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Distribution and antimicrobial susceptibility profile of extended-spectrum β-lactamase-producing *Proteus mirabilis* strains recently isolated in Japan

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ABSTRACT

Here we report on the prevalence of extended-spectrum β-lactamase (ESBL)-producing *Proteus mirabilis* from a nationwide antimicrobial resistance survey in different geographical regions of Japan. A total of 799 *P. mirabilis* isolates recovered between July 2009 and June 2010 from 314 healthcare facilities were characterised according to ESBL production, source, location and antimicrobial susceptibility pattern. ESBL production was found in 364 (45.6%) of the isolates, among which 354 (97.3%) produced CTX-M-2 group β-lactamases. Of the 349 ESBL-producing isolates in which the inpatient or outpatient status of the source was known, 324 (92.8%) were from inpatients and 25 (7.2%) were from outpatients ($P < 0.05$). Results of pulsed-field gel electrophoresis (PFGE) analysis performed on 66 of the ESBL-producers generated a distribution of PFGE patterns into 21 groups. Genetic relatedness was seen among isolates within a region, which is consistent with horizontal transmission. With respect to the frequency of ESBL-producers by specimen source, 12/14 (85.7%) central venous catheter specimens yielded ESBL-producing *P. mirabilis* compared with 159/405 (39.3%), 119/209 (56.9%), 42/77 (54.5%) and 20/49 (40.8%), respectively, for isolates from urine, sputum, decubitus ulcer and wound specimens. Among the ESBL-producers, non-susceptibility to ciprofloxacin was found in 74.2% of the ESBL-producing isolates compared with 17.7% of the ESBL-non-producing isolates. These results show that approximately one-half of the *P. mirabilis* isolates from clinical specimens in Japan are ESBL-producers and that the potential for concomitant fluoroquinolone resistance must also be considered.

1. Introduction

Proteus mirabilis is a causative agent of healthcare-associated infections (HCAIs), including urinary tract, respiratory tract and skin infections [1–3]. As HCAIs associated with this organism are often associated with horizontal transmission through urine specimens [4], monitoring is required for early detection and intervention. Since this organism is prevalent in the environment, including contaminated water, soil and animals [3], the spread of antimicrobial-resistant *P. mirabilis* is a widespread public health problem and is not restricted to the hospital environment.

Similar to reports of fluoroquinolone resistance in *Escherichia coli* and *Klebsiella* [5,6], fluoroquinolone resistance in *P. mirabilis* has also been reported [5–7]. Furthermore, there have also been reports of *Klebsiella pneumoniae* carbapenemase (KPC)-producing *P. mirabilis* strains from different geographical regions, which is consistent with the emerging resistance of this organism [8,9]. Whilst this organism was originally susceptible to β-lactams [1], emergence of resistance to penicillins, cephalosporins, cephamycins and monobactams has been reported in recent years in *P. mirabilis* strains producing extended-spectrum β-lactamases (ESBLs) [4,6,7,10–12].

The increased isolation of ESBL-producing *P. mirabilis* isolates in recent years is a concern, as infections caused by ESBL-producers tend to have a less satisfactory clinical outcome than infections caused by ESBL-non-producers [12,13]. In Europe, the USA and Asian countries excluding Japan, the prevalence of CTX-M ESBL-producing organisms has been increasing, reflecting a shift from ESBLs of TEM or SHV lineage [14,15]. In contrast, CTX-M has been

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the dominant ESBL in Japan [14–17]. It has also been reported that the prevalence of ESBL-producing *P. mirabilis* in Japan may exceed *E. coli* and *Klebsiella*. Despite these findings, the limitations of these reports were the small number of isolates studied in the investigation and the outcome being restricted to a limited geographical area or a single healthcare institution [10,16,17].

We previously reported our analysis of 74 *P. mirabilis* isolates from 54 healthcare institutions in Japan [7]. In the present study, we undertook a study of *P. mirabilis* isolates collected over a 1-year period, which represents the first large-scale study in Japan to determine the prevalence of ESBL-producing *P. mirabilis*.

2. Materials and methods

2.1. Bacterial isolates

In total, 799 non-blood culture isolates of *P. mirabilis* recovered from specimens at 314 hospitals located in different geographic regions in Japan between July 2009 and June 2010 were submitted to the clinical testing laboratory of Mitsubishi Chemical Medience Corporation (Tokyo, Japan). All isolates were recovered from specimens submitted for routine bacteriology testing. Only one isolate per patient was included in the study. Identification of *P. mirabilis* was by VITEK® 2 (SYSMEX bioMérieux Co. Ltd., Tokyo, Japan).

Of the *P. mirabilis* isolates, 405 were recovered from urine, 209 from sputum, 77 from decubitus ulcers, 49 from wounds, 14 from central venous catheters (CVC), 10 from nasal or throat swabs, 8 from ear discharges, 8 from vaginal swabs, 6 from skin, 5 from eye discharges, 5 from drain tubes, and 1 isolate each from gastric abscess, ascites fluid and joint fluid specimens. Of the 799 isolates, 557 (69.7%) were recovered from inpatients and 152 (19.0%) from outpatients; the inpatient or outpatient source of the remaining 90 isolates could not be determined. The mean age of patients from whom *P. mirabilis* isolates was recovered was 76.6 years.

2.2. Antimicrobial susceptibility testing

Screening and confirmatory testing for ESBL production were performed by the disc diffusion method using cefotaxime and ceftazidime with and without clavulanic acid (Eiken Chemical Co. Ltd., Tokyo, Japan) as described by the Clinical and Laboratory Standards Institute (CLSI) [18]. Minimum inhibitory concentrations (MICs) of cefotaxime (Sigma–Aldrich, St Louis, MO), ceftazidime (Sigma–Aldrich), meropenem (Sumitomo Dainippon Pharma, Osaka, Japan), ciprofloxacin (Bayer, Osaka, Japan), gentamicin (Sigma–Aldrich), piperacillin/tazobactam (TZP) (Sigma–Aldrich) and cefoxitin (Sigma–Aldrich) were determined by the CLSI agar dilution method [19]. MICs were interpreted according to CLSI standards [18].

2.3. PCR assay for extended-spectrum β-lactamase genes

Molecular characterisation of ESBLs was performed as follows: (i) PCR using specific primer sets for ESBL family enzymes (CTX-M-1, CTX-M-2 and CTX-M-9 groups) based on the methodology described by Dallenne et al. [20]; and (ii) PCR and sequencing using TEM- and SHV-specific primers according to the method described by Ishii et al. [21]. DNA sequencing was performed with a BigDye® Terminator v3.1 Cycle Sequence Kit using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Amino acid sequences were compared with the reported sequences from the Lahey Clinic website (http://www.lahey.org/Studies/).

2.4. Pulsed-field gel electrophoresis typing

Chromosomal DNA was prepared for pulsed-field gel electrophoresis (PFGE) analysis as described previously [7]. Following digestion with *Sfi*I (Takara, Otsu, Japan), DNA fragments were separated by electrophoresis on a CHEF-DR II system (Bio-Rad, Tokyo, Japan) at 6 V/cm with pulse times of 1.0–70 s for 20 h. A λ DNA ladder (New England Biolabs, Ipswich, MA) was used as the DNA size marker. The relatedness of PFGE patterns was determined by the unweighted pair-group method with arithmetic mean (UPGMA) and cluster analysis according to the Dice setting using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Relatedness of isolates was defined on the basis of PFGE profiles with ≥80% similarity.

3. Results

3.1. Antimicrobial susceptibilities

Based on the CLSI disc diffusion test, 364 (45.6%) of the 799 *P. mirabilis* isolates were ESBL-producers. The MICs of ESBL-producing and -non-producing *P. mirabilis* are shown in Table 1. Almost all of the ESBL-producers were non-susceptible to cefotaxime, with MIC_{50} and MIC_{90} values (MIC for 50% and 90% of the organisms, respectively) of 32 mg/L and 128 mg/L, respectively, compared with ≤0.06 mg/L for ESBL-non-producers. Against ceftazidime, both ESBL-producers and -non-producers exhibited low MIC_{50} and MIC_{90} values, with resistance seen in only 1.9% and 0.2% of the isolates, respectively. For meropenem, one ESBL-producing isolate showed a MIC of 2 mg/L, which would be interpreted as intermediate according to the CLSI. Non-susceptibility to ciprofloxacin was seen in 74.2% of the ESBL-producers compared with 17.7% of the ESBL-non-producers. No resistance to TZP was seen among ESBL-producing or -non-producing isolates. No differences in susceptibility to cefoxitin and gentamicin were seen between ESBL-producers and -non-producers.

3.2. Proteus mirabilis isolates and extended-spectrum β-lactamase genes

Among the 364 ESBL-producers, the CTX-M-2 group β-lactamase gene was detected in 354 isolates (97.3%) and 1 isolate possessed both a CTX-M-2 and CTX-M-1 group β-lactamase gene. Of the remaining nine isolates, four were shown to harbour a CTX-M-9 group β-lactamase gene. Five isolates produced an ESBL that could not be identified by the PCR primer sets used in this study.

None of the isolates possessed SHV-derived ESBLs. The TEM-type β-lactamase gene was detected in 21 isolates, of which 18 isolates also possessed a CTX-M-type β-lactamase gene. Of these 18 isolates, TEM-1 β-lactamase was observed in 12 isolates, TEM-191 in 3 isolates and TEM-135 in 3 isolates. None of the TEM-type β-lactamases were TEM-type ESBL. The remaining three isolates possessed a TEM-1 β-lactamase gene.

3.3. Specimens of extended-spectrum β-lactamase-producing P. mirabilis isolates

Of the specimens from which *P. mirabilis* was recovered, CVC specimens yielded the highest frequency of isolates from inpatients (14/14; 100%), followed by sputum (189/209; 90.4%), decubitus ulcer (59/77; 76.6%), wound specimens (34/49; 69.4%) and urine (242/405; 59.8%). The inpatient or outpatient status of the patient was determined for 349 of the ESBL-producing isolates, of which 324 (92.8%) isolates were recovered from inpatients and 25 (7.2%) were from outpatients ($P < 0.05$).

Table 1
Antimicrobial susceptibility of *Proteus mirabilis* isolated in different geographical regions of Japan (2009–2010).

Antimicrobial agent	ESBL-producing *P. mirabilis* (n = 364)				ESBL-non-producing *P. mirabilis* (n = 435)			
	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	n (%) non-susceptible[a]	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	n (%) non-susceptible[a]
Cefotaxime	1–128	32	128	362 (99.5)	≤0.06–64	≤0.06	≤0.06	3 (0.7)
Ceftazidime	≤0.06–128	0.25	1	7 (1.9)	≤0.06–16	≤0.06	≤0.06	1 (0.2)
Meropenem	≤0.06–2	≤0.06	≤0.06	1 (0.3)	≤0.06–0.12	≤0.06	≤0.06	0
Ciprofloxacin	≤0.06–128	4	32	270 (74.2)	≤0.06–128	≤0.06	4	77 (17.7)
Gentamicin	0.12–128	0.5	4	17 (4.7)	0.12–128	0.5	2	28 (6.4)
TZP	0.25–8	1	2	0	0.12–16	0.25	1	0
Cefoxitin	0.5–64	4	4	8 (2.2)	0.25–64	2	4	8 (1.8)

ESBL, extended-spectrum β-lactamase; MIC, minimum inhibitory concentration; $MIC_{50/90}$, MIC for 50% and 90% of the organisms, respectively; TZP, piperacillin/tazobactam. It is debatable whether any ESBL-producer should be counted as susceptible to ceftazidime or other cephalosporins.
[a] Interpreted according to Clinical and Laboratory Standards Institute guidelines [18].

The majority of *P. mirabilis* isolates were from urine and sputum specimens, with sputum isolates having a higher frequency of ESBL-producers (56.9%; 119/209) compared with urine isolates (39.3%; 159/405). With respect to isolates from decubitus ulcer, wound and CVC, ESBL-producers were found in 54.5% (42/77), 40.8% (20/49) and 85.7% (12/14) of the isolates, respectively.

3.4. Distribution of extended-spectrum β-lactamase-producing *P. mirabilis* in Japan

Fig. 1 shows the distribution of ESBL-producing *P. mirabilis* isolates among the five regions of the main islands of Japan (Region A, Hokkaido and Tohoku; Region B, Kanto; Region C, Chubu; Region D, Kinki; and Region E, Shikoku and Kyushu). Geographically, these regions span from Hokkaido Prefecture in the northeast to Kyushu Prefecture in the southwest. The highest rate of ESBL-producing *P. mirabilis* was seen in isolates recovered from healthcare facilities in Region A (52.8%), followed by Region B (48.3%) and Region C (44.7%). The isolation rates of ESBL-producing *P. mirabilis* in Regions D and E were 21.7% and 21.3%, respectively, which is approximately one-half that of Regions B and C.

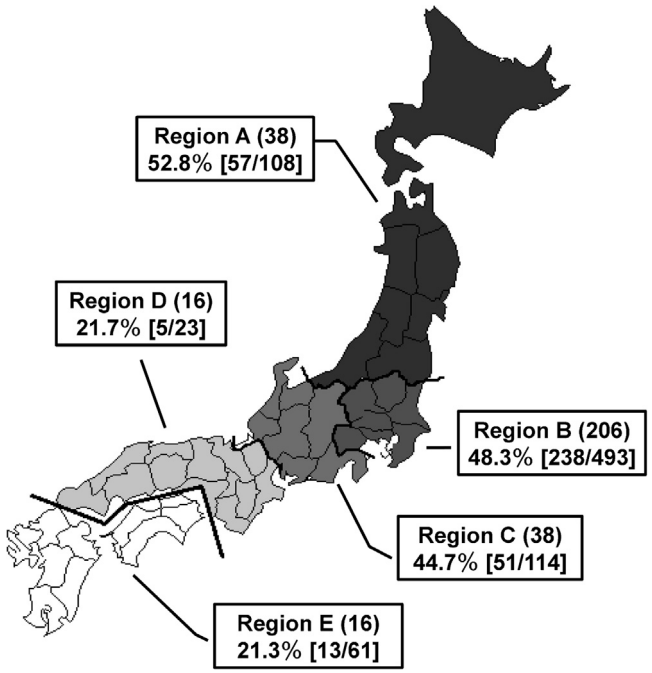

Fig. 1. Distribution of extended-spectrum β-lactamase (ESBL)-producing *Proteus mirabilis* isolates among regions of Japan. Region A, Hokkaido and Tohoku; Region B, Kanto; Region C, Chubu; Region D, Kinki; and Region E, Shikoku and Kyusyu. The number of healthcare facilities is shown in parentheses, and the number of ESBL-producers/number of *P. mirabilis* isolates is shown in square brackets.

3.5. Pulsed-field gel electrophoresis typing of extended-spectrum β-lactamase-producing isolates

Of the 364 ESBL-producing isolates, 71 (19.5%) were characterised by PFGE typing. To ensure that there were no potential duplicate isolates from the same healthcare facility, random numbers were generated using Microsoft Excel Random Generator (Microsoft Corp., Redmond, WA). Isolates from 141 healthcare facilities were assigned sequential numbers and randomised, resulting in analysis of 71 isolates from 71 facilities. All 71 of the isolates possessed the CTX-M-2 group β-lactamase gene.

Of the 71 ESBL-producing isolates analysed by PFGE, PFGE patterns were generated for 66 of the isolates (Fig. 2); 5 isolates with smear profiles were excluded. The 66 isolates generated chromosomal restriction fragment PFGE patterns leading to 21 groups, of which group III had the most isolates at 22. Of the 22 isolates in group III, 17 were from Region B. Moreover, two of three isolates from Region D and all three isolates from Region E that were analysed also exhibited PFGE patterns consistent with group III. Group XVII had the second highest number of isolates (9), comprising isolates mostly from Regions A and C.

With respect to the clonal diversity of PFGE groups by region, four groups (X, XIV, XVII and XVIII) were identified in isolates from Region A, whilst five groups (XI, XIII, XV, XVII and XX) were seen in isolates from Region C. Fourteen PFGE groups were found in Region B reflecting clonal diversity; however, it was observed that there were isolates belonging to groups III, VII, XVI and XIX that had identical PFGE patterns within Region B.

4. Discussion

Over the last few years, ESBL-positive *P. mirabilis* isolates have been recovered worldwide [6,7,10–12,22,23]. In Japan, the isolation rate of ESBL-producing *P. mirabilis* exceeds that of *E. coli* and *Klebsiella* spp., with reports of HCAIs as well spread within a geographical area, which has generated significant concern among healthcare professionals [10,16]. Chong et al. reported that 15.2% of *K. pneumoniae*, *E. coli* and *P. mirabilis* isolates from 2009 were ESBL-producers compared with 3.6% in 2003, which is consistent with the increasing prevalence of ESBL-producing bacteria in Japan in recent years [17]. In a large-scale study of 40,000 Enterobacteriaceae isolates recovered between 2000 and 2009 in a particular geographical region, the increased prevalence of ESBL-producing isolates was also confirmed [16].

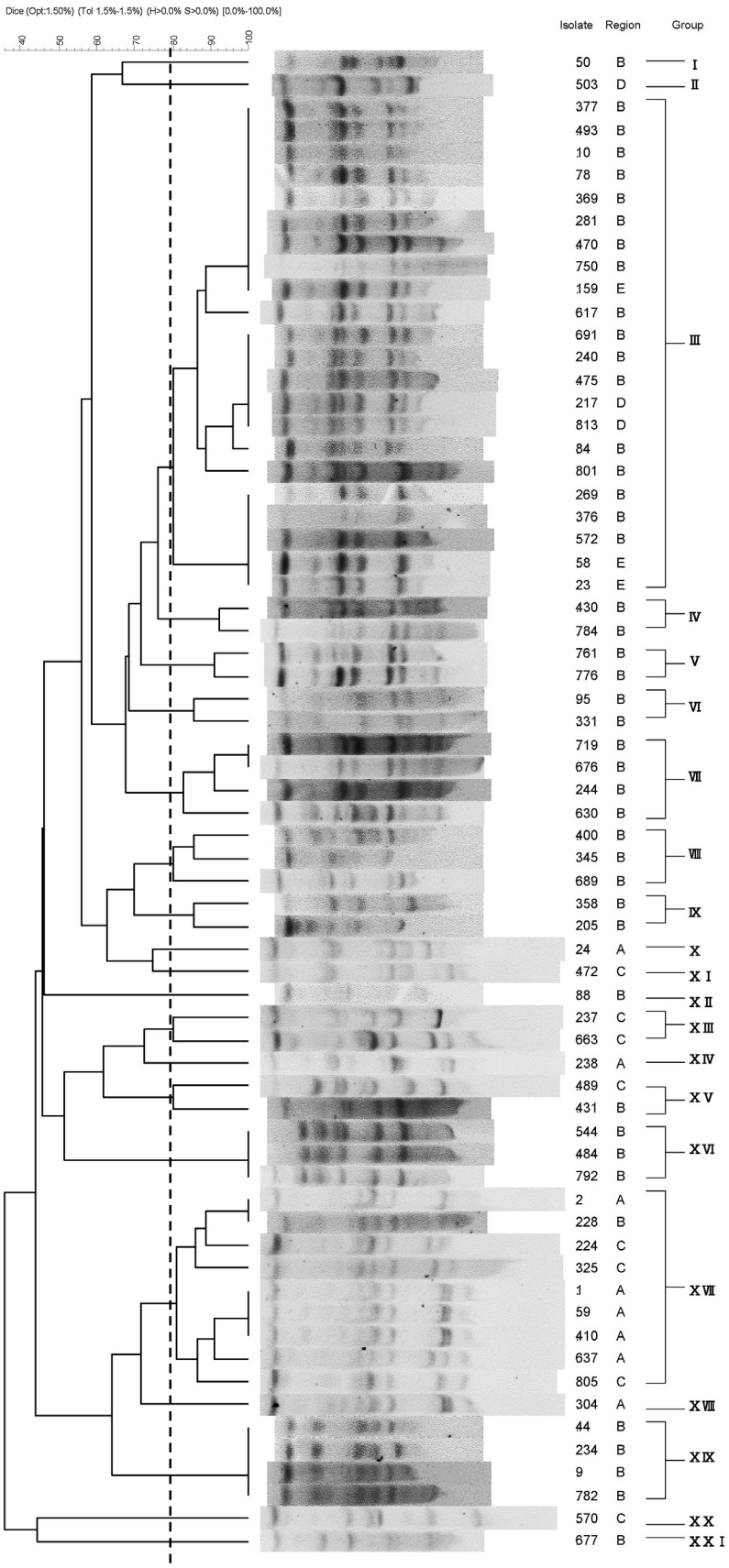

Fig. 2. Dendrogram based on pulsed-field gel electrophoresis (PFGE) typing of 66 extended-spectrum β-lactamase (ESBL)-producing *Proteus mirabilis* isolated from Japan. The vertical line indicates the ≥80% similarity score adopted to assign isolates to the same strain by the unweighted pair-group method with arithmetic mean (UPGMA) and cluster analysis according to the Dice setting using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

In this follow-up to our 2006 report [7], an expanded survey was conducted to better understand the prevalence of ESBL-producing *P. mirabilis* in Japan. The current findings show that 364 (45.6%) of the 799 *P. mirabilis* isolates from 314 geographically scattered facilities were ESBL-producers, with CTX-M-2 group ESBL predominating.

Patients from whom ESBL-producing Enterobacteriaceae are isolated are more likely to have risk factors such as underlying disease, longer length of hospitalisation, older age, use of invasive devices and antimicrobial therapy that can affect patient outcome [12,13,23,24]. The mean age of the patients from whom ESBL-producing *P. mirabilis* was isolated in this study was 76.6 years, with 69.7% of the isolates from hospitalised patients, which is consistent with the presence of risk factors associated with recovery of ESBL-producing Enterobacteriaceae. This study showed that isolation of ESBL-producing *P. mirabilis* was highest in patients with risks factors such as older age and extended hospitalisation and that CVC placement was an important risk factor as evidenced by the high rate of CVCs that yielded ESBL-producing *P. mirabilis*.

The findings of this study show that the percentage of ESBL-producing *P. mirabilis* has increased compared with our 2006 survey, with the highest rate of ESBL-producers found in northern Japan and the lowest rate in southern Japan [7]. The current findings are also consistent with a previous report confirming the diversity of ESBL-producing *P. mirabilis* in Japan [7]; however, genetic relatedness of isolates recovered within Regions A, C, D and E as well as a subset of isolates within Region B was also observed. This suggests the occurrence of horizontal transmission through healthcare workers or patients within a geographical area. The factors explaining the finding of isolates belonging to groups III and XVII in healthcare facilities separated by >500 km could not be determined.

This investigation found resistance to ciprofloxacin to be much higher in ESBL-producing isolates compared with ESBL-non-producing isolates. The high use of fluoroquinolones and cephalosporins in infectious disease treatment especially in inpatients has been reported as a risk factor for infection with ESBL-producing Enterobacteriaceae [13,24]. This raises the question of why there is such a strong correlation between resistance to two unrelated classes of antibiotics.

An explanation for the high isolation rate of ESBL-producing Enterobacteriaceae may be that exposure to fluoroquinolones provides the selective conditions necessary for selection of mutations in DNA gyrase and topoisomerase IV leading to elevated MICs [25,26].

Carbapenems are considered the drugs of choice in the treatment of infections caused by ESBL-producers [27]. In this study, one ESBL-producing *P. mirabilis* isolate exhibited a meropenem MIC of 2 mg/L, which was elevated compared with other isolates. As carbapenemase production has been reported in Enterobacteriaceae including *P. mirabilis* [8,9], the modified Hodge test [18] to screen for carbapenemase production was performed and was found to be positive in this isolate (data not shown).

Almost one-half of the *P. mirabilis* isolates from clinical specimens in Japan are ESBL-producers. As fluoroquinolone resistance is common among these isolates, antimicrobial therapy must take into account the potential of both ESBL production and fluoroquinolone resistance. Furthermore, as resistance prevalence differs by geographical region, a region-based antibiogram will provide insight into local resistance patterns. Finally, there is a need to monitor for changes to carbapenem resistance in *P. mirabilis*.

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