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Whole Genome Sequence Analysis of CTX-M-55 Producing *Escherichia coli* Isolates from Clinical Patients in Japan

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Abstract

Extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* is one of the most important drug resistant pathogen of clinical infection and is of global concern. CTX-M-55 type ESBL that has D240G amino acid substitution to confer ceftazidime resistance is often isolated from not only clinical patients, but also livestocks and retail meats. This study revealed molecular epidemiology and characteristics of CTX-M-55 producing *E. coli* isolated from medical facilities in Japan with less than 300 beds. Among the isolates possessing $bla_{CTX\text{-}M\text{-}55}$, antimicrobial susceptibility was examined, and resistant genes, sequence type, and incompatibility type of its plasmids were determined by next-generation sequencing. The proportion of *E. coli* harboring $bla_{CTX\text{-}M\text{-}55}$ ESBL was 12.5% (8/64) of the CTX-M-1 group ESBL genes positive isolates. Four and six isolates were resistant to fosfomycin and ciprofloxacin, respectively. Results of the whole genome sequencing for $bla_{CTX\text{-}M\text{-}55}$ positive strains, sequence type and plasmid incompatibility were dominant as ST131 and IncF, respectively. Further, two isolates determind as ST131 were likely to be isolated from the same facility, and are identical. This finding suggests that continuous surveillance for CTX-M-55 producing *E. coli* ST131 is needed. Moreover, the presence of *floR* gene in clinical strain implied that ingestion of contaminated meat and/or contact of livestocks transferred CTX-M-55 producing *E. coli* to community human. Additionally, the spread of *fosA* possessing *E. coli* combined with $bla_{CTX\text{-}M\text{-}55}$ may restrict therapeutic options against patients of urinary tract infection on which β-lactam has no therapeutic effect. In conclusion, this is the first study on the epidemiology and characteristics of CTX-M-55 ESBL producing *E. coli* isolates from regional medical facilities with less than 300 beds in Japan. These results show that CTX-M-55 producing *E. coli* originates from a different clone such as pandemic and veterinary clone.

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Introduction

Escherichia coli is the most important clinical pathogen of hospital infection, and its broad-spectrum β-lactam resistance is of global concern. Broad-spectrum β-lactam resistance is commonly expressed by production of β-lactamases. Certain genes encoding β-lactamases are located on the plasmids; thereby, considered to spread over many species and strains [1]. In particular, in order to prevent selection of carbapenem resistant *Enterobacteriaceae,* which is one of serious problem in the world, it should be necessary to keep up surveillance and control of extended spectrum β-lactamase (ESBL) producing gram negative organisms [2].

Currently, CTX-M-15 type ESBL producing *E. coli* has notably been disseminated all over the world. Moreover, CTX-M-16, -27, and -55 type ESBL producing *E. coli* that possesses D240G amino acid substitution and confers ceftazidime resistance was also found [3-4]. In comparison with CTX-M-15 enzyme, CTX-M-55 enzyme is substituted by a single amino acid in mutated sites, A77V, and is classified as CTX-M-1 groups [5]. Since CTX-M-55 producing *Enterobacteriaceae* is often isolated from livestock and retail meat, moreover, it is suspected that ESBL producing bacteria spread to human through meat ingestion. Indeed, several reports support the suspicion that ESBL producing bacteria spread through the meat ingestion [6], having been prevailing in clinical patients and healthy humans [7].

However, characteristics of CTX-M-55 producing *E. coli* strains isolated from clinical patients are still unclear. The aim of the present study is to investigate molecular epidemiology and characteristics of CTX-M-55 producing *E. coli* isolated from medical facilities with less than 300 beds in Japan using next-generation sequencing.

Materials and Methods

Bacteria collection

Sixty-four isolates including CTX-M-1 group ESBL genes, which were selected from 207 ESBL positive *E. coli* isolated from medical facilities with less than 300 beds from February to October in 2016 in Kanagawa prefecture, Japan, were used as samples [8]. Classification of CTX-M groups was performed by multiplex PCR according to the study of Woodford et al [9]. Indeed, these isolates were characterized in commercial laboratories. Only one isolate per patient per facility was collected. However, the strain isolated from the same patient in different facilities was probably included. The isolates were collected with their information regarding specimen, clinical department, and patient status (inpatient or outpatient), as well as with facility information about number of beds and location.

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Antimicrobial susceptibility testing

MICs were determined by the CLSI microdilution broth method [10]. Commercial microdilution broth plates "Neg EN MIC 1J" for antibiotic susceptibility testing were purchased from the Beckman Coulter.

Determination of bla_{CTX-M} gene typing

The isolates were cultured for enrichment in LB broth (Nacalai Tesque) including 2mg/L cefotaxime (sigma), and whole DNAs were extracted by DNeasy Blood & Tissue Kit (QIAGEN). The bla_{CTX-M} gene typing in CTX-M-1 group ESBL genes positive isolates was determined by direct sequencing. Primers for PCR were used as primers for direct sequencing [8-9].

Next-generation sequencing and data analysis

The isolates involving $bla_{CTX-M-55}$ gene were cultured for enrichment in LB broth including 2mg/L cefotaxime, and whole DNAs of the strains were extracted by PureLink Genome DNA Kit (Thermo). The fragment library was constructed by Ion Xpress Plus Fragment Library Kit (Thermo). The DNA fragment was ligated to barcode and P1 adapters, and the 480 bp library fragment was collected by size selection. Ion Chef and Ion S5 system (Thermo) and Ion 530 Chip (Thermo) was employed for next-generation sequencing. High-throughput sequencing data was analyzed for *de novo* assembly using Center for Genome Epidemiology (CGE) site [11]. Assembled contigs of these isolates were analyzed through bacterial analysis pipeline as CGE site. Multilocus sequence typing, resistant genes, plasmid incompatibility (Inc) typing, and plasmid sequence typing were also determined by CGE site.

Results

The proportion of $bla_{CTX-M-55}$ positive *E. coli* was 12.5% (8/64) of the CTX-M-1 group ESBL genes positive isolates (Table 1). Two of eight isolates were from outpatient, and hkk-129 and hkk-130 were likely to be collected from the same medical facility. Among the 8 $bla_{CTX-M-55}$ identified isolates, 7 isolates of *E. coli* were applied for further antimicrobial susceptibility and next-generation sequencing data. But, one isolate remaining was not available for re-growing from bacterial stock. The results were shown in Table 2 and Table 3. Four and six isolates were resistant to fosfomycin and ciprofloxacin, respectively. As a result, bla_{TEM-1B} and *tetA* were detected in 6 and 5 isolates. Some aminoglycoside modifying enzyme genes were detected in 4 isolates. All isolates harbored plasmid incompatibility type (Inc) F plasmid. IncN and IncI1 were detected in 2 and 2 isolates, respectively. Additionally, *E. coli* sequence type (ST) 131 was determined in 3 isolates, and ST70, ST1193, ST1771, and ST2003 was identified in each of 4 isolates. The isolate of hkk-61 harbored IncF, IncX, and IncI1, and carried *mphA, floR,* and *tetB* that provide trimethoprim, florphenicol, and tetracyclin resistance, respectively. Moreover, the sequence type, resistant genes, and harbored plasmids of hkk-129 corresponded to those of hkk-130.

Discussion

This study reveals the prevalence and molecular characteristics of CTX-M-55 producing *E. coli* isolated from medical facilities with fewer than 300 beds in Kanagawa prefecture, Japan. There were many studies on the prevalence of CTX-M-55 producing *E. coli* in tertiary hospitals in Japan [12], but not in middle and small medical facilities. This study dealing with the strain isolated from medical facilities with less than 300 beds demonstrates precisely the prevalence of CTX-M-55 producing gram-negative bacteria in community in Kanagawa prefecture.

Strain No	Sample	City	No. of bed	Department	In/Out
hkk-061	Cathererized urine	Yokohama	64	--	Inpatient
hkk-089	Urine	Kamakura	112	Dialysis hp.	Outpatient
hkk-127	Catheterized urine	Yokohama	190	--	Inpatient
hkk-129	Urine	Yokohama	41	--	Inpatient
hkk-130	Purulent fluid	Yokohama	41	--	Inpatient
hkk-141	Urine	Kamakura	198	Internal med.	Outpatient
hkk-152	Urine	Yokohama	52	Nephrology	Inpatient
hkk-199	Urine	Chigasaki	100	Internal med.	--

Table 1: Bacterial isolates.

Strain No.	A/C	P/T	CMZ	CAZ	CPR	C/S	FMOX	IPM	CPFX	LVFX	GM	AMK	FOM	ST	MINO
hkk-061	16/8	≤8	≤4	>8	>16	16/8	≤2	≤0.5	>2	>4	>8	≤4	≤4	>2/38	4
hkk-127	8/4	≤8	≤4	>8	>16	16/8	≤2	1	2	1	≤1	≤4	>16	≤2/38	8
hkk-129	8/4	≤8	≤4	8	>16	8/4	≤2	≤0.5	>2	>4	>8	≤4	≤4	≤2/38	≤1
hkk-130	8/4	≤8	≤4	>8	>16	≤4/2	≤2	≤0.5	>2	>4	>8	≤4	≤4	≤2/38	≤1
hkk-141	8/4	≤8	≤4	>8	>16	≤4/2	≤2	≤0.5	≤0.25	≤0.5	≤1	≤4	>16	≤2/38	≤1
hkk-152	8/4	≤8	≤4	>8	>16	16/8	≤2	≤0.5	>2	>4	>8	≤4	16	≤2/38	≤1
hkk-199	>16/8	>64	≤4	>8	>16	>32/16	≤2	1	>2	>4	2	8	>16	≤2/38	8

Table 2: Antimicrobial susceptibility of the isolates.

Strain	MLST	Resistant Genes							Plasmids	
		β-lactam	Aminoglycoside	Tetracycline	Trimethoprim	Sulphonamide	Phenicol	Macrolide	Fosfomycin	IncF	Others
hkk-061	ST-2003	$bla_{CTX-M-55}$, bla_{TEM-1B}	*aac(3)-IIa, aadA5, aph(3')-IIa, strA, strB*	*tea(A)*	*dfrA14, dfrA17*	*sul1, sul2*	*floR*	*mph(A)*		F31: A-:B6	IncI1 ST-18 IncX1
hkk-127	ST-1771	$bla_{CTX-M-55}$		*tet(B)*						Unknown ST	
hkk-129	ST-131	$bla_{CTX-M-55}$, bla_{TEM-1B}	*aac(3)-IId, strA, strB*	*Tet(A)*		*sul2*				F1:A2:B20	
hkk-130	ST-131	$bla_{CTX-M-55}$, bla_{TEM-1B}	*aac(3)-IID, strA, strB*	*tet(A)*		*sul2*				F1:A2:B20	
hkk-141	ST-70	$bla_{CTX-M-55}$, bla_{TEM-1B}		*tet(A)*					*fosA*	F33:A-:B-	IncN ST-5
hkk-152	ST-1193	$bla_{CTX-M-55}$, bla_{TEM-1B}	*aac(3)-IId*							F-:A1:B-	IncI1 Unknown ST
hkk-199	ST-131	$bla_{CTX-M-55}$, bla_{TEM-1B}		*tet(A)*					*fosA*	F29:A-:B10	IncN ST-8

Table 3: Resistant genes and plasmids detected and sequence types of seven *E. coli* harboring $bla_{CTX-M-55}$ isolates

It is well known that CTX-M-15 producing *E. coli* is widely disseminated to the world. Nonetheless, it is surprising that the prevalence rate of $bla_{CTX-M-55}$ positive isolate in CTX-M-1 group ESBL genes positive *E. coli* was greater than 10%. CTX-M-55 producing *Enterobacteriaceae* and its plasmids were isolated from livestock in China, Southeast Asia, and East Asia [13-14]. Shu et al. reported that CTX-M-55 producing *Enterobacteriaceae* was more common in community- and hospital-associated infection than CTX-M-15 in China [15]. Kameyama et al. also pointed out that CTX-M-55 was a secondary dominant type of CTX-M in Japanese broiler farms [16]. Therefore, it is suggested that the increment in prevalence of CTX-M-55 producing *E. coli* in human communities is due to its dissemination into livestock and retail meats.

The sequence type of *E. coli* harboring $bla_{CTX-M-55}$ gene were diverse. Three of the seven isolates corresponded to *E. coli* ST131. *E. coli* ST131 harboring $bla_{CTX-M-15}$ is disseminated to the world. It was reported, moreover, that *E. coli* ST131 harboring $bla_{CTX-M-27}$ also spread in Japan [12]. Unfortunately, the spread of *E. coli* ST131 carrying $bla_{CTX-M-55}$ gene may have the potential for dissemination of the strain that is resistant to ceftazidime. Two isolates, hkk-129 and hkk-130, were likely to be isolated from the same facility (Table 1), and are identical. This finding suggests that *E. coli* ST131 has a high risk of clonal spread in a facility and that continuous surveillance for the CTX-M-55 producing *E. coli* ST131 is needed.

One of the seven strains was recognized as *E. coli* ST1193. *E. coli* ST1193 harboring $bla_{CTX-M-55}$ dominates in China, which differs from the result of this paper [17]. *E. coli* ST1193 and ST2003 were also reported to be isolated from clinical patients [18]. In this study, these two strains were resistant to ciprofloxacin (CPFX). Particularly, a number of literatures have addressed the *E. coli* ST1193 having resistance to CPFX. As can be seen from Table 3, plasmid mediated quinolone resistant genes (PMQR) such as *qnr* and *aac(6')-Ib-cr* were not detected in any isolates. Hence, the resistance of CPFX in *E. coli* ST1193 seems to be conferred by the chromosomally mutation of quinolone resistant determined regions (QRDR) [19]. Meanwhile, there are few reports that the other ST, ST70 and ST1771, was that sporadically isolated from livestock and retail meats [19-20], and these strains were susceptible to CPFX. If the PMQR genes are acquired by these strains by means of horizontal transfer among veterinary fields, its spreading will be concern about the treatment using fluoroquinolone agants against several infections diseases such as pyelonephritis.

The resistant genes combined with $bla_{CTX-M-55}$ were bla_{TEM-1B} and *tetA*. This result is consistent with that of earlier studies [5]. Since the Inc type of plasmids harboring $bla_{CTX-M-55}$ was IncF, N, and I, $bla_{CTX-M-55}$ may be located on their Inc type plasmids in *E. coli* [21-23]. The resistant gene of *floR* responsible for resistant to florphenicol, which is generally used in a veterinary field, was detected from hkk-61. Meunier et al. suggested that florphenicol selects the strain producing β-lactamases in livestock [24]. Thus, the florphenicol and β-lactam co-resistance caused by *floR* and $bla_{CTX-M-55}$ on the same plasmid then might be related to dissemination of the strain. In this study, however, it was uncertain which Inc types of plasmid encode each resistant gene, because the contigs were not assembled completely. Besides, it was unclear whether the strain possessed one or more than 2 plasmids.

Moreover, hkk-141 and hkk-199 involving *fosA* gene harbored IncN plasmid. MacGann et al. reported that IncF with $bla_{CTX-M-55}$ and *mcr-1*

was determined in the strain isolated in US and that IncN plasmid coding *fosA* gene was retained simultaneously [25]. Additionaly, many reports in China described the determination of the *fosA* combined with IncN [26-27]. The spread of *fosA* possessing *E. coli* combined with $bla_{CTX\text{-}M\text{-}55}$ may restrict therapeutic options for treatments of urinary tract infection (UTI) patients on which β-lactam has no therapeutic response. On the other hand, the fosfomycin resistant rate of *E. coli* isolates from community UTI was 0 %. This finding then confirms that fosfomycin is available as a candidate antibiotic agent to community-UTI patients [28].

Conclusion

This is the first study on the epidemiology and characteristics of CTX-M-55 ESBL producing *E. coli* isolates from regional medical facilities with less than 300 beds in Japan. As a result, CTX-M-55 producing *E. coli* originates from a different clone. The ST of isolates harboring $bla_{CTX\text{-}M\text{-}55}$ in medical facilities, but not in central hospitals, was classified into several types including global clone ST131, but other STs of isolates from retail meats and livestocks was also determined. Unlike ST, the plasmid inc type in the clinical isolates corresponded to the type of the isolate from retail meats and livestocks.

The limitation and remaining question of this study is whether these strains directly spread to human through meat ingestion or not, that is to say, whether the strain transfers its β-lactamase genes to human strains through horizontal transfer. However, the occurrence of the *floR* gene detected from some isolates suggests that the dissemination of $bla_{CTX\text{-}M\text{-}55}$ is related to veterinary fields.

Among the isolates possessing $bla_{CTX\text{-}M\text{-}55}$, the resistance against fluoroquinolone and fosfomycin depending on spread of ST131 and/or ST1193 and of *fosA* has a clinical impact to restrict therapeutic options against infectious disease such as community UTI.

Competing Interests

The authors declare that they have no competing interests.

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