, *E. hormaechei* subsp. *oharae*; EH-S, *E. hormaechei* subsp. *steigerwalltii*; EK, *E. kobei*; EL, *E. ludwigii*.
[a]ESBL-positive isolates identified in this study are marked in bold.
[b]Different numbers following capital letters represent different hospitals in the region.
[c]Ages are in years unless specified otherwise.
[d]New STs identified in this study are indicated.
[e]The three isolates have been published.

Table 2. Susceptibility profiles and MICs for 62 ECC strains

Antibiotics	%R	%I[a]	%S	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)
Ampicillin	100	0	0	>256	>256	16–1024
Ampicillin/sulbactam	75.8	0	24.2	32	64	0.06–512
Piperacillin	41.9	3.2	54.9	8	>256	0.5–1024
Piperacillin/tazobactam	21.9	12.9	65.2	4	32	0.125–128
Cefoperazone/sulbactam	4.8	4.8	90.5	1	16	0.03–64
Cefazolin	83.9	3.2	12.9	256	256	0.5–256
Cefuroxime	69.4	0	30.6	128	256	1–256
Ceftazidime	29	3.2	67.8	0.5	128	0.125–256
Ceftriaxone	40.3	0	59.7	1	128	0.03–256
Cefepime	25.8	16.1	58.1	0.5	16	0.03–64
Biapenem	0	0	100	0.064	0.25	0.015–1
Imipenem	0	0	100	0.25	0.5	0.015–1
Meropenem	0	0	100	0.064	0.125	0.015–1
Amikacin	14.5	4.8	80.7	2	128	0.03–512
Gentamycin	41.9	0	58.1	1	128	0.25–512
Ciprofloxacin	21	6.5	72.5	0.125	16	0.015–128
Levofloxacin	21	1.6	77.4	0.25	16	0.015–64

S, susceptible; I, intermediate; R, resistant.
[a]MIC values not covered by the breakpoints are shown as intermediate here.

genes (8–24 genes) than the seven ESBL-positive isolates lacking the ESBL genes (3–4 genes) (Figure 3). In agreement with their genotypes, significant differences in antibiotic susceptibility were observed between the two groups. The ESBL producers were non-susceptible to amikacin (MIC value: 8 to >256 mg/L; ECC3137 was susceptible), gentamicin (8 to >256 mg/L), ciprofloxacin (4 to 32 mg/L; ECC3018, 3026 and 3047 were susceptible) and cefepime (8 to 64 mg/L), whereas the non-ESBL producers were susceptible (amikacin: 0.5 to 2 mg/L; gentamicin: 0.05 to 1 mg/L; ciprofloxacin: 0.03 to 0.125 mg/L; cefepime: ≥0.03 to 4 mg/L). Additionally, numerous genes associated with MDR efflux pumps were found in all isolates (Table S1), with no significant differences between the two groups.

Genetic environment of $bla_{CTX-M-3}$

Four different types of genetic environments of $bla_{CTX-M-3}$ were observed in 10 isolates (Figure 4). In all these genetic

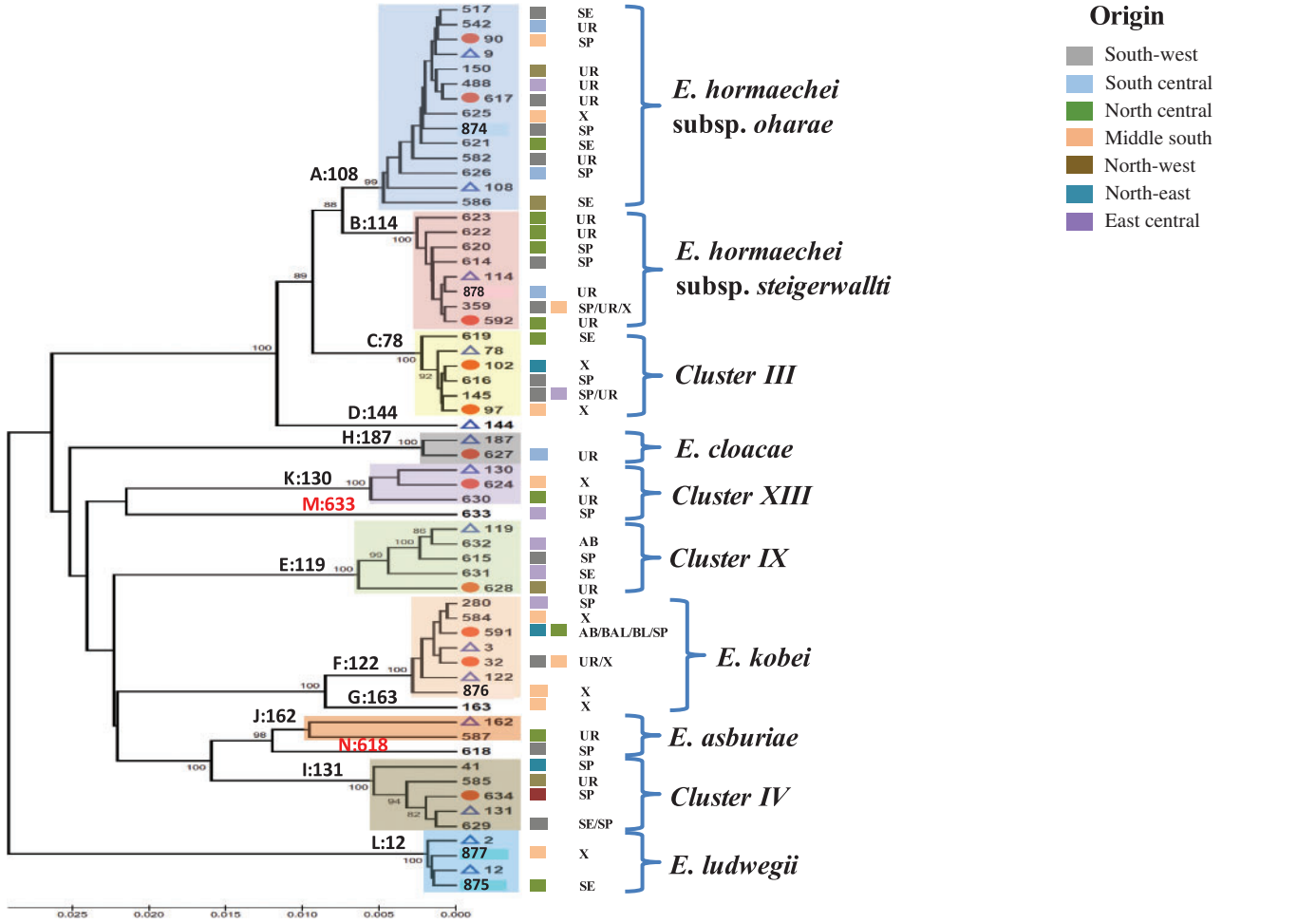

Figure 1. Unrooted tree of concatenated sequences from combinations of seven MLST loci. Phylogenetic analysis of 46 STs from 62 ECC isolates and 14 reference STs (marked with a triangle) from the literature as representatives of each cluster (letters A–L) previously reported. The STs of ESBL-positive isolates are marked with a red dot. Two novel clusters are indicated in red (M and N). The tree was constructed by bootstrap phylogenetic inference using MEGA6 software. The origins of isolates of each ST are represented as rectangles with different colours. The specimen of isolates of each ST is represented as SE (secretion), UR (urine), SP (sputum), AB (abscesses), BAL (bronchoalveolar lavage), BL (blood) and X (unknown source). The *hsp60* typing results are also shown here. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

environments, the varying-length IS*Ecp1* was located 127 bp upstream of $bla_{CTX\text{-}M\text{-}3}$ in the same orientation. In addition, the IS*Ecp1*-$bla_{CTX\text{-}M\text{-}3}$ transposition units were found in six different wider contexts.

IncHI2/2A plasmids are prevalent among the ESBL producers

Replicon typing identified numerous plasmid incompatibility (Inc) groups in 21 of the 22 ESBL-positive isolates, including IncHI ($n = 13$), IncA/C2 ($n = 4$), IncFIB ($n = 9$), IncFII ($n = 4$), IncFII (pECLA) ($n = 3$), IncL/M ($n = 2$), IncQ ($n = 1$), IncCol (RGK) ($n = 1$), IncN ($n = 1$), IncR ($n = 1$) and IncpSL483 ($n = 1$). Of note, IncHI (especially IncHI2/2A) was almost exclusively detected in the ESBL-producing isolates ($n = 12$). Conjugation assays demonstrated that most of the IncHI2/2A plasmids (11/12) were mobilizable. We therefore suspected that IncHI2/2A could be the major plasmid type mediating ESBL community dissemination in China. To verify this, the location of ESBL genes was inspected by sequencing of the conjugators harbouring one plasmid each. Except for the $bla_{CTX\text{-}M\text{-}12}$ gene,[34] the other ESBL genes were all detected on plasmids (Table 3). Specifically, most ESBL genes were carried on IncHI2/2A plasmids. The backbone analysis revealed that all IncHI2/2A plasmids shared a similar backbone to an IncHI2/2A plasmid p09-036813-1A_261 (Genbank accession number CP016526) recovered from *Salmonella enterica* in Canada (Figure 5).

Taxonomic status of the ECC determined by ANI

The ANI analysis was performed to determine the taxonomic status of *cluster III*, *IV*, *IX* and *XIII*. The ANI values of two *cluster III* strains ECC1840 and ECC3137 were >95% (95.52%–95.78%) versus the type strains *E. hormaechei* subsp. *oharae* DSM16687 and *E. hormaechei* subsp. *steigerwalltii* DSM16691, but <95% (94.21%–94.37%) versus *E. hormaechei* subsp. *hormaechei* ATCC

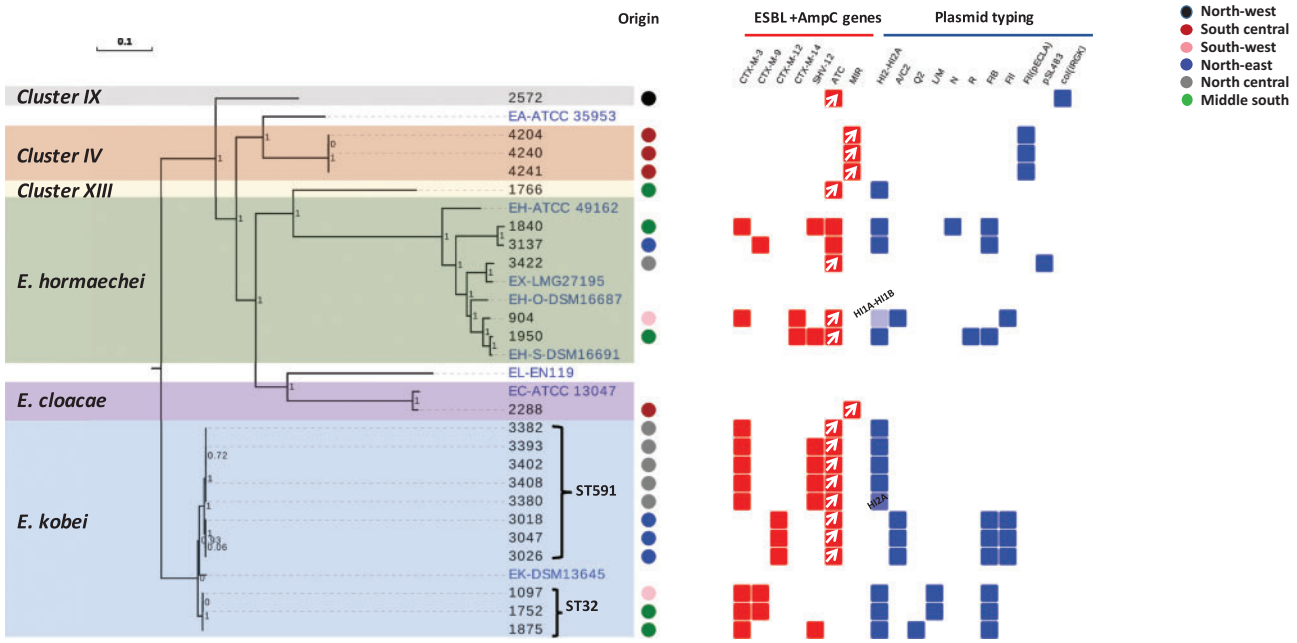

Figure 2. Core-genome phylogeny of ESBL-positive ECC isolates. The tree was constructed by using 37113 core-genome SNPs acquired from 22 ESBL-positive isolates and 8 type strains (in blue). The tree was mid-point rooted. The clades are named according to the type strains with the exception of *cluster IV*, *IX* and *XIII*, which have no available type strains. AmpC-overexpression phenotype is marked with a white arrow in the red rectangle. Plasmid Inc group is shown by a blue rectangle and different subgroups are marked in colour gradients. The origin of isolates is indicated with dots in different colours. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

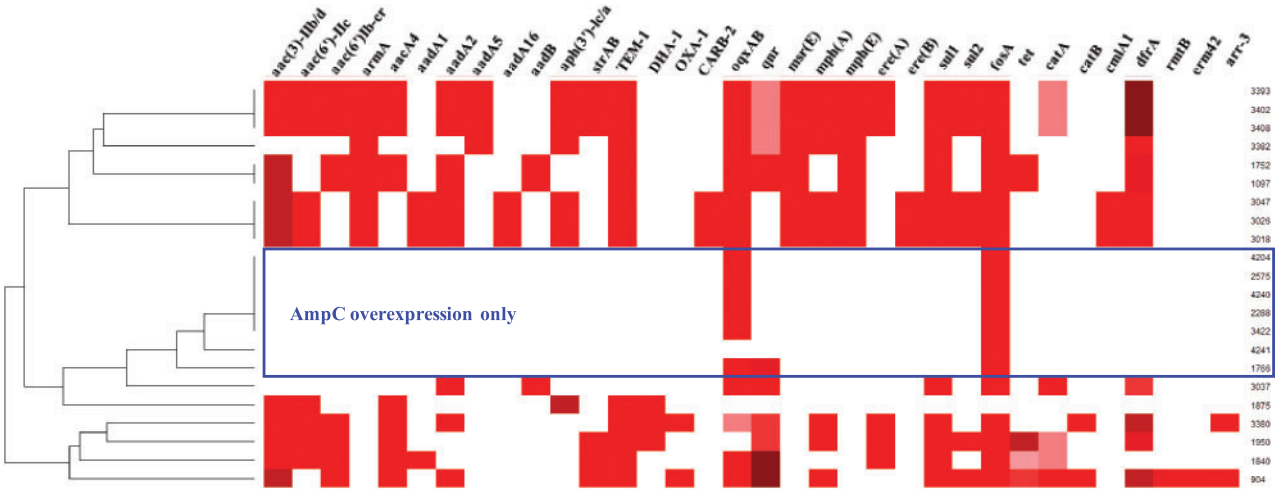

Figure 3. Resistome (without ESBL genes) of ESBL-positive ECC isolates collected in this study. The existence of a gene is shown as a red block. The colour gradients represent different alleles. ESBL-positive isolates without ESBL genes are marked in a blue frame. Isolates are clustered according to the similarity of the resistome. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

49162. This suggests that *cluster III* is a novel subspecies of *E. hormaechei*. The ANI values of the three *cluster IV* strains were 92.96% versus the type strain *E. asburiae* ATCC 35953, suggesting that *cluster IV* is a novel ECC species, closely related to *E. asburiae* according to the core-genome phylogeny (Figure 2). Furthermore, the ANI values of *cluster IX* strain ECC2572 and *cluster XIII* strain ECC1766 were <92% (87.73–91.55% and 86.87–90.16%, respectively) versus all available type strains, indicating that they are novel species of the ECC.

Discussion

The ECC includes multiple phylogenetically closely related species, resulting in a complicated taxonomic status. Accurate identification of ECC species by either phenotypic or genotypic methods is challenging. To date, *hsp60* typing is the only PCR-based method available for ECC species identification. Interestingly, our study revealed that the phylogeny based on concatenating the sequences of seven MLST loci corresponds to the

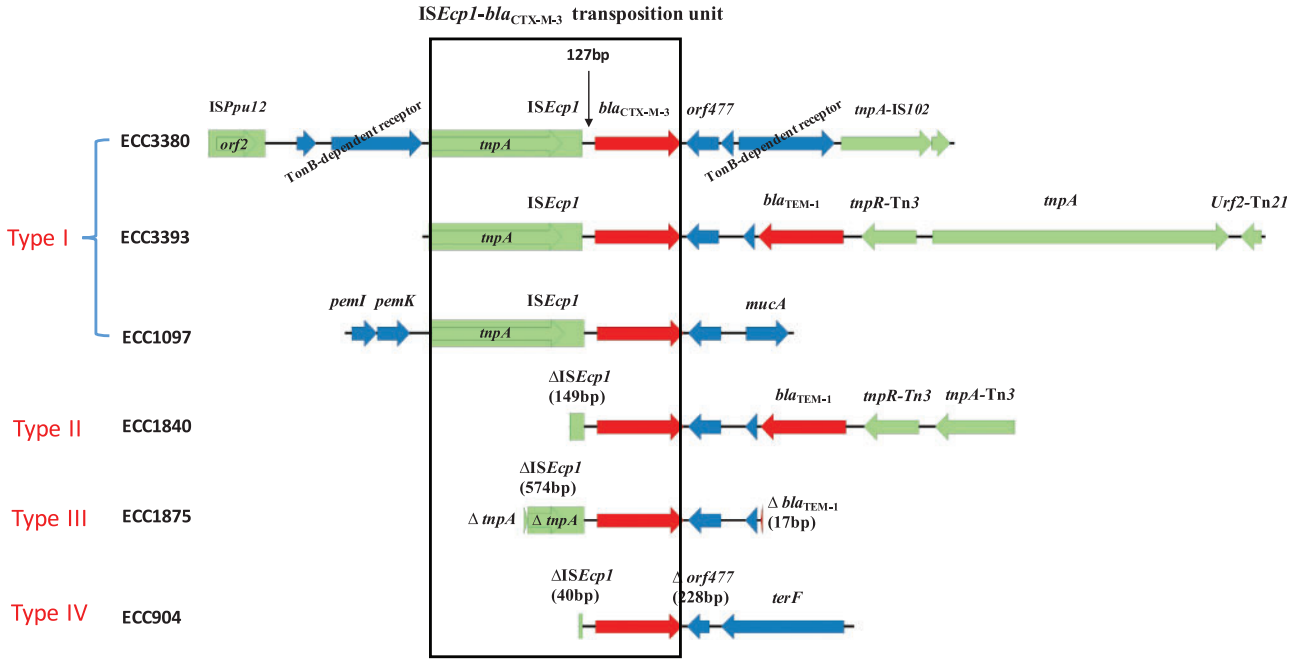

Figure 4. Schematic diagram of the genetic environment surrounding $bla_{CTX\text{-}M\text{-}3}$. Genes are shown as red (resistance genes), green (genes of mobile genetic elements) and blue (other genes) arrows. The annotation of hypothetical genes is not shown. The cassette IS*Ecp1*-$bla_{CTX\text{-}M\text{-}3}$ is marked with a black frame. The surrounding environment was typed according to the length of the *tnpA* sequence. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Table 3. Location of ESBL genes detected in ECC isolates

Isolates	CTX-M-3	CTX-M-9	CTX-M-12	CTX-M-14	SHV-12
ECC904	IncHI1A/B			IncA/C2	
ECC3380	IncHI2A				
ECC3393	IncHI2/2A				
ECC3402	IncHI2/2A				
ECC3382	IncHI2/2A				
ECC3408	IncHI2/2A				
ECC1875	IncQ2				
ECC1840	IncN				IncHI2/2A
ECC1097	IncL/M	IncHI2/2A			
ECC1752	IncL/M	IncHI2/2A			
ECC3137		IncHI2/2A			
ECC3018			chromosome		
ECC3026			chromosome		
ECC3047			chromosome		
ECC1950				IncHI2/2A	

results of *hsp60* typing, albeit resulting in a higher resolution (i.e. more clusters). We therefore suggest that *hsp60* typing can be replaced by the phylogenic analysis of MLST for ECC species identification in the future, since MLST but not *hsp60* typing is routinely performed in most laboratories.

The ECC species are often investigated at the complex level, thus largely impeding our understanding of their epidemiology and clinical significance at the species/subspecies level. In this study, *E. hormaechei* (33.9%) and *E. kobei* (24.2%) were identified as the predominant species among 62 CA-ECC strains collected across China. Although this is consistent with *E. hormaechei* being the most prevalent species in the clinical setting,[4,5,35] to the best of our knowledge, this is the first report identifying *E. kobei* as the predominant species within the ESBL-positive population, with a higher prevalence than *E. hormaechei* (50% versus 22.7%). Of more concern, dissemination of the ESBL-producing *E. kobei* clone ST591 was identified in northern China and SNP analysis additionally supports an inter-regional transmission of an ESBL-producing *E. kobei* clone ST32 between the middle south and the south-west. These results suggest that surveillance is required for controlling further spread of ESBL-producing *E. kobei* clones in China. Additionally, very few epidemiological data are available for *E. kobei*, probably due to its infrequent detection in the clinical setting. The limited information does not allow us to conclude whether this distribution is specific to community-associated infections or to the geographical epidemiology (i.e. China). More data are needed to understand the epidemiology of *E. kobei* in the future.

Before the current study, the drug-resistance profiles and clonality of CA-ECC on a national scale in China was unclear. We report here a high level of ESBL; 35.5% of CA-ECC. This is comparable with CA-*E. coli* (46.5%) and CA-*K. pneumoniae* (31.8%) from the same surveillance project,[36,37] whereas the prevalent ESBL genotypes differed among CA-ECC ($bla_{CTX\text{-}M\text{-}3}$), CA-*E. coli* ($bla_{CTX\text{-}M\text{-}14}$) and CA-*K. pneumoniae* ($bla_{CTX\text{-}M\text{-}15}$). Additionally, $bla_{CTX\text{-}M\text{-}9}$ was previously detected as the most prevalent ESBL gene in nosocomially acquired ECC in China[19] and $bla_{CTX\text{-}M\text{-}9}$ and $bla_{CTX\text{-}M\text{-}15}$ in other

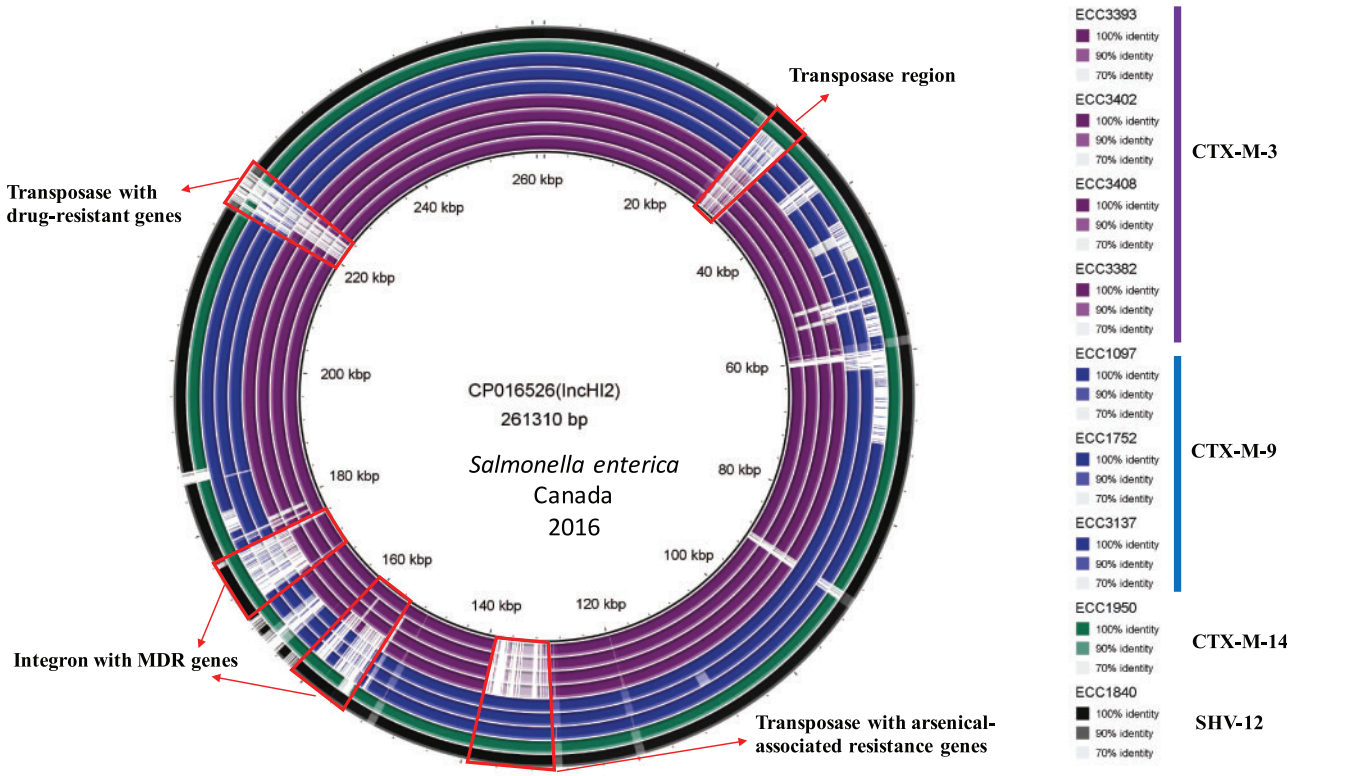

Figure 5. Comparison of IncHI2/2A plasmids detected in ECC strains of this study. One plasmid of each clone was selected and is shown here. Each ring corresponds to the BLASTn result of one plasmid relative to the reference p09-036813-1A_261 (CP016526) from *S. enterica*. From outer to inner, the rings are ordered as the sequence shown in the key (right-hand side of the figure). Plasmids are grouped in different colours according to the carried ESBL genes. The gradients (dark, pale and white) of each colour represent the sequence similarity (from 100% to 0%) between samples and reference. Some mobile genetic regions are marked in red frames. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

countries.[13,24,38,39] This indicates that the ESBL genotype of community-associated and nosocomially acquired ECC could be different. Currently, little is known about the clonality and/or global epidemiology of the ECC due to the relatively late establishment of the MLST scheme. More recently, international high-risk clones (ST66, ST78, ST108 and ST114) were detected in European countries.[23–25] However, none of these high-risk clones was found in the current study or our previous study,[19] indicating that the epidemiological characterization of the ECC could be geographically specific.

Intriguingly, we found a significant inter-species variation of the ESBL mechanism among the ESBL-positive ECC population. All ESBL producers were exclusively identified as *E. kobei* and *E. hormaechei*, while the ESBL-positive isolates of other species were caused by AmpC overexpression. Likewise, a recent study reports that colistin hetero-resistance of ECC is dependent on the cluster/species.[40] We further observed a significant inter-group difference in drug susceptibilities among the ESBL-positive isolates. Such a difference might significantly compromise clinical treatment, thus emphasizing the necessity of species-level identification of ECC in clinical settings. Additionally, all ESBL-positive isolates caused by AmpC overexpression encoded a very limited repertoire of resistance genes, since they carried no or only one plasmid. However, the ESBL-producing isolates harboured multiple plasmids, resulting in an MDR phenotype. Such broad-spectrum antibiotic resistance conferred by plasmids greatly facilitates the dissemination of ESBL genes in the hospital environment and thus affects the inter-species distribution diversity in clinical settings. Taken together, our results suggest that tailored surveillance at the species level is necessary for efficient control of the spread of drug-resistant ECC.

Although the ECC is one of the most important ESBL producers, unlike *E. coli* and *K. pneumoniae*, no epidemic ESBL-producing plasmids have yet been identified in the ECC. In this study, we identified that IncHI2 is the major plasmid type responsible for the dissemination of ESBL genes in the CA-ECC in China. We further found that the IncHI2-type plasmids were also prevalent among the nosocomially acquired ESBL-producing ECC (18/27) (data not shown). This is consistent with two previous studies showing that IncHI2-type plasmids are responsible for the dissemination of CTX-M genes in nosocomially acquired ECC isolates in Norway and Spain.[41,42] Taken together, the IncHI2-type plasmids may act as an important reservoir mediating the dissemination of CTX-M genes in ECC populations worldwide.

IncHI2-type plasmids are frequently involved in the acquisition of various antibiotic resistance genes in isolates of human and animal origin.[43–46] All IncHI2 plasmids identified in this study shared a similar backbone to the one recovered from *S. enterica* in Canada. This indicates that IncHI2 could act as a major vector transferring the resistance genes between human and animals. Further, IncHI2 frequently carries carbapenemase genes, especially *bla*$_{VIM-}$

1.[47–50] It would not be surprising if the ESBL-producing ECC isolates carrying IncHI2 plasmids would easily become carbapenem resistant by acquiring integron-borne carbapenemase genes in the future.

In conclusion, the detected high ESBL rate in the CA-ECC in China, especially the clonal dissemination of *E. kobei* identified in certain regions, suggests that tailored surveillance of this species is necessary in the future. The inter-species difference in drug susceptibility is mainly associated with the diverse ESBL mechanisms among the ECC population. Particular attention should be paid to the wide spread of IncHI2-type plasmids, which could be a reservoir of carbapenemase genes in the future.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 and S2 and Table S1 appear as Supplementary data at *JAC* Online.

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