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Isolation and molecular characterization of vancomycin-resistant *Enterococcus faecium* in Malaysia

R. Son[1], F. Nimita[2], G. Rusul[2], E. Nasreldin[1], L. Samuel[1] and M. Nishibuchi[3]

[1]Department of Biotechnology, [2]Department of Food Science, Faculty of Food Science and Biotechnology, University Putra Malaysia, Serdang, Selangor, Malaysia, [3]The Center for South-east Asian Studies, Kyoto University, Yoshida, Sakyo-ku, Kyoto, Japan

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R. SON, F. NIMITA, G. RUSUL, E. NASRELDIN, L. SAMUEL AND M. NISHIBUCHI. 1999. Nineteen strains of vancomycin-resistant *Enterococcus faecium* isolated from 10 of 75 (13·3%) tenderloin beef samples were examined for resistance to selected antibiotics, presence of plasmids, and genetic diversity by random amplification of polymorphic DNA analysis. All strains showed multiple resistant to the antibiotics tested. Multiple antibiotic indexing of the vancomycin-resistant *E. faecium* strains showed that all (100%) originated from high risk contamination environments where antibiotics were often used. Plasmids ranging in size from 1·5 to 36 megadalton were detected in 15 of 19 (79%) strains. Thus, three plasmid profiles and eight antibiotypes were observed among the *E. faecium* strains. A high degree of polymorphism was obtained by combining the results of the two primers used; with the 19 *E. faecium* strains being differentiated into 19 RAPD-types. These preliminary results suggest that RAPD-PCR has application for epidemiologic studies and that resistance patterns and plasmid profiling could be used as an adjunct to RAPD for the typing of *E. faecium* in the study area.

INTRODUCTION

In the last decade, enterococci have become increasingly important cause of nosocomial infections Murray 1990; Korten and Murray 1993). Ampicillin and aminoglycosides have been considered the drugs of choice for treatment of serious enterococcal infections (Calia 1996). However, the number of enterococci that are resistant to ampicillin and aminoglycosides has increased (Herman and Gerding 1991). The glycopeptide antibiotics vancomycin and teicoplanin are important substances for treatment of severe hospital infections. Diseases caused by enterococci which are resistant to the β–lactam antimicrobial agent ampicillin and aminoglycoside antibiotics can be treated only with glycopeptides (Lerner 1996). Unfortunately, resistance to vancomycin and teicoplanin has also been reported. In the United States, the Center for Disease Control and Prevention reported that there was a 20-fold increase (1989–90) in the occurrence of vancomycin-resistant enterococci associated with nosocomial infections from animals to humans. The source of glycopeptide-resistant enterococci is not known. One possibility is that these organisms are spread via the food chain. Some data have indicated that raw poultry and raw minced meat may harbour VRE (Bates *et al.* 1994). In this context, adding the glycopeptide avoparcin, a mycelial product of *Streptomyces candidus*, to animal feed was thought to be responsible for the development of glycopeptide resistance in enterococci in animals. This paper describes the characterization of *E. faecium* strains isolated from tenderloin beef retailed in Malaysia with respect to plasmid profiles, antimicrobial resistances and their random amplified polymorphic DNA profiles.

Correspondence to: Dr Son Radu, Department of Biotechnology, Faculty of Food Science and Biotechnology, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia (e-mail: son@fsb.upm.edu.my).

MATERIALS AND METHODS

Samples, isolation and identification of vancomycin-resistant Enterococci

Between July 1997 and March 1998, a total of 75 samples of tenderloin beef were purchased from supermarkets at various

locations in the state of Selangor and the Federal Territory, Malaysia. A 25 g portion from each sample was placed in 225 ml of SF-broth (*Streptococcus faecalis*) and homogenized with a stomacher for 1 min. Then 0·1 ml of the diluted sample was plated onto Slanetz and Bartley agar, supplemented with 20 μg of vancomycin per ml^{-1} (SBA, Merck, Germany). The agar plates were incubated for 24 h at 37 °C aerobically. From each SBA plate, typical red colonies were isolated randomly and used for further investigation. The vancomycin resistance of suspected enterococcal colonies was reconfirmed by subculturing the colonies on sectored Slantez and Bartley agar plates containing 20 μg of vancomycin per ml. The enterococcal isolates were identified by growth and biochemical reactions as described by Devriese *et al.* (1996)

Antibiotic sensitivity

The susceptibility to antimicrobial agents was tested by the standard disc diffusion method (National Committee for Clinical Laboratory Standards 1993). Discs containing the following antibiotics were spotted with a 3 cm interval: ampicillin at 10 μg, kanamycin at 30 μg, bacitracin at 10 μg, gentamicin at 15 μg, streptomycin at 30 μg, erythromycin at 15 μg, chloramphenicol at 30 μg, norfloxacin at 10 μg, nalidixic acid at 30 μg, and vancomycin at 30 μg (BBL Microbiology System, Cockeysville, MD). The plates were incubated for 24 h at 30 °C. The sensitivity or resistance of each tested strain to these antibiotics was measured by the diameter of the inhibition zone around the antibiotic disc. The multiple antibiotic resistance index of the isolates is defined as a/b where 'a' represents the number of antibiotics to which the particular isolate was resistant and 'b' the number of antibiotics to which the isolate was exposed (Krumperman 1983).

DNA extractions

Prior to amplification, total genomic DNA of the Enterococcus faecium strains were extracted by the method described by Ward *et al*. (1994). Small-scale preparation of plasmid DNA from the *E. faecium* strains were obtained by the rapid alkaline extraction procedure described by Sambrook *et al*. (1989), except that mutanolysin (Sigma) at a concentration of 200 units per ml was used. The approximate molecular mass of each plasmid was determined by comparison with plasmids of known molecular mass of *Escherichia coli* V517 (Macrina *et al*. 1978).

RAPD-PCR amplification

PCR assays were routinely performed in a 25 μl reaction mixture containing 20–30 ng of genomic DNA, 2·5 μl 10 X buffer, 1 unit Taq DNA polymerase, 2 μmol primer, 1 mmol each of dCTP, dGTP, dATP and dTTP and 2·5 mmol $MgCl_2$. PCR reactions were performed on a Perkin Elmer 2400 thermocycler (Perkin-Elmer, Norwalk, USA) under the following conditions: 30 cycles of 94 °C for 1 min, 36 °C for 1 min, and 72 °C for 2 min, with a final extension of 72 °C for 5 min. Amplified DNA fragments (15 μl) were electrophoresed through a 1·2% agarose gel according to the method of Sambrook *et al*. (1989). DNA ladder (Promega, USA) was used as DNA size markers. In preliminary experiments, two randomly designed 10-mer oligonucleotide set were obtained from Genosys Biotechnologies Inc. (TX, USA). There were 10 types of content in each set from Gen15001 to Gen15010 and Gen25010 to Gen25020, respectively. The Gen15006 (5'-AGGTTCTAGC-3') and Gen25011 (5'-AAATCGGAGC-3') primers were chosen for RAPD analysis, because on PCR they yielded clear patterns with a subset of five isolates to detect polymorphisms within *E. faecium*.

RESULTS AND DISCUSSION

In this study the isolation of vancomycin-resistant *Enterococcus faecium* in tenderloin beef retailed in Malaysia was investigated to determine the importance of beef as a possible vector for the transfer of vancomycin-resistant enterococci (VRE). Nineteen isolates with the typical characteristics of the species *Enterococcus faecium* were obtained from 10 of 75 beef samples (13·3%). Our results confirm and expand on those reported elsewhere (Bates *et al*. 1994; Devriese *et al*. 1996), who reported on the isolation of *E. faecium* strains from animals. The importance of animal products in the spread of *E. faecium* is well (Bates *et al*. 1994; *Thal* et al. 1995; Klare *et al*. 1995; *Aarestrup* 1995); however, in Malaysia little attention has been paid to the role of beef in disseminating enterococci. For this reason, it is important to monitor potential sources of this pathogen in beef to minimize product contamination. Learning about the ecology and epidemiology of *Enterococcus* spp. can help to identify potential sources of contamination and to trace the spread of vancomycin-resistant enterococci in food sources.

The results of the antimicrobial susceptibility tests are given in Table 1.

The highest prevalence of resistance among the total number of *E. faecium* isolates was shown for bacitracin, erythromycin and streptomycin (100%). The least resistance was observed for ampicillin (32%) and norfloxacin (42%) of the isolates, respectively.

Our results confirms data reported previously by other authors indicating that *E. faecium* are frequently and increasingly demonstrating multiple resistance to antimicrobial agents tested (Gordon *et al*. 1992; Boyle *et al*. 1993) The relatively high incidence of antibiotics resistance observed in our study suggests that these microorganisms are more likely to have a predisposition to develop resistance under con-

Table 1 Strains of Enterococcus faecium used in this study

Strain	Antibiotic resistance*†	MAR‡	Plasmid(s) size (MDa)§	RAPD-type with primers Gen15006	Gen25011
EF1	ApBCmErGmNorSm (1)	0·7	2·6, 1·5 (1)	1	UT
EF2	ApBCmErGmNorSm (1)	0·7	2·6, 1·5 (1)	2	1
EF3	ApBCmErGmNorSm (1)	0·7	36 (2)	3	UT
EF4	ApBCmErGmNorSm (1)	0·7	2·6, 1·5 (1)	4	2
EF5	BCmErGmKmNaNorSm (2)	0·8	2·6, 1·5 (1)	5	3
EF6	BCmErGmKmNaNorSm (2)	0·8	2·6, 1·5 (1)	1	3
EF7	ApBCmErGmKmNaNorSm (3)	0·9	36, 2·6, 1·5 (3)	6	4
EF8	BCmErGmKmNaSm (4)	0·7	36 (2)	6	5
EF9	BCmErGmKmNaSm (4)	0·7	ND	7	6
EF10	BCmErGmKmNaSm (4)	0·7	36 (2)	8	7
EF11	BCmErGmKmNaSm (4)	0·7	ND	8	8
EF12	BCmErGmKmNaNorSm (2)	0·8	ND	6	6
EF13	BCmErGmKmNaSm (4)	0·7	ND	8	9
EF14	BCmErGmKmNaSm (4)	0·7	2·6, 1·5 (1)	9	10
EF15	BErGmKmNaSm (5)	0·6	2·6, 1·5 (1)	10	6
EF16	BErKmNaSm (6)	0·5	2·6, 1·5 (1)	10	11
EF17	ApBErKmNaNorSm (7)	0·7	2·6, 1·5 (1)	10	12
EF18	BErGmKmNaSm (8)	0·6	2·6, 1·5 (1)	6	UT
EF19	BErGmKmNaSm (8)	0·6	2·6,1·5,(1)	11	6

*Tested for ampicillin (Ap), bacitracin (B), chloramphenicol (Cm), erythromycin (Er), gentamycin (Gm), kanamycin (Km), nalidixic acid (Na), norfloxacin (Nor), streptomycin (Sm) and vancomycin.
§†Number in parenthesis indicates antibiotypes group and plasmid patterns group, respectively.
‡MAR – multiple antibiotic resistance.
UT = untypeable.
ND = none detected.

ditions of antibiotic selective pressure or that they share a common environmental and a common mode for developing antibiotic resistance. This assumption was based on the results of the multiple antibiotic resistance index (MAR) of the *E. faecium* strains ranging from 0·5 to 0·9, as shown in Table 1. MAR index values higher than 0·2 are considered to have originates from high risk sources of contamination like humans, commercial poultry farm, swine and dairy cattle where antibiotics are often used. MAR index values of less than or equal to 0·2 indicate a strain originated from animals where antibiotics are seldom or never used (Krumperman 1983). It is generally accepted that bacterial resistance to antibiotics parallels the frequency of use of such agents. Hence, a MAR index of 0·5–0·9 indicates that the *E. faecium* used in this study originated from high risk sources. There is a dearth of information on antibiotic susceptibility of *E. faecium*, especially of animal origin in the study area. To our knowledge, this is the first report describing the isolation and characterization of vancomycin-resistant *E. faecium* from animals in Malaysia.

The results of plasmid profile studies among the *E. faecium* strains are shown in Table 1. It is interesting to note that small plasmids of 1·5 and 2·6 megadalton (MDa) occur in 15 of 19 strains examined. Plasmid of 36 megadalton were found in 4 of 19 strains. Plasmid analysis could provide useful epidemiology information, but plasmid carriage by *E. faecium* used in this study appears to be low, limiting the usefulness of plasmid typing for these isolates. Since all of the *E. faecium* strains we studied have not been examined for their ability to transfer their antibiotic resistance phenotypes, it is not possible to correlate the presence of plasmid with antibiotic resistance with certainty. During the last decade, multiple drug-resistant enterococci have become significantly important from a clinical point of view (Murray 1990; Gray and Pedler 1992; Korten and Murray 1993; Tailor *et al.* 1993), with *E. faecium* considered the second most clinically important species accounting for about 5–10% of all encountered clinical isolates of Enterococcus. Many attempts have been made to show the ability of enterococci to transfer genes encoding for antibiotic resistance with the same or different entero-

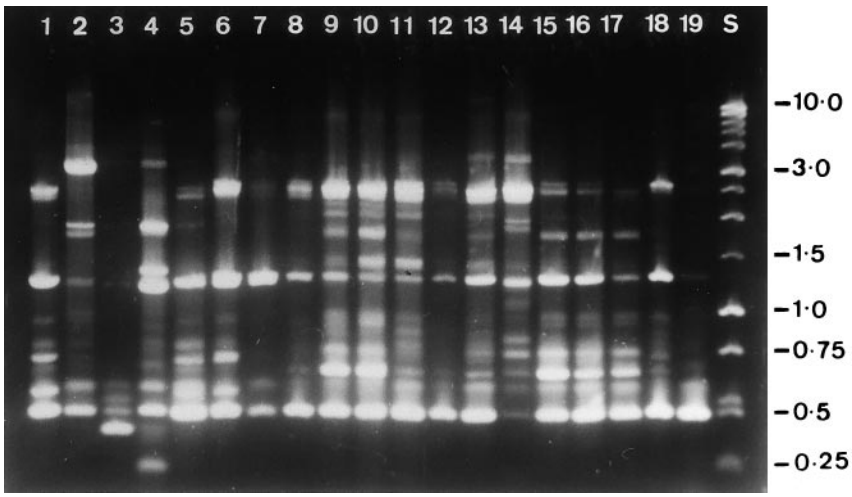

Fig. 1 RAPD fingerprints of vancomycin-resistant *E. faecium* with primer Gen15006. Lanes 1–19 represent strains EF1 to EF19. Lane S contain lambda ladder DNA molecular weight markers (in kilobase pairs, kbp).

coccal species, as well as to other members of other bacteria genera (Leclercq *et al.* 1989; Nicas *et al.* 1989; Noble *et al.* 1992; Boyle *et al.* 1993) Transferable drug-resistant represent a major threat to the treatment of infectious diseases in both humans and animals. Hence the findings in this study are worth taking into consideration, since the contamination of the beef with Enterococcus showing multiple resistance to antimicrobial agents would be a threat not only as a source of disease, but also as a source from which antibiotic resistance can easily spread to other pathogens.

Only two of the 20 primer used (Gen15006 and GEN25011) gave positive results under the PCR conditions used. In order to obtain a sufficient number of polymorphic bands that will permit reliable comparison, the two different 10-mer primers were used in amplification reactions. The results obtained indicate that RAPD provides a high degree of discrimination between *E. faecium* strain, with amplified products ranging from 0·25 to 3·0 kbp (Fig. 1 and 2). The results of RAPD-PCR with primer Gen25011 are shown in Fig. 2. A total of 16 of 19 *E. faecium* isolates generated appropriate fragments, while 3 of 19 isolates failed to produce any signal. The later result can be interpreted as the loss of primer binding sites in the chromosomal DNA of these isolates since these DNAs gave the appropriate bands when examined by using primer Gen15006 (Fig. 1). The discriminatory power was similar for each of the two primers; primers Gen25011 detected 12 RAPD-types, while primer Gen15006 detected 11 RAPD-types. A high degrees of discrimination was observed by combining the results of the two primers; 19 different RAPD-types were obtained for the 19 strains, confirming that all 19 *E. faecium* strains isolated were genotypically distinct. This result is in agreement with findings of other authors who reported on considerable genetic variability within *E. faecium* (Quednau *et al.* 1988). Our study demonstrates that *E. faecium* strains could be easily differentiated by RAPD-fingerprinting, thus supporting the validity of this fast and accurate technique in studying diversity of *E. faecium* populations.

On the basis of their resistance patterns and plasmid profiles, eight antibiotypes and three plasmid patterns were identified among the 19 strains of *E. faecium* (Table 1). The value of using the RAPD-PCR for strains differentiation is self-evident, as the 19 *E. faecium* strains were separated into 19 RAPD-types, indicating that beef is a common source of resistant or multiple resistant *E. faecium* strains belonging to different clonal lineages in the study area.

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REFERENCES

Aarestrup, F.M. (1995) Occurrence of glycopeptide resistance among *Enterococcus faecium* isolates from conventional and ecological poultry farms. *Microb Drug Resist* **1,** 255–257.

Bates, J., Jordan, J.Z. and Griffiths, D.T. (1994) Farm animals as a putative reservoir for vancomycin-resistant enterococcal infection in man. *Journal of Antimicrob Agents Chemother* **34,** 507–516.

Boyle, J.M., Soumakis, S.A., Rendo, A., *et al.* (1993) Epidemiologic analysis and genotypic characterization of a nosocomial outbreak of vancomycin-resistant enterococci. *Journal of Clinical Microbiology* **31,** 1280–1285.

Calia, F.M. (1996) Glycopeptides, polypeptides, tertiary amines, furans, nitroimidazoles, and other organic compounds. In: *Current*

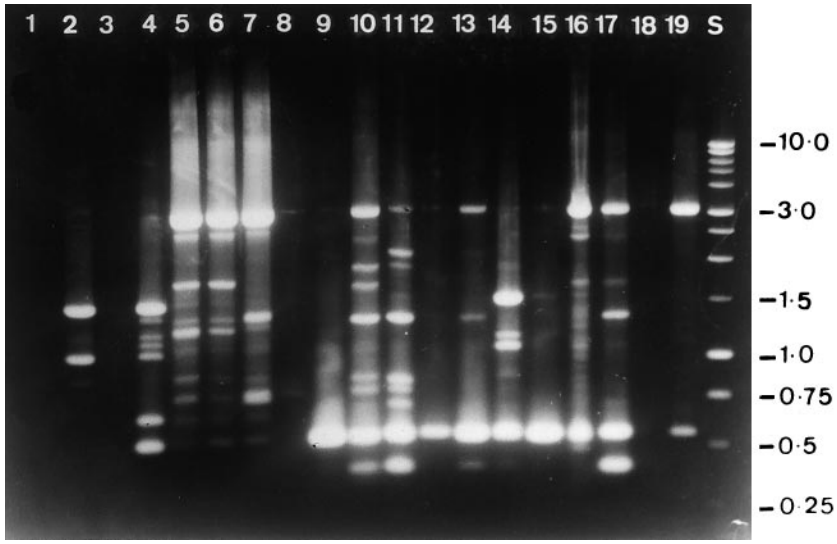

Fig. 2 RAPD fingerprints of vancomycin-resistant *E. faecium* with primer Gen25011. Lanes 1–19 represent strains EF1 to EF19. Lane S contain lambda ladder DNA molecular weight markers (in kilobase pairs, kbp).

Infectious Drugs Current Medicine (ed. Andriolet, V.T.), pp. 117–138. Philadelphia, Pa.

Devriese, L.A., Ieven, M., Goossens, H., *et al.* (1996) Presence of vancomycin-resistant enterococci in farm and pet animals. *Journal of Antimicrob Agents Chemother* **40,** 2285–2287.

Gordon, S., Swenson, J.M., Hill, B.C. *et al.* (1992) Antimicrobial susceptibility patterns of common and unusual species of enterococci causing infections in the United States. *Journal of Clinical Microb* **30,** 2373–2378.

Gray, J.W. and Pedler, S.J. (1992) Antibiotic-resistant enterococci. *Journal of Hosp Infection* **21,** 1–14.

Herman, D.J. and Gerding, D.N. (1991) Antimicrobial resistance among enterococci. *Journal of Antimicrob Agents Chemother* **35,** 1–4.

Klare, I.H., Heier, H.C., Bohme, G. *et al.* (1995) *Enterococcus faecium* strains with vanA-mediated high-level glycopeptide resistance isolated from animal food-stuffs and fecal samples of humans in the community. *Microb Drug Resist* **1,** 265–272.

Korten, V. and Murray, B.E. (1993) The nosocomial transmission of enterococci. *Current Opinions in Infection and Disease* **6,** 498–505.

Krumperman, P.H. (1983) Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Applied Environmental Microbiology* **46,** 165–170.

Leclercq, R., Derlot, E., Weber, M. *et al.* (1989) Transferable vancomycin- and teicoplanin-resistance in *Enterococcus faecium*. *Antimicrob Agents Chemother* **33,** 10–15.

Lemmen, R. and Daschner, F. (1996) Veranderung des Erregerspektrims bei schweren nosokomialen infektionen. *Chemother Journal* **5,** 2–4.

Lerner, S.A. (1996). Aminoglycosides. In: *Current Infectious Drugs Current Medicine* (ed. Andriolet, V.T.), pp. 1–12. Philadelphia, Pa.

Low, D.E., Willey, B.M., Betschel, S. *et al.* (1994) *Enterococcus*: pathogens of the 90s. *European Journal of Surgery* (Suppl.) (573), 19–24.

Macrina, F.L., Kopecko, D.J., Jones, K.R. *et al.* (1978) A multiple-plasmid-containing Escherichia coli strain: convenient source of size reference plasmid molecules. *Plasmid* **1,** 417–420.

Murray, B.E. (1990) The life and times of the *enterococcus*. *Clinical Microbiological Review* **3,** 46–65.

National Committee for Clinical Laboratory Standards (1993) Performance Standards for antimicrobial Disc Susceptibility Tests: tentative Standards, 13, 24 NCCLS document M2–A5. National Committee for Clinical Laboratory Standards, Vallinova, PA.

Nicas, T.I., Wu, C.Y.E., Hobbs, N.R. Jr, *et al.* (1989) Characterization of vancomycin resistance in *Enterococcus faecium* and *Enterococcus faecalis*. *Antimicrob Agents Chemother* **33,** 1121–1124.

Noble, W.G., Virani, Z. and Cree, R.G.A. (1992) Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to Staphylococcus faecalis. *FEMS Microbiological Letters* **93,** 195–198.

Quednau, M., Ahrne, S., Petersson, A.C. *et al.* (1998) Identification of clinically important species of *Enterococcus* within one day with random amplified polymorphic DNA (RAPD). *Current Microbiology* **36,** 332–336.

Sambrook, J., Fritsch, E.F. and Maniatis, M. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Tailor, S.A.N., Bailey, E.M. and Rybak, M.J. (1993) *Enterococcus*, and emerging pathogen. *Annals of Pharmacother* **27,** 1231–1242.

Thal, L.A., Chow, J.W., Mahayni, R. *et al.* (1995) Characterization of antimicrobial resistance in enterococci of animal origin. *Antimicrob Agents Chemother* **39,** 2112–2115.

Ward, L.J.H., Brown, J.C.S. and Davey, G.P. (1994) Application of the ligase chain reaction to the detection of nisin A and nisin Z genes in *Lactococcus lactis* ssp. *Lactis FEMS Microbiological Letters* **117,** 29–34.