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Detection of Non-transferable *van*C1 and *van*C2/3 Genes in Vancomycin Resistant Enterococci Isolated from Freshwater Fish Collected from Retail Markets

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Abstract

Enterococci are opportunistic pathogens that can cause life-threatening infections like nosocomial endocarditis, bacteremia, wound infection and urinary tract infection in humans. Vancomycin resistant enterococci (VRE) carrying *vanA* and *vanB* genes are recognized by World Health Organization (WHO) as high-risk category organisms. The aim of this study was to ascertain the status of VRE in freshwater fish being sold in retail fish markets for human consumption, and document the molecular mechanism of their vancomycin resistance. A total of 60 gill/muscle swabs collected from freshwater fish were enriched in bile esculin azide broth containing vancomycin (6 g/mL). Enterococci were isolated on Citrate-azide tween carbonate agar supplemented with vancomycin. Species-specific PCR was performed for molecular identification of enterococci. Out of 60 fish samples screened, 47 (78.3%) samples were positive for VRE. Among VRE the prevalence % of *E. casseliflavus* was maximum 28/47(59.5%), followed by *E. faecalis* 12/47 (25.5%), *E. gallinarum* 4/47 (8.5%) and *E. faecium* 3/47 (6.3%). The VRE isolates were screened by multiplex PCR for the detection of vancomycin resistance genes. The chromosomal encoded gene *van*C1 and *van*C2/3 were detected both in *E. faecalis* and *E. faecium.* While, *van*C2/3 gene was detected in *E. casseliflavus* and *E. gallinarum.* Notably, *van*A and *van*B genes were not detected in any of the VRE. Further, virulence genes (*asa1, cylA, esp, gelE* and *hyl*) or biofilm encoding gene (*Ebap*) were not detected in the VRE. The findings of our study indicate that vancomycin resistance in enterococci isolated from freshwater fish was mediated by chromosomal genes and not by acquired genes. VRE of freshwater fish may neither pose risk to public health nor contribute in the global spread of vancomycin resistance.

Keywords: Enterococci, VRE, *van*C1, *van*C2/3, Freshwater fish

Introduction

Enterococci are commensal microbiota of humans and animals that can survive high temperature and show tolerance to 6.5% salt (Lebreton, Willems, & Gilmore, 2014). Enterococci are inherently resistant to a number of antimicrobial drugs such as cephalosporins and aminoglycosides. During last decade, Vancomycin Resistant Enterococci (VRE) mainly *Enterococcus faecalis* and *E. faecium* have emerged as important nosocomial pathogen of humans across globe (Ayobami, Willrich, Reuss, Eckmanns, & Markwart, 2020). Some of the other species of Enterococci showing resistance to vancomycin are *E. durans*, *E. hirae*, *E. gallinarum* and *E. casseliflavus.* In India, significant increase in VRE infections has been observed in last two decades. From 2000-2010 the rate of increase was 4.8% which increased to 14.1% during 2011-2020 (Smout, Palanisamy, & Valappil, 2023). Nine clusters of vancomycin resistance genes (VRGs) have been

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identified (*van*A, *van*B, *van*C1, *van*C2/3, *van*D, *van*E, *van*G, *van*L, *van*M and *van*N) in VRE. Among these, *van*A, *van*B, *van*D, *van*E, *van*G, *van*L, *van*M and *van*N are known to be acquired mechanisms of vancomycin resistance in VRE. In contrast, *van*C1 and *van*C2/3 are inherent resistant mechanisms in enterococci (Miller, Munita, & Arias, 2014; Stogios & Savchenko, 2020; George et al., 2021). Detection of VRGs helps in understanding the mechanisms underlying glycopeptide resistance in enterococci. The *van*A and *van*B resistant genotypes are clinically important that have the ability to transfer their VRGs to other bacterial species by horizontal gene transfer. The *van*A gene cluster encodes proteins that confer high-level resistance to both vancomycin (VAN) and teicoplanin (TEI). The *van*B gene product provides moderate to severe resistance to VAN, but not to TEI. The *van*C1 and *van*C2/3 genes are specific to some species of VRE, such as *E. gallinarum, E. casseliflavus*. The other genes, *van*D, *van*E and *van*G, have been detected in only a few enterococcal strains.

Enterococci, that cause infections in human possess a variety of virulence factors that are associated with capsule formation, enterococcal surface protein, gelatinase, cytolysin, hyaluronidase, aggregation substance, etc. (Vankerckhoven et al., 2004; Hosseini et al., 2016). Biofilm formation promotes disease by lessening the impact of antimicrobials in human and other hosts (Farahani, 2016). Virulence factors likes enterococcal surface protein encoding gene, *esp* and cytolysin encoding gene *cyl* are associated with the increased virulence, colonization and persistence in the urinary tract along with biofilm formation. Aggregation substances encoded gene, *asa1* is responsible for increased bacterial adhesion to renal tubular cells and heart endocardial cell, while hyaluronidase gene *hyl*, is a degradative enzyme associated with tissue damage. Gelatinase is an extracellular zinc-containing metalloproteinase that can hydrolyze gelatin, collagen, fibrinogen, casein, hemoglobin and insulin. Studies reveal association of virulence genes to pathogenicity of enterococci in the human and animal models. Co-occurrence of virulence factors and biofilm formation increases the probability of VRE to cause infection in humans (Toledo-Arana et al., 2001; Jahan & Holley, 2014; Farahani, 2016).

Evidence shows that food-producing animals including farmed fish may act as a reservoir of VRE. Reports suggests spread of vancomycin resistance between human and animal isolates of enterococci can take place through horizontal transfer of VRGs or by clonal dissemination of resistant strains (Nilsson, 2012). Aquaculture is one of the fastest growing food producing sector with an annual growth of approximately 8.0% (FAO, 2018). Biswas, Sharma, & Joshi (2019) documented VRE in fish samples collected from retail markets of North-east India. Dwivedi et al. (2023) observed unhygienic conditions in the retail markets can lead to contamination of antimicrobial resistance (AMR) in fish. In this context, it is important to ascertain the status of VRE in freshwater fish being sold in retail fish markets for human consumption. Accordingly, the aim of our study was to i) assess the prevalence of VRE in freshwater fish being sold in retail fish markets of Lucknow, Uttar Pradesh, India ii) evaluate the antimicrobial susceptibility profile of the isolated VRE iii) and detect VRGs, putative virulence genes and biofilm forming gene in VRE.

Materials and Methods

Isolation of vancomycin-resistant enterococci from freshwater fish: A total of 60 gill or muscle swabs were collected from four retail fish markets (Market-1 26.785908^{o} N; 80.941089^{0} E, Market-2 26.800653^{o} N; 80.897331^{0} E, Market-3 26.89542^{o} N; 80.944324^{0} E and Market-4 26.72989^{o} N; 80.95957^{0} E) in Lucknow, Uttar Pradesh, India in bile esculin azide broth (Himedia) containing 6 μg/mL vancomycin. The broths were transported on ice and incubated at 40°C for 24 h. The tubes showing black colour were considered positive for presumptive enterococci and processed for bacterial isolation. Following broth incubation, the bacterial growth was streaked on citrate azide tween carbonate agar (CATC) plate supplemented with 1% 2,3,5-Triphenyl Tetrazolium Chloride and 6 μg/mL vancomycin (Lemcke & Bülte, 2000). The streaked plates were incubated at 37°C for 24 h. Two to three reddish colonies were picked and purified on Tryptic soya agar as presumptive VRE.

Identification of vancomycin-resistant enterococci: Pure cultures of presumptive VRE were subjected to biochemical tests like Gram staining, oxidase, catalase, 6.5% NaCl (Manero & Blanch, 1999) and VITEK 2 Compact system for bacterial identification. Further, molecular identification of VRE was done by species-specific PCR (Jackson, Fedorka-Cray, & Barrett, 2004).

Antimicrobial susceptibility testing (AST) of VRE: AST was done by disk diffusion method (Hudzicki, 2009). The antimicrobials tested were ampicillin 30µg, erythromycin 15µg, ciprofloxacin 5µg, vancomycin 30µg, teicoplanin 30µg, streptogramins 15µg, tetracycline 30µg, linezolid 30µg and chloramphenicol 30µg. *Staphylococcus aureus*, ATCC 25923 was used as the quality control strain in AST. In addition, Minimum inhibitory concentration (MIC) of vancomycin and gentamicin for VRE was estimated by broth micro dilution method (CLSI, 2021). *E. faecalis*, ATCC 29212 was used as quality control strain in the broth micro dilution method.

Screening of VRE for VRGs and virulence factors by PCR: VRGs namely *van*A, *van*B, *van*C1 and *van*C2/3 genes were screened by PCR as per previously described method (Dutka-Malen, Evers, & Courvalin, 1995). The VRE were also screened for five virulence encoding genes (*asa1, cylA, esp, gelE* and *hyl*) using multiplex PCR (Vankerckhoven et al., 2004; Kiruthiga et al., 2020) and biofilm encoding gene, *Ebap* by simplex PCR (Fallah et al., 2017).

Results and Discussion

Enterococci are normal microbiota present in the intestinal tract of humans and animals, and also abundant in environment. These organisms are used as indicators of faecal contamination in the aquatic environment and food. Importantly, few species of enterococci specially VRE cause nosocomial infections in humans. Studies indicate that food animals are important reservoir of VRE and can transfer antimicrobial resistance genes to the human gut bacteria through the food chain and/or animal husbandry (Poeta et al., 2005). In this context, understanding the prevalence of VRE in freshwater fish being sold in retail market for domestic consumption is important. This will help in identifying the source of VRE and understanding the molecular mechanisms responsible for vancomycin resistance.

Isolation and identification of VRE: In our study, out of 60 freshwater fish samples screened, 47 fish samples (78.33%) were positive for VRE. All the isolates of VRE were Gram positive cocci, oxidase and catalase negative, and showed growth in 6.5% NaCl. The presumptive isolates of VRE (n=47) were subjected to Vitek 2 for species identification. The species identified were *E. casseliflavus* (59.5%), *E. faecalis* (25.5%), *E. gallinarum* (8.5%) and *E. faecium* (6.3%). The probability of species identification by Vitek 2 was excellent (>99%). Multiplex PCR was also used for confirmation of the Enterococcal species. Species specific amplicons detected in multiplex PCR were *E. faecalis*-360bp, *E. faecium*-215bp, *E. casseliflavus*-288bp and *E. gallinarum*-173bp (Fig. 1). We noted concordant identification of all the four species between Vitek 2 and multiplex PCR.

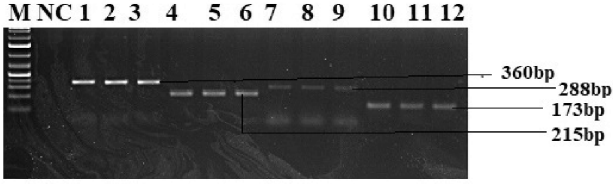

Fig. 1. Molecular identification of Enterococci by species specific PCR. (Lane; M- Ladder (100bp-3Kb), Lane; NC- Negative control, Lane;1-3 *E. faecalis* (360bp), Lane; 4-6 *E. faecium* (215bp), Lane; 7-9 *E. casseliflavus* (288bp) and Lane; 10-12 *E. gallinarum* (173bp).

Species wise distribution/prevalence: The species wise distribution of VRE in different retail markets of Lucknow, Uttar Pradesh, India is presented in Table 1. *E. casseliflavus* and *E. faecalis* together accounted for 85% of the VRE, while rest of 15% VRE were *E. gallinarum* and *E. faecium*. *E. casseliflavus* and *E. faecalis* were recovered from all the four markets. *E. faecium* was recovered from Market 1 and Market 2, while *E. gallinarum* was recovered from Market 2 and Market 4 (Table 1).

Our findings are similar to a previous study, where rectal swabs of bovines, equines and poultry were collected and analysed for VRE. The species wise prevalence of VRE in that study was *E. gallinarum* (48%), *E. casseliflavus*/*E. flavescens* (25%), *E. faecalis* (16%) and *E. faecium* (10%) respectively (Bathini, Arora, & Rai, 2018). Many studies have documented vancomycin resistance in enterococci of fish or aquaculture origin. A study from Northeast India reported isolation of *E. faecalis* from 50% of fermented fish samples collected from retail fish market, and vancomycin resistance was observed in 39.4% of the isolates (Biswas et al., 2019). Vignesh, Muthukumar, and James (2012) reported vancomycin resistance in 31.6 - 33.3% of the enterococci isolated from seawater and sediments samples collected from Chennai coast. A study from Egypt also revealed three out of eight (37.8%) *Enterococcus* isolates from fish were resistant to vancomycin

Table 1. Market wise distribution of vancomycin resistant Enterococci isolated from freshwater fish collected from retail market of Lucknow, Uttar Pradesh, India.

Enterococci species	Samples source				Distribution (%)
	Market-1	*Market-2*	*Market-3*	*Market-4*	
E. faecalis	5/18 (27.7%)	4/15 (26.6%)	1/6 (16.6%)	2/8 (25%)	12/47 (25.5%)
E. faecium	1/18 (5.5%)	2/15 (13.3%)	0/6	0/8	3/47 (6.3%)
E. casseliflavus	12/18 (66.6%)	7/15 (46.6%)	5/6 (83.3%)	4/8 (50%)	28/47 (59.5%)
E. gallinarum	0/18	2/15 (13.3%)	0/6	2/8 (25%)	4/47 (8.5%)
Total positive	18/20 (90%)	15/20 (75%)	6/10 (60%)	8/10 (80%)	47/60 (78.33%)
Total negative	2/20 (10%)	5/20 (25%)	4/10 (40%)	2/10 (20%)	13/60 (21.66%)

Table 2. Estimation of Minimum inhibitory concentration (MIC) of vancomycin and gentamicin and detection of vancomycin resistant genes in Enterococci.

Enterococci species	MIC (μg/mL)		Vancomycin resistance genes			
	Vancomycin	Gentamicin	*van*A	*van*B	*van*C1	*van*C2/3
E. faecalis (n=12)	32 μg/mL	64 μg/mL	-	-	+	+
E. faecium (n=3)	32 μg/mL	64 μg/mL	-	-	+	+
E. casseliflavus (n=28)	16 μg/mL	32 μg/mL	-	-	-	+
E. gallinarum (n=4)	16 μg/mL	32 μg/mL	-	-	-	+

Indicates; + Positive, - Negative

(Osman et al., 2016). Our results and previous findings both suggest wide distribution of VRE in fish and food animals. In several countries, high prevalence of VRE in livestock and food producing animals has been reported. This has been associated due to intensive use of avoparcin, a glycopeptide as an antimicrobial growth promotor (Bager, Madsen, Christensen, & Aarestrup, 1997; Wist et al., 2020). However, use of avoparcin in food producing animals is not reported from India.

Among the VRE, higher prevalence of *E. casseliflavus* and *E. gallinarum* in fish samples can be explained by the fact that both these species of enterococci are abundantly present in aquatic environment (Zhang et al., 2016; Araújo, Grassotti, & Frazzon, 2020). Among enterococci, inherent resistance to vancomycin is observed mostly in *E. casseliflavus* and *E. gallinarum* (Courvalin, 2006; Werner et al., 2008a). Thus, both these species have higher probability to grow in enrichment medium containing vancomycin. However, isolation of vancomycin resistant *E. faecalis* and *E. faecium* from freshwater fish intended for human consumption is concerning as both the species have the ability to cause nosocomial infections in humans. In North India, the prevalence of VRE (*E. faecalis* and *E. faecium*) in human patients symptomatic of urinary tract infection was reported as 16.95% (Das, Dudeja, Kohli, & Ray, 2022). In comparison to clinical samples, the combined prevalence of vancomycin resistant *E. faecalis* and *E. faecium* in our study from freshwater fish was higher (26%).

Antimicrobial susceptibility test: In our study, all the 4 species of enterococci, i.e., *E. casseliflavus, E. gallinarum E. faecalis* and *E. faecium* were resistant only to vancomycin. The MIC of vancomycin for all the isolates was ≤32 μg/mL (Table 2). MIC of vancomycin ranging between 2-32 μg/mL is referred as low-level resistance for enterococci. This shows that the isolates of VRE from freshwater fish carried only low level of vancomycin resistance. They were susceptible to ampicillin, erythromycin, ciprofloxacin, streptogramins, tetracycline, teicoplanin, linezolid and chloramphenicol in the disc diffusion assay.

Multidrug resistance in enterococcal isolates is often reported in several studies. High percentage of VRE from bovines and equines (30%) were reported as multidrug resistant (Bathini et al., 2018). Similarly, Osman et al. (2016) found that large proportion of *Enterococci* isolated from fish were resistant to at least three antibiotics, and all the vancomycin resistant isolates exhibited high level of vancomycin resistance (≤230 μg/ml). It is pertinent to note that low level resistance to vancomycin in enterococci is mediated by chromosomal *van*C gene and is considered inherent and non-transferable. The two most dominant species of VRE (*E. casseliflavus* & *E. gallinarum)* isolated in our study harboured low level of vancomycin resistance which is non-transferable. Hence, it is safe to assume that majority of the VRE from freshwater fish are relatively harmless as far as transmission of AMR in concerned.

E. faecalis and *E. faecium* are associated with life threating conditions in human beings like endocarditis, infections in wounds, urinary tract, teeth etc. Resistance to vancomycin in *E. faecalis* and *E. faecium* is not common and has therefore wider implications for human health. Many studies show that hospital-acquired *E. faecium* are mostly ampicillin-resistant or partly high-level ciprofloxacin-resistant (Werner et al., 2008a). A recent study from Bangladesh revealed 82% of *E. faecalis* isolated from cultured fish were resistant to ampicillin, 15% isolates showed intermediate resistance to ciprofloxacin and showed lower resistance to tetracycline and vancomycin (Rana et al., 2023). In our study, resistance to common antimicrobials was not observed in enterococci except vancomycin. This possibly suggests low use of antibiotics in freshwater aquaculture in the study area.

Screening of VRE for VRGs and virulence factors by PCR: In our study, all the isolates of vancomycin resistant *E. faecalis* and *E. faecium* carried chromosomal encoded gene *van*C1 and *van*C2/3. *E. casseliflavus* and *E. gallinarum* harboured only chromosomal encoding gene *van*C2/3 (Fig. 2).

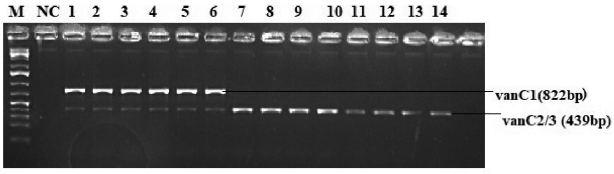

Fig. 2. Molecular detection vancomycin resistance genes in VRE samples of freshwater fish. (Lane; M-Ladder (100bp-3Kb), Lane; NC- Negative control, Lane;1-3 *van*C1 (822bp) and *van*C2/3 (439bp) genes in *E. faecalis*, Lane; 4-6 *van*C1 (822bp) and *van*C2/3 (439bp) genes in *E. faecium*), Lane; 7-10 *van*C2/3 (439bp) gene in *E. casseliflavus* and Lane; 11-14 *van*C2/3 (439bp) gene in *E. gallinarum*.

Importantly, *van*A and *van*B were not detected in any of the 47 isolates of VRE (Table 2). In a similar study, chromosomal encoding gene *vanC1* and *vanC2/3* were detected in VRE isolated from sewage and river water samples in provincial city of Miyazaki, Japan (Matsumoto et al., 2004; Nishiyama, Iguchi, & Suzuki, 2015). Similar to our findings, these isolates also had low-level resistance to vancomycin. Another study previously reported from Punjab, India, VRE isolated from bovines, equines and poultry were positive for *vanC1* and *vanC2/3* genes and did not harbor either *vanA* or *vanB* genes (Bathini et al., 2018). While investigating VRE, it is important to describe the molecular mechanism of vancomycin resistance. The *vanA* and *vanB* phenotypes correspond to high level vancomycin resistance in enterococci that is transferable, while *vanC1* and *vanC2/3* phenotypes are associated with low level of intrinsic resistance which is chromosomal mediated and non-transferable. Moreover, *vanC1* and *vanC2/3* phenotypes of VRE are not

Table 3. Detection of virulence and biofilm genes in Enterococci by PCR.

Enterococci species	Virulence genes (*asa1, gelE, esp, cyl & hyl*)		Biofilm encoding gene (*Ebap*)	
	Positive	Negative	Positive	Negative
E. faecalis	0/12	12/12	0/12	12/12
E. faecium	0/3	3/3	0/3	3/3
E. casseliflavus	0/28	28/28	0/28	28/28
E. gallinarum	0/4	4/4	0/4	4/4

associated with human nosocomial infection and only *vanA* and *vanB* genotypes of VRE are recognized as serious concern of VRE by World health organization (WHO). In this context, the key finding of our study, reveals that VRE recovered from freshwater retail fish markets belong to *vanC1* and *vanC2/3* phenotypes and have low-level vancomycin resistance. VRE were not resistant to any other class of antimicrobials tested in this study. Further, screening of VRE by PCR indicated absence of virulence genes, *asa1, gelE*, *cyl*, *esp* and *hyl* and biofilm forming gene *Ebap* (Table 3).

These results indicate VRE isolates of freshwater fish lack virulence potential. The VRE associated with nosocomial infections are known to possess putative virulence traits such as a gene for an enterococcal surface protein, *esp*, genes encoding different cell wall-anchored surface proteins, a putative hyaluronidase gene, *hyl* and a gene encoding a collagen-binding protein, *acm* (Werner et al., 2008b). Earlier studies have revealed that the "biological cost" associated with development of vancomycin resistance could be associated with decline in the virulence of enterococci (Foucault, Depardieu, Courvalin, & Grillot-Courvalin, 2010; Banerjee & Anupurba, 2015).

In conclusion, our findings suggest that VRE isolated from freshwater fish collected from retail markets of Lucknow, Uttar Pradesh do not possess the potential to cause negative impact on human health or have the ability to spread vancomycin resistance through horizontal gene transfer.

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