Molecular epidemiology of *Enterobacter cloacae* complex isolates with reduced carbapenem susceptibility recovered by blood culture

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Summary

The *Enterobacter cloacae* complex (ECC) is one of the most common causes of bacteremia and leads to poor clinical outcomes. The aim of this study was to clarify the antimicrobial susceptibility profiles and genetic backgrounds of non-carbapenemase-producing reduced-carbapenem-susceptible (RCS) ECC blood isolates in Japan using agar dilution antimicrobial susceptibility testing, whole-genome sequencing, and quantitative polymerase chain reaction for assays of *ampC*, *ompC* and *ompF* transcripts. Forty-two ECC blood isolates were categorized into RCS and carbapenem-susceptible groups based on imipenem minimum inhibitory concentration. RCS ECC blood isolates belonged to distinct species and sequence types and produced varying class C β-lactamases. The *E. roggenkampii, E. asburiae*, and *E. bugandensis* isolates belonged only to the RCS group. Some *E. hormaecheii* ssp. *steigerwaltii* isolates of the RCS group exhibited *ampC* overexpression caused by amino acid substitutions in AmpD and AmpR along with *ompF* gene downregulation. These findings suggest that non-carbapenemase-producing RCS ECC blood isolates are genetically diverse.

Introduction

Among the Gram-negative bacteria belonging to the *Enterobacteriaceae* family, antimicrobial resistance has become a global public health concern because of the worldwide spread of carbapenem-resistant *Enterobacteriaceae* (CRE) which are associated with treatment failure.

Carbapenem resistance among *Enterobacteriaceae* was first reported in the early 1990s. The effectiveness of carbapenems is being diminished by two major resistance mechanisms in this microbe: carbapenemase-producing *Enterobacteriaceae* (CPE) (1, 2) and non-CPE CRE which overproduce β-lactamases and have low levels of carbapenem-hydrolysing activity combined with alteration of the bacterial outer membranes, which affects the influx or efflux of carbapenems (1, 2).

According to the annual reports of the Japan Nosocomial Infections Surveillance (JANIS; https://janis.mhlw.go.jp/english/index.asp/), *Enterobacter cloacae* complex (ECC) was the second most common group of CRE in 2017–2018, and non-CPE ECC was dominant among the carbapenem-resistant ECC (3, 4).

The non-CPE CRE have acquired augmented production of AmpC β-lactamases or extended-spectrum β-lactamases (ESBLs) combined with membrane-associated mechanisms of resistance: decrease in porins involved with carbapenem influx and/or increase in the functions of membrane proteins implicated in the drug efflux systems through downregulation or upregulated expression of their genes. In ECC, the AmpC gene (*ampC*) is generally encoded on the chromosome and can be induced in the presence of muramyl peptides as inducer molecules, a precursor of peptidoglycan layer, made by β-lactams that block the functions of penicillin-binding proteins (5). Mutations in the gene encoding AmpD and AmpR proteins involved in the suppressive

regulation of *ampC* expression give rise to a stable increase in AmpC production. A recent review of AmpC β-lactamases highlighted the problematic role that *Enterobacter* species (including *Klebsiella aerogenes*) play because of their potential for AmpC β-lactamase induction (6).

Involvement of reduced production of major outer membrane porins (OMPs; e.g., OmpF and OmpC), through which drugs enter Gram-negative bacterial periplasmic space, and increased expression of genes for efflux systems that expel antimicrobials from the reduced-carbapenem-susceptible (RCS) ECC cells have been described (1, 7).

The emergence of resistance during antimicrobial therapy, which leads to poor clinical outcomes, was reported among patients with *Enterobacter* bacteremia in 1991 (8). According to recent studies, ECC is among the most common causes of CRE bacteremia in many parts of the world, including Japan (9, 10).

Acquisition of specific resistance determinants by successful epidemic host clones for the dissemination of carbapenem resistance was best characterized in many carbapenemase-producing microbes, such as *Klebsiella pneumoniae* sequence type (ST) 258, *Escherichia coli* ST131, ECC STs 171 and 78 (11-13), *Pseudomonas aeruginosa* ST235, and *Acinetobacter baumannii* ST2 (14, 15).

Although many reports on ECC have documented ECC CRE in Japan, they have focused mainly on bacterial and clinical risk factors for the emergence of CPE CRE and non-CPE CRE, and there is limited information regarding the invasive ECC isolates with an RCS phenotype recovered by blood culture (13, 16).

The emergence of bacterial antimicrobial resistance is linked to the selection of innate drug-resistant cells in the presence of relevant antimicrobials, which spontaneously

occurs owing to natural errors in the replication of genomic DNA, leading to phenotypic mutations. When bacteria are exposed to antimicrobials, only accidentally mutated bacterial cells can survive in the presence of antimicrobial pressure. If mutated cells can survive and have the ability to cause disease, antimicrobial-resistant mutants would become evident and they get the advantage to be predominant in particular environments (17).

Despite the clinical significance of *Enterobacter* bacteremia caused by non-CPE CRE, little attention has been paid to the contribution of specific clonal genetic lineages to the high prevalence of carbapenem-resistant clones in the clinical environment. Therefore, we conducted a study of multicentre isolates to clarify the genetic backgrounds and antimicrobial susceptibility profiles of the RCS ECC isolates from blood samples by excluding carbapenemase producers because plasmid-mediated genes for carbapenemases can spread among bacteria with different traits, making it difficult to analyse and interpret the genetic lineages of RCS ECC isolates.

Materials and methods

Frozen stored ECC blood isolates collected between 2017 and 2019 from 42 hospitals across Japan were sent to the Department of Bacteriology, Graduate School of Medicine, Nagoya University, by two private microbial laboratories: Miroku Medical Laboratory Co., Ltd, in Saku, Nagano, and Bio Medical Laboratories, Inc., in Tokyo.

Samples collected from different clinical settings located throughout Japan, were used in our study and only a single bacterial isolate was included from each clinical site to prevent sample bias due to outbreaks.

The minimum inhibitory concentrations (MICs) of cefmetazole, cefepime, imipenem, amikacin, ciprofloxacin, tigecycline, fosfomycin, and colistin were measured on each isolate using the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (18). Interpretation of the tigecycline results was performed according to the European Committee on Antimicrobial Susceptibility Testing criteria (19) and other antimicrobial results were interpreted according to the CLSI criteria (20). Quality control testing was performed using *Escherichia coli* ATCC 25922. ECC blood isolates for which imipenem MICs were 0.5 µg/mL or higher were selected as RCS group, and those for which imipenem MICs were 0.125 µg/mL or lower were selected as carbapenem-susceptible (CS) group. Indeed, MIC ≥4 µg/mL is the criterion for resistance to diagnose clinical resistance in imipenem and meropenem, but a cut-off value of imipenem MIC ≥0.5 µg/mL was used to define the RCS group for comparative characterization of bacterial isolates for which carbapenem MICs are just below the breakpoint for "Resistance" to exactly perform present study.

Carbapenemase producers were excluded using the carbapenem inactivation method (21). Bacterial species were primarily identified by the API 20E phenotypic method (BioMérieux, Marcy-l'Étoile, France), and confirmed by genetic analysis.

The draft whole-genome sequence of each of the 42 isolates was determined using the MiSeq platform (Illumina, Inc., CA, USA). DNA was extracted from the bacterial cells using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and DNA libraries were prepared using the Nextera XT DNA library preparation kit (Illumina) and

sequenced on a MiSeq system. Draft genome sequences were obtained using the A5-MiSeq assembler (22).

Species identification was performed by average nucleotide identity on the basis of the genome sequences of type strains (23). The cut-off values for identification of bacterial species and subspecies (ssp.) were set at 95% and 98%, respectively.

Antimicrobial resistance genes (with criterion of 90% identity, 60% minimum length) and STs were identified by submitting genomic sequences to the ResFinder, version 3.2, database (https://cge.cbs.dtu.dk/services/ResFinder/) and the *E. cloacae* PubMLST database (https://pubmlst.org/ecloacae/). Genetic relatedness between ECC blood isolates was determined using concatenated sequences of seven housekeeping genes and the maximum parsimony phylogenetic analysis method (BioNumerics 7.6-evaluation license, Applied Maths, Sint-Martens-Latem, Belgium).

An in-house python script was used to extract *ampC*, *ampD*, *ampR* and *ampG* gene sequences from draft genome sequences by using GenBank accession nos. X07274, JX482632, AB016612, AB194784 as query sequences accordingly. Presence of premature stop codon was applied to identify Broken AmpD, AmpR and AmpG.

To evaluate the expression levels of genes involved in carbapenem susceptibility, real-time qPCR was conducted on the most prevalent subspecies in both RCS and CS groups. The total RNA was extracted using RNeasy mini kit (Qiagen) from bacterial cultures. Real-time qPCR reactions were performed in triplicate using the Power SYBR Green RNA-to-CT kit (Life Technologies, Carlsbad, CA, USA) and previously described primers and conditions on the StepOne Plus instrument (Life Technologies) (24). The relative expression of the genes encoding AmpC β-lactamases, OmpF and

OmpC porin channels was determined by normalization to the endogenous reference gene (*rpoB*) and comparison to the *E. hormaechei* ATCC 700323 baseline isolate (25).

All statistical analyses were performed using EZR (26). The RCS and CS groups were compared using Fisher's exact test for categorical variables. The relative changes in gene expression were analysed using the Wilcoxon rank-sum test. A *P* value of <0.05 was considered statistically significant.

Results

A total of 42 ECC blood isolates representing eight geographic regions (Hokkaido, Tohoku, Kanto, Chubu, Kansai, Chugoku, Shikoku, Kyushu & Okinawa) in Japan were obtained of which 21 were assigned to each group. (The equal number of specimens in each group was by chance, not by design.)

The isolates were obtained from 42 patients admitted to 42 hospitals aged 53 to 97 years (median, 80 years; interquartile range, 71–88 years), of whom 45% were male and 55% were female. As summarized in Table 1, all the isolates in the RCS group were resistant to cefmetazole and susceptible to amikacin, while all the isolates in the CS group were susceptible to cefepime, amikacin, ciprofloxacin, and colistin. The prevalence of resistance was <25% to all the antimicrobials tested except cefmetazole and tigecycline in both groups.

A total of 42 genome sequences were obtained using the MiSeq platform at an average depth of 45.02 (standard deviation [SD], 10.66). The assembled genomes had an average number of 45.31 (SD, 28.55) contigs and an N_{50} value of 309,908 bp (SD, 219,316 bp).

Chromosomally encoded ACT type class C β-lactamase genes were detected in all the isolates in the CS group, and 16/21 isolates (76%) in the RCS group (Table 2). Two isolates of the RCS group harbored both class C (ACT) and class A (CTX-M or TEM) β-lactamase genes. The most common subtype of the ACT gene was ACT-7-like, which was present in 6/21 (28%) of the RCS group and 7/21 (33%) of the CS group. Analysis of amino acid insertions and deletions found that the AmpC enzymes produced by the RCS group were the same as in amino acid length of the original AmpC reported in the reference strain of ECC p99 while insertion at position 36 and deletions at positions 37 and 39 were detected in single isolate of CS group (27).

Forty-one of the 42 isolates were identified by API 20E as *E. cloacae*, and low discriminatory results were obtained for one isolate belonged to *E. kobei* species. The isolates in the RCS group belonged to six species (*E. hormaechei*, *E. kobei*, *E. ludwigii*, *E. roggenkampii*, *E. asburiae*, and *E. bugandensis*) and the isolates in the CS group belonged to three species (*E. hormaechei*, *E. kobei*, and *E. ludwigii*). The seven *E. hormaechei* isolates in the RCS group came from the Tohoku, Kanto, Chubu and Kyushu, and the 16 *E. hormaechei* isolates in the CS group came from all regions except Shikoku. Only the *E. roggenkampii* isolates belonging to the RCS group harbored genes for MIR (n=2) or CMG (n=3) type class C β-lactamases. Among them, ST41 and ST1259 were single locus variants carrying MIR type class C β-lactamases, whereas the other three isolates were different in at least four of seven loci and carrying CMG type class C β-lactamases. One isolate of *E. bugandensis* ST1052 was found in the RCS group.

Multilocus sequence type (MLST) analysis distinguished 36 STs among the 42 isolates, indicating high genetic diversity (Fig. 1, Table 3, Table 4). Among these 36 STs, 8 STs (1196, 1197, 1252, 1253, 1255, 1256, 1258, 1259) were newly identified in the RCS group and three new STs (1204, 1254, 1257) were identified in the CS group.

The *pyrG* gene, which functions in pyrimidine biosynthesis, was absent in *E. hormaechei* subsp. *steigerwaltii* isolate belonging to the RCS group, which allelic profile of *fusA*, *gyrB* and *rplB* genes was identical to ST112. ST50 and ST662 were single locus variants, belonging to allelic numbers 37 and 240 of the *pyrG* gene exhibiting two nucleotide differences.

The relative *ampC* gene expression in the RCS group and the CS group ranged from 5.165 to 12.607, with a median of 8.949, and from −4.896 to 1.621, with a median of −1.157, respectively. The difference in the relative *ampC* gene expression between the two groups was statistically significant ($p<0.001$). Strain 24017, which is a member of the RCS group, was excluded from statistical analysis of the *ampC*, *ompC*, *ompF* transcripts as we did not detect a relative increase in the expression of the *ampC* gene. We failed to identify Class A and/or D β-lactamases in strain 24017. Therefore, Factors contributing to RCS in strain 24017 should be further explored.

The relative *ompC* gene expression in the RCS group and the CS group ranged from 0.347 to 3.403 with a median of 2.25, and −12.70 to 2.20 with a median of −0.5, respectively. The difference in the relative *ompC* gene expression between the two groups was statistically significant ($p=0.005$).

The relative *ompF* gene expression in the RCS group and the CS group ranged from −4.31 to −0.12, with a median of −1.055, and from 0.9 to 3.371, with a median of 2.133, respectively. The difference in the relative expression of the *ompF* gene between the

two groups was statistically significant (*p*=0.003). Reduction in the expression of either major porin channel was not detected in RCS group member Strain 23635 (sample 1), but both ACT and CTX-M-9 type β-lactamases were identified. All the other members of the RCS group showed increased expression of the *ampC* gene and decreased expression of the *ompF* gene. However, no increased expression of the *ampC* gene or decreased expression of the *ompF* gene was detected in any isolates in the CS group (Fig. 2).

Analysis of *ampD* and *ampR* revealed an Ile259Val substitution in AmpR and Pro28His, Ile48Asp, Trp95Arg, His19Tyr, and Asp164Ala substitutions in AmpD exclusive to the *E. hormaechei* ssp. *steigerwaltii* RCS isolates while sample 5 had truncated AmpD caused by premature stop codon at position 95 (Fig. 2). The significance of these substitutions on derepression of *ampC* needs to be confirmed with more appropriate studies.

Discussion

ECC is one of the carbapenem-resistant pathogens that is increasingly implicated in nosocomial infections and is problematic because it has the ability to rapidly become resistant to antimicrobials.

Enterobacter spp. have emerged as one of the main causes of bacteremia after *E. coli* and *K. pneumoniae* (9, 28). *K. pneumoniae* ST14 and ST258 (29, 30), and *P. aeruginosa* ST235 are specific genetic lineages that cause invasive infections with carbapenem-resistant bacteria. However, there is limited information on the specific genetic lineages of ECC that cause invasive infections.

Genetic diversity of ECC blood isolates has been demonstrated in China by Wang et al. (31) with 51 STs from 53 isolates. The authors did not specify the carbapenem resistance and carbapenemase production of each isolate, but the majority of the CS isolates were non-carbapenemase-producers. Tetsuka et al. (16) demonstrated genetic divergence among three carbapenem-resistant ECC blood isolates producing no carbapenemases. Specific genetic lineages of carbapenem-resistant ECC clinical isolates with carbapenemases have been reported in Japan. Among IMP-1 producing ECC clinical isolates, ST78 is the most common, and several studies have described the contribution of IncHI2 plasmids to the spread of *bla*$_{\text{IMP-1}}$ (13, 32, 33).

In this study, we found that the non-carbapenemase-producing RCS ECC blood isolates belonged to distinct sequence types, species and produced distinct types of class C β-lactamases. This suggests that the selection of RCS mutant among ECC blood isolates is independent of sequence types, species and type of class C β-lactamases produced. Therefore, RCS phenotype might independently evolve from CS phenotypes of ECC with diverse genetic backgrounds. All ECC clinical isolates may have the potential to develop carbapenem resistance, so early microbial diagnosis of ECC blood isolates is important to ensure adequate treatment and infection control.

ECC Hoffmann cluster IV was defined as a novel species of *E. roggenkampii* by Sutton et al. in 2018 (34). Non-carbapenemase-producing carbapenem-resistant ECC Hoffmann cluster IV blood isolates have been reported in Nagoya University hospital (16). Three sputum isolates of ST634 harboring the *bla*$_{\text{MIR}}$ gene on IncFII plasmids have been reported in China (35), which showed resistance to cephems without ESBL genes, indicating AmpC hyperproduction. A RCS ECC Hoffmann cluster IV blood isolate belonged to ST997 carrying both IncFIB and IncN plasmids that mediated

genes for IMP-1 metallo-β-lactamase, CTX-M-14 ESBL, and MIR-6 type class C β-lactamase was reported in Japan (32). As *E. roggenkampii* was recently established as a novel species, knowledge of its carbapenem susceptibility is limited. We found that the five *E. roggenkampii* blood isolates with the RCS phenotype had different sequence types, suggesting polyclonal spread.

It has been shown that single nucleotide polymorphisms (SNPs) in the *ampD* gene associated with derepression of *ampC* leading to cephalosporin resistance (36). The involvement of global transcriptional regulators such as RamA to the expression of proteins implicated in the change of bacterial membrane permeability and adaptation to antimicrobial challenge have been recently demonstrated by Majewski et al. (37). Our study showed an association of augmented AmpC production with reduced-carbapenem susceptibility in *E. hormaechei* ssp. *steigerwaltii*. Therefore, it suggests that replacement of larger channel OmpF with smaller channel OmpC in *E. hormaechei* ssp. *steigerwaltii* might contribute to decreased efficacy of carbapenems by maintaining their concentration in the periplasm below saturation level. Although downregulated production of OmpF porins has a negative influence (38) on the virulence and fitness of RCS *E. hormaechei* ssp. *steigerwaltii* isolates, upregulated production of OmpC porins may have compensatory effects or may serve as a virulence factor.

In conclusion almost all ECC isolates have the potential to develop the RCS phenotype regardless of their genotype and species.

One limitation of this study is unavailability of patient clinical data due to ethical reasons therefore, it is difficult to draw conclusions regarding whether the development of carbapenem resistance depends on the selection of resistant mutants.

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Conflicts of interest

None to declare

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Table 1. Rates of antimicrobial resistance in reduced-carbapenem-susceptible and carbapenem-susceptible groups

Antimicrobial	Number of isolates (%)		*P*
	RCS group (N=21)	CS group (N=21)	
Cefmetazole	21 (100)	13 (61.9)	0.003
Cefepime	1 (4.7)	0 (0)	>0.99
Imipenem	0 (0)	0 (0)	
Meropenem	0 (0)	0 (0)	
Tigecycline	16 (76.1)	11 (52.3)	0.2
Amikacin	0 (0)	0 (0)	
Ciprofloxacin	1 (4.7)	0 (0)	
Fosfomycin	5 (23.8)	3 (14.2)	0.7
Colistin	2 (9.5)	0 (0)	

Abbreviations: CS, carbapenem-susceptible; RCS, reduced-carbapenem-susceptible.

Table 2. Identified antimicrobial resistance genes among ECC blood isolates

Types of resistance genes			Number of isolates (%)		
			Total	RCS group	CS group
			(N=42)	(N=21)	(N=21)
AmpC					
ACT/MIR					
	ACT				
		ACT-7-like	13 (30.9)	6 (28.5)	7 (33.3)
		ACT-15-like	7 (16.6)	1 (4.7)	6 (28.5)
		ACT-9-like	5 (11.9)	2 (9.5)	3 (14.2)
		ACT-12-like	4 (9.5)	2 (9.5)	2 (9.5)
		ACT-5-like	2 (4.7)	1 (4.7)	1 (4.7)
		ACT-1-like	1 (2.3)	1 (4.7)	0 (0)
		ACT-2-like	1 (2.3)	1 (4.7)	0 (0)
		ACT-6-like	1 (2.3)	1 (4.7)	0 (0)
		ACT-10-like	1 (2.3)	1 (4.7)	0 (0)
		ACT-14-like	1 (2.3)	0 (0)	1 (4.7)
		ACT-16-like	1 (2.3)	0 (0)	1 (4.7)
	MIR		2 (4.7)	2 (9.5)	0 (0)
CMG	CMG		3 (7.1)	3 (14.2)	0 (0)
ESBL					
	CTX	CTX-M-9 group	1 (2.3)	1 (4.7)	0 (0)
Others					
	TEM	TEM-1-like	1 (2.3)	1 (4.7)	0 (0)

Abbreviations: CS, carbapenem-susceptible; ESBL, extended-spectrum β-lactamases; RCS, reduced-carbapenem-susceptible.

Table 3. Genotypes in the reduced-carbapenem-susceptible group identified by multilocus sequence type analysis (N=21[†])

		Resistance genes		Species	AmpD		AmpR		AmpG	
ST	Total	AmpC	ESBL							
ST116	2	ACT-7-like		*E. hormaechei* ssp. *steigerwaltii*	E	I/B*	E	I	E	I
ST113	1	ACT-15-like	CTX-M-9 group	*E. hormaechei* ssp. *steigerwaltii*	E	I	E	I	E	I
ST927	1	ACT-7-like		*E. hormaechei* ssp. *steigerwaltii*	E	I	E	I	E	I
ST1115	1	ACT-2-like		*E. hormaechei* ssp. *steigerwaltii*	E	I	E	I	E	I
ST1196	1	ACT-7-like		*E. hormaechei* ssp. *steigerwaltii*	E	I	E	I	E	I
Unknown[†]	1	ACT-7-like		*E. hormaechei ssp. steigerwaltii*	E	I	E	I	E	I
ST120	1	ACT-7-like		*E. hormaechei* ssp. *xiangfangensis*	E	I	E	I	E	I
ST1197	1	ACT-5-like		*E. hormaechei* ssp. *hoffmanii*	E	I	E	I	E	I
ST41	1	MIR-3-like		*E. roggenkampii*	E	I	E	I	E	I
ST1255	1	CMG-like		*E. roggenkampii*	E	I	E	I	E	I
ST1256	1	CMG-like		*E. roggenkampii*	E	I	E	I	E	I
ST1258	1	CMG-like		*E. roggenkampii*	E	I	E	I	E	I
ST1259	1	MIR-3-like		*E. roggenkampii*	E	I	E	I	E	I
ST1253	1	ACT-1-like		*E.asburiae*	E	I	E	I	E	I
ST1057	1	ACT-10-like		*E.asburiae*	E	I	E	I	E	I
ST1052	1	ACT-6-like		*E. bugandensis*	E	I	E	I	E	I
ST99	1	ACT-9-like		*E. kobei*	E	I	E	I	E	I
ST563	1	ACT-9-like		*E. kobei*	E	I	E	I	E	I
ST1252	1	ACT-12-like		*E. ludwigii*	E	I	E	I	E	I
ST20	1	ACT-12-like		*E. ludwigii*	E	I	E	I	E	I

[†] One isolate of *E. hormaechei* ssp. *steigerwaltii* ST was not identified due to the absence of *the pyrG* gene.
*Among the two isolates Intact AmpD was identified in only one isolate.
Abbreviations: ESBL, extended-spectrum β-lactamases, ST, sequence type, E, Exist, N, Not Exist, I, Intact, B, Broken.

Table 4. Genotypes in the carbapenem-susceptible group identified by multilocus sequence type analysis (N=21)

		Resistance genes	Species	AmpD		AmpR		AmpG	
ST	Total	AmpC							
ST50	3	ACT-15-like	*E. hormaechei* ssp. *steigerwaltii*	E	I	E	I	E	I/B*
ST116	1	ACT-7-like	*E. hormaechei* ssp. *steigerwaltii*	E	I	E	I	E	I
ST48	1	ACT-7-like	*E. hormaechei* ssp. *steigerwaltii*	E	I	E	I	E	I
ST62	1	ACT-7-like	*E. hormaechei* ssp. *steigerwaltii*	E	I	E	I	E	I
ST112	1	ACT-7-like	*E. hormaechei* ssp. *steigerwaltii*	E	I	E	I	E	I
ST151	1	ACT-7-like	*E. hormaechei* ssp. *steigerwaltii*	E	B	E	I	E	I
ST346	1	ACT-15-like	*E. hormaechei* ssp. *steigerwaltii*	E	I	E	I	E	I
ST636	1	ACT-7-like	*E. hormaechei* ssp. *steigerwaltii*	N	NA	E	I	E	I
ST662	1	ACT-7-like	*E. hormaechei* ssp. *steigerwaltii*	E	I	E	I	E	I
ST744	1	ACT-15-like	*E. hormaechei* ssp. *steigerwaltii*	E	I	E	I	E	I
ST1254	1	ACT-15-like	*E. hormaechei* ssp. *steigerwaltii*	E	I	E	I	E	I
ST728	1	ACT-16-like	*E. hormaechei* ssp. *xiangfangensis*	E	I	E	B	E	I
ST78	1	ACT-5-like	*E. hormaechei* ssp. *hoffmanii*	E	I	E	I	E	I
ST1257	1	ACT-14-like	*E. hormaechei* ssp. *hoffmanii*	E	I	E	I	E	I
ST32	1	ACT-9-like	*E. kobei*	N	NA	E	I	E	B
ST1001	1	ACT-9-like	*E. kobei*	E	I	E	I	E	I
ST1204	1	ACT-9-like	*E. kobei*	E	B	E	I	E	I
ST20	1	ACT-12-like	*E. ludwigii*	E	I	E	I	E	I
ST781	1	ACT-12-like	*E. ludwigii*	E	I	E	I	E	I

Abbreviations: ST, sequence type, E, Exist, N, Not Exist, I, Intact, B, Broken, NA, Not applicable.

* Among the three isolates broken AmpG was identified in one isolate.

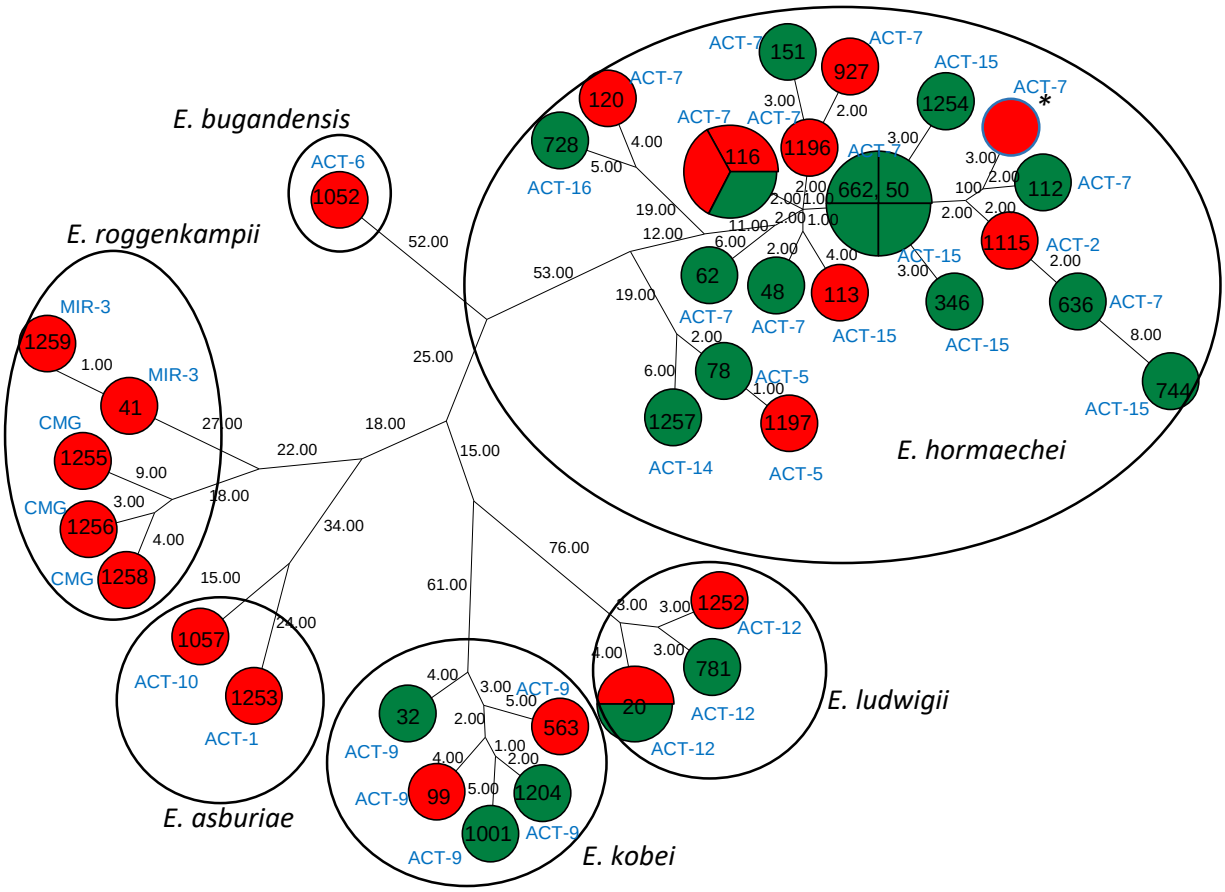

Fig. 1. Genetic relatedness of non-carbapenemase-producing reduced-carbapenem-susceptible and carbapenem-susceptible *E. cloacae* complex blood isolates.
The RCS and CS group isolates are represented by red and green segments, respectively. The number of isolates attributable to particular ST is indicated by the division of each node into a relevant number of segments. The genetic distance between the different STs is reflected by the branch length.
*ST was not identified due to the absence of the pyrG gene.
Abbreviations: CS, carbapenem-susceptible; RCS, reduced-carbapenem-susceptible; ST, sequence type.

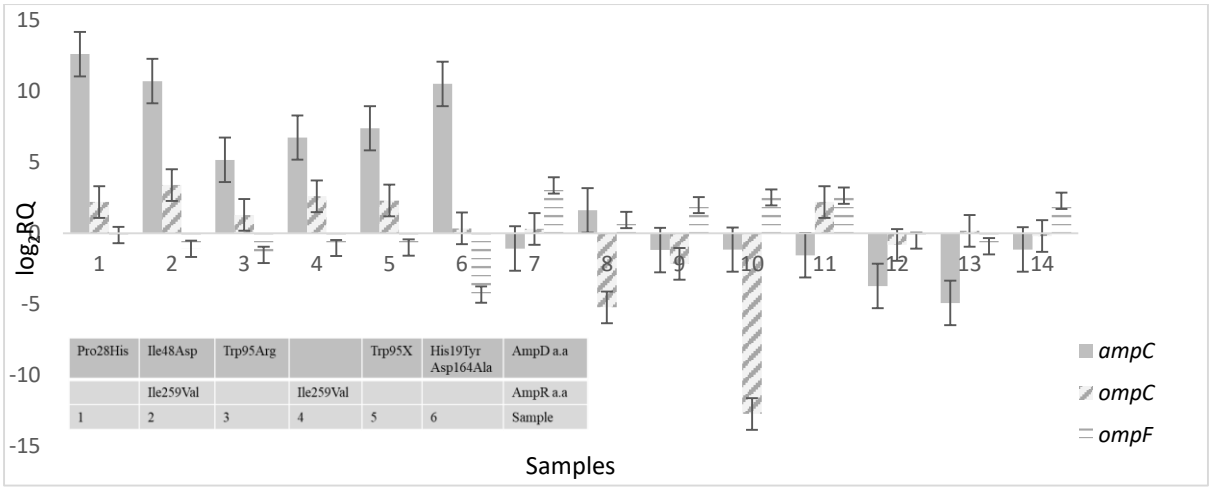

Fig. 2. Expression of *ampC*, *ompC* and *ompF* genes of *E. hormaechei* ssp. *steigerwaltii* blood isolates relative to *E. hormaechei* ATCC 700323.
Sample numbers 1–6 and 7–14 represent RCS group and CS group respectively.