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Hemolytic and Nonhemolytic Vancomycin-Resistant *Enterococcus faecalis* Isolated from Beef Imported to Malaysia

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ABSTRACT

Twenty-two strains of vancomycin-resistant *Enterococcus faecalis* were isolated from 9 (6%) of 150 samples of frozen beef and beef products imported to Malaysia. The isolates were obtained from eight samples of beef and one sample of minced beef patty. No *E. faecalis* was isolated from frankfurters. Twelve of the 22 isolates (54.5%) were beta-hemolytic, and all isolates harbored the *vanA* gene. All vancomycin-resistant isolates were also resistant to streptomycin, erythromycin, kanamycin, bacitracin, ceftazimide, gentamycin, tetracycline, nalidixic acid, and teicoplanin; 95.4% were resistant to trimethoprim-sulfamethoxazole; 68.8% were resistant to chloramphenicol; and 41% were resistant to ampicillin and penicillin. Small plasmids ranging in size from 1.5 to 5.8 kb were detected in 8 (36.4%) of 22 strains. The 22 isolates were classified into 20 random amplified polymorphic DNA types. Isolates were divided into two groups, each containing subclusters, that may reflect their clonal lineages. It is concluded that several clones of vancomycin-resistant *E. faecalis* are represented in the isolates obtained from beef imported to Malaysia.

Reservoirs for antibiotic-resistant enterococci have been described *(10, 15, 16, 28)*. There are two types of suspected sources of vancomycin-resistant enterococci (VRE): clinical sources arising from the use of vancomycin in human therapy *(20, 23)*, and foods of animal origin *(3, 5, 25, 36, 40, 41)*. For the VRE isolated from food animal sources, most of the parent vancomycin-susceptible strains were originally endogenous and nonpathogenic to animals. When animals consume avoparcin-supplemented feeds, vancomycin-susceptible strains may undergo selection for resistance to the antibiotic. Vancomycin-resistant strains may not necessarily be pathogenic, because the genes for vancomycin resistance and virulence may be different. Resistant strains can serve as a reservoir pool for vancomycin-resistant genes at the interspecies, intraspecies, and genus levels *(43)*.

Enterococcus faecalis is an opportunistic human pathogen, and a significant percentage of clinical isolates of this microorganism are hemolytic *(6, 21)*. Various factors have been shown to contribute to virulence *(7, 9, 43)*. Only a few studies have been conducted to determine the phenotypic and genotypic relationships of VRE originating from foods of animal origin. Beef is a major food of animal origin in Malaysia, and most beef and beef products are imported. The present study was undertaken to survey frozen beef and beef products imported to Malaysia for the presence of VRE. VRE isolates were characterized with respect to resistance to antibiotics, hemolysin production, plasmid profiles, and genetic diversity by random amplified polymorphic DNA (RAPD) analysis.

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MATERIALS AND METHODS

Isolation and antibiotic susceptibility. One hundred fifty packaged frozen samples of imported beef (87 samples), imported beef frankfurters (24 samples), and imported minced beef patties (39 samples) were collected from supermarkets, wet markets, retail shops, and hawker stalls in Malaysia from July 1997 to July 1998. Twenty-five grams of each sample was pummeled for 2 min in a stomacher (Stomacher Lab-Blender 400, Seward Medical, Ltd., London, UK) in 225 ml of sterile azide dextrose broth (Merck and Co., Inc., Whitehouse Station, N.J.) supplemented with 50 μg of vancomycin (Sigma Chemical Co., St. Louis, Mo.) per ml (ADV) and in 225 ml of tryptic soy broth (TSB; Merck). Mixtures were enriched for 24 h at 37°C.

All samples enriched in ADV and TSB were serially diluted (10^{-1} to 10^{-8}) in ADV and TSB, respectively, and surface plated (0.1 ml) on Slanetz and Bartley agar (SBA; Merck) and bile asculin azide (BAA) agar (Merck), both supplemented with vancomycin (50 μg/ml). Plates were incubated aerobically for 24 to 48 h at 37°C. All typical *Enterococcus*-like colonies *(11)* were subcultured on deMan Rogosa Sharpe (MRS) agar (Merck) and in MRS broth (Merck) and subsequently identified by standard methods (conventional biochemical tests whose results were confirmed with the Biolog system). Isolates were tested for resistance to vancomycin (30 μg) and teicoplanin (30 μg) as described by Wegener et al. *(46)*. Susceptibility to antimicrobial agents was determined by a standard disk diffusion method *(32)*. Disks containing ampicillin (10 μg), kanamycin (30 μg), bacitracin (10 μg), gentamycin (15 μg), streptomycin (30 μg), erythromycin (15 μg), chloramphenicol (30 μg), ceftazimide (30 μg), nalidixic acid (30 μg), penicillin G (10 μg), trimethoprim-sulfamethoxazole (0.25 mg), tetracycline (30 μg), teicoplanin (30 μg), and vancomycin (30 μg) (BBL/Difco, Sparks, Md.) were placed 3 cm apart on the surface of Mueller-Hinton agar (Oxoid, Basingstoke, UK) on which 0.1 ml of an 18-h MRS broth culture was surface spread. Plates were incubated for 24 h at 37°C before they were examined

TABLE 1. *Enrichment broth and selective media from which presumptive positive and confirmed isolates of vancomycin-resistant* E. faecalis *were recovered*

Sample no.	Sample type[a]	Enrichment broth[b]	Selective agar[c]	No. of presumptive positive isolates	No. of confirmed isolates	Isolate(s)
1	FIB	ADV	SBA+Va	8	3	E1–E3
2	FIB	ADV	SBA+Va	7	4	E4–E7
3	FIB	ADV	SBA+Va	8	2	E8, E9
4	FIB	ADV	SBA+Va	5	1	E10
5	FIB	ADV	SBA+Va	5	2	E11, E12
6	FIB	TSB	SBA+Va	6	1	E13
7	FIB	TSB	BAA+Kan	12	2	E14, E15
8	FIB	TSB	BAA+Kan	20	4	E16–E19
9	BP	ADV	SBA+Va	5	3	E20–E22

[a] FIB, frozen imported beef; BP, beef (minced) patty.
[b] ADV, azide dextrose broth supplemented with 50 μg vancomycin per ml; TSB, tryptic soy broth.
[c] SBA+Va, Slanetz and Bartley agar supplemented with 50 μg of vancomycin per ml; BAA+Kan, bile asculin azide agar supplemented with 50 μg of kanamycin per ml.

for zones of inhibition around disks. The multiple antibiotic resistance (MAR) index for each isolate, defined as *a/b,* where *a* is the number of antibiotics to which a particular isolate is resistant and *b* is the number of antibiotics to which the isolate is exposed *(27),* was determined.

Hemolysin activity. Twenty-two isolates of VRE from 150 samples of imported beef and beef products were examined for their ability to produce alpha- and/or beta-hemolysin. Cultures of all strains in the log growth phase were streaked on blood agar (Oxoid) plates containing fresh defibrinated horse blood (5%). Following incubation at 37°C for 24 to 48 h, hemolysin activity, as evidenced by an opaque or clear zone around a colony *(21),* was recorded.

DNA and plasmid extraction. Genomic DNA of *E. faecalis* was extracted by the method described by Ward et al. *(45).* A rapid alkaline extraction procedure was used for small-scale preparation of plasmid DNA from *E. faecalis* as described by Ward et al. *(45)* except that mutanolysin (Sigma) at a concentration of 200 U/ml was used. The approximate molecular size of each plasmid was determined by comparisons with *Escherichia coli* V517 plasmids of known molecular size *(30).* Plasmids were analyzed by assigning isolates to individual plasmid profiles to determine whether common plasmid patterns could be discerned.

***vanA* gene.** The *vanA* gene fragments of *E. faecalis* isolates from genomic DNA preparations were amplified by the procedure described by Klare et al. *(24).* Oligonucleotides used as primers for the amplification of the 377-bp fragment *(13)* of the *vanA* gene and the 433-bp fragment *(18)* of the *vanB* gene were *vanA 1* (5′-TCT GCA ATA GAG ATA GCC GC-3′), *vanA 2* (5′-GG AGT AGC TAT CCC AGC ATT-3′), *vanB 1* (5′-GTG ACA AAC CGG AGG CGA-3′), and *vanB 2* (5′-CCG CCA TCC TCC TGC AAA A-3′). Primers were obtained from Genosys Biotechnologies Inc., (Woodlands, Tex.). Vancomycin-resistant *Enterococcus faecium* BM4147 and *E. faecium* UAA522 were used as positive controls for *vanA* and *vanB* genotypes, respectively. These strains were kindly supplied by Dr. Patrice Courvalin, Institute Pasteur (Paris, France). *E. faecium* ATCC 19434 was used as a negative control for PCR studies. The use of positive control strains with all test isolates provided confidence in PCR results.

RAPD-PCR amplification. Ten 10-mer oligonucleotide sets, each with a G+C content of 50% (Genosys Biotechnologies), were screened for their ability to exhibit a suitable banding pattern. Two of the 10 sets (Gen1-50-01 [5′-GTGCAATGAG-3′] and Gen1-50-10 [5′-CCATTTACGC-3′) produced a clear, reproducible pattern with a subset of five *E. faecalis* strains and were used to analyze all 22 *E. faecalis* isolates. A dendrogram was constructed with the use of the Gel Compare Version 4.1 software package. Cluster analysis of a collection of genomic fingerprints was carried out with this software. Fingerprints were analyzed on a band-based pattern. The similarity index and the percentage of similarity were calculated from the dendrogram with the use of published equations *(34).*

PCR assays were carried out with 25 μl of a reaction mixture containing 20 to 30 ng of genomic DNA, 2.5 μl of 10× PCR buffer (pH 8.8), 1 U of *Taq* DNA polymerase, 2 μmol of primer, 1 mmol of dCTP, 1 mmol of dGTP, 1 mmol of dATP, 1 mmol of dTTP, and 2.5 mmol of $MgCl_2$. PCR reactions were carried out with a Perkin Elmer 2400 thermocycler (Perkin-Elmer, Norwalk, Conn.) with 30 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min followed by a final extension of 72°C for 5 min. Amplified DNA fragments (15 μl) were electrophoresed in a 1.2% agarose gel *(39).* The DNA ladder was used as a size marker (1Kb DNA ladder, Promega, Madison, Wis.).

RESULTS

Twenty-two strains of vancomycin-resistant *E. faecalis* isolates were isolated from 9 (6%) of 150 packaged frozen beef and beef product samples imported to Malaysia (Table 1). Eight isolates were obtained from imported beef and one isolate was obtained from minced beef patties. VRE were isolated from six samples by enrichment in ADV broth and from three samples by enrichment in TSB. No VRE were isolated from frankfurters. It is evident that the use of TSB for enrichment yielded more false-positive VRE isolates. Presumptive positive *E. faecalis* isolates were vancomycin sensitive. Comparative studies involving the use of modified media for the selective isolation of VRE are needed.

All 22 isolates were resistant to streptomycin, erythromycin, kanamycin, bacitracin, ceftazimide, gentamycin, tetracycline, nalidixic acid, teicoplanin, and vancomycin; 95.4% were resistant to trimethoprim-sulfamethoxazole;

TABLE 2. *Characteristics of* E. faecalis *strains isolated from frozen imported beef*

Strain no.	MAR index	Plasmid size (kb)[a]	Hemolytic activity	*van* gene	No. of bands (RAPD type) for primer[b]	
					Gen1-50-01	Gen1-50-10
E1	0.85	ND	None	*vanA*	3 (2)	4 (4)
E2	0.85	ND	None	*vanA*	6 (5)	9 (8)
E3	0.85	ND	Beta	*vanA*	3 (2)	7 (7)
E4	0.93	ND	Beta	*vanA*	9 (8)	4 (4)
E5	0.93	ND	None	*vanA*	6 (5)	9 (8)
E6	0.93	ND	None	*vanA*	7 (6)	2 (2)
E7	0.85	ND	None	*vanA*	5 (4)	11 (10)
E8	1.00	ND	Beta	*vanA*	8 (7)	4 (4)
E9	1.00	ND	None	*vanA*	UT	1 (1)
E10	0.78	3.9 (1)	Beta	*vanA*	6 (5)	7 (7)
E11	0.85	1.5 (2)	Beta	*vanA*	9 (8)	1 (1)
E12	0.85	1.5 (2)	Beta	*vanA*	1 (1)	15 (11)
E13	0.78	ND	Beta	*vanA*	UT	3 (3)
E14	1.00	ND	None	*vanA*	4 (3)	5 (5)
E15	1.00	ND	None	*vanA*	9 (8)	6 (6)
E16	0.85	1.5, 4.2, 5.8 (3)	None	*vanA*	UT	10 (9)
E17	0.93	ND	Beta	*vanA*	6 (5)	2 (2)
E18	0.93	ND	Beta	*vanA*	1 (1)	6 (6)
E19	0.85	1.5 (2)	None	*vanA*	5 (4)	1 (1)
E20	0.78	4.2, 2.3 (4)	Beta	*vanA*	6 (5)	2 (2)
E21	0.78	4.2 (5)	Beta	*vanA*	UT	4 (4)
E22	0.85	4.2, 2.3 (4)	Beta	*vanA*	10 (9)	4 (4)

[a] ND, not detected. The number in parentheses indicates the assigned plasmid profile.
[b] UT, untypeable.

68.8% were resistant to chloramphenicol; and 41% were resistant to both ampicillin and penicillin G. The antibiotic sensitivity, MAR index, plasmid profile, and hemolysin production characteristics of VRE isolates are shown in Table 2. All three isolates obtained from minced beef were positive for beta-hemolysis and harbored a 4.2-kb plasmid. The sizes of plasmids in isolates from frozen beef ranged from 1.5 to 5.8 kb. DNA fragments similar in size to the *vanA* gene (377 bp) and the *vanB* gene (433 bp) in the positive controls were visualized on agarose gels after the amplification of extracted DNA from genomic preparations. The *vanA* gene (377 bp) was detected in all 22 VRE isolates by a specific PCR assay. No fragments corresponding to the *vanB* genotype were detected.

The number of RAPD patterns for each isolate was estimated on the basis of the number of bands. Nine and 11 RAPD types were made apparent by primers Gen1-50-01 and Gen1-50-10, respectively (Table 2). The number of RAPD bands produced by a given primer for an isolate ranged from 0 to 15, with molecular sizes ranging from 0.25 to 10.0 kb. The same RAPD patterns were consistently observed for each isolate in three replicate analyses. Figure 1 shows that the percentages of similarity for the 22 *E. faecalis* isolates ranged from 10 to 100%. Figure 1 also shows that isolates E9 and E11, from two different imported beef samples, had identical RAPD patterns. Isolates E21 and E22 (Table 2), from the same minced beef patty, were beta-hemolytic; isolate E21 contained a 4.2-kb plasmid, while isolate E22 contained 2.3- and 4.2-kb plasmids. Isolates E20 and E22, also from the same minced beef patty, were beta-hemolytic and contained 4.2- and 2.3-kb plasmids but did not have similar RAPD patterns. Thus, plasmid profiles, hemolysin activity, and RAPD profiles proved to be very sensitive and efficient indicators for use in discriminating isolates from the same sample. Similarity percentages for isolates from imported beef ranged from 10 to 95%. Isolates from the same sample as well as those from different samples formed common as well as different clusters, indicating a wide diversity among them. At a 10% similarity level, the 22 strains can be divided into two major groups, with group I comprising isolates E1 through E12, E14 through E17, and E19 and group II comprising isolates E13, E18, and E20 through E22 (Fig. 1). Group I can be further divided into three subgroups (subgroups S1, S2, and S3) and group II can be further divided into two subgroups (subgroups S4 and S5). Table 2 shows the RAPD results obtained with the two primers. The use of the results from both primers increased the number of typing patterns to 20 (9 + 11), demonstrating the discrimination power of RAPD analysis.

DISCUSSION

Bates et al. *(4),* who suspected that VRE were entering the food chain as a result of animal husbandry practices, were among the first authors to discuss animals as a source of VRE. Later reports confirmed the presence of VRE in meats analyzed in Denmark *(46)* and Germany *(17, 25).* In Asia, there is a dearth of information on VRE from animal sources, although there are reports of cases of clinical VRE infections *(37).* Investigations in Japan have revealed the

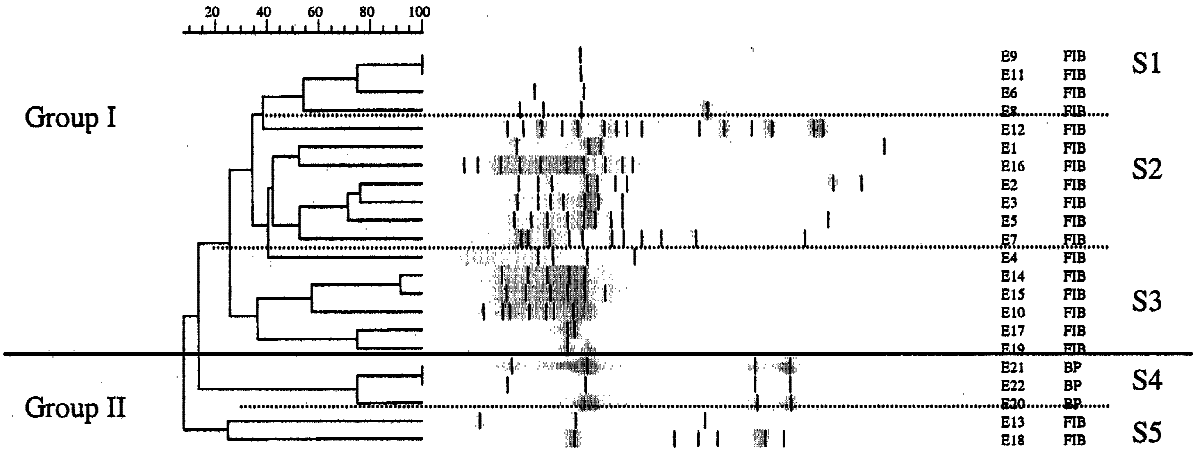

FIGURE 1. *Dendrogram showing similarity percentages for 22* E. faecalis *isolates (isolates E1 through E22) as determined with primer Gen1-50-10. Twenty-two strains are divided into two groups; group I consists of three subgroups (subgroups S1, S2, and S3), and group II consists of two subgroups (subgroups S4 and S5). The scale shows the percentage of similarity. FIB, frozen imported beef; BP, beef (minced) patty.*

presence of VRE in clinical sources *(19)* and imported chicken *(22).* Beef was selected for analysis in our study, and only vancomycin-resistant *E. faecalis* was isolated. This outcome was not unexpected, because it has been reported that one vancomycin-resistant species often predominates in a specific host *(11).* Our results are in agreement with those of studies *(17, 25, 26)* showing that *E. faecalis* dominates pig carcasses and minced beef. The presence of VRE in frozen minced beef imported to Malaysia provides evidence of their ability to survive in foods of animal origin and is consistent with observations by other investigators who have reported retention of viability in adverse environments and on dry surfaces, fingertips, fabrics, and plastics *(33, 35, 47).* Retention of viability in stress environments may facilitate the survival of VRE in beef and beef products and the transmission of VRE to beef by workers in processing plants, in butcher shops, or in the home. VRE may survive processing and remain viable in beef at the point of consumption. Strobbingh et al. *(42)* provided evidence of the transmission of VRE from turkey, turkey farms, and turkey slaughterhouses to humans.

The relatively high prevalence of VRE in beef imported to Malaysia suggests that the strains isolated were likely to have been predisposed to the development of resistance under conditions involving antibiotic selective pressure. This assumption is based on the high MAR indices (0.78 to 1.0) of the isolates (Table 2). MAR index values of >0.2 are considered indicators of high-risk sources of selective pressure for the development of antibiotic-resistant bacteria *(27).* Examples of such high-risk sources include commercial poultry farms, swine, and dairy cattle for which antibiotics are often used. Hence, a MAR index of 0.78 to 1.0 indicates that there is a high risk that beef imported to Malaysia is a source of VRE. The possibility of cross-contamination of some of the test samples exists, even though precautions were taken to avoid such cross-contamination. Notwithstanding this possibility, the presence of VRE in beef imported to Malaysia has been demonstrated. Plasmid analysis can provide useful epidemiologic information *(29),* but no particular plasmid was common among isolates in our study, thus limiting the usefulness of plasmid typing. Only small plasmids, ranging in size from 1.5 to 5.8 kb, were detected. In addition, the ability of bacterial cells to acquire or lose plasmids under various conditions may make plasmid analysis of less value than PCR-based methods in epidemiologic investigations of the incidence of specific VRE in beef and beef products.

There have been reports that urinary tract infections caused by *E. coli* are more likely to involve hemolytic strains *(8, 12, 31). Enterococcus* is also a frequent causative agent of urinary tract infections and can be involved in other diseases, such as endocarditis. It has been reported that strains of enterococci from dairy products do not produce hemolysin *(1, 14),* and it has been suggested that the absence of hemolytic activity should be a criterion for the selection of starter strains to be used in fermented dairy products *(14).* Our results are in agreement with those of Ike et al. *(21),* who reported that 60% of the clinical isolates of *E. faecalis* showed hemolytic activity and only 17% of the fecal specimens from healthy individuals showed hemolysin production. Although the *vanA* and *vanB* genes have been detected in *E. faecalis,* the detection of only the *vanA* gene was expected, since this gene confers a high level of resistance to vancomycin and teicoplanin, whereas the *vanB* gene results in low to very high resistance to vancomycin but susceptibility to teicoplanin.

Examination of the dendrogram constructed on the basis of the RAPD analysis reveals that strains from frozen imported beef and minced beef patties can be divided into two groups that may reflect the clonal lineages of these strains. Strains from the same sample were further observed to differentiate into subclusters within a group. This finding is in agreement with findings by others indicating considerable genetic variability among *Enterococcus* species *(2, 38, 44).* In our study, strains showing 100% similarity to primer Gen1-50-10 did not show 100% similarity to primer Gen1-50-01, illustrating the value of using more than one primer to discriminate strains. Results obtained in the pres-

ent study indicate that RAPD analysis represents a tool for investigating VRE isolates in epidemiologic studies. Our results also further extend the utility of the RAPD technique to the analysis of foodborne VRE.

On the basis of genetic relatedness shown in the phylogenetic tree, substantial heterogeneity exists among strains of VRE isolated from beef and beef products imported to Malaysia. These observations will be valuable in the design of comprehensive studies to determine differences between VRE isolates from clinical and food sources.

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