

# Multicenter Survey for Carbapenemase-Producing Enterobacteriales in Central Japan

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## Research article

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# Abstract

**Background:** Carbapenemase-producing Enterobacteriales (CPE) are resistant to many antimicrobial agents and raise concerns about its treatment options as well as its infection control.

**Methods:** It was conducted a multicenter study between 2015 and 2019 in order to clarify the molecular epidemiology of CPE in the Central Japan region.

**Results:** Out of the 56,494 Enterobacteriales strains detected during the timeframe of this study, 341 (0.6%) strains which met our criteria were analyzed. 65 out of 341 strains were determined to be CPE, and the incidence rate of CPE in Enterobacteriales was 0.12% (65/56,494). Most of which were IMP-1 and only three were IMP-6 types. Three *Escherichia coli* strain producing NDM-5 were detected and MLST analysis also showed the ST78 type was most predominant in the *E. cloacae* complex CPE (n = 14, 60.9%). On the other hand, various STs were detected in CP-*K. pneumoniae*, in which ST37 and ST517 were the most common ST types.

**Conclusion:** The incidence rate of CPE was slightly higher than the national data registers for this region, and it was possible to detect the spread of ST78 of carbapenemase-producing *E. cloacae* complex across hospitals by using this limited three-month follow-up analysis, which indicated the need to strengthen regional infection control programs.

## Background

The rapid spread of carbapenemase-producing Enterobacteriales (CPE) is a worldwide public health concern and poses a serious threat within clinical settings [1] as there are limited antimicrobial agents that can be used for infectious diseases caused by CPE, which is associated with high mortality and expensive medical costs [2, 3]. At present, CPE is endemic in Southeast Asia and some parts of Europe, but it still is rare in Japan [4]. Although the prevalence of carbapenem-resistant Enterobacteriales (CRE) is low according to the report of Japan Nosocomial Infections Surveillance (JANIS) [5], epidemiology of each region based on molecular biological analysis is not fully understood. The lack of knowledge regarding the levels of epidemiology of CPE due to the exclusive consideration of CRE on nationwide studies, leaving the presence or absence of carbapenemase genes and its production behind. The implementation of multi-institutional regional surveys is particularly important to clarify the epidemiology of CPE in each region and to develop the design of regional infection control plan against CPE [6]. In 2014, we initiated the present regional supervision analysis among major acute-care hospitals in the Aichi prefecture in the central region of Japan.

The aim of this study was to analyze the epidemiological, phenotypic and molecular characteristics of CPE collected in this survey within a time span ranging from 2015 to 2019.

## Methods

## Study setting and study populations

A total of 24 microbiological laboratories from major acute-care hospitals in the Aichi prefecture participated in the study on a voluntary basis. The hospitals that participated in the study were certified as meeting the national infection control standards and they are currently participating in the prefectural infection control network (Prefectural Infection Control Kasan-1 Network Inter-Conference). This study comprises of 24 hospitals, 3 with more than 800, 6 municipal hospitals, 3 national hospitals and 12 private hospitals.

## Bacterial isolates

The present study has a timeframe between January and March of the years ranging 2015 to 2019, in which the laboratories involved collected all non-duplicate *Escherichia coli*, *Klebsiella* spp. (*K. pneumoniae*, *K. oxytoca*, *K. aerogenes*) and *E. cloacae* complex isolates which met the susceptibility criteria attached below (Table 1). Even if the isolates were from the same species, multiple ones from the same patient were considered for this analysis under the condition of being identified more than one year apart. All isolates were sent to our laboratory for their due analysis. The participating laboratories provided the following data: specimen source, bacterial identification, antimicrobial susceptibility testing results, number of isolates of each Enterobacteriales species during each study period / individual timeframe.

## Bacterial identification and antimicrobial susceptibility profile

The identification of the species of all isolates enrolled in this study was re-confirmed by matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) by using the Vitek MS system (bioMérieux) as previously described [7]. If the data collected from this re-identification is not consistent with the original one, the result of re-identification is selected and adapted into the corresponding susceptibility criteria for the isolate. If the isolate did not meet the criteria, it was excluded from the study. Minimum inhibitory concentrations (MICs) of cefotaxime (CTX), ceftazidime (CAZ), cefmetazole (CMZ), flomoxef (FMOX), imipenem (IPM), meropenem (MEPM), cefepime (CFPM), ceftazopran (CZOP), or cefpirome (CPR) were determined according to the version of the Clinical Laboratory Standards Institute (CLSI) M100 document applied by each laboratory.

## Detection of carbapenemase

All collected strains were screened for carbapenemase production by the modified carbapenem inactivation method (mCIM) according to the CLSI description [8]. According to the CLSI guidelines, the results were classified as negative for a zone diameter  $\geq 19$  mm and as positive for a zone diameter of 6–15 mm, and the result was regarded as intermediate if it detected the presence of pinpoint colonies within a 16–18 mm zone.

## PCR and DNA sequence analysis of *bla* genes

The DNAs of all isolates were obtained by using a Cica Geneus<sup>R</sup> DNA extraction reagent (Kanto Chemical Holdings, Tokyo, Japan) and they were used as PCR templates. The carbapenemase genes (*bla*<sub>KPC</sub>, *bla*<sub>GES</sub>, *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, *bla*<sub>NDM</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>OXA-23</sub>, *bla*<sub>OXA-24</sub>, and *bla*<sub>OXA-58group</sub>) were screened in all mCIM positive and intermediate strains and carbapenem-resistant strains regardless of the results of mCIM. Common ESBL genes (*bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-2</sub>, *bla*<sub>CTX-M-9</sub>, *bla*<sub>CTX-M-8/25</sub>, *bla*<sub>TEM</sub>, and *bla*<sub>SHV</sub>) and AmpC genes (*bla*<sub>MOX</sub>, *bla*<sub>DHA</sub>, *bla*<sub>CIT</sub>) were screened in all clinical strains using previously described primers [9–11]. The positive PCR products were screened by electrophoresis on 2.0% agarose gel, and the subsequent analysis (of PCR products for carbapenemase genes) was sent to Eurofins Genomics Inc. (Ohta-ku, Tokyo, Japan). Nucleotide sequences were compared and analyzed using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>).

## Multi-locus Sequence typing

The multi-locus sequence typing (MLST) analysis was performed with all carbapenemase-producing *K. pneumoniae* and *E. cloacae* complex isolates and the DNA was isolated with Cica Geneus<sup>D</sup> DNA Extraction Reagent (Kanto Chemical, Tokyo, Japan). Seven housekeeping genes were amplified using primer sets according to the method previously reported [12, 13]. The DNA sequencing was performed at a commercial laboratory (FASMAC, Kanagawa, Japan) and the consensus regarding the sequence type (ST) was determined with *Enterobacter cloacae* locus/sequence definitions database ([https://pubmlst.org/bigsub?db=pubmlst\\_ecloacae\\_seqdef](https://pubmlst.org/bigsub?db=pubmlst_ecloacae_seqdef)), and *Klebsiella pneumoniae* database (<https://bigsub.pasteur.fr/klebsiella/klebsiella.html>).

## Whole-genome sequencing

In this study 29 representative isolates from carbapenemase-producing *E. cloacae* complex and *K. pneumoniae* were subject to a whole-genome sequencing analysis on a MiniSeq system (Illumina, San Diego, CA, USA) and on a MinION nanopore sequencer (Oxford Nanopore Technologies, Oxford, UK) by using the SQK-RBK004 kit and R9.4 flowcells in order to obtain complete sequences of plasmids carrying the *bla*<sub>IMP-1</sub> gene. De novo sequence assembly was performed by using Unicycler or Miniasm and error correction was provided by Illumina reads with Unicycler or CLC Genomics Workbench v9.5.3 (QIAGEN, Hilden, Germany). Coding sequence (CDS) was annotated and registered with the PATRIC server (<https://www.patricbrc.org>) and, specifically, the linear comparison of *bla*<sub>IMP-1</sub>-carrying plasmid sequences was performed by using BLAST and visualized with Easyfig (<http://mjsull.github.io/Easyfig/>). Finally, we have also indicated the *bla*<sub>IMP-1</sub> gene and other antimicrobial resistance genes, type IV secretion system-associated genes for conjugation were detected by using the T346Hunter server, whereas mobile gene elements were detected through CDS annotations.

# Results

## Survey data

During the period of the study, 56,494 strains of Enterobacteriales were detected in twenty-four participating hospitals (, which ranged from 250 to 1435 beds), and 360 strains were sent to our laboratory from which nineteen strains were excluded from the study as 10 isolates did not match the antimicrobial susceptibility criteria, 7 isolates were misidentified and 2 isolates did not grow from the stock. More importantly, 341 strains were consistent with the inclusion criteria, the number of which was most predominant in *E. cloacae* complex (n = 134), followed by *E. coli* (n = 96) and *K. pneumoniae* (n = 62). A total of 65 out of 341 strains (19.1%) were identified as CPE and subsequently isolated from 14 hospitals. The number of hospitals where CPE was detected was only four in 2015, a fact which increased to seven hospitals in the years of 2016 and 2017. The annual trend of the CPE detection rates is shown below on Table 2, which also shows *K. pneumoniae* and *E. cloacae* complex were increasingly detected until the year of 2017, where it peaked began decreasing ever since. No significant changes were observed for carbapenemase-producing *E. coli* and no carbapenemase-producing isolates were found in *K. aerogenes*.

### **Carbapenemase-producing Enterobacteriales species and carbapenemase diversity**

Among the 65 CPE strains detected in this study, *K. pneumoniae* (n = 24) was the most dominant, followed by *E. cloacae* complex (n = 23), *K. oxytoca* (n = 10) and *E. coli* (n = 8) (Table 3). IMP-type carbapenemase genes were found in 62 of all 65 CPE isolates, and 3 NDM-type were found in the years of 2016 and 2019. Regarding the IMP type, () the majority of carbapenemases were predominantly IMP-1 (n = 58) and only 3 *K. pneumoniae* isolates in 2015 and 2017 were found to be IMP-6.

### **Multi-locus Sequence typing of Carbapenemase-producing *Klebsiella pneumoniae* and *Enterobacter cloacae* complex**

The result of the MLST analysis *E. cloacae* complex identifies 7 unique sequence types (ST). as shown in Table 4 and 5. The most predominant isolates belonged to ST78 and account for 15 (65.2%) of all isolates (n = 23), and are followed by ST513 (2/23, 8.7%) and ST113 (2/23, 8.7%). Each of ST25, ST29, ST53 and ST133 were also found to be simultaneously present on one isolate.

Even though most ST78 *E. cloacae* complex isolates were detected in hospital A in 2015, there were also findings in Hospital J in 2018 and in Hospital H in 2019. *K. pneumoniae* CPE strains were divided into 8 different STs by using MLST, of which the most frequent types were the ST517 (6/23, 26.1%) and the ST37 (6/23, 26.1) %, followed by ST716 (4/23, 17.4%), ST3012 and 592 (both 2/23, 8.7) %. Accordingly, ST70, ST2158 and ST461 were identified in one isolate. Each ST was found mostly in a specific hospital, but ST592 strains were detected in Hospitals F & G in 2017, and one ST517 isolate was detected in Hospital G along with Hospital A in 2019.

## **Discussion**

### **Incidence rates of carbapenemase-producing Enterobacteriales**

In this study we have shown the incidence rate of CPE from 2015 through 2019 in the central district of Japan. The Japan Nosocomial Infections Surveillance (JANIS), which is a nosocomial infection survey program conducted by the Ministry of Health, Labor and Welfare [14], has been collecting the data on CRE since 2015 and show according to the JANIS data a downward trend in the percentage of CRE among patients who underwent microbiological from the year of 2015 to 2017, with 0.36%, 0.29%, and 0.27% respectively. However, the trend appears to be of an increase after 2018, which attests to a different trend from the results collected with the JANIS data. Since JANIS data includes both carbapenemase-producing and carbapenemase non-producing CRE, it is difficult to precise the trend of CPE.

Another study conducted by the National Institute of Infectious Diseases (NIID) shows the detection rate of CRE in Japan was 0.3%, of which one third was CPE. Presumably, the detection rate of CPE in Japan was considered to be about 0.1%, which is comparable to this study's average detection rate of CPE over five years of 0.11%. In our study CPE was mostly detected in *E. cloacae* complex and is followed by *K. pneumoniae* and *K. oxytoca*, which illustrates a similar pattern to the NIID study. Additionally, another study shows the rates of positive for the carbapenemase gene were the following: 0.92% for *K. pneumoniae*, 0.80% for *E. cloacae*, 0.55% for *K. oxytoca*, 0.81% for *K. aerogenes* and 0.50% for *P. mirabilis* [15]. These numbers reveal the regional differences regarding the epidemiology of CPE in Japan. Therefore, in the era of complex patient traffics within all regions regional molecular epidemiology is considered to instrumental for/in designing an infection control program in each hospital.

### **Carbapenemase genotypes**

Most of the carbapenemase produced by CPE isolates in this study were IMP-1, accounting for 58 (89.2%) out of 65 CPE isolates. The wide spread of IMP-type carbapenemase has been reported mainly in Japan, Taiwan, and eastern China while there are also sporadic reports from other countries [16]. IMP-1 was firstly detected in a *Serratia marcescens* isolate in Japan [17], and Arakawa has reported that the predominance of IMP-type carbapenemases is characteristic in Japan, having rarely found other types of carbapenemase [18]. IMP-1 is followed by IMP-6 producers (3/65, 4.6%) and NDM-5 in three *E. coli* isolates. The IMP-6 enzyme, which shows to be highly resistant to meropenem, was found in Japan in 2001 [19]. IMP-6 type CPE was found mainly in the western region and was hardly found in other regions of Japan [20]. In this study, only three IMP-6 type suggest the influx of this type of CPE in low prevalence.

### **Multi-locus sequencing typing**

In the present study, *E. cloacae* complex CPE isolates were detected in six hospitals. Among them, ST78 is the most prevalent ST, a result which was also reported to be detected in other regions of Japan [21]. Until 2017, the ST78 was detected in only two hospitals but since 2018 it has been detected in two other hospitals, which suggest a spread in this region. Since *E. cloacae* complex ST78 is considered to be a high-risk clone [22], the following monitorization of the trends of detection of this specific clone in our region is necessary. In contrast to carbapenemase-non-producing or carbapenem-resistant *E. cloacae* complex, according to Tetsuka et al.'s study [23] CPE was likely to be transmitted horizontally in a

hospital setting, which might warn hospitals about strict contact precautions so that transmission of this clone are prevented.

Specific STs such as ST258 and ST147 of carbapenemase-producing *K. pneumoniae*, which are recognized as high-risk clones, were detected widely in the world [24, 25]. However, in this study we have not detected such highly pathogenic ST in *K. pneumoniae*. Various ST types of *K. pneumoniae* CPE were detected, which were not only in university hospitals but also in community acute-care hospitals. ST37, which is widely distributed worldwide with outbreaks having been reported in Europe [26, 27], was one of the most dominant clones in this study. In-hospital transmission of carbapenem-resistant *K. pneumoniae* ST37 has also been reported in Japan [28], but current epidemiological studies in this region show ST37 was detected in only one hospital with no apparent spread between hospitals. For the other STs, ST517 and ST592 were detected in two separate participant hospitals where ST592 strains producing IMP-6 were detected in 2017.

We suggest attention should be paid to the further spread of these clones across hospitals in this region. In a limited, three-months survey study such as this molecular epidemiology of CPE showed signs of spread of specific STs, especially ST78 of carbapenemase-producing *E. cloacae* complex. These results suggest that it is important to strengthen the nosocomial infection control programs in each hospital while also continuing the molecular supervision of CPE in this region.

## Limitations

There were some limitations in this study. We have not re-measured the susceptibility test. Therefore, some strains may have been excluded from the collection, and the detection rate of CPE may have been estimated to be lower than it actually is. However, we believe that this is within the expected range of the study and therefore poses little impact on the results.

Additionally, we have collected isolates by using a timeframe of three months, which raises the desire for a yearly analysis in the future. Even though there are 60 microbiology laboratories in this region, twenty-four laboratories participated in this study, most of which belonged to acute-care hospitals. Therefore, we were not able to cover full the situation of CPE in this region.

Lastly, the measurement of the levels of epidemiology of CPE in the region on future analysis need to include nursing homes or long-term care facilities.

## Conclusion

In conclusion, we have revealed molecular epidemiology and resistance genes for CPE in central Japan. In our region, the incidence rate of CPE was slightly higher than the national data, and signs of the spread of ST78 of carbapenemase-producing *E. cloacae* complex were detected even with a limited, three-month yearly survey such as the present one. What remains and is strongly suggested by this study is the need

for strengthening the regional infection control programs and promoting regional survey /control programs and highlights the importance of registering molecular epidemiological data on CPE in Japan.

## **Abbreviations**

CPE: carbapenemase-producing Enterobacteriales; CRE: carbapenem-resistant Enterobacteriales; JANIS: Japan Nosocomial Infections Surveillance; MALDI-TOF: matrix-assisted laser desorption ionization–time of flight; MICs: minimum inhibitory concentrations; CTX: cefotaxime; CAZ: ceftazidime; CMZ: cefmetazole; FMOX: flomoxef; IPM: imipenem; MEPM: meropenem; CFPM: cefepime; CZOP: ceftazopran; CPR: cefpirome; CLSI: Clinical Laboratory Standards Institute; mCIM: modified carbapenem inactivation method; MLST: multi-locus sequence-typing; ST: sequence type; NIID: National Institute of Infectious Diseases

## **Declarations**

### **Ethics approval and consent to participant**

The institutional review boards at the Nagoya University Hospital have approved the study (approval number 2017-0396). Informed consent was obtained from all individual participants included in the study.

### **Consent to publish**

The participant has consented to the submission of this original article to the journal.

### **Availability of data and materials**

The datasets for the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare they hold no conflict of interest in relation to this project.

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### **Authors' contributions**

All authors have contributed to the study conception and design. Material preparation, data collection and analysis were performed by YH, MI, AH and TY. The first draft of the manuscript was written by YH and all

authors have commented on previous versions of the manuscript. Finally, all the authors have read and approved the final manuscript.

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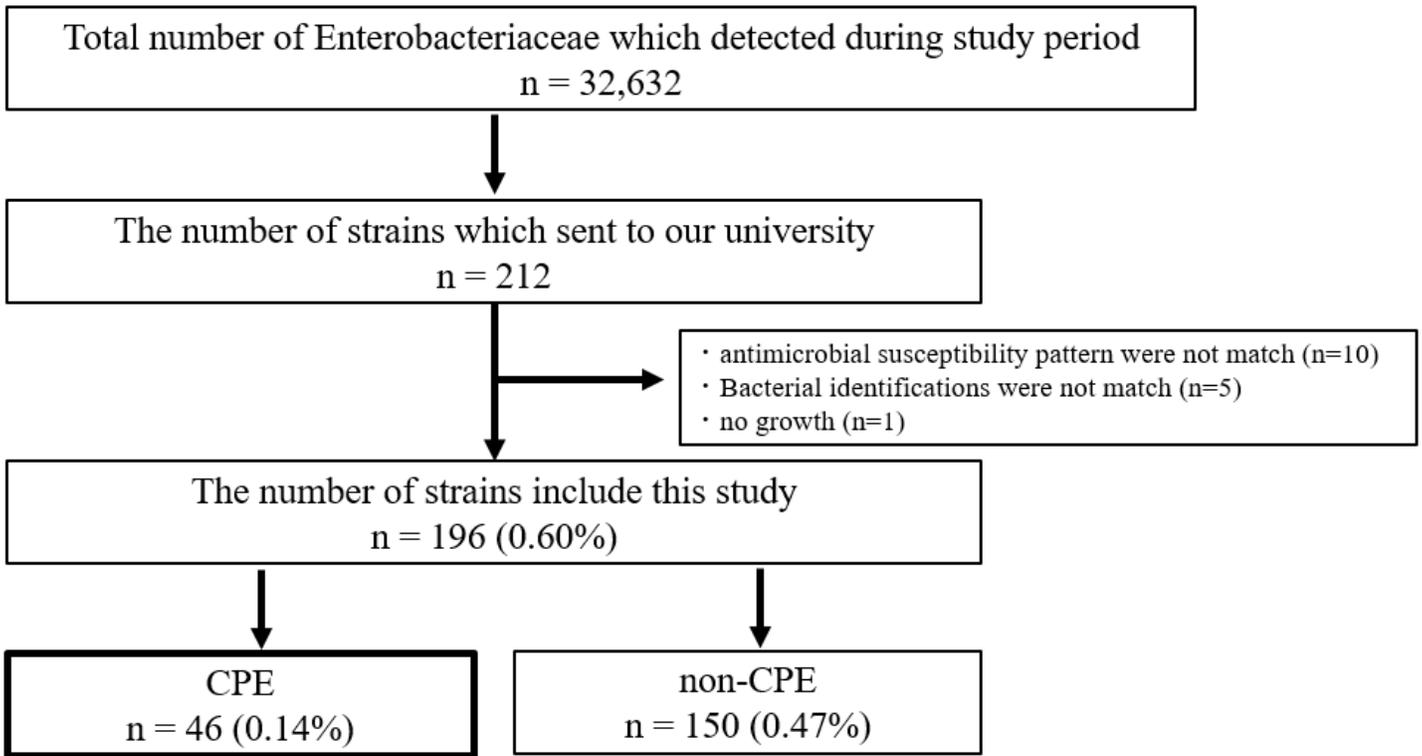
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## Tables

Due to technical limitations, table 1 to 5 is only available as a download in the Supplemental Files section.

## Figures

**Figure 1**



**Figure 1**

Number of strains to be analyzed.

## Supplementary Files

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