Detection of vanC1 and vanC2 Genes in an Enterococcal Isolate and vanC Genes in non-Motile Enterococcus spp.

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Received: September 9, 2014; Revised: September 23, 2014; Accepted: September 25, 2014

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 report 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 vanC1 or vanC2 gene. One resistant E. casseliflavus isolate with pig origin (MIC > 8 μg/mL) contained both vanC1 and vanC2 genes. Furthermore, one vanC1 was found in a sensitive E. faecalis isolate of pig origin (MIC ≤ 4 μg/mL) and one vanC2 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 vanA in 11 E. faecium isolates of chicken origin (MIC > 32 μg/mL). No vanB 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 vanC1 and vanC2 genes in one enterococcal species with pig origin. This report is important as it proves the horizontal transfer of various vanC genes to one species possibly due to the compatibility class of plasmids. Furthermore, detection of vanC 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 vanA, vanB, vanC, vanD, vanE and vanG (11, 12). vanA, vanB, vanD and vanE are usually found in E. faecalis and E. faecium and vanG in E. falcis. The vanC-encoded vancomycin resistance is restricted to motile enterococcal species, with a limited prevalence. Literature review shows no report on the presence of multiple vanC genes in an entrococcal species with pig origin. Furthermore, detection of vanC genes in E. falcis and isolates is important as it 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 undifferentiated 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% CO₂. 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 MgCl₂, 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 | vanA | F: GGGAAAAACGACAATGCC | 732   | (19)      |
|        |      | R: GTACAATGCGGCGCTTA |       |           |
| VAN    | vanB | F: ATGGGAAGCCGATAGTC | 635   | (19)      |
|        |      | R: GATTTCGTTCCTCGACC |       |           |
| VAN    | vanC1| F: GGTATCAGGAACCTTC | 822   | (19)      |
|        |      | R: CTTCCGCACATGACCT |       |           |
| VAN    | vanC2| F: CTCTTAAGATCCCTTG | 439   | (19)      |
|        |      | R: CGAGCAAGACCTTAAAG |       |           |

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). 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. faecalis and one E. faecium with human origin (MIC ≥ 16 μg/mL). All avoparcin resistant isolates (except one E. faecalis and one E. faecium of human origin) were from chickens (Table 2). Of the total isolates, 19% were phenotypically resistant to vancomycin, while nearly 15% contained either vanC1 or vanC2 gene. VanC1 was found in 22 isolates (10%) (including three E. casseliflavus, one E. faecalis and 18 E. gallinarum), vanC2 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 vanC1 and vanC2. Furthermore, one vanC1 was found in a sensitive E. faecalis isolate of pig origin (MIC ≤ 4 μg/mL). No vanC1 was found in chicken isolates but one vanC2 E. faecium of chicken origin (MIC > 32 μg/mL). No vanC2 gene was found in enterococcal isolates of human origin. vanA 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 vanB 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 vanA 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

| 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 | 9 (10) |
| E. faecium (88)  | 79 | 1 | 8 | 0 (0) |
| E. gallinarum (22) | 22 |    |    |    |
| E. casseliflavus (14) | 14 |    |    |    |
| E. hirae/durans (19) | 16 |    |    |    |
| E. raffinosus (2) | 2 |    |    |    |
| N/D (1) | 1 |    |    |    |
| Vancomycin |   |   |   |   |    |    |    |    |    |     |     |     |
| E. faecalis (84) | 84 |    |    |    |
| E. faecium (88)  | 76 | 12 | 21 (95) |
| E. gallinarum (22) | 2 | 12 | 0 (0) |
| E. casseliflavus (14) | 19 | 0 (0) |
| E. raffinosus (2) | 2 |    |    |    |
| N/D (1) | 1 |    |    |    |

* N/D, not announced or applicable.

### Table 3. Numbers of van Genes Detected by PCR

| Gene | Total (n = 230) | Pig (n = 207) | Chicken (n = 15) | Human (n = 8) |
|------|-----------------|---------------|------------------|--------------|
| vanA | 13 (6)          | 0 (0)         | 11 (73)          | 2 (25)       |
| vanB | 0 (0)           | 0 (0)         | 0 (0)            | 0 (0)        |
| vanC1 | 22 (10)        | 21 (10)       | 0 (0)            | 1 (12)       |
| vanC2 | 13 (6)         | 12 (6)        | 1 (7)            | 0 (0)        |

* 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 vanC) 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, vanC 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 vanC1 or vanC2/3 was associated with low-level resistance to vancomycin in E. gal-

Acknowledgements

We wish to thank microbiology lab staff within the School of Pharmacy and Medical Sciences, University of South Australia.

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.

Funding/Support
This project was a part of a PhD thesis supported by the University of South Australia.

References
1. Larson E. Community factors in the development of antibiotic resistance. Annu Rev Public Health. 2007;28:43–47.
2. Levy SB, Marshall B. Antibacterial resistance worldwide: causes, challenges and responses. Nat Med. 2004;10(12 Suppl):S22–9.
3. Murray BE. Vancomycin-resistant enterococcal infections. N Engl J Med. 2000;342(16):120–21.
4. Wisell KT, Kahlmeter G, Giske CG. Trimethoprim and enterococci in urinary tract infections: new perspectives on an old issue. J Antimicrob Chemother. 2008;62(1):35–40.
5. Patel R, Rouse MS, Piper KE, Steckelberg JM. Linezolid therapy of vancomycin-resistant Enterococcus faecium experimental endocarditis. Antimicrob Agents Chemother. 2001;45(2):624–3.
6. Iaria C, Stassi G, Costa GB, Di Leo R, Toscano A, Caccio A. Enterococcal meningitis caused by Enterococcus casseliflavus. First case report. BMC Infect Dis. 2003;3(1):1.
7. Cooper EE. Antimicrobials: use in animal husbandry & resistance in humans. AIC. 2000;5(5):216–19.
8. Collignon PJ. Vancomycin-resistant enterococci and use of avoparcin in animal feed: is there a link? Med Aust. 1999;171(3):344–6.
9. Lu K, Asano R, Davies J. Antimicrobial resistance gene delivery in animal feeds. Emerg Infect Dis. 2004;10(4):679–83.
10. Levy SB. The 2000 Garrod lecture. Factors impacting on the problem of antibiotic resistance. J Antimicrob Chemother. 2002;49(2):25–30.
11. Domingo MC, Hulettsky A, Giroux R, Picard FJ, Bergeron MG. vanD and vanG-like gene clusters in a Ruminococcus species isolated from human bowel flora. Antimicrob Agents Chemother. 2007;51(2):419–27.
12. Fraimow H, Knob C, Herrero IA, Patel R. Putative VanRS-like two-component regulatory system associated with the inducible glycopeptide resistance cluster of Paenibacillus popilliae. Antimicrob Agents Chemother. 2005;49(7):2625–33.
13. Clinical Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically: Approved Standard. 7 ed: Wayne; 2008.
14. Wilder MA, Clinical, Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically: Approved Standard. National Committee for Clinical Laboratory Standards; 2009.
15. The National Antimicrobial Resistance Monitoring System. Human Isolates Final Report. NARMS website; 2004.
16. The Norwegian Monitoring Programme for Antimicrobial Resistance. Usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway: NORM/NORM-VET web site; 2004.
17. Hartman AB, Essiet J, Isenbarger DW, Lindler LE. Epidemiology of tetracycline resistance determinants in Shigella spp. and enteroinvasive Escherichia coli: characterization and dissemination of tet(A)-L. J Clin Microbiol. 2001;39(1):3023–32.
18. Jackson CR, Fedorka-Cray PJ, Barrett JB. Use of a genus-and species-specific multiplex PCR for identification of enterococci. J Clin Microbiol. 2004;42(3):3585–6.
19. Durka-Malen S, Evers S, Courvalin P. Detection of glycopeptidase resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol. 1995;33(12):4–7.
20. Padiglione AA, Grabsch EA, Olden D, Hellard M, Sinclair MI, Fairley CK, et al. Fecal colonization with vancomycin-resistant enterococci in Australia. Emerg Infect Dis. 2000;6(5):534–6.
21. Padiglione AA, Wolfe R, Grabsch EA, Olden D, Pearson S, Franklin C, et al. Risk factors for new detection of vancomycin-resistant enterococci in acute-care hospitals that employ strict infection control procedures. Antimicrob Agents Chemother. 2003;47(8):2492–8.
22. Tenover FC, McDonald LC. Vancomycin-resistant staphylococci and enterococci: epidemiology and control. Curr Opin Infect Dis. 2005;18(4):390–9.
23. Barton M, Wilkins J. Antibiotic resistance in bacteria isolated from poultry. Record of Rural Industries Research and Development Corporation Publication No 1/05. RRDC Project No USA-9A website; 2001.
24. National Drugs and Poisons Schedule Committee. Record of the Reasons Meeting 37. NDPSC website; 2003.
25. Hart WS, Heuzenroeder MW, Barton MD. Antimicrobial resistance in Campylobacter spp., Escherichia coli and enterococci associated with pigs in Australia. J Vet Med B Infect Dis Vet Public Health. 2004;51(5):216–21.
26. Australian Government Department of Agriculture FAE. Pilot Surveillance Program for Antimicrobial Resistance in Bacteria of Animal Origin: DAFW, Department of Agriculture, Fisheries and Forestry; 2007.
27. See KS, Lim JY, Yoo HS, Baek WK, Park YH. Comparison of vancomycin-resistant enterococci isolates from human, poultry and pigs in Korea. Vet Microbiol. 2005;106(1-4):225–33.
28. Batista Xavier D, Moreno Bernal FE, Titze-de-Almeida R. Absence of vanA and vanB-containing enterococci in poultry raised on non-intensive production farms in Brazil. Appl Environ Microbiol. 2006;72(4):3072–3.
29. Lencene R, Buite M. Occurrence of the vancomycin-resistant genes vanA, vanB, vanC1, vanC2 and vanC3 in Enterococcus strains isolated from poultry and pork. Int J Food Microbiol. 2000;50(2-3):185–94.
30. Patel R, Uhl JR, Kohnner P, Hopkins MK, Cockerill FR, 3rd. Multiplex PCR detection of vanA, vanB, vanC, and vanC2-3 genes in enterococci. J Clin Microbiol. 1997;35(3):703–7.
31. Ferguson JK. Vancomycin-resistant enterococci: causes and control. Med J Aust. 1999;171(3):17–8.
32. Schooneveldt JM, Marriott RK, Nimmo GR. Detection of a VanB determinant in Enterococcus gallinarum in Australia. J Clin Microbiol. 2000;38(10):3902.
33. Bell JM, Paton C, Turnidge J. Emergence of vancomycin-resistant enterococci in Australia: phenotypic and genotypic characteristics of isolates. J Clin Microbiol. 1998;36(8):2887–90.
34. Burrell LJ, Grabsch EA, Padiglione AA, Grayson ML. Prevalence of colonisation with vancomycin-resistant enterococci (VRE) among haemodialysis outpatients in Victoria: implications for screening. Med J Aust. 2005;182(9):492.