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Isolation and molecular characterization of enterococci from different animal species

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ABSTRACT

The study was undertaken to determine the occurrence of *Enterococcus* spp. with special reference to vancomycin resistant enterococci (VRE) in bovines, equines and poultry; detection of vancomycin resistance gene markers by polymerase chain reaction (PCR) and to compare VRE isolates by means of pulse field gel electrophoresis (PFGE). In this study, 60 VRE were isolated from 150 faecal samples. Besides vancomycin sensitivity, isolates were also tested for 6 more antimicrobial drugs by MIC. Significant number of isolates exhibited intermediate level of resistance to vancomycin (70%), norfloxacin (50%) and cephalothin (43.3%). Multidrug resistance (MDR) was observed in 37 (61.6%) isolates. Multiplex PCR revealed *vanC1* (14), *vanC2/3* (9), *van C1* + *vanC2/3* (11) but no *vanA* or *vanB* genes. Further genotyping of the VRE isolates carrying *vanC* genes using pulse field gel electrophoresis revealed ~73% homology.

Key words: *Enterococcus* spp., Pulse field gel electrophoresis (PFGE), Vancomycin resistant enterococci (VRE), *VanA, VanB, VanC1, VanC2/3*

Enterococcus spp., which form part of the natural intestinal flora of most mammals and birds, have emerged as an important nosocomial and community-acquired pathogen. They are Gram positive, facultative anaerobes, catalase negative and non-spore-forming cocci occurring singly, in pairs or short chains. *Enterococcus faecalis, E. faecium, E. durans* found in intestinal tract of many species cause opportunistic infections such as septicaemia in chickens, mastitis in bovine, endocarditis in cattle and lambs, and urinary-tract infections in dogs. Enterococci may acquire resistance to antimicrobial agents through transfer of plasmids and transposons, chromosomal exchange, or mutation. The major concern is the emergence of vancomycin-resistant enterococci (VRE), since vancomycin is considered one of the last resort in humans after most other antibiotics have failed when treating infections caused by Gram positive bacteria (Khan *et al*. 2005). The risk of transfer of resistance genes from VRE to other pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus* is also a cause for concern (Kaszanyitzky *et al*. 2007).

Antimicrobial agents are used in large amounts in feed to promote growth and for the production of food animals for the therapy and prophylaxis of bacterial infections. A link between the use of antibiotics in animal husbandry and the rise of antibiotic resistance has been observed. Resistance pattern of enterococci in food animals is very similar to enterococci isolated from nosocomial infections (including resistance to aminoglycosides, macrolides, lincosamides, penicillins, nitrofurans, streptogramins, quinolones, tetracycline, and rarely vancomycin) (Frye and Jackson 2013). Alarming rise of vancomycin-resistant enterococci (VRE) is a cause of global concern. Prevalence of multidrug resistant enterococci in a tertiary care hospital in India had been reported as a growing threat (Gangurde *et al*. 2014).

There is paucity of information on vancomycin resistance in enterococci in animals from India. Therefore, this study was carried out to study the occurrence of *Enterococcus* spp. with special reference to vancomycin resistance enterococci (VRE) in bovines, equines and poultry, detection of vancomycin resistance gene markers by polymerase chain reaction (PCR) and to compare VRE isolates by means of pulse field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Faecal samples (150) were collected from bovines (50), equines (50) and poultry (50) from animal farms in Punjab and those brought to the university veterinary clinics for treatment.

Isolation of vancomycin-resistant enterococci from fecal samples: Fecal samples were inoculated in HiCrome Enterococci Broth (Himedia, India), supplemented with 6 µl/ml vancomycin hydrochloride (Himedia). Cultures

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showing colour change (bluish green) in broth were sub-cultured onto Bile Esculin Azide Agar (Himedia) as described by Baðcigil *et al*. (2014).

Identification of vancomycin-resistant enterococci: Colonies with morphological characteristics of enterococci were identified following the procedures and biochemical key for *Enterococcus* spp. (Facklam and Collins 1989).

Antimicrobial susceptibility testing: All the isolates were tested by agar disc diffusion method using 30 µg vancomycin discs (Clinical and Laboratory Standards Institute 2015). Minimal inhibition concentration (MICs) for the antibiotics, viz. vancomycin, gentamycin, tetracycline, norfloxacin, cephalothin, streptomycin and enrofloxacin were performed using an E-test as described by the manufacturer. A reference strain of *Enterococcus faecalis* ATCC 29212 was used as a control.

DNA preparation and PCR: Template DNA was prepared by using snap chill method. Multiplex PCR was optimized for the detection of vancomycin resistance genes (Kariyama *et al*. 2000). The sequence of primers is given in Table 1. The DNA of strains *E. faecium* BM4147 (*vanA*), *E. faecalis* V583 (*vanB*), *E. gallinarum* BM4174 (*vanC1*), *E. casseliflavus* DSMZ 20680 (*vanC2/C3*), *E. faecium* CCUG542 (vancomycin susceptible), provided by Dr. Serkan Ikiz (Istanbul University, Turkey), were used as positive controls in PCR assays.

Pulse field gel electrophoresis (PFGE): Genetic relatedness of selected isolates of vancomycin resistant enterococci was determined by pulse field gel electrophoresis using CHEF MAPPER (Bio-Rad). Enterococci containing *vanC1* and *vanC2/3* genes were chosen for typing following protocol of Murray *et al*. (1990) with slight modifications. Single isolated colony from brain heart infusion (BHI) agar plate was inoculated in 5 ml BHI broth and incubated at 37°C overnight in incubator. Bacterial cells were harvested by centrifugation at 5,000 × g for 15 min. Pellet was suspended with equal volume PIV buffer [1 M NaCI, 10 mM Tris hydrochloride (pH 7.6)] and centrifuged at 5,000 × g for 15 min. The cells were harvested and resuspended in half the volume of PIV buffer and warmed at 37°C. Plugs were made and lysed with 10 ml fresh lysis solution (6 mM Tris hydrochloride [pH 7.6], 1 M NaCl, 100 mM EDTA [pH 7.5], 0.5% Brij58, 0.2% sodium deoxycholate, 0.5% sodium lauroyl sarcosine and 1 µg of lysozyme/ml). Following overnight incubation at 37°C with gentle shaking, this solution was replaced with 10 ml of ESP (0.5 M EDTA [pH 9 to 9.5], 1% sodium lauryl sarcosine, 50 µg of proteinase K/ml) and again incubated overnight at 50°C with gentle shaking. The plugs were washed thrice for 30 min each with 15 ml of TE buffer (10 mM Tris hydrochloride [pH 7.5], 0.1 mM EDTA) and stored at 4°C until use. Digestion of plugs with *SmaI* (10 U/µl) (New England Biolabs) was performed by incubating for 4 h at 25°C. The slices were washed with TE buffer (1 ml) at 37°C for 1 h. Plugs with digested genomic DNA were separated for 19–20 h by electrophoresis in CHEF MAPPER (Bio-Rad) at 200 volts (6 V/cm) with the conditions: 14°C temperature, 3.5 sec initial switch time, 23.5 sec final switch time, 120° angle and linear ramping. A lambda ladder (Bio-Rad Laboratories, USA) and 5 kb ladder (Bio-Rad Laboratories, USA) were used as molecular weight marker. After the run, gel was stained and visualised using gel documentation system (Syngene, USA). The gel image was subjected to analysis using Genysis software.

RESULTS AND DISCUSSION

Isolation and identification of vancomycin resistant enterococci: Out of 150 faecal samples, 60 were able to grow in vancomycin supplemented BEA agar and thus were presumed to be vancomycin resistant. In this study, the prevalence of *Enterococcus* spp. was as follows: *E. gallinarum* (48%), *E. casseliflavus/E. flavescens* (25%), *E. faecalis* (16%) and *E. faecium* (10%) respectively. *E. casseliflavus* and *E. gallinarum* represent significant percentage of the faecal enterococci population of various animal species (Seo *et al.* 2005, Lopez *et al*. 2011). Khan *et al.* (2005) isolated *E. gallinarum* from milk samples of animals with mastitis and litters from 28 different flocks. Pruksakorn *et al.* (2016) reported that *Enterococcus gallinarum* was the most prevalent species for VRE in all

Table 1. Primers used for amplification

Target gene	Primer pair sequence (5'-3')	Amplicon (bp)	Reference
vanB	AAGCTATGCAAGAAGCCATG CCGACAATCAAATCATCCTC	536	Lopez *et al*. (2011)
vanA	CATGAATAGAATAAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	1,030	Devriese *et al*. (1996)
vanC1	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	822	
vanC2/C3	CGGGGAAGATGGCAGTAT CGCAGGGACGGTGATTTT	484	
E. faecalis	ATCAAGTACAGTTAGTCTTTATTAG ACGATTCAAAGCTAACTGAATCAGT	941	
E. faecium	TTGAGGCAGACCAGATTGA CG TATGACAGCGACTCCGATTCC	658	
rrs (16SrRNA)	GGATTAGATACCCTGGTAGTCC TCGTTGCGGGACTTAACCCAAC	320	

Table 2. Phenotype, MIC values and *van* gene occurrence among *Enterococcus* spp. isolates

Enterococcus spp.	Animal spp.	Genotype					R	I	S	MIC
		vanA	*vanB*	*van C1*	*van C2/3*	*Van C1+ C2/3*				
E. gallinarum	Poultry	0	0	2	0	0	–	–	–	–
	Bovine			6	0	0	–	6	–	3–8
	Equine			5	0	0	2	3	–	3–8
E. casseliflavus or	Poultry	0	0	0	0	0	–	–	–	–
E. flavescens	Bovine				8	0	2	7	–	3–12
	Equine				1	0	–	1	–	8
E. gallinarum or	Poultry	0	0	0	0	1	–	1	–	3
E. casseliflavus	Bovine	0	0	0	0	8	2	5	–	4-8
or *E. flavescens*	Equine	0	0	0	0	0	2	3	–	6

R, Resistant; I, intermediate; S, sensitive.

age groups, followed by *Enterococcus casseliflavus*.

Antimicrobial susceptibility test: All the VRE isolates containing *van* genes exhibited resistance and intermediate phenotype by disc diffusion method. VRE negative for *van* genes showed all the three phenotypes, i.e. susceptible, resistant and intermediate. By E-test, isolates carrying *van* gene exhibited intermediate resistance pattern having MIC values ranging from 6 to 12 µg/ml and susceptible isolates having MIC ranging from 3 to 4 µg/ml (Tables 2 and 3). Pruksakorn *et al*. (2016) detected VRE with MICs ranging from 8 µg/ml to 16 µg/ml in 43 of 179 (24%) pigs. VRE carriers were identified in 12 of 61 (19.7%) suckling pigs, 15 of 60 (25%) fattening pigs and 16 of 58 (27.6%) breeding sows respectively. In addition to vancomycin, the isolates were also tested for susceptibility to gentamycin, tetracycline, norfloxacin, cephalothin, streptomycin and enrofloxacin (Table 4). Our results showed strong presence of multidrug resistance (MDR) in 37 (61.6%) isolates to vancomycin, enrofloxacin, norfloxacin and cephalothin. Significantly high percentage of *Enterococcus* sp. isolates from bovines (31.6%) and equines (30%) exhibited multidrug resistance as compared to those from poultry (10%). Occurrence of MDR among enterococcal isolates had been reported by other workers also. This might be due to diverse selective pressure from the different usage of antibiotics within the 3 species. Our results were in contrast to other workers (Jung *et al*. 2007) who reported broader spectrum of resistance of *Enterococcus* sp. from poultry faeces than bovine and swine isolates. Similarly, Lozano *et al*. (2015) found MDR *E. gallinarum* isolates from red kites that showed higher rates of resistance to tetracycline (66.3%) and aminoglycosides: kanamycin (22.9%), streptomycin (11.5%), and gentamicin (9.4%) than those of other animal species.

Table 3. Phenotype, MIC values of *Enterococcus* isolates not carrying any *van* genes

Animal spp.	Resistant	Intermediate	Sensitive	MIC (µg/ml)
Poultry	1	8	1	2-8
Bovine	-	5	-	4-6
Equine	-	11	-	4-6
Total	1	24	1	

Amplification of van *genes by multiplex PCR:* Multiplex PCR was optimized at an annealing temperature of 54°C. All the 7 primer sets used in multiplex PCR targeting the 7 genes, viz. 16S rRNA, *E. faecalis, E. faecium, vanA, vanB, vanC1* and *vanC2/vanC3,* revealed amplified PCR products of expected size corresponding to the genes. All the 60 isolates showed positive amplification for 16S rRNA gene; 14 isolates were positive for *vanC1* and 9 for *vanC2/3* genes (Fig. 1); 11 isolates contained both *vanC1* and *van C2/3* genes; 10 were confirmed as *E. faecalis* and 6 as *E. faecium* on the basis of PCR. However none of these harboured the *vanA* or *vanB* genes. While *vanA* and *vanB* phenotypes correspond to high level resistance in enterococci, *vanC1/ vanC2/3* are associated with low level of intrinsic resistance. All the isolates of VRE containing *vanC1* and *vanC2/3* genes showed intermediate level of resistance by E-test. Also it was noted that VRE isolates having no *van* genes showed intermediate resistance phenotypically by E-test. Because of difference of *van* phenotypes in transferability and susceptibility, status of *van* type in clinical settings is epidemiologically important for control of VRE. Xavier *et al.* (2006) who also reported that none of the *Enterococcus* species isolated by them were carrying *vanA* or *vanB* genes, but they detected *vanC1* in 13% and *vanC2/C3* in 5.5% of the isolates.

In our study, none of the 60 isolates were found positive for *vanA* or *vanB*. Although others reported widespread

Table 4. Sensitivity pattern of *Enterococcus* isolates to different antibiotics

Antibiotic	Isolate		
	Sensitive	Intermediate	Resistant
Vancomycin	18 (30)	42 (70)	0
Gentamycin	55 (91.6)	0	5 (8.3)
Tetracycline	37 (61.6)	14 (23.3)	9 (15)
Norfloxacin	24 (40)	30 (50)	6 (10)
Cephalothin	27 (45)	26 (43.3)	7 (11.6)
Streptomycin	51 (85)	0	9 (15)
Enrofloxacin	22 (36.6)	24 (40)	14 (23.3)

Figure in parenthesis indicates %.

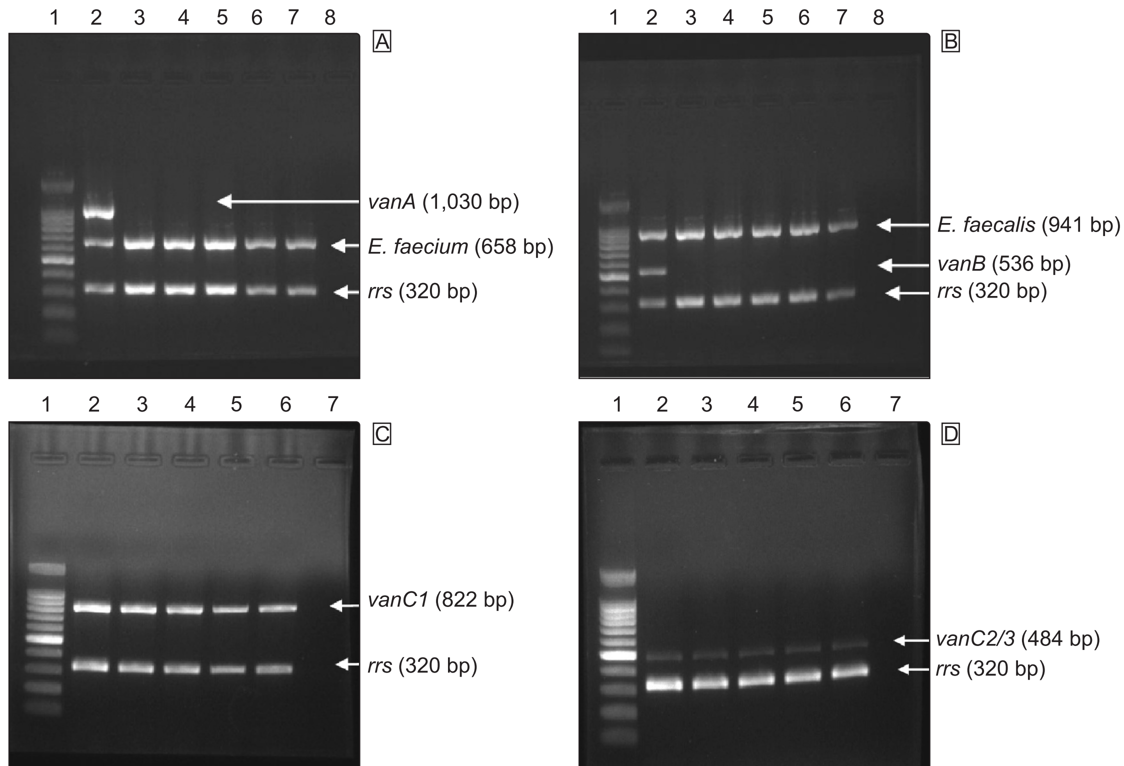

Fig. 1(A–D). Multiplex PCR of *Enterococcus* spp. isolates for identification of species and detection of *van* gene. **A**. Lane 1, 100 bp ladder; lane 2, *rrs, E. faecium, vanA* positive control; lanes 3–7, samples; lane 8, non-template control. **B**. Lane1, 100 bp ladder; lane 2, *rrs, van B, E. faecalis* positive control; lanes 3–7, samples; lane 8, non-template control. **C**. Lane 1, 100 bp ladder; lane 2, *rrs, vanC1* positive control; lanes 2–6, samples; lane 7, non template control. **D**. Lane 1, 100 bp ladder; lane 2, *rrs, van C2/3* positive control; lanes 3–6, samples; Lane 7, non-template control.

occurrence of *vanA* and *vanB* genotypes, there is no particular protocol suggested by CDC for the patients that are infected or colonized by *E. gallinarum*. However, most researchers emphasized that in spite of the lack of any instructions for those patients, the ability of *E. gallinarum* strains to catch the genes encoding high level of vancomycin resistance and to transfer them to important clinical strains such as *E. faecium* should not be omitted (Seo *et al.* 2005, Lopez *et al.* 2011, Khan *et al.* 2005, Corso *et al.* 2005). Although the potential for horizontal gene transfer is low, the transmission risk of the *vanC* strains to humans could not be excluded.

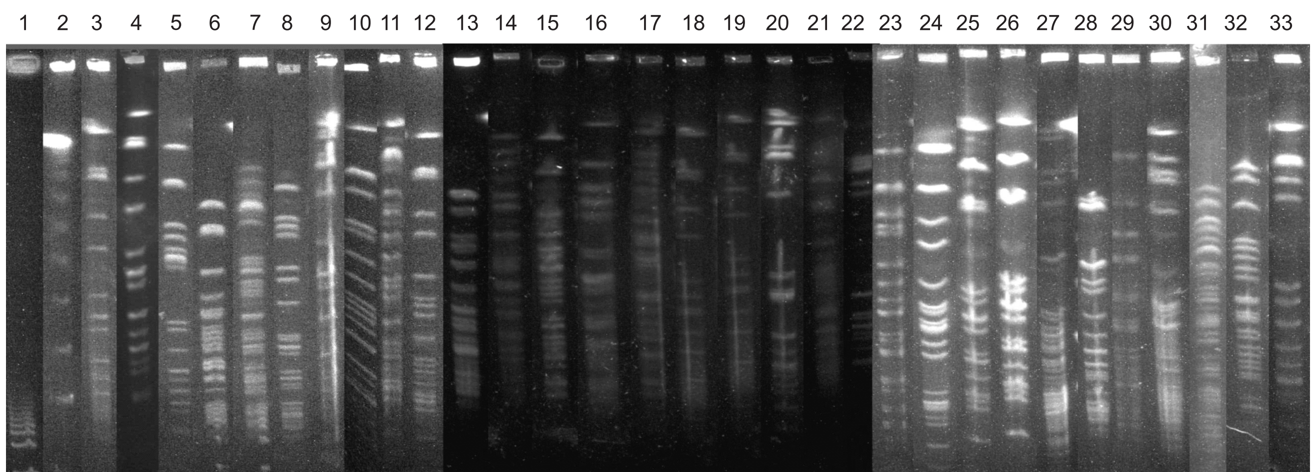

Fig. 2. Pulse field gel electrophoretic (PFGE) pattern of DNA from *Enterococcus* spp. strains after *SmaI* digestion. Lane 1, Biorad 5 kb ladder; lane 2, l ladder; lane 3, E25; lane 4, E21; lane 5, B24; lane 6, B23; lane 7, B22; lane 8, B21; lane 9, B20; lane 10, B19; lane 11, B18; lane 12, P40; lane 13, SK; lane 14, B7; lane 15, B5; lane 16, B3; lane 17, B17; lane 18, B16; lane 19, B15; lane 20, B14; lane 21, B13; lane 22, B12; lane 23, B11; lane 24, B10; lane 25, B9; lane 26, V; lane 27, H512; lane 28, E27; lane 29, P25; lane 30, B8; lane 31, E24; lane 32, E22; lane 33, E9.

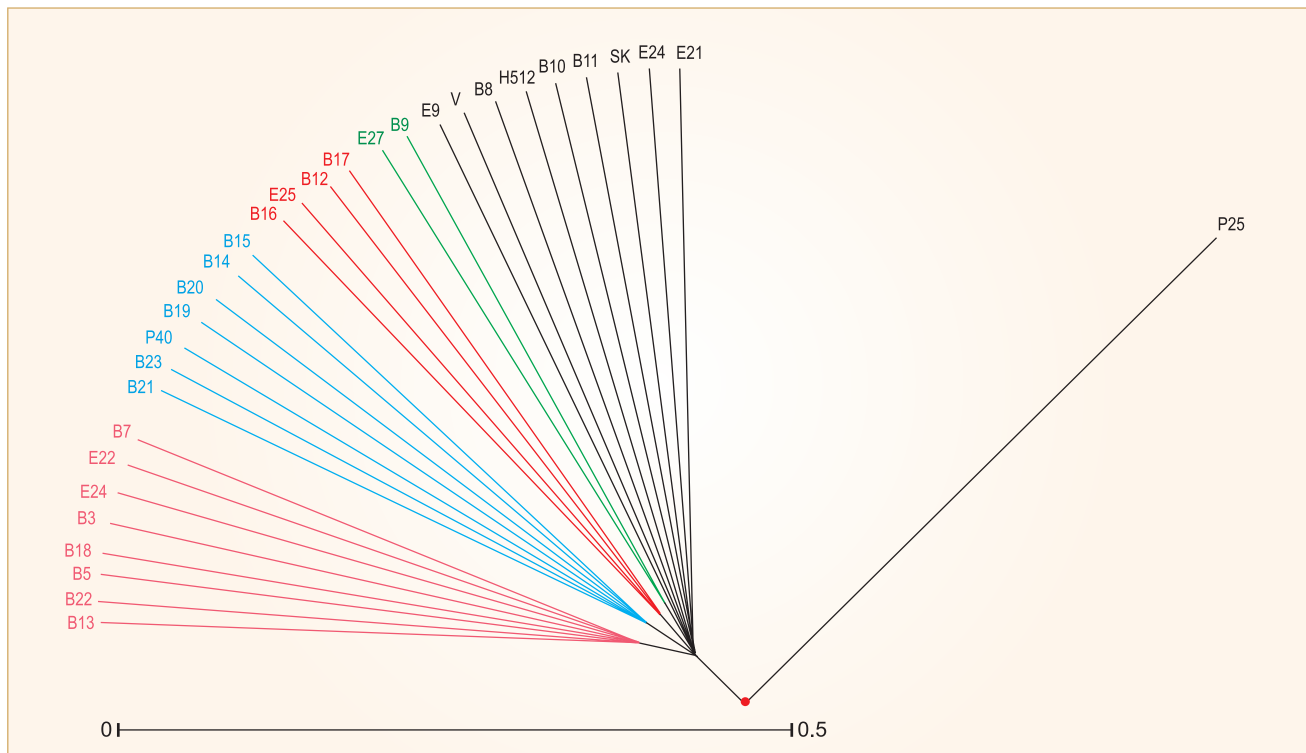

Fig. 3. Dendrogram showing similarity index (five clusters) among *Enterococcus* isolates carrying *vanC1* and *vanc2/3* genes.

Pulse field gel electrophoresis (PFGE) analysis: The pulse field gel electrophoresis pattern and the dendrogram derived using Genysis software and population genetic analysis (POPGEN ver.1.32) are depicted in Figs 2 and 3, respectively. On the basis of PFGE analysis, 31 isolates of *Enterococcus* spp. could be grouped in 5 clusters showing homology of ~73% among themselves. Among different clusters, the isolates from bovine, poultry and equine were fairly interspersed, thereby indicating widespread prevalence of *vanC* genes bearing enterococci in different areas. That indicates the possibility of genetic mechanism other than *vanC* gene being responsible for development of resistance. Results of Khan *et al*. (2005) also corroborate the current observation. They performed PFGE on 22 *E. gallinarum* isolates carrying *vanC1* gene along with 5 *E. faecalis strains* (MIC, 2 μg/ml) and 3 *E. gallinarum* strains (MIC, 8 μg/ml) not having any of the *van* genes and showed 11 different PFGE groups. In current study, we found intrinsically vancomycin-resistant enterococci, which cause rare but significant opportunistic infections in animals and acquire transferable *vanA* and *vanB* genes. It is suggested to routinely screen intrinsically vancomycin-resistant enterococci for virulence factors and transferable antibiotic resistance markers as they could also be transferred to methicillin-resistant *Staphylococcus aureus*. Also there is a need to maintain a constant vigil and regularly screen *Enterococcus* isolates from more areas for the presence of transferable resistant markers.

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REFERENCES

Baðcigil A F, Ikiz S A K S and Özgür N Y. 2015. Isolation of vancomycin resistant enterococci from animal faeces, detection of antimicrobial resistance profiles and vancomycin resistant genes. *Kafkas Universitesi Veteriner Fakultesi Dergisi* **21**(1): 35–40.

Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement.* CLSI Document M100-S25 (ISBN 1-56238-989-0 [Print]; ISBN 1-56238-990-4 [Electronic]). Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

Corso A, Faccone D, Gagetti P, Togneri A, Lopardo H, Melano R, Rodriguez V, Rodriguez M and Galas M. 2005. First report of VanA *Enterococcus gallinarum* dissemination within an intensive care unit in Argentina. *International Journal of Antimicrobial Agents* **25**: 51–56.

Devriese L A, Ieven M, Goossens H, Vandamme P, Pot B, Hommez J and Haesebrouck F. 1996. Presence of vancomycin-resistant enterococci in farm and pet animals. *Journal of Antimicrobial Agents and Chemotherapy* **40**(10): 2285–87.

Facklam R R and Collins M D. 1989. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. *Journal of Clinical Microbiology* **27**: 731–34.

Frye J G and Jackson C R. 2013. Genetic mechanisms of antimicrobial resistance identified in *Salmonella enterica, Escherichia coli* and *Enteroccocus* spp. isolated from US food animals. *Frontiers in Microbiology* **4**: 135.

Gangurde N, Mane M and Phatale S. 2014. Prevalence of multidrug resistant enterococci in a tertiary care hospital in India: A growing threat. *Open Journal of Medical Microbiology* **4**(1): 11.

Jung W K, Lim J Y, Kwon N H, Kim J M, Hong S K, Koo H C and Park Y H. 2007. Vancomycin-resistant enterococci from animal sources in Korea. *International Journal of Food Microbiology* **113**(1): 102–07.

Kaszanyitzky E J, Tenk M, Ghidan A, Fehervari G Y and Papp M. 2007. Antimicrobial susceptibility of enterococci strains isolated from slaughter animals on the data of Hungarian resistance monitoring system from 2001 to 2004. *International Journal of Food Microbiology* **115**: 119–23.

Kariyama R, Mitsuhata R, Chow J W, Clewell D B and Kumon H. 2000. Simple and reliable Multiplex-PCR assay for surveliance isolates of vancomycin-resistant enterococci. *Journal of Clinical Microbiology* **38**: 3092–95.

Khan S, Nawaz M, Khan A, Hopper S, Jones R and Cerniglia C. 2005. Molecular characterization of multidrug-resistant *Enterococcus* spp. from poultry and dairy farms: Detection of virulence and vancomycin resistance gene markers by PCR. *Journal of Molecular and Cellular Probes* **19**(1): 27–34.

Lozano C, Gonzalez-Barrio D, Camacho M C, Lima-Barbero J F, de la Puente J, Höfle U and Torre C. 2016. Characterization of fecal vancomycin-resistant enterococci with acquired and intrinsic resistance mechanisms in wild animals, Spain. *Microbial Ecology* **72**(4): 813–20.

Lopez M, Tenorio C and Torres C. 2011. Study of vancomycin resistance in faecal enterococci from healthy humans and dogs in Spain a decade after the avoparcin ban in Europe. *Zoonoses and Public Health* **60**: 160–67.

Murray B E, Singh K V, Heath J D, Sharma B R and Weinstock G M. 1990. Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. *Journal of Clinical Microbiology* **28**(9): 2059–63.

Pruksakorn C, Pimarn C, Boonsoongnern A and Narongsak W. 2016. Detection and phenotypic characterization of vancomycin-resistant enterococci in pigs in Thailand. *Agriculture and Natural Resources* **50**(3): 199–203.

Seo K S, Lim J Y, Yoo H S, Bae W K and Park Y H. 2005. Comparison of vancomycin-resistant enterococci isolates from human, poultry and pigs in Korea. *Journal of Veterinary Microbiology* **106**: 225–33.

Xavier D B, Bernal F E M and Titze-de-Almeida R. 2006. Absence of vanA- and vanB- containing enterococci in poultry raised on nonintensive production farms in Brasil. *Applied Environmental Microbiology* **72**: 3072–73.