JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2005, p. 458–461 Vol. 43, No. 1
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.1.458–461.2005

Clonal Diversity of Metallo-β-Lactamase-Possessing *Pseudomonas aeruginosa* in Geographically Diverse Regions of Japan

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Received 24 March 2004/Returned for modification 30 April 2004/Accepted 1 June 2004

The aim of this study was to determine the distribution of metallo-β-lactamase-producing *Pseudomonas aeruginosa* in Japan and to investigate the molecular characteristics of resistance gene cassettes including the gene encoding this enzyme. A total of 594 nonduplicate strains of *P. aeruginosa* isolated from 60 hospitals throughout Japan in 2002 were evaluated. This study indicated that although the prevalence of imipenem-resistant *P. aeruginosa* has not increased compared to that found in previous studies, clonal distribution of the same strain across Japan is evident.

Class A, B, and D β-lactamases, as defined by Ambler et al., can hydrolyze carbapenems (1, 9). In particular, class B β-lactamases, termed metallo-β-lactamases, are an increasingly serious clinical problem because they have a very broad substrate profile that includes penicillins, expanded-spectrum cephalosporins, and carbapenems and excludes only monobactams, such as aztreonam. It has been reported that IMP-1 metallo-β-lactamase-producing *Serratia marcescens* was first isolated in Japan in 1991 (10). Recently, metallo-β-lactamase-producing *Pseudomonas aeruginosa* and *S. marcescens* probably have the highest incidence of isolation in Japan (7).

Most metallo-β-lactamase genes are located on integrons, which are genetic elements containing gene cassettes that can facilitate their spread and mobilize the genes to other integrons or to other sites. The gene cassettes often encode clinically important antibiotic resistance genes, including those encoding β-lactamases such as extended-spectrum β-lactamases and carbapenemases, and also aminoglycoside-modifying enzymes (12).

Little is known about the distribution of the clone(s) that produces metallo-β-lactamases in Japan. Therefore, we conducted a surveillance study covering a wide geographic area with the aim of determining the distribution of metallo-β-lactamase producers in Japan and to investigate the molecular characteristics of the resistance gene cassettes that included the gene encoding a metallo-β-lactamase.

A total of 594 nonduplicate strains of *P. aeruginosa* isolated from 60 hospitals throughout Japan in the year 2002 were evaluated. The susceptibility of *P. aeruginosa* to several antibiotics was measured with the Etest strip, and the strains were stored on Casitone medium (Eiken Chemical Co. Ltd., Tokyo, Japan) (data not shown). After 6 months, the antibiotic susceptibility of these isolates was reassessed by the National Committee for Clinical Laboratory Standards broth microdilution method with cation-adjusted Mueller-Hinton broth (Difco, Detroit, Mich.). The isolates were screened for the presence of metallo-β-lactamase by a double-disk synergy test reported by Arakawa et al. (2). Integron analysis was performed by PCR mapping (5′-conserved segment *int*I to 3′-conserved segment *qacEΔ1*) of the typical antibiotic resistance genes and integron with specific primer sets (Table 1). The specificity of the primer sets for bla_{IMP-1}-like and bla_{VIM-2}-like gene was confirmed with positive-control strains producing IMP-1 or VIM-2 metallo-β-lactamase. The specificity of amplicons obtained by specific primer sets (*aacA4*, *aadA1*, *aadA2*, and bla_{OXA-2}) was also partially verified with the automatic sequencer ABI Prism 310 genetic analyzer (Applied Biosystems/Perkin-Elmer Biosystems). PCR with Ex *Taq* polymerase (Takara Bio, Inc., Tokyo, Japan) were carried out by standard methodology (13). pulsed-field gel electrophoresis analysis was performed by a modified method of the standard protocol (6). The restriction enzyme used was SpeI (15). By use of the dendrogram, isolates with a genetic relatedness of >80% were

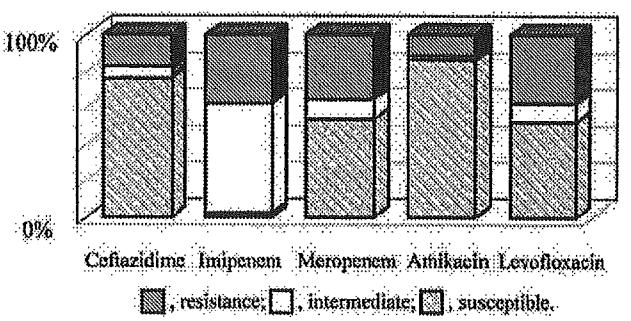

FIG. 1. Antimicrobial susceptibilities of imipenem-nonsusceptible *P. aeruginosa* isolates.

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TABLE 1. Nucleotide sequences of PCR primers used in this study

Gene[a]	Primer sequence (5′ to 3′)	T_m (°C)	Reference
intA(S)	ATC ATC GTC GTA GAG ACG TCG G	67.4	11
intB(AS)	GTC AAG GTT CTG GAC CAG TTG C	66.9	11
bla_{IMP-1}(S)	CTA CCG CAG CAG AGT CTT TG	62.7	This study
bla_{IMP-1}(AS)	AAC CAG TTT TGC CTT ACC AT	59.9	This study
bla_{VIM-2}(S)	AAA GTT ATG CCG CAC TCA CC	63.9	This study
bla_{VIM-2}(AS)	TGC AAC TTC ATG TTA TGC CG	64.5	This study
aacA4(S)	GAC CTT GCG ATG CTC TAT GAG TGG CTA AAT	73.0	This study
aacA4(AS)	TTC GCT CGA ATG CCT GGC GTG TT	76.9	This study
aadA1(S)	TGA TCG CCG AAG TAT CGA CTC	66.3	This study
aadA1(AS)	CCT TGG TGA TCT CGC CTT TC	65.8	This study
aadA2(S)	TTC GAA CCA ACT ATC AGA GGT GCT AA	67.4	This study
aadA2(AS)	AAA GCG AAT AAA TTC TTC CAA GTG ATC T	66.4	This study
bla_{OXA-2}(S)	CAA TCC GAA TCT TCG CGA TAC TT	66.9	This study
bla_{OXA-2}(AS)	AAG TAT CGC GAA GAT TCG GAT TG	66.9	This study
qacEΔ1	CTC TCT AGA TTT TAA TGC GGA TG	60.6	This study

[a] (S), sense; (AS), antisense.

considered to represent the same pulsed-field gel electrophoresis type (4).

Eighty-eight (15%) of 594 isolates were not susceptible (MIC ≥ 8 mg/ml) to imipenem. Among 88 isolates, 88 (100%), 21 (24%), 41 (47%), 12 (14%), and 42 (48%) were not susceptible to imipenem, ceftazidime, meropenem, amikacin, and levofloxacin, respectively (Fig. 1). Screening of metallo-β-lactamase producers was carried out for these isolates by the double-disk synergy test. Eleven (1.9%) of 594 isolates were found to produce metallo-β-lactamase. Ten of these isolates were IMP-1-like, and the other was a VIM-2-like metallo-β-lactamase producer.

The type of metallo-β-lactamase gene was also confirmed by PCR. The genetic relatedness of these isolates was also evaluated by pulsed-field gel electrophoresis as described above (Fig. 2, Table 2). Strains TUM1683, TUM1708, TUM1709, TUM1710, and TUM1732 had related electrophoresis chromosomal DNA banding patterns, whereas other strains (TUM1672, TUM1673, TUM1682, TUM1721, TUM1733, and TUM1757) showed different banding patterns. Strain TUM1708, TUM1709, and TUM1710 were isolated from same hospital, suggesting nosocomial spread. Interestingly, although strains TUM1683, TUM1708 (or TUM1709 and TUM1710), and TUM1732 has been isolated in different hospitals, Kawasaki, Saitama, and Nara, respectively, these isolates had related patterns. Since the distance from Okayama to Saitama and from Saitama to Nara is about 800 and 400 km, respectively, the results observed suggested clonal spread of metallo-β-lactamase-producing strains.

Several researchers have reported an incidence of metallo-β-lactamase-producing *P. aeruginosa* of between 0.4 and 1.3% in Japan from 1992 to 2002 (5, 7, 14, 16). In this study, we isolated 1.9% metallo-β-lactamase-producing *P. aeruginosa* strains from geographically diverse regions in Japan. We suggest that the incidence of metallo-β-lactamase-possessing *P. aeruginosa* has not increased during the past decade. However, the same clone of metallo-β-lactamase-carrying *P. aeruginosa* has now spread throughout Japan.

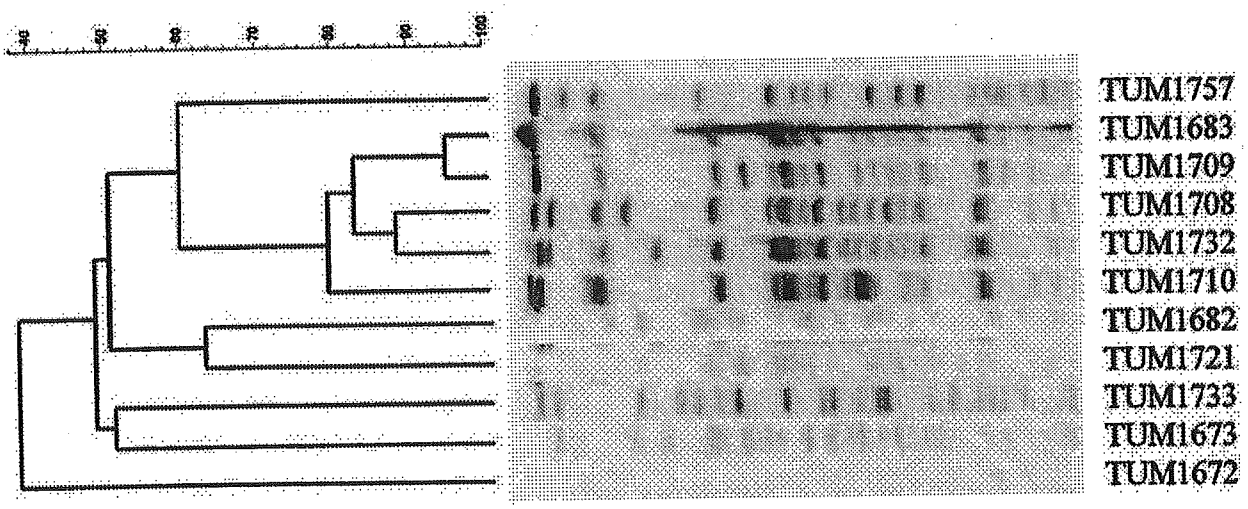

FIG. 2. Pulsed-field gel electrophoresis profiles obtained with SpeI chromosomal digestion of metallo-β-lactamase-carrying *P. aeruginosa*. The second through sixth lanes contained related strains TUM1683, TUM1709, TUM1708, TUM1732, and TUM1710, respectively. Lanes first and seventh to eleventh lanes contained unrelated strains TUM1757, TUM1682, TUM1721, TUM1733, TUM1673, and TUM1672, respectively.

TABLE 2. Characteristics of bla_{IMP}-containing non-imipenem-susceptible *P. aeruginosa* isolates

Strain	Hospital no.	Material	Type of enzyme	Pattern[b]	Integron structure[c]	MIC (μg/ml)[a]									
						CAZ	IPM	MEM	LVX	AZT	AMK	NET	GEN	KAN	ABK
TUM1672	1	Urine	VIM-2-like	A	I	64	>128	>128	16	32	0.06	0.5	0.5	8	0.06
TUM1673	1	Sputum	IMP-1-like	B	II	>128	8	32	16	8	64	>128	4	>128	16
TUM1682	2	Sputum	IMP-1-like	C	III	>128	64	>128	32	32	32	>128	2	>128	2
TUM1683	2	Sputum	IMP-1-like	D	IV	>128	64	>128	32	64	16	>128	2	>128	2
TUM1708	3	Urine	IMP-1-like	D	IV	>128	64	>128	32	32	32	>128	4	>128	4
TUM1709	3	Urine	IMP-1-like	D	IV	>128	64	>128	32	32	32	>128	4	>128	2
TUM1710	3	Urine	IMP-1-like	D	IV	>128	64	>128	32	64	32	>128	2	>128	4
TUM1721	4	Urine	IMP-1-like	E	V	>128	64	>128	32	32	32	>128	>128	>128	64
TUM1732	5	Urine	IMP-1-like	D	IV	>128	64	>128	32	128	32	>128	4	>128	2
TUM1733	5	Pus	IMP-1-like	F	VI	>128	64	>128	64	32	2	>128	>128	>128	1
TUM1757	6	Sputum	IMP-1-like	G	VII	>128	64	>128	16	16	32	>128	1	>128	16

[a] CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; LVX, levofloxacin; AZT, aztreonam; AMK, amikacin; NET, netilmicin; GEN, gentamicin; KAN, kanamycin; ABK, arbekacin.

[b] PEGE profiles obtained with SpeI chromosomal digestion of *P. aeruginosa* carrying a metallo-β-lactamase gene as recommended by Tenover et al. (15).

[c] Integron structures possessed by each gene as mentioned in the text. I, bla_{VIM-2}-like, *aacA4* and *aadA2*; II, bla_{IMP-1}-like, *aadA1* and *orfG*; III, bla_{IMP-1}-like, *aadA1* and unknown gene; IV, bla_{IMP-1}-like, *aadA1* and unknown gene; V, bla_{IMP-1}-like, *aacA4*, *aadA1* and bla_{OXA-2}; VI, bla_{IMP-1}-like, *aacA4*; VII, only bla_{IMP-1}-like gene.

It has been reported that genetic analysis of bla_{IMP-1} revealed features typical of an integron-located gene (9). The detection of a type 1 integron was confirmed in 11 strains. In these strains, bla_{IMP-1}-like or bla_{VIM-2}-like genes were located immediately downstream of the *IntI*1 integrase gene. However, these isolates possessed a variety of gene cassettes, such as the *aacA4* aminoglycoside 6′-*N*-acetyltransferase gene and *aadA1* and *aadA2* aminoglycoside adenyltransferase genes between the metallo-β-lactamase gene and *qacΔE1*. Therefore, these isolates are likely resistant not only to β-lactams but also to aminoglycosides. Interestingly, strain TUM1721 possessed not only the bla_{IMP-1}-like genes *aacA4* and *aadA1* but also an OXA-type β-lactamase gene on the integron gene cassette.

Little is known about optimal chemotherapy for infection due to metallo-β-lactamase-producing *P. aeruginosa*. To detail the antibiotic susceptibility of *P. aeruginosa* possessing a metallo-β-lactamase, the MICs of several antibiotics were evaluated (Table 2). All of the isolates were resistant to ceftazidime, meropenem, and levofloxacin. Ten of the 11 were resistant to imipenem and netilmicin, nine were resistant to aztreonam, and eight were not susceptible to amikacin. Bellais et al. reported that chemotherapy with high aztreonam doses effectively reduced viable cells of a metallo-β-lactamase-producing strain of *P. aeruginosa* in a rat pneumonia model (3). In general, although metallo-β-lactamases do not hydrolyze aztreonam, 9 of 11 isolates were resistant to aztreonam in this study (MIC ≥ 32 μg/ml). On the other hand, arbekacin was found to suppress the growth of some isolates in this study. In Japan, arbekacin, which has fewer side effects than vancomycin, has been used against methicillin-resistant *Staphylococcus aureus* (8). Recently, arbekacin-resistant *P. aeruginosa* possessing the 16S rRNA methylase gene *rmtA* was isolated in Japan (17). However, the incidence of these isolates is still low (0.8%, 9 of 1,113 clinical isolates). Therefore, arbekacin could be used as treatment against metallo-β-lactamase-possessing *P. aeruginosa*.

In conclusion, this study indicates that although the prevalence of metallo-β-lactamase-producing *P. aeruginosa* has not increased, this pathogen has spread from a single source to a wide geographic area of Japan. Further surveillance and monitoring of multidrug-resistant *P. aeruginosa* should be a high priority.

This study was supported by grants from the Ministry of Health, Labor and Welfare of Japan during 2003 (H15-Iyaku-003 and H15-shinkou-009). S.K. was supported by a grant from the Society of Japanese Pharmacopoeia.

We thank Kenneth S. Thomson, Creighton University School of Medicine, for useful advice. We also thank Kunimoto Hotta, National Institute of Infectious Diseases, for helpful discussions.

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JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2005, p. 1072–1075 Vol. 43, No. 3
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.3.1072–1075.2005

Extended-Spectrum β-Lactamase-Producing Shiga Toxin Gene (stx_1)-Positive *Escherichia coli* O26:H11: a New Concern

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Received 4 August 2004/Returned for modification 24 September 2004/Accepted 3 October 2004

***Escherichia coli* strain TUM2139 was isolated from a stool sample from a 9-year-old girl on 16 June 2004. This strain was categorized as Shiga toxin-producing *Escherichia coli* (STEC) because the Shiga-like toxin gene stx_1 was detected by immunochromatography and PCR assay. The strain was highly resistant to cefotaxime (256 μg/ml) and was also resistant to cefepime, cefpodoxime, ceftriaxone, and aztreonam. In the presence of 4 μg of clavulanic acid per ml, the MIC of cefotaxime decreased to ≤0.12 μg/ml, indicating that this strain was an extended-spectrum β-lactamase (ESBL) producer. Cefotaxime resistance was transferred to *E. coli* C600 by conjugation at a frequency of 3.0×10^{-6}. A PCR assay was performed with primer sets specific for TEM-type and SHV-type ESBLs and for the CTX-M-2 (Toho-1), CTX-M-3, and CTX-M-9 groups of ESBLs. A specific signal was observed with the primer set specific for the CTX-M-9 group of β-lactamases. This β-lactamase was confirmed to be the ESBL CTX-M-18 by DNA sequencing. This is the first report of an ESBL-producing STEC isolate.**

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is an important cause of waterborne and food-borne illnesses. STEC is ingested most commonly with undercooked ground beef (22). Human infection with STEC is potentially fatal and may be associated with serious complications such as hemolytic-uremic syndrome (HUS) and hemorrhagic colitis (21). The production of Stx is the unifying feature of all STEC strains. Various types of Stxs are produced, but they fall into two main types: Stx1 and Stx2 (4, 5, 6, 8). The clinical significance of four serological or biological variants of Stx2 (Stx2c, Stx2d, Stx2e, and Stx2f) is unknown (17). The majority of Stx genes are encoded by bacteriophages. The most severe or important pathogen among STEC strains is *E. coli* O157:H7. The cases of HUS in approximately 90% of children in the developed part of the world are associated with infections caused by Stx-producing bacteria; among these, 70% are caused by *E. coli* O157:H7 (21). Epidemiologic data suggest that isolates that produce Stx2 alone are more likely to cause severe disease than those that produce only Stx1 or a combination of Stx1 and Stx2 (20).

Recently, Schroeder et al. (23) reported on the antimicrobial resistance of 752 STEC strains from animals and humans. They found that 50% of *E. coli* O26, O103, O111, O128, and O145 strains from humans were resistant to ampicillin, cephalothin, tetracycline, streptomycin, or sulfamethoxazole but that no strain was resistant to expanded-spectrum β-lactams, including cefotaxime, cefpodoxime, or aztreonam. A recent study suggested that antibiotic therapy for the early stage of STEC infection is able to prevent progression of the disease to HUS (24). However, antimicrobial therapy for STEC infection is still regarded as controversial, because antibiotics induce increased levels of Stx production in vivo (18). On the other hand, many patients with diarrhea receive empirical antibiotic therapy (21).

E. coli TUM2139 was isolated from a clinical stool specimen at Toho University Ohashi Hospital. This strain was resistant to cefotaxime. On the other hand, the O-antigen type of this strain was determined to be O26. In this study, the resistance of *E. coli* TUM2139 to β-lactam antibiotics and the presence of virulence factors in this strain were confirmed by PCR assay and the direct sequencing technique.

MATERIALS AND METHODS

Bacterial strain, media, and culture. On 16 June 2004, *E. coli* TUM2139 was isolated from a stool sample from a 9-year-old female patient at Toho University Ohashi Hospital. She had diarrhea, vomiting, and a low-grade fever. The strain was identified with the Phoenix system (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan). *E. coli* C600 (F⁻ *lacY1 leuB6 supE44 thi-1 thr-1 tonA21* nalidixic acid resistant) was used as the recipient strain for the conjugation experiments. The bacterial strains were stored at −70°C in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, Mich.) containing 30% glycerol. Subsequently, the bacterial strains were inoculated onto Mueller-Hinton agar plates and incubated overnight at 35°C.

Serotyping. *E. coli* TUM2139 was screened for Vero toxin (Stx) with a Capillia O157 immunochromatography assay kit for Vero toxin (Nippon Becton Dickinson Company, Ltd.). The O- and H-antigen types of the strain were determined with neutralizing antisera. A total of 181 types of antisera against the O antigen (O1 to O181) and also 56 different kinds of antisera against the H antigen were used. These antisera were made by the National Institute of Infectious Diseases, Tokyo, Japan, which is the typing center for *E. coli* in Japan.

PCR assay for stx_1, stx_2, *eaeA*, and *hlyA*. The use of multiplex PCR for the detection of stx_1, stx_2, *eaeA*, and enterohemorrhagic *E. coli hlyA* was reported by Fagan et al. (18). DNA amplification was performed by a PCR method directly with the colonies (26). A small amount of the test organism was picked up with a toothpick and transferred directly to 50 μl of the PCR mixture. The primers and the predicted sizes of the PCR amplicons are listed in Table 1. PCR assays were performed with EX*taq* DNA polymerase (Takara Bio Inc., Shiga, Japan) and a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, Conn.). The PCR

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TABLE 1. Primer sequences and predicted lengths of PCR amplification products

Primer specificity	Direction	Primer sequence (5′ to 3′)	Fragment size (bp)	Reference or source
Virulence factors[a]				
stx_1	Sense	ACGATGTGGTTTATTCTGGA	165	8
	Antisense	CTTCACGTGACCATACATAT		
stx_2	Sense	ACACTGGATGATCTCAGTGG	614	10
	Antisense	CTGAATCCCCCTCCATTATG		
EHEC *hlyA*	Sense	CCATGACAACGGACAGCAGTT	779	10
	Antisense	CCTGTCAACTGAGCAGCACTTTG		
eaeA	Sense	GTGGCGAATACTGGCGAGACT	890	9
	Antisense	CCCCATCTTTTTCACCGTCG		
β-Lactamases[a]				
CTX-M-2	Sense	GCGAACAGCGTGCAACAGCAGCTGG	521	This study
	Antisense	GCCAGCGCTTTACCCAGCGTCAG		
CTX-M-3	Sense	GAGCATATGGTTAAAAAATCACTGCGTCAGTTC	891	This study
	Antisense	CAGGGATCCTTACAAACCGTCGGTGACGATTTTAGCC		
CTX-M-9	Sense	GTTTGAGCATATGGTGACAAAGAGAGGCAACGG	895	11
	Antisense	CAGGGATCCTTACAGCCCTTCGGCGATG		
TEM	Sense	GGGGAGCTCATAAAATTCTTGAAGAC	1,199	23
	Antisense	GGGGGATCCTTACCAATGCTTAATCA		
SHV	Sense	GTTCATATGCGTTATATTCGCCTGTG	876	This study
	Antisense	ATAGGATCCTTAGCGTTGCCAGTGCT		
CTX-M-18[b]				11
	Sense	AGAGAGTGCAACGGATGATGTT		
	Sense	GTTGCAGTACAGCGACAATACC		
	Sense	GCTGGTTCTGGTGACCTATTTTAC		
	Antisense	GCCATAACTTTACTGGTACTGCAC		
	Antisense	CTGGGTAAAATAGGTCACCAGAAC		

[a] Primers for PCR.
[b] Primers for sequencing. DNA template amplified by primers for CTX-M-9 was used for sequencing.

conditions were as follows: incubation 95°C for 3 min, followed by 35 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 90 s, with a final cycle of 72°C for 7 min. The amplified DNA fragments of the target genes were identified by electrophoresis on a 2% agarose gel. After electrophoresis, the gels were stained with 0.5 mg of ethidium bromide per ml and visualized with UV illumination.

Antimicrobial susceptibility testing. MICs were determined by a broth microdilution method with cation-adjusted Mueller-Hinton broth. The dilution ranges of the antibiotic agents were 0.25 to 512 μg/ml. Quality control was done by using *E. coli* ATCC 25922 as a reference strain for antibiotic susceptibility. All procedures were done and the results were interpreted as described by the National Committee for Clinical Laboratory Standards (15, 16). The organisms were inoculated at about 5×10^5 cells per well by using a MIC2000 inoculation device (Dynatech, McLean, Va.). The MIC was defined as the lowest concentration that prevented visible growth after incubation for 18 h at 35°C.

Antibiotics. The following agents, all with known potencies, were used in this study: piperacillin (Toyama Chemical Co., Ltd., Tokyo, Japan); cephalothin (Sigma-Aldrich Japan Co., Ltd., Tokyo, Japan); cefoxitin, imipenem, and ciprofloxacin (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan); ceftazidime and clavulanic acid (Glaxo Smith Kline Ltd., Tokyo, Japan); cefotaxime (Aventis Japan Ltd., Tokyo, Japan); aztreonam (Eisai Co., Ltd., Tokyo, Japan); tazobactam (Taiho Pharmaceutical Co., Ltd, Tokyo, Japan); cefepime (Bristol Pharmaceutical Co., Ltd., Tokyo, Japan); cefotetan (Yamanouchi Pharmaceuticals Co. Ltd., Japan); cefmetazole and cefpodoxime (Sankyo Co., Ltd., Tokyo, Japan); ceftriaxone (Roche Japan K.K., Tokyo, Japan); faropenem (Suntory Ltd., Tokyo, Japan); gentamicin (Nihon Shering K.K. Osaka, Japan); kanamycin (Meiji Seika Ltd., Tokyo, Japan); minocycline (Wyeth K.K. Tokyo, Japan); and nalidixic acid (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan).

Conjugation experiment. Conjugation experiments were performed by the broth method (11). A nalidixic acid-resistant marker was added to recipient cells (*E. coli* C600). Then, the recipient cells and the donor cells (*E. coli* TUM2139) were mixed in a ratio of 1:9. The same volume of fresh LB broth was added to the cell mixture and the mixture was incubated for 90 min at 35°C. After 90 min, the cells were plated onto LB agar plates containing 5 μg of cefotaxime per ml and 25 μg of nalidixic acid per ml and incubated at 35°C overnight. Donor cells were also plated on LB agar in the absence of antibiotics. After incubation, the colonies were counted and the frequency of conjugation of a plasmid was calculated.

PCR assay for ESBLs and ESBL DNA sequence analysis. The total DNA template was obtained and the amplification products were identified by the same procedure used for the PCR assay of the bla_{TEM} type, bla_{SHV} type, bla_{Toho-1} group, $bla_{CTX-M-3}$ group, and $bla_{CTX-M-9}$ group of extended-spectrum β-lactamases (ESBLs). The primers used for PCR, the predicted sizes of the PCR amplicons, and the primers used for sequencing are listed in Table 1. PCR conditions were as follows: incubation at 94°C for 3 min and 30 cycles of amplification (denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min), with a final extension at 72°C for 7 min.

Both strands of the PCR products obtained were sequenced by using the same set of primers used to obtain the PCR amplification products and then by using primers synthesized by primer walking. All primers used for PCR and DNA sequencing are listed in Table 1. DNA sequencing was carried out with a BigDye (version 3.1) Terminator Cycle Sequencing kit and a model 310 DNA sequencer (Applied Bio-

TABLE 2. Antimicrobial susceptibilities of donor, recipient, and conjugant strains

Antibiotic	MIC (μg/ml)		
	E. coli TUM2319	Recipient	Conjugant
Piperacillin	128	≤0.25	64
Piperacillin-tazobactam	2/4	≤0.25/4	1/4
Cephalothin	512	0.5	512
Cefoxitin	4	≤0.25	2
Cefmetazole	1	≤0.25	1
Cefotaxime	256	≤0.25	32
Cefotaxime-clavulanic acid	≤0.25/4	≤0.25/4	≤0.25/4
Ceftazidime	2	≤0.25	2
Ceftazidime-clavulanic acid	≤0.25/4	≤0.25/4	≤0.25/4
Cefpodoxime	256	≤0.25	128
Ceftriaxone	256	≤0.25	128
Cefotetan	≤0.25	≤0.25	≤0.25
Cefepime	8	≤0.25	4
Aztreonam	8	≤0.25	8
Faropenem	≤0.25	≤0.25	≤0.25
Imipenem	≤0.25	≤0.25	≤0.25
Gentamicin	≤0.25	≤0.25	≤0.25
Kanamycin	2	≤0.25	≤0.25
Minocyclin	≤0.25	≤0.25	≤0.25
Nalidixic acid	4	64	64
Ciprofloxacin	≤0.25	≤0.25	≤0.25

systems, Foster City, Calif.). The deduced amino acid sequences were examined by using the BLAST program at the DNA Data Bank of Japan (Shizuoka, Japan).

RESULTS

Characterization of *E. coli* TUM2319. *E. coli* TUM2319 was confirmed to be a O26:H11 strain with antisera. The Capillia O157 immunochromatography assay kit was used to determine whether the Vero toxin (Stx) produced by this strain is Vero toxin 1 (VT1; Stx1) and/or VT2 (Stx2). After this screening, the strain was investigated for the presence of the stx_1 and stx_2 genes by the PCR assay directly with the bacterial colonies. Only the stx_1 gene was amplified. The stx_2 gene was not detected in this strain. The *eaeA* and enterohemorrhagic *E. coli* (EHEC) *hlyA* genes were also detected by PCR. From these results, *E. coli* TUM2319 was confirmed to be an STEC strain.

Antimicrobial susceptibility testing of *E. coli* TUM2319. The antimicrobial susceptibility testing results for *E. coli* TUM2319 are presented in Table 2. *E. coli* TUM2319 was resistant to piperacillin, cephalothin, cefotaxime, ceftriaxone, and cefpodoxime but was susceptible to all other agents tested. The MIC of cefotaxime was reduced from 256 to ≤ 0.25 μg/ml in the presence of 4 μg of clavulanic acid per ml.

Conjugation experiments. Conjugation experiments showed that conjugants grew on LB agar plates in the presence of 25 μg of nalidixic acid per ml and 5 μg of cefotaxime per ml. The frequency of conjugation was 3.0×10^{-6}. The MICs of piperacillin, cephalothin, cefotaxime, cefpodoxime, and ceftriaxone for the conjugants were significantly increased compared with those for *E. coli* C600, the recipient strain (Table 2). These results show that this resistance marker could move from a resistant strain to a sensitive strain by conjugation. *E. coli* TUM2319 and its conjugants harbored a plasmid of the same size, approximately 80 kbp (data not shown).

Type of ESBL. A class A β-lactamase gene of the CTX-M-9 group was detected by PCR in *E. coli* TUM2319; but genes for ESBLs of the TEM type, SHV type, Toho-1 (CTX-M-2) group, and CTX-M-3 group were not detected. DNA sequencing confirmed that the enzyme of the CTX-M-9 group was the CTX-M-18 ESBL. This β-lactamase gene was also detected on an 80-kbp plasmid in the conjugant. Moreover, the EHEC *hlyA* gene was detected in the conjugants.

DISCUSSION

E. coli strain TUM2319 was confirmed to be an O26:H11 strain and an stx_1-, *eaeA*-, and EHEC *hlyA*-positive but stx_2-negative STEC strain by serological tests and PCR assays. *E. coli* O157:H7 infection in humans sometimes leads to HUS; however, almost all *E. coli* O26:H11 infections are mild (5).

Antibiotic treatment for STEC infections is not recommended (18). However, chemotherapy might be initiated before the diagnosis of an STEC infection. It is most widely accepted, however, that empirical therapy with antibiotics be started for children with acute diarrhea. β-Lactam antibiotics, especially expanded-spectrum β-lactams, such as cefotaxime, ceftazidime, ceftriaxone, and cefoperazone, are the most useful clinically because they combine safety with high potency against gram-negative bacteria, such as members of the family *Enterobacteriaceae*, including *E. coli*. Accordingly, expanded-spectrum β-lactams are one of the groups of antibiotics recommended for the treatment of serious *E. coli* infections (21).

Expanded-spectrum β-lactams are very stable to class A, class C, and class D β-lactamases of the Ambler classification (2); however, ESBLs can easily hydrolyze this group of β-lactam antibiotics, such as cefpodoxime, ceftriaxone, cefotaxime, and ceftazidime (7). In Japan, CTX-M-type β-lactamase-producing *E. coli* strains, such as cefotaxime-resistant *E. coli*, are often isolated from clinical specimens, with the CTX-M-2 or CTX-M-18 β-lactamases becoming the most commonly encountered ESBLs (11, 14, 25, 28, 29). The CTX-M-18 enzyme, which was initially reported to be Toho-3 (GenBank accession number AB038771) when the sequence was electronically published in the DNA database on 23 February 2000, does not hydrolyze ceftazidime. CTX-M-19 is a derivative of CTX-M-18 that can hydrolyze ceftazidime (19). If a single amino acid residue change occurs in the CTX-M-18 enzyme, this mutant (CTX-M-19) can acquire the capability to hydrolyze ceftazidime (12, 19). Accordingly, we believe that CTX-M-18 poses a higher risk when it is encountered in clinical isolates due to this potential ability to mutate into a ceftazidime-hydrolyzing enzyme.

ESBL-producing *Salmonella* and *Shigella* strains have been reported worldwide. Some 0.8 to 3.4% of clinical isolates of *Salmonella* recovered from 1997 to 1999 expressed the ESBL phenotype (29). Recently, investigators have also reported on CTX-M-3 (3), TEM-3 (1), and SHV-2a (13) ESBL-producing *Salmonella* isolates. However, to our knowledge, no ESBL-producing STEC or EHEC isolate has been described, until now.

In conclusion, this is the first report of an ESBL-producing STEC isolate. The possibility that this isolate may be an unsuspected reservoir of CTX-M-18 capable of spreading the plasmid carrying this ESBL to other bacteria might be worth

considering. It is necessary to extend antimicrobial resistance surveillance programs for *E. coli*, including STEC, not only to the clinical field but also to the agricultural field.

ACKNOWLEDGMENTS

This work was supported in part by two grants from the Ministry of Health, Labor and Welfare of Japan (Molecular Analyses of Drug-Resistant Bacteria and Establishment of Rapid Identification Methods [grant H15-Shinkou-9]) and by Toho University Project Research grant 15–4.

We are grateful to Kenneth S. Thomson, Creighton University School of Medicine, for useful advice and discussion.

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International Journal of Antimicrobial Agents 25 (2005) 296–301

INTERNATIONAL JOURNAL OF Antimicrobial Agents

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Evaluation of antimicrobial activity of β-lactam antibiotics using Etest against clinical isolates from 60 medical centres in Japan

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Received 14 September 2004; accepted 17 December 2004

Abstract

An antimicrobial resistance surveillance study was carried out in 60 medical centres across Japan. Resistance to piperacillin was 10.8% in clinical isolates of *Escherichia coli*, while 1.3% or fewer isolates were resistant to other β-lactams. *Klebsiella* spp. were more susceptible to imipenem, cefepime and cefpirome. Isolates of *Enterobacter* spp., *Citrobacter* spp., indole-positive *Proteus* and *Serratia* spp. were susceptible to imipenem, cefepime and cefpirome, while *Acinetobacter* spp. were most susceptible to cefoperazone/sulbactam, imipenem, ceftazidime (5.8% resistance) and cefepime (7.6%). Isolates of *Pseudomonas aeruginosa* were more susceptible to ceftazidime (12.3% resistance), cefoperazone/sulbactam (12.5%) and cefepime (12.6%) than to piperacillin (15.0%), cefpirome (22.6%) and imipenem (30.8%). The percentage of Japanese imipenem resistant *P. aeruginosa* clinical isolates was around 30%.

Keywords: Cefepime; β-Lactams; Etest; Susceptibility; Drug resistance

1. Introduction

β-Lactam antibiotics are widely used in the clinical field, because the incidence of cytotoxity with these antibiotics is very low compared with other antibiotics, such as aminoglycosides, quinolones or chloramphenicols. β-Lactamases are the major resistance mechanism to β-lactam antibiotics in Gram-negative and Gram-positive bacteria [1]. Other known resistance mechanisms include decreased outer membrane permeability [2–4], over expression of efflux-pumps [3] and the change of cell wall biosynthesis enzymes as the target of β-lactam antibiotics [5–8].

The presence of class B β-lactamase producers, such as *Serratia marcescens*, *Pseudomonas aeruginosa*, *Citrobacter freundii* or *Acinetobacter* spp., has been reported in Japanese hospitals [9]. However, ESBL producers are generally not as common in Japanese hospitals as in those in the United States or Europe [10].

Previous antimicrobial surveillance data suggests that class B β-lactamases are produced by *P. aeruginosa*, *S. marcescens* and *Acinetobacter* spp. strains [11–13]. This surveillance programme was therefore designed to provide more extensive β-lactam antibiotic susceptibility data by including a wide range of Gram-negative organisms and staphylococci.

2. Materials and methods

2.1. Bacterial isolates

The collection and subsequent testing of clinical isolates by the 60 participating centres (Fig. 1) began in July and concluded in September of 2002. Each laboratory was instructed to collect 10 consecutive non-duplicate isolates from each of 10 designated species groups as stated in a prevalence format. These 10 organism groups were: *Escherichia coli*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., indole-positive *Proteus* spp., *Serratia* spp., *Acinetobacter* spp., *P. aeruginosa*, oxacillin-susceptible *Staphylococ-*

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doi:10.1016/j.ijantimicag.2004.12.003

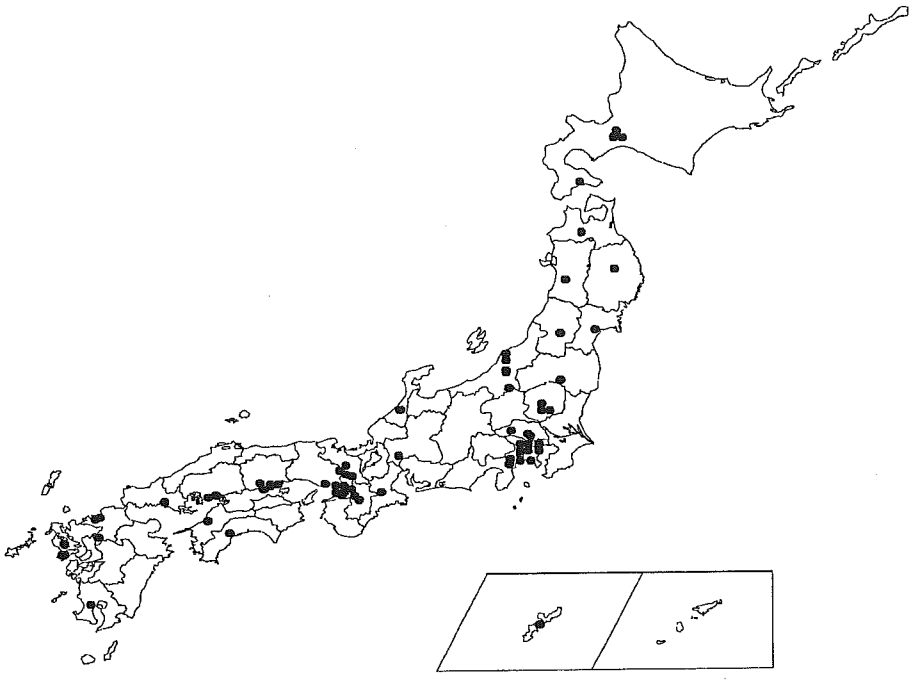

Fig. 1. Geographical location of 60 participating hospitals in this study.

cus aureus (MIC, ≤2 mg/L) and oxacillin-susceptible coagulase-negative staphylococci (MIC, ≤0.25 mg/L). Identification of isolates was determined utilizing routine methods used in each individual clinical laboratory. The combined overall collection of bacterial strains from the 60 centres totalled 5523 strains including 594 *E. coli*, 596 *Klebsiella* spp., 575 *Enterobacter* spp., 492 *Citrobacter* spp., 508 indole-positive *Proteus* spp., 549 *Serratia* spp., 536 *Acinetobacter* spp., 584 *P. aeruginosa*, 594 oxacillin susceptible *S. aureus* and 485 oxacillin-susceptible coagulase-negative staphylococci. Although compliance was complete, one strain of *S. aureus* was omitted from analysis because of documented oxacillin-resistant *S. aureus* that was redefined by the National Committee for Clinical Laboratory Standards (NCCLS) during the protocol period [14].

2.2. Antimicrobial susceptibility testing

Susceptibility of each isolate was determined using Etest (AB Biodisk, Solna, Sweden) according to the manufacture's instruction manual and the National Committee for Clinical Laboratory Standard [14,15]. Bacteria were cultured on 90-mm diameter Mueller Hinton agar plates (Difco Laboratories, Michigan, USA) for 16 h at 35 °C. Isolated colonies were resuspended in sterile saline to obtain a turbidity of 0.5 MacFarland. Each cell suspension was spread on a 135-mm diameter Mueller Hinton agar plate (Becton Dickinson, Maryland, USA) with a cotton swab. Etest strips were placed on the plates according to the manufacturer's instructions. The following strips were used: oxacillin (for Gram-positive bacteria), piperacillin (for Gram-negative bacteria), ceftazidime, cefepime, cefpirome, cefoperazone/sulbactam and imipenem. Results were recorded after 16–20-h incubation at 35 °C except for *S. aureus* and coagulase-negative staphylococci. MIC value for oxacillin of *S. aureus* and coagulase-negative staphylococci strains were determined by incubation for 24 h. MIC values were interpreted as the point of intersection of the inhibition ellipse with the Etest strips edge. All clinical laboratories used Etest strips, Mueller Hinton agar plates and reference strains from the same lot number. NCCLS does not have criteria (susceptible, intermediate or resistant) for cefpirome and cefoperazone/sulbactam. In this study, as the expedient breakpoints, the same values for cefepime were used as for cefpirome and the value for cefoperazone alone was used for cefoperazone/sulbactam. All 60 hospitals provided not only their results but also the resistant strains to the Department of Microbiology, Toho University School of Medicine. Identification by Vitek II system (bioMerieux, Marcy l'Etoile, France) was used for problem isolates and MIC values were reconfirmed using Etest at the Department of Microbiology, Toho University School of Medicine.

2.3. Quality control

For quality control of the Etest strips, the following reference strains were used: *E. coli* ATCC25922, *S. aureus* ATCC29213 and *P. aeruginosa* ATCC27853 [14]. NCCLS does not give a value for cefpirome and cefoperazone/sulbactam minimal inhibitory concentration. Medians of the MIC values of previous study [11–13] were used as

MIC values for reference strains. Laboratories tested all organisms in duplicate.

3. Results

3.1. Quality assurance

Validity of the data generated was checked by using appropriate quality control (QC) and quality assurance measures. Participating laboratories were required to test a set of three reference strains for QC composed of the following organisms: *S. aureus* ATCC29213, *E. coli* ATCC25922 and *P. aeruginosa* ATCC27853. Values obtained for the challenge set of strains resulted in 186 of the 4851 values failing to make the appropriate susceptibility category (3.8%). Of these 3.8%, 1.7% ($n = 82$ strains) were very major (false-susceptible) errors, 0.4% ($n = 20$ strains) were major (false-resistant) errors (Table 1). Overall, this meant 96.2% of MIC categorical results were acceptable (Tables 2 and 3).

3.2. Activity against staphylococci

As the NCCLS recommends that oxacillin susceptible staphylococci be considered as resistant to all β-lactam antibiotics, only oxacillin-susceptible strains were collected in this study. Of all isolates tested, 594 *S. aureus* and 485 oxacillin-susceptible coagulase-negative staphylococci strains were susceptible to cefepime, cefpirome, cefoperazone/sulbactam and imipenem. However, 14 *S. aureus* (2.4%) and 19 oxacillin-susceptible coagulase-negative staphylococci (3.9%) were resistant to ceftazidime. Rank order of activity for all tested agents using MIC_{90} values was imipenem > oxacillin > cefpirome > cefoperazone/sulbactam > cefepime > ceftazidime.

3.3. Activity against E. coli and K. pneumoniae

A total of 594 *E. coli* and 596 *K. pneumoniae* isolates were tested. Generally, all agents tested except piperacillin (10.8% resistant) were highly active against *E. coli* and *K. pneumoniae*. No-imipenem resistant strains of *E. coli* and *K. pneumoniae* were observed in this study.

Table 2
Categorical accuracy of 60 hospitals Etest results three strains against six antibiotics (total 4851 tests)

Categorical accuracy (%)	Errors (%)			
	Very major	Major	Minor	Total
96.2	1.7	0.4	1.7	3.8

3.4. Activity against other Enterobacteriaceae

Enterocbacter spp. and *Citrobacter freundii* showed lower rates of susceptibility to piperacillin (76.8–77.9%), ceftazidime (76.5–78.5%) and cefoperazone/sulbactam (88.3–89.6%) compared with the other tested β-lactams. Susceptibility rates for cefepime (97.2–98.6%) and imipenem (98.8–99.8%) were superior to that of cefpirome (93.4–96.5%). For the indole-positive *Proteus* spp., susceptibility rates to piperacillin (90.5%) and imipenem (91.1%) were lower than for other β-lactam antibiotics. *Serratia* spp. showed lower rates of susceptibility to piperacillin (83.6%) and cefoperazone/sulbactam (85.4%) compared with the other tested β-lactams (92.0–96.2%).

3.5. Activity against non-fermentative Gram-negative bacilli

For *Acinetobacter* spp., cefoperazone/sulbactam was the most active antibiotic (96.6% susceptible), followed by imipenem (95.0%), ceftazidime (88.6%), cefepime (85.4%) and cefpirome (85.3%). A lower susceptibility rate was seen for piperacillin (69.8%).

The resistance of *P. aeruginosa* strains was high for all tested antibiotics except piperacillin and ceftazidime. The breakpoint of piperacillin for *P. aeruginosa* is 128 mg/L (13), which is higher than that of other organisms.

Table 1
Quality control Etest results from 60 hospitals participating the trial

Antibiotics	Percent of study results with in quality control ranges for					
	S. aureus ATCC29213		*E. coli* ATCC25922		*P. aeruginosa* ATCC27853	
Cefepime	99.6	(1–4)[a]	95.5	(0.016–0.06)	99.2	(1–8)
Cefpirome	90.8	(0.5–2)[b]	72.1	(0.016–0.06)[b]	98.4	(1–4)[b]
Ceftazidime	98.7	(4–16)	99.6	(0.12–0.5)	100	(1–4)
Cefoperazone/sulbactam	88.5	(1–4)[b]	98.8	(0.12–0.5)[b]	100	(1–8)[b]
Imiepenem	100	(0.016–0.06)	97.5	(0.06–0.25)	99.6	(1–4)
Oxacillin	100	(0.12–0.5)	NA	–	NA	–
Piperacillin	NA	–	100	(1–4)	99.6	(1–8)
Total	96.3		95.4		99.4	

NA: not available.
[a] The acceptable limits for quality control strain used to monitor accuracy of minimal inhibitory concentration (μg/ml).
[b] Range was determined that near (±1 tube) the middle of the concentration for cefepime and cefoperazone/sulbactam by our previous reports.

Table 3
Antimicrobial activity of seven tested β-lactams against clinical isolates (2002)

Organism (no. tested)	Antibiotics	MIC (mg/L)		MIC (mg/L)			Category (mg/L)			Category (%)	
		50%	90%	Range			S[a]	I[b]	R[c]	S[a]	R[c]
S. aureus (594)	Oxacillin	0.38	0.5	0.019	–	2	≤2	–	≥4	100.0	0.0
	Ceftazidime	16	24	1	–	>256	≤8	16	≥32	14.3	2.4
	Cefepime	3	4	0.38	–	16	≤8	16	≥32	99.8	0.0
	Cefpirome[d]	1.0	1.5	0.38	–	4	≤8	16	≥32	100.0	0.0
	CP_SB[e]	2	3	0.25	–	32	≤16	32	≥64	99.8	0.0
	Imipenem	0.032	0.064	0.003	–	0.5	≤4	8	≥16	100.0	0.0
Coagulase negative staphylococci (485)	Oxacillin	0.19	0.5	<0.016	–	2	≤0.25	–	≥0.5	100.0	0.0
	Ceftazidime	8	16	1.5	–	96	≤8	16	≥32	71.5	3.9
	Cefepime	1	2	0.047	–	12	≤8	16	≥32	99.8	0.0
	Cefpirome	0.5	1	0.032	–	4	≤8	16	≥32	100.0	0.0
	CP_SB	1.5	2	0.047	–	8	≤16	32	≥64	100.0	0.0
	Imipenem	0.032	0.064	<0.002	–	0.75	≤4	8	≥16	100.0	0.0
E. coli (594) (CP_SB: 585)	Piperacillin	2	128	0.032	–	>256	≤16	32–64	≥128	79.6	10.8
	Ceftazidime	0.19	0.75	0.032	–	>256	≤8	16	≥32	99.0	0.5
	Cefepime	0.047	0.125	<0.016	–	64	≤8	16	≥32	98.8	0.7
	Cefpirome	0.064	0.19	0.016	–	>256	≤8	16	≥32	98.7	1.3
	CP_SB	0.19	1.5	<0.016	–	>256	≤16	32	≥64	98.8	1.0
	Imipenem	0.25	0.38	0.019	–	2	≤4	8	≥16	100.0	0.0
Klebsiella spp. (596)	Piperacillin	6	24	0.25	–	>256	≤16	32–64	≥128	89.6	7.4
	Ceftazidime	0.25	0.75	0.032	–	>256	≤8	16	≥32	98.7	1.0
	Cefepime	0.047	0.125	<0.016	–	>256	≤8	16	≥32	99.7	0.2
	Cefpirome	0.064	0.25	<0.016	–	>256	≤8	16	≥32	98.8	0.3
	CP_SB	0.25	2	0.016	–	>256	≤16	32	≥64	97.1	2.5
	Imipenem	0.25	0.5	0.064	–	4	≤4	8	≥16	100.0	0.0
C. freundii (492)	Piperacillin	3	>256	0.5	–	>256	≤16	32–64	≥128	76.8	18.7
	Ceftazidime	0.75	>256	0.094	–	>256	≤8	16	≥32	78.5	19.7
	Cefepime	0.064	1.5	<0.016	–	>256	≤8	16	≥32	98.6	0.6
	Cefpirome	0.094	4	0.023	–	>256	≤8	16	≥32	96.5	1.6
	CP_SB	0.50	24	0.047	–	>256	≤16	32	≥64	89.6	2.0
	Imipenem	0.50	1.5	0.125	–	>32	≤4	8	≥16	99.8	0.2
Enterobacter spp. (575)	Piperacillin	2	>256	0.125	–	>256	≤16	32–64	≥128	77.9	15.0
	Ceftazidime	0.38	>256	0.064	–	>256	≤8	16	≥32	76.5	20.2
	Cefepime	0.064	2	<0.016	–	>256	≤8	16	≥32	97.2	2.1
	Cefpirome	0.125	4	0.023	–	>256	≤8	16	≥32	93.4	3.7
	CP_SB	0.5	32	<0.016	–	>256	≤16	32	≥64	88.3	5.9
	Imipenem	0.5	1.5	0.125	–	>32	≤4	8	≥16	98.8	0.5
Indole-positive *Proteae* (508) (Piperacillin and CP_SB:507)	Piperacillin	0.5	16	0.032	–	>256	≤16	32–64	≥128	90.5	5.5
	Ceftazidime	0.125	2	0.023	–	>256	≤8	16	≥32	93.1	4.5
	Cefepime	0.047	0.19	<0.016	–	>256	≤8	16	≥32	97.6	1.4
	Cefpirome	0.094	0.5	<0.016	–	>256	≤8	16	≥32	98.0	0.6
	CP_SB	1	3	0.032	–	>256	≤16	32	≥64	97.4	2.4
	Imipenem	2	4	0.25	–	>32	≤4	8	≥16	91.1	3.3
Serratia spp. (549)	Piperacillin	2	96	0.38	–	>256	≤16	32–64	≥128	83.6	9.8
	Ceftazidime	0.25	4	0.023	–	>256	≤8	16	≥32	92.0	7.1
	Cefepime	0.094	3	0.016	–	>256	≤8	16	≥32	92.9	5.3
	Cefpirome	0.094	3	0.023	–	>256	≤8	16	≥32	92.5	4.7
	CP_SB	1.5	64	0.032	–	>256	≤16	32	≥64	85.4	10.9
	Imipenem	0.50	1.5	0.094	–	>32	≤4	8	≥16	96.2	3.6
Acinetobacter spp. (536)	Piperacillin	12	64	0.016	–	>256	≤16	32–64	≥128	69.8	9.3
	Ceftazidime	4	12	0.38	–	>256	≤8	16	≥32	88.6	5.8
	Cefepime	2	16	0.064	–	>256	≤8	16	≥32	85.4	7.6
	Cefpirome	2	32	0.064	–	>256	≤8	16	≥32	85.3	11.6
	CP_SB	1.5	4	0.125	–	>256	≤16	32	≥64	96.6	1.5
	Imipenem	0.38	0.75	0.064	–	>32	≤4	8	≥16	95.0	5.0

Table 3 (*Continued*)

Organism (no. tested)	Antibiotics	MIC (mg/L)		MIC (mg/L)			Category (mg/L)			Category (%)	
		50%	90%	Range			S[a]	I[b]	R[c]	S[a]	R[c]
P. aeruginosa (594)	Piperacillin	4	>256	0.38	–	>256	≤64	–	≥128	84.5	15.0
(CP_SB: 584)	Ceftazidime	2	48	0.19	–	>256	≤8	16	≥32	82.0	12.3
	Cefepime	4	32	0.064	–	>256	≤8	16	≥32	75.4	12.6
	Cefpirome	6	256	0.064	–	>256	≤8	16	≥32	62.3	22.6
	CP_SB	4	96	0.25	–	>256	≤16	32	≥64	78.4	12.5
	Imipenem	2.0	>32	0.19	–	>32	≤4	8	≥16	65.7	30.8

[a] Susceptible.
[b] Intermediate.
[c] Resistant.
[d] The brake point of cefpirome used was the same value of cefepime.
[e] Cefoperazone:sulbactam (2:1) and the brake point of this combination used was of cefoperazone alone.

4. Discussion

We report a surveillance programme using Etest strip, a simple and reproducible method [16]. Since the same lot of Etest strips, quality control strains and Mueller Hinton Agar plates were used at all hospitals, the QC assurance kept almost total accuracy (Table 2). However, the results of cefpirome within QC ranges for *E. coli* ATCC25922 were only 72.1%. This could be due to an inoculum effect, thickness of media, control of Etest strip, condition of incubation or a misread MIC value.

Compared with previous studies [11–13,17] imipenem maintained antibiotic activity against Gram-positive and Gram-negative bacteria except for indole-positive *Proteus* spp., *Acinetobacter* spp. and *P. aeruginosa*. Against *Acinetobacter* spp., the combination of cefoperazone and sulbactam was the most potent antimicrobial agent. Sulbactam inhibits the peptidoglycan biosynthesis of *Acinetobacter* spp. [18]. Cefpirome had the best activity against indole-positive *Proteus* spp. of the tested antibiotics. Overall, cefepime was active against all organisms, including all Gram-negative bacteria such as *P. aeruginosa*. Affinity of cefepime and class C β-lactamases is very low [19], thus it is very stable against class C β-lactamases.

Enterobacter spp., *Citrobacter freundii*, *Serratia marcescens* and indole-positive *Proteus* encode AmpC β-lactamase gene on their chromosome. AmpC type β-lactamases hydrolyze penicillins and cephalosporins [1], but not expanded-spectrum cephalosporins. Resistant strains producing a large amount of AmpC β-lactamase have been found in the Enterobacteriaceae. This is because the AmpD enzyme, a precursor from the cell wall encoded by *ampD* gene, does not work in these strains, a phenomenon called de-repressed or constitutive production of AmpC β-lactamase. We interpreted all Enterobacteriaceae with lower MICs for cefepime than for cefpirome as AmpC overproducers.

ESBL producing Enterobacteriaceae are well known as expanded-spectrum cephalosporin resistant strains. In this study, 2.9% (17 strains) of *E. coli* and 2.0% (12 strains) of *K. pneumoniae* show resistance to ceftazidime, respectively (data not shown). In Japan, ceftazidime hydrolyzing ESBL producers are not common in the clinical field, but cefotaxime hydrolyzing ESBL producers, CTX-M-type or Toho-type β-lactamases, are sometimes isolated from clinical specimens [10,20–22] For the surveillance of ESBL producers, ceftazidime and other β-lactam antibiotics such as cefotaxime, cefpodoxime, ceftriaxone or aztreonam should be used [14].

Class B β-lactamase producing *P. aeruginosa* known as multi-antibiotic resistant strains, are resistant to many antibiotics, including carbapenems [23]. Eleven strains of *P. aeruginosa* (1.9%) were confirmed as metallo-β-lactamase producers by the double-disk synergy test in this surveillance programme, as previously stated by Kimura et al. [23]. This study found 30.8% (184 isolates) of imipenem-resistant *P. aeruginosa*. This suggests that class B β-lactamase is not the main resistance mechanism for carbapenem in *P. aeruginosa*. Prevalence of ceftazidime-resistant *P. aeruginosa* was 12.3% (73 isolates) in this study. Ceftazidime does not use OprD on the outer membrane as a main penetration pathway [2]. These results suggest that the imipenem resistance mechanism for *P. aeruginosa* is the result of a decrease or lack of this outer membrane protein molecule. In the clinical field, penicillin use is decreasing yearly and this study showed numbers of piperacillin susceptible *P. aeruginosa* are increasing. This fact reflects the recovery of drug susceptibility of *P. aeruginosa* towards piperacillin.

Six months after the strains from this study were collected and studied using Etest, Kimura et al. tested antibiotic susceptibility to imipenem or ceftazidime using a broth microdilution method [23]. They reported 14.8% (88 isolates) of imipenem resistant *P. aeruginosa*. Although the same strains were used, the percentage in this study is 30.8%. This discrepancy could be explained by the method for stock used. We adopted casitone media (Eiken Co. Ltd., Japan) to stock and deliver resistant bacteria from each station to Toho University School of Medicine. Sometimes bacteria lose the gene markers encoded by plasmid DNA in casitone media. Thus, to reconfirm drug susceptibilities or genes causing antibiotic resistant bacteria, another process for stock and delivery should be used.

In conclusion, imipenem resistant *P. aeruginosa* increased compared with previous reports. The results of this study suggest that the participation of OprD is a major resistance mechanism against imipenem in *P. aeruginosa*. Susceptibility of *P. aeruginosa* to piperacillin has increased compared with previous data. Overall, cefepime is maintaining antibiotic activity against Gram-positive and Gram-negative bacteria. It is necessary to continue this surveillance programme to evaluate commercial antibiotics.

Acknowledgements

We thank the working group of technical staff and directors in the 60 hospitals without whom this study could not have been performed. This work was supported by a grant from Bristol-Myers K.K., a grant from the Ministry of Health, Labor and Welfare of Japan (H15-Shinkou-009) and in part by grant from project research grant 15-4 from Toho University School of Medicine. We thank Professor Kenneth S. Thomson, Creighton University School of Medicine for his useful advice and discussion, and also Reiko Shimatsu Rie Shibuya and Fusako Kashitani for their technical assistance.

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Res. Adv. in Microbiology 3, 2003

DNA-based diagnosis method for typhoid fever and paratyphoid fever, and the screening method for *Salmonella enterica* serovar Typhi and serovar Paratyphi A with decreased susceptibility to fluoroquinolones by PCR-restriction fragment length polymorphism (RFLP).

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Abstract

DNA-based diagnosis method for typhoid fever and paratyphoid fever were designed by using multiplex PCR, which used five pair of primers for detecting Vi antigen gene (*viaB*), H antigen gene (*fliC-d*, *fliC-a*) and O antigen synthesis gene (*tyv*, *prt*). Clinical isolates of *Salmonella enterica* serovar Typhi and Paratyphi A were correctly identified by this method. We also designed PCR- restriction fragment length polymorphism (RFLP) method for screening of the *gyrA* mutations of *S. enterica* serovar Typhi and serovar Paratyphi A with decreased susceptibility to fluoroquinolones. These two methods were useful for earlier diagnosis of typhoid fever and paratyphoid fever and earlier screening for *S. enterica* serovar Typhi and serovar Paratyphi A with decreased susceptibility to fluoroquinolones than ordinary culture methods.

Introduction

Enteric fever remains an important public health problem in many countries of the world. Typhoid fever is a sometimes fatal infection of adults and children that causes bacteremia and inflammatory destruction of the intestine and other organs. Typhoid fever is endemic in developing countries, especially in southeast Asia and Africa. Chloramphenicol has been a choice of treatment for typhoid fever for about 40 years, but alternative drugs were required for the treatment by the emergence of multidrug-resistant (MDR) *Salmonella enterica* serovar Typhi, that is resistant to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole. Fluoroquinolones have proven to be effective for the treatment of typhoid fever caused by MDR strains in early 1990's, and have become the first line drugs of treatment for typhoid fever at moment (1, 5). But, *S. enterica* serovar Typhi strains resistant to

fluoroquinolones have already been reported by many researchers (4, 11, 23). Further, several failures of clinical treatment of typhoid patients with ciprofloxacin and other fluoroquinolones have also been reported (3, 26, 27). The emergence and spread of these resistant organisms have been reported from developing countries, particularly from Vietnam (21, 28), the Indian subcontinent (4, 15, 22, 26), and Tajikistan (16, 17). Typhoid fever and paratyphoid fever are sometimes fatal infection, and require the urgent diagnosis and treatment by the administration of appropriate antibiotics. We describe here that the diagnosis by PCR and screening method for *S. enterica* serovar Typhi and serovar Paratyphi A reduced susceptibility to several fluoroquinolones by PCR- restriction fragment length polymorphism (RFLP) method. Both methods require less time than ordinary culture methods.

Multiplex PCR for *S. enterica* serovar Typhi and serovar Paratyphi A.

Typhoid fever and paratyphoid fever are sometimes fatal infection of adults and children, and require the urgent treatment by the administration of appropriate antibiotics. The diagnosis of typhoid fever and paratyphoid fever is now performed by ordinary blood or stool culture methods and biochemical tests. The classical diagnosis method of typhoid fever and paratyphoid fever requires at least 4 or 5 working days for the positive results. The more rapid alternative diagnosis method is required for the diagnosis of typhoid fever and paratyphoid fever. Some researchers have already reported the *S. enterica* serovar Typhi detection methods with PCR by using the *fliC-d* gene (25), Vi capsular antigen gene (7)and 16S rRNA gene (33), however PCR diagnosis method for paratyphoid fever has not reported yet. As only one gene was targeted for the identification of *S. enterica* serovar Typhi in these methods, they detected other *Salmonella* serotype strains than *S. enterica* serovar Typhi in some cases. We recently developed and reported more specific diagnosis method based on a multiplex PCR technique for both typhoid fever and paratyphoid fever, detecting Vi antigen gene (*viaB*), H antigen gene (*fliC-d*, *fliC-a*) and O antigen synthesis gene (*tyv*, *prt*) (9). This system enabled us to identify and differentiate *S. enterica* serovar Typhi and serovar Paratyphi A, that are clinically important *Salmonella* serovars as human pathogens, by only a single PCR reaction, when we isolated the bacteria from blood or stool culture of clinical patients.

The primer sequences used in this study are listed in Table1. We designed the primers tyv-s and tyv-as for detection of the tyvelose epimerase gene (*tyv*, previously called *rfbE*) and the primers fliCcom-s and fliCd-as for detection of the *fliC-d* gene (phase-1 flagellin gene for d antigen [H:d]) of *S. enterica* serovar Typhi. The primers parat-s and parat-as were designed for detection of a paratose synthase gene (*prt*, previously called *rfbS*), and the primers fliCcom-s and fliCa-as were designed for detection of a *fliC-a* gene (phase-1 flagellin; H:a). The gene *prt* encodes CDP-paratose synthase, which converts CDP-4 –keto -3,6-dideoxyglucose to CDP-paratose. The gene *prt* is present in both *S. enterica* serovar Typhi and serovar Paratyphi A. The gene *tyv* encodes CDP-tyvelose epimerase, which converts CDP-paratose to CDP-tyvelose. The *tyv* gene is present in both serovar Typhi and serovar Paratyphi A, but the *tyv* gene of *S. enterica* serovar Paratyphi A does not produce active CDP-tyvelose epimerase due to the 1-bp deletion which causes the frameshift mutation and converts codon 4 of Tyv to a stop codon. Our

Table 1. Primers for the multiplex PCR amplification of *Salmonella enterica* serovar Typhi and Paratyphi A.

Oligonucleotide sequence	Length (bp)	Amplified fragment size (bp)	Primer designed from (Accession number).[b]
tyv(rfbE)			
tyv-s, 5'-gag gaa ggg aaa tga agc ttt t-3'	22	615bp	M29682
tyv-as, 5'- tag caa act gtc tcc cac cat ac-3'	23		M29682
prt(rfbS)			
parat-s, 5'-ctt gct atg gaa gac ata acg aac c-3'.	25	258bp	M29682
parat-as, 5'-cgt ctc cat caa aag ctc cat aga-3'.	24		M29682
viaB			
vi-s, 5'-gtt att tca gca taa gga g-3'.	19	439bp	D14156
vi-as, 5'-ctt cca tac cac ttt ccg-3'.	18		D14156
fliC			
fliCcom-s, 5'-aat caa caa caa cct gca gcg-3'.	21		L21912
fliCd-as, 5'- gca tag cca cca tca ata acc-3'.	21		L21912
fliCa-as, 5'-t[illegible] tgc tta atg tag ccg aag g-3'.	22		X03393
fliCcom/fliCd-as		750bp(489bp)[a]	
fliCcom/fliCa-as		329bp	

a: Number in parenthses represents size of PCR product of H:j gene.

b: Primers were designed using sequences corresponding to indicated GenBank/EMBL/DDBJ nucleotide sequence database accession number.

primers detect only *S. enterica* serovar Typhi *tyv* gene. We designed the primers for the *viaB* gene which specifically detect the *Salmonella* Vi antigen gene, because *viaB* primers previously reported by other researchers detected both *S. enterica* serovar Paratyphi C, *S. enterica* serovar Dublin and the *Citrobacter freundii* Vi antigen genes.

The multiplex PCR using five sets of primer pairs, which were targeted for the *viaB*, *prt*, *tyv*, *fliC-d*, and *fliC-a* genes, correctly identified *S. enterica* serovar Typhi and serovar Paratyphi A and differentiated the two serovars by the combinations of the different-size bands produced: four positive bands, which consist of *viaB*, *prt*, *tyv* and *fliC-d* PCR products, in *S. enterica* serovar Typhi and two positive bands, which consist of *prt* and *fliC-a* PCR products, in *S. enterica* serovar Paratyphi A (Fig. 1).

The primers for *tyv* specifically detected the *tyv* gene of *S. enterica* serovar Typhi. The *prt* primers also detected strains belonging to the O2 and O9 groups of *Salmonella*, and the *tyv* primers detected isolates of the *Salmonella* O9 group (Table 2). The primer pairs for *fliC-d* and *fliC-a* specifically detected the *fliC-d* and *fliC-a* genes, respectively, for the *Salmonella* serovars, and were able to distinguish *fliC-d* and *fliC-a* genes from other *Salmonella* serovar *fliC* genes. The primers for *fliC-d* also detected the *fliC-j* gene, which is an alternate phase of *S. enterica* serovar Typhi H-1 antigen genes (6, 14). Since *fliC-j* is a 261-bp deletion derivative of the *fliC-d* gene (ref), the PCR product was smaller than fliC-d

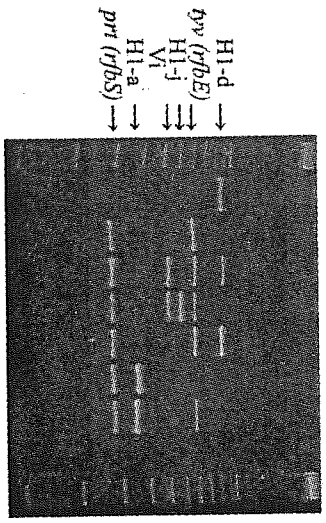

Fig. 1. Identification of *S. enterica* serovar Typhi and serovar Paratyphi A by multiplex PCR. After PCR, the PCR products were separated by 15% polyacrylamide gel electrophoresis.

gene (Fig.1).

To examine possible cross-reactions of the selected *viaB, prt, tyv*, and *fliC* primers among major enteric pathogens, including the several genera of the family *Enterobacteriaceae*, some strains were tested by the multiplex PCR assay; none showed positive results (Table 2). To further evaluate the primer specificities for *Salmonella* species, we tested several kinds of salmonella serovars. The primer combinations of *prt* and *fliC-a*, and the combinations of *viaB, tyv*, and *fliC-d*, correctly identified *S. enterica* serovar Typhi and serovar Paratyphi A respectively.

The methods described here may make possible the identification of clinically important strains of *S. enterica* serovar Typhi and serovar Paratyphi A strains within a few working days of the arrival of specimens in the diagnostic microbiology laboratory.

S. enterica serovar Typhi and serovar Paratyphi A with decreased susceptibility to fluoroquinolones.

Fluoroquinolones are used for the treatment of MDR *S. enterica* serovar Typhi infections, and now they have become the first-line drugs for the treatment of typhoid fever (5, 20, 24, 29). However, some *S. enterica* serovar Typhi strains that exhibit resistance to several fluoroquinolones and decreased susceptibilities to fluoroquinolones have been already reported. Several clinical treatment failures after the administration of ciprofloxacin and other fluoroquinolones to patients with typhoid fever due to strains with decreased susceptibilities to fluoroquinolones have also been reported (23). The emergence and spread of these organisms have been reported in developing countries. The incidence of strains that are resistant to nalidixic acid and that exhibit decreased susceptibilities to the most fluoroquinolones used for the treatment of typhoid fever is also increasing in Japan (Fig. 2)

Table 2. Bacterial strains used to evaluate the specificity of multiplex PCR and the multiplex PCR results.

Strain No.		Serotype	Antigen structure				PCR results [1]				
			Phage Type	O antigen	H-1	H-2 [2]	*tyv*	*fliC-d*	*viaB*	*fliC-a*	*prt*
990116	*Salmonella*	Typhi	D1	9,12,[Vi]	d	-	+	+	+	-	+
990120	*Salmonella*	Typhi	E1	9,12,[Vi]	d	-	+	+	+	-	+
990005	*Salmonella*	Typhi	UVS1	9,12,[Vi]	d	-	+	+	+	-	+
990006	*Salmonella*	Typhi	A	9,12,[Vi]	d	-	+	+	+	-	+
990007	*Salmonella*	Typhi	E1	9,12,[Vi]	d	-	+	+	+	-	+
990008	*Salmonella*	Typhi	E1	9,12,[Vi]	d	-	+	+	+	-	+
990009	*Salmonella*	Typhi	E1	9,12,[Vi]	d	-	+	+	+	-	+
990012	*Salmonella*	Typhi	E1	9,12,[Vi]	d	-	+	+	+	-	+
990014	*Salmonella*	Typhi	E1	9,12,[Vi]	d	-	+	+	+	-	+
990037	*Salmonella*	Typhi	D1	9,12,[Vi]	d	-	+	+	+	-	+
980096	*Salmonella*	Typhi	46	9,12,[Vi]	d	-	+	+	+	-	+
980111	*Salmonella*	Typhi	DVS	9,12,[Vi]	d	-	+	+	+	-	+
980077	*Salmonella*	Typhi	UVS1	9,12,[Vi]	d	-	+	+	+	-	+
980014	*Salmonella*	Typhi	UVS1	9,12,[Vi]	j	-	+	+[3]	+	-	+
GIFU9954	*Salmonella*	Typhi		Rough	d	-	+	+	+	-	+
000055	*Salmonella*	Paratyphi A	1	1,2,12	a	[1,5]	-	-	-	+	+
000056	*Salmonella*	Paratyphi A	1	1,2,12	a	[1,5]	-	-	-	+	+
990110	*Salmonella*	Paratyphi A	2	1,2,12	a	[1,5]	-	-	-	+	+
970083	*Salmonella*	Paratyphi A	2	1,2,12	a	[1,5]	-	-	-	+	+
960007	*Salmonella*	Paratyphi A	3	1,2,12	a	[1,5]	-	-	-	+	+
000001	*Salmonella*	Paratyphi A	4	1,2,12	a	[1,5]	-	-	-	+	+
000041	*Salmonella*	Paratyphi A	4	1,2,12	a	[1,5]	-	-	-	+	+
990081	*Salmonella*	Paratyphi A	5	1,2,12	a	[1,5]	-	-	-	+	+
970032	*Salmonella*	Paratyphi A	5	1,2,12	a	[1,5]	-	-	-	+	+
990046	*Salmonella*	Paratyphi A	6	1,2,12	a	[1,5]	-	-	-	+	+
990103	*Salmonella*	Paratyphi A	6	1,2,12	a	[1,5]	-	-	-	+	+
99023	*Salmonella*	Chester		1,4,[5],12	e,h	e,n,x	-	-	-	-	-
99076	*Salmonella*	Agona		1,4,[5],12	f,g,s	[1,2]	-	-	-	-	-
99026	*Salmonella*	Oranienburg		6,7,14	m,t	[z_{57}]	-	-	-	-	-
99063	*Salmonella*	Infantis		6,7,14	r	1,5	-	-	-	-	-
99087	*Salmonella*	Litchfield		6,8	l,v	1,2	-	-	-	-	-
99114	*Salmonella*	Hadar		6,8	z_{10}	e,n,x	-	-	-	-	-
99109	*Salmonella*	Enteritidis		1,9,12	f],g,m,[p	[1,7]	+	-	-	-	+
99112	*Salmonella*	Javiana		1,9,12	l,z_{28}	1,5	+	-	-	-	+
99017	*Salmonella*	Senftenberg		1,3,19	g,[s],t	-	-	-	-	-	-
99089	*Salmonella*	Grumpensis		13,23	d	1,7	-	+	-	-	-
99108	*Salmonella*	Poona		1,13,22	z	1,6	-	-	-	-	-
1363	*Salmonella*	Typhimurium		1,4,[5],12	i	1,2	-	-	-	-	-
1364	*Salmonella*	Enteritidis		1,9,12	f],g,m,[p	[1,7]	+	-	-	-	+
1365	*Salmonella*	Weltevreden		3,10[15]	r	z_6	-	-	-	-	-
S-222	*Salmonella*	Durban		9,12	a	e,n,z_{15}	+	-	-	+	+
S-214	*Salmonella*	Strasbourg		9,46	d	1,7	+	+	-	-	+
S-154	*Salmonella*	Ndolo		1,9,12	d	1,5	+	+	-	-	+
GIFU12823	*Salmonella*	Paratyphi C		6,7,[Vi]	c	1,5	-	-	+	-	-
GIFU13011	*Salmonella*	Dublin		1,9,12[Vi]	g,p	-	+	-	+	-	+
	Citrobacter	*freundii*		Vi+			-	-	-[4]	-	-
	Yersinia	*pseudotuberculosis*		1b			-	-	-	-	-
	Yersinia	*pseudotuberculosis*		2a			-	-	-	-	-
	Yersinia	*pseudotuberculosis*		2b			-	-	-	-	-
	Yersinia	*pseudotuberculosis*		4a			-	-	-	-	-
	Yersinia	*pseudotuberculosis*		4b			-	-	-	-	-
	Yersinia	*pseudotuberculosis*		5b			-	-	-	-	-
	Yersinia	*enterocolitica*		O3			-	-	-	-	-
	Yersinia	*enterocolitica*		O5			-	-	-	-	-

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Table 2. Bacterial strains used to evaluate the specificity of multiplex PCR and the multiplex PCR results. (*continued*)

Yersinia	*enterocolitica*	O8	-	-	-	-	-
Yersinia	*enterocolitica*	O9	-	-	-	-	-
Vibrio	*cholerae* eltor Ogawa	O1	-	-	-	-	-
Vibrio	*cholerae* eltor Inaba	O1	-	-	-	-	-
Vibrio	*cholerae*	O139	-	-	-	-	-
Vibrio	*cholerae*	non-O1,non-O139	-	-	-	-	-
Vibrio	*mimicus*		-	-	-	-	-
Vibrio	*parahaemoliticus*		-	-	-	-	-
Vibrio	*fluvialis*		-	-	-	-	-
Aeromonas	*hydrophila*		-	-	-	-	-
Aeromonas	*sobria*		-	-	-	-	-
Aeromonas	*caviae*		-	-	-	-	-
Escherichia	*coli*		-	-	-	-	-
Shigella	*dysenteriae*		-	-	-	-	-
Shigella	*flexneri*		-	-	-	-	-
Shigella	*boidyii*		-	-	-	-	-
Shigella	*sonnei*		-	-	-	-	-

1) +; PCR-positive, -; PCR-negative.
2) -; no H-2 phase.
3) H1-j antigen.
4) Our primers for *viaB* gene did not react with Vi antigen genes of *C. freundii* .

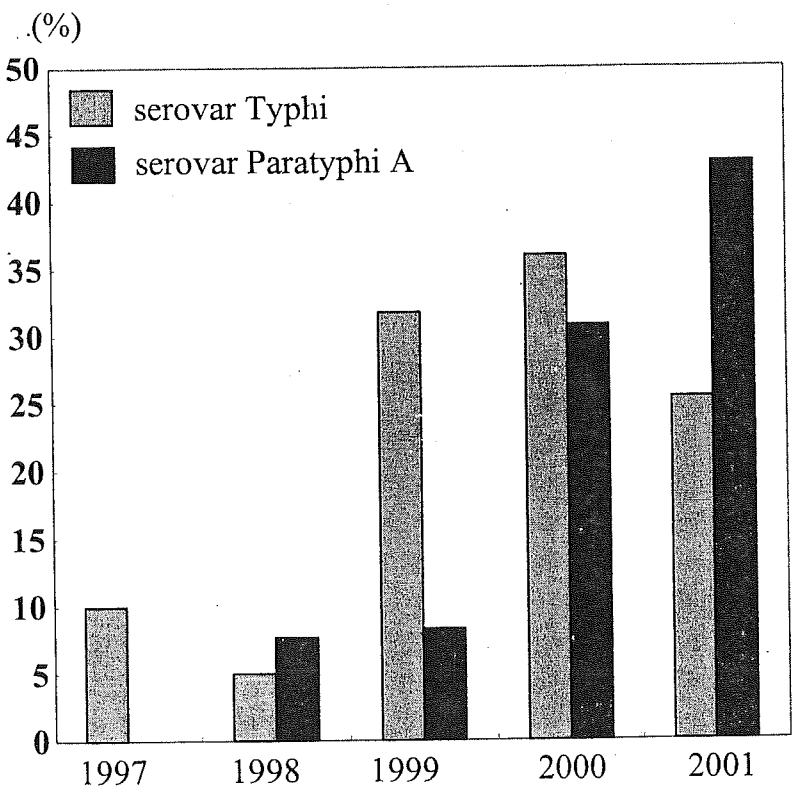

Fig. 2 Incidence of *S. enterica* serovar Typhi and serovar Paratyphi A with decreased susceptibility to fluoroquinolones in Japan.