International Journal of Veterinary Sciences and Animal Husbandry

ISSN: 2456-2912
VET 2024; 9(6): 509-514

www.veterinarypaper.com
Received: 14-11-2024
Accepted: 13-12-2024

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Detection of Van A-mediated vancomycin resistance in *E. faecium* isolated from seafood retailed in Wayanad, Kerala

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DOI: https://doi.org/10.22271/veterinary.2024.v9.i6h.1922

Abstract
Seafood is an integral part of diets worldwide, valued for its rich flavours and exceptional nutritional benefits. *Enterococcus faecium* is a significant nosocomial pathogen known for its increasing antibiotic resistance and association with severe infections in healthcare settings. This study aimed to detect the presence of vancomycin-resistant *E. faecium* in seafood retailed in Wayanad district, Kerala, India. A total of 204 samples (seafood and environmental samples) were collected from six retail fish outlets and analysed using conventional culture methods, followed by genotypic confirmation of *E. faecium* using *PCR.* Vancomycin resistance in isolates was assessed both phenotypically and genotypically. The results revealed an occurrence rate of 28.73 per cent for *E. faecium* in the seafood samples and 30.00 per cent in the environmental samples. Phenotypically, 3.39 per cent of the isolates exhibited vancomycin resistance, while genotypically, 1.69 per cent of the isolates harboured the *van* A gene. The detection of the *van* A gene in seafood samples is particularly concerning as it suggests the potential spread of vancomycin resistance beyond healthcare settings. These findings underscore the need for enhanced food safety practices and surveillance to mitigate the public health risks associated with vancomycin-resistant *E. faecium* in the food chain.

Keywords: *Enterococcus faecium*, seafood, environmental samples, Kerala, vancomycin resistance

1. Introduction
Enterococci are Gram-positive, facultative anaerobic bacterium commensal in the gastrointestinal tract of humans, animals and insects (Heim *et al*., 2023) [1]. Their adaptability to adverse environmental conditions enables them to thrive not only in host organisms but also in natural environments such as plants, soil, freshwater, marine ecosystems, and various food products (Lopes *et al*., 2024 [2]; Kumar *et al.*, 2024) [3]. Certain Enterococcus species, particularly the *E. faecalis* and *E. faecium*, have evolved into opportunistic pathogens capable of causing a wide spectrum of infections like urinary tract infections, endocarditis, and bacteraemia, particularly in elderly, neonates and individuals with weakened immune systems (Del Turco *et al*., 2021) [4].

In recent years, *E. faecium* has garnered significant attention as a nosocomial pathogen due to its remarkable capacity to acquire multiple antibiotic resistance determinants, distinguishing it from other faecal bacteria in the environment. The *E. faecium* possesses intrinsic resistance to certain antibiotics and can both acquire and transfer resistance to other bacteria through mobile genetic elements (Kumar *et al.*, 2024) [3]. In particular, vancomycin-resistant *E. faecium* (VREfm) has emerged as a major concern in healthcare settings, given its association with severe, difficult-to-treat infections (Lopes *et al*., 2024) [2]. The resistance is primarily mediated by specific gene clusters, including *van*A and *van*B, which alter peptidoglycan precursors and reduce vancomycin's binding affinity to the bacterial cell wall (Raza *et al*., 2018) [5]. Since the first report of vancomycin resistance in *E. faecium* in the 1980s, these resistance mechanisms have spread, making *E. faecium* a critical public health challenge, both in hospital and

community settings (Igbinosa and Beshiru, 2019) [6]. According to Murray *et al.* (2022) [7], vancomycin-resistant Enterococci (VRE) was responsible for between 100,000 and 250,000 deaths globally linked to antimicrobial resistance (AMR) and Diekema *et al*., 2019 [8] reported that VRE ranks as the fourth most common pathogen causing bloodstream infections. The rise of vancomycin resistance has become especially prominent in India, where the prevalence of VRE infections increased from 4.8 per cent between 2000 and 2010 to 14.1 per cent between 2011 and 2020. Although *E. faecalis* was the most frequently isolated species in India, among the VRE strains, *E. faecium* was the most prevalent, accounting for a significant proportion of VRE cases (Smout *et al*., 2023) [9].

To date, nine gene clusters associated with vancomycin resistance have been identified in Enterococcus species (Raza *et al*., 2018) [5]. Among these, the *van*A and *van*B genotypes are of particular clinical significance due to their ability to transfer vancomycin resistance genes (VRGs) to other bacterial species via horizontal gene transfer (Kumar *et al*., 2024) [3]. The *van*A gene cluster is of major concern as it encodes proteins that confer high-level resistance to both vancomycin and teicoplanin. The ability of *van*A to spread resistance poses a significant challenge to the treatment of infections, particularly in healthcare settings.

Reports of VRE isolated from non-clinical sources, including seafood, suggest the potential for the spread of vancomycin resistance between human and animal isolates of Enterococcus. This transmission can occur through the horizontal transfer of VRGs or via clonal dissemination of resistant strains (Nilsson, 2012) [10]. These findings highlight the importance of monitoring non-clinical environments, like seafood, as potential reservoirs for the spread of antimicrobial resistance. The rapidly expanding aquaculture and fisheries sector in India, where the country is the third-largest fish producer globally, has led to a significant increase in fish consumption, from 4.9 kg to 8.89 kg per person over the past 15 years (Padiyar *et al*., 2024) [11]. Given the increasing recognition of the marine environment as a critical route for the dissemination of AMR, retail seafood markets are emerging as important points of exposure for consumers. So, the aim of our study was i) to detect the occurrence of *E. faecium* in seafood and retail seafood markets in Wayanad, Kerala, India and ii) to assess the prevalence of VREfm in these samples using both phenotypic and genotypic methods.

2. Materials and Methods

2.1 Isolation and molecular confirmation of *E. faecium*: A total of 174 seafood (molluscs, finfish and crustaceans) and 30 environmental samples (ice, wastewater, handwashing of fish handlers, surface swabs of knife and cutting boards) were collected from six retail fish outlets located in Wayanad district, Kerala, India with a minimum distance of 10 km between each outlet. The study was conducted for a period of 10 months (September 2023 to July 2024). All samples were aseptically collected and transported to the lab under chilled conditions. The samples were subjected to isolation and identification of *E. faecium.* Twenty-five grams of each sample was transferred to buffered peptone water and incubated at 37 °C overnight. Enriched samples were streaked onto Slanetz and Bartley agar and incubated for a period of 48 h at 37 °C (Russo *et al*., 2022) [12]. Colonies exhibiting characteristic red or maroon pigmentation were selected, and species-level identification was carried out using Polymerase Chain Reaction (PCR) targeting the *ddl E. faecium* gene (Table 1and Fig. 1). The DNA was extracted from all the isolates using the phenol-chloroform method and a PCR with the following conditions was carried out: 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 sec, 55 °C for 1min, and 72 °C for 1min, and a final elongation step at 72 °C for 7 min (Jackson *et al*., 2004) [13]. The amplicons were visualised following electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

2.2 Detection of vancomycin resistance: The Kirby-Bauer disc diffusion test, employing vancomycin (VA 30 µg) antibiotic discs, was used for the phenotypic detection of vancomycin resistance as per CLSI (2023) standards. A PCR assay was employed to detect the *van*A gene, following a method outlined in prior research (Seo *et al*., 2011) [14].

Table 1: Details of oligonucleotides used in the study

$ddl_{E. faecium}$	F: 5'- GAAAAAACAATAGAAGAATTAT-3' R: 5'- TGCTTTTTTGAATTCTTCTTTA-3'	215	Jackson *et al*., 2004 [13]
*van*A	F: 5'- GGGAAAACGACAATTGC-3' R: 5'- GTACAATGCGGCCGTTA-3'	732	Seo *et al*., 2011 [14]

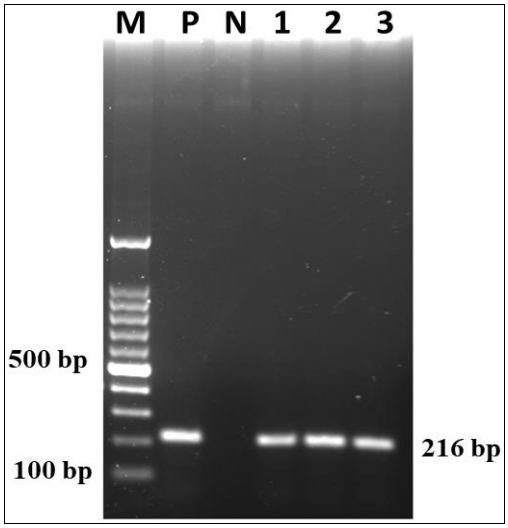

Fig 1: Molecular identification of *E. faecium.*

(Lane M: Marker, Lane P: Positive control, Lane N: Negative control, Lane 1, 2, 3: Samples)

3. Results and Discussion

Enterococci are resilient microorganisms that dominate the bacterial flora in food, largely due to their ability to endure harsh conditions during food production and adapt effectively to various storage environments (Elisa *et al.*, 2024) [15]. Studies have reported the presence of *E. faecium* in diverse habitats including marine environments and seafood (Ullah *et al.*, 2023) [16]. Owing to its importance as a nosocomial pathogen and its alarming ability to develop resistance, especially to vancomycin, *E. faecium* poses a significant challenge as a foodborne pathogen. Food animals serve as important reservoirs of vancomycin-resistant enterococci (VRE), facilitating the transfer of antimicrobial resistance genes to humans through the food chain, contaminated environments, or direct human contact (Cebeci, 2024[17]; Kumar *et al.*, 2024) [3].

Out of the 174 seafood samples screened in the current study, 28.73 per cent (50/174) were positive for *E. faecium*. The presence of *E. faecium* in seafood has been documented globally, yet data specific to Kerala remains scarce. Previous studies in Kerala have reported the isolation of *E. faecium* from diverse sources, including poultry faeces (Peter *et al*., 2012) [18], human clinical samples (Peter *et al*., 2013 [19]; Varghese *et al*., 2020) [20], river water (Manjusha *et al*., 2012) [21], and human pus samples (Rajalakshmy *et al*., 2024) [22]. However, to the best of our knowledge, this study is the first to report the occurrence of *E. faecium* in seafood retailed in Kerala. The prevalence observed in our study (28.73%) aligns closely with the findings of Ullah *et al*. (2023) [16], who reported a comparable prevalence rate of 27.30 per cent in raw seafood samples from Bangladesh. Globally, the prevalence of *E. faecium* in seafood varies widely, from as low as 7.70 per cent in processed seafood in Turkey (Çardak *et al*., 2022) [23] to as high as 52.23 per cent in bivalves from the Norwegian coast (Heim *et al*., 2023) [1]. Even in India, there is a report of *E. faecium* isolation from fish, with Kumar *et al*. (2024) [3] documenting a 6.3 per cent prevalence in freshwater fish sold in retail markets in Lucknow, Uttar Pradesh. Various factors like the type of seafood, geographic locations, processing and storage conditions, and the detection methods employed influence the prevalence estimates of *E. faecium* in seafood samples. In our study, among different seafood categories, a higher occurrence rate was observed in crustaceans (23/58; 39.66%) compared to finfish (14/58; 24.14%) and mollusc samples (13/58; 22.41%) (Table 2). A similar high prevalence of *E. faecium* (49.20%) was reported in shrimp sold in Nigeria (Igbinosa and Beshiru, 2019) [6]. The significant presence of *E. faecium* in crustaceans and molluscs can be attributed to faecal contamination in aquatic ecosystems. Crustaceans, due to their bottom-dwelling habitat and scavenging behaviour, are in frequent contact with sediment, which may contain high levels of faecal bacteria. Similarly, molluscs, through their filter-feeding behaviour, tend to accumulate bacteria present in the surrounding water, including faecal contaminants. Additionally, cross-contamination during transport and retail sales, originating from various environmental sources, could also contribute to the presence of *E. faecium* in finfish, highlighting the role of improper handling and hygiene practices throughout the supply chain. This was supported by its isolation from various environmental samples in our study (Table 2). The persistence of *E. faecium* in such environments is further evidenced by the detection of *E. faecium* on the hands of personnel and surfaces of equipment in retail fish markets in northern Greece, as reported by Sergelidis *et al*. (2013) [24]. Its ability to withstand environmental stressors, coupled with its biofilm-forming capacity and other virulence factors, enables *E. faecium* to thrive in fish market environments, underscoring the importance of strict hygiene protocols in preventing contamination and ensuring food safety.

Table 2. Occurrence of *E. faecium* in seafood and environmental sample

Type of samples	Total no. of samples	Positive isolates	
		No.	Percentage
Finfish	58	14	24.14
Crustaceans	58	23	39.66
Mollusc	58	13	22.41
Ice	6	1	16.67
Wastewater	6	3	50.00
Hand washings	6	0	0
Cutting boards swab	6	4	66.67
Knife swab	6	1	16.67
Total	204	59	28.92

In this study, phenotypic analysis revealed that 3.39 per cent of the isolates exhibited resistance to vancomycin (Fig. 2) (Table 3). Although the occurrence of vancomycin resistance in *E. faecium* isolated from non-hospital environments is noteworthy, studies have reported considerable variability in the prevalence of VREfm in seafood across different regions. For instance, research by Ben *et al*. (2017) [25] in Tunisia and Hammad *et al*. (2014) [26] in Japan found no evidence of vancomycin resistance among *E. faecium* isolates recovered from seafood. In contrast, Ullah *et al*. (2023) [16] reported a significantly higher prevalence of 73.20 per cent in raw seafood samples from Bangladesh. The detection of VREfm in seafood underscores the importance of understanding the molecular mechanisms behind vancomycin resistance. Screening of isolates for the *van*A gene revealed that only 1.69 per cent of isolates harboured this resistance determinant (Fig. 3) (Table 3). Similarly, Kumar *et al*. (2024) [3] reported that *E. faecium* isolated from freshwater fish sold in retail markets in Lucknow, Uttar Pradesh exhibited resistance to vancomycin on antibiotic susceptibility testing. However, the resistance in their study was not mediated by *van*A or *van*B genes but instead was attributed to chromosomally encoded, non-transferable *van*C1 and *van*C2/3 genes. These findings indicate the presence of diverse mechanisms of vancomycin resistance among *E. faecium* isolates, driven by both transferable and chromosomal genetic elements.

Fig 2: Phenotypic characterisation of *E. faecium* isolates for vancomycin resistance

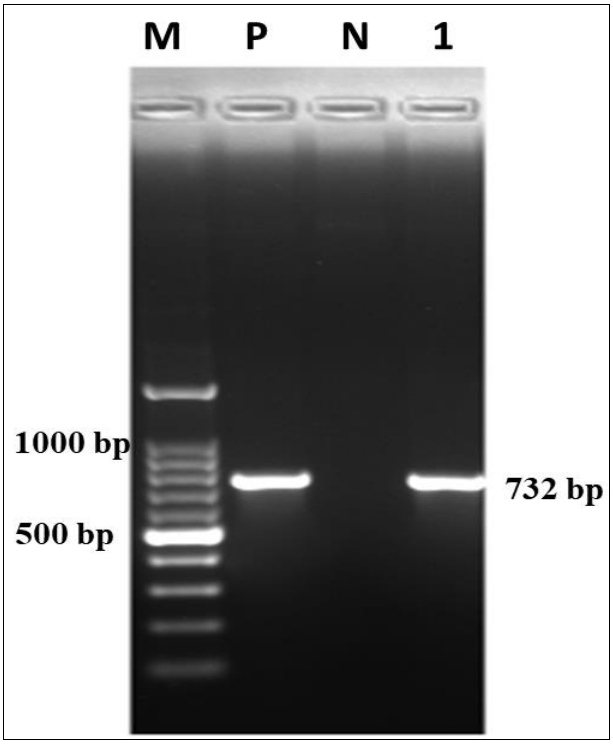

Fig 3: Molecular identification of *van*A gene. (Lane M: Marker, Lane P: Positive control, Lane N: Negative control, Lane 1: Sample)

Table 3: Characterisation of *E. faecium* isolates for vancomycin resistance

Sl. No.	Type of sample	No. of *E. faecium* isolates	Genotypic assay	Phenotypic assay (VA Disc)	
			*van*A gene	S	R
1	Finfishes	14	0	14	0
2	Crustaceans	23	0	21	1
3	Molluscs	13	1	12	1
4	Ice	1	0	1	0
5	Wastewater	3	0	3	0
6	Hand washings	0	0	0	0
7	Cutting board swab	4	0	4	0
8	Knife swab	1	0	1	0
Total		59	1	56	2

S- Sensitive R- Resistant VA- Vancomycin

The presence of the *van*A genotype, associated with high-level resistance to vancomycin and its transferability, represents a significant public health concern. In this study, *E. faecium* isolates recovered from seafood retailed in Wayanad district harboured the *van*A gene, posing not only a direct threat to human health but also the potential to spread resistance genes through horizontal gene transfer. The *van*A gene cluster encodes proteins that confer high-level resistance to both vancomycin and teicoplanin, further complicating efforts to control antimicrobial resistance. This highlights the critical importance of monitoring and addressing the presence of *van*A-associated vancomycin resistance in *E. faecium* from seafood, as it emphasizes the need for enhanced food safety practices and antimicrobial stewardship to combat the escalating problem of antimicrobial resistance.

4. Conclusion

The findings of this study highlight the significant public health implications of *E. faecium* as a potential contaminant in seafood, particularly given its association with severe infections and antimicrobial resistance. The detection of the *van*A gene, associated with high-level resistance to vancomycin and teicoplanin in retailed seafood, suggests its potential spread beyond hospital settings and into the community. Given the ability of *E. faecium* to persist in harsh environments, coupled with its virulence factors, the presence of this pathogen in seafood underscore the importance of strict food safety protocols and antimicrobial stewardship to mitigate the public health risks of AMR. Furthermore, this study calls attention to the need for enhanced monitoring and control measures to limit the spread of VRE beyond hospital settings and into the broader community through the food chain.

5. Conflict of interest

The authors declare that no actual or potential conflict of interest could inappropriately influence in this work.

6. Acknowledgements

The authors express their sincere gratitude to the College of Veterinary and Animal Sciences, Pookode, Wayanad, Kerala for providing all research facilities to complete the study successfully.

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How to Cite This Article
Renukuttan A, Arya VS, Asha K, Vinod VK, Jess V, Nayar R, Latha C, Hariharan R. Detection of *van*a-mediated vancomycin resistance in *e. Faecium* isolated from seafood retailed in wayanad, Kerala. International Journal of Veterinary Sciences and Animal Husbandry. 2024;9(6):509-514.