allergic to penicillin. The rates of GBS resistance to erythromycin and clindamycin in vaginal specimens in Japan around 2000 reportely were 3% and 1%, respectively (16). However, in the present study, the rates of resistance against these agents were 10.1% and 5.0%, respectively, which implies recent increases in GBS resistance to these antibiotics.

Figure 1 illustrates the serotype distributions as determined by the agglutination and PCR methods. Serotypes VI and VIII accounted for 60% of GBS recovered from pregnant women in Japan in the 1990s and early 2000s (15-17; Fig. 1D). In the present study, however, the incidence of serotypes VI and VIII clearly have decreased, and account for only 26% of GBS between 2007 and 2008 (Fig. 1C). The development of multivalent polysaccharide conjugate GBS vaccines, which are effective against 5 major serotypes (Ia, Ib, II, III, and V), was proposed in the United States (4). According to our results, this serotype coverage of these vaccines would account for 71%.

Despite the inclusion of a few GBS isolates from only 1 hospital in this study, our results provide important information concerning GBS isolates from pregnant women in Japan. The serotype shift among GBS isolates from pregnant women may continue; therefore, serotype distribution among GBS isolates from neonates and pregnant women should be monitored continuously. Careful monitoring of antimicrobial susceptibility also will become increasingly necessary among these types of patients.

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Conflict of interest None to declare.

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Short Communication

Screening for Group B Streptococci with Reduced Penicillin Susceptibility in Clinical Isolates Obtained between 1977 and 2005

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SUMMARY: Group B streptococcus (GBS; *Streptococcus agalactiae*) is a leading cause of neonatal invasive infections, and until recently, it was thought to be completely susceptible to penicillin. However, we recently identified several clinical GBS isolates with reduced penicillin susceptibility (PRGBS) whose minimum inhibitory concentrations of penicillin were $>0.12\,\mu g/ml$, which is above the susceptibility breakpoint set by the Clinical and Laboratory Standards Institute. These PRGBS were isolated between 1995 and 2005 in Japan; whether these PRGBS existed in Japan before 1995 is unknown. In the study described here, we screened for PRGBS among 349 clinical GBS isolates obtained in Japan between 1977 and 2005 using the previously developed disk diffusion method for the detection of PRGBS. With this method, we selected 6 PRGBS candidates and confirmed that 1 isolate was PRGBS, using agar dilution method, including oxacillin, ceftizoxime, and penicillin-binding protein 2X (PBP2X) gene sequencing analysis. This isolate was obtained from sputum in 2005, and we could not detect PRGBS isolates before 1995 in this investigation.

Group B streptococcus (GBS; *Streptococcus agalactiae*) is a leading cause of neonatal sepsis and meningitis and is also an important pathogen causing illness among elderly people and those suffering from underlying medical disorders (1–4). The highest GBS mortality and morbidity result from invasive infections in neonates, particularly in those with very low-birth weight (5–7). Approximately 5% of GBS-infected infants die, and survivors often suffer from severe neurological sequelae such as mental retardation and vision and/or auditory disabilities (8). Vaccination against GBS is still under investigation (9,10). Therefore, intrapartum antibiotic prophylaxis has been recommended by the Center for Disease Control and Prevention (8), and the rate of early onset GBS infection, during the first postnatal week, has declined.

Penicillins are the first-line agents in the prophylaxis and treatment of GBS infections because all clinical GBS isolates have been considered to be uniformly susceptible to β-lactams, including penicillins (8,11). However, we recently identified and molecularly characterized several clinical GBS isolates obtained between 1995 and 2005 that demonstrated reduced penicillin susceptibility (PRGBS) through acquisition of multiple mutations in the penicillin-binding protein 2X (PBP2X) gene (12), and similar PRGBS isolates were recently reported from the United States (13), Canada (14,15), and Japan (from our group [16–20] and other group [21]). However, it is unclear whether PRGBS has existed undetected for several years or emerged only recently.

In this study, we used the previously reported disk diffusion method (22) to detect PRGBS among 349 clinical isolates that were collected from various sources including blood and cerebrospinal fluid in Japan between 1977 and 2005.

A total of 349 clinical isolates (324 clinical isolates between 1977 and 1994, and 25 clinical isolates between 1995 and 2005) were recovered from patients who visited Meijyo Hospital in Japan between 1977 and 2005. These were isolated from various sources such as vaginal specimens, respiratory specimens, blood, and cerebrospinal fluid. Lancefield grouping was performed using Lancefield grouping anti-serum (Denka-Seiken, Tokyo, Japan). The disk diffusion method for detecting PRGBS using oxacillin, ceftizoxime, and ceftibuten disks was used as reported previously (22). The minimum inhibitory concentrations (MICs) of penicillin G, oxacillin, and ceftizoxime were determined by the agar dilution using method *Streptococcus pneumoniae* ATCC49619 as a quality control for MIC measurements, in accordance with Clinical and Laboratory Standards Institute (CLSI) recommendations (11). Sequencing analysis of PBP 2X genes was performed as reported previously (12).

The disk diffusion method uses 3 Kirby-Bauer disks of oxacillin, ceftizoxime, and ceftibuten. The diameters of the growth-inhibitory zones around each Kirby-Bauer disk for PRGBS isolates are smaller than those for penicillin-susceptible GBS. Although penicillin-susceptible GBS produce an apparent growth-inhibitory

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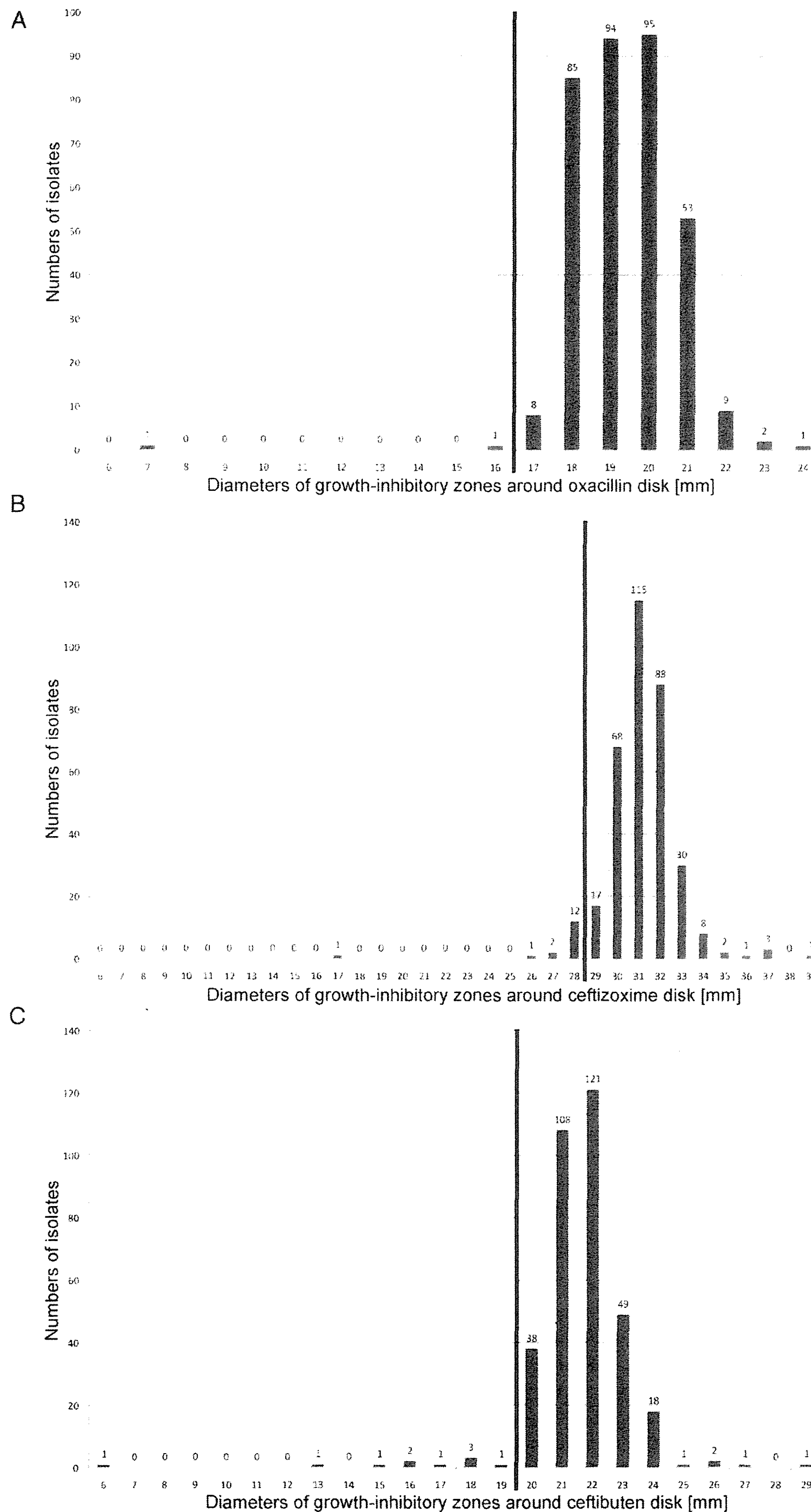

Fig. 1. Diameters of the growth-inhibitory zones and the number of clinical isolates. The diameters of the growth-inhibitory zones measured by the disk diffusion method using oxacillin (A), ceftizoxime (B), and ceftibuten disks (C) (X-axis) and the number of isolates (Y-axis) are plotted. The numbers on the bars indicate the number of clinical isolates. Diameters of growth-inhibitory zones are given in millimeters. The vertical lines indicate the tentative cutoff values for the growth-inhibitory zones around oxacillin (< 17 mm), ceftizoxime (< 29 mm), and ceftibuten (< 20 mm) disks.

Table 1. Results of disk diffusion method for detecting PRGBS and MICs of penicillin G, oxacillin, and ceftizoxime for PRGBS candidates

Isolate	Diameters of growth-inhibitory zones around Kirby-Bauer disks (mm)			MICs determined by the agar dilution method (μg/ml)		
	Oxacillin disk	Ceftizoxime disk	Ceftibuten disk	Penicillin G	Oxacillin	Ceftizoxime
MRY08-469	17	26	16	0.06	1	1
MRY08-470	17	28	18	0.06	1	1
MRY08-471	19	28	18	0.06	1	1
MRY08-472	18	28	17	0.06	1	1
MRY08-474	17	27	13	0.06	1	1
MRY08-517	7	17	6	0.5	8	16

MICs, minimum inhibitory concentrations.

zone around the ceftibuten disk, most PRGBS strains do not produce these zones.

We applied this disk diffusion method to the 349 clinical isolates described above. Fig. 1 illustrates the distribution of the isolates in terms of the diameters of the growth-inhibitory zones around the oxacillin (Fig. 1A), ceftizoxime (Fig. 1B), and ceftibuten disks (Fig. 1C), which were 7-24 mm, 17-39 mm, and 6-29 mm, respectively. We previously established tentative cutoff values for the growth-inhibitory zones around oxacillin (<17 mm), ceftizoxime (<29 mm), and ceftibuten (<20 mm) disks (22). We selected 6 PRGBS candidates with diameters lower than these cutoff values for more than 2 disks (Table 1).

The MICs of penicillin G for PRGBS (0.25-1 μg/ml) are close to the susceptibility breakpoint ($\leq$0.12 μg/ml) set by the CLSI. However, the MICs of oxacillin (2-8 μg/ml) and ceftizoxime (4-128 μg/ml) for PRGBS are higher than those of penicillin-susceptible GBS (12). Therefore, the 6 PRGBS candidates, selected by the disk diffusion method, were subjected to the MIC determination of penicillin G, oxacillin, and ceftizoxime by the agar dilution method (Table 1). Five isolates, MRY08-469, 470, 471, 472, and 474, did not exhibit high MICs of penicillin G, oxacillin, and ceftizoxime. However, MRY08-517 showed high MICs of oxacillin (8 μg/ml), ceftizoxime (16 μg/ml), and penicillin G (0.5 μg/ml), which were above the CLSI breakpoint. Therefore, we thought that MRY08-517 might be a PRGBS. We performed sequencing analysis on the PBP2X gene of MRY08-517. This gene harbored 3 amino acid substitutions and one silent mutation—C1201T (H401Y), T1214C (V405A), C1408A (R470S), and C1617T (A539A) (GenBank accession no. AB775806), compared with that (SAG0287) of *S. agalactiae* strain ATCC BAA-611/2603 V/R (Fig. 2). Among these, V405A amino acid substitution is a typical amino acid substitution of PRGBS located near the conserved motif of PBP2X. Therefore, we concluded that MRY08-517 was a PRGBS.

In this study, we detected 1 PRGBS among 349 clinical isolates obtained between 1977 and 2005. This indicates that the disk diffusion method is useful for detecting PRGBS, and the advantage of this method is that it does not require special reagents or equipments. Therefore, this method may be useful for detecting PRGBS in clinical laboratories worldwide.

We have previously compared the disk diffusion method for detection of PRGBS with the agar dilution method for determination of the MICs of penicillin G

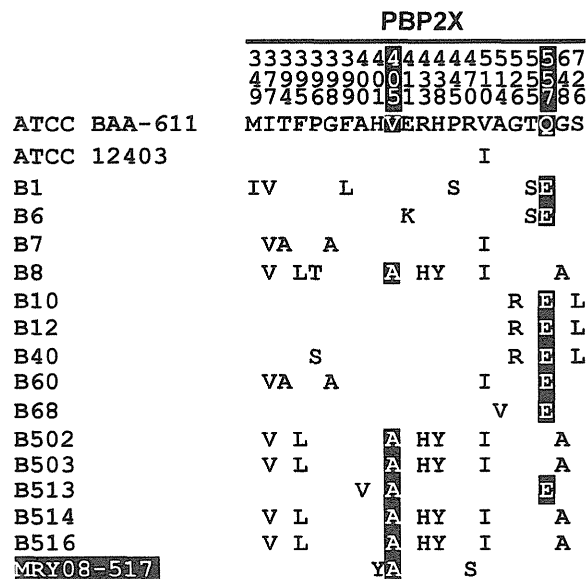

Fig. 2. Amino acid sequence of the penicillin-binding protein (PBP) 2X of control strains (ATCC BAA-611 and ATCC 12403), PRGBS previously analyzed (B1-B516 [12]), and MRY08-517. Numbers above sequence indicate the positions of amino acid substitution. Common amino acid substitutions, V405A and Q557E, exist adjacent to the active site motifs, $_{402}$SSN and $_{552}$KSG, respectively.

(22,23). These studies confirmed that isolates that did not exhibit small diameters of growth-inhibitory zones around the 3 disks were penicillin-susceptible GBS. This suggests that it is unlikely that any PRGBS remained undetected in this study.

The only PRGBS detected in this study was isolated in 2005. The oldest PRGBS previously analyzed was isolated in 1995; no PRGBS was detected prior to 1995. To the best of our knowledge, the oldest PRGBS was isolated in 1995, suggesting that PRGBS emerged after the mid-1990s in Japan. One limitation of this study was that isolates were obtained from patients admitted to only one hospital. However, obtaining old clinical isolates can be challenging, and the samples that were procured for this study provided valuable evidence concerning the emergence of PRGBS.

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Original Article

Comparison of Test Methods for Detecting Metallo-β-Lactamase-Producing Gram-Negative Bacteria

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SUMMARY: The recent increase in gram-negative bacteria coproducing multiple classes of β-lactamases has made it difficult to accurately identify metallo-β-lactamase (MBL) producers. In the present study, six methods for detecting MBL producers were compared using 56 gram-negative bacterial isolates that produce various β-lactamases. Sodium mercaptoacetic acid (SMA) and EDTA were used as inhibitors for a double-disk synergy test (DDST), and antimicrobial agents, including ceftazidime (CAZ), imipenem, and meropenem (MEPM), along with the distance between the disks, were compared. The Etest®, dry-plate DPD1®, Cica-Beta®, and modified Hodge test were also compared. Among the six methods compared, DDST using the SMA disk showed the highest sensitivity (Se) and specificity (Sp). In DDST, the clearest appearance of growth inhibition was observed when the distance between the disks was maintained at approximately 5 mm. A combination of CAZ and SMA successfully detected only MBL-producing isolates (Se, 87.5%; Sp, 100%), and MEPM exhibited the best performance in combination with SMA in the detection of MBL producers coproducing other classes of β-lactamases such as CTX-M- and CMY-type enzymes (Se, 79.1%; Sp, 100%). DDST using SMA and CAZ and/or MEPM is a simple, specific, and cost-effective method to screen MBL producers in routine clinical laboratory testing.

INTRODUCTION

Metallo-β-lactamases (MBLs) have been globally isolated from numerous bacterial species; more than 80 distinct types have been identified worldwide, with over 75% occurring as plasmid-encoded enzymes (1). The major families of acquired MBLs, including IMP- and VIM-type β-lactamases, were initially found in *Serratia marcescens* and *Pseudomonas aeruginosa* isolates (2,3) but have since been encountered in various bacterial species belonging to the genus *Pseudomonas* and the family *Enterobacteriaceae* (1). MBLs are worrisome because they virtually hydrolyze all β-lactams, including the oxyimino cephalosporins, cephamycins, and carbapenems, and are not inhibited by any therapeutically utilized β-lactamase inhibitors. Moreover, a growing concern is the rapid spread of a novel MBL group (NDM-types), which are often coproduced with CTX-M- and/or CMY-enzymes (4,5), and some of the NDM-1 producers demonstrate low resistance to carbapenems (MIC, $<2\,\mu$g/ml). In the early stage of admission, exact identification of MBL producers by routine microbiological tests is therefore sometimes difficult.

To date, several different methods have been developed to detect MBL-producing gram-negative bacteria, including the following three tests that are available in Japan: a double-disk synergy test (DDST) using inhibitors, such as sodium mercaptoacetic acid (SMA) or ethylenediaminetetraacetic acid (EDTA), a microdilution test using 2,6-pyridinedicarboxylic acid (dipicolinic acid [DPA]) as an inhibitor, and the Etest using EDTA as an inhibitor. However, these tests are based on different combinations and concentrations of antimicrobials and inhibitors, as well as interpretation criteria; thus, no standardized protocol for reliable detection of MBL producers is available. In addition, recent emergence and global dissemination of gram-negative bacteria producing new MBLs, such as NDM-1, together with CTX-M-type extended spectrum β-lactamases (ESBLs) and CMY-type cephalosporinases, have muddled the interpretation of the results of these detection methods (6).

In an era where the epidemiology of MBL is constantly changing, it remains unclear which detection methods are the most reliable and which inhibitors are the most suitable. Therefore, the aim of this study was to compare test methods that can be routinely applied in microbiological laboratories for detecting MBL-producing gram-negative bacteria, particularly bacterial isolates that coproduce plural classes of β-lactamases.

MATERIALS AND METHODS

Bacterial strains: The bacterial strains tested in the present study are listed in Table 1 and included genetically well-characterized strains that produce various

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Table 1. Control strains with well-characterized resistance mechanisms used for evaluating test methods in this study

Isolate (n = 56)		No. of isolates	MIC (μg/ml)[1]	
			CAZ	IPM
MBL-producing strains (n = 24)				
Plural classes of β-lactamases (n = 3)				
NDM-1, CTX-M-1, CMY-4 and TEM-1	*E. coli*	1	128<	32
NDM-1 and TEM-1	*K. pneumoniae*	1	128<	4
IMP-1 and CTX-M-2	*K. pneumoniae*	1	32	0.5
IMP- or VIM-type β-lactamase (n = 21)				
IMP-1	*E. coli*	3	128<	4–8
IMP-1	*K. pneumoniae*	3	128<	4–64
IMP-1	*Acinetobacter* sp.	6	128<	32–128<
IMP-1	*P. aeruginosa*	6	128<	64–128<
IMP-1	*P. putida*	1	128<	128<
VIM-1	*P. aeruginosa*	1	128<	128<
VIM-2	*P. aeruginosa*	1	128<	128<
Non-MBL-producing strains (n = 32)				
CMY-type β-lactamase (n = 5)				
CMY-2	*E. coli*	2	128<	0.5–1
CMY-8	*K. pneumoniae*	1	64	0.25>
CMY-9	*E. coli*	1	128<	0.5
CMY-9	*K. pneumoniae*	1	128<	0.5
CTX-M-type β-lactamase (n = 15)				
CTX-M-15	*E. coli*	5	8–32	0.25–0.5
CTX-M-2	*E. coli*	5	0.5–32	0.25
CTX-M-14	*E. coli*	4	0.5–8	0.25
CTX-M-8	*E. coli*	1	1	0.25
KPC-type β-lactamase (n = 1)				
KPC-3	*K. pneumoniae*	1	128<	128<
Non-β-lactamase-producing strains (n = 11)				
	P. aeruginosa	6	32–128<	8–64
	Acinetobacter sp.	5	2–16	0.25–0.5

[1]: Antimicrobial susceptibility test was performed using the agar dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI), and the interpretation of minimal inhibitory concentration (MIC) results was in accordance with CLSI criteria in document M7-A9.
CAZ, ceftazidime; IPM, imipemem.

MBLs, including IMP-type, VIM-type, and NDM-1-type. Clinical isolates producing CTX-M-type ESBLs, CMY-type cephalosporinase, or KPC-3 carbapenemase, which belong to different classes of β-lactamases, and non-β-lactamases-producing isolates with decreased ceftazidime (CAZ) susceptibility were also used to examine specificities (Sps) of six methods as the negative control strains.

DDSTs: DDST was performed using SMA or EDTA as inhibitors as described previously (7). A colony of each bacterial strain was suspended and adjusted with Mueller-Hinton (MH) broth (Becton Dickinson, Sparks, Md., USA) to the turbidity of McFarland standard No. 0.5 and then spread on an MH agar plate (Becton Dickinson) using a cotton swab in accordance with the protocol of the Clinical and Laboratory Standards Institute (CLSI) (8). After the agar surface dried, a CAZ disk (30 μg/disk), an imipenem disk (IPM; 10 μg/disk), or a meropenem disk (MEPM; 10 μg/disk) (Eiken Co., Ltd., Tokyo, Japan) was placed on the agar plate together with a SMA disk (Eiken) or a blank filter disk. The distance between the two antimicrobial disks was maintained at approximately 4–5 cm, and the SMA disk or blank filter disk was placed in close proximity of one of the antimicrobial disks within an edge-to-edge distance of 5, 10, 15, or 20 mm. Twenty microliters of 0.5 M EDTA-2Na solution (Nacalai Tesque, Inc., Kyoto, Japan) was added to the blank filter disk placed on the agar plate, which was then incubated for 18 h at 35°C. In DDST with the SMA disk, strains were considered to be MBL-positive when an expansion (≥5 mm) of the growth-inhibition zone was observed around the antimicrobial disk near the SMA disk compared with the growth-inhibition diameter around the control disk containing the same antimicrobial agent (Fig. 1A–a). In the test with EDTA disk, the bacterial strain was considered to be an MBL producer when the result met at least one of the following three interpretation criteria: (i) the observed growth-inhibition zone was 5 mm greater than that of the control antimicrobial disk, similar to DDST using the SMA disk; (ii) the growth-inhibition zone was 2.5 mm greater than that of the control antimicrobial disk (Fig. 1B–b) based on the mean expansion of the growth-inhibition zones of 2.5 mm for IMP-1 producers (19 isolates) in the present study; and (iii) when no apparent expansion of the growth-inhibition zone was observed, the strain was considered MBL-positive if the shape of the growth-inhibition zone expanded toward the CAZ, IPM, or MEPM disk or when a characteristic growth-inhibition zone was observed (Fig. 1B–c).

Etset MBL IP/IPI: Etest (bioMerieux, Lyon, France) was performed usnig MH agar plates according to the manufacturer's guidelines and interpretation criteria, and the isolates were considered MBL-positive when a reduction in MIC by three doubling dilutions (2^3) was observed in the presence of EDTA (i.e., MIC ratio ≥ 8).

Cica-Beta test MBL: Cica-Beta test MBL (Kanto Chemical, Inc., Tokyo, Japan) was performed according to the manufacturer's guidelines, which was based on hydrolysis of the chromogenic cephalosporin and nitrocefin on paper strips. Two strips were needed: a control strip with no inhibitor (Cica-Beta I) and a second with SMA to detect the inhibition of MBL function (Cica-Beta MBL). Results were obtained within 15 min using colonies grown on agar plates by these two tests. Isolates were considered to be MBL-positive when nitrocefin hydrolysis was observed with the Cica-Beta I test but not the Cica-Beta MBL test. Isolates were deemed as MBL-negative when no nitrocefin hydrolysis was observed using the Cica-Beta I test or when nitrocefin hydrolysis was observed with both the Cica-Beta I and Cica-Beta MBL tests.

Dry-plate DPD1 test: Dry-plate DPD1 test was performed according to the manufacturer's guidelines. This method is based on the microdilution test using DPA as an MBL inhibitor. A colony of each bacterial strain was suspended in MH broth and adjusted to the turbidity of McFarland standard No. 1.0. A 25-μl ali-

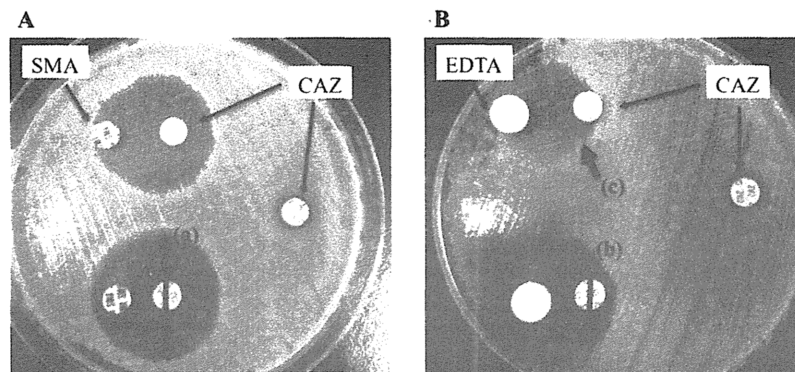

Fig. 1. (Color online) Interpretation criteria of double-disk synergy tests (DDSTs) for detecting MBL production. (A) CAZ and SMA disk. Isolates were considered MBL-positive when the inhibition zone of the antimicrobial disk placed near the SMA disk was at least 5 mm greater than that of corresponding antimicrobial disk (a). (B) CAZ and EDTA disk. Isolates were considered as MBL-positive when they satisfied interpretation criteria as following: (b) The mean expansion of the growth-inhibition zone for IMP-1 producers was 2.5 mm in this study. Then, isolates were considered MBL-positive if the growth-inhibition zone was 2.5 mm greater than that of corresponding antimicrobial disk. (c) If no expansion of the growth-inhibition zone was observed, isolates were considered MBL-positive when the shape of the growth inhibition had changed, or when a characteristic shaped-zone was observed. CAZ, ceftazidime; EDTA, ethylenediaminetetraacetic acid; SMA, sodium mercaptoacetic acid.

quot of the bacterial suspension was added to 12 ml of MH broth, followed by addition of a 100-μl aliquot of the test suspension to each well of the dry-plate DPD1, which was then incubated for 18 h at 35°C. Bacterial isolates were considered MBL-positive when MIC of CAZ and/or IPM was reduced by three doubling dilutions (2^3) in the presence of 175 μg/ml of DPA.

Modified Hodge test: The modified Hodge test was performed according to the CLSI protocol (8). *Escherichia coli* ATCC 25922 was used as an indicator strain. The *E. coli* culture suspension to be tested was adjusted to one-tenth the turbidity of McFarland standard No. 0.5 and spread on an MH agar plate using a cotton swab. An MEPM disk (10 μg/disk) was placed at the center of the MH agar plate, and both a positive (*Klebsiella pneumoniae* ATCC BAA-1705) and a negative (*K. pneumoniae* ATCC BAA-1706) control strain were heavily streaked from the edge of the MEPM disk to the periphery of the MH agar plate, which was then incubated for 18 h at 35°C. Appearance of a distorted growth inhibition zone was interpreted as positive carbapenemase production.

Statistical analysis: The sensitivity (Se) of each phenotypic test was calculated from the number of true positive bacteria, whereas the Sp was calculated from the number of true negative bacteria. In addition, the positive and negative predictive values were also calculated as described previously (9).

RESULTS AND DISCUSSION

The Se and Sp of DDST using SMA or EDTA as an inhibitor and three kinds of β-lactams, including CAZ, MEPM, and IPM, were evaluated when the edge-to-edge distances between the disks containing a β-lactam and an inhibitor, respectively, were maintained at approximately 5, 10, 15, and 20 mm. In the IMP- or VIM-type MBL producers (21 isolates), the clearest appearance of a growth-inhibition zone was observed when the distance between the two disks was maintained at approximately 5 mm so that the combination of CAZ-SMA (Se, 100%) yielded a better result than other combinations (Table 2). No distinct change in the appearance of the growth-inhibition zone was observed for strains producing only the KPC- or CMY-type β-lactamase so that DDST using SMA provided the highest Sp (100%) among the non-MBL producers (32 isolates) (Table 2). In DDST using EDTA, interpretation criteria substantially affected Se and Sp. Among the three interpretation criteria compared, those based on alteration in the shapes of growth-inhibition zones toward the antimicrobial agent disk yielded the highest Se (Table 3). Meanwhile, when the distance between the two disks was maintained at approximately 5 mm, the criteria yielded false-positive results in multidrug-resistant *P. aeruginosa* strains that did not produce MBL, which was likely because the expansion of the growth-inhibition zone was determined to be greater than that of the control antimicrobial disk by larger alterations in the shape of the inhibition zone. As a result, the interpretation criteria using the expansion ($\geq$2.5 mm) of the growth-inhibition zone presented the most reproducible results so that the combination of EDTA-IPM yielded better results (Se, 81.0%; Sp, 87.5%) than other combinations (Table 3). However, DDST using EDTA failed to detect IMP-1-producing *Acinetobacter* sp. because EDTA itself disrupted the growth of this organism (Fig. 2B), resulting in a decreased Se compared with those of SMA. Moreover, the use of EDTA yielded false-positive results in the CMY-type β-lactamase-producing *E. coli* strains because EDTA probably disrupted the growth of this strain by chelating biologically important metals in the MH agar (Fig. 3B). These results indicated that EDTA is not an appropriate inhibitor for detecting MBL producers. EDTA behaves as a strong chelator of biologically important cations, including zinc, in the medium, but it is not a specific MBL inhibitor. However, SMA reportedly binds to the active center of MBLs (10) and blocks their functions, whereas EDTA indirectly inhibits the functions of metallo-enzymes, including MBL, by reducing the free-zinc concentration in the MH agar. In this case, EDTA would merely dis-

Table 2. Summary of sensitivities and specificities of the DDSTs using of SMA and EDTA in the phenotypic detection of IMP- or VIM-producing strains

Agent	Distance between disks (mm)	DDSTs using SMA disk[1)]						DDSTs using EDTA disk[1)]					
		MBL-producing strains (n = 21)		non-MBL-producing strains (n = 32)		Se (%)	Sp (%)	MBL-producing strains (n = 21)		non-MBL-producing strains (n = 32)		Se (%)	Sp (%)
		Positive	Negative	Positive	Negative			Positive	Negative	Positive	Negative		
CAZ	5	21	0	0	32	100	100	16	5	7	25	76.2	78.1
	10	20	1	0	32	95.2	100	11	10	0	32	52.4	100
	15	20	1	0	32	95.2	100	1	20	0	32	4.8	100
	20	5	16	0	32	23.8	100	0	21	0	32	0	100
MEPM	5	16	5	0	32	76.2	100	15	6	5	27	71.4	84.4
	10	16	5	0	32	76.2	100	3	18	1	31	14.3	96.9
	15	14	7	0	32	66.7	100	0	21	2	30	0	93.8
	20	3	18	0	32	14.3	100	0	21	2	30	0	93.8
IPM	5	18	3	0	32	85.7	100	17	4	4	28	81.0	87.5
	10	18	3	0	32	85.7	100	8	13	0	32	38.1	100
	15	17	4	0	32	81.0	100	1	20	0	32	4.8	100
	20	1	20	0	32	4.8	100	0	21	0	32	0	100

[1)]: Isolates were considered MBL-positive if the growth-inhibition zone was 2.5 mm larger than that of corresponding antimicrobial disk.
MBL, metallo-β-lactamase; DDSTs, double-disk synergy tests; Se, sensitivity; Sp, specificity; CAZ, ceftazidime; MEPM, meropenem: IPM, imipenem; SMA, sodium mercaptoacetic acid; EDTA, ethylenediaminetetraacetic acid.

Table 3. Comparison of sensitivity and specificity of the DDSTs using EDTA obtained by three interpretation criteria in IMP- or VIM-producing strains (21 strains)

Criteria	EDTA-CAZ (5 mm)			EDTA-MEPM (5 mm)			EDTA-IPM (5 mm)		
	Expansion (mm)		Shape of zone[3)]	Expansion (mm)		Shape of zone	Expansion (mm)		Shape of zone
	≥5[1)]	≥2.5[2)]		≥5	≥2.5		≥5	≥2.5	
Se (%)	66.7	76.2	76.2	61.9	71.4	81.0	57.1	81.0	100
Sp (%)	87.5	78.1	34.3	90.6	84.4	78.1	93.8	87.5	68.8

DDSTs were performed using EDTA as inhibitor, and the distance between the two disks was maintained at approximately 5 mm.
[1)]: Isolates were considered MBL-positive when the growth-inhibition zone was observed 5 mm larger than that of corresponding antimicrobial disk.
[2)]: Isolates were considered MBL-positive when the growth-inhibition zone was observed 2.5 mm larger than that of corresponding antimicrobial disk.
[3)]: Isolates were considered MBL-positive when the shapes of growth inhibition were changed or characteristic shaped-zone was observed.
Abbreviations are in Table 2.

rupt nonspecific bacterial growth in some strains or species through the shortage of metals in the media.

When the distance between the disks maintained at 15 or 20 mm as recommended by the manufacturer, two NDM-1 producers isolated in Japan were not detected regardless of the antimicrobial agents used as the indicator. The combination of SMA and CAZ failed to detect NDM-1 producers because the NDM-producers coproduced CAZ-hydrolyzing enzymes, such as CTX-M-15, CMY-type cephalosporinases, and/or SHV-12 (Fig. 4A). However, the combination of SMA and MEPM yielded positive results when the distance between the two disks was maintained at approximately 5 mm (Fig. 4C; Table 4) because MEPM is barely hydrolyzed by SHV-derived ESBLs, CTX-M-type ESBLs, and CMY-type cephalosporinases. Moreover, under the same experimental conditions, the best appearance of a growth-inhibition zone was observed in the *K. pneumoniae* isolates that coproduced IMP-1 and CTX-M-2 β-lactamase (Table 4). Thus, MEPM showed the best performance in combination with SMA in detecting MBL-producing strains that coproduce CTX-M-type and/or CMY-type enzymes. The combination of EDTA and MEPM also produced positive results in NDM-1-producing *E. coli* and *K. pneumoniae* isolates. However, EDTA disrupted growth of these NDM-1-producers to some extent, and ambiguous growth-inhibition zones appeared for both isolates of NDM-1-producing *E. coli* and *K. pneumoniae* (Fig. 4D). Thus, when two disks containing MEPM and EDTA were placed in close proximity of each other at a distance of approximately 5 or 10 mm, it became difficult to interpret the expansion of the growth-inhibition zones due to the formation of an apparent growth-inhibition zone around the EDTA disk. Therefore, unlike DDST using SMA, it should be noted that the combination of EDTA and MEPM may occasinally overlook MBL-producing strains coproducing MBL and other classes of β-lac-

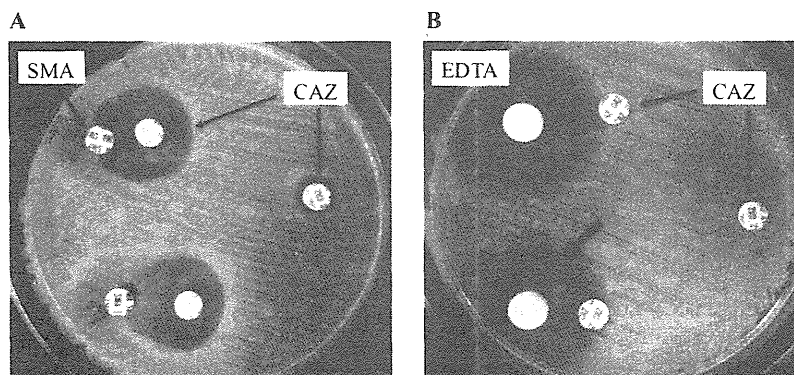

Fig. 2. (Color online) Appearance of growth-inhibition zone in IMP-1-producing *Acinetobacter* sp. The DDSTs using EDTA failed to detect IMP-1-producing *Acinetobacter* sp. because EDTA disrupted the growth of this organism. Abbreviations are in Fig. 1.

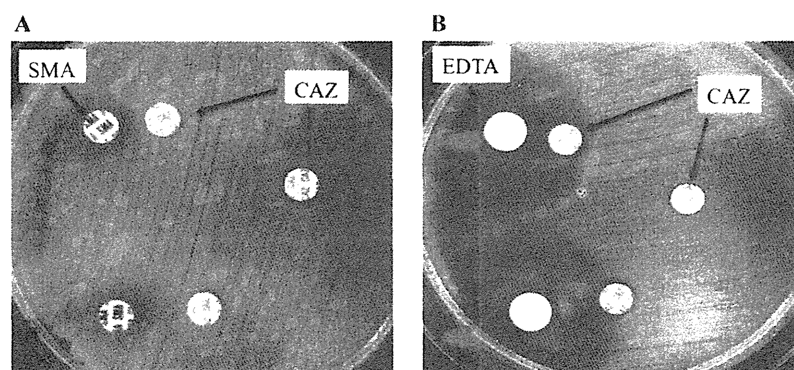

Fig. 3. (Color online) Appearance of growth-inhibition zone in CMY-producing strains. In DDSTs using SMA, no distinct change in the appearance of the growth-inhibition zone was observed for strains producing CMY. However, the use of EDTA yielded false-positive results in the CMY-type β-lactamases producing strains, since EDTA itself disrupted the growth of this organism. Abbreviations are in Fig. 1.

tamases, particularly when the disk distance is maintained at approximately 5 mm (Table 4).

The DDST results of 56 strains were compared with four other test methods. The DPD1 plate test provided the highest Se (87.5%) and Sp (96.9%) among the four compared screening methods (Table 5). In contrast, the Etest showed false-negative results in IMP-1-producing *E. coli* and *K. pneumoniae* strains isolated in Japan; the Cica-Beta test produced false-negative results in IMP-1-producing *Acinetobacter* sp.; and the modified Hodge test also showed false-negative results in IMP-1-producing *P. aeruginosa*, resulting in Se values of 58.3%, 66.7%, and 70.8%, respectively (Table 5). These results were due to the fact that MIC of IPM (i.e., the substrate used in the MBL Etest) is occasionally low (MIC, <2 μg/ml) for the IMP-1-producing *E. coli* and *K. pneumoniae* strains (11). Meanwhile, both the Cica-Beta I and Cica-Beta MBL tests yielded positive results in IMP-1-producing *Acinetobacter* strains, indicating false-negative results. Because many *Acinetobacter baumannii* strains coproduce OXA-type carbapenemases, such as OXA-23-like and OXA-58-like enzymes, together with or without production of intrinsic OXA-51-like enzymes, nitrocefin hydrolysis by the OXA-type carbapenemases may yield false-negative results in the Cica-Beta MBL test despite MBL inhibition by SMA. In addition, as a matter of course, both the Etest and Cica-Beta test failed to specify carbapenemase types produced by each of three strains coproducing plural classes of β-lactamases, and the modified Hodge test also failed to detect NDM-1-producing *E. coli*. Our results support the findings of previous studies; which found that the modified Hodge test occasinally lacks Se (e.g., weak detection of NDM producers) (11). Both the Etest and Cica-Beta test are indeed simple and rapid screening methods, which are easy to implement into a clinical laboratory, although they are not specific for the detection of MBL producers, particularly strains coproducing plural classes of β-lactamases. The modified Hodge test is also a simple and inexpensive assay, but it may produce false-positive results in ESBL-producing clinical isolates (12,13) and negative or weakly positive results in NDM producers. Although MEPM showed the best performance in combination with SMA in detecting MBL-producing strains coproducing other classes of β-lactamases, the combination of SMA and CAZ among 56 strains provided the highest overall Se (87.5%) and Sp (100%) for the detection of MBL producers. CAZ appears to be the most suitable substrate at present for the identification of only MBL-producing isolates because almost all isolates usually demonstrated high resistance to CAZ (MIC, >64 μg/ml), even if the IPM MIC was low, as reported previously (14,15). As shown in the present study, addi-

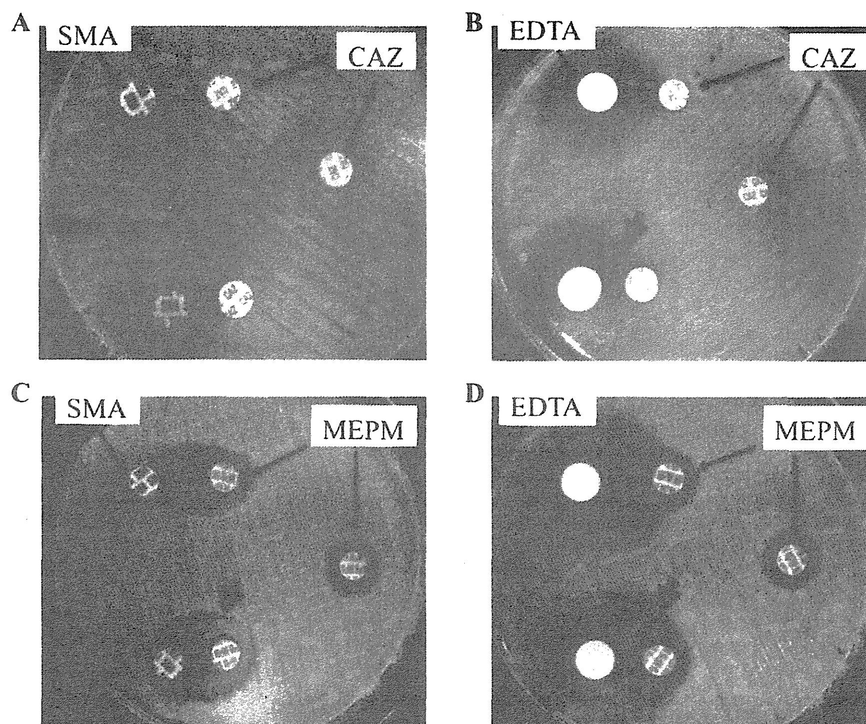

Fig. 4. (Color online) Appearance of growth-inhibition zone in NDM-1-producing strains. The combination of SMA and CAZ failed to detect NDM-1 producers (A). The combination of SMA and MEPM gave positive results when the distance between the two disks was maintained at approximately 5 mm (C). The combination of EDTA and MEPM also produced positive results in NDM-1 producers. However, EDTA disrupted the growth of this organism, and the ambiguous growth-inhibition zones appeared for NDM-1 producers (D). MEPM, meropenem. Other abbreviations are in Fig. 1.

Table 4. Detection of MBL producers by DDSTs in bacteria coproducing plural classes of β-lactamases

Strain	Combination of antimicrobial agent and inhibitor[1]											
	CAZ-SMA		CAZ-EDTA		MEPM-SMA		MEPM-EDTA		IPM-SMA		IPM-EDTA	
	Distance (mm)		Distance (mm)		Distance (mm)		Distance (mm)		Distance (mm)		Distance (mm)	
	5	10	5	10	5	10	5	10	5	10	5	10
NDM-1-producing *E. coli*	−	−	−	−	+	−	+	+	+	−	+	+
NDM-1-producing *K. pneumoniae*	−	−	+	+	+	−	+	+	−	−	+	−
IMP-1 and CTX-M coproducing *K. pneumoniae*	−	−	−	−	+	+	−	−	−	−	−	−

[1]: Isolates were considered MBL-positive if the growth-inhibition zone was 2.5 mm larger than that of corresponding antimicrobial disk. Abbreviations are in Table 2.

tion of the MEPM disk was effective for successful detection of MBL-producing isolates coproducing other classes of cephalosporin-hydrolyzing enzymes while performing DDST, although such strains remain rare in Japan. The DPD1 plate test also showed fine results similar to those of DDST using SMA and CAZ in 56 strains (Se, 87.5%; Sp, 96.9%), as shown in Table 5, suggesting that SMA and DPA may be the most suitable inhibitors for detecting MBL producers. Thus, both the SMA disk method and DPD1 plate test are easy to perform and interpret and can easily be incorporated into the daily workflow of clinical microbiological laboratories. However, the DPD1 plate test is too expensive for use in routine testing.

In conclusion, we demonstrated that CAZ and MEPM showed the best performance in combination with SMA when the distance between two disks was maintained at approximately 5 mm, even though the clinical isolates produced MBL, together with broad-spectrum serine β-lactamases, such as CMY- and CTX-M-type enzymes. A combination of CAZ with SMA successfully detected isolates producing only MBL, and MEPM showed the best performance in combination with SMA in the detection of MBL-producing strains coproducing other classes of β-lactamases. DDST using SMA and CAZ and/or MEPM is a simple, specific, and

Table 5. Comparison of sensitivities and specificities of six screening test methods to detect MBL producers

Screening test method		All strains (n = 56)				IMP- or VIM-producers (n = 21)	Plural classes of β-lactamases producers (n = 3)	non-MBL-producers (n = 32)
		Se (%)	Sp (%)	PPV (%)	NPV (%)	TP/FN[3]	TP/FN	FP/TN[3]
DDSTs								
(5 mm)[1]	SMA-CAZ	87.5	100	100	91.4	21/0	0/3	0/32
	SMA-MEPM	79.1	100	100	100	16/5	3/0	0/32
	SMA-IPM	79.1	100	100	94.1	18/3	1/2	0/32
DDSTs								
(5 mm)[1,2]	EDTA-CAZ	70.8	78.1	70.8	92.5	16/5	1/2	7/25
	EDTA-MEPM	70.8	84.4	77.2	96.4	15/6	2/1	5/27
	EDTA-IPM	79.1	87.5	82.6	96.6	17/4	2/1	4/28
Dry-plate DPD1 test		87.5	96.9	95.4	91.2	19/2	2/1	1/31
Etest MBL IP/IPI		58.3	96.9	93.3	75.6	14/7	0/3	1/31
Cica-Beta test MBL		66.7	100	100	80.0	14/7	2/1	0/32
Modified Hodge test		70.8	100	100	82.1	16/5	1/2	0/32

[1]: In DDSTs, the distance between the two disks was maintained at 5 mm from edge-to-edge.
[2]: Isolates were considered MBL positive if the growth-inhibition zone was 2.5 mm larger than that of corresponding antimicrobial disk.
[3]: Results shown are number of isolates that yielded true-positive (TP), true-negative (TN), false-positive (FP), and false-negative (FN) results, respectively.
PPV, positive-predictive value; NPV, negative-predictive value. Other abbreviations are in Table 2.

cost-effective method that is feasible for screening MBL-producing clinical isolates in routine clinical laboratory analysis. Because the data analyses in the present study were performed with a limited number of strains, we plan to further evaluate these methods using additional clinical isolates that produce multiple classes of β-lactamases.

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Conflict of interest None to declare.

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Original Article

Molecular Epidemiology of Extended-Spectrum β-Lactamases and *Escherichia coli* Isolated from Retail Foods Including Chicken Meat in Japan

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Abstract

Contamination of retail meat with extended-spectrum β-lactamase (ESBL)–producing *Escherichia coli* has been reported, but only limited data have been documented in Japan. One hundred fifty-three retail foods including chicken meat, beef, pork, and vegetables were purchased from 29 supermarkets between January and October in 2010. ESBL producers were recovered from each food sample using McConkey agar plate supplemented with 1 mg/L of cefotaxime. ESBL type was identified by DNA sequencing analysis after polymerase chain reaction amplification. Antibiogram, O serotype, plasmid replicon type, pulsotype, and multilocus sequence type were also determined. Fifty-two epidemiologically unrelated *Escherichia coli* isolates producing ESBL were recovered from 35 (22.9%) of 153 samples, all of which were chicken meat. ESBL types were mainly CTX-M-2 group followed by CTX-M-1 group and CTX-M-8 group. The numbers of bacterial isolates (8 of 21, 38.1%) harboring $bla_{CTX\text{-}M\text{-}8}$ recovered from imported meat samples were significantly larger than those of domestic ones (one of 31, 3.2%) ($p < 0.05$). Nine O serotypes (mainly O8, O25, and O1) were found, together with O-antigen untypable (OUT). Four *E. coli* belonging to the O25b:H4-ST131 clone were recovered from domestic ($n = 1$) and imported meat samples ($n = 3$), respectively. These four isolates were susceptible to fluoroquinolones, although the *E. coli* O25b:H4-ST131 clone producing CTX-M-15, which is predominant in human isolates, is usually resistant to fluoroquinolones. By contrast, five CTX-M-15-producing *E. coli* strains were recovered only from domestic meat samples, and their serotypes were O8 or OUT instead of predominant serotype O25b. Our results showed that ESBL-producing *E. coli* isolates recovered from retail chicken meat samples in Japan are generally divergent in both genetic and serological aspects. Further comparative analyses of $bla_{CTX\text{-}M}$-mediating genetic elements would be continued in the next step to characterize the ESBL producers from retail foods in Japan.

Introduction

Production of extended-spectrum β-lactamases (ESBLs) is the most common cephalosporin resistance mechanism in bacteria belonging to the members of family *Enterobacteriaceae* (Paterson *et al.*, 2005). CTX-M-type ESBL-producing *Escherichia coli* especially have been increasing among clinical isolates recovered from community-acquired infections such as urinary tract infections and bacteremia (Cantón *et al.*, 2008). Increasing isolation rates of these microbes alert us to a probably growing health risk for public health (Pitout and Laupland, 2008). The reason for the rapid spread of CTX-M-producing *E. coli* in the community remains uncertain. Recent investigations have revealed that previous use of antimicrobials, especially oxyimino-cephalosporins and fluoroquinolones, was a risk factor for community-onset bloodstream infections caused by ESBL-producing *E. coli* (Rodríguez-Baño *et al.*, 2010). Additionally, several studies have reported a high prevalence of ESBL-producing *E. coli* isolates in fecal samples of broilers and chickens (Costa *et al.*, 2009; Li *et al.*, 2010). These findings suggested that prescriptions of oral cephalosporins for outpatients and/or extensive veterinary use of antimicrobials may well promote the rapid increase of epidemic clones of *E. coli* that produce CTX-M-type ESBL in both human and animals.

In many food animals such as chickens, pigs, and cattle, certain antimicrobials such as colistin sulfate, avilamycin, and monensin were approved to add to animal feeds for preventing infections in many countries. By contrast, the veterinary use of fluoroquinolones such as enrofloxacin and cephalosporins such as ceftiofur and cefquinome have been approved only for therapeutic purpose for livestock infected with pathogenic bacteria. Indeed, the veterinary use of these antimicrobials has been strictly restricted, but the use of such

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antimicrobials may well contribute to the emergence and spread of ESBL producers in livestock farming environments through selection of bacterial strains harboring genetic determinants responsible for cephalosporin resistance such as $bla_{CTX\text{-}M}$ and bla_{CMY}. Actually, Ma *et al.* have reported the occurrence of ESBL-producing *E. coli* in fecal samples of healthy ducks and environmental samples from a duck farm in South China (Ma *et al.*, 2012), and an increasing amount of data regarding this issue has been accumulated to date (Dhanji *et al.*, 2010; Ewers *et al.*, 2010; Ho *et al.*, 2011). Food animals and their products are now suspected to be one of the potential sources in the dissemination of the antimicrobial-resistant bacteria, especially ESBL-producing *E. coli* (Overdevest *et al.*, 2011). In Japan, Hiroi *et al.* and Asai *et al.* have reported the prevalence of ESBL-producing bacteria in food-producing animals (Asai *et al.*, 2011; Hiroi *et al.*, 2012), however, there have been few reports about the dissemination of ESBL-producing *E. coli* in samples derived from food animals and/or their products collected since 2007. The aim of our study was to assess the recent state of contamination with ESBL-producing *E. coli* in retail food samples and to characterize the types of ESBLs, together with the serotype and genotype of ESBL-producing *E. coli* isolates in Japan. Moreover, we also compared the prevalence of ESBL producers in domestic and imported meat samples, since imported meat products have recently become a commonplace in the Japanese market.

Materials and Methods

Sample collection and bacterial isolation

One hundred fifty-three retail food samples (chicken, beef, pork, and vegetables) were purchased from 29 separate supermarkets in Aichi Prefecture, Japan, between January and October 2010. As for raw chicken meat, 42 samples were domestic products and 26 samples were ones imported mainly from South America, and the remaining samples were mincemeat mixed of domestic and imported meat. Approximately 10 g of each sample was incubated in brilliant green lactose bile broth (SYSMEX bioMérieux Co., Ltd., Tokyo, Japan) at 35°C for 24 h; aliquots (100 μL) of the incubated bacterial preculture were inoculated onto McConkey agar plates (Eiken Chemical Co., Ltd., Tokyo, Japan) supplemented with 1 mg/L of cefotaxime (Jouini *et al.*, 2007), and the plates were incubated at 35°C for 24 h. The two to four colonies growing on the CTX-MacConkey agar plate were picked up and identified in terms of bacterial species by using the API-20E system (SYSMEX bioMérieux). These isolates were further subjected to screening of ESBL producers and pulsed-field gel electrophoresis (PFGE) analysis.

Screening and genetic identification of ESBL

ESBL screening was performed on the basis of the double-disk synergy test by using three different commercially available discs: ceftazidime, cefotaxime, and amoxicillin/clavulanic acid, according to the protocol recommended by the Clinical and Laboratory Standards Institute (CLSI, 2009b) and confirmed using Etest (SYSMEX bioMérieux).

The presence of CTX-M-type β-lactamase genes was determined by polymerase chain reaction (PCR) using primers specific to the CTX-M-1 group, CTX-M-2 group, CTX-M-8 group, or CTX-M-9 group as described elsewhere (Shibata *et al.*, 2006; Dallenne *et al.*, 2010). The TEM- and SHV-type β-lactamase genes were detected by PCR, and their genotypes were further determined by sequencing analysis (Yagi *et al.*, 2000). The nucleotide sequences were analyzed with BLAST software (http://blast.ddbj.nig.ac.jp/top-j.html).

Cluster analysis by PFGE

PFGE typing of the ESBL-producing isolates was performed as described elsewhere (Barrett *et al.*, 1994). Plug containing whole genomic DNA was digested with *Xba*I (Takara Bio. Inc., Tokyo, Japan), and electrophoresis was performed using a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA) with pulses ranging from 2.2 to 54.2 s at a voltage of 6 V/cm at 14°C for 19 h. A dendrogram showing genetic relatedness among the isolates was constructed with Fingerprinting II software (Bio-Rad Laboratories). In the isolates from each food sample, when $\geq$85% genetic similarity was observed, these were classified as a clone with a common genetic background, and one representative isolate was further studied. When $<$85% genetic similarity was observed, these were considered as a different clone and separately treated in this study (Carrico *et al.*, 2005).

Serotyping of ESBL-producing E. coli

Serotyping of each *E. coli* isolate was performed with the *E. coli* antisera "SEIKEN" Set 1 (Denka Seiken, Tokyo, Japan) for O-antigen and Set 2 (Denka Seiken) for H-antigen according to the manufacturer's instructions. Serotypes that could not be distinguished by this method were designated OUT (O-antigen untypable) or HUT (H-antigen untypable). Genetic serotyping of each bacterial isolate was performed according to the procedures described elsewhere (Clermont *et al.*, 2008).

Antimicrobial susceptibility testing

The antimicrobial susceptibility of each isolate was determined by the agar dilution method, according to the protocol recommended by the CLSI in document M07-A9 (CLSI, 2009a). The antimicrobial agents were obtained from the following sources: piperacillin, cefotaxime, ceftazidime, imipenem, aztreonam, gentamicin, minocycline, fosfomycin, Wako Pure Chemical Co., Inc., Tokyo, Japan; cefmetazole, amikacin, chloramphenicol, Sigma-Aldrich Japan, Tokyo, Japan; ciprofloxacin, levofloxacin, Daiichi-Sankyo Company, Tokyo, Japan; and flomoxef, Shionogi & Co., Ltd., Tokyo, Japan. Susceptibilities of ESBL-producing *E. coli* to each antimicrobial agent were categorized into susceptible, intermediate, or resistant according to the CLSI criteria. Type Culture Collection (ATCC) 25922 (Microbiologics, Inc., MN) was used as reference strain.

Multilocus sequence typing

Multilocus sequence typing (MLST) of ESBL-producing *E. coli* isolates was performed with seven conserved housekeeping genes (*adfK, fumC, gyrB, icd, mdh, purA*, and *recA*). The MLST procedure, including allelic type and sequence type (ST) assignment methods, was done according to the website (http://mlst.ucc.ie/mlst/dbs/Ecoli).

Conjugation studies and plasmid replicon typing

The conjugation experiments were performed with rifampin-resistant *E. coli* CSH-2 (*metB* F⁻, nalidixic acid resistant, rifampicin resistant) as the recipient strain by the filter mating methods. Transconjugants were selected on LB agar plates supplemented with rifampin (100 mg/L) and cefotaxime (2 mg/L) (Wako Pure Chemical Co., Inc., Tokyo). For resultant transconjugants and the parent isolates, plasmid replicon typing was performed as described elsewhere (Carattoli *et al.*, 2005).

Statistical analysis

Comparisons of proportions of ESBL producers recovered from both imported meat samples and domestic products and comparisons of proportions of susceptible isolates between imported and domestic products were made by continuity-adjusted x^2 test with SPSS software (version 20.0 for Windows; SPSS Inc., Chicago, IL); $p < 0.05$ was considered as denoting a significant difference.

Results

Isolation of ESBL producers from food samples

Thirty-five (22.9%) of 153 food samples were contaminated with ESBL-producing bacteria. The ESBL producers were only isolated from chicken meat samples, and the isolation rate (15 of 26, 57.7%) of imported chicken meat samples was slightly higher than that (19 of 42, 45.2%) of domestic chicken meat samples. Of 85 isolates recovered from 35 chicken meat samples, clonally related isolates recovered from the same food samples were excluded based on the cluster analysis by PFGE. The range of clonally different isolates was one to three per chicken sample. As a result, 53 clonally unrelated isolates (domestic products, 32; imported products including one mix product, 21) were recovered from 35 chicken meat samples as probable ESBL producers. Of the 53 isolates, 52 were identified as *E. coli* and the remaining one was *Klebsiella pneumoniae*. Fifty-two *E. coli* isolates were subjected to further studies.

Genotyping of ESBL-producing E. coli

Forty-three *E. coli* isolates produced CTX-M-type ESBLs, while the remaining nine isolates produced TEM-derived or SHV-derived ESBLs (Table 1). Of the bla_{CTX-M} genes, 22 (51.2%), nine (20.9%), five (11.6%), five (11.6%), and two (4.7%) coded for CTX-M-2, CTX-M-8, CTX-M-1, CTX-M-15, and CTX-M-3 ESBL, respectively. The number of bacterial isolates (eight of 21, 38.1%) harboring $bla_{CTX-M-8}$ recovered from imported meat samples was significantly larger than that of domestic ones (one of 31, 3.2%) ($p < 0.05$), but the number of the isolates harboring $bla_{CTX-M-2}$ in imported ones (11 of 21, 52.4%) was slightly higher than that of domestic products (11 of 31, 35.5%). Of the 52 ESBL-producing *E. coli* isolates, seven and one were SHV-12 and SHV-2 producers, respectively, and seven of eight SHV-producers were from domestic meat samples (Table 1).

Serotype of 52 ESBL-producing E. coli *isolates*

PFGE profiles of 52 ESBL-producing *E. coli* isolates were dissimilar to each other (Fig. 1). However, several strains carrying the same ESBL-genes were found to be genetically different (e.g., S7, S416), while closely related strains (e.g., S150, S424) harbored different ESBL genes. Thirty-one (59.6%) were O untypeable, and the serogroups of the remaining isolates were O8 (seven of 52, 13.5%), followed by O25 (five of 52, 9.6%) and O1 (two of 52, 3.8%), respectively. Of the five *E. coli* O25 isolates, four and one isolates were identified as O25b and O25a types, respectively, by PCR. In addition, all four O25b isolates, which harbor $bla_{CTX-M-2}$ ($n=2$), $bla_{CTX-M-8}$ ($n=1$), and bla_{SHV-12} ($n=1$), belonged to ST131 by MLST analysis.

Antimicrobial susceptibility profiles of ESBL-producing E. coli

As shown in Table 2, ESBL-producing *E. coli* isolates were susceptible to cefmetazole, flomoxef, imipenem, amikacin, and fosfomycin. On the other hand, these isolates tended to be resistant to ceftazidime, aztreonam, and minocycline as well as cefotaxime. Among antimicrobial agents including β-lactams tested, susceptibility profiles to chloramphenicol and fluoroquinolones of isolates from domestic products were similar to those from imported products, although the resistance rate to gentamicin in bacterial isolates from imported products was significantly higher than those from domestic ones ($p < 0.05$) (Table 2). In addition, they showed resistance to β-lactams such as piperacillin, cefotaxime, and/or ceftazidime, whereas they were not resistant to fluoroquinolones such as ciprofloxacin and levofloxacin.

Plasmid replicon types

CTX-M-15-producing *E. coli* belonging to the O25b:H4-ST131 is a global epidemic clone, and their isolation rate has been increased recently in Japan. Therefore, conjugation experiments were performed for five isolates (non-O25) harboring $bla_{CTX-M-15}$ and four O25b:H4-ST131 isolates. The conjugal transfer of the bla_{CTX-M}-carrying plasmid to recipient cells was successful in four non-O25 and two O25b isolates.

TABLE 1. GENOTYPES OF 52 EXTENDED-SPECTRUM β-LACTAMASES PRODUCED BY *ESCHERICHIA COLI* ISOLATES

	CTX-M-1 group						*SHV*	
Products	*CTX-M-1*	*CTX-M-3*	*CTX-M-15*	*CTX-M-2*	*CTX-M-8*	*TEM-52*	*SHV-12*	*SHV-2*
Domestic products (31)	4	2	5	11	1	1	7	—
Imported products (21)	1	—	—	11[a]	8[b]	—	—	1
Total (52)	5	2	5	22	9	1	7	1

[a]One sample was an imported product mixed with domestic product, and classified into the imported products.
[b]One isolate harbored both $bla_{CTX-M-8}$ and $bla_{TEM-135}$.

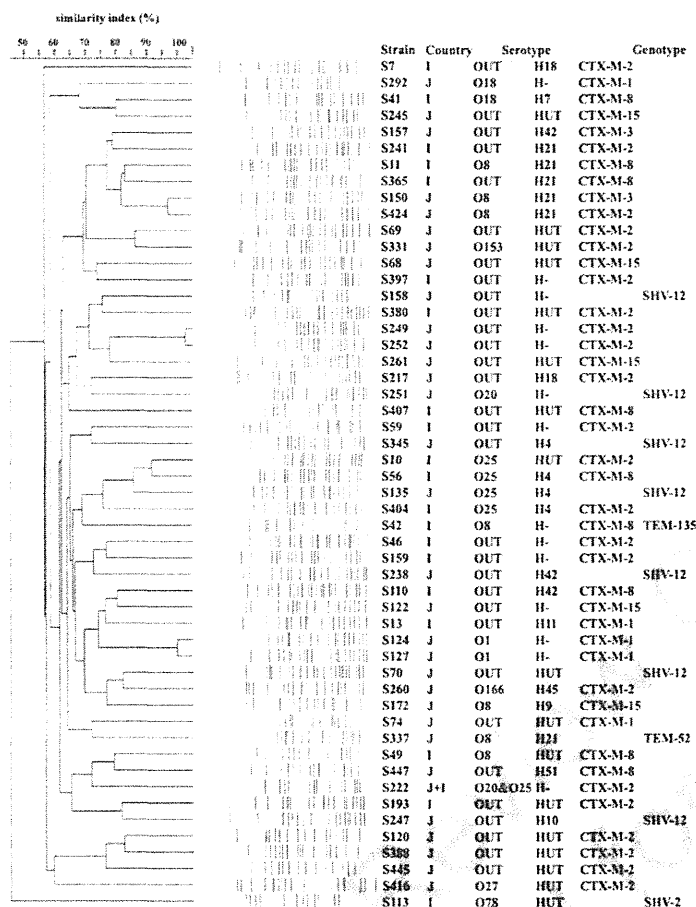

FIG. 1. Dendrogram of pulsed-field gel electrophoresis (PFGE) pattern among bla_{CTX-M}-harboring *Escherichia coli* (based on unweighted pair group method with arithmetic averages cluster analysis). Ladder patterns were analyzed by Fingerprinting II software (Bio-Rad Laboratories) to calculate the Dice similarity index. Isolates were considered to belong to a group of clonally related strains if the dice similarity index of the PFGE pattern was ≥85% (Carrico *et al.*, 2005). When two isolates were recovered from separate parts such as leg and chest purchased from the same supermarket at intervals of more than 3 weeks, the isolates were subjected to further studies as clonally unrelated isolates in the present study, even if they demonstrated 100% similarity by PFGE. J, domestic chicken meat samples; I, imported chicken meat samples; OUT, O-antigen untypeable; HUT, H-antigen untypeable.

TABLE 2. ANTIBIOTIC SUSCEPTIBILITY PROFILES OF 52 EXTENDED-SPECTRUMβ-LACTAMASE (ESBL)–PRODUCING *ESCHERICHIA COLI* ISOLATES

Agents	*Domestic products (n=31)*			*Imported products (n=21)*			*Continuity-adjusted chi-square test (p-value)*[a]
	S (%)	*I (%)*	*R (%)*	*S (%)*	*I (%)*	*R (%)*	
PIPC	1 (3.2)	7 (22.6)	23 (74.2)	0 (0)	0 (0)	21 (100)	1.00
CMZ	31 (100)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	—[b]
CTX	0 (0)	2 (6.5)	29 (93.5)	0 (0)	0 (0)	21 (100)	—
CAZ	14 (45.1)	2 (6.5)	15 (48.4)	14 (66.7)	3 (14.3)	4 (19.0)	0.214
FMOX	31 (100)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	—
IPM	31 (100)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	—
AZT	12 (38.7)	6 (19.3)	13 (42.0)	12 (57.2)	2 (9.5)	7 (33.3)	0.305
GM	31 (100)	0 (0)	0 (0)	14 (66.7)	0 (0)	7 (33.3)	0.002
AMK	31 (100)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	—
MINO	12 (38.7)	0 (0)	19 (61.3)	6 (28.6)	0 (0)	15 (71.4)	0.648
CP	29 (93.5)	0 (0)	2 (6.5)	19 (90.5)	0 (0)	2 (9.5)	1.00
FOM	31 (100)	0 (0)	0 (0)	21 (100)	0 (0)	0 (0)	—
CPFX	28 (90.3)	0 (0)	3 (9.3)	19 (90.5)	0 (0)	2 (9.5)	1.00
LVFX	28 (90.3)	0 (0)	3 (9.3)	19 (90.5)	1 (4.75)	1 (4.75)	1.00

Susceptibilities of 52 ESBL-producing *E. coli* to each antimicrobial agent were categorized into susceptible, intermediate, and resistant in accordance with Clinical and Laboratory Standards Institute criteria.

[a]Proportions of R+I were compared between imported meat samples and domestic meat samples.

[b]*P*-value was not obtained.

S, susceptible; I, intermediate; R, resistant; PIPC, piperacillin; CMZ, cefmetazole; CTX, cefotaxime; CAZ, ceftazidime; FMOX, flomoxef; IPM, imipenem; AZT, aztreonam; GM, gentamicin; AMK, amikacin; MINO, minocycline; CP, chloramphenicol; FOM, fosfomycin; CPFX, ciprofloxacin; LVFX, levofloxacin.

TABLE 3. REPLICON TYPES OF FOUR O25B ISOLATES AND FIVE CTX-M-15-PRODUCING ISOLATES

Sample number	*O serotype*	*Replicon types (patient)*	*Genotype of ESBL*	*Replicon types (transconjugant)*
S10	O25b	I1-γ, N, FIB, F, P	CTX-M-2	P
S56	O25b	I1-γ, FIB, F	CTX-M-8	I1-γ
S135	O25b	I1-γ, FIB, F	SHV-12	ND
S404	O25b	FIB, F	CTX-M-2	ND
S68	OUT	I1-γ, FIB, F	CTX-M-15	I1-γ
S122	OUT	I1-γ, FIB, F	CTX-M-15	I1-γ
S172	O8	FIB, F	CTX-M-15	ND
S245	OUT	F	CTX-M-15	F
S261	OUT	I1-γ, FIB, F	CTX-M-15	I1-γ

OUT, O-antigen untypeable; ND, conjugal transfer of bla_{CTX-M} to recipient cells did not succeed.

Replicon types of the plasmid were mainly I1-γ (4 of 6) followed by IncF, and IncP (Table 3).

Discussion

Contamination of foods with ESBL-producing *E. coli* has recently become a worldwide concern. ESBL-producing *E. coli* isolates were recovered from 62 (29.5%) among 210 batches in raw chicken meat imported by the United Kingdom from South America (Dhanji *et al.*, 2010). In Tunisia, the distribution of isolation rates of ESBL-producing *E. coli* were between 12.6% and 26% among the food samples including chicken and turkey (Jouini *et al.*, 2007). In the present study, the isolation rate of ESBL producers was 50.7% (35 of 69) in chicken meat samples. The high prevalence of ESBL producers in chicken meat in the present study was consistent with Japanese previous reports and was higher than reports from the United Kingdom and Tunisia (Kojima *et al.*, 2005; Jouini *et al.*, 2007; Dhanji *et al.*, 2010; Asai *et al.*, 2011; Hiroi *et al.*, 2012). In Japan, the oxyimino-cephalosporins such as ceftiofur and cefquinome are approved to treat bacterial diseases in cattle and pigs, but are not allowed for use in broilers and chickens. At present, the reason for the high prevalence of ESBL producers in chicken meat samples remains unclear.

Some investigators have reported that CTX-M-type ESBLs detected from chicken meat consisted of CTX-M-1, −2, −14, and −15 (Belgium), CTX-M-2 and −8 (United Kingdom), and CTX-M-1, −8, and −14 (Tunisia), respectively, but the CTX-M-types were somewhat different from those isolated from patients admitted to clinical settings (Jouini *et al.*, 2007; Smet *et al.*, 2008; Dhanji *et al.*, 2010). By contrast, in the Netherlands, a probable relationship between the contamination of chicken meat with drug-resistant bacteria and the appearance of ESBL-producers in humans has been reported (Leverstein-van Hall *et al.*, 2011; Overdevest *et al.*, 2011). In the present study, 43 bla_{CTX-M}-harboring *E. coli* isolates were recovered from 35 chicken meat samples, and their CTX-M-types were mainly CTX-M-2 in both domestic and imported meat samples, followed by CTX-M-8 in imported meat samples. These findings are consistent with those reported from the United Kingdom, suggesting that the genotypes of ESBLs detected from chicken meat samples were very similar to those from chicken meat in the United Kingdom, which would reflect the fact that both countries import chicken meat from South America. In Japan, the predominant CTX-M-types were CTX-M-14 and CTX-M-9 belonging to the CTX-M-9-group enzymes in clinical isolates (Suzuki *et al.*, 2009). Thus, the CTX-M-types of ESBL-producing *E. coli* recovered from chicken meat samples in the present study were inconsistent with those of clinical isolates detected more recently in Japanese hospital settings. The most probable reason for the increasing isolation of CTX-M-9-group ESBL producers from humans in Japan would be the acquisition of *E. coli* that produce CTX-M-9-group enzymes from different routes, such as human-to-human or pets-to-human transmissions (Harada *et al.*, 2012).

In the present study, several genes belonging to the $bla_{CTX-M-1}$-group, such as $bla_{CTX-M-3}$ and $bla_{CTX-M-15}$, were found from retail chicken meat samples, but the O serotypes of *E. coli* harboring the $bla_{CTX-M-1-group}$ gene were somewhat different from those of ESBL-producing *E. coli* isolated in Japanese clinical settings (Matsumura *et al.*, 2012). PFGE analysis showed that strains carrying the same ESBL genes were genetically different, while closely related strains harbored different ESBL genes, suggesting that clonal spread is less important for the distribution of antimicrobial-resistant *E. coli*. Recently, Zheng *et al.* have reported that IS*Ecp*1-like elements were found in the upstream of the $bla_{CTX-M-9-group}$ genes of ESBL producers obtained from food animals, and such genetic structures were very similar to those of humans (Zheng *et al.*, 2012). Further comparative analyses of environmental genetic elements mediating bla_{CTX-M} would be necessary to assess the probability of transmission of ESBL producers via retail foods in Japan.

The pandemic of CTX-M-15-producing *E. coli* in the past decade has been reported, especially in Europe (Woodford *et al.*, 2004; Rogers *et al.*, 2011). The epidemic *E. coli* O25b:H4-ST131 clone producing CTX-M-15 was consistently resistant to fluoroquinolones as well as various β-lactams (Nordmann and Poirel, 2005). In the present study, no CTX-M-15-producing O25b:H4 isolate was found, but four *E. coli* O25b:H4-ST131 isolates that produced CTX-M-2 or CTX-M-8 and five CTX-M-15 producers, serotypes of which were O8 or O-untypable, were recovered from retail chicken meat samples. Interestingly, these isolates were unexpectedly susceptible to fluoroquinolones. Although Inc types of plasmids encoding genes for CTX-M-15 in *E. coli* clinical isolates were usually IncFII, FIA, or FIB (Carattoli, 2009), the replicon types of three CTX-M-15-producing *E. coli* isolates were IncI1-γ plasmids in our study. Although no direct comparison between chicken meat and human isolates was performed in the present study, these results indicated that the CTX-M-15-producing *E. coli* isolates recovered from chicken meat samples have different genetic backgrounds.

Our study presents several limitations. The number of samples processed was small, and systematic surveillance was not used. However, we purchased food samples from 29 separate supermarkets between January and October 2010, and ESBL producers were excluded when the isolates were suggested to belong to the same clone by the cluster analysis of PFGE profile. This study seems to indicate the recent state of contamination with ESBL-producing microbes in retail foods. Indeed, it should be considered that plasmid and mobile genetic elements conferring resistance to third-generation cephalosporins might spread within *E. coli* via gene transfer, but the cluster analysis of PFGE profile could not exclude the possibility described above. It seems very important to

investigate horizontal gene transfer such as exchanges of plasmids or mobile genetic elements carrying genes for ESBLs between bacteria isolated from foods. In the next step of our investigation, we will carefully check the genetic structures of the mobile elements carrying ESBL genes recovered from retail chicken meat and compare them to the relevant mobile elements from human isolates accumulated in the database.

Conclusions

We found a higher-than-expected prevalence of ESBL-producing *E. coli* in retail chicken meat samples in Japan. No clonal spread of ESBL-producing isolates contaminating food samples was observed in the present study, but the high prevalence rate of ESBL producers recovered from food samples might well depend on frequent horizontal gene transfer between bacterial isolates, through exchanges of plasmids and/or mobile elements carrying ESBL genes. Further surveillance and molecular epidemiological investigations conducted in an interdisciplinary way so as to consider not only the human but also the veterinary fields would be essential to predict the future spread of ESBL producers in both humans and animals.

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Disclosure Statement

No competing financial interests exist.

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MECHANISMS

First Detection of Fosfomycin Resistance Gene *fosA3* in CTX-M-Producing *Escherichia coli* Isolates from Healthy Individuals in Japan

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We examined the prevalence and mechanism of fosfomycin resistance in CTX-M-producing *Escherichia coli* isolates from healthy Japanese individuals. One hundred thirty-eight CTX-M-producing *E. coli* isolates were subjected to fosfomycin susceptibility testing. The presence of acquired fosfomycin resistance genes such as *fosA*, *fosA3*, and *fosC2* was explored, and the transmissibility of fosfomycin resistance, replicon type of plasmid, and genetic environment of *fosA3* were investigated. Eight isolates (5.8%) showed resistance to fosfomycin, five of which harbored *fosA3*, which was in genetic linkage with $bla_{CTX\text{-}M}$. The replicon types of the five transferred *fosA3*-carrying plasmids were as follows: IncI1 ($n=2$), IncN ($n=1$), and IncFII ($n=2$). Each *fosA3* gene was located close to the $bla_{CTX\text{-}M}$ gene and was flanked by IS*26* elements. These genetic environments of *fosA3* in *E. coli* from healthy individuals were quite similar to those observed in the clinical and veterinary settings. Our results indicate that *fosA3* genes possibly inserted by small mobile genetic elements flanked by two IS*26* elements have already spread throughout the plasmids along with the $bla_{CTX\text{-}M}$ genes of commensal *E. coli* colonizing in healthy Japanese people.

Introduction

Increase in the incidence of multidrug-resistant Gram-negative bacteria and depletion of newly developed antibiotics provide a new opportunity for use of "old" antibiotics in treating infectious diseases.[1] Fosfomycin, an antibiotic approved for oral and parenteral administrations at 1980 in Japan, has been recently reconsidered a potent therapeutic option for urinary tract infections (UTIs) caused by *Escherichia coli*, which has now been reported to show resistance to various oxyimino-cephalosporins through production of extended-spectrum β-lactamases (ESBLs).[6]

Meanwhile, fosfomycin-resistant bacterial strains have been occasionally observed in clinically isolated *E. coli*. Fosfomycin resistance in *E. coli* is mainly attributable to the depression of fosfomycin influx transporters, glycerol-3-phosphate transporter (GlpT) and/or hexose phosphate transporter (UhpT), and the substitution of amino acids in UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA), which is the target enzyme to which fosfomycin binds.[23] In addition, the acquired fosfomycin resistance determinants, namely *fosA3* and *fosC2*, which inactivate fosfomycin by exerting glutathione-*S*-transferase activities, were identified in *E. coli* clinical isolates.[26] These resistance determinants were located along with the $bla_{CTX\text{-}M}$ genes on transferable plasmids.[26] The presence of *fosA3* with genetic linkage to $bla_{CTX\text{-}M}$ was subsequently revealed in *E. coli* isolates from human, food animal, and domestic animal sources exclusively in East Asian countries.[9–11,13,14]

We recently investigated the fecal carriage of ESBL-producing *E. coli* in healthy individuals and found that most of the isolates resistant to cefotaxime were categorized as CTX-M-type ESBL producers. Moreover, it has been reported that CTX-M-producing *E. coli* from clinical samples and domestic animals often carry the *fosA3* gene in its plasmids.[10,12,14] These findings, thus, raise the possibility that the CTX-M-producing *E. coli* dwelling in the intestinal tract of healthy individuals have also acquired *fosA3*. Therefore, in this study, we investigated the prevalence and mechanisms of fosfomycin resistance in CTX-M-producing *E. coli* isolates from the stool samples of healthy individuals.

Materials and Methods

Bacterial isolation and pulsed field gel electrophoresis

A total of 4,314 stool specimens were collected from 2,563 healthy adult volunteers at a City Public Health Center in Japan from January to August 2010. Written informed

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