Mechanism of resistance and antibacterial susceptibility in extended-spectrum \( \beta \)-lactamase phenotype \textit{Klebsiella pneumoniae} and \textit{Klebsiella oxytoca} isolated between 2000 and 2010 in Japan

Takafumi Sato, Takafumi Hara, Tsukasa Horiyama, Sachi Kanazawa, Takahiro Yamaguchi and Hideki Maki

Clinical isolates of \textit{Klebsiella pneumoniae} and \textit{Klebsiella oxytoca} collected from 20 Japanese medical facilities between 2000 and 2010 were analysed to evaluate the mechanisms of resistance and antibacterial susceptibilities to 14 antimicrobials. Overall, eight of 484 (1.6\%) \textit{K. pneumoniae} and 19 of 359 (5.3\%) \textit{K. oxytoca} were determined to be extended-spectrum \( \beta \)-lactamase (ESBL) phenotype isolates, and the identified ESBLs amongst the \textit{K. pneumoniae} isolates were CTX-M-2, -3, -14 and -15, and SHV-12. In contrast, overproduction of chromosomal \( \beta \)-lactamase OXY-2, which was due to a distinct mutation at the \( -235 \) and/or \( -210 \) promoter regions of this gene, conferred the ESBL phenotype to all the \textit{K. oxytoca} isolates except one. Based on the Clinical and Laboratory Standards Institute breakpoints, all the ESBL phenotype \textit{K. pneumoniae} were susceptible to doripenem, flomoxef, moxalactam (latamoxef), cefmetazole and tazobactam/piperacillin, whereas the ESBL phenotype \textit{K. oxytoca} were susceptible to ceftazidime and ceftibuten with the exception of tazobactam/piperacillin. Amongst the oral antimicrobials, ceftibuten was relatively effective against both ESBL phenotype \textit{Klebsiella} species compared with levofloxacin and amoxicillin/clavulanic acid.

INTRODUCTION

Antimicrobial resistance due to rapid spread of extended-spectrum \( \beta \)-lactamases (ESBLs) in \textit{Enterobacteriaceae} is an emerging problem worldwide. The ESBLs confer resistance to various classes of \( \beta \)-lactam such as oxyimino-cephalosporin (e.g. cefotaxime, ceftriaxone and ceftazidime) and monobactam (e.g. aztreonam). Moreover, ESBL-producing \textit{Enterobacteriaceae} have a tendency toward cross-resistance to non-\( \beta \)-lactam classes of antimicrobials such as quinolone and aminoglycoside agents (Pitout, 2010; Livermore, 2012).

The currently dominant ESBLs belonging to class A in the Ambler classification are TEM, SHV and CTX-M types. Although the dominant types of ESBLs in the 1990s were SHV and TEM, the rapid spread of CTX-Ms (especially CTX-M-1,-2,-3,-14 and -15) has also been observed in \textit{Escherichia coli} as well as \textit{Klebsiella pneumoniae} since the early 2000s (Pitout, 2010; Livermore, 2012). In \textit{Klebsiella oxytoca}, the species-specific chromosome-encoded class A \( \beta \)-lactamases called OXY (mainly divided into OXY-1 and -2) are also associated with \( \beta \)-lactam resistance (Fournier et al., 1996). These enzymes are constitutively produced at low levels, which are sufficient to confer resistance to penicillins but no significant resistance to other \( \beta \)-lactam classes (Livermore, 1995). However, overproduction of OXYs due to distinct point mutations in the \( -35 \) and/or \( -10 \) promoter regions of the gene results in additional resistance to aztreonam, combinations of penicillins with \( \beta \)-lactamase inhibitors and oxyimino-cephalosporins, with the exception of ceftazidime (Fournier et al., 1996).

Several epidemiology studies on ESBL-producing \textit{K. pneumoniae} in Japan have been reported (Nakamura et al., 2012; Chong et al., 2013), but little is known (especially in \textit{K. oxytoca}) about the epidemiology and molecular characteristics of ESBLs. The aim of this study was to investigate the resistance mechanisms to \( \beta \)-lactams amongst ESBL phenotype \textit{K. pneumoniae} and \textit{K. oxytoca} isolated in Japan through the 2000s, including antimicrobial susceptibility to parenteral and oral antimicrobials.
METHODS

Screening for ESBL phenotype isolates. Confirmation of ESBL phenotype according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) was performed using the following two susceptibility tests: (i) MIC of either cefotaxime, ceftazidime, ceftriaxone or aztreonam was \( \leq 1 \) mg l\(^{-1} \) and (ii) an eightfold or greater MIC reduction of either cefotaxime or ceftazidime occurred in the presence of clavulanic acid (4 mg l\(^{-1} \)) (CLSI, 2013). The ESBL phenotype isolates used in this study were screened from 484 \( K. \) pneumoniae (80 isolates per year) and 359 \( K. \) oxytoca (60 isolates per year) that were randomly collected from 20 medical facilities in Japan every 2 years between 2000 and 2010. These were mainly isolated from urine and sputum for \( K. \) pneumoniae, and urine, pus and blood for \( K. \) oxytoca.

Detection of ESBL genes. All isolates positive in ESBL phenotype screening tests were subjected to conventional PCR analysis for the detection of \( \beta \)-lactamase genes. DNA templates were prepared by suspending a freshly grown colony in distilled water, heating at 94 °C for 10 min and then centrifuging to collect supernatant. The PCR amplification with \( \text{Ex Taq} \) (TaKaRa Bio) consisted of a pre-PCR stage at 94 °C for 5 min, and then 25 cycles at 94 °C for 30 s, at 55 °C for 30 s and at 74 °C for 1 min, and a final extension stage at 74 °C for 7 min using a C1000 Thermal Cycler (Bio-Rad). Direct sequencing of amplified PCR products including \( \beta \)-lactamase genes and partly their promoter region was performed by Eurofins Genomics. The eight sets of primers used for amplification and sequencing are shown in Table 1.

Susceptibility testing. MICs were determined by the broth microdilution method with cation-adjusted Mueller–Hinton broth (Becton Dickinson) according to the recommendations of the CLSI (CLSI, 2012). The following antimicrobials were used: cefotaxime, ceftriaxone, ampicillin, clavulanic acid, sulbactam and tazobactam (US Pharmacopeia); doripenem, moxalactam (latamoxef), flomoxef, ceftibuten and sulfamethoxazole (Shionogi); ceftazidime and cefepime (Chem-Impex International); piperacillin and levofloxacin (LKT Laboratories); amoxicillin (Wako Pure Chemical Industries); cefmetazole (Sigma-Aldrich); and trimethoprim (NacalaiTesque). MIC interpretation of susceptibility was determined based on the CLSI breakpoint (as the MIC breakpoint of flomoxef was unavailable, that of moxalactam was applied instead) (CLSI, 2013).

RESULTS

Identification and molecular characterization of ESBL phenotype isolates

Of 484 isolates of \( K. \) pneumoniae collected between 2000 and 2010, eight (1.6%) were identified as ESBL phenotype

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Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Target*</th>
<th>Sequence (5′→3′)</th>
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| CTX-M-1 group | Forward: CCGACATATGGTTAAAAATCACTGGTGCA  
Reverse: CCGTGAATTCTTACAAAAACGTGGTGACGATT |
| CTX-M-2 group | Forward: CCGACATATGATGACTCAGAGCATTCGCC  
Reverse: CCGTGAATTCTTACAAAAACGTGGTGACGATT |
| CTX-M-8 group | Forward: CCGACATATGATGCTATAGCAACAGGCAGG  
Reverse: CCGTGAATTCTTACAAAAACGTGGTGACGATT |
| CTX-M-9 group | Forward: CCGACATATGATGCTAGCAACAGGCAGG  
Reverse: CCGTGAATTCTTACAAAAACGTGGTGACGATT |
| CTX-M-25 group | Forward: CCGACATATGATGCTATAGCAACAGGCAGG  
Reverse: CCGTGAATTCTTACAAAAACGTGGTGACGATT |
| TEM | Forward: CCGACATATGATGCTATAGCAACAGGCAGG  
Reverse: CCGTGAATTCTTACAAAAACGTGGTGACGATT |
| SHV | Forward: CCGACATATGATGCTATAGCAACAGGCAGG  
Reverse: CCGTGAATTCTTACAAAAACGTGGTGACGATT |
| Promoter and OXY-1/2 | Forward: CCGACATATGATGCTATAGCAACAGGCAGG  
Reverse: CCGTGAATTCTTACAAAAACGTGGTGACGATT |

*CTX-M-1 group includes CTX-M-3 and CTX-M-15; CTX-M-9 group includes CTX-M-14.

Table 2. \( \beta \)-Lactamases detected in \( Klebsiella \) isolates by ESBL phenotype screening

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<td>SHV-12</td>
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<td>( K. ) oxytoca (n=359)</td>
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<td>4</td>
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<td>OXY-2 [TATAAT]</td>
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<td>OXY-1 [GATAAT], CTX-M-3</td>
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*Square brackets present the nucleoside sequence of the −10 promoter region of OXY \( \beta \)-lactamase; the common sequence is GATAAT and the mutation is in italic.
Fig. 1. MIC distribution of 10 parenteral antimicrobials for ESBL phenotype *K. pneumoniae* (open bars; eight isolates) and *K. oxytoca* (solid bars; 19 isolates). S, susceptible; I, intermediate; R, resistant. *Fixed tazobactam concentration of 4 μg ml⁻¹.*

*Fixed ratio of 2 : 1.*
strains (Table 2). The positive rates after 2006 ranged from 2.4 to 3.7% each year. The \(\beta\)-lactamase genes in these eight isolates were sequenced. Almost all of the ESBLs were the CTX-M type; \(\text{bla}_{\text{CTX-M-2}}\) was identified in all three strains isolated before 2006, and \(\text{bla}_{\text{CTX-M-3}}, \text{bla}_{\text{CTX-M-14}}, \text{bla}_{\text{CTX-M-15}}\) or \(\text{bla}_{\text{SHV-12}}\) was identified in the rest of the strains isolated between 2008 and 2010.

For \(K.\) oxytoca, ESBL phenotype isolates were present in 19 of the total 359 (5.3%) isolates (Table 2). The positive rates (except for 2004) ranged from 3.3 to 12.1% each year. The ESBL genes were not positive for any other \(\text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}}\) or \(\text{bla}_{\text{CTX-M}}\) except for one strain carrying \(\text{bla}_{\text{CTX-M-3}}\). Focusing on the chromosome-encoded \(\text{bla}_{\text{OXY}}\) and its promoter region between -35 and -10, the \(\text{bla}_{\text{OXY-2}}\) gene with a single mutation (either G→A of the fifth base or G→T of the first base) in the -10 promoter sequence was identified in all isolates other than the \(\text{bla}_{\text{CTX-M-3}}\)-carrying strain. This \(\text{bla}_{\text{CTX-M-3}}\)-carrying strain was positive for \(\text{bla}_{\text{OXY-1}}\) with a common -10 promoter sequence (GATAGT). Also, no mutation was found in -35 and gap promoter sequences.

**Antimicrobial susceptibility of ESBL phenotype isolates**

The results of antimicrobial susceptibility of the ESBL phenotype isolates to 10 parenteral and four oral commonly used antimicrobials are shown in Figs. 1 and 2, respectively. Based on the CLSI breakpoints, all the \(K.\) pneumoniae isolates were susceptible to doripenem, moxalactam, floxof, cefmetazole and tazobactam/piperacillin, whereas the ESBL phenotype \(K.\) oxytoca were susceptible to ceftazidime and ceftibuten in addition to the above. Amongst the oral antimicrobials tested, ceftibuten showed potent *in vitro* activity against both ESBL phenotype *Klebsiella* species compared with levofloxacin and amoxicillin/clavulanic acid. The three \(K.\) pneumoniae isolates less susceptible to both ceftazidime and ceftibuten were the strains producing either CTX-M-15 or SHV-12, which were cross-resistant to sulfamethoxazole/trimethoprim. All the \(K.\) oxytoca isolates with a mutated promoter of OXY-2 showed high-level resistance to tazobactam/piperacillin as well as other \(\beta\)-lactam/\(\beta\)-lactamase inhibitor combinations, consisting of either sulbactam or clavulanic acid.

**DISCUSSION**

The recent long-term epidemiology study by Chong *et al.* (2013) reported that the ESBL-positive rate amongst \(K.\) pneumoniae in Japan ranged from 0 to 9.2% between 2003 and 2011. Nakamura *et al.* (2012) also demonstrated that the rates of ESBL-positive \(K.\) pneumoniae in the Kinki region of Japan were from 0 to 2.4% between 2000 and 2009. Similar results were observed in this study, i.e. the
detection rate of ESBL phenotype isolates was 1.6% in total through the observation period. These data indicate that the prevalence of ESBL-producing *K. pneumoniae* is still low compared with those rates in surrounding countries, because recent worldwide surveillance shows global ESBL-positive rates were 20.1% for *K. pneumoniae*, with the rate in the Asia/Pacific region being relatively high compared with other areas (Hawkey, 2008; Lob et al., 2013). The two previous studies did not determine the detailed subtypes of CTX-Ms, but our molecular genotyping revealed a subtype in ESBL phenotype *K. pneumoniae*, i.e. we found the appearance of CTX-M-15, known as the world pandemic ESBL in *E. coli*. Recently, several researchers have reported acceleration of the increase of the CTX-M-15-producing *E. coli* ST131 clone in Japan (Nakamura et al., 2012; Yano et al., 2013). The detection of CTX-M-15-producing *K. pneumoniae* isolates has also been reported in Asian and Middle East countries, such as South Korea, India and Pakistan (Hawkey, 2008; Livermore, 2012). This prevalence shift in *E. coli* is likely to have influenced the appearance of the world pandemic plasmid-mediated CTX-M-15 amongst *K. pneumoniae* in Japan.

Our study revealed that the ESBL phenotype *K. oxytoca* isolates in Japan were constantly isolated every year (except for 2004) and the positive rate accounted for 5.3% of all *K. oxytoca* isolates tested. Although the numbers of isolates in 2008 and 2010 were slightly high, they would be considered insufficient to support the regional outbreaks because these isolates were from seven facilities located up to 1200 km apart. The ESBL-phenotype-positive rate in *K. oxytoca* was around three times that in ESBL phenotype *K. pneumoniae*. However, the mechanism conferring ESBL phenotypes is thought to be quite different between *K. oxytoca* and *K. pneumoniae*. Our results demonstrate that there was only one strain carrying *bla*<sub>CTX-M-3</sub> was screened amongst *K. oxytoca* isolates during the observation period. The overproduction of OXY-2 is due to a distinct mutation in the promoter region, which increases the promoter strength by >10-fold (Fournier et al., 1999). This resistance mechanism of *K. oxytoca* has been reported since the 1990s (Livermore, 1995; Fournier et al., 1996). In addition, isolates carrying plasmid-mediated ESBLs occurred in *K. pneumoniae*. Limited in the plasmid-mediated ESBLs, only one strain carrying *bla*<sub>CTX-M-3</sub> was screened amongst *K. oxytoca* isolates during the observation period. The overproduction of OXY-2 is due to a distinct mutation in the promoter region, which increases the promoter strength by >10-fold (Fournier et al., 1999). This resistance mechanism of *K. oxytoca* has been reported since the 1990s (Livermore, 1995; Fournier et al., 1996). In addition, isolates carrying plasmid-mediated ESBLs have been reported in several countries (Zhang et al., 2008; Castanheira et al., 2013). Our results demonstrate that there was only one strain carrying plasmid-mediated ESBL (CTX-M-3) with a non-mutated OXY promoter region amongst the identified ESBL phenotype isolates, suggesting overproduction of OXY-2 to be the major cause of ESBL phenotypes amongst *K. oxytoca* isolates in Japan. Intriguingly, in our study, all the isolates harbouring the mutated OXY promoter region were not OXY-1 producers, but OXY-2 producers. The most likely explanation is that substrate catalytic activity between OXY-1 and -2 is somewhat different. We found several isolates of *K. oxytoca* carrying the *bla*<sub>OXY-1</sub> gene with the mutation conferring an increase of promoter activity that were not judged as the ESBL phenotype by the confirming test using cefotaxime or ceftazidime with clavulanic acid, in spite of lesser susceptibility to tazobactam/piperacillin (MIC > 64 µg ml<sup>-1</sup>) and aztreonam (MIC 4–16 µg ml<sup>-1</sup>). Although overproduction of OXY-1 may confer resistance to limited classes of β-lactams such as penicillins, there is the possibility of the emergence of strains overproducing OXY variants with extended-substrate catalytic profiles by specific amino acid substitutions, as reported previously (Rodriguez-Martinez et al., 2008). To monitor them, further study focusing on the prevalence of OXY variants and/or mutations conferring overproduction of OXYs that affect antimicrobial susceptibility needs to be continuously addressed.

Doripenem (carbapenem) as well as two cephalosporin classes of antimicrobials, moxalactam and flomoxef (oxa-cephem) and cefmetazole (cephamycin), were thoroughly effective in vitro against these ESBL phenotype *K. pneumoniae* and *K. oxytoca*. We also revealed that ceftibuten (a third-generation cephalosporin) was relatively effective amongst oral antimicrobials against these ESBL phenotype *K. pneumoniae* and *K. oxytoca*, ovulating resistance in vivo. These data indicate that the prevalence of ESBL-producing *K. pneumoniae* is still low compared with those rates in surrounding countries, because recent worldwide surveillance shows global ESBL-positive rates were 20.1% for *K. pneumoniae*, with the rate in the Asia/Pacific region being relatively high compared with other areas (Hawkey, 2008; Lob et al., 2013). The two previous studies did not determine the detailed subtypes of CTX-Ms, but our molecular genotyping revealed a subtype in ESBL phenotype *K. pneumoniae*, i.e. we found the appearance of CTX-M-15, known as the world pandemic ESBL in *E. coli*. Recently, several researchers have reported acceleration of the increase of the CTX-M-15-producing *E. coli* ST131 clone in Japan (Nakamura et al., 2012; Yano et al., 2013). The detection of CTX-M-15-producing *K. pneumoniae* isolates has also been reported in Asian and Middle East countries, such as South Korea, India and Pakistan (Hawkey, 2008; Livermore, 2012). This prevalence shift in *E. coli* is likely to have influenced the appearance of the world pandemic plasmid-mediated CTX-M-15 amongst *K. pneumoniae* in Japan.

In summary, this report describes the trends of isolation of ESBL phenotype *K. pneumoniae* and *K. oxytoca* between 2000 and 2010 in Japan, and the difference in genes conferring the ESBL phenotype between these two species. Although there are still some active antibacterials left, the further emergence of antibacterial resistance by inappropriate use of antibacterials must be avoided.

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