

F. 健康危険情報

該当無し

1. 研究発表

論文発表

原著論文

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該当なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表 平成20年度(1)

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Yamane K, Wachino J, Suzuki S, Arakawa Y	Plasmid-Mediated <i>qepA</i> Gene among <i>Escherichia coli</i> Clinical Isolates from Japan	Antimicrob Agents Chemother	52(4)	1564-1566	2008
Sekiguchi J, Morita K, Kitao T, Watanabe N, Okazaki M, Miyoshi-Akiyama T, Kanamori M, Kirikae T	KHM-I, a novel plasmid-mediated metallo- β -lactamase from a <i>Citrobacter freundii</i> clinical isolate	Antimicrob Agents Chemother.	52(11)	4194-4197	2008

IV. 研究成果の刊行物・別冊

Change in the prevalence of extended-spectrum- β -lactamase-producing *Escherichia coli* in Japan by clonal spread

Satowa Suzuki^{1*}, Naohiro Shibata¹, Kunikazu Yamane¹, Jun-ichi Wachino¹,
Kenitiro Ito² and Yoshichika Arakawa¹

¹Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Musashi-murayama, Tokyo 208-0011, Japan; ²Infectious Disease Surveillance Centre, National Institute of Infectious Diseases, 4-7-1 Musashi-murayama, Tokyo 208-0011, Japan

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Introduction: In the early 2000s, there was a rapid increase in extended-spectrum β -lactamase (ESBL)-producing *Escherichia coli* in hospital settings throughout Japan. The reasons for this rapid increase are unclear.

Methods: Between 2002 and 2003, 142 clinical isolates of *E. coli* suspected of producing ESBL were obtained from 37 hospitals and commercial clinical laboratories in geographically distinct regions throughout Japan. They were tested for ESBL types and further subtyped for serogroups, *fimH* single nucleotide polymorphism, pulsed-field gel electrophoresis patterns and multilocus sequence type (MLST). Representative isolates were also subjected to plasmid analysis.

Results: Of 142 *E. coli* isolates suspected of producing ESBL, 130 were confirmed as harbouring *bla*_{CTX-M} by PCR analysis and sequencing. Of these, 84 (65%) harboured CTX-M-9-group *bla*_{CTX-M}. Two serogroups O25 and O86 accounted for 41% of the 130 *bla*_{CTX-M}-positive *E. coli*. All O86 serogroup strains belonged to ST38 by MLST and they formed 18% of all the *bla*_{CTX-M}-positive *E. coli*. Serogroup O25 strains belonged to ST131 and ST73, and formed 21% and 1% of *bla*_{CTX-M}-positive *E. coli*, respectively. Seven characterized plasmids carrying *bla*_{CTX-M} genes belonged to three distinct incompatibility groups: IncF, IncN and Inc11.

Conclusions: In this study, clonally related strains of *E. coli* accounted for a large proportion of *bla*_{CTX-M}-positive *E. coli*. This high proportion of clonal groups identified in different regions of Japan suggests their recent spread by mechanisms other than healthcare-associated transmission. These observations imply that restricting antimicrobial use in human clinical settings may have limited impact on the spread of ESBL-producing *E. coli*.

Keywords: *E. coli*, ESBLs, CTX-M

Introduction

The incidence of hospital and community-acquired extraintestinal infections caused by multidrug-resistant *Escherichia coli* is increasing worldwide. The oxymino-cephalosporins, such as cefotaxime, ceftazidime and ceftriaxone, have potent activity against *E. coli* clinical isolates resistant to other β -lactam agents. However, even these antimicrobial agents have come to be challenged by the emergence of strains that produce extended-spectrum β -lactamases (ESBLs).¹

ESBLs confer resistance by hydrolysing oxymino-cephalosporins but not cephamycins (e.g. ceftiofur, cefotetan) or carbapenems (e.g. imipenem, meropenem). Most ESBLs can be classified into three main groups: TEM, SHV and CTX-M.^{2,3} Worldwide, ESBL-producing organisms are most frequently found among the members of the family Enterobacteriaceae, especially *E. coli* and *Klebsiella pneumoniae*. There are now more than 60 different variants of CTX-M-type ESBLs, which can be further classified into five different subgroups based on their amino acid sequences: CTX-M-1, CTX-M-2, CTX-M-8,

*Corresponding author. Tel: +81-42-561-0771; Fax: +81-42-561-7173; E-mail: suzukiss@nih.go.jp

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CTX-M-9 and CTX-M-25 groups. Of these, CTX-M-1, CTX-M-2 and CTX-M-9 groups are the most common.^{4,5}

Community-acquired extraintestinal infections caused by *E. coli* and hospital-acquired *K. pneumoniae* strains harbouring plasmid-encoded *bla*_{CTX-M} have been increasingly reported worldwide, from both developed and developing countries in the last decade.^{2,6} They have become the most prevalent type of ESBL-producing isolates belonging to the family Enterobacteriaceae during the past 5 years. In Japan, ESBL-producing *E. coli* were rarely isolated until the late 1990s, although strains producing FEC-1 and Toho-1, which were subsequently classified under the CTX-M-type ESBLs, were identified earlier.^{7,8} A report of nosocomial spread of Toho-1-like β -lactamase-producing *E. coli* and a preliminary survey in 1997–98 showed that *E. coli* strains producing CTX-M-2 were the predominant ESBL-producing *E. coli* strains in Japan.⁹ However, in the early 2000s, the dominant CTX-M group shifted from CTX-M-2 to CTX-M-9 among the *E. coli* isolates from clinical facilities.¹⁰ This change in the prevalence of the CTX-M group occurred nationwide in a relatively short period and cannot be solely explained by local and multiple person-to-person nosocomial transmissions. Thus, we characterized these *E. coli* isolates further to determine a possible explanation for this rapid emergence of CTX-M-9-group *bla*_{CTX-M}-positive *E. coli* in hospital settings all over Japan.

Methods

Surveillance data and bacterial strains

To estimate the prevalence of oxymino-cephalosporin resistance among the clinical isolates of *E. coli* in Japan, we reviewed data maintained by the Japan Nosocomial Infections Surveillance (JANIS), which includes participation of more than 200 hospitals across Japan.¹¹ For microbiological characterization, a total of 142 clinical isolates of *E. coli* phenotypically positive for ESBL production were obtained between 2002 and 2003 from 37 hospitals and commercial clinical laboratories located in 15 Prefectures throughout Japan. ESBL production was screened by oxymino-cephalosporin resistance and double-disc diffusion synergy tests as recommended by the CLSI (formerly the NCCLS).¹²

PCR and sequencing analysis

To determine the genotype of ESBLs, we performed PCR using the TEM-, SHV-, CTX-M-1, CTX-M-2 and CTX-M-9 group-specific primers as reported previously.^{9,10} Sequencing was performed with PCR primers as follows: CTX-M-2sequenceF (5'-TTA ATG ATG ACT CAG AGC ATT C-3') and CTX-M-2sequenceR (5'-GAT ACC TCG CTC CAT TTA TTG-3'); CTX-M-9sequenceF (5'-GAT TGA CCG TAT TGG GAG TTT G-3') and CTX-M-9sequenceR (5'-ATT TAC TTC CAT TAC TTT GCG G-3'); CTX-M-14sequenceF (5'-GAT TGA CCG TAT TGG GAG TTT G-3') and CTX-M-14sequenceR (5'-TTG AAC TTT TGC TTT GCC ACG G-3'). PCR amplicons were purified with Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA) and were subsequently sequenced with the appropriate primers.

Serotyping

Serotyping 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.

Antimicrobial susceptibility testing

MICs were determined by the Etest (AB Biodisk, Solna, Sweden) method according to the manufacturer's instructions. Resistance was interpreted based on the recommended breakpoints of the CLSI (formerly the NCCLS).¹³

Genotyping

Genotypic analysis based on *fimH* single nucleotide polymorphism (SNP) was performed as reported previously, and multilocus sequence typing was performed according to the protocol described on the Max-Planck Institut für Infektionsbiologie web site.^{13,14} Pulsed-field gel electrophoresis (PFGE) analysis was performed as reported previously.¹⁵ A dendrogram was generated from the distance matrix by the unweighted pair-group method using arithmetic averages.

Plasmid analysis

Broth mating method was used for conjugation experiments with *E. coli* DH10B as described previously.¹⁶ Transconjugants were selected on Luria-Bertani agar plates containing 16 mg/L cefotaxime and 800 mg/L streptomycin. Plasmid DNA was purified from transconjugant cells with the PureYield Plasmid Miniprep System (Promega), according to the manufacturer's instructions. Purified plasmid DNA was digested with *Pst*I and *Eco*RI restriction enzymes (New England Biolabs, Beverly, MA, USA) for 2 h at 37 °C. The digested plasmid DNA was electrophoresed in a 0.8% agarose gel for the restriction fragment length polymorphism (RFLP) analysis and hybridized with a digoxigenin-labelled DNA probe specific for CTX-M-2- or CTX-M-9-group *bla*_{CTX-M} with the PCR DIG detection system (Roche Diagnostics, Indianapolis, IN, USA). The plasmids were also classified according to their incompatibility group by a PCR-based replicon-typing method.¹⁷

Statistical analysis

Comparisons of proportions were made by χ^2 test with SPSS, version 14.0J for Windows (SPSS, Chicago, IL, USA).

Results

A temporal change in prevalence of *E. coli* resistance to ceftazidime and cefotaxime

The JANIS, a sentinel hospital-based surveillance system established in Japan in 2000, shows that the prevalence of *E. coli* non-susceptible to cefotaxime increased from 2001 through 2006. In 2001, 3.8% and 0.7% of the *E. coli* isolates were non-susceptible to ceftazidime and cefotaxime, respectively. However, in 2004, the prevalence reversed, when ceftazidime-non-susceptible *E. coli* decreased to 1.1% and cefotaxime-non-susceptible isolates increased to 1.7%. Since then, the prevalence of *E. coli* non-susceptible to cefotaxime has continued to increase, reaching 4.6% in 2006. The prevalence of *E. coli* non-susceptible to ceftazidime remained virtually unchanged at 1.4% in the last 2 years of the surveillance.

PCR classification of CTX-M-type β -lactamases and serotyping

PCR analysis showed that 130 of the 142 *E. coli* isolates phenotypically positive for ESBL production harboured *bla*_{CTX-M}. Among the 12 *bla*_{CTX-M}-negative isolates, nine harboured

*bla*_{SHV-12} and three were positive for *bla*_{TEM}; in one of these, the sequence of the PCR amplicon was identical to the gene that encodes TEM-28. The sequence of the *bla*_{TEM} PCR amplicon from the other two isolates was identical to that of the gene that encoded TEM-1, but the molecular mechanism for its resistance to oxymino-cephalosporin has not yet been determined.

Among the 130 *bla*_{CTX-M}-positive *E. coli* isolates 84 (65%) strains harboured *bla* genes from the CTX-M-9 group, 25 (19%) from the CTX-M-1 group and 21 (16%) from the CTX-M-2 group. The most predominant serogroups O25 and O86 comprised 29 (22%) and 24 (18%) of the 130 *bla*_{CTX-M}-positive isolates, respectively (Table 1). All of the O86 strains were H18, while three of the O25 isolates were non-motile and H untypeable. Others were H4. These two most prevalent *E. coli* serogroup isolates were, therefore, subjected to further genotypic analysis as outlined below.

Among the 130 *bla*_{CTX-M}-positive *E. coli* isolates, all 24 *E. coli* O86 isolates harboured CTX-M-9-group *bla*_{CTX-M}, while

only 60 (57%) of the non-O86 isolates harboured the same gene ($P < 0.001$). CTX-M-9-group *bla*_{CTX-M} was found in 22 (76%) of the 29 O25 *E. coli* isolates and in 62 (61%) of non-O25 isolates ($P > 0.05$).

Antimicrobial susceptibility profiles

Tests for susceptibility to 11 antimicrobial agents were conducted for 25 unduplicated *E. coli* O86 and O25 isolates harbouring *bla*_{CTX-M} (Table 2). Duplication was defined as isolates belonging to the same O serogroup and CTX-M group from the same hospital. The drug susceptibility patterns between O25 and O86 isolates were distinct. All O86 isolates were resistant to sulfamethoxazole/trimethoprim, while O25 isolates were more frequently resistant to ceftazidime, ceftoxitin, ciprofloxacin and chloramphenicol. There was no imipenem and fosfomycin resistance in any O86 or O25 strains.

Table 1. O serogroup and CTX-M-group typing of 130 *E. coli* isolates

O serogroup	CTX-M group			Total
	CTX-M-9 group	CTX-M-1 group	CTX-M-2 group	
O25	22		7	29 (22.3%)
O86	24			24 (18.5%)
O1	7	6		13 (10.0%)
O166	3		1	4 (3.1%)
O146	2	1		3 (2.3%)
O153	1		1	2 (1.5%)
O8		2		2 (1.5%)
O125		1		1 (0.8%)
O15	1			1 (0.8%)
O18	1			1 (0.8%)
O44	1			1 (0.8%)
OUT	22	15	12	49 (37.7%)
Total	84 (64.6%)	25 (19.2%)	21 (16.2%)	130

OUT, O-antigen untypeable.

Table 2. Antimicrobial resistance profiles of *E. coli* O86 and O25

Antimicrobial agent	Number of isolates resistant (%)		P value
	<i>E. coli</i> O86	<i>E. coli</i> O25	
Cefotaxime	11 (68.8)	8 (88.9)	0.258
Ceftazidime	0 (0)	3 (33.3)	<0.05
Ceftoxitin	0 (0)	2 (22.2)	<0.05
Imipenem	0 (0)	0 (0)	—
Ciprofloxacin	0 (0)	3 (33.3)	<0.05
Chloramphenicol	1 (6.3)	4 (44.4)	<0.05
Sulfamethoxazole/trimethoprim	16 (100.0)	4 (44.4)	<0.001
Gentamicin	14 (87.5)	6 (66.7)	0.211
Amikacin	4 (25.0)	0 (0)	0.102
Minocycline	2 (12.5)	1 (11.1)	0.918
Fosfomycin	0 (0)	0 (0)	—
Total	16	9	

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Genotyping of *E. coli* O86 and O25 isolates

The *fimH*-sequence-based SNP analysis of all 53 *E. coli* O86 and O25 isolates revealed five distinct genotypes (designated A, B, C, D and E; Figure 1). All CTX-M-9-group *bla*_{CTX-M}-positive *E. coli* O86 isolates ($n = 24$) belonged to a single *fimH* sequence type (cluster A). By multilocus sequence type (MLST), they were all found to belong to ST38. In contrast, 29 *E. coli* O25 isolates fell into four distinct *fimH* SNP types (cluster B, C, D and E). By MLST analysis, the *fimH* cluster C, D and E strains

fell into ST131, while cluster B isolates belonged to ST73. By PFGE analysis of selected O86 and O25 isolates, the O86 isolates had similar electrophoretic banding patterns, but the O25 isolates showed more diverse patterns (Figure 2).

Sequence analysis of *bla*_{CTX-M}

Half of 46 CTX-M-9-group *bla*_{CTX-M}-positive *E. coli* O86 and O25 isolates harboured *bla*_{CTX-M-9} that was 100% identical in

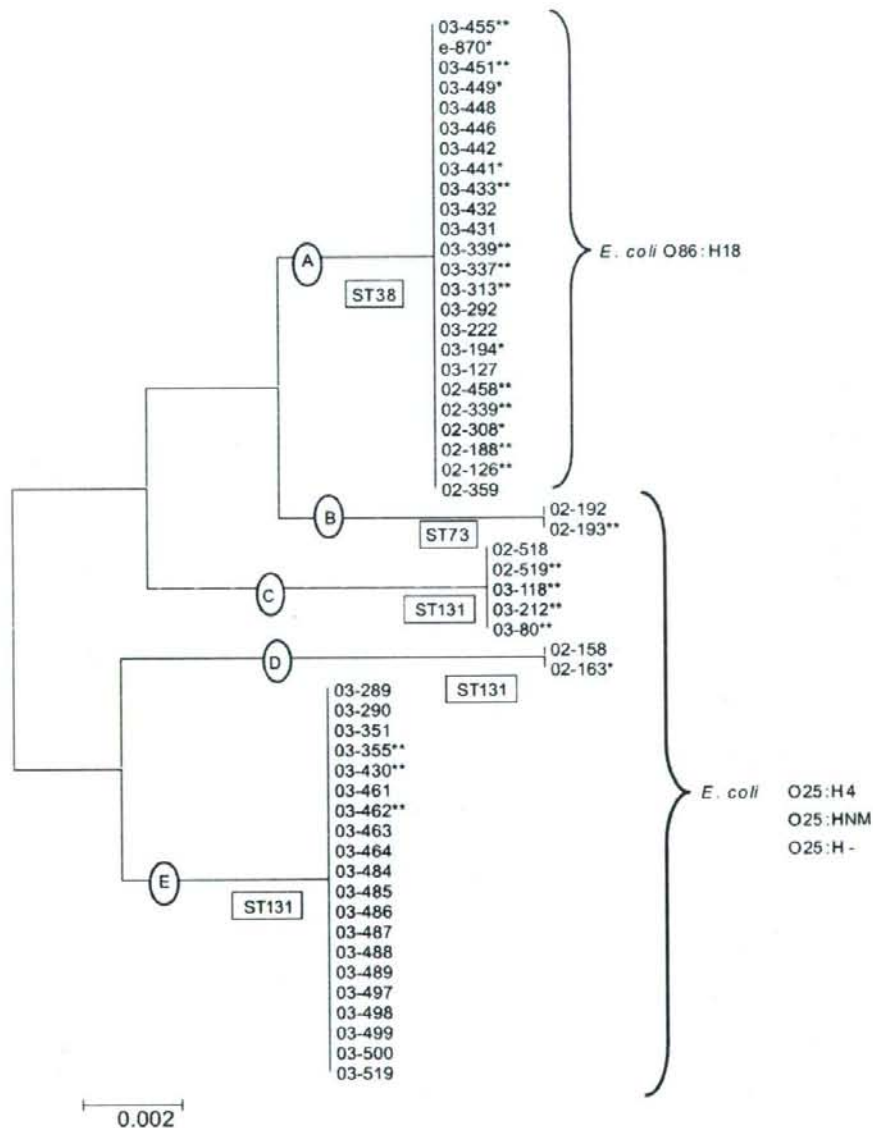


Figure 1. Neighbour-joining trees constructed from the *fimH* sequences and multilocus sequence type (MLST) of *bla*_{CTX-M}-positive *E. coli* O86 and O25. Isolates with single asterisk were subjected to PFGE analysis. Isolates with double asterisk were subjected to both PFGE and MLST analyses. Five *fimH* SNP clusters designated as A–E were identified.

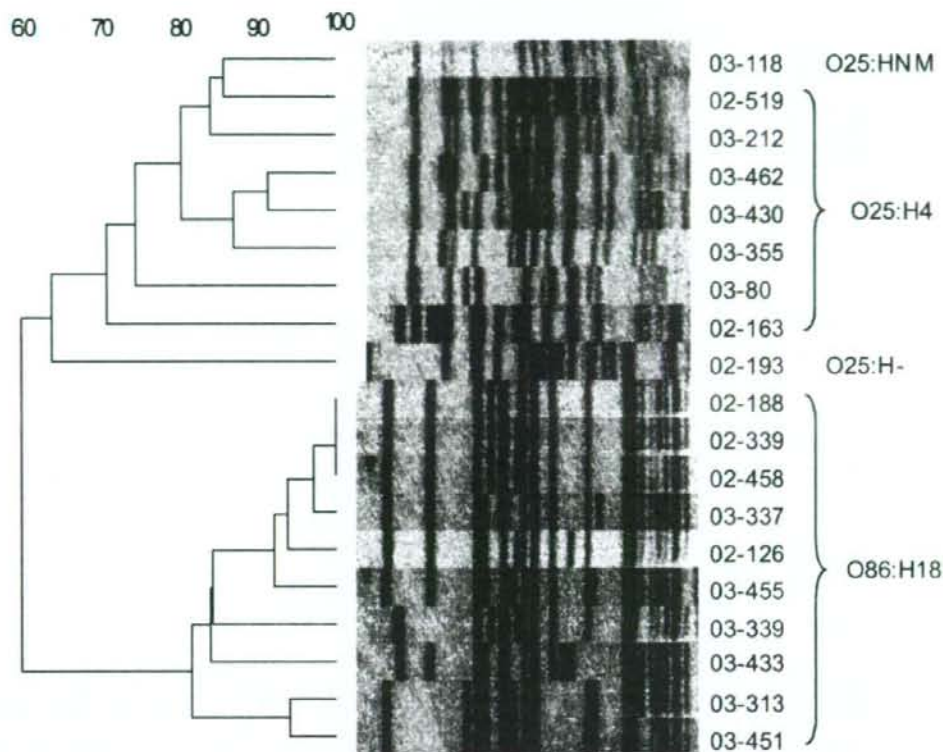


Figure 2. Dendrogram of PFGE patterns among *bla*_{CTX-M}-harboring *E. coli* O86 and O25 serogroups (based on unweighted pair group method with arithmetic averages cluster analysis).

sequence; the rest had a variant *bla*_{CTX-M-9} sequence designated as *bla*_{CTX-M-14}^{18,19}. All but one of the *E. coli* O86 isolates harboured the prototype *bla*_{CTX-M-9}, and all O25 *E. coli* isolates positive for CTX-M-9-group ESBL harboured the variant *bla*_{CTX-M-14}. The enzyme CTX-M-14 differs from CTX-M-9 by only one amino acid at position 234 (Ala→Val) and two silent mutations at nucleotide positions 372 (A→G) and 570 (G→A). However, the nucleotide sequence of a 20–30 bp downstream region of *bla*_{CTX-M} differed completely between *bla*_{CTX-M-9} and *bla*_{CTX-M-14} genes (data not shown).

In isolates positive for the CTX-M-2-group ESBL gene, which were all *E. coli* O25, four and three isolates harboured *bla*_{CTX-M-35} and *bla*_{CTX-M-2}, respectively (GenBank accession number AB176534).²⁰ The enzyme encoded by *bla*_{CTX-M-35} differs from that encoded by *bla*_{CTX-M-2} also by only one amino acid substitution at position 170 (Pro→Ser).

Plasmid analysis

Eight isolates from five *finH* sequence type clusters were subjected to conjugation experiments and seven transconjugants were obtained. Transconjugants were not obtained from isolates belonging to *finH* sequence type B. Among seven plasmids purified from the transconjugants, four carrying CTX-M-9-group *bla*_{CTX-M} belonged to the IncF group, two carrying CTX-M-2-

group *bla*_{CTX-M} belonged to the IncN group and one carrying *bla*_{CTX-M-14} belonged to the Inc11-group plasmids according to the PCR-based replicon typing. Restriction fragment length polymorphism analysis of the plasmid DNA showed different patterns and digoxigenin-labelled *bla*_{CTX-M} probe also hybridized to bands of different molecular weight in all seven plasmids (data not shown).

Epidemiological information on the *E. coli* O86 and O25 isolates

Epidemiological and clinical information on the 53 *E. coli* O86 and O25 isolates harbouring *bla*_{CTX-M} are summarized in Table 3. Twenty-four *E. coli* O86:H18 isolates, which belonged to a single *finH* cluster A, ST38 by MLST analysis and having a similar PFGE band pattern with higher than 90% similarity, were isolated from 14 different hospitals and a commercial laboratory in seven different Prefectures. These Prefectures are located in geographically distant regions of Japan. However, 11 (46%) of these were isolated from six hospitals located in one Prefecture, Ishikawa Prefecture, over a 1 year period, which suggests a regional outbreak. Two *E. coli* O25:H- isolates belonging to *finH* cluster B were isolated from two different patients in one hospital, and two *E. coli* O25:H4 isolates belonging to *finH* cluster D were isolated from different patients

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Table 3. Epidemiological, bacterial and genotypic characteristics of the 53 CTX-M type ESBL-positive *E. coli* O86 and O25 isolates

<i>fimH</i> cluster	Number of isolates	Serotype; MLST	Year	Location	Specimen type	CTX-M-type
A	24	O86:H18; ST38	2002, 2003	14 hospitals and a commercial laboratory/7 prefectures	urine (7), blood (5), endotracheal aspirate (2), vaginal secretion (1), faeces (1), ascites (1), unknown (7)	CTX-M-9 (23), CTX-M-14 (1)
B	2	O25:H-; ST73	2002	1 hospital	unknown (2)	CTX-M-14 (2)
C	5	O25:H4 (4), O25:HNM (1); ST131	2002, 2003	4 hospitals/3 prefectures	urine (1), sputum (1), unknown (3)	CTX-M-2 (3), CTX-M-14 (2)
D	2	O25:H4; ST131	2002	1 hospital	unknown (2)	CTX-M-14 (2)
E	20	O25:H4; ST131	2002, 2003	3 hospitals/3 prefectures	faeces (12), sputum (4), urine (3), blood (1)	CTX-M-35 (4), CTX-M-14 (16)

in another hospital, which suggests nosocomial transmissions. Four *E. coli* O25:H4 isolates and one *E. coli* O25:HNM isolate, which belonged to a single *fimH* cluster C and ST131, were isolated from four different hospitals in three Prefectures. Twenty *E. coli* O25:H4 isolates in *fimH* cluster E were obtained from three hospitals located in three different Prefectures. Of these, 15 (75%) were isolated during an outbreak in a haematology unit. Four (20%) isolates with *bla*_{CTX-M-35} were isolated from different patients admitted to a single hospital.

Discussion

CTX-M-9-group ESBL-producing *E. coli* were not common in Japan prior to 2000.⁹ According to the JANIS, *E. coli* isolates non-susceptible to cefotaxime increased by more than 6-fold between 2001 and 2006. In this study, we found a cluster of *E. coli* O86 isolates, obtained from recognized outbreaks as well as from sporadic cases from hospitals all across Japan. These isolates were highly clonal, as evidenced by the multiple subtyping tests we used. This single clonal group accounted for 18% of all the *bla*_{CTX-M}-positive *E. coli* isolates obtained during 2002–03. Its high clonality suggests that this epidemic strain spread throughout Japan relatively recently in a short period. As far as we know, this is the first documented report of a clonal spread of *E. coli* O86:H18-ST38 harbouring CTX-M-9-group *bla*_{CTX-M} anywhere.

On the other hand, *E. coli* O25:H4-ST131 has been already recognized as an emerging intercontinental clonal group expressing CTX-M-type ESBL.^{6,21} In our study, we also found a cluster of O25 isolates but they were composed of multiple lineages—two different MLST groups (ST131 and ST73), four distinct *fimH* SNP genotypes, diverse PFGE patterns and two different flagella types (H4 and HNM). This suggests that *E. coli* O25 serogroup strains may have been circulating in Japan for a longer period. *E. coli* O25 is increasingly recognized in recent decades and is now the second to third most frequent serogroup among clinical *E. coli* isolates in Japan.²²

In addition, all the *E. coli* O25:H4-ST131 isolates in our study harboured CTX-M-2-group *bla*_{CTX-M} or *bla*_{CTX-M-14}, which are the dominant CTX-M types in Asia including Japan. Most of the previously reported *E. coli* O25:H4-ST131 strains are CTX-M-15 producers. CTX-M-15 is a member of the CTX-M-1 group, which is dominant in European countries.²³ In addition, plasmid analyses in this study revealed that even among the clonal *E. coli* strains, *bla*_{CTX-M} genes were carried by different plasmids. Thus, *E. coli* O25:H4-ST131 strains could have multiple reservoirs from which they spread to different regions of the world acquiring different plasmids carrying different *bla*_{CTX-M} types.

Clonal spread of other antimicrobial-resistant *E. coli* strains causing community- and hospital-acquired extraintestinal infections has been reported previously. Clusters of multidrug-resistant community-acquired *E. coli* O15:K52:H1 and O78:H10 extraintestinal infections have been reported in the 1990s in the UK and continental Europe.^{24,25} A clonally related group of multidrug-resistant uropathogenic *E. coli* called CgA has been reported throughout the USA and some parts of Europe, as well as from animals and the environment in the USA.^{26,27} The nationwide and intercontinental spread, as well as sporadic occurrences of unrelated clusters of community-acquired

infections caused by ESBL-producing *E. coli* strains belonging to identical clonal lineages suggest that such strains are spread not just by person-to-person transmission or in hospital settings, but by some widely distributed contaminated ingestible products. Indeed, CTX-M-type ESBL-producing *E. coli* isolates have been isolated from food animals, usually from chickens but also from calves.²⁸⁻³¹ This observation further supports the idea that the use of antimicrobial agents as growth promoters in animal food or as veterinary medicines contributes to the initial selection of these resistant organisms.

Our findings that a single clonal strain can abruptly change the prevalence of infections with ESBL-producing *E. coli* in different regions of a country suggest that restricting antimicrobial use in human clinical settings may have minimal impact on the spread of ESBL-producing *E. coli* that cause healthcare-associated infections in Japan and elsewhere. The control of antimicrobial resistance may require monitoring of antimicrobial use and surveillance of drug-resistant pathogens in the veterinary environment.

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Transparency declarations

None to declare.

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First Molecular Characterization of Group B Streptococci with Reduced Penicillin Susceptibility[†]

Kouji Kimura,¹ Satowa Suzuki,¹ Jun-ichi Wachino,¹ Hiroshi Kurokawa,¹ Kunikazu Yamane,¹ Naohiro Shibata,¹ Noriyuki Nagano,^{1,2} Haru Kato,¹ Keigo Shibayama,¹ and Yoshichika Arakawa^{1*}

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Disease, Tokyo, Japan,¹ and Medical Microbiology Laboratory, Funabashi Medical Center, Chiba, Japan²

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Group B streptococci (GBS; *Streptococcus agalactiae*) are the leading cause of neonatal invasive diseases and are also important pathogens for adults. Penicillins are the drugs of first choice for the treatment of GBS infections, since GBS have been regarded to be uniformly susceptible to penicillins so far. Here we characterize the first strains of GBS with reduced penicillin susceptibility (PRGBS) identified in Japan. Fourteen PRGBS strains were clinically isolated from the sputa of elderly patients from 1995 to 2005; and the MICs of penicillin, oxacillin, and ceftizoxime ranged from 0.25 to 1 µg/ml, 2 to 8 µg/ml, and 4 to 128 µg/ml, respectively. Moreover, some strains were also insusceptible to ampicillin, cefazolin, cefepime, and cefotaxime. All the PRGBS isolates tested possessed a few amino acid substitutions adjacent to the conserved SSN and KSG motifs (amino acids 402 to 404 and 552 to 554, respectively) of PBP 2X, and the amino acid substitutions could be classified into two types, Q557E and V405A. Western blotting analysis of the 14 clinical isolates with anti-PBP 2X-specific serum suggested that the amount of PBP 2X among the 14 PRGBS isolates was reduced, although the 2 ATCC strains produced a significant amount of PBP 2X. The introduction of PRGBS-derived PBP 2X genes into penicillin-susceptible strains through allelic exchange elevated their penicillin insusceptibility, suggesting that these altered PBP 2X genes are responsible for the penicillin insusceptibility in PRGBS strains. In this study, we characterized for the first time PRGBS strains on a molecular basis, although several reports have so far mentioned the existence of β-lactam-insusceptible GBS from a phenotypic standpoint.

Group B streptococci (GBS; *Streptococcus agalactiae*) are the leading cause of neonatal sepsis and meningitis. Because GBS cause high rates of mortality and morbidity in neonates and no licensed vaccines are available, the use of intrapartum antibiotic prophylaxis has been recommended by the Centers for Disease Control and Prevention and others (1, 2, 5, 7, 10, 14, 15, 16, 21, 22, 23), and the rate of early-onset GBS infections (during the first postnatal week) but the rate of not late-onset GBS infections has been lowered (20).

On the other hand, GBS are also important pathogens that infect both pregnant women and nonpregnant adults, especially elderly people and those with underlying medical disorders (2, 10, 15, 22). Elderly adults account for >40% of persons with invasive GBS disease and for >50% of GBS-associated deaths (9). Moreover, GBS disease in adults is frequently nosocomial and may be related to the placement of an intravenous catheter (11).

Penicillins, including penicillin G, are the first-line agents for intrapartum antibiotic prophylaxis and also the treatment of GBS infections in adults, since all clinically isolated GBS have been considered to be uniformly susceptible to β-lactams, including penicillins (2, 22). Actually, no criteria for "penicillin resistance" have been established so far by the Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS)

(6), and no report of penicillin nonsusceptibility or insusceptibility in a GBS strain has been clearly confirmed on a molecular basis to date.

In this study, we identified and characterized 14 clinical GBS isolates that acquired reduced penicillin susceptibility (MICs, 0.25 to 1 µg/ml) and ceftizoxime insusceptibility (MICs, 4 to 128 µg/ml) and that were isolated in 12 geographically separate hospitals in Japan from 1995 to 1998 and in 2005 (17).

MATERIALS AND METHODS

Strains and identification of GBS. The properties of 3 control strains and 14 strains of GBS with reduced penicillin susceptibility (PRGBS) isolated clinically are listed in Table 1. Nine PRGBS strains were isolated from 1995 to 1998 and were kept until this study. *Streptococcus pneumoniae* ATCC 49619 was a quality control strain used for measurement of the MICs of the antimicrobial agents tested. To obtain candidate PRGBS strains, we screened all 159 clinical isolates sent to a clinical laboratory from various Japanese clinical facilities over 3 days in 2005. We initially predicted that the PRGBS candidates would show reduced susceptibility to some β-lactams as a result of mutations in penicillin-binding protein (PBP) genes, since penicillin-resistant strains of *Streptococcus pneumoniae* usually demonstrate resistance to oxacillin. Thus, we selected from among the 159 clinical isolates 49 isolates which showed oxacillin MICs of >2 µg/ml by the microdilution method. We further selected from among the 49 isolates 5 isolates that demonstrated high levels of resistance to β-lactams. We analyzed both the nine clinical isolates stocked before 1998 and the five new clinical strains isolated in 2005. All 14 strains were isolated from the sputa of individual patients, most of whom were elderly patients, at 12 geographically separate hospitals in Japan from 1995 to 1998 and in 2005. None of the PRGBS candidates were isolated from sterile body sites such as blood or cerebrospinal fluid. All clinical isolates were subjected to multiple tests for the exact identification of GBS, including Gram staining, 16S rRNA PCR followed by restriction enzyme-digested fingerprinting, biochemical identification, surface antigen detection and the CAMP test, with *Streptococcus agalactiae* ATCC BAA-611 and *S. agalactiae* ATCC 12403 used as the positive controls. PCR amplification of 16S

* Corresponding author. Mailing address: Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771, ext. 500. Fax: 81-42-561-7173. E-mail: yarakawa@nih.go.jp.

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TABLE 1. Strains used in this study

Strain	Characteristic	Serotype	Place of isolation ^a	Yr of isolation
<i>S. pneumoniae</i> ATCC49619	Quality control strain for MIC measurements			
<i>S. agalactiae</i> ATCC BAA-611	A genome project strain	V		
<i>S. agalactiae</i> ATCC 12403	A genome project strain	III		
<i>S. agalactiae</i> strain B1	Clinical isolate (sputum)	III	Tokyo A	1995
B6	Clinical isolate (sputum)	VIII	Shizuoka A	1997
B7	Clinical isolate (sputum)	III	Kanagawa A	1997
B8	Clinical isolate (sputum)	VI	Kanagawa B	1997
B10	Clinical isolate (sputum)	III	Toyama A	1997
B12	Clinical isolate (sputum)	III	Toyama A	1997
B40	Clinical isolate (sputum)	III	Toyama A	1997
B60	Clinical isolate (sputum)	III	Kanagawa C	1998
B68	Clinical isolate (sputum)	VI	Akita A	1998
B502	Clinical isolate (sputum)	VI	Tochigi A	2005
B503	Clinical isolate (sputum)	Ib	Shizuoka B	2005
B513	Clinical isolate (sputum)	III	Chiba A	2005
B514	Clinical isolate (sputum)	VI	Kanagawa D	2005
B516	Clinical isolate (sputum)	III	Kanagawa E	2005

^a The place of isolation is expressed anonymously by using the prefecture name and a letter.

rRNA and restriction enzyme-digested fingerprinting were performed as described previously (18). Biochemical identification was performed with the API 20 Strep system (bioMérieux) and surface antigen detection was performed by the Slidex strep test (bioMérieux) according to the manufacturer's instructions. Serotyping was performed with anti-GBS serotype-specific serum (Denka Seiken). Notably, 8 of the 14 strains were serotype III (Table 1), a well-known major serotype found in strains causing neonatal meningitis in Western countries, although serotypes VI and VIII are the predominant types among Japanese clinical isolates.

Measurement of MICs. Measurement of the MICs of penicillin G, penicillin V, ampicillin, oxacillin, cefazolin, cefepime, cefotaxime, ceftizoxime, and meropenem were performed by the agar dilution method, as recommended by the CLSI (6). A penicillin-susceptible *S. pneumoniae* strain, ATCC 49619, was used as the quality control strain for measurement of the exact MICs.

PFG. Pulsed-field gel electrophoresis (PFGE) of the 14 clinical isolates of PRGBS was performed as described previously (13), with minor modifications. The restriction enzyme used in the PFGE experiment was *Apal*. Run conditions were generated by the autoalgorithm mode of the CHEF Mapper PFGE system with a size range of 20 to 200 kb.

Sequencing of high-molecular-weight PBP genes. Five genes encoding high-molecular-weight PBPs (PBP 1A, PBP 1B, PBP 2A, PBP 2B, and PBP 2X) were amplified by DNA polymerase Pyrobest (Takara) by using genomic DNA as the template. The PCR conditions were as follows: 1 cycle of 98°C for 1 min; 30 cycles of 98°C for 10 s, 55°C for 1 min, and 72°C for 2 min 30 s; and 1 cycle of 72°C for 7 min. The mixture was then held at 4°C. Purified PCR products were obtained with a Wizard SV gel and PCR clean-up system (Promega), followed by sequencing reactions and analysis with an ABI Prism 3100 genetic analyzer (Applied Biosystems). The primers used for amplification of the PBP genes and sequencing are listed in Table 2.

Visualization and Western blotting analysis of PBPs. Membrane fractions were prepared from the ATCC strains and the clinical isolates as described previously (24). The membrane fraction (400 µg) was incubated with 12.5 mM of fluorescence-conjugated penicillin V (Bocillin FL; Molecular Probes) at 37°C for 30 min and a 3× sodium dodecyl sulfate (SDS) sample buffer was added, followed by SDS-polyacrylamide gel electrophoresis (PAGE) analysis with a fluorescent image analyzer (LAS-3000 multicolor; Fuji).

Western blotting analysis was performed with rabbit anti-PBP 2X serum raised against a keyhole limpet hemocyanin-conjugated peptide corresponding to the 14 amino acid residues of PBP 2X (amino acids 237 to 250), which is a very conserved region that lacks mutations even in PRGBS.

Allelic exchange experiments. To generate *S. agalactiae* ATCC BAA-611 integrant strains harboring the chromosomally encoded PBP 2X genes derived from the clinically isolated PRGBS strains, we performed allelic exchange experiments, as described previously (4). A thermosensitive *Escherichia coli*-streptococcus shuttle vector, pG+host6, was modified by removing the *Eam11051*-*Aat* II fragment to delete its penicillinase gene, and the resultant vector was desig-

nated pG+host6amp. To generate targeting vectors, fragments containing the nucleotide region from positions 295821 to 297819 of the GBS ATCC BAA-611 genome were amplified from the chromosomal DNA of clinical isolates B12 and B503 with Pyrobest DNA polymerase (Takara) and ligated into pG+host6amp. Several inserts were sequenced, and no additional mutation was found in the inserts. Electrocompetent cells were made from *S. agalactiae* ATCC BAA-611 as described previously (12). One microgram of the targeting vector was introduced into electrocompetent cells derived from GBS strain BAA-611, as described previously (12), and transformants were selected on agar plates containing 0.5 µg/ml of erythromycin at 30°C. To obtain integrant cells in which the targeting vectors had integrated into the chromosomes of the recipient GBS cells, transformants were incubated in liquid medium containing 5 µg/ml of erythromycin at 37°C, as described elsewhere (4). Such integrant strains were successively cultivated for 3 days in liquid medium at 30°C without erythromycin selection to facilitate the excision of vector pG+host6amp. Integrant strains were selected on an agar plate containing 4 µg/ml of ceftizoxime, and susceptibility to erythromycin was confirmed by use of an agar plate containing 5 µg/ml of erythromycin. The presence of the altered PBP 2X gene in each integrant strain was confirmed by sequencing, and the integrant strains were subjected to MIC measurements.

Nucleotide sequence accession numbers. All high-molecular-weight PBP genes derived from the 14 clinical isolates were deposited in the EMBL/GenBank database through the DDBJ database and were assigned accession numbers AB279794 to AB279863.

RESULTS

Confirmatory identification of GBS. From among the 159 clinical strains isolated in 2005, we selected 49 isolates for which the oxacillin MICs were >2 µg/ml by the microdilution method. We also selected from among these 49 isolates 5 isolates that demonstrated high-level resistance to β-lactams. We analyzed nine stocked from 1995 to 1998 and five clinical isolates isolated in 2005. The identities of the 14 clinical isolates were confirmed to be *S. agalactiae* by Gram staining, 16S rRNA PCR followed by restriction enzyme-digested fingerprinting (18), biochemical identification, surface antigen detection, and the CAMP test.

These 14 strains were gram-positive cocci, and no apparent morphological abnormalities were observed at the light microscopy level when the strains were cultured without penicillins (data not shown). The partial 16S RNA gene amplified

TABLE 2. Primers used for PCR amplification and sequencing

Target gene	Sequence	Amplicon size (bp)
<i>pbp1a</i> (SAG0298, gbs0288)	f1 5'-CGGAATTCATGGGATTATTATCTTAGCTA-3'	2,209
	r1 5'-ACGTCGACTTAATACCGTTAGGTAAGTACTGTA-3'	
	f1b 5'-ACACCAAAGAAGAAATTTCTTAC-3'	
	f2 5'-TAAAGCAAAAATCTACTTATCC-3'	
	f2b 5'-GTAGTGAGAAAATGGCAGCGGC-3'	
	f3 5'-GCCTACATGATGACGGATATGC-3'	
	f3b 5'-CAAAATTCTGGACAGTCAAGTC-3'	
	r2 5'-TCCAACTGCACTGTATCCGCC-3'	
	r3 5'-TAGCTGCTTTAGTACCAGTACC-3'	
	r4 5'-CAGCGGCTTCAAGTGCTCTGAC-3'	
	r5 5'-TGACTTTACCATTAGTCGCATC-3'	
	r6 5'-TTTTATCTTGATACATCTGCTG-3'	
<i>pbp1b</i> (SAG0159, gbs0155)	f1 5'-CGGAATTCATGTTTTAAAGGTAATAAGAAGT-3'	2,314
	r1 5'-ACGTCGACTTATCGTTTTCCACCACAAAGTA-3'	
	f1b 5'-GGTTTGGAGAGAGTAGCGG-3'	
	f2 5'-CTATTGTATATTCTCCTTATAC-3'	
	f2b 5'-GTATACTATTAATACTACTATC-3'	
	f3 5'-TGATGTAAAAAATATATGGAG-3'	
	f3b 5'-CCTGTCCGTGCTTTTCGAAAG-3'	
	r2 5'-GTGTAGAAAGCATCAACCAAAAC-3'	
	r3 5'-GAGCAACTGACGTATCAATACC-3'	
	r4 5'-GATCAATAGCAATTCGTAAGG-3'	
	r5 5'-TTTTTAAATCATGCTCTGAAAC-3'	
	r6 5'-GTAAACCTGCAAGGAAAGCTGC-3'	
<i>pbp2a</i> (SAG2066, gbs2020)	f1 5'-CGGGATCCATGAAATTTTGATAAGTTTA-3'	2,338
	r1 5'-ACGTCGACTTCTAAAGTAGTCCTTTAGA-3'	
	f1b 5'-TGCTCTAAAAACAACCACCACC-3'	
	f2 5'-ATCTTAATAACTCTTATTTTGG-3'	
	f2b 5'-GGTATGAAAAATAGATTAGCAG-3'	
	f3 5'-TCCGTGCTTTTACTTTAGAC-3'	
	f3b 5'-ACTCGAATTGAGACAGCTAATG-3'	
	r2 5'-CTGTCAAATAATGGTGTATC-3'	
	r3 5'-ATGAGCGCGATGCATTATACCG-3'	
	r4 5'-GTTCTTTATCTATTGACCATCC-3'	
	r5 5'-TATAGCCATTATTGACAATATC-3'	
	r6 5'-TCAAATTAGCAGCACTGGTTCC-3'	
<i>pbp2b</i> (SAG0765, gbs0785)	f1 5'-CGGAATTCATGTTGAATCGTAAAAAAGGT-3'	2,062
	r1 5'-ACGTCGACTTATTGCTCTGTGAACTGTGAA-3'	
	f1b 5'-TTCATCTCAGTCTATCAAAGAG-3'	
	f2 5'-CTATTTCTACAGAAAAGGCAGG-3'	
	f2b 5'-AGAAAGTATCTTGAACAATAC-3'	
	f3 5'-CAACTCTAATGGAATCGTTCGG-3'	
	f3b 5'-TGGACAAACAGTTTCTACCTAC-3'	
	r2 5'-CTATCTTATTTAGTGTTTTAGG-3'	
	r3 5'-GATAGCCTCGATCAGTTAAAGC-3'	
	r4 5'-CATGATCATTTTTCAGACCAGC-3'	
	r5 5'-CTCGGTCAATTCAGTGAATAGCC-3'	
	r6 5'-TAGCGCTCACTGGAAGTGCAGC-3'	
<i>pbp2x</i> (SAG0687, gbs0277)	f1 5'-CGGAATTCGTGACTTTTTTTAAAAAGCTAA-3'	2,275
	r1 5'-ACGTCGACTTAATCTCCTATTGTAATTTTG-3'	
	f1b 5'-AACTATACGACAGCTACAGGTC-3'	
	f2 5'-GTAGTGGGAATGTTCTTTTAGG-3'	
	f2b 5'-TCTAAGCATTTTAACTCTACTG-3'	
	f3 5'-AAGAAGCAGCTAGTAAAACACG-3'	
	f3b 5'-GAAAATCCAGGTCATGTAGCGG-3'	
	r2 5'-GAACCAGATTACGACGTAATTC-3'	
	r3 5'-CAGATTTTACTGCAACTGATTG-3'	
	r4 5'-ATGAGCTCATAGCGATAGTTAC-3'	
	r5 5'-TTGCAGAGGCTAGAGTCATTAC-3'	
	r6 5'-CCGCCCTACGTTCTGTTGTTGC-3'	
r7 5'-AAGACAATCCTGAACTGAACTTCC-3'		
r8 5'-TATCTGTACCAACGATGATGAC-3'		

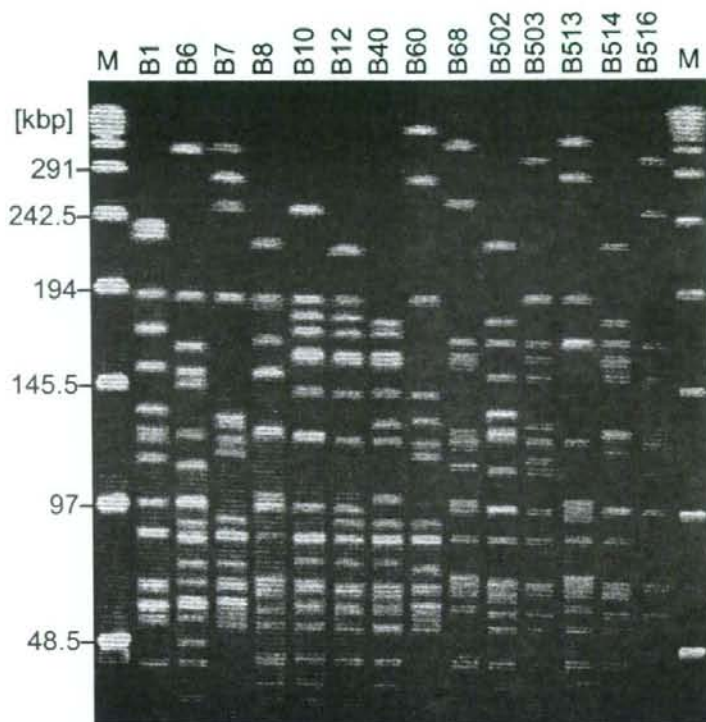


FIG. 1. PFGE of 14 clinical isolates (isolates in lanes B1 to B516). Lanes M, molecular markers.

from chromosomal DNA was fingerprinted with the HaeIII restriction enzyme. The fingerprinting patterns of the 14 strains were identical to those of the standard strains of *S. agalactiae* obtained from ATCC (ATCC BAA-611 and ATCC 12403) (data not shown). All 14 clinical isolates showed biochemical properties consistent with those of *S. agalactiae*. We further performed the streptococcus grouping latex agglutination test with anti-streptococcus-specific surface antigen serum and confirmed the validity of the identification. Finally, the CAMP test, a GBS-specific identification test which detects the production of GBS-specific CAMP factor, confirmed that all the clinical isolates showed the typical phenotype of GBS. The several assays mentioned above clearly showed that these strains must be *S. agalactiae*.

PFGE. We performed PFGE with the 14 clinical isolates (Fig. 1). All clinical isolates except B10 and B12 showed different DNA band patterns, suggesting that all clinical isolates except B10 and B12 were genetically nonrelated strains. Interestingly, B10 and B12, which were clinical isolates from the same hospital, showed very similar DNA band patterns, implying their clonal relatedness.

Measurement of β -lactam MICs. We determined the MICs of several β -lactams for the 14 strains by the agar dilution method recommended by the CLSI (Table 3). The MICs for the control strain, *S. pneumoniae* ATCC 49619, fulfilled the criteria for "quality control" established by the CLSI. All 14 strains, however, showed reduced susceptibilities to penicillin

G (0.25 to 1 μ g/ml), oxacillin (2 to 8 μ g/ml), and ceftizoxime (4 to 128 μ g/ml). Some strains were also "insusceptible" to ampicillin (0.12 to 0.5 μ g/ml), cefazolin (0.5 to 2 μ g/ml), cefepime (0.25 to 1 μ g/ml), and cefotaxime (0.12 to 2 μ g/ml).

Genes for high-molecular-weight PBPs. Alterations in the high-molecular-weight PBPs are widely acknowledged to be the leading molecular mechanisms of resistance to β -lactams in *S. pneumoniae*. To obtain deduced amino acid sequences of the PBPs of the 14 GBS strains, we analyzed the nucleotide sequences of the coding regions for five high-molecular-weight PBPs (PBP 1A, PBP 1B, PBP 2A, PBP 2B, and PBP 2X) (Fig. 2). Some of the 14 clinical isolates possessed a few mutations in PBP 1A (0 to 3 amino acid substitutions and 0 to 4 amino acid deletions and 0 to 10 nucleotide substitutions and 0 to 12 nucleotide deletions), PBP 1B (0 to 1 amino acid substitution and 0 to 4 nucleotide substitutions), PBP 2A (0 to 2 amino acid substitutions and 2 to 11 nucleotide substitutions), and PBP 2B (0 to 1 amino acid substitution and 0 to 2 nucleotide substitutions) (Fig. 2A). On the other hand, all 14 clinical isolates possessed several deduced amino acid substitutions in PBP 2X (2 to 7 amino acid substitutions and 2 to 10 nucleotide substitutions) adjacent to the conserved SSN and KSG motifs (amino acids 402 to 404 and 552 to 554, respectively), considered to form the active site of the enzyme (Fig. 2B and C). PBP 2X of PRGBS did not have a mosaic structure like that of the PBPs of penicillin-resistant *S. pneumoniae* strains. The amino acid substitutions found in PBP 2X of PRGBS could be clas-

TABLE 3. MICs of nine β -lactams for clinical PRGBS isolates

Strain	MIC ($\mu\text{g/ml}$) ^a								
	PEN	PCV	AMP	OXA	CFZ	FEP	CTX	ZOX	MEM
<i>S. pneumoniae</i> ATCC 49619	0.25	0.5	0.12	1	1	0.03	0.03	0.12	0.06
<i>S. agalactiae</i> ATCC BAA-611	0.06	0.03	0.12	0.25	0.12	0.06	0.06	0.12	0.03
<i>S. agalactiae</i> ATCC 12403	0.06	0.03	0.12	0.25	0.12	0.06	0.06	0.12	0.03
<i>S. agalactiae</i>	0.5	0.25	0.12	4	2	0.5	2	128	0.06
B1									
B6	0.25	0.5	0.12	4	1	0.25	1	32	0.06
B7	0.25	0.12	0.12	2	0.5	0.25	0.12	4	0.25
B8	0.25	0.25	0.5	4	1	0.5	0.25	64	0.12
B10	0.5	0.25	0.12	4	1	0.25	0.5	16	0.06
B12	0.25	0.5	0.25	4	1	0.5	0.5	32	0.25
B40	0.5	0.5	0.12	8	1	0.25	0.5	32	0.06
B60	0.25	0.25	0.25	4	1	0.25	0.25	32	0.12
B68	0.5	0.25	0.5	4	0.5	0.5	0.25	4	0.12
B502	0.5	0.25	0.5	4	0.5	0.5	0.25	16	0.25
B503	0.25	0.12	0.5	2	0.5	0.25	0.25	16	0.12
B513	1	1	0.5	8	1	1	1	64	0.25
B514	0.25	0.25	0.5	4	1	0.5	0.25	32	0.25
B516	0.25	0.25	0.25	4	0.5	0.5	0.25	16	0.12

^a Abbreviations: PEN, penicillin G; PCV, penicillin V; AMP, ampicillin; OXA, oxacillin; CFZ, cefazolin; FEP, cefepime; CTX, cefotaxime; ZOX, ceftizoxime; MEM, meropenem.

sified into two types, Q557E and V405A. Among the 14 clinical isolates, all isolates except isolate B7 possessed at least one of these two amino acid substitutions, which existed adjacent to the conserved active-site motifs of PBP 2X. The Q557E of PBP 2X in PRGBS corresponded to the Q552E of PBP 2X found in penicillin-resistant *S. pneumoniae* strains because the amino acid sequence of PBP 2X in GBS showed 74% similarity to that in *S. pneumoniae* R6, and the three active-site motifs in PBP 2X were well conserved between the GBS strains and *S. pneumoniae*. These results indicate that alterations in PBP 2X existing around the regions corresponding to the active-site motifs are the critical determinants responsible for the reduced susceptibility to penicillin G, oxacillin, and ceftizoxime among the penicillin-insusceptible GBS strains.

Visualization and identification of PBPs. To elucidate the resistance mechanisms on a molecular basis, we next visualized the PBPs using fluorescent-conjugated penicillin V, Bocillin FL. Membrane fractions prepared from the 14 clinical strains and the 2 ATCC strains were incubated with Bocillin FL, followed by SDS-PAGE and detection with a fluorescent image analyzer (Fig. 3A and B). The two ATCC strains possessed six apparent PBPs, but one band was eliminated from among all 14 clinical strains, demonstrating penicillin insusceptibility. To identify the eliminated band, we generated rabbit anti-PBP 2X serum by immunization with a synthetic peptide corresponding to the 14 amino acid residues of PBP 2X (amino acids 237 to 250), which is a very conserved region lacking mutations even in PRGBS. Western blotting analysis of the membrane fraction prepared from the two ATCC strains revealed that the band that was commonly eliminated among the 14 clinical strains corresponded exactly to PBP 2X (data not shown).

Western blotting analysis of the 14 clinical isolates with anti-PBP 2X-specific serum revealed that the level of produc-

tion of PBP 2X among the 14 clinical isolates was reduced or eliminated, although the two ATCC strains possessed a significant amount of PBP 2X (Fig. 3C).

Allelic exchange with PRGBS-derived PBP 2X gene. Finally, to assess the contribution of the altered PBP 2X genes to the elevation of penicillin insusceptibility, the PBP 2X genes derived from penicillin-insusceptible clinical isolates were introduced into a β -lactam-susceptible GBS strain, strain ATCC BAA-611, through allelic exchange experiments. One clinical isolate with either type of altered PBP 2X, strain B12 for the Q557E type and strain B503 for the V405A type, was selected, because these strains showed the least number of alterations in the PBP 2X genes. Each type of altered PBP 2X gene from a penicillin-insusceptible strain was transformed into ATCC BAA-611 by integration (Table 4). The MICs for these integrated strains revealed that the reduced susceptibilities to penicillin G, oxacillin, and ceftizoxime were comparable to those of the parental clinical isolates. These results confirmed that both types of altered PBP 2X genes (types Q557E and V405A) are the major determinants of reduced susceptibility to penicillin G, oxacillin, and ceftizoxime in GBS.

DISCUSSION

According to the CLSI criteria, the existence of β -lactam-insusceptible strains of beta-hemolytic *Streptococcus* spp. other than *S. pneumoniae* has not been recognized so far (6). In the present study, however, the 14 clinical isolates, which were donated by geographically separate hospitals and which were not genetically related, obviously showed properties of "insusceptibility" to penicillin G. The MIC measurements revealed that the 14 clinical isolates investigated have MICs indicating penicillin-insusceptible properties (MICs, 0.25 to 1.0 $\mu\text{g/ml}$) greater than the MIC criteria for "susceptibility" to penicillin