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Characterization of CTX-M type ESBL-producing *Enterobacteriaceae* isolated from asymptomatic healthy individuals who live in a community of the Okinawa prefecture, Japan

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ABSTRACT

This study was performed to characterize CTX-M type extended spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* carriage in asymptomatic health individuals, which has not been well investigated, in a community of the Okinawa prefecture, Japan. Fecal samples were voluntary collected from asymptomatic healthy individuals who were going to take a routine medical checkup. The collected fecal samples were inoculated on MacConkey agar supplemented with 2 μg/ml of cefotaxime and incubated at 37 °C. Randomly selected three lactose-fermented colonies per each sample were analyzed. Genetic relatedness among the CTX-M type ESBL-producing *Enterobacteriaceae* isolates were performed by pulsed-field gel electrophoresis (PFGE) after confirmation of ESBL phenotype and determination of bacterial species. Location of bla_{CTX-M} was confirmed by S1-PFGE, I-CeuI-PFGE and the Southern blotting hybridization. ESBL-producing *Enterobacteriaceae* was isolated from 32 (12.2%) of the collected 263 fecal samples, and 96 ESBL-producing *Enterobacteriaceae* isolates were obtained. CTX-M type ESBL-producing *Escherichia coli* B2 were major (67 isolates, 72.0%) and 40 (59.7%) of the 67 CTX-M type ESBL -producing *E. coli* B2 were *E. coli* B2-ST131. Three CTX-M type ESBL-producing *E. coli* B2-ST131 isolates from asymptomatic healthy individuals showed similar PFGE band patterns as five CTX-M type ESBL -producing *E. coli* B2-ST131 isolates from a hospital locates in the same area of the target community. Chromosomally-transferred bla_{CTX-M} was observed in 10.0% of the examined CTX-M type ESBL-producing *Enterobacteriaceae* isolates. We report current situation CTX-M type ESBL-producing *Enterobacteriaceae* carriage in asymptomatic healthy individuals of the Okinawa prefecture, Japan. In addition, our results indicated that worldwide distributed CTX-M type ESBL-producing *E. coli* B2-ST131 has been spread in a community. Therefore monitoring of ESBL-producing *Enterobacteriaceae* in healthy individuals is important.

There are many studies describing community-acquired ESBL-producing *Enterobacteriaceae* infection which was based on results obtained from outpatient specimens in Japan. However, status of ESBL-producing *Enterobacteriaceae* carriage in Japanese asymptomatic healthy individuals has not been well investigated and characterized. In this regard, Karanika et al. reviewed already published 17,479 studies to extract studies describing status of ESBL-producing *Enterobacteriaceae* in asymptomatic healthy individuals and to exclude community-acquired outpatient infections [1]. The meta-analysis indicated that there is increasing prevalence of ESBL-producing *Enterobacteriaceae* in asymptomatic healthy individuals and that CTX-M type ESBL was prevalent enzyme (69%) in subjects reported in the extracted sixty-six studies [1]. From Japan, only one study was included in the extracted sixty-six studies as

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study describing ESBL-producing *Enterobacteriaceae* carriage in Japanese asymptomatic healthy individuals in the Kinki region, Japan [2]. After that, one study investigating carriage of ESBL-producing *E. coli* in Japanese healthy food handlers was published [3]. However, there is still less information about distribution of ESBL-producing *Enterobacteriaceae* in Japanese asymptomatic healthy individuals.

We previously investigated status of ESBL-producing *Enterobacteriaceae* in a hospital of the Okinawa prefecture, and observed ESBL-producing Enterobacteria detection rate of 6.8% [4]. This study was conducted in order to explain the current status of ESBL-producing *Enterobacteriaceae* in asymptomatic healthy individuals living in a community which is in the same area as the hospital in our previous study.

Asymptomatic healthy individuals, who were going to take a routine medical checkup in a target community of the Okinawa prefecture (at December 2017, population of the target community was approximately 35,000), were recruited to this study between June and December 2017. Finally, 263 fecal samples were voluntarily collected from asymptomatic healthy individuals after obtaining informed consent. Research proposal of this study was evaluated and approved by Ethics committee in the University of the Ryukyus (Approved study number: #371).

The collected fecal samples were cultured on MacConkey agar supplemented with cefotaxime 2 μg/ml, and incubated at 37 °C for 24 h. Randomly selected three different lactose-fermented colonies of each samples were subjected to ESBL phenotype confirmation test using double disc diffusion according to the Clinical and Laboratory Standards Institute standard, M100S27. Bacterial species of the ESBL phenotype-positive isolates were determined by the API 20 (bioMérieux, Marcy l'Etoile, France) by following the company's instruction manual.

ESBL-producing *Enterobacteriaceae* was isolated from 32 (12.2%) of the collected 263 fecal samples. Consequently, 96 ESBL-producing *Enterobacteriaceae* isolates (NCE series isolates) which included 93 (96.9%) *Escherichia coli* isolates from 31 fecal samples and 3 (3.1%) *Klebsiella pneumoniae* isolates from one fecal sample were isolated. Previously, in Japan, detection rate of ESBL-producing *Enterobacteriaceae* among healthy individuals was reported as 6.4% in healthy adult volunteers in the Kansai area in 2010 [2] and as 15.6% in healthy food handlers in the Aichi prefecture between 2010 and 2011 [3]. Therefore, the detection rate (12.2%) in this study was comparable to these two previous studies which were carried out in the main island of Japan. Then, bacterial DNA was extracted from ESBL-producing isolates and was used as template for PCR-based genotypings such as *E. coli* phylogenetic group with genetic markers (*chuA*, *yjaA*, TspE4.C2) [5]. Consequently, 3 isolates (3.2%), 8 isolates (8.6%), 67 isolates (72.0%) and 15 isolates (16.1%) of the ESBL-producing *E. coli* isolates were classified into phylogenetic group A, B1, B2 and D, respectively. In addition, conventional multilocus sequence typing [6], PCR detection of *E. coli* O25b-ST131 clone [7], detection of $bla_{CTX\text{-}M}$ and grouping of $bla_{CTX\text{-}M}$ to $bla_{CTX\text{-}M\text{-}1}$-, $bla_{CTX\text{-}M\text{-}2}$-, $bla_{CTX\text{-}M\text{-}9}$- and $bla_{CTX\text{-}M\text{-}25}$-subgroups as previously described [4,8]. The amplified DNA fragments were subjected to sequencing analysis. Results obtained in this study was summarized in Table 1.

Antibiotic susceptibility of CTX-M type ESBL-producing *Enterobacteriaceae* were evaluated by disc diffusion test using 11 different antibiotic discs (Eiken Chemical Co., Ltd., Tokyo, Japan), amikacin (AMK), clavulanic acid/amoxicillin (CVA/AMPC), chloramphenicol (CP), ciprofloxacin (CPFX), gentamicin (GM), imipenem (IPM), meropenem (MEPM), nalidixic acid (NA), trimethoprim/sulfamethoxazole (ST), tazobactam/piperacillin (TAZ/PIPC) and tetracycline (TC). Antibiotic susceptibilities test indicated that the CTX-M type ESBL-producing *Enterobacteriaceae* isolates were resistance to NA (53 isolates, 55.2%), TC (48 isolates, 50.0%), CPFX (44 isolates, 45.8%) and ST (38 isolates, 39.6%), and that more than 90% of isolates were susceptible to AMK, CP, GM, IPM and MEPM. In average, CTX-M type ESBL-producing *Enterobacteriaceae* isolates were resistance to 1.55 antibiotics examined in this study.

Table 1
Distribution of $bla_{CTX\text{-}M}$s in CTX-M type ESBL-producing *Enterobacteriaceae* isolates obtained from asymptomatic healthy individuals in the Okinawa prefecture, Japan.

	n	No. of the detected *bla* CTX-M subgroups.			
		1	2	9	25
All	96	12	0	84	0
E. coli	93	12	0	81	0
B2-O25b-ST131	40	3	0	37	0
Other than B2-O25b-ST131	53	9	0	44	0
K. pneumoniae	3	0	0	3	0

Genetic relatedness among three CTX-M type ESBL-producing isolates obtained from each fecal sample was evaluated by XbaI-Pulsed Field Gel Electrophoresis (PFGE) as previously described [9]. As shown in Fig. 1A, exactly same PFGE band patterns were observed in three CTX-M type ESBL-producing *E. coli* isolates originated from 25 (80.6%) of the 31 fecal samples. PFGE patterns of other five (16.1%) fecal samples, *i.e.*, NCE046, NCE119, NCE199, NCE253 and NCE261, were not exactly same (Fig. 1B). We could not obtain PFGE patterns from one sample (NCE054). Next, we examined PFGE whether the 40 CTX-M type ESBL-producing *E. coli* B2-ST131 NCE series isolates were related to 33 clinical CTX-M type ESBL-producing *E. coli* B2-ST131 isolates which were obtained from a hospital (N series isolates) in same area [4]. Consequently, similar PFGE band patterns were observed only among three NCE isolates from healthy individuals (NCE137A, NCE234A and NCE241A) and five N series isolates from a hospital (N1118, N1315, N1546, N1009, N1647). Consequently, only 8 (11.0%) of the 73 examined CTX-M type ESBL-producing *E. coli* B2-ST131 isolates showed similar PFGE band patterns (Fig. 1C), suggesting that the CTX-M type ESBL-producing *E. coli* B2-ST131 isolates were originated from common ancestral clone(s).

Finally, we evaluated location of $bla_{CTX\text{-}M}$ in randomly selected 50 CTX-M type ESBL-producing *Enterobacteriaceae* isolates by S1-PFGE, I-CeuI-PFGE and the Southern blotting [9,10]. Consequently, 45 CTX-M type ESBL-producing *Enterobacteriaceae* isolates (90.0%) possessed only plasmid $bla_{CTX\text{-}M}$. Chromosomal-located $bla_{CTX\text{-}M}$ was observed in five (10.0%) CTX-M type ESBL-producing *E. coli* isolates and one of the five isolates possessed both plasmid $bla_{CTX\text{-}M}$ and chromosomal-located $bla_{CTX\text{-}M}$. All chromosomally-located $bla_{CTX\text{-}M}$ was belonged to $bla_{CTX\text{-}M\text{-}9}$ subgroup (Fig. 2). The detection rate of chromosomally-transferred $bla_{CTX\text{-}M}$ in this study (10.0%) was lower than detection rate in CTX-M type ESBL-producing *E. coli* isolates obtained from a hospital (27.2%) which locates in the same area of the target community (unpublished data). Chromosomally-transferred antibiotics resistant genes could be more stable than antimicrobial resistant plasmid. Therefore, the chromosomally-transferred $bla_{CTX\text{-}M}$ could be risk factor to wider distribution and transfer of CTX-M type ESBL-producing *Enterobacteriaceae* between healthcare-associated facilities and communities. At this point, we do not have any evidence regarding to stability of chromosomally-transferred $bla_{CTX\text{-}M}$ in bacterial cells; therefore, it is not able to conclude that chromosomally-transferred $bla_{CTX\text{-}M}$ can be one index for long-lasting carriage of CTX-M type ESBL-producing *Enterobacteriaceae*.

In this study, we did not take interviews of healthy individuals who voluntarily provided their fecal samples from epidemiological point of view. Therefore, further precise analysis of the CTX-M type

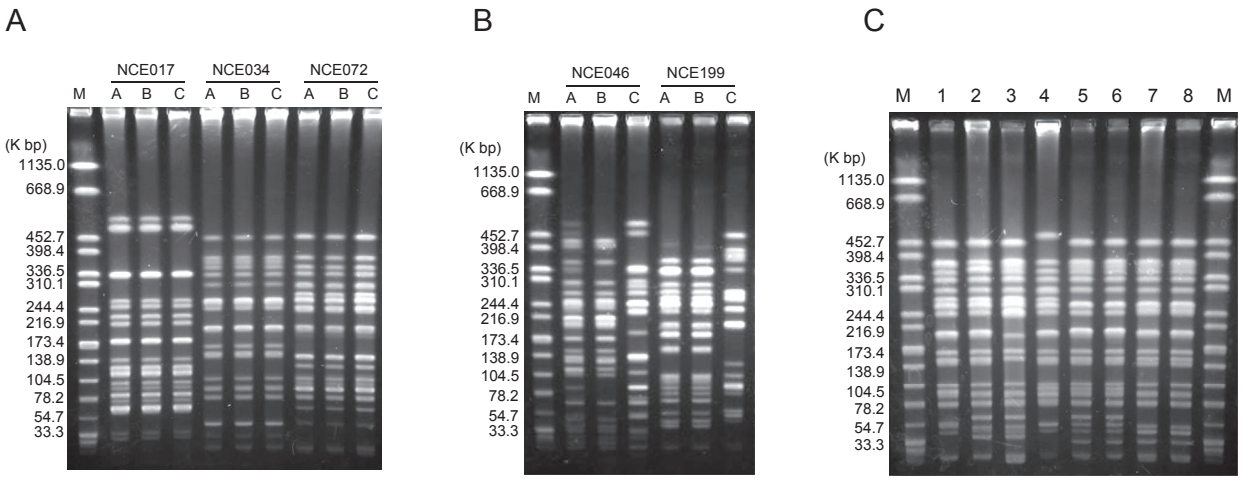

Fig. 1. Genetic relatedness of CTX-M type ESBL-producing *Enterobacteriaceae* obtained from asymptomatic healthy individuals (**A** and **B**). Three CTX-M type ESBL-producing *Enterobacteriaceae* isolates from each fecal samples were examined whether there was genetic relatedness among the three CTX-M type ESBL-producing *Enterobacteriaceae* isolates by XbaI-PFGE. Representative results of same (**A**) and different (**B**) DNA band patterns among three CTX-M type ESBL-producing *Enterobacteriaceae* isolates by XbaI-PFGE are shown. (**C**) CTX-M type ESBL-producing *E. coli* B2-ST131 isolates obtained from asymptomatic healthy individuals (NCE137A, NCE234A and NCE241A) and a hospital (N1118, N1315, N1546, N1009, N1647) were examined whether there was genetic relatedness among the isolates by XbaI-PFGE. M, XbaI-digested genomic DNA of *Salmonella enterica* subsp. *enterica* serovar Braenderup (ATCC BAA-664); 1, NCE137A; 2, N1118; 3, N1315; 4, N1546; 5, NCE234A; 6, NCE241A; 7, N1009; 8, N1647.

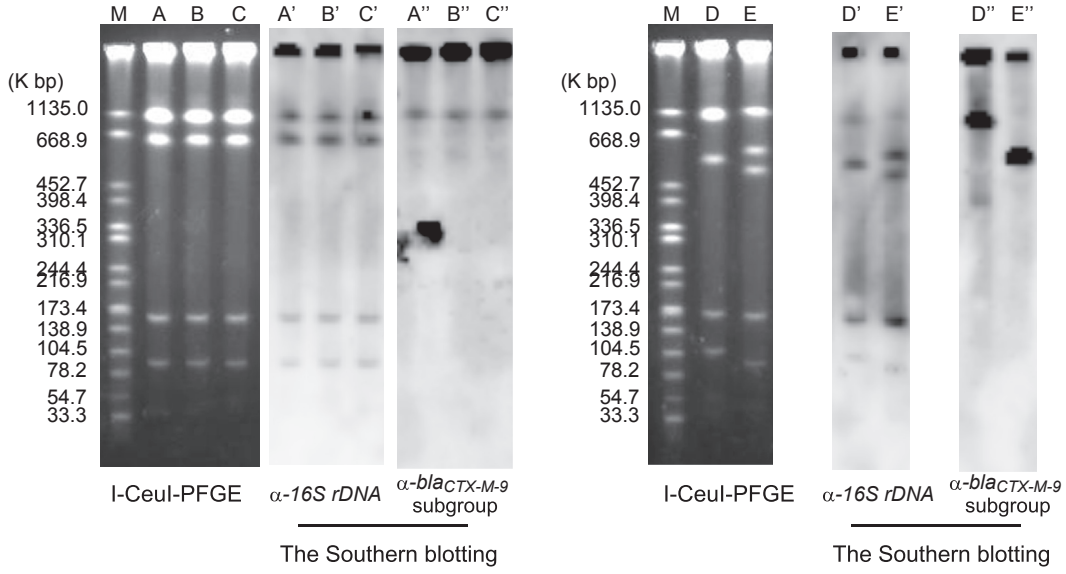

Fig. 2. Confirmation of chromosomally-located bla_{CTX-M} in CTX-M type ESBL-producing *E. coli* obtained from asymptomatic healthy individuals by I-CeuI-PFGE and the Southern blotting. Chromosomally-located bla_{CTX-M} in five CTX-M type ESBL-producing *Enterobacteriaceae* isolates from fecal samples were confirmed by I-CeuI-PFGE and the Southern blotting using specific probes specific for *16S rRNA* and $bla_{CTX-M-9}$ subgroups. A, NCE017A; B, NCE017B; C, NCE017C; D, NCE199C; E, NCE180A; M, XbaI-digested genomic DNA of *Salmonella enterica* subsp. *enterica* serovar Braenderup (ATCC BAA-664).

ESBL-producing *Enterobacteriaceae* isolates and detail epidemiological investigation will be important to explain transmission of CTX-M type ESBL-producing *Enterobacteriaceae* isolates between community and healthcare-associated facilities.

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Declaration of interest

None.

ICMJE statement

All authors meet the ICMJE authorship criteria.

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