Regional outbreak of CTX-M-2 \(\beta\)-lactamase-producing \textit{Proteus mirabilis} in Japan

Ryuichi Nakano,¹,² Akiyo Nakano,³ Michiko Abe,⁴ Matsuhisa Inoue,¹,⁵ and Ryoichi Okamoto¹,⁵

¹Kitasato University Graduate School of Medical Sciences, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0374, Japan
²Department of Microbiology and Immunology, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan
³School of Veterinary Medicine Azabu University, 1-17-71 Fuchinobe, Chuoh-ku, Sagamihara, Kanagawa 252-5201, Japan
⁴Department of Medical Laboratory Sciences, School of Allied Health Sciences, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan
⁵Department of Microbiology, Kitasato University School of Medicine, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0374, Japan

\textit{Proteus mirabilis} is a common cause of urinary tract infection. Wild-type \textit{P. mirabilis} strains are usually susceptible to penicillins and cephalosporins, but occurrences of \textit{P. mirabilis} producing extended-spectrum \(\beta\)-lactamases (ESBLs) have been recently reported. Here, we surveyed the prevalence of cefotaxime resistance among \textit{P. mirabilis} strains at seven different hospitals in Kanagawa Prefecture, Japan, and investigated their molecular epidemiology to explain the mechanism of their spread. The prevalence of cefotaxime resistance among \textit{P. mirabilis} increased annually, from 10.1 % in 1998 to 23.1 % in 2003, and increased drastically in 2004, exceeding 40 %. We collected 105 consecutive and non-duplicate cefotaxime-resistant \textit{P. mirabilis} isolates (MIC 16 to \(\geq 256\) mg l\(^{-1}\)) from these hospitals from June 2004 to May 2005 and characterized their profile. PCR and sequence analysis revealed that all resistant strains produced exclusively CTX-M-2 \(\beta\)-lactamase. PFGE analysis identified 47 banding patterns with 83 % or greater similarity. These results indicated that a regional outbreak of \textit{P. mirabilis} producing CTX-M-2 \(\beta\)-lactamase has occurred in Japan and suggest that the epidemic spread occurred within and across hospitals and communities by extended clonal strains. Plasmid analysis revealed that 44.8 % of plasmids harboured by \(\text{bla}_{\text{CTX-M-2}}\) isolates had common profiles, encoding ISEcp1, IS26 and \text{Int1}, and belonged to incompatibility group T. Spread of the resistant isolates in Japan resulted from dissemination of narrow-host-range plasmids of the IncT group encoding \(\text{bla}_{\text{CTX-M-2}}\). These findings indicate the rapidly developing problem of treating the species to prevent dissemination of ESBL producers.

INTRODUCTION

Plasmid-borne extended-spectrum \(\beta\)-lactamases (ESBLs) in members of the \textit{Enterobacteriaceae} are becoming increasingly frequent at clinical sites worldwide (Paterson & Bonomo, 2005). Most have been described in nosocomial isolates, predominantly of \textit{Escherichia coli} and \textit{Klebsiella pneumoniae}, although some have been described in outpatients. Detection and specific targeting of ESBLs are clinically important, because ESBL producers may be clinically resistant to many \(\beta\)-lactams. Most ESBLs are derivatives of TEM and SHV enzymes, but CTX-M enzymes have also been disseminated worldwide and are increasingly important (Bradford, 2001). CTX-M enzymes predominantly hydrolyse cefotaxime, and most are weakly active against ceftazidime. More than 130 CTX-M-type enzymes have been classified into four clusters according to their amino acid sequences: CTX-M-1 group, CTX-M-2 group, CTX-M-8 group and CTX-M-9 group (Rossolini \textit{et al.}, 2008; http://www.lahey.org/studies/other.asp).

\(\text{bla}_{\text{CTX-M}}\) genes are often associated with transferable plasmids, and some of them are parts of transposons or constitute cassettes in integrons (Cantón & Coque, 2006; Eckert \textit{et al.}, 2006). Insertion sequences, especially ISEcp1, have been repeatedly found adjacent to \(\beta\)-lactamase genes.

Abbreviation: ESBL, extended-spectrum \(\beta\)-lactamase.
Within the CTX-M β-lactamases (CTX-M-1, CTX-M-2 and CTX-M-9 groups) and plasmid-borne AmpC β-lactamases (CMY-2 and CMY-4) (Canét & Coque, 2006; Eckert et al., 2006; Nakano et al., 2007). This ISEsp1 element contains typical ~35 and ~10 putative promoter regions and could mobilize such genes (Poiré et al., 2003; Nakano et al., 2007). Insertion sequence IS26 and unusual class 1 integrons (Int1) with ORF513 (GenBank accession no. AJ416343) have also been reported to associate with blaCTX-M-2 and blaCTX-M-9 genes (Saladín et al., 2002; Canét & Coque, 2006; Eckert et al., 2006). CTX-M enzymes have been found predominately in members of the Enterobacteriaceae, most prevalently in E. coli, K. pneumoniae and Salmonella enterica.

Among the Enterobacteriaceae, Proteus mirabilis is one of the most common causes of urinary tract infection, can cause pneumonia in debilitated or immunocompromised patients, and is also an important cause of nosocomial infections (Rózalski et al., 1997). Wild-type strains of P. mirabilis are usually susceptible to penicillins and cephalosporins. However, occurrences of the species that are resistant to β-lactams, mediated by the production of acquired β-lactamases, have been reported recently in Europe and the US (Chanal et al., 2000). Since the 1990s, P. mirabilis producing TEM-derived ESBLs (TEM-15, TEM-24, TEM-52 and TEM-66) has been reported (Bonnet et al., 1999; Luzarzo et al., 2001; Biendo et al., 2005). Non-TEM-derived ESBLs such as VEB-1 (Kim et al., 2004), CTX-M β-lactamases (CTX-M-1, CTX-M-2, CTX-M-13 and CTX-M-31) (Ho et al., 2005; Karapavlidou et al., 2005) and plasmid-borne AmpC β-lactamases (CMY-2 and CMY-4) (Verdet et al., 1998; Park et al., 2006) have also been reported infrequently. The prevalence of ESBL-positive strains has been reported at 6.9% in France (de Champs et al., 2000), 7.0% in Argentina (Quintero et al., 2003), 8.8% in Italy (Luzarzo et al., 2001) and 9.5% in the US (Saurina et al., 2000). An outbreak of ESBL-producing P. mirabilis has been reported as a nosocomial infection caused by clonal spread (Nagano et al., 2003; Kim et al., 2004). However, less is known about the regional epidemiology and prevalence of intra- and inter-hospital spread of these organisms.

In this study, we conducted a survey to assess the prevalence of P. mirabilis strains producing ESBLs and investigate the molecular epidemiology of resistant strains. We found that cefotaxime-resistant P. mirabilis has increased since 2000. Molecular analysis revealed that all resistant isolates produced exclusively CTX-M-2 enzyme, and that the plasmids encoding blaCTX-M-2 genes have some common profiles. We report here that a community outbreak of CTX-M-2 enzyme-producing P. mirabilis has occurred in Japan that may be caused by dissemination of R plasmids.

**METHODS**

**Epidemiological study of cefotaxime-resistant P. mirabilis from 1998 to 2004.** An epidemiological study was conducted to identify and compare trends in annual prevalence of cefotaxime resistance among P. mirabilis isolates. The strains were isolated from seven hospitals (D, H, F, S, T, M and Y) that were located in distinct cities in Kanagawa Prefecture, Japan, from 1998 to 2004. The bed capacity of all hospitals is more than 300. A total of 1656 clinical isolates of P. mirabilis were identified through hospital records and underwent susceptibility testing. The antimicrobial susceptibilities were determined with the Microscan WalkAway system (Dade Behring). The annual prevalence of cefotaxime resistance was calculated as the annual mean percentage of all P. mirabilis isolates resistant to 16 µg cefotaxime ml⁻¹.

**Clinical isolates for molecular analysis.** Two hundred and ninety-six consecutive and non-duplicate P. mirabilis isolates were obtained from the seven hospitals from June 2004 to May 2005. They were isolated from hospital patients and outpatients. The bacterial strains resistant to cephalosporins were collected and screened with the Clinical and Laboratory Standards Institute (CLSI) screening test for ESBL detection. The criteria for strain inclusion were MIC >1 µg ml⁻¹ of ceftazidime, cefotaxime or cefpodoxime. The isolates were examined by molecular analysis to identify the resistance gene.

**Antimicrobial susceptibility testing and screening for ESBL.** Antimicrobial susceptibility of the collected isolates was determined by the agar dilution method using Mueller-Hinton agar (Eiken Chemical) according to CLSI guidelines (CLSI, 2005). The antimicrobials tested were cefpodoxime and cefmetazole (Sankyo); ceftazidime (Sigma Chemical); and imipenem (Banyu Pharmaceutical). The MICs of ampicillin and cefotaxime (Sigma Chemical) with and without 5 µg clavulanic acid ml⁻¹ (SmithKline Beecham Pharmaceuticals) were also determined. As a non-β-lactam, the fluoroquinolone levofloxacin (LKT Laboratories) was also used.

In addition, ESBL production was inferred by analysis of the β-lactam susceptibility profile. A twofold dilution decrease in the MIC of cefotaxime tested in combination with clavulanic acid versus the MIC of the agent tested alone ≥3 was considered indicative of ESBL production, in accordance with CLSI guidelines.

**PFGE typing.** PFGE analysis was carried out according to the manufacturer’s protocol (Bio-Rad) (Hosaka et al., 2005). The whole-cell DNA of P. mirabilis isolates embedded in 1.6% low-melt agarose plugs (Bio-Rad) was digested with the SfiI restriction enzyme (Takara) for 18 h at 50 °C. DNA fragments were electrophoresed on a 1.0% SeaKem Gold agarose gel (FMC Bioproducts) on the CHEF-MAPPER system (Bio-Rad) with 0.5 × Tris/borate-EDTA running buffer at 14 °C using the following protocol: 6.0 V cm⁻¹ gradients for 20.18 h ramped with an initial switching time of 0.47 s and a final switching time of 1 min 33.7 s.

The PFGE patterns were analysed with Molecular Analysis software (Bio-Rad) and compared using the average linkage matching method (UPGMA) with the Dice coefficient and a 1.0% tolerance. A dendrogram was constructed and interpreted according to the criteria described by Tenover et al. (1995). Isolates with a coefficient ≥83% were considered to represent the same PFGE type (≤three bands different).

**PCR identification and sequencing of blaCTX-M genes.** Plasmid DNA was isolated from clinical isolates by the alkaline lysis method and used as a template for PCR (Birnboim & Doly, 1979). The ESBL types were determined by PCR using specific primers for blaTEM, blalAP and blaxalM using methods described elsewhere (Nagano et al., 2003). PCR experiments were also performed to detect the
plasmid-encoded AmpC β-lactamases and metallo-β-lactamases according to a previously described method (Yano et al., 2001; Pérez-Pérez & Hanson, 2002). PCR was performed with HotStarTaq DNA polymerase (Qiagen) according to the manufacturer’s instructions. PCR products were subjected to electrophoresis on a 1.2% agarose gel (GibcoBRL agarose) to identify the amplified DNA fragments. Both strands of the PCR products were sequenced with an ABI PRISM 310 DNA sequencer (Applied Biosystems) (Sanger et al., 1977).

DNA sequences were analysed with DNASTAR software, and comparisons with other sequences were performed with the BLAST and FAST programs of NCBI (Burland, 2000).

**Genetic environment of blaCTX-M genes.** Some bla genes have been revealed to associate with mobile insertion sequences such as IS26 and IS3ECp1 (Saladin et al., 2002; Ford & Avison, 2004; Nakano et al., 2004). Detection of insertion sequences IS26 and IS3ECp1 associated with β-lactamase genes was performed by PCR analysis. For** blaCTX-M** associated with IntI1, the primers INT2F and 3′-CS were also used for amplification (Poirel et al., 2003). The genetic environment of the** blaCTX-M** genes was investigated by PCR and sequencing of the regions surrounding these genes according to a previously described method (Eckert et al., 2006). The regions upstream of the** blaCTX-M** genes were amplified with forward primers hybridizing to the insertion sequences IS26 and IS3ECp1 and to the ORF513 region and the CTX-M reverse primer (Radice et al., 2002). Both strands of the PCR products were sequenced.

**Identification of plasmid incompatibility.** Plasmids were classified according to their incompatibility (Inc) group using the PCR replicon-typing scheme described by Carattoli et al. (2005). PCR replicon typing assays have been used to identify plasmids bearing CTX-M β-lactamase genes (Canton & Coque, 2006; Lavallée et al., 2006). This PCR assay discriminates plasmids of known Inc groups by the presence of specific genes involved in plasmid maintenance using an inc/rep PCR-based method (5-multiplex and 3-simplex).

## RESULTS

**Prevalence of cefotaxime-resistant *P. mirabilis* isolates from 1998 to 2004**

A 7-year survey of cefotaxime resistance among 1656 clinical isolates of *P. mirabilis* was conducted. Of the 401 isolates that were resistant to cefotaxime, the annual mean prevalence of resistance was as follows: 10.1% in 1998, 9.8% in 1999, 14.2% in 2000, 19.4% in 2001, 26.5% in 2002, 23.1% in 2003 and 46.2% in 2004. Until 1999, the prevalence of cefotaxime resistance was approximately 10%; this then gradually increased from 2000, and substantially increased to 46.2% in 2004. The isolates were also resistant to ampicillin and were considered potential CTX-M-type ESBL producers.

**Clinical isolates and antibiotic susceptibility**

During a 1-year survey, 296 strains of *P. mirabilis* were isolated from seven hospitals. Of these, 105 strains (35.5%) were collected as potential ESBL producers based on CLSI screening criteria from 89 hospital patients and 16 community patients (Table 1). They were isolated from urine (57.1%), sputum (30.5%), blood (3.8%), pus (3.8%), wound and other samples.

Susceptibility testing was performed on the 105 strains, and the MIC range results are shown in Table 2. These isolates demonstrated similar susceptibility profiles and were highly resistant to ampicillin, cefodoxime and cefotaxime, although they remained susceptible to ceftazidime and imipenem. The presence of clavulanic acid significantly reduced (≥3 twofold dilutions) the MICs of ampicillin and cefotaxime. These resistance profiles identified the isolates as ESBL producers according to CLSI criteria. They also showed that the MIC of cefotaxime was more than eightfold higher than that of ceftazidime, suggesting that the resistant isolates produced CTX-M-type ESBLs. In addition to extended-spectrum cephalosporins, 61 of the CTX-M producers were resistant to levofloxacin (MICs >2 µg ml⁻¹).

**Characterization of the ESBL-producing strains: PCR and DNA sequencing of *bla* genes**

The genotypes of ESBLs were determined by PCR and DNA sequencing of the PCR products (Table 2). Of the 105

<table>
<thead>
<tr>
<th>Hospital (no. of isolates)</th>
<th>No. (%) of resistant isolates*</th>
<th>No. of clusters/no. of other types†‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>D (15)</td>
<td>2 (13.3)</td>
<td>Others (2)</td>
</tr>
<tr>
<td>H (26)</td>
<td>11 (42.3)</td>
<td>I, X–XII (4) and others (3)</td>
</tr>
<tr>
<td>F (38)</td>
<td>3 (7.9)</td>
<td>IX and XI (2)</td>
</tr>
<tr>
<td>S (34)</td>
<td>4 (11.8)</td>
<td>IX, X (2) and others (2)</td>
</tr>
<tr>
<td>T (99)</td>
<td>53 (53.5)</td>
<td>II–VIII, XII, XIII–XVI, XX (13) and others (15)</td>
</tr>
<tr>
<td>M (34)</td>
<td>9 (26.5)</td>
<td>XIX, XXXI (2) and other (1)</td>
</tr>
<tr>
<td>Y (50)</td>
<td>23 (46.0)</td>
<td>XVII, XVIII, XX, XXXI (4) and others (3)</td>
</tr>
<tr>
<td>Total (296)</td>
<td>105 (35.5)</td>
<td>I–XXI (21) and others (26)</td>
</tr>
</tbody>
</table>

*Strains were resistant to 1 µg ml⁻¹ ceftazidime, cefotaxime or cefpodoxime.
†Strains were defined as isolated with PFGE banding patterns showing >83% similarity.
‡Underlined clusters were defined as isolated at other hospitals.
Table 2. Susceptibilities of collected *P. mirabilis* isolates to β-lactams, characterized by β-lactamase production and number of levofloxacin-resistant isolates

| Hospital (no. of isolates tested) | No. with associated LVX resistance† | β-Lactamase type‡ | MIC (µg·ml⁻¹) | AMP | IMP | CMZ | CTX | CTX+CLA§ | Ecp1
|----------------------------------|-------------------------------------|-------------------|----------------|-----|-----|-----|-----|----------|-----
| P (2)                            | 1                                   | CTX-M2            | ≥256           | ≥256| ≥256| ≥256| ≥256| ≥256     | 1.32
| H (1)                            | 1                                   | CTX-M2            | ≥256           | ≥256| ≥256| ≥256| ≥256| ≥256     | 1.32
| F (3)                            | 2                                   | CTX-M2            | ≥256           | ≥256| ≥256| ≥256| ≥256| ≥256     | 1.32
| S (4)                            | 3                                   | CTX-M2            | ≥256           | ≥256| ≥256| ≥256| ≥256| ≥256     | 1.32
| M (9)                            | 4                                   | CTX-M2            | ≥256           | ≥256| ≥256| ≥256| ≥256| ≥256     | 1.32
| Y (23)                           | 10                                  | CTX-M2            | ≥256           | ≥256| ≥256| ≥256| ≥256| ≥256     | 1.32
| Total (105)                      | 61                                  | CTX-M2            | ≥256           | ≥256| ≥256| ≥256| ≥256| ≥256     | 1.32

*AMP, Ampicillin; CPD, cepodiamide; CAZ, ceftazidime; CTX, cefotaxime; CMZ, cefamandole; IMP, imipenem; CLA, clavulanic acid.
†LVX, Levofloxacin.
‡Identified by PCR and sequencing.
§MICs were determined in the presence of clavulanic acid (5 µg·mL⁻¹).

isolates examined by PCR, all of the ESBL-producing *P. mirabilis* isolates were positive for the CTX-M-2-group β-lactamase gene, whereas no amplicons were observed for *blaTEM*, *blaSHV*, *blaCTX-M-1*, *blaCTX-M-9*, plasmid-encoded AmpC β-lactamases or metallo-β-lactamases. Sequence analysis of the *blaCTX-M* genes yielded 873 bp products that were 100% identical to the CTX-M-2 β-lactamase gene. These results indicated that cephalosporin-resistant *P. mirabilis* isolates predominantly produced CTX-M-2 enzyme and did not produce an additional β-lactamase.

Exploration of the regions surrounding *blaCTX-M* genes

PCR and sequencing were performed on all isolates to identify the regions surrounding the *blaCTX-M* genes. PCR analysis demonstrated that the entire IScep1 sequence was amplified in all 105 isolates, IS26 was amplified in 59 isolates and *Int1* was amplified in 50 isolates. The IScep1 sequence was inserted in regions upstream of the *blaCTX-M* genes, and ORF513 was not detected in any isolates. IScep1 was observed 49 bp upstream of *blaCTX-M* genes, and the sequences contained typical −35 and −10 putative promoter regions. IS26 was not found in the upstream region of the *blaCTX-M* genes. Therefore, CTX-M-2 β-lactamase genes of all isolates were associated with insertion sequence IScep1, and none was found to be associated with the class 1 integron containing ORF513.

Epidemiological typing of strains

PFGE banding patterns were obtained for 98 of the 105 CTX-M-2 enzyme producers. DNA from the remaining seven producers consistently autodigested, and no banding patterns were obtained from these isolates (Fig. 1). The isolates represented 47 banding patterns with a similarity of more than 38.6 ± 13.9%. The analysis identified 72 strains that demonstrated the same patterns or >83% similarity and defined 21 clusters, and the other 26 strains were defined as unique (Table 1). No major cluster with a high rate of spreading was observed among these strains; at most nine strains, in cluster XXI (8.6%), were present (Table 3). No hospital demonstrated only one banding pattern; several strains of genetic types were isolated from each hospital (Table 1).

Of these clusters, 13 (I–V, VII, VIII, XII, XIV, XVII–XIX and XXI) were distributed within isolated hospitals and considered to represent clonal strains (Fig. 1, Table 3). As an example, the five strains of cluster IV were isolated from hospital T. They were identified as a clonal spread among their respective hospitals: clusters III–V, VII, VIII and XIX were in hospital T; clusters I and XII were in hospital H; clusters XVII, XVIII and XXI were in hospital Y; and cluster XIX was in hospital M. These clusters may represent small-scale hospital-acquired infections.

The remaining five clusters (IX–XII and XX) were each distributed with isolates from two hospitals. All hospitals
except hospital D shared a cluster with another hospital (Table 2, Fig. 1). For example, cluster IX was identified in hospital F and hospital S. These isolates were defined as multiclonal strains with interhospital occurrence caused by community-acquired infections. The 16 strains from the community were also identified on the basis of their PFGE profiles (Table 3, Fig. 1). These isolates had various PFGE banding patterns (11 genetic types) comprising eight clusters (VI–VIII, XI, XIII, XV, XVI and XIX) and three unique strains. Six of these clusters (VII, VIII, XI, XIII, XV and XIX) also included strains from hospital patients (Table 3). Community-acquired CTX-M-2 producers were of various genetic types, and some were also spread across the hospitals. These findings suggest that epidemic clusters and strains were spread in hospitals and communities without any clonal spread.

Characterization of plasmid profiles

In total, 86 CTX-M-2-producing isolates were identified as belonging to Inc groups, and Inc classification could not be determined for the remaining isolates. Among these, 84 isolates belonged to a single Inc group: IncT (n=58), IncW (n=21), IncK (n=3), IncHI 1 (n=1) and IncX (n=1). The remaining two strains contained both IncN and IncW. A large number of isolates, about half of the CTX-M-2-producing strains, carried IncT plasmids. They were isolated from inpatients and outpatients of all hospitals except hospital D, from which the two isolates belonged to the IncW group.

IncT plasmids were detected from 34 of 47 PFGE types. Among them, nine clusters (I–VIII and XIII) contained only IncT plasmids and were identified as clonal strains. Another seven clusters (X–XII, XV, XVII, XIX and XXI) contained IncT or another plasmid; they were identified not to be clonal strains although their PFGE patterns were the same. These results indicate that various types of CTX-M-2-producing strains were identified, with the majority containing IncT but with other Inc groups also represented.

In silico analysis revealed that 53 of the 59 isolates positive for IS26 and 47 of the 50 isolates positive for IntI1 were identified among the 58 isolates harbouring IncT plasmids. Forty-seven of the isolates (44.8%) were co-positive for IS26 and IntI1. They had a common profile and may share the same plasmid. Strains representing the 34 PFGE types harbouring IncT plasmids may have the same plasmid encoding bla_CTX-M-2.

DISCUSSION

This study revealed an emergence and high prevalence of ESBL-producing P. mirabilis in Japan. ESBL-producing P. mirabilis has gradually increased since 2000 and dramatically increased with a very high prevalence (46.2%) in 2004. The observed prevalence of ESBL producers is remarkably high compared with previous reports: 6.9% in France and 9.5% in the US (Verdet et al., 1998; Luzzaro et al., 2001). Among the Enterobacteriaceae isolated from these hospitals during the same periods, E. coli and K. pneumoniae produced ESBLs at a very low frequency; 43 of 3926 E. coli strains (1.1%) and 7 of 1575 K. pneumoniae strains (0.4%) were ESBL producers (data not shown). These findings reveal that ESBL-producing P. mirabilis spread coincidently in Japan.

Molecular analysis indicated that all of the P. mirabilis isolates produced only CTX-M-2 β-lactamase, without any additional β-lactamase production. A diversity of ESBLs such as TEM-derived mutants was reported to be produced by P. mirabilis in Europe (Bonnet et al., 1999; Luzzaro et al., 2001; Biendo et al., 2005). CTX-M-2-, CTX-M-3-, CTX-M-13- and CTX-M-14-producing P. mirabilis has been reported in Hong Kong, Taiwan and Japan (Nagano et al., 2003; Ho et al., 2005; Wu et al., 2006). These data indicate a geographical feature of ESBL-producing P. mirabilis, with TEM types in Europe and CTX-M types in Eastern Asia. Uniquely, the species isolated in Japan possessed the CTX-M-2 enzyme frequently and predominantly in the Enterobacteriaceae. Previous studies have reported that ESBL-producing P. mirabilis in hospitals always encoded additional β-lactamase genes (Ho et al., 2005; Wu et al., 2006), with the exception of nosocomial outbreaks by ESBL-producing clonal strains (Nagano et al., 2003; Kim et al., 2004).

The clonal relationship among ESBL producers was assessed by PFGE, and 47 genetic types were identified from 98 P. mirabilis isolates. Each hospital had strains representing several genetic types, indicating that the producers were not clonal strains. The same PFGE profiles were also determined by SmaI digestion of the strains (data not shown). Five of seven examined hospitals (hospitals T, M, Y, H and F) demonstrated some intrahospital clonal strains that may have been spread by nosocomial infections (Table 3). Each clonal strain was isolated within 3 months (data not shown), which means the clusters were spread transiently among their hospitals. Five clusters were isolated from outpatients; however, each cluster consisted of only two isolates. This observation may suggest that no clonal spread occurred among the community-acquired infections, unlike the situation in hospitals. We also isolated clusters in patients across hospitals and outpatients, which may have been caused by interhospital- and community-acquired infections. In our research, there were no possibilities of contact with doctors or patients from other hospitals. These findings indicate that the CTX-M-2 enzyme has spread among multiclonal strains and unique strains, and the outbreaks were not the result of a clonal strain.

This regional outbreak may be explained by horizontal transfer of plasmids carrying the bla_CTX-M-2 gene among P. mirabilis as well as clonal dissemination of microorganisms via nosocomial infection. Plasmid analyses indicated some common features: all bla_CTX_M genes were associated with IS26cp1 and not located upstream of IS26. Although almost all P. mirabilis isolates were reported to
possess class 1 integron, 44.8% of the plasmids harboured by the isolates belonged to incompatibility group IncT and possessed IS\textsubscript{26} and \textit{Int1}. IncT plasmids were first reported and characterized in \textit{P. mirabilis} in Japan (Odakura \textit{et al.}, 1977). The mutual or predominant R plasmids encoding \textit{bla\textsubscript{CTX-M}} are thought to have disseminated among the species by transfer of the plasmids to a multitude of susceptible strains. However, transconjugant strains were not obtained by transferring the ESBL phenotype to \textit{E. coli} in this study (data not shown), as was previously reported (Karapavlidou \textit{et al.}, 2005). We also found that only 5 of 3926 \textit{E. coli} strains (0.1%) isolated from the same hospitals during the same period produced CTX-M-2-group enzyme (data not shown).

Although CTX-M-2 was not detected among these \textit{E. coli} strains as previously reported (Lavollay \textit{et al.}, 2006; Novais \textit{et al.}, 2006), incompatibility group IncT is a narrow-host-range plasmid harboured by \textit{P. mirabilis} as well as \textit{E. coli} (Shapiro, 1977). These results demonstrate that \textit{P. mirabilis} produces the CTX-M-2 enzyme exclusively and predominantly, with high prevalence among the \textit{Enterobacteriaceae}. \textit{P. mirabilis} isolates may possess common R plasmids encoding \textit{bla\textsubscript{CTX-M}} spread by horizontal transfer independent of any other members of the \textit{Enterobacteriaceae}, which may be one of the reasons for the spread of the species. The dissemination of the plasmids among \textit{P. mirabilis} is inferred to result from species features such as swarming. Further research characterizing the spread of these R plasmids among \textit{P. mirabilis} will help to clarify the associations of the outbreak.

\textit{P. mirabilis} has been implicated in contamination and colonization, and is occasionally isolated in severe infections (Rózalski \textit{et al.}, 1997). The species has biofilm-forming capacity on urinary catheters and may be long-term indwelling in hospitals (Jones \textit{et al.}, 2005). The majority of CTX-M-2-producing \textit{P. mirabilis} isolates were also recovered from urinary specimens (57.1%), which may be one of the reasons underlying the spread of the species. Dissemination of CTX-M-producing \textit{E. coli} among community-acquired urinary tract infections has also been

**Table 3.** PFGE fingerprints of CTX-M-2-producing \textit{P. mirabilis} among inpatients and outpatients

<table>
<thead>
<tr>
<th>Cluster (no. of isolates)</th>
<th>Isolates from inpatients (no. of isolates)</th>
<th>Isolates from outpatients (no. of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (2)</td>
<td>H (2)</td>
<td>T (2)</td>
</tr>
<tr>
<td>II (2)</td>
<td>T (2)</td>
<td>T (2)</td>
</tr>
<tr>
<td>III (2)</td>
<td>T (2)</td>
<td>T (2)</td>
</tr>
<tr>
<td>IV (5)</td>
<td>T (5)</td>
<td>T (2)</td>
</tr>
<tr>
<td>V (2)</td>
<td>T (2)</td>
<td>T (2)</td>
</tr>
<tr>
<td>VI (2)</td>
<td></td>
<td>T (2)</td>
</tr>
<tr>
<td>VII (3)</td>
<td>T (2)</td>
<td>T (2)</td>
</tr>
<tr>
<td>VIII (4)</td>
<td>T (2)</td>
<td>T (2)</td>
</tr>
<tr>
<td>IX (2)</td>
<td>S (1), F (1)</td>
<td></td>
</tr>
<tr>
<td>X (2)</td>
<td>S (1), H (1)</td>
<td></td>
</tr>
<tr>
<td>XI (3)</td>
<td>H (1), F (1)</td>
<td>F (1)</td>
</tr>
<tr>
<td>XII (5)</td>
<td>T (1), H (4)</td>
<td></td>
</tr>
<tr>
<td>XIII (3)</td>
<td>T (1)</td>
<td>T (2)</td>
</tr>
<tr>
<td>XIV (2)</td>
<td>T (2)</td>
<td>T (2)</td>
</tr>
<tr>
<td>XV (2)</td>
<td>T (1)</td>
<td>T (1)</td>
</tr>
<tr>
<td>XVI (2)</td>
<td>T (2)</td>
<td></td>
</tr>
<tr>
<td>XVII (4)</td>
<td>Y (4)</td>
<td></td>
</tr>
<tr>
<td>XVIII (7)</td>
<td>Y (7)</td>
<td></td>
</tr>
<tr>
<td>XIX (7)</td>
<td>M (5)</td>
<td></td>
</tr>
<tr>
<td>XX (2)</td>
<td>T (1), Y (1)</td>
<td></td>
</tr>
<tr>
<td>XXI (9)</td>
<td>M (1), Y (8)</td>
<td></td>
</tr>
<tr>
<td>Others (26)</td>
<td>D (2), H (2), S (2), M (1), T (13), Y (3)</td>
<td>H (1), T (2)</td>
</tr>
</tbody>
</table>
reported in the UK (Woodford et al., 2004). Antibiotics used in therapy are usually able to reach high urinary concentrations, which are likely to be clinically effective. The fluoroquinolones always used for urinary tract infection are an appropriate therapy; however, almost all of the CTX-M-2-producing *P. mirabilis* isolates were resistant to levofloxacin. The emergence of multidrug-resistant *P. mirabilis* is difficult to eradicate and may restrict the therapeutic options of antimicrobial uses. Thus, treatment of those strains is often limited to carbapenems. This emergence represents a very serious problem for treating the species in Japan.

In conclusion, our findings indicate that a regional outbreak of CTX-M-2-producing *P. mirabilis* occurred coincidentally in several hospitals in Kanagawa, Japan, and was caused by intrahospital-, interhospital- and community-acquired CTX-M-2 among *P. mirabilis*. Only CTX-M-2-type enzyme was produced by *P. mirabilis*, and the producers were highly prevalent in Japan. Spread of *bla*<sub>CTX-M-2</sub> in Japan occurred via dissemination of narrow-host-range plasmids of the IncF group. These data suggest that the species should always be regarded as resistant to extended-spectrum cephalosporins and fluoroquinolones in Japan, which is necessary to control their spread in hospitals and community environments. We must be on high alert against *P. mirabilis*, because ESBL producers may spread rapidly and widely when detected at clinical sites.

**ACKNOWLEDGEMENTS**

We thank Yuko Kakinuma for excellent technical assistance. We also thank Odawara Medical Hospital, Kitasato University East Hospital, Showa University Fujigaoka Hospital, St Marianna University School of Medicine Hospital, Chigasaki Tokushukai Medical Center, Yokohama Minami Kyousai Hospital and Yokohama Stroke and Brain Center for providing the clinical strains used in this study. This work was supported by the Charitable Trust Clinical Pathology Research Foundation of Japan and Grant-in-Aid for Young Scientists (B) of the Japan Society for the Promotion of Science.

**REFERENCES**


