Molecular Characterization of Vancomycin-Resistant Enterococcus faecium Isolates from Mainland China

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Little is known about vancomycin-resistant enterococci in China. Thirteen pulsed-field gel electrophoresis-confirmed heterogeneous VanA-type vancomycin-resistant Enterococcus faecium (VRE) isolates were obtained from five Chinese hospitals from 2001 to 2005. The isolates were typed by multilocus sequence typing into nine different sequence types (STs), including five new STs (ST18, ST25, ST78, ST203, ST320, ST321, ST322, ST323, and ST335). Vancomycin resistance in each isolate was encoded on conjugative plasmids; two of the plasmids, pZB18 (67 kbp) and pZB22 (200 kbp), were highly conjugative and were able to transfer at high frequencies of around 10^{-4} and 10^{-7} per donor cell in broth mating, respectively. None of the plasmids identified in these isolates carried traA, which is usually conserved in the pMG1-like highly conjugative plasmid for E. faecium, implying that pZB18 and pZB22 were novel types of a highly conjugative plasmid in enterococci. Thirteen Tn1546-like elements encoding VanA-type VRE on the conjugative plasmids were classified into six types (types I to VI), and most of them contained both IS1216V and IS1542 insertions. The isolates carrying the type II element were predominant. The six type elements were different from that of a VanA-type Enterococcus faecalis strain isolated from Chinese chicken meat. The results suggested that the disseminations of VRE in these areas were by Tn1546-like elements being acquired by the conjugative plasmids and transferred among E. faecium strains.

The isolation of vancomycin-resistant enterococci (VRE) was first reported in 1988 in the United Kingdom and France (17, 29) and then in hospitals in the United States (20). VRE are now encountered in many countries, especially in Europe and the United States (2). There are several reports describing the isolation of VRE in East Asian regions and countries, including Japan, Korea, and Taiwan, and isolation frequencies of VRE from patients and food animals have been increasing both in Korea and in Taiwan (15, 34). Since the first Japanese report of a VanA-type VRE (Enterococcus faecium) clinical isolate in 1996, the frequencies of VRE isolation from patients have also increased (7, 18). However, little information is available on the prevalence of VRE and their molecular makeup from mainland China, although glycopeptide antimicrobials have been used there for decades.

VanA-type resistance, characterized by high-level inducible vancomycin resistance (MICs of 64 to >1,024 μg/ml) and teicoplanin resistance (MICs of 16 to >512 μg/ml), is most frequently encountered (19). The genes encoding VanA-type vancomycin resistance are located on mobile Tn1546-like elements; therefore, the horizontal transfer of resistance genes among enterococci has a more significant impact on the dissemination of VRE than does the clonal spread of resistant enterococci (14). Epidemiological studies of VanA-type enterococci indicate that there are geographic differences (22). Considerable diversity has been identified in the Tn1546-related elements. This variation, in the form of point mutations, insertion sequence (IS) elements, and deletions, has been exploited in several epidemiological studies (33, 31).

Vancomycin has been used in patient care in mainland China for 40 years, and its usage is increasing. Our group has previously described vancomycin-dependent VanA-type VRE strains isolated in Japan from retail chicken meat imported from China (25). Clinical isolates of VanA-type VRE (E. faecium) are rarely obtainable from China. Over the past five years, we have obtained a total of 13 clinical strains of VanA-type VRE (E. faecium) from China. The current report is the first to describe molecular characterization of VanA-type VRE from mainland China.

MATERIALS AND METHODS

Bacterial strains and culture media. Thirteen clinical isolates of vancomycin-resistant E. faecium recovered from blood cultures and urine and sputum samples from patients in China were used in this study (Table 1). VanA-type vancomycin-resistant Enterococcus faecalis strain KC122.1, isolated in Japan from chicken meat imported from China in 2001, was used for the comparative analysis of Tn1546-like elements (25). E. faecium strains BM4105RF and BM410SS were used as recipient strains for transfer experiments (13). Enterococci were grown in Todd-Hewitt broth (THB).

Antimicrobial susceptibility testing. Glycopeptide resistance levels were determined by the agar dilution method. An overnight pure culture of each strain grown in Mueller-Hinton broth (Nissui, Tokyo, Japan) was diluted 100-fold with fresh broth. An inoculum of approximately 5 × 10^5 cells was plated on a series of Mueller-Hinton agar plates (Eiken, Tokyo, Japan) containing a range of concentrations of the test drug. The plates were incubated at 37°C, and the susceptibility results were finalized at 24 h of incubation. Susceptibility testing and interpretation of results were in compliance with standards recommended by...
PCR amplification was performed as previously described with specific primers.

**RESULTS AND DISCUSSION**

**Isolation and characterization of VanA-type vancomycin-resistant E. faecium.** The characteristics of the 13 isolates of vancomycin-resistant E. faecium are listed in Table 1. Each VRE strain was isolated from an individual patient in the hospital. In all cases, glycopeptide antibiotics and/or broad-spectrum cephalosporins were administrated to the patient before the isolation of VRE. All isolates were multidrug resistant, with MICs of 256 to 512 µg/ml and 16 to 512 µg/ml for vancomycin and teicoplanin, respectively. All 13 E. faecium isolates were resistant to erythromycin, and 11 isolates (85%) also showed high-level resistance to ampicillin and gentamicin. All isolates were sensitive to linezolid.

The PFGE profiles of Smal-digested chromosomal DNA demonstrated that strain ZB23 was closely related to strain ZB24, differing by only one band; the other 11 isolates were largely heterogeneous in nature (Fig. 1), indicating that these isolates were unrelated and suggesting that vancomycin resistance was able to emerge in different E. faecium strains.

**MLST analysis.** All isolates were analyzed by the MLST scheme for E. faecium described previously (10, 32; http://www.mlst.net). Allelic profiles of these E. faecium isolates were obtained by sequencing of internal fragments of seven housekeeping genes—*atpA, ddl, gdh, purK, gys, putS*, and *adkC*—and STs were determined (Table 1). In strain ZB18, three of seven alleles, *atpA, adh*, and *pdkC*, belonged to the new alleles, and the closest homologue alleles found were between *atpA* and *atpA15* (81% identity), *adh* and *gdh19* (99% identity), and *pdkC* and *adkC8* (81% identity). Based on the allelic profiles of the 13 isolates, three belonged to ST78 and three to ST203.
for CCS. All isolates were ampicillin resistant, except for two genetically linked to each other and were close to CC17, except (16, 30, 32). The clonal complexes of the Chinese isolates were ZB16, ZB22, ZB19, ZB23, ZB24, ZB21, ZB18, and ZB20, respectively.

and one isolate belonged to ST18 and another to ST25. The remaining five isolates were new STs, designated as ST320, ST321, ST262, and ST17, respectively. In total, nine STs (ST323, ST18, ST322, ST25, ST78, ST203, ST320, ST321, and ST335) of the Chinese isolates were categorized into seven molecular mass marker (Midrange Molecular Marker; New England Bio-labs); lanes 2 to 12, plasmid DNAs from strains ZB11, ZB14, ZB15, ZB16, ZB22, ZB19, ZB23, ZB24, ZB21, ZB18, and ZB20, respectively.

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from the other CCs. Most previously reported hospital outbreak isolates are ampicillin resistant and positive for the esp gene (16). However, only 6 of the 11 ampicillin-resistant isolates in this study were found to carry the esp gene.

Analysis of VanA-type vancomycin resistance genes encoded on Tn1546-like elements. Since, most Tn1546-like elements encoding VanA-type vancomycin resistance are plasmid borne (4), all of the isolates in this study were examined for plasmid content and location of the vanA gene by Southern hybridization. The EcoRI restriction profiles of total plasmid DNAs separated from the VRE strains showed that the plasmids of strain ZB23 were identical to those of strain ZB24 and that the plasmids of strain ZB14 were closely related to those of strain ZB15 (Fig. 2A). Other strains showed heterogeneous plasmid patterns. The vanA probe hybridized to an EcoRI fragment in plasmid DNA from each of the strains (Fig. 2B). These results indicated that all of the VanA determinants (Tn1546-like elements) were carried on plasmids in these VRE strains. The transferabilities of the vancomycin-resistant traits of the strains were examined by broth mating and filter mating with E. faecium BM4105RF used as the recipient strain (Table 1). Vancomycin resistance was transferred at frequencies from $10^{-1}$ to $10^{-7}$ per donor cell by filter mating among the strains studied. The vancomycin resistance of ZB18 and ZB22 was transferred at frequencies of $10^{-4}$ and $10^{-7}$ per donor cell by broth mating, respectively. All of the vancomycin resistance plasmids were self-transferable or mobile. Two highly conjugative vancomycin resistance plasmids, pZB18 (67 kbp) and pZB22 (200 kbp), were isolated from strains ZB18 and ZB22, respectively (Fig. 3). There are two kinds of highly conjugative plasmids found in enterococci, including pheromone-responsive plasmids of E. faecalis and pMG1-like plasmids of E. faecium (3, 13). We previously discovered the highly conjugative gentamicin resistance plasmid pMG1 (65 kbp) from an E. faecium clinical isolate in Japan (13). pMG1-like plasmids were widely disseminated in vancomycin-resistant E. faecium clinical isolates obtained from a hospital in the United States (27). Recently, we also isolated pMG1-like plasmids carrying Tn1546-like transposons that encode vancomycin resistance in E. faecium clinical isolates in Japan (26, 28). All of the pMG1-like plasmids carry a conserved traA gene which is involved in the tra gene.
system for conjugation and is pMG1 specific (24). pZB18 and pZB22 were examined by PCR amplification to determine whether traA was conserved in each of these plasmids. Neither of plasmids carried the traA gene. The result implied that both pZB18 and pZB22 were different from the pMG1-like plasmids and could be a new type of highly conjugative E. faecium plasmid, as previously reported (23).

The DNA sequences of the Tn1546-like elements encoding the vanA operon for vancomycin resistance on the plasmids were determined (Fig. 4) (1). Specific primers for the insertion sequences IS1216V (809bp) and IS1542 (1,324bp), which are often found in Tn1546-like elements, were used in the sequence analysis (8, 31, 33).

A summary of the sequence analysis of the plasmid Tn1546-like elements and their comparison to the prototype element (designated type I in this study) are shown in Fig. 4. The vanS genes of all of the Chinese isolates were identical to that of the BM4147 strain and had no substitutions. Three specific substitutions within VanS result in low-level teicoplanin resistance, which is frequently found in East Asian VRE isolates (9, 15, 18, 34). The Tn1546-like elements of the 13 isolates were classified into six types based on sequence analysis and were designated type I to type VI (Fig. 4). We have reported two VanA-type VRE (E. faecium) clinical isolates, C264 and I125, which were originally isolated from patients in China (35). The Tn1546-like elements of both strains contained the insertion sequences IS1216V and IS1542 and are classified as type V and type VI, respectively (Fig. 4).

Our group reported the first case of VanA-type Enterococcus faecalis: strain KC122.1, isolated in Japan from chicken meat imported from mainland China in 2001 (25). The Tn1546-like element was encoded on a conjugative plasmid and had three specific substitutions of VanS, resulting in low-level teicoplanin resistance, as mentioned above. The Tn1546-like element of KC122.1 was also examined in this study (Fig. 4). The element was identical to the prototype (type I) except for five point mutations, including the three substitutions within VanS, and had no insertions, suggesting that the origins of the elements of VRE clinical isolates were different from that of the VRE isolate from food animals.

Analytical data for the Tn1546-like elements of Chinese VRE isolates can be summarized as follows: (i) all of the elements are plasmid borne; (ii) 12 of the 13 isolates had multiple insertions of IS1216V and IS1542 in the Tn1546-like elements; (iii) IS1542 was inserted into the 8-bp target sequence CTATAATC, from bp 3817 to 3924 of Tn1546; (iv) the origins of the two IS1216V elements differed from each other, and the IS1216V in the vanXY region had one base pair substitution (T662C); (v) the distributions and insertions of IS1216V and IS1542 associated with the Tn1546-like elements of the Chinese isolates were similar to those previously re-

FIG. 2. Agarose gel electrophoresis of EcoRI-digested plasmid DNAs from E. faecium isolates (A) and Southern hybridization analysis with the vanA probe (B). Lanes 1, HindIII-digested lambda DNA; lanes 2 to 12, plasmid DNAs from strains ZB11, ZB14, ZB15, ZB16, ZB22, ZB19, ZB23, ZB24, ZB21, ZB18, and ZB20, respectively.

FIG. 3. Agarose gel electrophoresis of the EcoRI-digested highly conjugative plasmid DNA of pZB18 and pZB22. Lane 1, HindIII-digested lambda DNA; lane 2, pZB18; lane 3, pZB22.
ported for European and Korean VanA-type VRE isolates (11, 22, 31, 34); (vi) Tn1546-like elements were classified into six types, based on DNA sequencing (type I to VI), and type II elements were predominantly isolated from hospitals in Beijing and could be disseminated among different E. faecium strains; and (vii) there was no linkage between VRE isolates from humans (patients) and the VRE isolate from a food animal (chicken meat).

This study is the first to provide detailed molecular analyses of VRE clinical isolates from mainland China. To further elucidate the characteristics of Chinese VRE strains, a nationwide surveillance of VRE and systemic analyses of other types of VRE strains are necessary. In the meantime, the current recommended hospital infection control measures for developed countries may be readily implemented to prevent further spread of VRE in mainland China.

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