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NOTE

Isolation, Identification and Characterization of Vancomycin-Resistant Enterococci from Raw Milk

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To determine the occurrence of vancomycin-resistant Enterococci in a raw milk sample, raw milk samples were examined for a period of 6 months. Enterococci were isolated directly from Enterococcal selective agar plates supplemented with 2 mg of vancomycin per liter. Nineteen strains were selected and identified by applying the Vitek system. To determine resistance patterns, 19 isolates were tested with vancomycin and teicoplanin. Vancomycin-resistant Enterococci were genotyped by using a PCR analysis and 5 out of 19 isolates were of the *Van*C type.

Key words: vancomycin-resistant, enterococci, Vitek system, PCR analysis

In the past, enterococcus was the main cause of surgical infections, urinary tract infections and bacteremia in hospitals (Schaberg *et al.*, 1991; Lemmen and Daschner, 1996). Ampicillin and aminoglycoside antibiotics were considered to be appropriate antibiotics to treat infections caused by enterococcus (Calia, 1996), but the rate of resistant bacteria to these antibiotics has increased (Rhinenhart *et al.*, 1990; Herman and Gerding, 1991; Boyce *et al.*, 1992), and as a result, vancomycin and teicoplanin (a type of glycopeptide antibiotics) have become important therapeutic medicines.

Glycopeptide antibiotics are currently the only cure for enterococcus-originated infections, which are resistant to ampicillin and aminoglycoside antibiotics (Calia, 1996; Lerner, 1996). Since vancomycin and teicoplanin-resistant enterococci were discovered, its population has continuously increased, therefore the use of glycopeptide-related antibiotics should be strictly prohibited, otherwise infection resulting from multiple drug-resistant enterococcus will be impossible to treat (Uttley *et al.*, 1988; Bondnar *et al.*, 1996).

High levels of resistance to glycopeptide antibiotics (MIC 512 μg/ml) are inducible. They are found in *Van*A gene clusters which are located in transposons (Shales and Binczeroski, 1990). Although the origin of glycopeptide-resistant enterococci is still unknown, several possibilities are being investigated.

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The increased rate of bacteria that are resistant to glycopeptide antibiotics seems fairly natural, when compared to other antibiotic resistant bacteria (Uttley *et al.*, 1988). It has been suggested that glycopeptide resistant bacteria might have originated in the food chain. According to some reports made recently, fresh chicken or ground meat retains Vancomycin Resistant Enterococcus (VREs) (Bates *et al.*, 1994; Klare *et al.*, 1995). Occasionally, certain glycopeptide antibiotics such as Avoparcin, is added as a growth stimulating agent for livestock feed. The use of glycopeptide antibiotics are, therefore, strictly prohibited in Germany and other European countries for the purpose of preventing the occurrence of bacteria that is resistant to growth stimulating agents containing glycopeptide antibiotics (Bates *et al.*, 1994). Another possible source of VRE is *Enterococcus faecium*, which is used as a starter strain for manufacturing cheese (Auwera *et al.*, 1996).

In this study we determined the occurrence of vancomycin-resistant enterococci in a raw milk sample in an attempt to verify the possibility of the food chain being a source of VRE. We isolated several VRE from raw milk samples and determined the genotypes of the isolates. It is thought based on this study that VRE may be transmitted to humans from raw milk.

Raw milk sample

For this experiment, raw milk samples were received once a week from the Sahmyook milk plant for 6 months.

Isolation and identification of vancomycin resistant Enterococcus

From March to August of 2000, 19 strains of VRE were isolated from raw milk samples obtained from the milk plant. Enterococci were isolated directly from Enterococcus selective agar (Difco) plates supplemented with 2 mg of vancomycin per liter. The isolates were identified using Vitek gram positive idenitfication kit(GPI, version R10-1). Among the 19 isolates, 4 of 19 were *E. faecium*, 10 of 19 were *E. faecalis* and 5 of 19 were *E. casseliflavus/gallinarun* (Table 1).

Antibiotic sensitivity test

Nineteen strains which were isolated from the raw milk samples were tested for resistance to vancomycin and teicoplanin. The MICs of the various antibiotics were determined by the agar dilution method according to the guidelines made by the National Committee for Clinical Laboratory Standards (NCCLS) (National Committee for Clinical Laboratory Standards, 1993). According to the NCCLS guideline (Tenover *et al.*, 1998) 5 of 19 isolates (F-1-4, F-2-1, F-2-2, F-2-4 and F-2-4-4) were shown to have an intermediate level of resistance to vancomycin. Of these isolates, vancomycin MICs of two strains (F-2-2 and F-2-4) were 12.5 μg/ml and those for three strains (F-1-4, F-2-1 and F-2-4-4) were 6.25 μg/ml (Table 2).

Table 1. Species of vancomycin-resistant enterococci isolated from raw milk

Strains	Species
S-1-1	*E. faecium*
S-1-2	*E. faecalis*
S-1-3	*E.faecalis*
S-1-4	*E. faecalis*
S-1-5	*E.faecium*
S-1-6	*E. faecalis*
F-1-4	*E. casseliflavus/gallinarum*
S-2-1	*E. faecalis*
S-2-2	*E. faecalis*
S-2-3	*E. faecalis*
S-2-4	*E. faecalis*
S-2-5	*E. faecium*
S-2-6	*E. faecium*
F-2-1	*E. casseliflavus/gallinarum*
F-2-2	*E. casseliflavus/gallinarum*
F-2-3	*E. faecalis*
F-2-4	*E. casseliflavus/gallinarum*
F-2-4-4	*E. casseliflavus/gallinarum*
F-2-9	*E. faecalis*

PCR analysis of gene confirmation

In an attempt to verify the presence of vancomycin

Table 2. Antibiotic resistant patterns of enterococci isolated from raw milk

Strains	MIC (μg/ml)				
	VAN	TEI	OXA	CFTX	GM
S-1-1	1.57	3.13	6.25	12.5	3.13
S-1-2	1.57	3.13	3.13	12.5	6.25
S-1-3	1.57	3.13	3.13	6.25	6.25
S-1-4	3.13	3.13	12.5	1.6	6.25
S-1-5	3.13	3.13	12.5	1.6	6.25
S-1-6	3.13	3.13	6.25	0.8	6.25
F-1-4	6.25	0.8	12.5	50	3.13
S-2-1	1.57	1.57	3.13	3.13	3.13
S-2-2	3.13	3.13	6.25	1.6	6.25
S-2-3	1.57	3.13	6.25	1.6	1.6
S-2-4	1.57	1.57	6.25	1.6	6.25
S-2-5	3.13	3.13	3.13	1.6	3.13
S-2-6	3.13	6.25	6.25	12.5	1.6
F-2-1	6.25	1.57	12.5	3.13	3.13
F-2-2	12.5	1.57	6.25	1.6	6.25
F-2-3	3.13	0.8	3.13	1.6	6.25
F-2-4	12.5	0.8	3.13	3.13	6.25
F-2-4-4	6.25	0.8	3.13	1.6	3.13
F-2-9	1.57	3.13	3.13	1.6	6.25

VAN; vancomycin, TEI; teicoplanin, OXA; oxacillin, CFTX; cefotaxim, GM; gentamicin

Table 3. PCR primer for detection of VanA, VanB, VanC-1, VanC-2, VanD and VanE

Target gene	Primer designation	Primer 5'-3'	Product size (bp)
VanA	VanA1	GCT ATT CAG CTG TAC TC	783
	VanA2	CAG CGG CCA TCA TAC GG	
VanB	VanB1	CAT CGC CGT CCC CGA ATT TCA AA	297
	VanB2	GAT GCG GAA GAT ACC GTTG GCT	
VanC-1	VanC1-1	GGT ATC AAG GAA ACC TC	822
	VanC1-2	CTT CCG CCA TCA TAG CT	
VanC-2	VanC2-1	CTC CTA CGA TTC TCT TG	439
	VanC2-2	CGA GCA AGA CCT TTA TG	
VanD	VanD1	TAA GGC GCT TGC ATA TAC CG	461
	VanD2	TGC AGC GAA GTA TCC GGT AA	
VanE	VanE1	TGT GGT ATC GGA GCT GCA AG	604
	VanE2	GTC GAT TCT CGC TAA TCC	

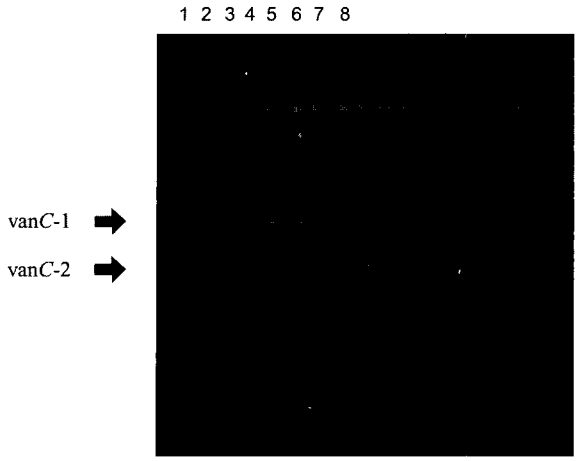

Fig. 1. Detection of the *Van*C gene in VRE from raw milk. Lane1; DNA size marker, 2; standard strain *E. casseliflavus* VanC-2 (439 bp), 3; standard strain *E. gallinarum* VanC-1 (822 bp),4; F-1-4, 5; F-2-1, 6; F-2-2, 7; F-2-4, 8; F-2-4-4, respectively. Each PCR mixture contained a PCR buffer [10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.01% (w/v) of gelatin], each dNTPs at a concentration of 200 μM, each primer at a concentration of 2 bp M (C1,1, C1,2, C2,1, C2,2,), 1unit of *Taq* polymerase (Takara Shuzo Co. Japan) and 20 ng of template DNA ($CHCl_3$ extracts) including water to bring the volume up to 50 μl. A Mastercycler personal cycler (Eppendorf, Germany) was programmed for 30 cycles; predenaturation for 10 min at 95°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 58°C, 1 min at 72°C, and finally 10 min at 72°C. Gel electrophoresis was performed for 90 min in a 2% agarose gel at 100 V.

resistant genes among the VRE isolates a PCR was performed using specific primers for each *van* gene (Table 3) as described by Ausubel *et al.* (Ausubel *et al.*, 1991; Klare *et al.*, 1995). *E. casseliflavus* (*Van*C-2) and *E. gallinarum* (*Van*C-1) were used as reference strains.

Out of 15 isolates, we detected the presence of only *VanC* in 5 isolates: no other *van* gene was amplified using various specific primers (Fig. 1). 3 out of 5 isolates that showed the presence of vanC gene, were identified to be type 1 and the other 2 to be type 2. It is thought that others may contain unidentified vancomycin resistant genes. We concluded that it is possible that vancomycin resistant entercoccus may be originated from a food source such as raw milk. Thus, we recommend periodical examinations of raw milk samples to determine the occurrence of VRE's.

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