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 (Kasznanjitzky 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 subcultured onto Bile Esculin Azideagar (Himedia) as described by Baçcigil 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 İkiz (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 NaCl, 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 lauryl 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. Pruskakorn et al. (2016) reported that Enterococcus gallinarum was the most prevalent species for VRE in all animal species (Seo et al. 2011). Khan et al. (2005) isolated E. gallinarum from milk samples of animals with mastitis and litters from 28 different flocks. Pruskakorn 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        | AACGCTATGCAAGAAGCCATG        | 536          | Lopez et al. (2011) |
|             | CCGACAATCAATCATTCCCT         |              | Devries et al. (1996) |
| vanA        | CATGAAATGATAAAAGTTGCAATA     | 1,030        | Devries et al. (1996) |
|             | CCCCTTTAAGGCTAATACGATCAA     |              |           |
| vanC1       | GTATCAAGGAAAACCTC            | 822          |           |
|             | CTTCGCCGCATCATGCT            |              |           |
| vanC2/C3    | CGGGGAAGATGGCGCAGTAT         | 484          |           |
|             | CGCGGAGCGCTGTTT            |              |           |
| E. faecalis | ATCAAGTACAGTTAGCTTTATTAG    | 941          |           |
|             | AGCATCAAAAGCTAATGCAATG     |              |           |
| E. faecium  | TGAGGCCAGCCAGATTGA           | 658          |           |
|             | CGTATGACAGGCGCTTCCGATTCC    |              |           |
| rrs (16srrRNA) | GAATTAGATACCCGTGGATGCC      | 320          |           |
|             | TCGTTCGCGGACTTACCCCAAC      |              |           |
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, cephalexin, streptomycin and enrofloxacin (Table 4). Our results showed strong presence of multidrug resistance (MDR) in 37 (61.6%) isolates to vancomycin, enrofloxacin, norfloxacin and cephalexin. 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 different selective pressure from the different usage of antibiotics within the 3 species. Our results were in contrast to those from 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.

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 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         |             |

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

Table 4. Sensitivity pattern of Enterococcus isolates to different antibiotics

| Antibiotic | Sensitive | Intermediate | Resistant |
|------------|-----------|--------------|-----------|
| Vancomycin | 18 (30)   | 42 (70)      | 0         |
| Gentamicin | 55 (91.6) | 0            | 5 (8.3)   |
| Tetracycline| 37 (61.6)| 14 (23.3)    | 9 (15)    |
| Norfloxacin| 24 (40)   | 30 (50)      | 6 (10)    |
| Cephalexin | 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 %.
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.

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**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. Lane 1, 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.

**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, 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.
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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