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Short Communication

Co-occurrence of antimicrobial resistance and virulence determinants in enterococci isolated from traditionally fermented fish products

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ABSTRACT

Objectives: Fermented foods frequently consumed in Northeast India can act as a reservoir for disseminating pathogenic organisms. Enterococci are often responsible for contamination of food products. This study investigated the antimicrobial resistance and co-existing virulence determinants of enterococci found in traditionally processed foods in India.
Methods: A total of 38 enterococci isolates identified as *Enterococcus faecalis* isolated from fermented fish samples from retail markets of Northeast India were selected for screening of pathogenic traits.
Results: Of the 38 isolates, 8 (21%) were able to hydrolyse gelatin and 13 (34%) showed protease activity. Screening for haemolytic activity of the isolates showed no positive test on sheep blood. The presence of virulence genes (*gelE*, *agg*, *esp*, *cpd*, *efaAfs* and *cylA*) was investigated by PCR. *gelE*, *agg* and *esp* were present in 17, 13 and 4 isolates, respectively. *cpd* and *efaAfs* were found in all isolates, whereas *cylA* was not detected. High resistance percentages to various antibiotics included kanamycin (63%), vancomycin and gentamicin (58%), tetracycline (53%) and rifampicin (50%). The *vanA* genotype was confirmed in 15 multidrug- and vancomycin-resistant strains.
Conclusion: The simultaneous occurrence of virulence determinants and antimicrobial resistance in enterococci prevalent in the fermented fish products studied poses a potential threat of transmission to humans through the food chain. This study highlights the importance of *E. faecalis* as a reservoir of antimicrobial resistance and virulence factors and their potential transfer to humans. The findings reopen the issue of food safety regarding enterococci prevalent in traditionally processed fish products in the region.

1. Introduction

In recent times, enterococci have emerged as clinically important nosocomial pathogens affecting immunosuppressed patients and those in intensive care units [1]. Vancomycin-resistant enterococci (VRE) are a significant concern in treating human infections since vancomycin is considered as the last-resort antibiotic for multidrug-resistant enterococci [1]. The increasing frequency of antimicrobial-resistant *Enterococcus* spp. is attributed to the indiscriminate use of antibiotics as animal growth promoters and in human health care [2].

Apart from enhanced antimicrobial resistance, nosocomial enterococci may possess other virulence factors that aid in human colonisation and pathogenesis [3]. In addition, certain virulence traits can promote the dissemination of virulence and antimicrobial resistance both within and outside hospital environments, especially through contaminated food [3].

The microbial composition of traditionally fermented foods in Northeast India has received considerable attention [4], but the characterisation and safety of *Enterococcus* has not garnered much interest from researchers. Some species of enterococci have been used as probiotics because of their possible health-promoting characteristics [5]. However, the selection of *Enterococcus* strains for such applications in the food industry should be based on the absence of any potential pathogenic traits or transferable antimicrobial resistance genes.

The present study was performed to characterise *Enterococcus* spp. isolates recovered from traditionally fermented and processed fish products, focusing on the presence of antimicrobial resistance determinants and virulence factors, in order to evaluate potential food-borne transmission of pathogenic enterococci to healthy individuals.

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2. Materials and methods

2.1. Enumeration of enterococci from selected samples

A total of 50 tungtap (fermented *Puntius* sp.) samples were obtained from retail markets of Northeast India and were homogenised in sterile saline (0.85% w/v NaCl). The diluted inoculum was spread on m-*Enterococcus* agar (HiMedia, Mumbai, India) plates and was incubated at 37 °C for 48–72 h for enumeration of enterococci.

2.2. Phenotypic characterisation of Enterococcus isolates

Presumptive identification of isolates was carried out by Gram staining, catalase and oxidase production, growth at 10 °C and 45 °C, growth in the presence of 6.5% NaCl and at pH 9.6, as well as esculin hydrolysis on bile esculin azide agar (HiMedia). The pyrrolidonyl arylamidase (PYR) test was used to confirm *Enterococcus*.

2.3. Phylogenetic analysis

Genomic DNA was extracted and the identity of the bacterial isolates was determined by first digesting the PCR-amplified 16S rRNA gene with 5 U each of the restriction enzymes *Hae*III, *Taq*I and *Hind*III (Fermentas, Mumbai, India), followed by sequencing of the 16 s rRNA gene. Phylogenetic analysis of the sequences was performed following the protocol of Rapsang et al. [6].

2.4. Phenotypic detection of virulence traits

Gelatinase and casein production was determined following the protocol of Kumar Patidar et al. [7]. Isolates were assessed for haemolytic activity on sheep blood agar (HiMedia) plates. Single colonies of the isolates were inoculated onto agar plates and were incubated at 37 °C for 24–48 h. The presence of a zone of clearance around the colonies was interpreted as a positive result for β-haemolysis.

2.5. Antimicrobial resistance profile

The antibiotic susceptibility patterns of the isolates to different antibiotics (kanamycin, gentamicin, streptomycin, ciprofloxacin, vancomycin, ampicillin, penicillin G, tetracycline, rifampicin, polymyxin B and chloramphenicol) was determined by the disk diffusion method according to Clinical and Laboratory Standards (CLSI) guidelines [8].

2.6. PCR detection of virulence and vancomycin resistance genes

The virulence factor genes *gelE* (gelatinase), *esp* (extracellular surface protein), *agg* (aggregation substance), *efaAfs* (cell wall adhesin expressed in *Enterococcus faecalis*), *cylA* (activation of cytolysin, bacterial toxin) and *cpd* (sex pheromone) were identified by PCR using specific primers [9]. Genes for resistance to vancomycin (*vanA*, *vanB*, *vanC1*, *vanC2/C3* and *vanD*) [10] were tested in all enterococcal isolates that showed resistance or reduced susceptibility to vancomycin.

PCR amplification was performed in a 50 μL final volume containing 5 μg of DNA, 15 mM $MgCl_2$, 20 pmol of each primer, 0.2 mM of deoxynucleoside triphosphates (dNTPs) and 1 U of *Taq* DNA Polymerase (Merck Life Sciences Pvt. Ltd., Bengaluru, India) under the following conditions: initial denaturation at 94 °C for 4 min; followed by 25 cycles of denaturation at 94 °C for 2 min, annealing at 55 °C for 2 min and elongation at 72 °C for 2 min. Amplification products were electrophoresed in 1.2% agarose gel, were stained with ethidium bromide (HiMedia) and were visualised using a gel documentation system (UVitec, Cambridge, UK).

2.7. Transferability of virulence genes to E. coli

All bacterial isolates harbouring *agg*, *gelE*, *cpd*, *efaAfs*, *esp* and *vanA* were cultured in MRS broth (HiMedia) and plasmids were purified by the standard alkaline lysis method. Transformation of plasmids by the heat-shock method was carried out using *E. coli* DH5α as recipient. Transformants were selected on Luria–Bertani agar plates (HiMedia) containing kanamycin (0.5 mg/L).

3. Results and discussion

A total of 76 lactic acid bacteria (LAB) isolates were obtained from fermented fish samples, of which 38 were Gram-positive cocci able to hydrolyse esculin and positive for the PYR test. PCR products of the 16S rRNA gene were digested with three restriction enzymes (*Hae*III, *Taq*I and *Hind*III), yielding identical amplified ribosomal DNA restriction analysis (ARDRA) banding patterns for all the isolates. The undigested amplicons were subsequently sequenced and BLAST analysis was performed against the EzTaxon database to identify the nearest phylogenetic neighbours. BLAST analysis revealed that all 38 isolates were *E. faecalis* (Fig. 1). This finding is in agreement with a previous study of 416 enterococci strains isolated from 155 food samples of animal origin, in which the majority (72%) were *E. faecalis* [11]. Enterococci have been isolated from the intestine of fish from integrated fish farming and from shellfish. In addition, it has been reported that processed fish products also contain large numbers of LAB, particularly enterococci [12]. The dominance of *E. faecalis* in such food products may be due to their exposure during processing of such samples and also because of their natural occurrence.

Analysis of the phenotypic traits of the 38 *E. faecalis* isolates revealed that 8 (21%) could hydrolyse gelatin, in agreement with a previous report [13]. Protease activity in skim milk media was demonstrated by 13 (34%) of the 38 isolates (Table 1). However, none of the isolates showed haemolytic activity on sheep erythrocytes, in contradiction to a previous report of a high incidence of β-haemolysis among *E. faecalis* isolates [14]. One possible reason is that sheep erythrocytes are less susceptible to haemolysin-mediated lysis compared with horse and human erythrocytes [15].

Enterococci are intrinsically resistant to cephalosporins, aminoglycosides and polymyxins, but acquired resistance to chloramphenicol, erythromycin, high levels of clindamycin and aminoglycosides, tetracycline, high levels of β-lactams and glycopeptides such as vancomycin is mediated by genes residing on plasmids or transposons [2]. The antimicrobial resistance rates of the 38 *E. faecalis* isolates were as follows: kanamycin, 63%; vancomycin, 58%; gentamicin, 58%; tetracycline, 53%; rifampicin, 50%; penicillin G, 18%; ampicillin, 13%; polymyxin B, 13%; streptomycin, 13%; and chloramphenicol, 3%. All of the isolates were susceptible to ciprofloxacin. The highest rates of resistance were observed against kanamycin and gentamicin, potentially increasing the likelihood of transmission of resistance determinants to these two antibiotics to the human food chain. Similarly, in agreement with previous findings, the present study identified a high prevalence of resistance to tetracycline, whereas only a small percentage of isolates demonstrated resistance to penicillin G and ampicillin [16,17] and only one isolate showed resistance to chloramphenicol, although there are some reports on the emergence of chloramphenicol resistance in enterococci [18]. In the present study, a high percentage of isolates (58%) were resistant to vancomycin. Significantly, most of the VRE were also

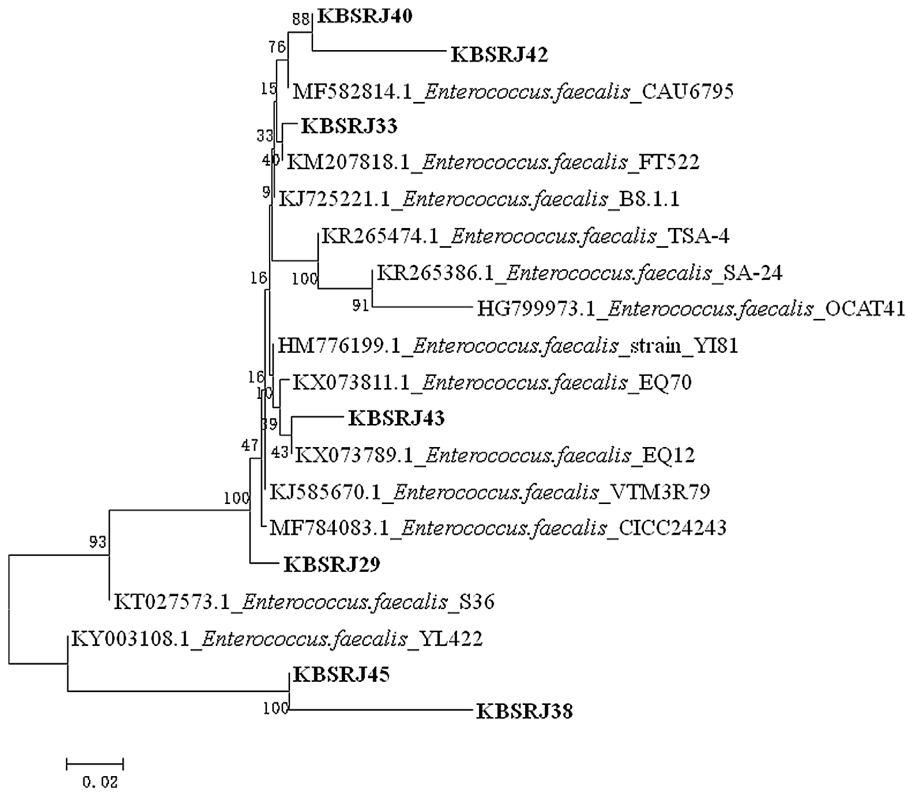

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence comparison showing the position of representative *Enterococcus faecalis* isolates (KBSRJ29, KBSRJ33, KBSRJ38, KBSRJ40, KBSRJ42, KBSRJ43 and KBSRJ45). Bootstrap values are indicated at branch points based on 1000 replicates.

resistant to other clinically relevant antibiotics, a cause of concern in terms of diminishing therapeutic options. Both natural and intrinsic antimicrobial resistance as well as acquired or transferable resistance are found in enterococci.

The occurrence of *gelE*, *efaAfs*, *esp*, *agg*, *cylA* and *cpd* genes in bacteria isolated from fermented fish in this study was consistent with previous studies [10–12,15,16] (Fig. 2; Table 1). The 402-bp *gelE* gene was detected in 17 isolates (45%). The *cylA* gene was not found in any of the isolates, in contrast to genes encoding the sex pheromone determinant Cpd and cell wall adhesin EfaAfs, both of which were found in all 38 *E. faecalis* isolates (100%). The aggregation substance-encoding gene *agg* was present in only 13 (34%) of the 38 isolates. Only 4 (11%) of the 38 enterococci isolates contained the *esp* gene that encodes the enterococcal surface protein, some of which also contained the *agg* gene. Gelatinase activity was not detected in some of the isolates possessing the *gelE* gene. Presence of the gene but lack of corresponding gelatinase activity in the isolates may suggest the presence of silent genes, which may be due to non-induction or repression of gene expression in response to environmental signals [19]. The *cylA* gene was not detected in any of the isolates. The sex pheromone determinant *cpd* was found to be present in all of the *E. faecalis* isolates. In some of these isolates, the aggregation substance gene *agg* was also present. The Agg protein causes aggregation of donor and recipient cells when it is produced in response to pheromones secreted by potential recipient *E. faecalis* cells, corroborating an earlier study [15]. Production of the adhesin-like *E. faecalis* endocarditis antigen EfaAfs is considered to be a potential virulence determinant [13]. Barbosa et al. reported a low incidence of the *esp* gene (16.5%) in their study [13], whereas this gene is found more frequently in clinical isolates [20]. The *E. faecalis* isolates in the current study contained the *efaAfs*, *gelE*, *agg*, *cpd* and *esp* genes, along with the vancomycin resistance-encoding *vanA* gene, with some isolates carrying all six genes. Interactions of these strains with susceptible hosts may contribute to the spread of virulence and antimicrobial resistance genes by horizontal gene transfer among the associated co-inhabitant bacterial microflora of processed fish products. Acquisition of new genes facilitates the emergence of enhanced bacterial virulence in the host as a result of acquired pathogenic properties.

Of the 22 isolates displaying vancomycin resistance, the *vanA* and *vanB* genes were detected in 15 (68%) and 6 (27%), respectively (Fig. 2; Table 1). No vancomycin-resistant isolate harboured *vanC1*, *vanC2/C3* or *vanD*. Vancomycin resistance consists of five known phenotypes, namely VanA, VanB, VanC, VanD and VanE [21]. Of these, VanA and VanB are distinct forms of transferable

Table 1
Distribution of virulence and vancomycin resistance genes in *Enterococcus faecalis* isolates from fermented fish samples.

No. (%) of isolates with positive phenotypic assay ($n = 38$)			No. (%) of isolates harbouring virulence genes ($n = 38$)						No. (%) of isolates harbouring vancomycin resistance genes ($n = 22$)[a]				
Gelatinase activity	Protease activity	Haemolytic activity	*agg*	*gelE*	*efaAfs*	*cpd*	*esp*	*cylA*	*vanA*	*vanB*	*vanC1*	*vanC2/C3*	*vanD*
8 (21)	13 (34)	0	13 (34)	17 (45)	38 (100)	38 (100)	4 (11)	0	15 (68)	6 (27)	0	0	0

[a] Among 22 isolates with resistance or reduced susceptibility to vancomycin.

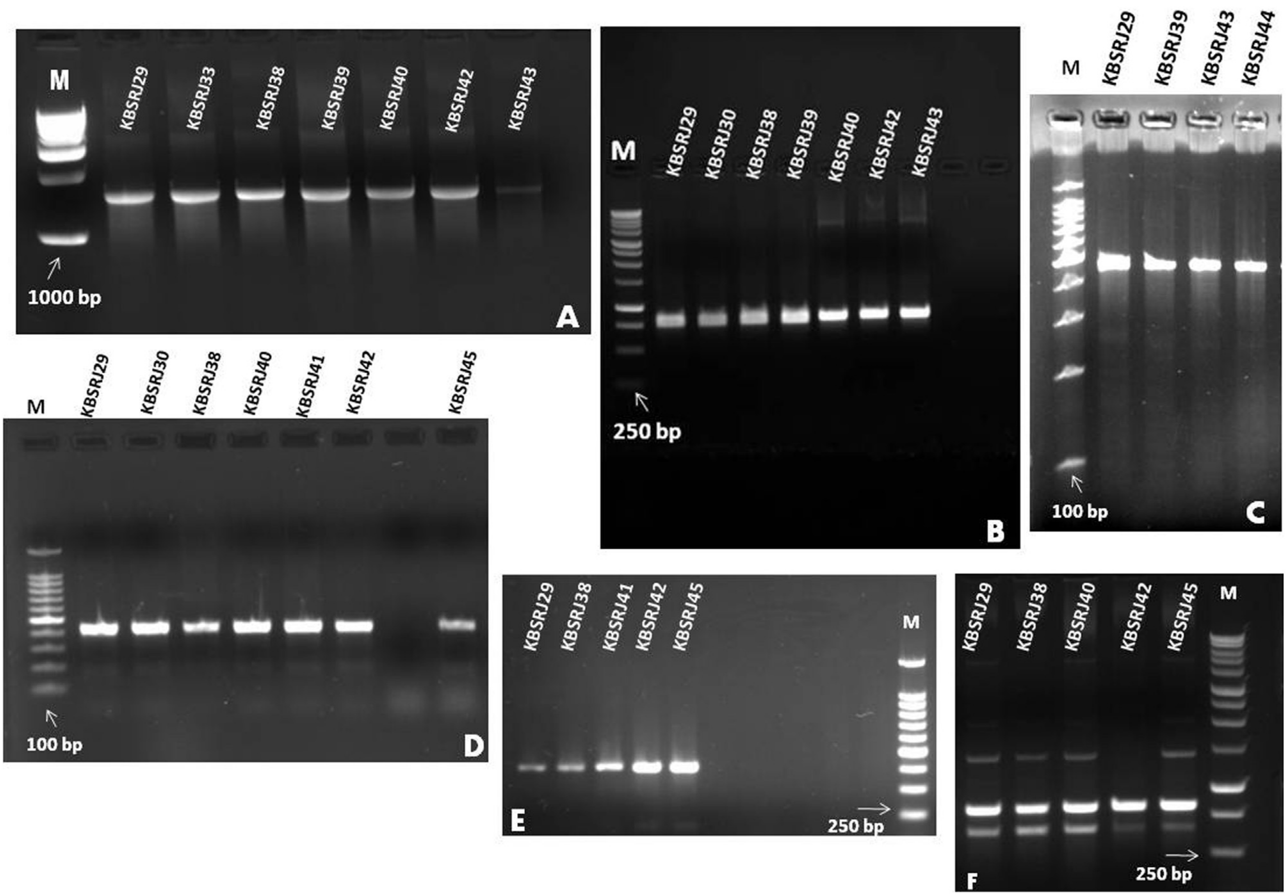

Fig. 2. Amplification of virulence-associated genes and vancomycin resistance genes in representative *Enterococcus faecalis* isolates: (A) *agg*; (B) *cpd*; (C) *esp*; (D) *gelE*; (E) *vanA*; and (F) *vanB*. M, molecular size marker (100 bp).

phenotypes and are associated with a high level of inducible vancomycin resistance [1]. In the current study, 21 isolates harboured plasmid-borne *vanA* or *vanB* genes. This significant finding highlights the fact that traditionally fermented products can be contaminated with vancomycin-resistant bacteria, with the potential to transfer the resistance genes. Transferability assays demonstrated that *agg*, *cpd*, *esp* and *vanA* genes were plasmid-encoded and could be horizontally transferred from a diverse range of enterococci isolates to recipient *E. coli* DH5α cells. However, plasmids harbouring *gelE* and *efaAfs* could not be transferred to *E. coli* DH5α.

4. Conclusion

This study suggests that enterococci from traditionally processed products should be considered with caution since they may be a reservoir for antimicrobial resistance and virulence genes enabling the propagation of these genes to the human microbiota through the food chain. This is especially true for *E. faecalis* since this species was isolated more frequently and was found to carry the determinants. This study is the first of its kind and raises concern about the safety aspect of enterococci isolated from traditional fish products, which is a component of the diet of ethnic tribes. Further research is needed to evaluate the expression of such factors and to monitor the increasing antimicrobial resistance and virulence determinants among these micro-organisms in the human system. This will facilitate successful treatment of enterococcal infections and map the potential transfer of such factors to other intestinal organisms.

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Competing interests

None declared.

Ethical approval

Not required.

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