Molecular Characterization of Virulence Genes in Vancomycin-Resistant and Vancomycin-Sensitive Enterococci

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ABSTRACT

Background: The aim of this study was to find out the correlation between presence of virulence (gelatinase [*gel E*], enterococcal surface protein [*esp*], cytolysin A [*cyl A*], hyaluronidase [*hyl*], and aggregation substance [*asa1*]) and vancomycin-resistant genes (*van A* and *van B*) in enterococci, with their phenotypic expression. **Materials and Methods:** A total of 500 isolates (250 each clinical and fecal) were processed. Enterococci were isolated from various clinical samples and from fecal specimens of colonized patients. Various virulence determinants namely *asa1*, *esp*, *hyl*, *gel E*, and *cyl* were detected by phenotypic methods. Minimum inhibitory concentration (MIC) of vancomycin was determined by agar dilution method. Multiplex polymerase chain reaction (PCR) was used to detect the presence of virulence and *van* genes. **Results:** Out of all the samples processed, 12.0% (60/500) isolates carried *van A* or *van B* genes as confirmed by MIC test and PCR methods. Genes responsible for virulence were detected by multiplex PCR and at least one of the five was detected in all the clinical vancomycin-resistant enterococci (VRE) and vancomycin-sensitive enterococci (VSE). *gel E*, *esp,* and *hyl* genes were found to be significantly higher in clinical VRE. Of the fecal isolates, presence of *gel E*, *esp,* and *asa1* was significantly higher in VRE as compared to VSE. The presence of *hyl* gene in the clinical VRE was found to be statistically significant ($P = 0.043$) as against the fecal VRE. Correlation between the presence of virulence genes and their expression as detected by phenotypic tests showed that while biofilm production was seen in 61.1% (22/36) of clinical VRE, the corresponding genes, i.e., *asa1* and *esp* were detected in 30.5% (11/36) and 27.8% (10/36) of strains only. **Conclusion:** *Enterococcus faecium* isolates were found to carry *esp* gene, a phenomenon that has been described previously only for *Enterococcus faecalis*, but we were unable to correlate the presence of *esp* with their capacity to form biofilms.

Key words: Aggregation substance, cytolysin A, enterococcal surface protein, gelatinase, hyaluronidase

INTRODUCTION

Enterococcus species are now recognized as important causes of urinary tract infections, postsurgical wound infections, bacteremia, endocarditis, meningitis, neonatal sepsis, and infections in transplant patients with *Enterococcus faecalis* and *Enterococcus faecium* responsible for the majority of these infections.[1] Nevertheless, the incidence of other species of enterococci from clinical sources shows an alarming increase. This is attributable to their acquisition of various putative virulence determinants and multidrug resistance.[2]

A number of genes encoding for virulence factors including aggregation substance (*asa1*), enterococcal surface protein (*esp*), hyaluronidase (*hyl*), gelatinase (*gel E*), and cytolysin (*cyl*) in *E. faecalis* and *E. faecium* have been described and their effects have been shown in human and animal studies.[3] *asa1*, a surface protein adhesin encoded by the gene *asa1* has a contribution to virulence together with cyl. It facilitates the aggregation of the donor and recipient bacteria for efficient transfer of transmissible

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conjugative plasmids.[4] Another enterococcal adhesin is the "*esp*," encoded by *esp* gene that plays a role in biofilm formation and adherence to abiotic surfaces.[5] *hyl*, which is expressed by the *hyl* gene, acts on hyaluronic acid and increases bacterial invasion.[6] The *gel E* gene encodes for an extracellular Zn-metalloendopeptidase that is capable of hydrolyzing gelatin, collagen, casein, hemoglobin, and other biological peptides.[7] The cyl is a cellular toxin, and is capable of lysing a range of prokaryotic and eukaryotic cells.[8]

There is a paucity of information on the virulence genes distributed among enterococcal species.[9,10] The putative virulence genes in enterococcal strains isolated from various clinical sources and colonized patients, and also the possible link between the presence of virulence markers and virulence genes was therefore investigated.

MATERIALS AND METHODS

Study population

The study population included patients of both sexes and all age groups attending the outpatient and inpatient departments of a Tertiary Care Hospital in Eastern Bihar, India. A total of 500 strains of enterococci were collected from samples submitted to the Microbiology laboratory for culture and sensitivity and were used in the present study. This included 250 enterococcal strains collected from clinical samples of the patients, attending the hospital with infections of different types. Another 250 enterococcal strains were isolated from among another 300 patients (otherwise not suffering from any infections), who had been admitted to the hospital and screened for gastrointestinal carriage of vancomycin-resistant enterococci (VRE). Clearance from Institutional Ethics Committee was obtained to carry out this study.

Isolation and identification

Two hundred and fifty enterococci were isolated from various clinical samples (118 isolates from urine, 79 isolates from pus, 34 isolates from blood, and 19 isolates from catheter tip). 300 fecal samples were collected from other patients (as mentioned above) on three separate occasions, i.e., at the time of admission, after 48 h, and after 5 days of admission to screen for VRE. Of the 300 fecal samples, 50 samples showed culture negativity and another 250 samples showed the growth of *Enterococcus* species. The isolates were identified to species level using standard procedures.[11-13]

Hemolytic-assay

Hemolytic activity of enterococci was assessed on two blood agar plates prepared with Muller-Hinton agar (Hi-Media, Mumbai, India) containing 5% defibrinated sheep and human blood, by observation of zone of hemolysis around colonies after incubation for 24 h at 37°C.[14]

Hemagglutination test

Enterococci were grown on brain heart infusion agar supplemented with 10% sheep blood. A loopful of bacteria was mixed on a glass slide with 25 μl of a 3% suspension of sheep, rabbit, human group A, human group O, and human group B erythrocytes. *Vibrio cholerae* was used as positive control. After 5 min at room temperature, results were recorded as positive or negative.[15]

Physico-chemical properties of the culture filtrates

The effects of physic-chemical agents on hemagglutination test (HA) were investigated by performing HA test after treatment of the bacteria with trypsin, protease K, and pepsin (Hi-Media, Mumbai, India). Bacterial suspensions of test strains were centrifuged and the deposit was added to separate test tubes containing trypsin (1 μg/ml), pepsin (1 μg/ml), and protease K (1 μg/ml) in phosphate-buffered saline (PBS). The test tubes were incubated at 37°C for 60 min. For heat treatment, bacterial suspensions were heated at 50°C for 30 min. HA test was carried out with 20 μl of 3% erythrocyte suspension and 20 μl of enzyme treated and heated culture suspensions on glass slides. The suspensions were mixed, rotated gently for 30 s, and results were recorded as either strong agglutination (+++ −), agglutination (− ++), or no agglutination.[16]

Caseinase production

Casein hydrolysis was detected on Muller-Hinton agar (Hi-Media, Mumbai, India) containing 3% skimmed milk. Plates were streaked with test strains followed by incubation at 37°C for 24 h. The presence of a transparent zone around the colonies indicated caseinase activity. *gel E* production was detected by stab inoculating the test strain on nutrient agar supplemented with 3% gelatin (Hi-Media, Mumbai, India) kept at 37°C for 24 h followed by refrigeration at 4°C for half an hour. Liquefaction of gelatin was considered as positive.[13,14]

Lipase production

Egg yolk agar (Hi-Media, Mumbai, India) was used for lipase production. The test organism was spot inoculated on the

medium and incubated at 37°C for 24-48 h. Positive test result was read as formation of thin iridescent pearly layer overlying the colonies and a confined opalescence in the medium, which was seen when the colonies were scraped off.[12]

Slime layer formation

Brain heart infusion agar (Hi-Media, Mumbai, India) supplemented with 5% sucrose was used to determine the ability of *Enterococcus* species to produce extracellular polysaccharide on the agar. Test strains grown in Todd-Hewitt broth (Hi-Media, Mumbai, India) were used as the inoculum. The colonies appeared mucoidal, runny, or slimy due to the production of polysaccharide.[13]

Deoxyribonuclease test

Test strains were inoculated on deoxyribonuclease agar (Hi-Media, Mumbai, India). Clearing of the medium around the colonies indicated a positive test.[17]

Biofilm detection assay

The test strains were grown overnight at 37°C in Brain Heart Infusion broth (Hi-Media, Mumbai, India) plus 0.25% glucose. Culture was diluted 1:20 in the same media. 200 μL of this suspension was used to inoculate sterile 96 well polystyrene microtiter plates. After 24 h at 37°C of static incubation, wells were washed with PBS, dried in inverted position, and stained with 1% crystal violet for 15 min. The cells were rinsed once more and solubilized in 200 μl ethanol/acetone (80:20 v/v). The A_{630} was determined using microtiter plate reader. Biofilm formation was scored as nonbiofilm forming (−), weak - (+), moderate - (++), and strong - (+++) corresponding to the A_{630} values ≤1, 1-≤2, 2-≤3, and >3, respectively.[18]

Antimicrobial susceptibility and minimum inhibitory concentration tests

Antibiotic susceptibility test was done by Kirby-Bauer disc diffusion method on Mueller-Hinton agar. Minimum inhibitory concentration (MIC) of VRE was determined by agar dilution method using the following concentration of vancomycin 0.5-64 μg/mL. The test was quality controlled using *E. faecalis* ATCC 51299 and *E. faecalis* ATCC 29212.[19,20]

Protocol for multiplex polymerase chain reaction

DNA extraction method

Genomic DNA used as template for polymerase chain reaction (PCR) amplification was prepared using conventional phenol-chloroform DNA extraction method. PCR was performed in PCR system, model number T1 Thermoblock. The oligonucleotide primer pairs used to amplify the virulence genes *asa1, cyl A, gel E, esp,* and *hyl* as well as the vancomycin-resistant genes *van A*, *van B,* and the expected amplicon sizes are as follows [Table 1]:

The amplification of virulence genes was carried out as follows: Initial denaturation at 95°C for 15 min followed by denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. The PCR amplification of the *van* genes were carried out as follows: Predenaturation at 95°C for 4 min followed by denaturation at 95°C for 30 cycles of 30 s each; 1 min for annealing at 52°C and elongation at 72°C for 1 min. Both positive control and negative control, consisting solely of the PCR reaction mixture without DNA template were included to check the validity of the technique utilized in the study.[21,22]

Analysis of DNA by agarose gel electrophoresis

Twenty-five microliters of respective amplified products were loaded into the wells and electrophoresed at a constant current of 50V for about 45 min using 1.5% agarose gel. A 100 bp DNA ladder marker was included as the standard molecular weight marker. The electrophoresed gel was later subjected to ethidium bromide staining and photographed under UV transillumination [Figures 1-3].

Statistical analysis

Statistical analysis was performed by Chi-square test. $P \leq 0.05$ was considered to be significant and $P \leq 0.001$ was considered to be highly significant. All statistical analyses

Table 1: Polymerase chain reaction primers and products for the detection of virulent genes and vancomycin-resistant genes

Target product	Oligonucleotide sequence (5′-3′)	Size
asa1	ASA11-GCACGCTATTACGAACTATGA ASA12-TAAGAAAGAACATCACCACGA	375
cyl A	CYT I-ACTCGGGGATTGATAGGC CYT IIb-GCTGCTAAAGCTGCGCTT	600
gel E	GEL 11-TATGACAATGCTTTTTGGGAT GEL 12-AGATGCACCCGAAATAATATA	100
esp	ESP 14F-AGATTTCATCTTTGATTCTTGG ESP 12R-AATTGATTCTTTAGCATCTGG	570
hyl	HYL n1-ACAGAAGAGCTGCAGGAAATG HYL n2-GACTGACGTCCAAGTTTCCAA	200
van A	F14-CATGAATAGAATAAAAGTTGCAATA R14-CCCCTTTAACGCTAATACGATCAAA	1030
van B	F15-GTGACAAACCGGAGGCGAGGA R15-CCGCCATCCTCCTGCAAAAAA	433

asa1: Aggregation substance; *cyl A*: Cytolysin A; *gel E*: Gelatinase; *esp*: Enterococcal surface protein; *hyl*: Hyaluronidase

were carried out using online statistical software at http://www.physics.csbsju.edu/stats/contingency_NROW_NCOLUMN_form.html. Statistical analysis has been done to find out the prevalence of different virulence markers and virulence genes (*gel E*, *esp*, *cyl A*, *hyl*, *asa1)* in VRE, and vancomycin-sensitive enterococci (VSE) were statistically significant/insignificant.

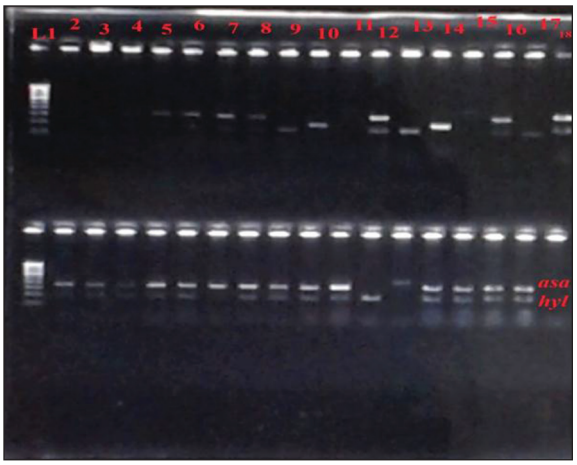

Figure 1: Polymerase chain reaction products of *asa1* gene 375bp and *hyl* gene 200bp. L1 stands for Lane 1 and corresponds to molecular markers from 100bp to 1000bp. asa1: Aggregation substance 1; *hyl:* Hyaluronidase

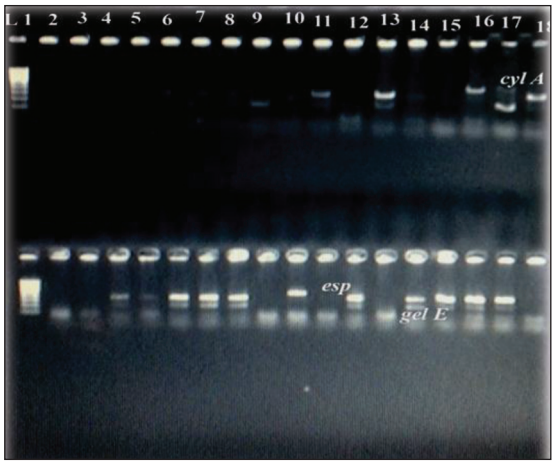

Figure 2: Polymerase chain reaction products of *cyl A* gene 600bp; *esp* gene 570bp; *gel E* 100bp. *cyl A:* Cytolysin A; *esp:* Enterococcal surface protein; *gel E*: Gelatinase

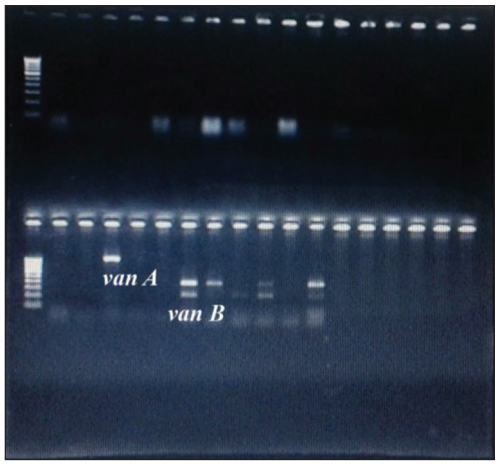

Figure 3: Bands corresponding to 1030 bp (*van A*) and 433 bp (*van B*)

RESULTS

A total of 500 enterococci (250 each clinical and fecal) were processed, out of which 37 (7.4%) isolates were vancomycin-resistant and 23 (5.6%) showed reduced susceptibility to vancomycin by phenotypic agar dilution method. Thus, a total of 60 strains (36 clinical and 24 fecal) which were VRE/VIE by MIC tests were also confirmed to carry *van A* or *van B* genes by PCR methods.

Various virulence factors of enterococci were detected by phenotypic methods for both clinical and fecal isolates. The differences in the presence of virulence factors in clinical VSE and VRE were found to be statistically insignificant for most of the virulence factors except for the production of caseinase, adhesins, and *gel E* which was significantly higher in VRE than VSE ($P = 0.000$). For the fecal isolates, production of slime layer and *gel E* was significantly higher in VRE than VSE ($P = 0.05$; $P = 0.03$) [Table 2].

The presence of genes encoding for potential virulence factors was studied by multiplex PCR in both the clinical and fecal isolates. The predominant virulence gene in clinical VRE was *gel E* with 16 (44.4%) clinical VRE isolates having the *gel E* gene as compared to 35 (16.4%) VSE isolates. This finding was found to be statistically significant ($P = 0.000$). The prevalence of *esp* ($P = 0.001$) and *hyl* ($P = 0.047$) genes were significantly higher among VRE isolates (27.8% and 36.1%) than among VSE isolates (8.9% and 21.0%). The presence of other virulence genes such as *cyl A* and *asa1* were not found to be statistically significant [Table 3].

Like the clinical isolates, *gel E*, as well as *esp*, was the predominant gene detected in the fecal VRE which was seen in 29.2% of isolates each followed by *asa1*, seen in 25.0% of strains. The presence of *gel E*, *esp,* and *asa1* was found to be significantly higher in the VRE than VSE ($P = 0.012$, 0.001, and 0.000, respectively). The presence of other virulence genes was statistically insignificant [Table 3].

The presence of virulence genes in clinical and fecal VRE was compared. All the virulence genes were encountered more frequently in clinical VRE than in fecal VRE except for *esp* which was found in 29.2% (7/24) of fecal VRE as compared to 27.8% (10/36) of clinical VRE. However,

Table 2: Comparative evaluation of the virulence factors in clinical and fecal isolates of vancomycin-sensitive enterococci and vancomycin-resistant enterococci

Phenotypic virulence markers of clinical isolates	Clinical isolates			Fecal isolates		
	VSE (*n* = 214) (%)	VRE (*n* = 36) (%)	*P*	VSE (*n* = 226) (%)	VRE (*n* = 24) (%)	*P*
Hemolysis of sheep RBC	42 (19.6)	9 (25.0)	0.459	20 (8.8)	2 (8.3)	0.932
Hemolysis of human RBC	30 (15.0)	9 (25.0)	0.093	12 (5.3)	2 (8.3)	0.540
Production of deoxyribonuclease	41 (19.2)	14 (38.9)	0.008	30 (13.3)	8 (33.3)	0.009
Slime layer	48 (22.4)	10 (27.8)	0.482	46 (20.4)	9 (37.5)	0.054
Lipase	41 (19.2)	4 (11.1)	0.245	22 (9.7)	5 (20.8)	0.096
Caseinase	16 (7.5)	11 (30.6)	0.000	19 (8.4)	4 (16.7)	0.183
Adhesins (responsible for biofilm formation)	20 (9.3)	22 (61.1)	0.000	22 (9.7)	7 (29.2)	0.005
Gelatinase	26 (12.1)	14 (38.9)	0.000	24 (10.6)	6 (25.0)	0.03

P ≤ 0.05 or ≤0.001 was considered statistically significant; VSE: Vancomycin-sensitive enterococci; VRE: Vancomycin-resistant enterococci; RBC: Red blood cell

Table 3: Presence of virulence genes in vancomycin-sensitive enterococci and vancomycin-resistant enterococci (clinical and fecal)

Genes of virulence markers	Clinical isolates			Fecal isolates			Clinical VRE (*n* = 36)	Fecal VRE (*n* = 24)	*P*
	VSE (*n* = 214)	VRE (*n* = 36)	*P*	VSE (*n* = 226)	VRE (*n* = 24)	*P*			
gel E	35 (16.4)	16 (44.4)	0.000	25 (11.1)	7 (29.2)	0.012	16 (44.4)	7 (29.2)	0.233
cyl A	20 (9.3)	9 (25.0)	0.007	19 (8.4)	2 (8.3)	0.990	9 (25.0)	2 (8.3)	0.102
esp	19 (8.9)	10 (27.8)	0.001	18 (7.9)	7 (29.2)	0.001	10 (27.8)	7 (29.2)	0.907
hyl	45 (21.0)	13 (36.1)	0.047	28 (12.4)	3 (12.5)	0.988	13 (36.1)	3 (12.5)	0.043
asa1	37 (17.3)	11 (30.6)	0.062	12 (5.3)	6 (25.0)	0.000	11 (30.6)	7 (29.2)	0.908

P ≤ 0.05 or ≤0.001 was considered statistically significant; VSE: Vancomycin-sensitive enterococci; VRE: Vancomycin-resistant enterococci; *gel E*: Gelatinase; *asa1*: Aggregation substance; *esp*: Enterococcal surface protein; *cyl A*: Cytolysin A

Table 4: Distribution of virulence genes in clinical vancomycin-resistant enterococci and vancomycin-sensitive enterococci

Type of isolates	gel E (%)	cyl A (%)	esp (%)	hyl (%)	asa1 (%)
Clinical VRE					
Enterococcus fecalis, n=14	8 (57.1)	4 (28.6)	4 (28.6)	3 (21.4)	7 (50.0)
Enterococcus faecium, n=17	6 (35.3)	4 (23.5)	6 (35.3)	9 (53.0)	4 (23.5)
Enterococcus gallinarum, n=5	2 (40.0)	1 (20.0)	0	1 (20.0)	0
Total=36	16 (45.4)	9 (25.0)	10 (27.8)	13 (36.1)	11 (30.6)
Clinical VSE					
Enterococcus fecalis, n=50	13 (26.0)	8 (16.0)	11 (22.0)	14 (28.0)	11 (22.0)
Enterococcus faecium, n=43	7 (16.3)	6 (13.9)	8 (18.6)	20 (46.5)	9 (20.9)
Enterococcus mundtii, n=33	5 (15.2)	2 (6.0)	0	3 (9.0)	6 (18.1)
Enterococcus raffinosus, n=28	3 (10.7)	1 (3.6)	0	2 (7.1)	4 (15.3)
Enterococcus gallinarum, n=14	2 (15.3)	1 (7.1)	0	2 (15.3)	4 (28.6)
Enterococcus malodoratus, n=20	2 (10)	1 (5.0)	0	1 (5.0)	0
Enterococcus solitarius, n=9	3 (33.3)	0	0	2 (22.2)	3 (33.3)
Enterococcus durans, n=17	0	1 (5.9)	0	1 (5.9)	0
Total=214	35 (16.4)	20 (9.3)	19 (8.9)	45 (21.0)	37 (17.3)

gel E: Gelatinase; *asa1*: Aggregation substance; *esp*: Enterococcal surface protein; *cyl A*: Cytolysin A; VSE: Vancomycin-sensitive enterococci; VRE: Vancomycin-resistant enterococci

only the presence of *hyl* gene was found to be significantly higher in clinical VRE than fecal VRE ($P = 0.043$) [Table 3].

Table 4 shows the presence of various virulence genes in VRE species. The predominant genes in *E. faecalis* were *gel E* 57.1%; followed by *asa1* 50.0%, *cyl A* and *esp* being 28.6% each, and *hyl* 21.4%. The predominant gene in *E. faecium* was *hyl* gene (53.0%) followed by *gel E*, *esp* 35.3% each, *cyl A*, and *asa1* 23.5% each. *Enterococcus gallinarum* showed the presence of *gel E* 40.0% followed by *cyl A* and *hyl* 20.0% each.

Out of the 14 VR *E. faecalis,* all the strains produced one or two virulence genes. Twelve (85.7%) strains of *E. faecalis* produced two virulence genes: 35.7% (5/14) strains showed the concomitant presence of *gel E* and *asa*; another 21.4% (3/14) strains expressed *gel A* and *hyl* genes; and 28.6% (4/14) strains presented *cyl A* and *esp* genes. Of the 17 VR *E. faecium*, 12 (70.6%) strains were found to produce two genes. The various combination of two virulence genes seen in *E. faecium* were 35.3% (6/17) expressed *gel A* with *hyl*; 17.6% (3/17), each, expressed *cyl A* with *esp* and *asa1* with *esp*.

Of the clinical VSE, the predominant genes in *E. faecalis* were *hyl* (28.0%); followed by *gel E* (26.0%), *esp* and *asa1* (22.0%) each, and *cyl A* (16.0%). *hyl* (46.5%) was the main gene in *E. faecium* followed by *asa1* (20.9%), *esp* (18.6%), *gel E* (16.3%), and *cyl A* (13.9%). Apart from the *esp* gene, the remaining four virulence genes were detected in all the nonfaecalis and nonfaecium strains. *Enterococcus durans* was the least virulent among VSEs as it showed the expression of only *cyl A* and *hyl* (5.9%) each [Table 4].

Table 5 shows the distribution of virulence genes in fecal VRE. *gel E* (33.3%) was the predominant virulence gene in *E. faecalis* followed by *esp* (33.3%), *asa1* (16.7%), *cyl A,* and *hyl* (8.3%) each. The main genes in *E. faecium* were *gel E*, *esp,* and *asa1* (37.5%) each. *E. gallinarum* showed the presence of only *hyl* and *asa1* (25.0%) each. The simultaneous presence of two virulence genes was seen between *gel E* and *asa1* in 16.2% (2/12) of *E. faecalis*, while 37.5% (3/8) *E. faecium* carried *asa1* and *esp* gene combination. Of the fecal VSE, *E. faecalis* and *E. faecium* showed the presence of all virulence genes. *Enterococcus dispar* was least virulent species as it did not express any virulence genes. The *esp* gene was totally absent in all nonfaecalis and nonfaecium strains [Table 5].

Of the 500 *Enterococcus* species isolated from clinical and fecal sources, 71 (14.2%) strains were positive for biofilm formation whereas 83 (16.6%) strains were found to show the presence of *gel E* gene and only 54 (10.8%) strains expressed the *esp* gene. Of *E. faecalis* strains, 25.3% (37/146) produced biofilm, 23.3% (34/146) showed the presence of *gel E,* and 22.6% (33/146) only expressed *esp*. *E. faecium* 21.2% (25/118) produced biofilm among which 17.9% (21/118 each) expressed both *gel E* and *esp* genes. 9.5% (9/95) *E. gallinarum* strains that produced biofilm also expressed *gel E* along with some additional strains accounting 11.6% (11/95). However, these strains lacked the *esp* gene. An additional 24 strains that included *Enterococcus mundtii* 15.2% (5/33), followed by *Enterococcus raffinosus* 11.4% (4/35), *Enterococcus solitaries* 33.3% (3/9), *Enterococcus malodoratus* 10.0% (2/20), *E. dispar* 20.0% (2/10), and *Enterococcus hirae* 5.9% (1/17) also expressed the gel *E* gene [Table 6].

Correlation between the virulence markers and virulence genes as detected by phenotypic and genotypic tests, for clinical and fecal VRE is shown in Table 7. It was seen that those strains that were positive for the production of *gel E* and hemolysin also showed the presence of the corresponding genes by multiplex PCR for both clinical and fecal strains. However, the number of strains positive for *gel E* genes by PCR was more than the number of strains showing positive reaction by phenotypic tests [Table 7]. As far as the production of biofilm (adhesin molecule) and slime layer was concerned, the number of strains positive

Table 5: Distribution of virulence genes in fecal vancomycin-resistant enterococci and vancomycin-sensitive enterococci

Type of isolates	*gel E* (%)	*esp* (%)	*cyl A* (%)	*hyl* (%)	*asa1* (%)
Fecal VRE					
Enterococcus fecalis, *n*=12	4 (33.3)	4 (33.3)	1 (8.3)	1 (8.3)	2 (16.7)
Enterococcus faecium, *n*=8	3 (37.5)	3 (37.5)	1 (12.5)	1 (12.5)	3 (37.5)
Enterococcus gallinarum, *n*=4	0	0	0	1 (25.0)	1 (25.0)
Total=24	7 (29.1)	7 (29.2)	2 (8.3)	3 (12.5)	6 (25.0)
Fecal VSE					
Enterococcus gallinarum, *n*=72	7 (9.7)	0	4 (5.6)	8 (11.1)	4 (5.6)
Enterococcus fecalis, *n*=70	9 (12.9)	8 (11.4)	7 (10.0)	10 (15.3)	6 (8.6)
Enterococcus faecium, *n*=50	5 (10.0)	3 (6.0)	6 (12.0)	6 (12.0)	2 (5.0)
Enterococcus raffinosus, *n*=7	1 (1.4)	0	2 (28.6)	3 (42.9)	0
Enterococcus hirae, *n*=17	2 (11.8)	0	0	1 (5.9)	0
Enterococcus dispar, *n*=10	0	0	0	0	0
Total=226	25 (11.1)	18 (7.9)	19 (8.4)	28 (12.4)	12 (5.3)

gel E: Gelatinase; *asa1*: Aggregation substance; *esp*: Enterococcal surface protein; *cyl A*: Cytolysin A; VSE: Vancomycin-sensitive enterococci; VRE: Vancomycin-resistant enterococci

Table 6: Correlation between biofilm formation and the presence of *esp* and *gel E* genes in all enterococcus species (clinical and fecal)

Enterococcus species	Biofilm formation (%)	Strains expressing *esp* gene (%)	Strains expressing gel *E* gene (%)
Enterococcus fecalis, *n*=146	37 (25.3)	33 (22.6)	34 (23.3)
Enterococcus faecium, *n*=118	25 (21.2)	21 (17.9)	21 (17.9)
Enterococcus gallinarum, *n*=95	9 (9.5)	0 (0)	11 (11.6)
Enterococcus mundtii, *n*=33	0	0	5 (15.2)
Enterococcus raffinosus, *n*=35	0	0	4 (11.4)
Enterococcus solitaries, *n*=9	0	0	3 (33.3)
Enterococcus malodoratus, *n*=20	0	0	2 (10.0)
Enterococcus dispar, *n*=10	0	0	2 (20.0)
Enterococcus hirae, *n*=17	0	0	1 (5.9)
Enterococcus durans, *n*=17	0	0	0
Total=500	71 (15.2)	54 (10.8)	83 (16.6)

esp: *Enterococcal surface protein*; *gel E*: *Gelatinase*

Table 7: Correlation between virulence markers and virulence genes in clinical and fecal vancomycin-resistant enterococci

Virulence factors/ genes	Number of strains positive for the production of virulence markers by phenotypic tests in clinical VRE, (*n* = 36) (%)	Number of strains positive for virulence genes by multiplex PCR in clinical VRE, (n=36) (%)	Number of strains positive for the production of virulence markers by phenotypic tests in fecal VRE, (*n* = 24) (%)	Number of strains positive for virulence genes by multiplex PCR in fecal VRE, (*n* = 24) (%)
gel E	14 (38.8)	16 (44.4)	6 (25.0)	7 (29.2)
Slime layer/*asa1*	10 (27.8)	11 (30.5)	9 (37.5)	6 (25.0)
Adhesin molecule/*asa1*	22 (61.1)	11 (30.6)	7 (29.2)	6 (25.0)
Adhesin molecule/*esp*	22 (61.1)	10 (27.8)	7 (29.2)	7 (29.2)
Slime layer/*esp*	10 (27.8)	10 (27.8)	9 (37.5)	7 (29.2)
Hemolysin/*cyl A*	9 (25.0)	9 (25.0)	2 (8.3)	2 (29.2)

gel E: Gelatinase; *asa1*: Aggregation substance; *esp*: Enterococcal surface protein; *cyl A*: Cytolysin A; VRE: Vancomycin-resistant enterococci; PCR: Polymerase chain reaction

by phenotypic tests was more than the number of strains showing the presence of the corresponding genes by PCR namely *asa1* and *esp*.

DISCUSSION

Very few reports regarding the distribution of virulence genes in various species of enterococci from clinical samples are available.[23] This study was designed to identify various virulence genes in VRE and VSE as well as to evaluate the correlation between virulence markers and virulence genes both phenotypically and genotypically.

Only *gel E*, *esp,* and *hyl* genes were found to be significantly higher in clinical VRE than VSE. As for fecal isolates, presence of *gel E, esp,* and *asa1* was significantly higher in VRE than VSE. Other studies show that prevalence of *esp* ($P = 0.001$) and *hyl* ($P = 0.04$) genes were significantly higher among VRE isolates (44.4% and 27.7%) than among VSE isolates (16.4% and 8.8%).[24]

Authors reported *esp* of 80.0% (32/40) to be the predominant virulence factor followed by *gel E* of 50.0% (20/40) among VRE. Considering vancomycin resistance as variable, the authors did not find any significant difference in the presence of activity of virulence factors between resistant and susceptible enterococci.[25]

It has been reported that the *esp* gene has been restricted to vancomycin-resistant strains.[26] In contrast, our result showed the presence of *esp* gene in both VSE and VRE strains. The presence of *esp* in isolates susceptible and resistant to different antibiotics indicate that this trait probably emerged prior to the acquisition of resistance not only to vancomycin but also to other antibiotics used in hospital settings.

Of the clinical VRE, all the five virulence genes were detected in *E. faecalis* and *E. faecium*. However, virulence genes namely *asa1* and *esp* were absent in *E. gallinarum*. Until recently, a majority of the infection derived isolates were *E. faecalis* strains and was regarded as the most pathogenic species. It is known that *Enterococcus* possess highly efficient gene transfer mechanism. The virulent genes are associated with highly transmissible plasmids which might have led to the dissemination of virulent genes in less virulent *E. faecium* and *E. gallinarum*.

Of the VSE, all the five different virulence genes were detected in vancomycin-sensitive *E. faecalis* and *E. faecium*. The predominant genes in *E. mundtii, E. raffinosus, E. solitaries,* and *E. gallinarum* were *asa1* being 18.1%, 15.3%, 33.3%, and 28.6% (6/33, 4/28, 3/9, and 4/14), respectively. The higher prevalence of *hyl* gene in *E. faecalis* and *E. faecium* and *asa1* gene in nonfaecalis and nonfaecium strains from our setup depicts that this virulence marker may have permeated more deeply into the species by horizontal transfer and would have acquired it comparatively earlier, thereby enhancing the ability of the organism to cause disease beyond that intrinsic to the species. We observed a considerable number of *E. faecium* strains expressed *hyl* and *asa1* genes as compared to *esp*, *gel E,* and *cyl A* genes. At present, we cannot say with certainty whether, and to what extent, *E. faecium* actually makes *hyl*, and under what conditions this protein may be synthesized or exported. Northern hybridization experiments indicate that the *hyl* open reading frame is transcribed under nonselective growth conditions *in vitro*. Therefore, we have compelling reasons to believe that the protein is synthesized at least under some environmental conditions. *asa* too has an important effect on biofilm formation because this substance promotes the adherence of microorganisms to a surface.[27]

In another study, the distribution of virulence genes in various species was viz: *E. faecalis* 53.0% (35/66), *Enterococcus casseliflavus* 50.0% (2/4), *E. faecium* 40.6% (13/32), and *E. mundtii* 25.0% (1/4). *Enterococcus durans* showed the total absence of all virulene genes. *E. casseliflavus* and *E. mundtii* showed the presence of only two genes - *asa1* and *esp* 25.0% (1/4).[28] The permeation of these virulence genetic characteristics into different species differs according to the setup, patient demographics, and other extrinsic factors. Thus, the nonfaecalis and nonfaecium strains isolated from our study were more virulent as compared to the strains seen in the above study which may be due to the presence of *asa1* in nonfaecalis and nonfaecium strains that promote cell-to-cell contact and help in the transfer of other virulence genes through plasmids.

Of the clinical VRE, 85.7% (12/14) *E. faecalis* produced two virulence genes: 35.7% (5/14) strains showed the concomitant presence of *gel E* and *asa1*. Another 21.4% (3/14) strains expressed *gel E* and *hyl* genes and 28.6% (4/14) strains presented *cyl A* and *esp* genes. Of the 17 VR *E. faecium*, 12 (70.5%) strains were found to produce two genes. The various combination of two virulence genes seen in *E. faecium* were: 35.3% (6/17) *gel E* with *hyl*; 17.6% (3/17), each expressing *cyl A* with *esp,* and *asa1* with *esp*. Contrasting reports were seen in another study, where the most common combination of genes was between *asa1* and *gel E* from infections of hospitalized patients. This suggests that these traits entered the species earlier than did

other toxins such as hemolysin, bacteriocin, and *gel E*. *asa1* is an integral component of the pheromone-responsive plasmid exchange system. Therefore, nosocomial strains of *Enterococcus* species may be those best equipped to participate in genetic exchange and may be selected by the presence of antibiotic resistance determinants on such plasmids.[25]

Of the fecal VRE, the predominant genes in *E. faecalis* were *gel E* (33.3%). The main genes in *E. faecium* were *gel E, esp,* and *asa1* (37.5% each). *E. gallinarum* showed the presence of *hyl* and *asa1* (25.0% each) only. Vancomycin-sensitive *E. faecalis* and *E. faecium* isolated from feces showed the presence of all five virulence genes. The *esp* gene was totally absent in nonfaecalis and nonfaecium strains. Contrasting reports were seen in another study from Portugal where virulence genes were detected only in *E. faecalis* (*gel E* 75.3%, *asa1* 30.1%, and *esp* 4.1%), but rarely found in *E. faecium* and other unusual species.[29]

When correlation between presence of virulence genes and their expression as detected by phenotypic tests was done, it was found that while biofilm production was seen in 61.1% (22/36) of clinical VRE, but the corresponding genes, i.e., *asa1* and *esp* were detected in 30.5% (11/36) and 27.8% (10/36) of strains only. This suggests the presence of other genes (not detected by multiplex PCR in the present study) responsible for the formation of biofilm *in vitro*.

Some authors reported that 74.0% (37/50) *Enterococcus* species were biofilm producers. However, *esp* genes were detected in 76.0% (38/50) and *gel E* gene in 60.0% (30/50) isolates. As with *esp*, *gel E* gene participation in biofilm formation is controversial. Authors say that presence of *gel E* enzyme can affect the virulence and the process of biofilm formation in *Enterococcus* species.[25]

Our study showed that the number of strains that were positive for the production of hemolysin also showed the presence of corresponding gene by multiplex PCR both for clinical and fecal VRE. However, the number of strains positive for this *gel E* gene by PCR was more than the number of strains showing positive reaction by phenotypic tests. In our hands, results obtained by phenotypic tests always revealed a lower percentage of strains that produced *gel E* compared to genotypic characterization. This may be due to the presence of silent genes that are expressed only under *in vivo* conditions or due to the presence of undetected gene mutations.

In another study, it was observed that the *asa1* and *esp* genes were equally identified in phenotypic and genotypic assays. *cyl A* gene (hemolysin) was detected in 82 strains genotypically as compared to only 54 in phenotypic assay. Similarly for *gel E*, *gel E* gene was identified in 83 strains in comparison to 77 phenotypically *gel E* positive strains.[30]

CONCLUSION

Multiplex PCR protocol used in the study for simultaneous detection of five different virulence genes and *van* genes proved to be a reliable and rapid alternative to phenotypic testing and uniplex PCR. The number of strains positive for *gel E* gene by PCR was more than the number of strains showing positive reaction by phenotypic tests, which might be due to the presence of silent genes. Thus, it seems necessary to perform both phenotypic and genotypic assays for better characterization of the strains. Biofilm formation could not be linked to any specific gene. In fact, this phenomenon is multifactorial and depends on a number of genes working together along with extrinsic factors. So far, several other genes or gene sets have been reported as auxiliaries in biofilm formation in enterococcus, which highlights the complexity and the multifactorial nature of this trait. We found the presence of *esp* in the vancomycin-resistant isolates as well as in the sensitive isolates. The prevalence of various virulence genes in nonfaecalis and nonfaecium strains proves widespread dissemination of virulence genes through horizontal gene transfer mechanism among the less virulent species.

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Conflicts of interest

There are no conflicts of interest.

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