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Detection of *van*C1 and *van*C2 Genes in an *Enterococcal* Isolate and *van*C Genes in non-Motile *Enterococcus* spp.

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Background: In recent decades, bacterial antibiotic resistance (especially in *enterococci*) has become a significant problem for human and veterinary medicine. One of the most important antibiotic resistances in *enterococci*, vancomycin resistance, is encoded by *van* gene family.
Objectives: The aim of this study was to investigate antibiotic resistance to vancomycin in *enterococci* and the genes responsible for this resistance.
Materials and Methods: Two-hundred and thirty *enterococcal* isolates from pigs (207 isolates), chickens (15 isolates) and humans (eight isolates) were phenotypically and genotypically tested for resistance to vancomycin by minimum inhibitory concentration (MIC) and polymerase chain reaction (PCR). The *van* genes were confirmed by gene sequencing.
Results: Of the total isolates, 19% were phenotypically resistant to vancomycin, while nearly 15% contained either *van*C1 or *van*C2 gene. One resistant *E. casseliflavus* isolate with pig origin (MIC > 8 μg/mL) contained both *van*C1 and *van*C2 genes. Furthermore, one *van*C1 was found in a sensitive *E. faecalis* isolate of pig origin (MIC ≤ 4 μg/mL) and one *van*C2 in a resistant *E. faecium* isolate of chicken origin (MIC > 32 μg/mL). These genes were not accompanied by other *van* genes. Other detected genes were *van*A in 11 *E. faecium* isolates of chicken origin (MIC > 32 μg/mL). No *van*B genes were found. Gene sequencing results showed 100% identity with GenBank reference genes.
Conclusions: The current report is the first report on the detection of *van*C1 and *van*C2 genes in one *enterococcal* species with pig origin. This report is important as it proves the horizontal transfer of various *van*C genes to one species possibly due to the compatibility class of plasmids. Furthermore, detection of *van*C genes in *E. faecalis* and *E. faecium* isolates is important as it suggests that resistance to vancomycin in non-motile *enterococci* can be encoded by several mechanisms.

Keywords:Enterococcus; Antibiotic Resistance; Vancomycin

1. Background

In recent decades, bacterial resistance to antibiotics has become a significant problem for patients and for medical and veterinary practitioners (1, 2). One group of these resistant bacteria, *enterococci*, is found among multiple-resistant opportunistic pathogens isolated from long-term hospitalized patients (3). These bacteria most commonly infect urogenital tract, bloodstream, endocardium, abdomen, pelvis, biliary tract, burn wounds and in-dwelling foreign devices (such as intravascular catheters) (4, 5). Less commonly, *enterococci* can infect central nervous system, lungs, soft tissues, paranasal sinuses, ears, eyes and periodontal tissues (6). One of the most important antimicrobials against *enterococci* is glycopeptide class of antibiotics. Glycopeptides such as vancomycin and avoparcin show bacteriostatic activity against a broad-spectrum of Gram-positive bacteria. Glycopeptides inhibit the biosynthesis of the major structural cell wall polymer, peptidoglycan, by forming bonds with the D-alanyl-D-alanine terminal of muramyl dipeptides (7, 8). This mechanism of resistance to avoparcin is similar to that of vancomycin, both encoded by the "*van*" genes (9). The resistance is associated to both the antibiotics and the genetic determinants (genes) (10). Six types of vancomycin resistance genes are found in *enterococci*, including *van*A, *van*B, *van*C, *van*D, *van*E and *van*G (11, 12). *van*A, *van*B, *van*D and *van*E are usually found in *E. fecalis* and *E. faecium* and *van*G in *E. fecalis*. The *van*C-encoded vancomycin resistance is restricted to motile entrococcal species, with a limited prevalence. Literature review shows no report on the presence of multiple *van*C genes in an entrococcal species with pig origin. Furthermore, detection of *van*C genes in *E. fecalis* and isolates is important as this suggests that resistance to vancomycin and avoparcin in non-motile *enterococci* can be encoded by several mechanisms.

2. Objectives

The aim of this study was to access antibiotic resistance

to two glycopeptide-type antibiotics in *enterococci* and the genes responsible for this resistance.

3. Materials and Methods

Two-hundred and thirty *enterococcal* isolates from pig (207 isolates including 80 *E. faecalis*, 71 *E. faecium*, 13 *E. casseliflavus*, 21 *E. gallinarum*, 17 *E. hirae/durans*, two *E. hirae*, one undefferintiated and two *E. raffinosus*), chicken (15 isolates including one *E. faecalis* and 14 *E. faecium*) and human (eight isolates including three *E. faecalis*, three *E. faecium*, one *E. casseliflavus* and one *E. gallinarum*) fecal specimens, collected by the University of South Australia and the Women's and Children's Hospital (WCH), Adelaide, were used in this study. Bacterial cultures were transferred into glycerol broth and stored at -80°C for long-term maintenance. These bacteria were recovered by culturing on blood Columbia agar or tryptone soy agar (TSA) plates and overnight incubation at 37°C with 5% CO_2. All isolates had previously been identified to the species level using differential culture media and biochemical tests and in the case of *E. faecalis* and *E. faecium*, by species-specific PCR primers.

3.1. Antibiotic Susceptibility Testing

All *enterococcal* isolates were phenotypically tested for susceptibility to vancomycin and avoparcin (Sigma-Aldrich, USA) by minimum inhibitory concentration (MIC), according to the approved standard procedure of Clinical and Laboratory Standards Institute (CLSI) (13). This was carried out using agar dilution method. Results were interpreted based on the breakpoints published by CLSI and other authorities, including Danish Integrated Antimicrobial Resistance Monitoring and Research (DANMAP) (14), National Antimicrobial Resistance Monitoring System (NARMS) (15) and Norwegian Monitoring Program for Resistance in Microbes (NORM/NORM-VET) (16). These well-established references were chosen when isolates from animal origin were tested, since CLSI mostly publishes breakpoints for human isolates. Briefly, various concentrations of the antibiotics were added to Mueller-Hinton agar media and poured into petri dishes. Then, a single colony of a pure culture on tryptone soy agar (TSA) was selected and suspended in sterile normal saline to a turbidity equivalent to 0.5 McFarland. This suspension was diluted again 1:10 in sterile saline to make a final concentration of 107 cfu/mL and inoculated onto the Mueller-Hinton plates by a replicator. The inoculated plates were incubated at 35-37°C for 16-24 hours and then were read.

3.2. Molecular Identification

3.2.1. PCR Amplification of Resistance Genes

Polymerase chain reaction (Single and Multiplex PCR) was used for the detection of antibiotic resistant genes in *enterococci*, using specific primers (Table 1). The generally modified protocol used for the PCR is given as follows: A few fresh *enterococcal* colonies on blood Columbia agar were suspended in 200 μL of sterile distilled water. Bacterial cells were heated at 95°C for 20 minutes for DNA extraction. The mixture was then centrifuged at 7500 g for five minutes and the supernatant was collected. To prepare 25 μL of master mix for each sample, 5 μL of 5 × PCR buffer, 1 μL of 25 mM $MgCl_2$, 0.2 μL of 25 mM dNTPs, 1 μL of each primer in 10 pmol concentration and 0.2 μL of 5 U/μL Taq DNA polymerase were mixed in a sterile microtube. Sufficient amount of sterile distilled water was added to this mixture to reach the total volume of 23 μL and then 2 μL of the extracted DNA was added to the mixture (17). Following an initial denaturation at 95°C for three minutes, products were amplified by 30 cycles of denaturation at 95°C for 30 seconds, annealing at different temperatures for 30 seconds and extension at 72°C for one minute. Amplification was followed by a final extension at 72°C for five minutes (18). PCR products were detected by electrophoresis in 1 μg/mL ethidium bromide-stained 1% agarose gels in 0.5× TBE buffer at 100 V for 90 minutes and then using visualizing technique under the UV light.

Table 1. Overview of Target Genes and PCR Primers Used in This Study [a]

Drug	Gene	Sequences (5' → 3')	bp	Reference
AVO, VAN	*van*A	F: GGGAAAACGACAATTGC	732	(19)
		R:GTACAATGCGGCCGTTA		
VAN	*van*B	F:ATGGGAAGCCGATAGTC	635	(19)
		R:GATTTCGTTCCTCGACC		
VAN	*van*C1	F:GGTATCAAGGAAACCTC	822	(19)
		R: CTTCCGCCATCATAGCT		
VAN	*van*C2	F: CTCCTACGATTCTCTTG	439	(19)
		R: CGAGCAAGACCTTTAAG		

[a] AVO, Avoparcin; VAN, vancomycin.

3.2.2. Gene Sequencing

Amplified DNA products from isolates with putative van genes were sequenced and the results were compared with GenBank and ExPASy genomic databases. Sequencing was carried out at the SouthPath Sequencing Facility (Flinders University, Adelaide) using Sanger method.

4. Results

Overall, most of the isolates were resistant to vancomycin (including 12 *E. casseliflavus*, 21 *E. gallinarum* and 12 *E. faecium*) and avoparcin (including one *E. faecalis* and 9 *E. faecium*). No resistance to vancomycin was seen in human isolates. Of the total isolates, 6% were phenotypically resistant to avoparcin. Avoparcin resistant isolates included 11 *E. faecium* of chicken origin (MIC ≥ 32 μg/mL) and one *E. fecalis* and one *E. facium* with human origin (MIC ≥ 16 μg/mL). All avoparcin resistant isolates (except one *E. faecalis* and one *E. faecium* isolates of human origin) were from chickens (Table 2). Of the total isolates, 19% were phenotypically resistant to vancomycin, while nearly 15% contained either *van*C1 or *van*C2 gene. *Van*C1 was found in 22 isolates (10%) (Including three *E. casseliflavus*, one *E. faecalis* and 18 *E. gallinarum*), *van*C2 in 13 isolates (6%) (including ten *E. casseliflavus*, one *E. faecium* and two *E. gallinarum*). One resistant *E. casseliflavus* isolate with pig origin (MIC > 8 μg/mL) contained both *van*C1 and *van*C2. Furthermore, one *van*C1 was found in a sensitive *E. faecalis* isolate of pig origin (MIC ≤ 4 μg/mL). No *van*C1 was found in chicken isolates but one *van*C2 *E. faecium* of chicken origin (MIC > 32 μg/mL). No *van*C2 gene was found in *enterococcal* isolates of human origin. *van*A was found in 13 isolates (6%) (including one *E. faecalis* and 12 *E. faecium*); all (except two) belonged to *E. faecium* of chicken origin. No *van*B was found (Table 3). Nine resistant *E. faecium* isolates with chicken origin (MIC ≥ 32 μg/mL), one resistant *E. faecalis* isolates with human origin (MIC ≥ 16 μg/mL), one sensitive *E. faecium* isolate with human origin (MIC ≤ 8 μg/mL) and two sensitive *E. faecium* isolates with chicken origin (MIC ≤ 8 μg/mL) contained *van*A gene within avoparcin resistant isolates. Sequencing of the detected genes showed at least 98% identity with GenBank reference genes.

Table 2. MIC of *Enterococcus* spp. to Avoparcin and Vancomycin [a]

Drug	No. of Isolates With MIC, μg/mL												Resistance, No. (%)
	1	2	4	8	12	16	20	32	64	128	512	1024	
Avoparcin													
E. faecalis (84)				83		1							1 (1)
E. faecium (88)				79		1		8					9 (10)
E. gallinarum (22)				22									0 (0)
E. casseliflavus (14)				14									0 (0)
E. hirae/durans (19)				16									0 (0)
E. raffinosus (2)				2									0 (0)
N/D (1)				1									0 (0)
Vancomycin													
E. faecalis (84)			84										0 (0)
E. faecium (88)			76					12					12 (14)
E. gallinarum (22)			1	21									21 (95)
E. casseliflavus (14)			2	12									12 (86)
E. hirae/durans (19)			19										0 (0)
E. raffinosus (2)			2										0 (0)
N/D (1)			1										0 (0)

[a] N/D, not announced or applicable.

Table 3. Numbers of *van* Genes Detected by PCR [a]

Gene	Total (n = 230)	Pig (n = 207)	Chicken (n = 15)	Human (n = 8)
***van*A**	13 (6)	0 (0)	11 (73)	2 (25)
***van*B**	0 (0)	0 (0)	0 (0)	0 (0)
***van*C1**	22 (10)	21 (10)	0 (0)	1 (12)
***van*C2**	13 (6)	12 (6)	1 (7)	0 (0)

[a] Data are presented as No. (%).

5. Discussion

Vancomycin is of high importance to human medicine. Vancomycin has never been used in animal feed; therefore, all resistance to this antibiotic in chickens must be due to cross-resistance with avoparcin; both of which belong to the glycopeptides class of antibiotics (9). There are a few reports on vancomycin-resistant *enterococcal* isolates of animal origin. Furthermore, studies have shown a significant decrease in the prevalence of vancomycin resistance in *Enterococcus* spp. In Australia, Padiglione (2000, 2003) (20, 21) is routinely used and molecular assays to assess the prevalence of vancomycin resistant *enterococci* (VRE). These studies suggested that a fecal colonization with VRE was present but uncommon in Australia. In the current study, 45 isolates (including 12 *E. casseliflavus*, 12 *E. faecium* and 21 *E. gallinarum*) with vancomycin resistance were investigated; of which, 12 were isolated from chickens. In chicken isolates, vancomycin resistance was detected more frequently in *E. faecium* than *E. faecalis* (12 instead of zero) ($P < 0.05$) as reported by other researchers (22). Differences in results for vancomycin and avoparcin were found. In general, a low percentage (6%) of resistance to avoparcin was detected; all in chicken with *E. faecium* (100%), except one *E. faecalis* of human origin. This is not surprising because a similar difference has been reported in variety of papers. This might be seen due to a second mechanism for vancomycin resistance. Another reason for this difference could be the difference between MIC breakpoints, which are ≥ 8 and ≤ 4 μg/mL for vancomycin and ≥ 6 and < 16 μg/mL for avoparcin, and also assessment of resistance/susceptibility of microorganisms with MICs close to breakpoint values. Avoparcin was used in Australian livestock (23), but has been banned since 2000 (24). A study carried out by Hart (2004) (25) showed no resistance to vancomycin or avoparcin in pig-related *Enterococcal* spp., two years after the ban of avoparcin use in Australian livestock. In a similar study carried out by Department of Agriculture, Fisheries and Forestry (DAFF) (26), no resistance to vancomycin was found in *enterococci* isolated from either pigs or chickens (except one chicken *E. faecalis* isolate with low-level resistance encoded by *van*C) three years after the ban of avoparcin use in animal husbandry in Australia. The resistance to avoparcin in chicken isolates was possibly due to the previous use of avoparcin in animal husbandry (7, 8) and the fact that these isolates had been collected before the ban on use of avoparcin. However, the two other *enterococcal* species, *E. casseliflavus* and *E. gallinarum*, are known to be naturally resistant to vancomycin.

Relatively, *van*C gene was found in intrinsically resistant *E. casseliflavus* and *E. gallinarum*, in accordance with previous studies. Seo (2005) (27) compared vancomycin resistance in 67 *enterococcal* isolates from poultry and pigs in Korea and found that *van*C1 or *van*C2/3 was associated with low-level resistance to vancomycin in *E. gallinaum*, *E. casseliflavus* and *E. flavescens* isolates. Reports by Xavier (2006) (28), and Lemcke and Bulte (2000) (29) indicate the prevalence of *van*C1 and *van*C2/3 in *enterococcal* samples of pig and chicken origins; mostly *van*C1 in *E. gallinarum* and *van*C2/3 in *E. casseliflavus*. For example, Patel (1997) (30) detected *van*C1 from one *E. faecalis* and one *E. faecium* strains; clinically isolated from humans. Only a low number of genes (13 *van*A) encoding resistance to avoparcin was detected; all in chicken isolates. Use of avoparcin has been reported to be largely responsible for the amplification of *van*A VRE in animals (31). Xavier (2006) (28) found no *van*A or *van*B in *enterococci* of poultry in Brazil. Similarly, Lemcke and Bulte (2000) (29) did not find *van*B in VRE *enterococcal* strains isolated from poultry and pork in Germany. However, they reported *van*A in nearly half of the isolates. In the current study, 11 of *van*A genes were found in *E. faecium* of chickens. Two *van*A genes (one in *E. faecalis* and one in *E. faecium*) were also found in human isolates. Not surprisingly, vancomycin resistance was also seen in all chicken isolates resistant to avoparcin, carrying *van*A. Although avoparcin was used in chickens - and to a lesser extent, pigs - before 2000, *van*A was either not identified in *E. faecium* in their study or was reported in low numbers. *E. faecium* carrying *van*B, the most commonly clinical VRE species in Australia, has been identified in healthy Australians (20, 21, 32). Similar findings were reported by Bell (1998) (33) and Burrell (2005) (34) in Adelaide and Melbourne, respectively. Moreover, Borhani et al. recovered 40 VRE isolates from an urban sewage treatment plant during 2009-2010 and reported that all the *E. faecium* isolates (100%) harbored *van*A and five isolates (13%) harbored both *van*A and *van*B (35). In contrast, no *van*B was found in the current study. The fact that *van*B-type vancomycin resistance is not associated to the use of avoparcin in animals may explain the difference between the clinical and livestock isolates (19). In general, vancomycin resistant isolates were found in this study; mostly in *E. faecium*, *E. gallinarum* and *E. casseliflavus*. Furthermore, avoparcin resistant isolates were detected, mostly belonged to *E. faecium*. The encoding *van* genes (*van*C1/C2 and *van*A) were reported in six to 10% of all isolates. This report is important as it proves the horizontal transfer of various *van*C genes to one species possibly due to the compatibility class of plasmids. Furthermore, detection of *van*C genes in *E. faecalis* and *E. faecium* isolates is important as it suggests that resistance to vancomycin in non-motile *enterococci* can be encoded by several mechanisms.

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Authors' Contributions

Study concept and design: Ramin Mazheri Nezhad Fard,

Mary D. Barton, Michael W. Heuzenroeder. Acquisition of data: Ramin Mazheri Nezhad Fard, Analysis and interpretation of data: Ramin Mazheri Nezhad Fard, Drafting of the manuscript: Ramin Mazheri Nezhad Fard, Critical revision of the manuscript for important intellectual content: Mary D. Barton, Michael W. Heuzenroeder. Administrative, technical, and material support: Mary D. Barton, Michael W. Heuzenroeder. Study supervision: Mary D. Barton, Michael W. Heuzenroeder.

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