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Research Article

Genetic Diversity of Vancomycin Resistance *Enterococcus* Spp. Isolated from Animal, Human and Environmental Origin

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Abstract

Enterococci are the common opportunistic pathogens having a worldwide food safety concern. The present study was undertaken to characterize the vancomycin resistant *Enterococcus* species of animal, human and environmental origin by using ERIC-PCR and REP-PCR assays. Out of 608 *Enterococcus* spp. isolates recovered by phenotypic and genotypic methods, 125 *Enterococcus* isolates were identified as Vancomycin resistance *Enterococcus* genotypically. The vancomycin resistant genes vanB, vanC1 and vanC2 were detected in 14 (11.20%), 69 (55.20%) and 42 (36.60%) Enterococcus isolates, respectively. A greater degree of heterogeneity was observed among 124 VRE isolates (one *E. gallinarum* isolate did not yield any bands for both ERIC-PCR and REP- PCR) of four species of Enterococcus from different sources as revealed by presence of 122 genotypes and 123 genotypes by ERIC and REP-PCR analysis, respectively. Nineteen different *E. faecalis*, 15 E. faecium, 57 *E. gallinarum* and 31 *E. casseliflavus* subtypes were differentiated by ERIC-PCR, whereas 21 different *E. faecalis*, 15 *E. faecium*, 56 *E. gallinarum* and 31 *E. casseliflavus* subtypes by REP- PCR. Genotyping of VRE species by ERIC- PCR and REP- PCR were found to be highly significant since discriminatory power > 0.9 are considered highly significant (0.9997 for ERIC-PCR and 0.9999 for REP-PCR). Cluster analysis also revealed a great degree of homogeneity among some VRE isolates recovered from different sources and implied at the chance of cross-contamination of foods of animal origin..

Keywords: Enterococci; VRE; ERIC-PCR; REP-PCR; Discriminatory Power; Cluster Analysis

Introduction

Vancomycin has proved to be active against most Gram-positive pathogens and is used in the treatments of infections due to *Staphylococci, Streptococci, Enterococci, Clostridia and Corynebacter* [1]. Gram-negative bacteria are, in general, resistant to glycopeptides because these antibiotics are unable to cross the outer cell envelope. Among the glycopeptide family, vancomycin and teicoplanin are the only two currently in clinical practice. VRE are often compounded by the use of antibiotics in animal farms as growth promoters. A link between the use of avoparcin as a growth promot-

er in poultry and swine farms and an increased occurrence of VRE in humans is well documented [2-5] and so is the evidence for transmission of VRE from animals to humans [6].

The first isolates of glycopeptide resistant enterococci (GRE) were reported by investigators in the UK in 1986 [7]. Resistance to glycopeptides in enterococci, as understood to date, is phenotypically and genotypically heterogeneous. Six glycopeptide resistance phenotypes *vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG*, have been described in enterococci; the first two types are the most clinically relevant [8,9].

In general, typing/differentiation of enterococci has been accomplished by analysis of proteins, biochemical profiles, antibiotic susceptibility and virulence patterns. Reliable molecular typing methods for purpose of finding the relatedness between bacterial isolates have become progressively important to evaluate outbreak and endemic conditions with food borne pathogens. Different techniques like Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR [10], Repetitive Extergenic Palindromic (REP) PCR [11], PFGE-Pulsed-field gel electrophoresis [12,13], determination of 16S *rRNA* sequences [14,15], RFLP- Restriction Fragment Length Polymorphism [16], MLST- Multilocus Sequence Typing [17-20] and AFLP-Amplified Fragment Length polymorphisms [21,22] are generally used for typing of *Enterococcus* spp. PCR-based techniques like REP and ERIC PCR are accurate, rapid, reproducible, sensitive, specific and reliable diagnostics, which are used for determining different DNA fingerprints [23].

Among several PCR-based tools, the ERIC-PCR is a simple, sharp and cost-effective genotyping technology for discriminating different types of strains. Indeed, ERICs are recognized as mobile DNA particles in association with Miniature Inverted Transposable Elements (MITEs) [24-26]. Repetitive Extragenic Palindromic (REP) elements first discovered in the genome sequences of *E. coli* and *Salmonella*. Hiett and Seal [27] reported the usage of REP-PCR in microorganisms is a proven discriminatory and reproducible tool for microbial subtype analyses and for microbial ecology investigations.

Recent studies have even revealed remarkable resistance of enterococci to the glycopeptide antimicrobials like vancomycin and teicoplanin in clinical samples of human origin. The presence of VRE has also been recorded in foods [28,29]. Vancomycin resistance has also been transferred *in vitro* by conjugation or transformation from enterococci to *Streptococcus sanguis*, *Lactococcus lactis*, *Streptococcus pyogenes* and *Listeria monocytogenes* [30,31]. The ability of enterococci to transfer vancomycin resistance to other common pathogens may pose further serious adverse public health consequences [32].

In this study, we attempted to determine the genetic relatedness of Vancomycin-resistant Enterococcus isolates isolated from foods of animal and fecal samples of animal, human and environmental origin. This is the first publication evaluating the effectiveness of ERIC-PCR and REP-PCR as molecular typing tools for Vancomycin-resistant *Enterococcus* spp.

Materials and Methods

A total of 608 *Enterococcus* isolates of different species isolated from different sources (234 food samples from poultry and quail; 324 food samples of animal origin; 85 faecal swabs; 25 water samples; 40 uterine discharges of cattle and 72 human faecal and clinical samples) were subjected for detection of vancomycin resistance both phenotypically and genotypically using m-PCR assays for detection of 4 major Vancomycin resistant markers like *vanA*, *vanB*, *vanC1* and *vanC2* because *vanC* mediated low level resistance is the intrinsic property of *E. gallinarum* and *E. casseliflavus* [33]. So all the *vanC* gene-carrying genotypes not show phenotypic resistance to vancomycin.

Molecular detection of vancomycin resistance genes in *Enterococcus* spp.

A single m-PCR assay [34] was standardized for the detection of vancomycin-resistant genes in enterococci. Primer sequence and standardized thermal cycling conditions used for detection of vancomycin-resistant genes were given in table 1. The band pattern was visualized using a UV transilluminator (BIO-RAD Gel Documentation system, USA) and the images were photographed using the supplied Image Lab software. Amplified PCR product size was determined by comparing it with the standard molecular weight marker.

Assessment of genetic diversity

Assessment of genetic diversity in the present study, rep-PCR was carried out using two different methods (ERIC-PCR and REP-PCR) which target two different sets of repetitive elements. About 100ng of DNA of each isolate was genotyped by two typing methods (ERIC-PCR and REP-PCR) in triplicate.

Genotyping of VRE by ERIC-PCR

VRE isolates from different sources were fingerprinted using ERIC-PCR assay as described by Blanco., *et al*. [10] with minor

Primer target gene	Sequence forward/ reverse	Size (bp)	PCR conditions
vanA	5'-TCT GCA ATA GAG ATA GCC GC-3' 5'-GG AGT AGC TAT CCC AGC ATT-3'	377	Initial Denaturation at 95^{0}C for 5 min 30 cycles of Denaturation at 95^{0}C for 30 sec Annealing at 54^{0}C for 30 sec Extension at 72^{0}C for 30 sec Final cycle elongation at 72^{0}C for 10 min
vanB	5'-CAT CGC CGT CCC CGA ATT TCA AA-3' 5'-GAT GCG GAA GAT ACC GTG GCT-3'	298	
vanC1	5'-GAC CCG CTG AAA TAT GAA G-3' 5'-CGG CTT GAT AAA GAT CGG G-3'	438	
vanC2	5'-CTC CTA CGA TTC TCT TG-3' 5'-CGA GCA AGA CCT TTA AG-3'	430	

Table 1: Primers and standardized thermal cycling conditions used for detection of VR genes in *Enterococcus* spp.

modifications in order to obtain a better band pattern. ERIC-1 (5^1-ATGTAAGCTCCTGGGGATTCAC-3^1) and ERIC-2 (5^1-AAGTAAGTGACTGGGGTGAGCG-3^1) primer pair was used for the amplification of conserved ERIC sequences in the chromosomal DNA of VRE isolates. ERIC-PCR was carried out in a 25 µl optimized reaction mixture under standardized thermal cycling conditions (Table 2).

Genotyping of VRE by REP-PCR

Enterococcus isolates showing vancomycin resistant genes from different sources were subjected to REP-PCR fingerprinting using single oligonucleotide primer $(GTG)_5$ (5^{1}GTGGTGGTGGTGGTG 3^1) as described by Bedendo and Pignatari [11] with slight modifications. PCR reactions were optimized in a 25 µl volume reaction mixture under standardized thermal cycling conditions given in table 2.

Analysis of ERIC-PCR and REP-PCR fingerprinting patterns of VRE

PCR products were subjected to 1.5% agarose gel electrophoresis and visualized using a gel documentation unit (BIORAD, USA). The ERIC-PCR and REP-PCR fingerprints (banding patterns) were compared visually with a 100 bp DNA ladder and transformed into

	Standardized cycling parameters		
Steps	**ERIC-PCR**	**REP-PCR**	**No. of cycles**
Primers	ERIC-1 (5^1-ATGTAAGCTCCTGGGGATTCAC-3^1) ERIC-2 (5^1-AAGTAAGTGACTGGGGTGAGCG-3^1)	$(GTG)_5$ (5^1 GTGGTGGTGGTGGTG 3^1)	
Initial denaturation	95˚C for 5 min	95˚C for 5 min	1
Denaturation	94˚C for 1 min	94˚C for 45 sec	40
Annealing	25˚C for 1 min	40˚C for 1 min	
Extension	72˚C for 2 min	65˚C for 10 min	
Final extension	72˚C for 10 min	65˚C for 20 min	1
Hold/stand by	4˚C for 10 min	4˚C for 10 min	---

Table 2: Standardized thermal cycling conditions for ERIC and REP-PCR for VRE.

a binary character matrix ('1' for the presence and '0' for the absence of a band at a particular position). The binary data were analyzed using the dollop program of phylip version 3.6 [35] software with default options. Dendrograms were constructed for the four *Enterococcus* species separately to establish genetic diversity or relatedness among the VRE isolates. The discriminatory power of ERIC-PCR and REP-PCR genotyping techniques for VRE by Simpson's index of diversity [36]. The standard culture *E. faecalis* (MTCC439) and *E. gallinarum* (MTCC 7049) were used as a standard for ERIC and REP-PCR.

Results and Discussion

Out of 608 *Enterococcus* isolates, 117 (19.24%) isolates showed resistance to vancomycin by disc diffusion (59 *E. faecalis*, 26 *E. faecium*, 16 *E. gallinarum* and 16 *E. casseliflavus*) and genotypically 125 (20.55%) were found to be VRE (Figure 1). Of 125 VRE positive genotypes, 21 were *E. faecalis* (3 *vanB,* 14 *vanC1* and 4 *vanC2*), 15 *E. faecium* (11 *vanB* and 4 *vanC2*), 58 *E. gallinarum* (52 *vanC1* and 6 *vanC2*) and 31 *E. casseliflavus* (3 *vanC1* and 28 *vanC2*) isolates. None of the isolates showed *vanA* gene. Out of 125 genotypically positive VRE isolates, the *vanB*, *vanC1* and *vanC2* were detected in 14 (11.20%), 69 (55.20%) and 42 (33.60%) VR *Enterococcus* isolates, respectively.

In the present study, all *vanB* genotypes showed phenotypic resistance to vancomycin in disc diffusion test but *vanC1* and C2 genotypes mostly showed phenotypic sensitivity to vancomycin. The *vanA* gene-mediated phenotype glycopeptide resistance is considered by acquired inducible high-level resistance to both vancomycin and teicoplanin which has been notified in several *Enterococcus* spp. and in certain *Staphylococcus aureus* isolates that were showing phenotypic vancomycin resistance. *vanB* gene mediated phenotype glycopeptide resistance is associated with acquired inducible low to high level resistance to various concentrations of vancomycin but typically not to teicoplanin but few isolates with resistance also to teicoplanin have been described [1]. *vanB* gene cluster was found predominantly in *E. faecalis* and *E. faecium* [8,9]. *vanC* gene mediated phenotype glycopeptide resistance is characterized by low-level vancomycin resistance and susceptibility to teicoplanin and has been described as an intrinsic property of *E. gallinarum* and *E. casseliflavus/flavescens* [33].

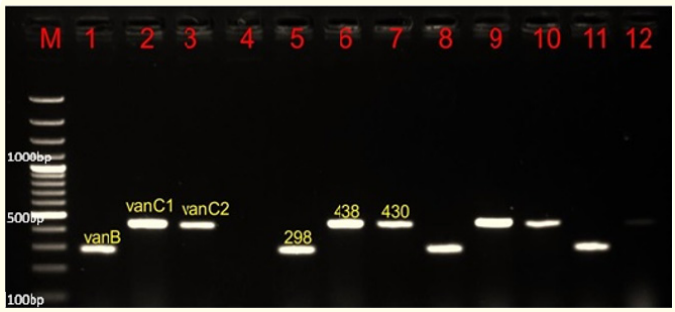


Figure 1: Gel photograph of PCR showing VR specific bands in Enterococcus spp.

Lane M Molecular weight marker (100bp)

Lane 1 Known positive standard of E. faecium showing gene vanB (298bp)

Lane 2 Positive control of E. gallinarum MTCC7049 showing gene vanC1 (438bp)

Lane 3 Known positive standard of E. casseliflavus showing gene vanC2 (430bp)

Lane 4 Negative control

Lane 5 E. faecium showing gene vanB isolated from water sample (298bp)

Lane 6 E. gallinarum showing gene vanC1 isolated from chicken sample (438bp)

Lane 7 E. casseliflavus showing gene vanC2 isolated from chicken sample (430bp)

Lane 8 E. faecalis showing gene vanB isolated from human diarrhoeic sample (298bp)

Lane 9 E. faecalis showing gene vanC1 from pork sample (438bp)

Lane 10 E. gallinarum showing gene vanC2 from chicken cloacal swab (430bp)

Lane 11 E. faecium showing vanB isolated from pork sample (298bp)

Lane 12 E. casseliflavus showing vanC2 isolated from fish sample (430bp)

Out of 59 and 26 phenotypically positive VR *E. faecalis* and *E. faecium*, only 21 and 15 isolates were found to be carried VR genes, respectively. This expression of phenotypic VR and absence of VR genes in the present study may be due to the presence of other VR genes which are not included in this study [37]. Out of 58 and 31 VR genotypes of *E. gallinarum* and *E. casseliflavus* only 16 isolates of both were phenotypically resistant to vancomycin. This may be due to the presence of a *vanC*-mediated intrinsic resistance mechanism [33].

In the present study *vanC1* (55.2%) is the predominant VR gene followed by *vanC2* (33.60%) and *vanB* (11.20%) which were in agreement with Xavier., *et al*. [38]. They also reported *vanC1* as the most prevalent vancomycin resistance gene (13.0%) followed by *vanC2/3* (5.5%). Further, they also reported that none of the isolates carried *vanA* or *vanB* genes of enterococci isolated from the chicken cloacal swab isolates obtained in Brazil. Nishiyama., *et al*. [39] reported that 92% of river water *Enterococcus* isolates from Japan were carrying *vanC2/3*. Latha., *et al*. [40] reported an increased rate of *vanB-associated* VRE isolates ranging from 22 to 100% from upstream to downstream in Gomati river water along the Lucknow city landscape.

Contrary to our findings Mac., *et al.* [41] reported the *vanA* gene in 21 *Enterococcus* isolates from foods of animal origin and they could not detect *vanB* gene in any of the isolates, however, the majority of isolates possessed either *vanC1* or *vanC2.*

Peculiar findings in the present study include *E. faecalis* with 18 *vanC* genes (14 *vanC1* and 4 *vanC2*) and *E. faecium* with 4 *vanC2* genes. The detection of these *vanC* genes in *E. faecalis* and *E. faecium* is remarkable because they were thought to acquire *vanC* genes by horizontal transfer from *E. gallinarum* and *E. casseliflavus*, natural inhabitants of the poultry gut in which *vanC* is intrinsic property. These findings were supported by Schwaiger., *et al*. [42], Moura., *et al*. [43] and Nishiyama., *et al*. [39], who also reported the presence of *vanC* genes in Enterococci isolates.

Among rep-PCR typing methods, as most ERIC-PCR and REP-PCR methods suffer from reproducibility problems, the PCR reactions in the present study were standardized for their reproducibility by the inclusion of DNA from *E. faecalis* (MTCC439) and *E. gallinarum* (MTCC7049). ERIC-PCR revealed genetic diversity between VRE species (*E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus*) with ERIC sequences found in all the *E. faecalis* isolates (4-9 distinct bands), *E. faecium* isolates (3-11 distinct bands), *E. gallinarum* isolates (1-11 distinct bands) and *E. casseliflavus* isolates (2-12distinct bands). REP-PCR revealed genetic diversity between the VRE species (*E. faecalis*, *E. faecium*, *E. gallinarum,* and *E. casseliflavus*) with REP sequences found in all the *E. faecalis* isolates (3-12 distinct bands), *E. faecium* isolates (3-14 distinct bands), *E. gallinarum* isolates (1-11 distinct bands) and *E. casseliflavus* isolates (3-12distinct bands).

Among 125 VRE, greater degree of heterogeneity was observed among 124 VRE isolates (one *E. gallinarum* isolate did not show any bands for ERIC and REP-PCR) of different species from different sources as revealed by presence of 122 genotypes and 123 genotypes under ERIC and REP-PCR analysis, respectively. Nineteen different *E. faecalis*, 15 *E. faecium*, 57*E. gallinarum* and 31 *E. casseliflavus* subtypes were differentiated by ERIC-PCR, whereas 21 different *E. faecalis*, 15 *E. faecium*, 56*E. gallinarum* and 31 *E. casseliflavus* subtypes were determined by REP-PCR, which revealed wide genetic diversity among the strains isolated.

Cluster analysis of ERIC-PCR profiles differentiated VR *E. faecalis* isolates from different sources into seven main clusters based on the genetic similarity cut-off value of 70% (Figure 2). Cluster I comprised of 3 isolates (c1, c9 and c17) of chicken samples obtained from Gannavaram, where c1 clustered separately from that of other two isolates (c17 and c9) recovered from same retail chicken shop. In cluster II, *E. faecalis* MTCC439 was closely clustered with isolate of mutton origin (m13) showing 90% similarity cut off. Clusters III and IV were having 2 sub clusters each with 2 isolates in each sub cluster. Cluster III comprised of four isolates of pork origin (p27, p31, p11 and p50) were recovered from pork processing unit, N.T.R CVSc. Gannavaram indicating the chances of cross contamination from equipment or lairage pen. Cluster V, VI and VII were having 2 isolates each. Cluster VI comprised of human diarrhoeic isolate (hd3) and chicken cloacal swab isolate (CC26) with the same ERIC-PCR band pattern. Cluster VII consisted of 2 isolates of human diarrhoeic origin (hd2 and hd6) having similar ERIC band patterns. Isolates from chicken meat origin (c21 and c136) and fish origin (f11) unclustered separately indicating wide genetic diversity. However, cluster analysis of REP-PCR profiles grouped into four main clusters (Figure 3). Cluster I was again divided into 2 sub clusters having 2 isolates each (p17 and f11; f22 and c110, respectively) and p53 isolate was distantly related. Cluster II has 3 isolates (hd2, hd6 and p53) where p53 isolate was

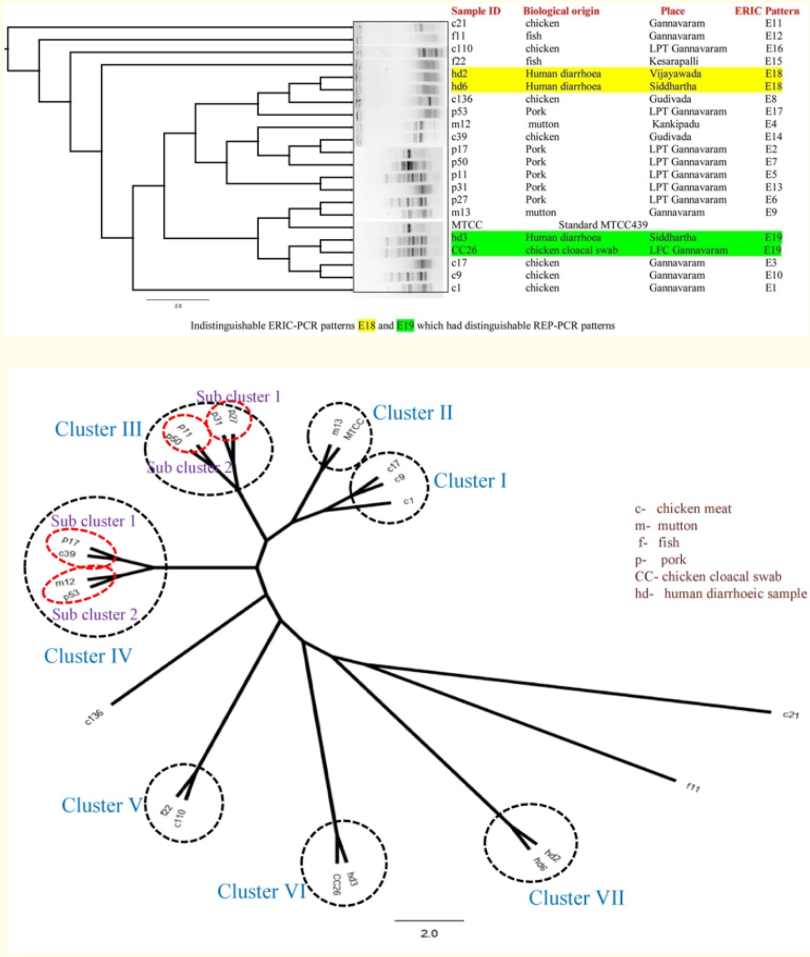
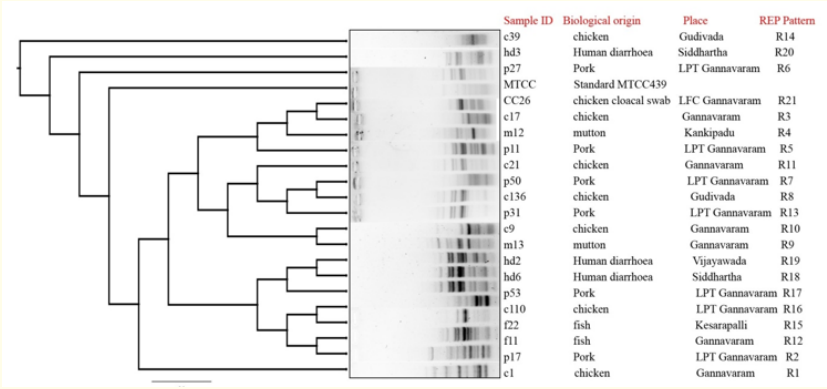


Figure 2: Dendrogram and Cluster analysis of ERIC-PCR fingerprints of VR E. faecalis from different Sources.

An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm).



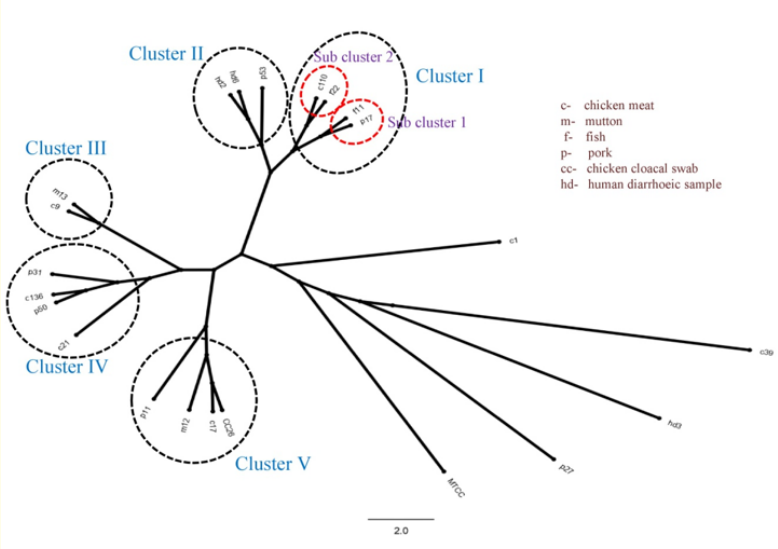


Figure 3: Dendrogram and Cluster analysis of REP-PCR fingerprints of VR E. faecalis from different Sources.

An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm).

distantly related. Cluster III has 2 isolates (m13 and c9) whereas Clusters IV and V have 4 isolates each. In cluster IV, isolate from chicken (c21) was distantly separated from other 3 isolates (p31, c136 and p50). In cluster V, isolate from pork (p11) was separated from other three isolates (m12, c17 and CC26). Four isolates (c1, c39, hd3 and p27) and *E. faecalis* MTCC749 were found to be unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates.

Cluster analysis of ERIC-PCR profiles differentiated VR *E. faecium* isolates from different sources into four main clusters for a similarity cut-off value of 70% (Figure 4). Cluster I again divided into 2 sub clusters, each sub cluster having 2 isolates. Cluster I (p14, c76, m10 and f39) and cluster II (p11, f14, c81 and c47) comprised isolates of meat origin. Within the cluster II, isolates p11 and c81 were clustered separately from that of other two isolates (f14 and c47). Cluster III comprised of 2 isolates recovered from samples of fish (f41 and f36) and 1 isolate recovered from water sample (w22) collected from Uppuluru showed closer genetic relatedness. It indicates the possibility of cross-contamination between the waterbodies and fish available in the retail market from where the fish samples were collected. In cluster IV, 1 isolate recovered from water sample (w12) in Kankipadu and 1 isolate recovered from chicken meat (c84) in Gannavaram showed genetic closeness. However, cluster analysis of REP-PCR profiles differentiated VR *E. faecium* isolates from different sources into four main clusters for a similarity cut-off value of 70% (Figure 5). Cluster I and II have 2 isolates in each cluster (c76 and p11; c84 and f39, respectively) of animal meat origin. Cluster III and IV comprised of isolates recovered from water and foods of animal origin sharing closer genetic relatedness. Cluster III comprised of 4 isolates. In cluster IV, two isolates from water (w22) and fish (f36) obtained from Kankipadu showed the close genetic relatedness and similar sub clustering pattern observed in ERIC-PCR also. In cluster IV, c81 and f14 were closely related and w21 and c62 were separated from other 2 isolates. Isolates c47, f41 and p14 were unclustered indicating presence of wide genetic diversity. But they were clustered with other VR *E. faecium* isolates in ERIC-PCR dendrogram.

Cluster analysis of ERIC-PCR profiles differentiated VR *E. gallinarum* isolates from different sources into 6 main clusters for a similarity cut-off value of 70% (Figure 6). Cluster I was divided into 4 sub clusters. Isolates c65, c31, m5 and h17 were sub clustered of which c31 and m5 showed 90% similarity. Within cluster I, quail isolate (q46) was distantly isolated from the 4 sub clusters. Sub clusters 2 and 3 have 2 isolates each (CC31 and p36; BR9 and q31,

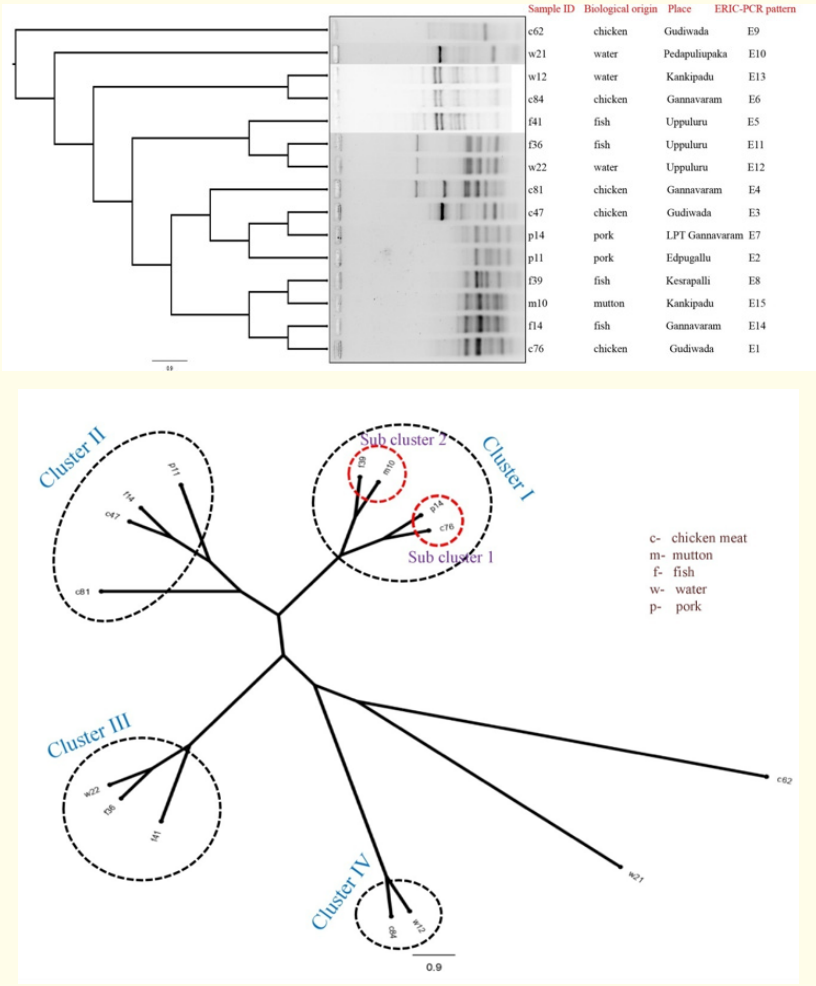
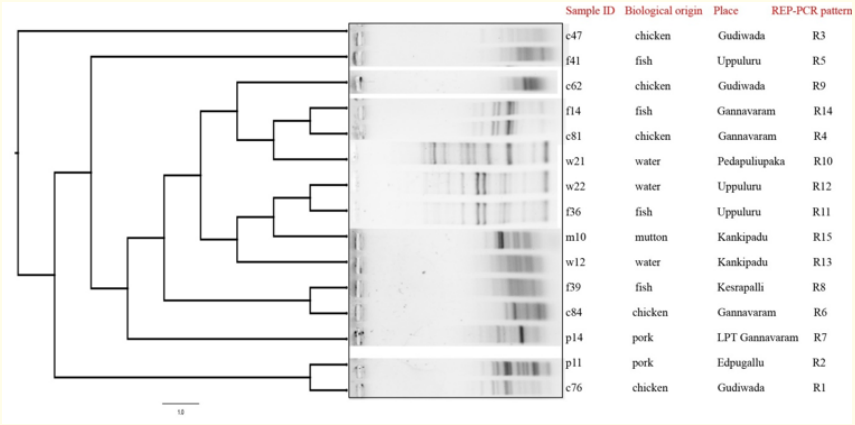


Figure 4: Dendrogram and Cluster analysis of ERIC-PCR fingerprints of VR E. faecium from different Sources.

An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm).



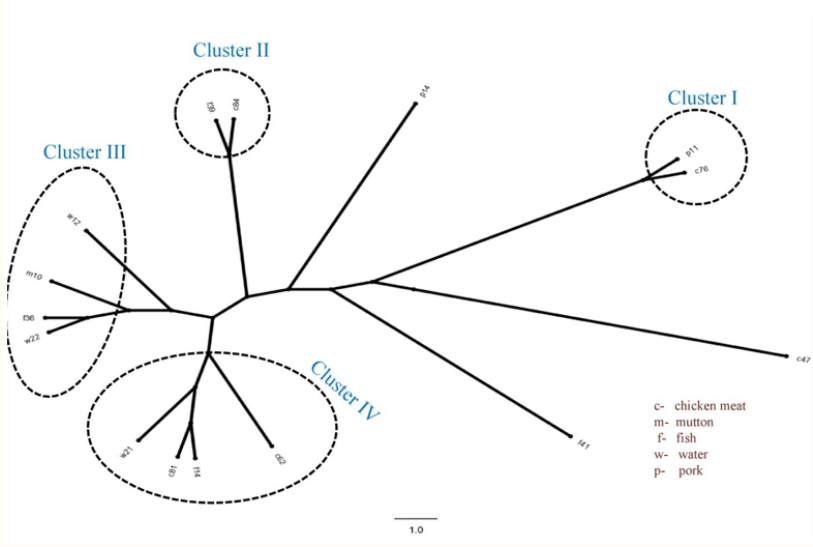


Figure 5: Dendrogram and Cluster analysis of ERIC-PCR fingerprints of VR E. faecalis from different Sources.
An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm).

respectively) and sub cluster 4 has 3 isolates (c3, q27 and m52). Cluster II comprised of 3 sub clusters where sub cluster 1 has 2 isolates (cb17 and M79), sub cluster 2 have 3 isolates (c101, M19 and *E. gallinarum* MTCC7049) whereas M19 and *E. gallinarum* MTCC7049 were closely related with > 90% similarity cut off and sub cluster 3 consisted of 4 isolates (cb11, CC10, c79 and M76). Cluster III has 5 isolates (M4, q50, c115, CC20 and hd5) and they were divided into 2 sub-clusters where human diarrhoeic isolate (hd5) and chicken cloacal isolate (CC20) expressed close genetic relatedness. Cluster IV consisted of 11 isolates (M43, cb21, q22, h20, M24, cb5, m26, hd1, M77, c83 and PR6). Cluster IV comprised of 3 sub clusters where sub cluster 1 has M43, cb21 and q22, sub cluster 2 has 6 isolates (h20, M24, cb5, m26, hd1 and M77) where cb5 and m26 were closely related with > 90% similarity cut off and sub-cluster 3 has 2 isolates (c83 and PR6) Within Cluster IV, 3 sub-clusters were noticed. In cluster IV, human diarrhoeic (hd1) and human stool isolates (h20) were clustered together with isolates of meat and milk origin. Cluster V is the largest cluster consisting of 14 isolates and was grouped into 5 sub-clusters where sub cluster 1 and 2 have 2 isolates each (c127 and CC13; c33 and m77, respectively), ssub-cluster3 has c112, c99, and c153 isolates where c99 and c153 were closely related, ssub-cluster4 has m25, c120 and w13 isolates of which c120 and m25 were closely related and ssub-cluster5 has cb29, M3 and c159 isolates in which M3 and c159 are closely related. Within cluster V, chicken cloacal isolate (CC19) was distantly away from other isolates that were sub-clustered. Isolates, c153 and c99 of sub-cluster 3 were obtained from the same chicken retail shop in Gudiwada, but the sampling interval between the 2 samples collected was 41 days. It indicates the possibility of a source of contamination from the surrounding environment (*Enterococcus* spp. can withstand adverse environment with minimal nutrient requirement). Cluster VI consisted of 2 isolates of chicken (c88) and chicken cloacal origin (CC8) which showed great genetic relatedness. Five isolates (w18, m21, q36, M39 and q24) were found to be unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates from different sources.

Dendrogram analysis of REP-PCR profiles discriminated VR *E. gallinarum* isolates into a 7 major clusters (Figure 7). Cluster I contained 4 isolates (c127, CC19, q22 and h17) where chicken cloacal isolate (CC19), showed closer genetic relation with isolate from quail (q22) origin. The clusters II and VII were the smallest clusters with 3 isolates each. Cluster II, human faecal isolate (h20) is distantly related with 2 isolates (cb21 and M79) that were sub-clustered in the same cluster. Cluster III contained 10 isolates (m77,

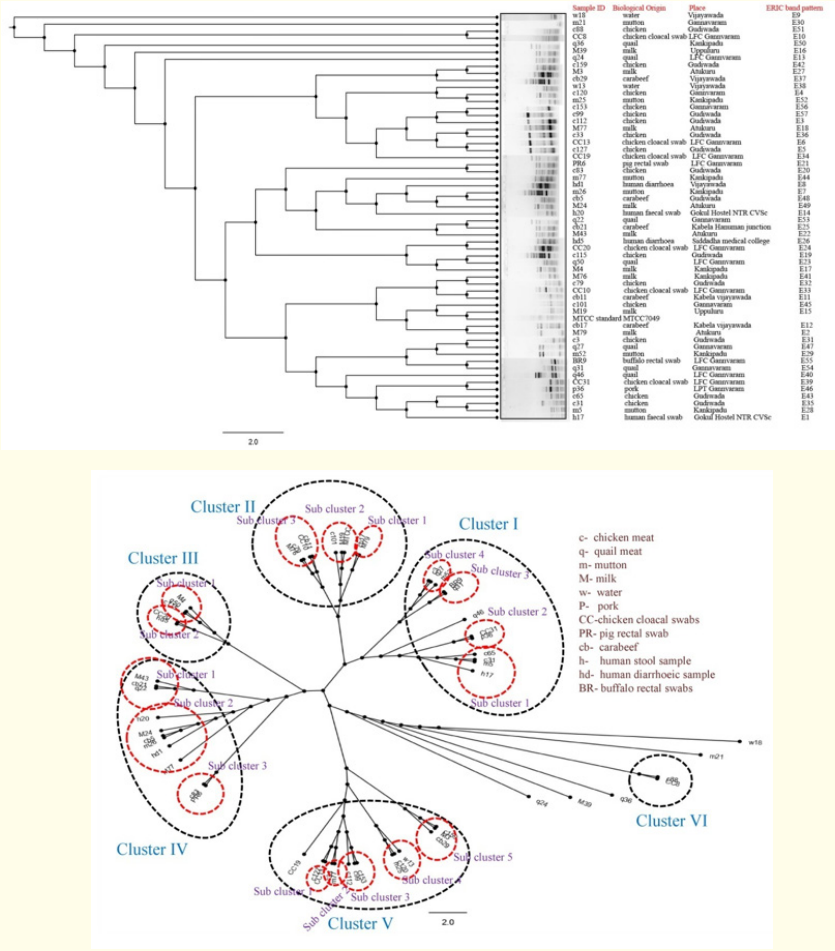
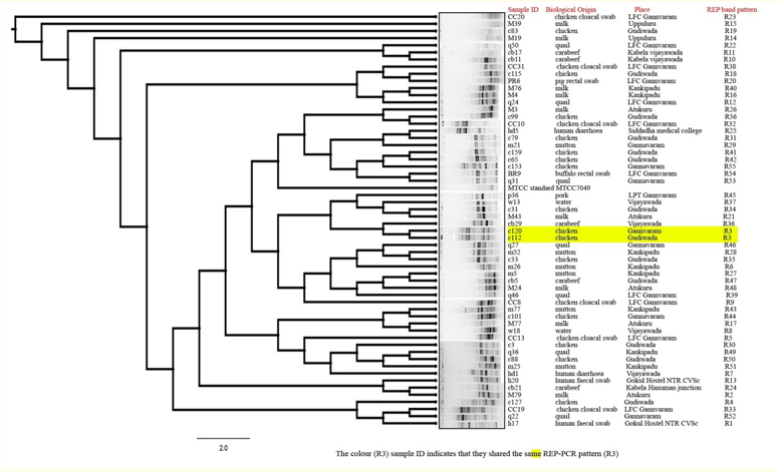


Figure 6: Dendrogram and Cluster analysis of ERIC-PCR fingerprints of VR E. gallinarum from different Sources.

An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm).



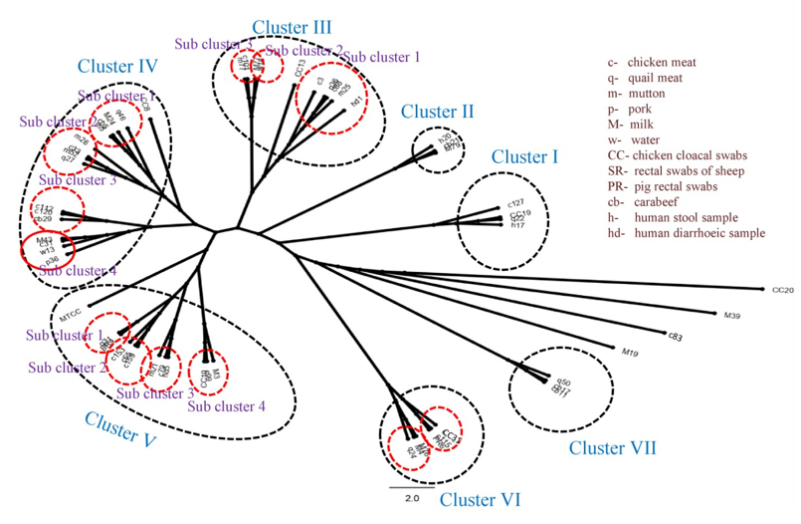


Figure 7: Dendrogram and Cluster analysis of REP-PCR fingerprints of VR E. gallinarum from different Sources.

An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm).

M77, w18, c101, CC13, c3, c36, c88, m25 and hd1). Within cluster III, 3 sub clusters were notified. Sub cluster 1 has hd1, m25, c88, q36 and c3 and sub cluster 2 and 3 had 2 isolates each (w18 and M77; c101 and m77). Chicken cloacal isolate (CC13) was distantly related with other isolates in the cluster III. Cluster IV was the largest cluster with 16 isolates and comprised of 4 sub clusters where sub cluster 1 (q46, M24, cb5 and m5), 2 (m26, c33, m52 and q27) and 4 (M43, c31, w13 and p36) had 4 isolates each and sub cluster 3 has 3 isolates (c112, c120 and cb29). Within the cluster IV, chicken cloacal origin isolate (CC8) showed distant relation with other isolates of the sub clusters. Cluster V was divided into 4 sub clusters where sub clusters 2 (c153, c65 and c159), 3 (m21, c79 and hd5) and 4 (CC19, c99 and M3) had 3 isolates each and sub cluster 1 has 2 isolates (q31 and BR9). Within the cluster V, *E. gallinarum* MTCC7049 showed wide distance with other isolates (4 sub clusters) within the cluster. Cluster VI contained 2 sub clusters where sub cluster 1 has q24, M4 and M76 and sub cluster 2 has PR6, c115 and CC31 isolates. Cluster VII contained 3 isolates (q50, cb17 and cb11) where quail isolate (q50) clustered separately from isolates of carabeef origin (cb17 and cb11). Isolates cb17 and cb11 showed close genetic proximity which were obtained from Kabela (Vijayawada) and it indicates possibility of contamination with similar *Enterococcus* strains occurred at slaughtering area. Four isolates (CC20, M39, c83 and M19) were found to be unclustered (UC) with other isolates.

Cluster analysis of ERIC-PCR profiles differentiated VR *E. casseliflavus* isolates from different sources into four main clusters for a similarity cut-off value of 70% (Figure 8). Cluster I comprised of 2 sub clusters where sub cluster 1 has c142, SR8 and M8 isolates and sub cluster 2 has 3 isolates of quail origin (q2, q50 and q15) showing close genetic relatedness. In cluster I, quail and sheep rectal isolate were obtained from LFC, Gannavram and remaining isolates were obtained from retail shops in Gannavarm. Cluster II comprised of 2 sub clusters where sub cluster 1 has c27, c94 and c66 (isolates of chicken origin) and sub cluster 2 has CC17, m6 and c109. Within cluster II, isolate of chicken origin (c139), genetically far distant from other isolates of the cluster. Cluster III was divided into 3 sub clusters where sub cluster 1 has q5, q49, m30 and c122, where isolate of chicken origin (c122) arranged distantly from other 3 isolates, sub cluster 2 has w10 and f13 and sub cluster 3 has c56, SR6 and c32. Cluster IV again divided into two sub clusters and each with 2 isolates (c11 and c13; f35 and m39). Five isolates (c119, q44, c93, c25 and c146) were found to be unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates. However, cluster analysis of REP-PCR profiles differentiated VR *E. casseliflavus* isolates from different sources into seven main clusters for a similarity cut-off value of 70% (Figure 9). Clusters I was grouped into 2 sub clusters where sub cluster 1 has c142, w10 and fm8 and sub cluster 2 has m39

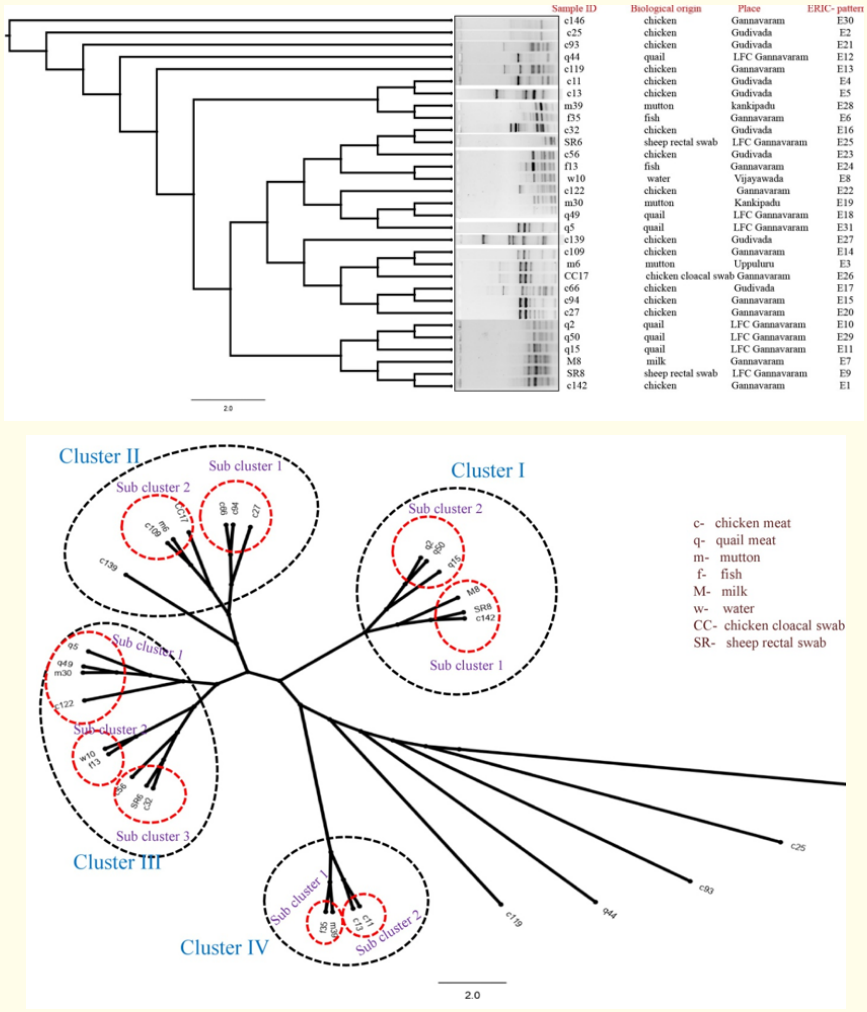
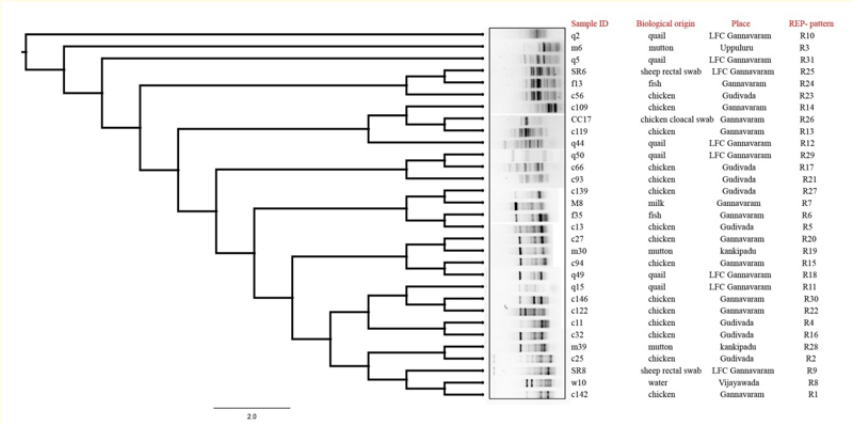


Figure 8: Dendrogram and Cluster analysis of ERIC-PCR fingerprints of VR E. casseliflavus from different Sources. An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm).



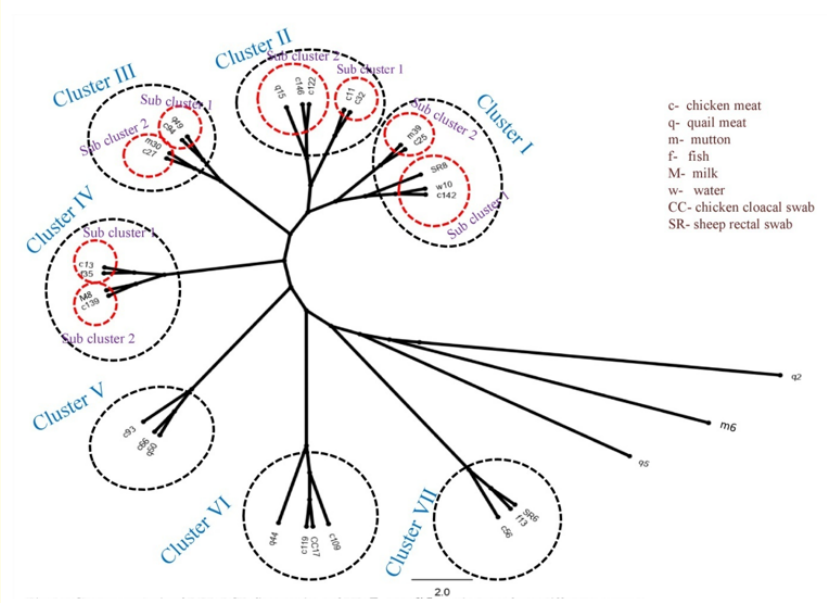


Figure 9: Dendrogram and Cluster analysis of REP-PCR fingerprints of VR E. casseliflavus from different Sources.

An unrooted phylogenetic tree constructed using dollop program of phylip 3.6 version (branch-and-bound algorithm).

and c25. Cluster II comprised of isolates recovered from samples of chicken (c11, c32, c122 and c146) and quail (q15). Within the cluster II, c32 and c11 were closely clustered which were obtained from same chicken retail shop in Gudiwada. It indicated the possible cross contamination either by handlers or equipment used for processing. In the same cluster II, chicken isolates (c146 and c122) collected from Gannavaram were also showing close proximity. Cluster III comprised of 2 sub clusters where sub cluster 1 has q49 and c94 and sub cluster 2 has m30 and c27. Similarly cluster IV again divided into 2 sub clusters where sub cluster 1 has c13 and f35 and sub cluster 2 has m8 and c139. Cluster V contained isolates of chicken (c93 and c66) and quail origin (q50) where c66 and q50 isolates showed great genetic relatedness. Cluster VI had 4 isolates (q44, c119, CC17 and c109) of which CC17 and c119 were showing close genetic association. Cluster VII has 3 isolates where isolates SR6 and f13 were showing close genetic relation (> 90% similarity cut off value and c56 was distantly related with the other isolates f13 and SR6). Three isolates (q5, M6 and q2) were found to be unclustered (UC) with other isolates. Cluster analysis indicated wide genetic diversity among the isolates.

The discriminatory power of two typing methods i.e., ERIC-PCR and REP-PCR for *Enterococcus* isolates was found to be 0.9997 and 0.9999, respectively. Present study results fall in line with Bedendo and Pignatari [11], who reported discriminatory power of 0.9722 for 8 *E. faecium* isolates from Stanford University. They also reported that PCR-based genetic diversity studies is of lower cost and are easier to perform than PFGE. However, PCR results are more difficult to analyze, since the presence of multiple weak bands in the PCR profiles makes it difficult to interpret the results. Similar results were also observed by Blanco., *et al*. [10], who conducted genetic diversity studies on one *E. hirae* and 67 *E. faecalis* strains by ERIC-PCR. They also reported that *E. fecalis* strains were clustered into five major groups and one strain was unclustered whereas *E. hirae* strain was distantly related to the rest of the strains.

Conclusion

The present study indicated both ERIC and REP-PCR to be the highly suitable genotyping method since discriminatory powers above 0.90 are considered highly significant (Hunter and Gaston, 1988). Thus rep-PCR (both ERIC and REP-PCR) fingerprinting

Source	*E. faecalis*			*E. faecium*		*E. gallinarum*		*E. casseliflavus*	
Gene marker	***vanB* (%)**	***vanC1* (%)**	***vanC2* (%)**	***vanB* (%)**	***vanC 2* (%)**	***vanC1* (%)**	***vanC2* (%)**	***vanC1*(%)**	***vanC2* (%)**
Foods of animal origin									
Chicken (43)	0	5 (11.62)	2 (4.65)	5 (11.62)	0	13 (30.23)	5 (11.62)	3 (6.97)	10 (23.25)
Quail (13)	0	0	0	0	0	7 (53.84)	0	0	6 (46.15)
Mutton (13)	0	0	2 (15.38)	1 (7.69)	0	5 (38.46)	0	0	5 (38.46)
Pork (9)	0	6 (66.66)	0	2 (22.22)	0	1 (11.11)	0	0	0
Fish (8)	0	2 (25)	0	0	4 (50)	0	0	0	2 (25)
Carabeef (6)	0	0	0	0	0	6 (100)	0	0	0
Milk (10)	0	0	0	0	0	9 (90)	0	0	1 (10)
Animal faecal swabs									
Chicken cloacal swabs (5)	0	1 (20)	0	0	0	2 (40)	1 (20)	0	1 (20)
Sheep rectal swabs (2)	0	0	0	0	0	0	0	0	2 (100)
Buffalo rectal swabs (2)	0	0	0	0	0	2 (100)	0	0	0
Pig rectal swabs (1)	0	0	0	0	0	1 (100)	0	0	0
Human samples									
Human Stool samples (2)	0	0	0	0	0	2 (100)	0	0	0
Human diarrheic stool samples (5)	3 (60)	0	0	0	0	2 (40)	0	0	0
Environmental samples									
Water (6)	0	0	0	3 (50.00)	0	2 (33.33)	0	0	1 (16.66)
Total (125)	3 (2.40)	14 (11.2)	4 (3.2)	11 (8.8)	4 (3.2)	52 (41.6)	6 (4.8)	3 (2.4)	28 (22.4)

Table 3: Vancomycin-resistant markers among different *Enterococcus* spp. isolated from different sources.

methods can be used when large numbers of isolates are needed to be investigated. The present study reportage the genotyping and genetic diversity of VRE isolates recovered from animals, foods of animal origin, environment and humans in India adds to the heterogeneity reports among *Enterococcus* species worldwide, supporting diversity among the same species. The ERIC and rep-PCR analysis also indicated the genetic similarity among diarrheic humans and meats of animals, which reveals the possibility of epidemiological relationship and evolutionary pattern between *Enterococcus* isolates of animal and human origin and its feasible zoonotic significance.

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