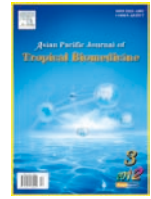




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## Characterization of vancomycin resistant *Enterococcus faecium* from clinical and chicken sources

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### ABSTRACT

**Objective:** The study was undertaken to investigate the genomic and phenotypic relationship among *E. faecium* strains isolated from chicken and clinical sources. **Methods:** Enterococci were isolated and identified by conventional biochemical methods and the antibiotic susceptibility was determined by disk diffusion methods. Phenotypes and genotypes of vancomycin resistance were detected by E tests and PCR amplification techniques respectively. Genotyping of the vancomycin resistant *E. faecium* from two sources were done by RAPD typing. **Results:** The Vancomycin resistant *E. faecium* identified was selected for this comparative study. Among the VREF from two sources minor biochemical difference with regards to raffinose fermentation and haemolytic properties was observed. The RAPD tests using random primers also showed polymorphism. **Conclusion:** The results of the study showed that the strains from two different sources were not identical.

## 1. Introduction

Enterococci are known to cause serious diseases like urinary tract infections, bacteremia, endocarditis, intra abdominal and pelvic infections, skin and soft tissue infections, meningitis etc [1]. Although *E. faecalis* was the most common species isolated earlier, a shift to *E. faecium* is observed in the reports for the last two decades [2]. *E. faecium* has become an important, nosocomial pathogen and a significant increase in infections by them has been reported recently [3]. *E. faecium* possesses a broad spectrum of natural and acquired antibiotic resistance and this has enabled Enterococci to emerge as a crucial nosocomial pathogen. *E. faecium* is found to be more phenotypically heterogeneous than all the other enterococci.

Vancomycin resistance has become an alarming clinical problem and majority of vancomycin resistant enterococcus (VRE) infections are due to the multidrug resistant vancomycin resistant *E. faecium* (VREF) as described previously from different parts of the world [4]. But the global epidemiology of vancomycin resistant

*E. faecium* is yet to be well understood. Difference in opinion exists among the investigators regarding the emergence of vancomycin resistance. There are reports on the nosocomial spread of VREF[5] while others have found that commensal microbiota of some animals and humans act as reservoirs[6,7], for vancomycin resistant enterococci. Evidence from Europe suggests that food borne VRE may cause human colonization [8]. However the role played by non human sources and reservoirs other than hospitalized patients in the spread of Enterococci is ambiguous [9]. For the epidemiological investigation of enterococcal outbreaks, several typing methods like serotyping, phage typing, biotyping and molecular methods like RFLP, plasmid profile analysis, Random amplification of polymorphic DNA (RAPD), PFGE, ribotyping etc. can be used. RAPD has been used to type Enterococci successfully by many investigators [10, 11]. RAPD involves less time and is cost beneficial.

Since the occurrence of vancomycin resistant enterococci from the clinical sources is less and an increasing frequency of this could be observed from poultry sources in South India, this study was conducted with the objective of investigating the phenotypic properties and genetic relationship of vancomycin resistant *E. faecium* from clinical sources and chicken faeces from that region.

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## 2. Materials and methods

### 2.1. Isolation of Enterococci

Chicken faeces were collected from 35 farms in Kerala state, South India. 1gm of faeces was suspended in 100ml of phosphate buffered saline and vortexed. 0.1ml of it was then transferred into streptococcus faecalis broth (HiMedia) and incubated at 37°C for 12 hours and was subcultured on bile esculin azide agar containing bile, esculin and azide. The black colored colonies observed were subjected to morphological and biochemical studies as described by Facklam and Collins [12]. Enterococcal isolates obtained from clinical sources were also identified as above.

### 2.2. Detection of beta haemolysin

Production of haemolysin was detected by streaking them on Brain heart infusion agar (HiMedia) plates supplemented with 5% defibrinated human blood. Production of haemolysin was indicated by the formation of clear zones surrounding the colonies on blood agar plates.

### 2.3. Antibiotic susceptibility testing

Antibiotic susceptibility tests were performed by disk diffusion techniques and interpreted according to guidelines from National Committee for Clinical Laboratory Standards [13].

### 2.4. Determination of vancomycin resistance phenotype and genotype

The MICs of the isolates were detected by the E-test (HiMedia Laboratories Pvt Limited). HiComb MIC strips were applied on the inoculated agar plates and incubated. The MIC values were detected from the concentration of antibiotic at which the zone intersects the test strip. Vancomycin resistance phenotypes were identified by detecting MIC of the vancomycin and teicoplanin. VanB phenotype, low levels of vancomycin resistance (MICs of 16–64 mg/L), but sensitivity to teicoplanin (MIC < 4 mg/L) were selected for the study. The corresponding genotypes of these were detected by the PCR amplification with specific primers for vancomycin resistance genes.

### 2.5. Genotyping of vancomycin resistant *E.faecium* by RAPD

One vanB harboring vancomycin resistant enterococci from chicken source and a vanB from blood were randomly selected and subjected to molecular typing by RAPD test. Primers used were OPK7, OPK11, OPA14, OPAA17, OPE6, OPL7, OPK12, and OPBG19. Reaction mixture (25 µl) contained template DNA (20–25 ng), 10X Taq buffer, MgCl<sub>2</sub> (25mM), dNTP mix (10mM), Primer (10pmol) Taq DNA polymerase (0.3U). Sterile water was used with an initial denaturation at 94°C followed by denaturation at 94°C. Subsequently annealing at 37°C for 1 minute was done followed by

polymerization at 72°C for 1 minute. The steps 2 to 4 were repeated 40 times. Finally it is subjected to extended polymerization at 72°C for 6 minutes.

## 3. Results

Among the 210 enterococci samples obtained from clinical sources, only three were found to be VREF with vanB phenotype. Whereas twenty out of the 200 isolates from chicken faeces were vanB type of VREF. All these resistant isolates were *E.faecium*. A comparative study of their antibiotic resistance and phenotypic properties are shown table 1.

**Table 1.**

Differentiating features of VREF from chicken and clinical sources

Characteristics tested	No. of VREF with +ve findings	
Fermentation Tests	Clinical Source	Faecal Source
Raffinose	0	20
Mannitol	3	18
Sorbitol	0	15
Adonitol	0	0
Sucrose	3	20
Arabinose	3	20
Lactose	3	20
Decarboxylation test	Clinical Source	Faecal Source
Arginine	3	20
Hemolysin Production	3	0
Antibiotic Sensitivity	Clinical Source	Faecal Source
Ampicillin	0	20
Gentamicin	0	20
Streptomycin	0	20
Amoxyclav	0	20
Ciprofloxacin	0	20
Vancomycin	0	0
Teicoplanin	3	20
Minimum Inhibitory Concentration of Antibiotic	Clinical Source	Faecal Source
Vancomycin	16 – 32 µg	32 µg
Teicoplanin	0.05 µg	0.1 – 1 µg

### 3.1. Results of haemolysin test

Beta haemolysin production was identified based on the formation of clear zones surrounding the colonies on blood agar plates. This property was exhibited only by VREF from clinical sources.

### 3.2. Antibiotic sensitivity profile

Antibiotic resistance profiles of VREF from both the sources showed difference in resistance towards antibiotics like gentamicin, streptomycin and amoxyclav. VREF tested were found to be resistant to most of the other antibiotics tested irrespective of the source of isolation.

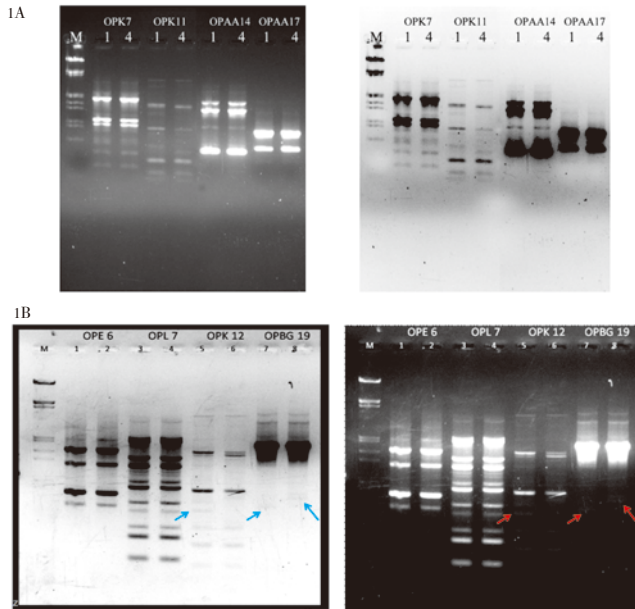
### 3.3. Phenotype and genotype of vancomycin resistance

MIC of vancomycin and teicoplanin against the VREF with

vanB phenotypes were also included in table 1. The genotypes of the selected two strains were detected by PCR and were found to be vanB (data is not shown).

### 3.4. RAPD analysis

The genotypic analysis of the two VREF, one from chicken and the other from clinical source using random primers and results are shown in fig. 1. The results of RAPD shows difference in bands among the two when primers like OPK11, OPK12 and OPBG19 were used.



**Figure 1:** Sample –1 is VREF from clinical and sample– 4 is VREF from chicken. Sample 1 in lane 2, 4, 6, and 8, Sample 4 in lane 3, 5, 7 and 9. Primers used in Fig. 1A were OPK7, OPK11, OPA14, and OPAA17 and for fig.1B were OPE6, OPL7, OPK12, and OPBG19.

Results of statistical analysis by Mann–Whitney rank sum test showed a significant difference with a  $P$  value of  $<0.01$  when the biochemical tests were compared and a significant difference of ( $P<0.05$ ) is obtained while comparing the antibiotic sensitivity of the chicken and clinical sources.

## 4. Discussion

In the present study *E.faecium* was found to be the most predominant species in chicken samples as described in previous reports on enterococci from animal sources [14,15]. In another study on clinical specimen *E.faecalis* was the dominant species. Biochemical reactions and Antibiotic sensitivity patterns demonstrated by Vancomycin resistant *E.faecium* from chicken and clinical sources were evaluated. We found that they exhibited similar results in fermentation of arabinose, lactose, adonitol, and sucrose and arginine deamination. Though there were some minor biochemical differences in the biochemical reactions as shown in table–1. Even if results of fermentation tests like mannitol and sorbitol were variable among isolates from two categories, raffinose fermentation was found to

be satisfactory to differentiate VREF from the two sources. All VREF isolates from chicken sources were found to be raffinose positive, whereas the same in clinical specimens came out raffinose negative. Devries and Pot have also made a similar observation regarding the raffinose fermentation by enterococcal isolates of chicken origin [16]. These results indicate a yet unidentified difference at genetic level between the *E.faecium* isolates from different sources. Raffinose fermentation was found to be constantly associated with the isolates from chicken and human faecal sources (unpublished data). Hence it is opined that further studies with more number of isolates from few more sources along with their properties can be useful for suggesting possible association of raffinose fermentation with some other phenotypic or genotypic characters. Such association if any will be of use in the characterization of the isolates.

Beta–haemolytic property was demonstrated only by the human isolates while the chicken isolates were found to be non haemolytic. Haemolysin plays an important role in enterococcal virulence and may increase the severity of infection [17, 18]. These observations suggest that hemolytic property can be used to differentiate VREF based on their origin and virulence.

Many reports are in supportive of the multiple resistances towards antimicrobials used for treatment of systemic infection caused by vancomycin resistant enterococci [19]. Antibiotic sensitivity patterns from the two sources were also dissimilar. VREF from clinical isolates displayed antibiotic resistance to most of antibiotics tested including streptomycin, gentamicin, amoxycylav and ciprofloxacin. But in this study, the VREF from chicken isolates were proved to be sensitive to the above mentioned antibiotics as described [20]. The antibiotic resistance towards amino glycosides and ciprofloxacin observed in Enterococci (non VREF) from chicken sources was much lesser in frequencies as compared to isolates from patient sources. This could be attributed to the possibility of the lack of selective pressure and proliferation of the resistant strains as chickens were not exposed to afore mentioned antibiotics.

In our study vanB type of vancomycin resistance was exhibited by all the *E.faecium* isolates from chicken sources, whereas vanA was the most frequently recovered vancomycin resistant phenotype observed from animal sources in earlier studies [21]. But the clinical VREF isolates in our study were of vanA and vanB phenotypes. Similar observation has been reported from other parts of India also [22]. In the present study the results of genotypic analysis of vancomycin resistance was in conformity with the phenotypes expressed. This shows that phenotypic methods are reliable to categorize the vancomycin resistance. The VREF strains harboring vanB genes from chicken and clinical sources were found to be polymorphic by RAPD typing. This clearly indicates that the strains from chicken samples and clinical sources are not identical. There are authors who have voiced similar observations indicating lack of evidence for the spread of strains from animals to humans [23]. However there are studies from other parts of the world with strong evidence of infection potential of animal enterococci [24]. Even though the present study excludes the possibility of such a spread from chicken sources, there is the possibility of horizontal transfer of vanB vancomycin resistance plasmids by conjugation to other enterococcal strains and these resistant

organisms may be a potential risk to consumers [25, 26].

## 5. Summary

The present study enterococcal isolates from two different sources. The observation in this study showed that VREF from these two sources were heterogeneous biochemically and based on haemolytic properties. Vancomycin resistant *E. faecium* from clinical sources were found to be resistant to most of the antibiotics than the chicken isolates. Genotypical analysis also showed they were diverse, suggesting the absence of evidence to conclude the possibility of clonal spread from chicken sources in this geographical area. Though the presence of VREF in chicken reflects the use of these antimicrobials in food animal production, the extent to which these population poses risk to the consumers is unknown. VREF from chicken sources may serve as reservoirs of vancomycin resistance genes. This is another possible route for the introduction of VRE to the community. Early detection and reporting of VRE is required for the immediate implementation of appropriate infection control measures.

## Reference

- [1] Sava IG, Heikens E, and Huebner J. Pathogenesis and immunity in enterococcal infections. *Clin Microbial Infect* 2010; **16**: 533–540
- [2] Willems RJ, van Schaik W. Transition of Enterococcus faecium from commensal organism to nosocomial pathogen. *Future Microbial* 2009; **4**: 1125–1135
- [3] Panesso D, Reyes J, Rincon S, Diaz L, Galloway–Pena J, et al. Molecular epidemiology of vancomycin–resistant Enterococcus faecium: a prospective, multicenter study in South American hospitals. *J Clin Microbiol* 2010; **48**: 1562–1569
- [4] Hayakawa K, Marchaim D, Martin ET, Tiwari N, Yousuf A, Sunkara B, Pulluru H, Kotra H, Hasan A, Bheemreddy S, Sheth P, Lee DW, Kamatam S, Bathina P, Nanjireddy P, Chalana IK, Patel S, Kumar S, Vahia A, Ku K, Yee V, Swan J, Pogue JM, Lephart PR, Rybak MJ, Kaye KS. Comparison of clinical characteristics and outcomes associated with vancomycin resistant Enterococcus faecalis and Enterococcus faecium bacteremia. *Antimicrob Agents Chemother*. 2012; **56**:2452–8.
- [5] Xu HT, Tian R, Chen DK, Xiao F, Nie ZY, Hu YJ, Zhang XZ, Li JM. Nosocomial spread of hospital–adapted CC17 vancomycin–resistant Enterococcus faecium in a tertiary–care hospital of Beijing, China. *Chin Med J* 2011; **124**: 498–503.
- [6] Hammerum AM, Lester CH, Heuer OE. Antimicrobial–resistant enterococci in animals and meat: a human health hazard? *Foodborne Pathog Dis* 2010; **7**:1137–1146
- [7] Wardwell LH, Huttenhower C, Garrett WS. Current concepts of the intestinal microbiota and the pathogenesis of infection. *Curr Infect Dis Rep* 2011; **13**: 28–34.
- [8] Aarestrup F M, Butaye P, and Witte W. Nonhuman reservoirs of enterococci, In M. S. Gilmore (ed.), The enterococci—pathogenesis, molecular biology, and antibiotic resistance. ASM Press, Washington D.C 2002. p. 55–99.
- [9] Phillips, L., M. Casewell, T. Cox, B. DeGroot, C. Friis, R. Jones, C. Nightingale, R. Preston, and J. Waddell. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J Antimicrob Chemother*. 2004; **53**:28–52.
- [10] Van den Braak N, Power E, Anthony R, Endtz, H P, Verbrugh H A, van Belkum A. Random amplification of polymorphic DNA versus pulsed field gel electrophoresis of SmaI DNA macrorestriction fragments for typing strains of vancomycin–resistant enterococci. *FEMS Microbiol Lett* 2000; **192**:45–52
- [11] Quednau M, Ahrne S, Petersson A C, Molin G. Antibiotic resistant strains of Enterococcus isolated from Swedish and Danish retailed chicken and pork. *J Appl Microbiol* 1998; **84**(6): 1163–1170
- [12] Facklam R R, Collins M D. Identification of Enterococcus species isolated from human infections by a conventional test scheme. *J Clin Microbiol* 1989; **27**: 731–734
- [13] National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, approved standard M7–A5, 5th ed. National Committee for Clinical Laboratory Standards, Wayne, Pa. 2000.
- [14] Diarra M S, Rempel H, Champagne J, Masson L, Pritchard J, and Topp E . Distribution of antimicrobial resistance and virulence genes in Enterococcus spp. and characterization of isolates from broiler chickens. *Appl Environ Microbiol* 2010; **76**:8033–8043.
- [15] Hayes J R, English L L, Carter P J, Proescholdt T, Lee K Y, Wagner D D, and White D G. Prevalence and antimicrobial resistance of Enterococcus species isolated from retail meats. *Appl Environ Microbiol* 2003; **69**:7153–7160
- [16] Devriese L A, Pot B. The genus Enterococcus. p. 327–367. In B. J. B. Wood and W. H. Holzappel (ed.), The lactic acid bacteria, vol. 2. The genera of lactic acid bacteria. Blackie Academic, London, United Kingdom, 1995
- [17] Clewell D B. Properties of Enterococcus faecalis plasmid pAD1, a member of a widely disseminated family of pheromone–responding, conjugative, virulence elements encoding cytolysin. *Plasmid* 2007; **58**: 205–227.
- [18] Jett B D, Huycke M M, Gilmore M S. Virulence of Enterococci. *Clin Microbiol Rev* 1994; **7**: 462–478
- [19] Fraser SL. Enterococcal Infections. [Serial on the internet] Updated on Jan 7, 2010. Available from <http://www.medscape.com/eMedicine/Specialties/InfectiousDiseases/BacterialInfections>
- [20] Seong CN, Shim ES, Kim SM, Yoo JC. Prevalence and characterization of vancomycin–resistant enterococci in chicken intestines and humans of Korea Arch Pharm Res 2004; **27**:246–253
- [21] Jung WK, Lim JY, Kwon NH, Kim JM, Hong SK, Koo HC, Kim SH, Park YH. Vancomycin–resistant enterococci from animal sources in Korea *Int J Food Microbiol* 2007; **113**: 102–7.
- [22] Mathur P, Kapil A, Chandra R, Sharma P, Das B. Antimicrobial resistance in Enterococcus faecalis at a tertiary care centre of northern India. *Indian J Med Res* 2003; **118**: 25–8
- [23] Hammerum AM, Lester CH, Heuer OE. Antimicrobial–resistant enterococci in animals and meat: a human health hazard? *Foodborne Pathog Dis*. 2010; **7**:1137–46.
- [24] Freitas AR, Coque TM, Novais C et al. Human and swine hosts share vancomycin–resistant Enterococcus faecium CC17, CC5, and Enterococcus faecalis CC2 clonal clusters harboring Tn1546 on indistinguishable plasmids. *J Clin Microbiol* 2011; **49**: 925–31
- [25] Dahl KH, Røkenes TP, Lundblad EW et al. Nonconjugative transposition of the vanB–containing Tn5382–like element in Enterococcus faecium. *Antimicrob Agents Chemother* 2003; **47**: 786–9.
- [26] Hanrahan J, Høyen C, Rice LB. Geographic distribution of a large mobile element that transfers ampicillin and vancomycin resistance between Enterococcus faecium strains. *Antimicrob Agents Chemother* 2000; **44**: 1349–51.