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Antimicrobial Resistance Patterns of Vancomycin-Resistant *Streptococcus equinus* Isolated from Animal Foods and Epidemiological Typing of Resistant *S. equinus* by Microbial Uniprimer Kit

Sung-Sook Choi, Jin Woo Lee[2], Byoung-Yong Kang[1], and Nam-Joo Ha[2]

Department of Food Science, Sahmyook College, Seoul 139-742, [1]Research Institute for Life Science, Sahmyook University, Seoul 139-742, Korea, and [2]Department of Pharmacy, Sahmyook University, Seoul 139-742, Korea

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Raw milk samples, and cow and chicken intestines were tested to isolate vancomycin-resistant, gram-positive bacteria. From these samples, we isolated seven vancomycin-resistant *Streptococcus equinus*, two vancomycin-resistant viridans *Streptococcus* and two vancomycin-resistant *Enterococcus faecium*. The MICs of several antibiotics, including vancomycin, against these strains were tested. Seven isolates of *S. equinus* showed high level resistance to vancomycin and teicoplanin (>100 μg/mL). The cell wall thickness of these strains was compared with that of the sensitive strain by TEM and no differences were obserbed between these strains. We compared the strains of vancomycin-resistant *Streptococcus equinus* using PCR with Microbial Uniprimer Kit. We concluded that it is necessary to combine other methods in order to cluster and identify all isolates of *S. equinus*.

Key words: Vancomycin-resistant gram-positive bacteria, *Streptococcus equinus*, MIC, Cell wall thickness, Epidemiological typing

INTRODUCTION

Glycopeptide antibiotics are currently used for the treatment of infections caused by gram-positive bacteria (Mainardi *et al.*, 1998). They form a complex with the C-terminal D-alanyl-Dalanine (D-Ala-D-Ala) of peptidoglycan precursors and block their incorporation into the bacterial cell wall (Reynolds, 1989). Following the emergence of glycopeptide-resistance in enterococci, a discussion on the use of growth promoting antibacterial agents has arisen. The fear of possible transfer of resistance genes from animal bacteria to human bacteria led to the ban on the use in animal feeds of most growth promoting antibiotics in the European Community (Bates *et al.*, 1994; Butaye *et al.*, 1999). 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 from the food chain. According to some recent report, 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, are added as a growth-stimulating agent for livestock feed. The use of glycopeptide antibiotics is strictly prohibited in Germany and other European countries to prevent 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 (Van der Auwera *et al.*, 1996). There is a possibility that glycopeptide antibiotics used as growth-stimulating agents create vancomycin-resistant bacteria in animals and further move through the human body in resistance gene movement. We have already reported that Glycopeptide Intermediate Coagulase Negative Staphylococci (GICNS) from raw milk samples have thickened cell wall and that *Enterococcus gallinarum/casseliflavus* from raw milk samples have *vanC*-1 and *vanC*-2 genes respectively. (Ha and

Correspondence to: Nam Joo Ha, Ph.D., Dept. of Pharmacy, Sahmyook University, 26-21, Kongreung-2Dong, Nowon-Gu, Seoul 139-742, Korea
Tel: 82-2-3399-3653, Fax: 82-2-948-5370
E-mail: hanj@syu.ac.kr

Choi 2001; Choi *et al.*, 2002). In order to further justify the experiment, we studied the intestine of cow, chicken, and raw milk samples. As a result, *Streptococcus equinus,* which have a high level of vancomycin-resistance, were isolated. *S. equinus* is group D streptococcus that does not necessarily cause serious disease in human, but be a moderate threat to patients with depressed immunity, possibly causing endocarditis or otitis (Farrow *et al.*, 1984). In this research we isolated and identified *S. equinus* from different samples and tested the antibiotic sensitivity and molecular epidemiological variations.

MATERIALS AND METHODS

Bacterial strains

Seven strains of *Streptococcus equinus* isolated from raw milk samples, and cow and chicken intestine, and *Streptococcus equinus* ATCC 9812 were used in this experiment.

Anmal food samples

Raw milk samples collected from Sahmyook University milk plant, cow intestines collected from a slaughterhouse, and chicken intestines, purchased from a conventional market, were used to isolate glycopeptide-resistant, gram-positive bacteria.

Isolation and identification of vancomycin-resistant, gram-positive bacteria

Vancomycin-resistant strains were isolated directly from Enterococci selective agar (Difco) plates supplemented with 2 μg/mL of vancomycin. The isolated strains were identified using Vitek gram-positive identification kit (GPI, version R10-1) (Stager and Davis 1992).

Antibiotics sensitivity test

Twelve strains, which were isolated from animal food samples, were tested to determine their sensitivity against the following antibiotics: vancomycin (Eli Lilly Benelux, Brussels, Belgium), teicoplanin (Hoeschst Marion Roussel, Romainville, France), gentamicin (Sigma), ampicillin (Sigma), ampicillin + sulbactam (Pfizer), cefotaxime (Hanil Pharm. Co.), chloramphenicol (sigma), oxacillin (Sigma) and oxazolidinone (Pharmacia Corp., Kalamazoo, Mich.). The minimum inhibitory concentration (MICs) of various antibiotics was determined by the agar dilution method according to the guidelines set by the National Committee for Clinical Laboratory Standards (NCCLS) (National Committee for Clinical Laboratory Standards, 1998).

Electron micrographs of vancomycin-resistant strains

To compare the cell wall thickness between vancomycin-sensitive control strain and vancomycin-resistant strains, transmission eletron microscope (TEM) was observed as described by Manin *et al.* (Manin *et al.*, 1994). The vancomycin-resistant *S. equinus* isolated from animal foods was cultured in the presence of vancomycin. The cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2), containing 2% paraformaldehyde for 6 h at 4°C. For the contrast amplification, the cells were treated with 1% oxonium tetroxide in 0.1 M sodium phosphate buffer (pH 7.2) for 2 h at 4°C. The cells were dehydrated with 70% ethanol for 18 h and embedded in Poly/bed 812 (Polyscience, Inc.). Ultrathin sections were stained with uranyl acetate and lead citrate and then examined with TEM (Hitachi, H-600). Calculation of cell wall thickness was performed by means of photographs taken at a final magnification of ×40,000.

Amplification of *vanA*, *vanB* genes by PCR

In an attempt to verify the presence of vancomycin-resistant genes (*vanA* and *vanB*) among the vancomycin-resistant *S. equinus* isolates, PCR was performed using specific primers for each *van* gene as described by Ausubel *et al.* (Ausubel *et al.*, 1991; Klare *et al.*, 1995). Genomic DNA was extracted by using DNA extraction kit (Promega, Co. Ltd., Madison, America). Cells were lysed with 5 mg/mL of lysostaphin for 2 h at 37°C. A Perkin-Elmer Cetus DNA thermocycler was programmed with the following condition: initial denaturation, 10 min. at 95°C; 30 cycles with a 30 s denaturation step at 94°C, a 30 s annealing step at 58°C and a 30 s extension step at 72°C; a 10 min extension step at 72°C; and a holding step at 4°C until the sample was analyzed. Samples of the PCR products were electrophoresed, stained with 10 M ethidium bromide and visualized and photographed by using UV transillumination. The control strains of enterococci used in this study included *E. faecalis* A256 (*vanA*) and *E. faecalis* V583 (*vanB*). Depending on the MIC results, *S. equinus* isolates were not tested for the presence of the *vanC* gene.

PCR-RAPD

Genomic DNA was extracted by using DNA extraction kit (Promega, Co. Ltd., Madison, America). Four primers of 20mer were synthesized by Seoulin Bioscience Institute, Seoulin Bioscience, Co. Ltd., Seoul, Korea (Table I). PCR reactions were carried out in 50 μL reaction mixtures containing the DNA template (20 to 50 ng of purified DNA), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM of each dNTP, 200 ng primer and 2.5 unit *Taq* DNA polymerase (Promega, Co. Ltd., Madison, USA). The reaction mixture was overlaid with a thin layer of sterile mineral oil to prevent evaporation. DNA amplification was performed in a programmable DNA thermal cycler (Perkin-Elmer Cetus, Inc., USA). The cycling parameters used

Table I. Description of oligonucleotide sequences of 4 microbial uniprimers

Primers	Sequence(5-3)
Microbial Uniprimer1	5-ATCCAAGGTCCGAGACAACC- 3
Microbial Uniprimer2	5-CCCAGCAACTGATCGCACAC- 3
Microbial Uniprimer3	5-GTGTGCGATCAGTTGCTGGG- 3
Microbial Uniprimer4	5-AGGACTCGATAACAGGCTCC- 3

were initial denaturation at 94°C for 4 min, followed by 35 cycles each consisting of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C (Kang *et al.*, 2002). After the last cycle, the PCR tubes were incubated at 72°C for 7 min and were held at 4°C. Amplified products were electrophoresed in a 1.5% agarose gel in TAE buffer and visualized by staining with ethidium bromide. Amplification reaction was performed twice to check the consistency and reproducibility of the method.

RESULTS AND DISCUSSION

Vancomycin resistant gram-positive bacteria isolated from animal foods

Twelve isolates of vancomycin-resistant, gram positive strains were isolated directly from animal foods samples and identified by Vitek gram-positive identification kit (GPI, version R10-1). Seven of 12 isolates were *Streptococcus equinus*, two were viridans *Streptococcus* and three were *Enterococcus faecium* (Table II).

Minimum inhibitory concentration

The MICs of several antibiotics, including vancomycin, against these 12 strains were tested. All of *S. equinus* showed high level resistance to vancomycin and teicoplanin (>100 μg/mL). In addition, viridans *Streptococcus* showed high-level resistance to vancomycin and teicoplanin. However, their resistance patterns to other antibiotics, which are not commonly used as growth promoting agents, were moderate or sensitive. This results means that the glycopeptide antibiotics might be used as growth promoting agents in Korea (Table III).

Table II. Species of Vancomycin-Resistant, Gram-Positive Bacteria Isolated from Animal Foods

Strains	Species	Origin
M-2	*Streptococcus equinus*	Raw milk
M-6	*Streptococcus equinus*	Raw milk
M-10	*Streptococcus equinus*	Raw milk
B-13	*Streptococcus equinus*	Intestine of cow
B-14	*Enterococcus faecium*	Intestine of cow
B-15	*Streptococcus equinus*	Intestine of cow
P-23	*Streptococcus equinus*	Intestine of chicken
P-25	*Streptococcus equinus*	Intestine of chicken
P-27	*Enterococcus faecium*	Intestine of chicken
P-43	viridans *Streptococcus*	Intestine of chicken
P-45	viridans *Streptococcus*	Intestine of chicken
P-59	*Enterococcus faecium*	Intestine of chicken

Electron micrographs of vancomycin-resistant strains

According to some reports, *S. aureus*, which showed reduced susceptibility to glycopeptide antibiotics, has

Table III. Antibiotic Resistance Patterns of Gram Positive Bacteria Isolated from Animal Foods

Strains	MIC (μg/mL)								
	VAN	TEI	OXA	OZD	CFTX	AMP	GM	AMP+sul	CM
S. equinus ATCC9812	0.8	<0.05	0.2	0.1	<0.05	0.2	<0.05	0.4	0.2
S. equinus M-2	>100	>100	12.5	3.13	0.4	6.25	0.4	12.5	25
S. equinus M-6	>100	>100	6.25	3.13	0.4	6.25	0.4	12.5	25
S. equinus M-10	>100	>100	12.5	3.13	25	12.5	3.13	25	50
S. equinus B-13	>100	>100	12.5	6.25	25	12.5	3.13	50	25
E. faecium B-14	12.5	1.6	>100	3.13	100	25	12.5	50	100
S. equinus B-15	>100	>100	12.5	6.25	12.5	12.5	0.8	25	25
S. equinus P-23	>100	>100	12.5	1.6	25	12.5	0.8	25	25
S. equinus P-25	>100	>100	6.25	3.13	12.5	12.5	0.8	12.5	25
E. faecium P-27	12.5	1.6	>100	1.6	>100	25	>100	50	>100
viridans *Streptococcus* P-43	>100	>100	25	3.13	25	12.5	0.1	25	25
viridans *Streptococcus* P-45	>100	>100	2.5	3.13	25	12.5	0.1	25	25
E. faecium P-59	12.5	1.6	>100	1.6	>100	25	12.5	50	>100

VAN: vancomycin, TEI: teicoplanin, OXA: oxacillin, OZD: Oxazolidinone, CFTX: cefotaxime, AMP: ampicillin, GM: gentamicin, Amp+su l: ampicillin + sulbactam, CM: chloramphenicol.

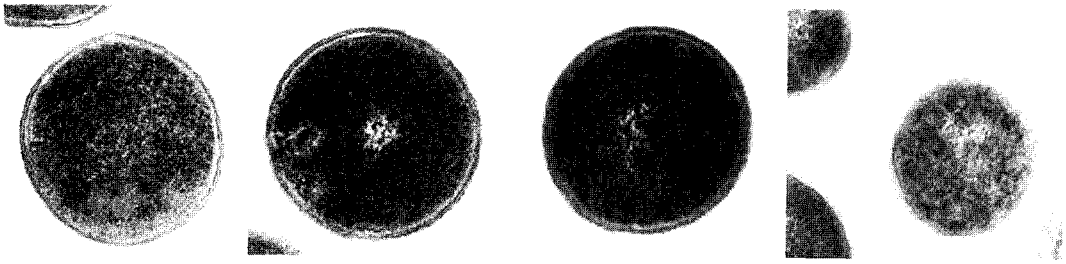

Fig. 1. Comparison of cell-wall thickness of the *S. equinus* isolates after cultivation in the presence of vancomycin. Magnification, x40,000. A, *S. equinus* M-6; B, *S. equinus* B-13; C, *S. equinus* P-23; D, *S. equinus* ATCC9812.

thickened cell wall (Manin *et al.*, 1994; Ha and Choi 2001). We tried to detect any differences of cell wall thickness between resistant strains and the sensitive strain. We observed the TEM of 3 of 7 *S. equinus* isolates according to their origin. The vancomycin-resistant *S. equinus* M-6 (isolated from raw milk), B-15 (isolated from cow intestine) and P-23 (isolated from chicken intestine) were cultured in the presence of vancomycin, and *S. equinus* ATCC 9812 was used as a glycopeptide-sensitive control strain. Fig. 1 shows photographs of ultrathin sections of both vancomycin-resistant *S. equinus* and vancomycin-sensitive control strain. The cell wall thicknesses of vancomycin-resistant *S. equinus* were 20-24 nm and 18-22 nm in the case of vancomycin sensitive control strain. There was no significant difference in cell wall thickness between resistant strains and the sensitive control strain. This result confirms that the resistance mechanism of *S. equinus* isolated from animal foods was not due to the increased thickness of cell wall.

Amplification of *vanA*, *vanB* genes by PCR

In the case of enterococci, which showed high level resistance to glycopeptide antibiotics having *vanA or vanB* genes (Uttley *et al.*, 1988), we tried to verify the presence of vancomycin-resistant genes among these 7 isolates of *S. equinus*. The 7 isolates of *Streptococcus equinus* and a standard strain were analyzed with *vanA* and *vanB* primers, and the presence of *vanA* and *vanB* genes was tested. No genes were detected in any of the 7 isolates, indicating that they have no *vanA* or *vanB* genes. So their high level resistance was not due to *vanA* or *vanB* genes (data not shown). Therefore, to certify the resistance mechanism of these strains, it is necessary to find out other *van* genes (*van D*) or other resistance determinants.

PCR-RAPD

The 7 isolates of *Streptococcus equinus* and the standard strain *S. equinus* ATCC9812 were analyzed with all four primers, and the RAPD profiles were compared. Two oligonucleotide primers (Microbial Primer 1 and 4) among the four tested were not found to generate any RAPD profiles from any isolates of *Streptococcus equinus.* The remaining two primers (Microbial Uniprimers 2 and 3) generated RAPD banding patterns with DNA from this species

Fig. 2 shows the genomic fingerprinting patterns of *Streptococcus equinus* isolates using Microbial Uniprimer 2. With PCR-RAPD, we found that the size of bands generated varied from 1.5 kb to 200 bp. All isolates of the *Streptococcus equinus* species tested produced unique RAPD profiles. Furthermore, all isolates of this species could be clearly distinguished from a standard strain from ATCC.

With PCR-RAPD using Microbial Uniprimer 3, we found that the number of bands generated varied from about 2 kb to 200 bp (Fig. 3). Like Microbial Uniprimer 2, all isolates of the *Streptococcus equinus* species could be clearly differentiated from one another as well as from a standard strain from ATCC.

RAPD technique has been extensively used to compare numerous bacterial species or strains within species (Welsh and McClelland, 1990; Mazurier *et al.*, 1992; McMillin and Muldrow, 1992; MacGowan *et al.*, 1993; Sandery *et al.*, 1994). However, this method requires very low annealing temperatures (about 35~37°C) as well as relatively short primer sequences (about 10 mer). As a result, non-specific

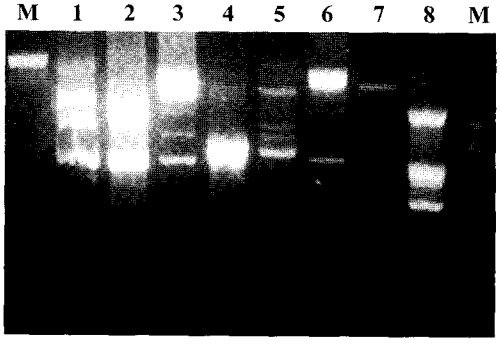

Fig. 2. Genomic fingerprint of *Streptococcus equinus* species by using microbial Uniprimer 2. Lane M, size marker; Lane 1, *S. equinus* M-2; Lane 2, *S. equinus* M-6; Lane 3, *S. equinus* M-10; Lane 4, *S equinus* B-13; Lane 5, *S equinus* B-15; Lane 6, *S equinus* P-23; Lane 7, *S equinus* P-25; Lane 8, *Streptococcus equinus* ATCC9812.

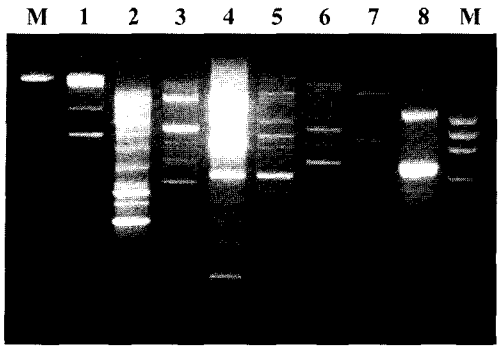

Fig. 3. Genomic fingerprint of *Streptococcus equinus* species by using microbial Uniprimer 3. Lane M, size marker; Lane 1, *S. equinus* M- 2; Lane 2, *S. equinus* M-6; Lane 3, *S. equinus* M-10; Lane 4, *S. equinus* B-13; Lane 5, *S. equinus* B-15; Lane 6, *S. equinus* P-23; Lane 7, *S. equinus* P-25; Lane 8, *Streptococcus equinus* ATCC9812.

bands are frequently formed by PCR reaction, and cause low reproducibility (Wu *et al.,* 1991). On the other hand, the fingerprinting technique using Microbial Uniprimer can solve this problem by use of relatively long primer and high annealing temperature.

In order to test the reproducibility of Microbial Uniprimer, all fingerprinting experiments were performed twice, and the two repeated experiments produced the similar results. Therefore, our results using Microbial Uniprimers showed the high reproducibility in the epidemiological typing of *Streptococcus equinus* isolates.

We also tested the applicability of PCR-RAPD using Microbial Uniprimers in typing the isolates of *Streptococcus equinus* from various origins. With PCR-RAPD by using Microbial Uniprimers 2 and 3, we found a high-level of diversity among banding patterns of the 7 isolates of *Streptococcus equinus* and a standard strain from ATCC. All samples yielded distinct RAPD patterns from one another. The finding that each of *Streptococcus equinus* isolates produced a unique RAPD profiles suggests that this species is genetically very heterogeneous. Therefore, our RAPD method with Microbial Uniprimer 2 and 3 may be an important tool for differentiating the clinical isolates of *Streptococcus equinus* species. However, our results could not confer any information for the clustering of the different isolates of this species or for precise identification of isolates through the comparison with a standard strain based on the RAPD patterns. Therefore, it is necessary to combine the PCR-RAPD method with other methods such as pulsed-field gel electrophoresis (PFGE), ribotyping or plasmid analysis in order to cluster and identify all isolates from *Streptococcus equinus* (Tang *et al.,* 1997).

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