Original Research Article

Physicochemical Properties Of Hemagglutinin Molecules In Enterococcus Species Isolated From Clinical And Fecal Samples Of Colonized Patients

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Abstract

Background: The aim of the study was to determine the prevalence of unusual Enterococcus isolates, investigate the physicochemical properties and the hemagglutination activity after various enzyme treatments of hemagglutinin molecules and determine multidrug resistance pattern in clinical and fecal sample. **Materials and Methods:** Enterococci isolated from clinical & fecal samples of colonized patients, were identified to species level. Hemagglutination activities were investigated by HA test after treatment of bacteria with trypsin, protease K and pepsin. MIC was determined and multiplex PCR was used to detect the presence of *van* genes. **Results:** The result of reduced HA activity of enzyme treated clinical strains that were VRE showed that 22.2% trypsin treated and 25.0% each of pepsin & protease treated bacterial cell suspensions agglutinated rabbit RBC. 16.6% trypsin treated and 19.4 % each of pepsin & protease K treated cell suspension agglutinated human "O" whereas 16.6% trypsin treated & 19.4% each of pepsin & protease K treated cell suspension agglutinated human "B" RBCs each. The rate of agglutination with enzyme treated cell suspensions in fecal VRE were 16.6%, followed by 20.8% each with rabbit RBC on trypsin, pepsin and protease K treatment. VR *E. gallinarum* showed 100% resistance to penicillin, ciprofloxacin & imipenem and to high level gentamicin. **Conclusion:** HA activity was more common with rabbit RBC in both clinical and fecal isolates, in VRE as well as VSE. Enzyme treatment of bacterial cell suspension was further found to reduce HA activity. The high antibiotic resistance seen is also suggestive of the possibility of circulation of transposable elements carrying resistant genes among clinical isolates.

Keywords: Hemagglutinationactivity, enzyme treated hemagglutinationactivity, vancomycin resistance, high level aminoglycoside resistance.

Introduction

Studies on adhesive properties of hemagglutinins which help enterococci to adhere to host cell surface may contribute towards understanding how these organisms interact wih host surfaces, as also the mechanism to attach to them[1]. The incidence of non-faecalis and non-faecium enterococci is underestimated because of frequent misidentification. On several instances only one phenotypic character differentiates one species from another, and to further complicate matters, some strains of enterococci do not possess identical phenotypic characters of the type strains, raising confusion over their exact taxonomic status[5]. Most of the laboratories do not perform the fundamental and additional tests to identify enterococci to the species level**.**

The present study attempts to investigates the profile of enterococcus species and to evaluate hemagglutinating activity after enzyme and heat treatment of enterococci isolated from clinical and fecal samples against different human RBC groups including A, B, AB, and O as well as.

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Materials And Methods

Study Population

Samples from patients of both genders of all age groups were included in the present cross- sectional study. 250 randomly selected clinical samples and another 250 strains isolated from colonized patients were included. The study was conducted over a period of two years from May 2019 to April 2021, after obtaining Clearance from Institutional Ethics Committee vide memo no. IEC/IRB No: KMC/IEC/Dept. Res./010/2019-2022 [Microbiology]; dated 20.04.2019

Isolation and Identification

Enterococci were identified using standard methods based on Gram staining, catalase reaction, hydrolysis of bile esculin, growth in 6.5% NaCl, growth at 10^0C and 40^0C, growth at pH 9.6, heat tolerance test, hydrolysis of leucine-beta-napthalamide [LAP] and L-pyrrolidonyl-β-napthalamide [PYR]. Further identification to species level was based on extensive phenotypic tests like carbohydrate fermentation using 1% solution of sugars, such as glucose, lactose, mannitol, sucrose, arabinose, sorbitol, raffinose, trehalose, xylose, melibiose, and glycerol; while, sugars such as sorbose and ribose were added to a final concentration of 1% directly into the broth base after sterilization. This was followed by testing for pigment production, performing motility test, pyruvate utilization test [in 1% pyruvate broth], acidification of methyl-alpha-D-glucopyranoside, Voges-

Proskauer test, arginine decarboxylation test, performing hippurate hydrolysis test, and detection of reduction of potassium tellurite & tetrazolium chloride[2,3,4].Fecal specimens were inoculated on KF-Streptococcus agar supplemented with 0.01% tetrazolium chloride by intermittent streaking process[2,3,4].

Detection of biofilm formation

The test strains were grown overnight at 37ºC in BHIB [HiMedia, Mumbai, India] with 0.25% glucose. Culture was diluted 1:20 in the same media. 200µl of this suspension was used to inoculate 96 well sterile polystyrene microtitre plates. After 24 hour at 37ºC of static incubation, wells were washed with PBS, dried in inverted position and stained with 1% crystal violet for 15 min. The wells were rinsed once more and solubilized in 200 µl ethanol/acetone [80:20 v/v]. The A_{630} was determined using microtitre plate reader. Biofilm formation was scored as non biofilm forming [-], weak [+], moderate [++], strong[+++] corresponding to the A_{630} values ≤1, 1- ≤2, 2- ≤ 3 and > 3 respectively[5].

Effect of enzyme and heat treatment of bacterial cells via Hemagglutination [HA] test

The effects of physicochemical agents on hemagglutination test were investigated by performing HA test after treatment of the bacteria with trypsin, protease K and pepsin. Bacterial suspensions of test strains were centrifuged and the deposit was added to separate test-tubes containing trypsin [1µgm/ml], pepsin [1µgm/ml] and protease K [1µgm/ml] in phosphate buffered saline [PBS]. The test-tubes were incubated at 37ºC for 60 minutes. For heat treatment bacterial suspensions were heated at 50ºC 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 seconds and results were recorded as either strong agglutination [+++], agglutination [++] or no agglutination[6,7].

Antimicrobial susceptibility testing

Antibiotic susceptibility test was done by Kirby-Bauer disc diffusion method on Mueller-Hinton agar. The inoculum was adjusted to 0.5 McFarland's turbidity standard. Antibiotic disc supplied by HiMedia Laboratories; Mumbai was used for the study[8]. When testing vancomycin against enterococci, plates were incubated for full 24 hours for accurate detection of resistance.

HLAR detection in enterococci by disc-diffusion [DDM] and agar screen method [ASM]:

High level [120µg] gentamicin and streptomycin [300µg] disks were used in disc-diffusion method. In ASM, BHIA was supplemented with 500µg/ml gentamicin and 2000µg/ml of streptomycin. Interpretation was done as per CLSI guidelines[8,9].

Minimum Inhibitory Concentration [MIC] determination by agar dilution method [ADM]

MIC was determined by ADM for vancomycin [0.125-64µg/ml] and gentamicin [8-2048µg/ml] in two fold serial dilutions. Enterococci which had MIC≥32µg/ml and ≥1024µg/ml to vancomycin and gentamicin, respectively were considered resistant. The following quality control strains were used: *Enterococcus facealis* ATCC 51299 [resistant], *Enterococcus faecalis* ATCC 29212 [sensitive][8,9].

DNA extraction method

Conventional phenol-chloroform DNA extraction method was used. PCR amplification was done using Genomic DNA as template[10]. Oligonucleotide primers were obtained from MerckSpecialities, Lucknow, India. The primers were used for amplification of the 1030bp of the van A gene and the 433bp of the van B geneas described by earlier workers[10]

PCR assay for van A and, Band virulent genes

1.5% agarose gelwas used for electrophoresis of the amplified products. A 100bp DNA ladder marker was included as the standard molecular weight marker. The electrophoresed gel was photographed under UV transillumination after treatment with ethidium bromide[10].

Statistical Analysis

Statistical analysis comprised of chi-square test. As is accepted, P<0.05 was considered statistically significant, while P<0.001 was highly significant.

Results

A total of 6024 clinical samples were received in the Departmental laboratory, during the study period, out of which only 2508 showed growth of various microorganisms. Out of the 2508 samples, 2402 showed unimicrobial growth and 106 showed polymicrobial growth. A total of 2614 organisms were isolated from the outdoor and indoor patient departments. Enterococcus species grew in 774 samples, out of which 250 strains were randomly taken up for further study. Out of the 250 isolates, 95.6% [239/250] were isolated in pure culture while the remaining 4.4% [11/250] were in combination with gram negative bacteria, and showed polymicrobial enterococcal infections.

A total 12%[60/500] clinical& fecal strains that were VRE/VIE by MIC test, were found to carry either van A or van B genes.

Table 1 shows the results of various biochemical tests used to differentiate enterococcal isolates. Our test results showed that Enterococcus faecalis [25.6%; 64/250] was the predominant species followed by Enterococcus faecium [24.0%; 60/250]. The biochemical phenotypic results revealed 6 different unusual species viz: Enterococcus mundtii [13.2%; 33/250], followed by Enterococcus raffinosus [11.2%; 28/250], Enterococcus malodoratus [8.0%; 20/250], Enterococcus gallinnarum [7.6%; 19/250], Enterococcus durans [6.8%; 17/250] and Enterococcus solitarius [3.6%; 9/250].

Table 1: Panel of biochemical tests used for their identification of clinical enterococcal isolates and the breakup of their isolation

Enterococcus isolates		*Enterococcus faecalis*	*Enterococcus faecium*	*Enterococcus gallinarum*	*Enterococcus mundtii*	*Enterococcus raffinosus*	*Enterococcus malodoratus*	*Enterococcus solitarius*	*Enterococcus durans*
Acid Production	Glucose	100	100	100	100	100	100	100	100
	Mannitol	100	97	100	100	100	100	40	0
	Sucrose	100	99	100	100	100	100	90	0
	Arabinose	0	100	100	100	100	0	10	0
	Melibiose	0	100	90	90	90	90	0	0

	Raffinose	0	0	100	100	100	100	0	0
	Ribose	0	0	100	90	90	90	0	0
	Sorbitol	98	47	0	70	100	100	10	0
	Glycerol	89	V*	V*	V*	89	V*	89	V*
	Trehalose	100	100	100	0	100	90	90	100
	Xylose	0	0	0	0	10	V*	0	0
	Sorbose	0	0	0	0	0	100	90	0
Arginine decarboxylation			100	100	100	90	0	0	90
Pyruvate utilization			100	0	0	0	40	100	0
Hippurate hydrolysis			100	100	100	100	100	60	V*
Growth tolerance to tellurite			100	0	70	0	0	0	0
Growth tolerance to tetrazolium			100	100	70	0	19	0	0
Motility			0	0	100	0	0	0	0
Pigment production			0	0	0	100	0	0	0

***V =Variable**

Table 2 shows the presence of HLSR, HLGR and HLAR in both VSE and VRE [both clinical and fecal] by HPDDT. For VSE, 32.7% strains showed HLGR and 39.7% strains showed HLSR. 21.4% strains showed high level resistance to both streptomycin and gentamicin. In comparison the number of HLGR strains in case of fecal VSE was 30.08% whereas the a slightly lower number of strains were HLSR 27.4% and HLAR 15.0%.

Among clinical VRE, 36.1% [13/36] strains showed HLGR and 30.5%[11/36] showed HLSR. HLAR was seen in 19.4% [7/36] of the strains. Of the fecal VRE, 29.1% strains were HLGR and HLSR, respectively. HLAR was seen in 12.5% of the isolates[**Table 2]**.

Table 2: HLGR, HLAR & HLSR in VSE/VRE isolated from clinical & fecal strains

Vancomycin sensitive clinical isolates	**No. of resistant strains [%]**		
	HLGR	**HLSR**	**HLAR***
E. faecalis, n=50	22[44.0%]	25[50.0%]	9[18%]
E. faecium, n=43	20[46.5%]	29[67.4%]	11[25.5%]
E. mundtii, n=33	10[30.3%]	12[36.3%]	8[25.2%]
E. raffinosus, n=28	2[25%]	5[17.8%]	6[21.4%]
E. gallinarum, n=14	6[42.8%]	7[50%]	5[35.7%]
E. malodoratus, n=20	7[10%]	2[10%]	3[15%]
E. solitarius, n= 9	2[22.2%]	4[45.4%]	2[22.2%]
E. durans, n=17	1[5.8%]	1[5.8%]	2[11.6%]
Total = 214	70[32.7%]	85[39.7%]	46[21.4%]
Vancomycin sensitive fecal isolates	**No. of resistant strains [%]**		
	HLGR	**HLSR**	**HLAR**
E. faecalis, n =70	19[27.1%]	19[27.1%]	10[15.2%]
E. faecium, n =50	19[38.0%]	19[38.0%]	11[22.0%]
E. gallinarum, n =72	19[26.3%]	19[26.3%]	9[12.5%]
E. raffinosus, n =7	3[42.8%]	1[15.2%]	1[15.2%]
E. hirae, n =17	6[35.2%]	2[11.8%]	3[17.6%]
E. dispar, n =10	2[20%]	2[20%]	0[0%]
Total=226	68[30.08%]	62[27.4%]	34[15.0%]
Vancomycin resistant clinical isolates	**No. of resistant strains [%]**		
	HLGR	**HLSR**	**HLAR**
E. faecalis, n = 14	5[35.7%]	6[42.8%]	3[21.4%]
E. faecium, n =17	7[41.1%]	4[23.5%]	3[20.0%]
E. gallinarum, n= 5	1[20.0%]	1[20%]	1[20.0%]
Total = 36	13[36.1%]	11[30.5%]	7[19.4%]
Vancomycin resistant fecal isolates	**No. of resistant strains [%]**		
	HLGR	**HLSR**	**HLAR**
E. faecalis, n = 12	3[25%]	4[33.3%]	2[16.6%]
E. faecium, n = 8	3[37.5%]	2[25%]	1[12.5%]
E. gallinarum, n =4	1[25%]	1[25%]	0[0%]
Total = 24	7[29.1%]	7[29.1%]	3[12.5%]

***Strains resistant to both antibiotic**

90.9% & 77.2% strains of clinical VS E. faecalis were resistant to ampicillin & penicillin as compared to resistance in E. faecium [ampicillin 75.0% and penicillin 65.0%]. Of the E. gallinarum strains 66.6% showed resistance to ciprofloxacin. Of the unusual enterococcal strains,100% resistance to penicillin was shown by E. mundtii, E. raffinosus, E. solitaries, E. durans and E. malodoratus. On the other hand, out of the 85 HLSR strains retained sensitivity to vancomycin, E. durans showed 100% resistance to ampicillin,penicillin,piperacillin,tetracycline & erythromycin.Out of the VRE complete resistance to high-gentamicin, ampicillin, penicillin, piperacillin, tetracycline, erythromycin was shown by *E. faecalis*. A single strain of VRE *E. gallinarum* was multidrug resistant exhibiting 100% resistance to gentamicin, ampicillin, penicillin, piperacillin, ciprofloxacin and imipenem. Likewise of the VRE that were resistant to streptomycin, 100% resistance to ampicillin, penicillin and imipenem was shown by *E. gallinarum* **[Table 3]**.

Table 3: Resistance pattern of clinical HLGR&HLSR among VSE and VRE strains

Antibiotics	HLGR strains amongst VSE								HLSR strains amongst VSE							
	E. faecalis, n=22	E. faecium n=20	E. gallinarum, n = 6	E. mundtii n=10	E. raffinosus n=2	E. solitarius n=2	E. durans n=1	E. malodoratus n=7	E. faecalis, n=25	E. faecium n=29	E. gallinarum, n = 7	E. mundtii n=12	E. raffinosus n=5	E. solitarius n=4	E. durans n=1	E. malodoratus n=2
Ampicillin	20[90.9%]	15[75.0%]	3[50.0%]	5[50.0%]	2[100%]	0	1[100%]	4[57.1%]	16[64.0%]	19[65.5%]	0	4[33.3%]	5[100%]	2[50.0%]	1[100%]	0
Penicillin	17[77.2%]	13[65.0%]	4[66.6%]	10[100%]	2[100%]	2[100%]	1[100%]	7[100%]	12[48.0%]	19[65.5%]	1[14.2%]	7[58.3%]	2[40.0%]	2[50.0%]	1[100%]	2[100%]
Piperacillin	12[54.5%]	14[70.0%]	1[16.6%]	6[60.0%]	0	0	1[100%]	3[42.8%]	8[32.0%]	10[34.4%]	4[57.1%]	8[66.6%]	5[100%]	2[50.0%]	1[100%]	0
Tetracycline	11[50.0%]	12[60.0%]	2[33.3%]	5[50.0%]	0	0	1[100%]	3[42.8%]	16[64.0%]	16[55.1%]	5[71.4%]	12[100%]	5[100%]	1[25.0%]	1[100%]	2[100%]
Erythromycin	15[68.1%]	14[70.0%]	2[33.3%]	6[60.0%]	2[100%]	0	1[100%]	0	13[52.0%]	10[34.4%]	4[57.1%]	7[58.3%]	3[100%]	0	1[100%]	0
Ciprofloxacin	9[40.9%]	11[55.0%]	4[66.6%]	4[40.0%]	0	0	0	0	12[48.0%]	10[34.4%]	2[28.5%]	0	0	0	0	0
Teicoplanin	2[9.0%]	1[5.0%]	0	0	0	0	0	0	3[12.0%]	2[6.8%]	0	0	0	0	0	0
Vancomycin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Imipenem	8[36.3%]	4[20.0%]	1[16.6%]	0	0	0	0	1[14.2%]	5[20.0%]	3[10.3%]	1[14.2%]	0	0	0	0	1[50.0%]
Linezolid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Antibiotics	HLGR strains amongst VRE			HLSR strains amongst VRE		
	E. faecalis, n= 5	E. faecium, n= 7	E. gallinarum,n =1	E. faecalis, n=6	E. faecium, n=4	E. gallinarum,n=1
Ampicillin	5[100%]	7[100%]	1[100%]	4[66.6%]	2[50.0%]	1[100.0%]
Penicillin	5[100%]	7[100%]	1[100.0%]	6[100%]	4[100%]	1[100.0%]
Piperacillin	5[100%]	5[71.4%]	1[100%]	4[66.6%]	0	0
Tetracycline	5[100%]	5[71.4%]	0	3[50.0%]	3[75.0%]	0
Erythromycin	5[100%]	5[71.4%]	0	4[66.6%]	2[50.0%]	0
Ciprofloxacin	2[40.0%]	5[71.4%]	1[100.0%]	3[50.0%]	1[50.0%]	0
Teicoplanin	2[40.0%]	0	0	0	0	0
Imipenem	3[60.0%]	0	1[100.0%]	3[50.0%]	2[50.0%]	1[100.0%]
Linezolid	0	0	0	0	0	0

Table 4 shows the result of reduced HA activity of enzyme treated bacterial cell suspensions. Trypsin treated vancomycin sensitive bacterial cells agglutinated 17.8% rabbit RBC, followed by 8.4% each of human "O" and human "B" RBCs. However the rate of agglutination with pepsin and protease K treated bacterial cells was much lower as compared to trypsin treated HA test results with various RBCs.

Table 4: Enzyme treated bacterial cells [clinical and fecal VSE &VRE] showing Haemagglutination activity:

Clinical VSE	Hemagglutination test of trypsin treated bacterial cells			Hemagglutination test of pepsin treated bacterial cells			Hemagglutination test of protease treated bacterial cells		
	Rabbit RBC	Human "O" RBC	Human "B" RBC	Rabbit RBC	Human "O" RBC	Human "B" RBC	Rabbit RBC	Human "O" RBC	Human "B" RBC
E. faecalis, n=50	24[48.0%]	10[20.0%]	10[20.0%]	20[40.0%]	17[34.0%]	17[34.0%]	20[40.0%]	17[34.0%]	17[34.0%]
E. faecium, n=43	12[27.9%]	3[6.9%]	3[6.9%]	7[16.3%]	2[4.6%]	2[4.6%]	7[16.3%]	2[16.3%]	2[16.3%]
E. mundtii, n=33	0	0	0	0	0	0	0	0	0

E. raffinosus, n=28	2[7.1%]	3[10.7%]	3[10.7%]	2[7.1%]	1[3.6%]	1[3.6%]	2[7.1%]	1[3.6%]	1[3.6%]
E. solitaries,n=9	0	0	0	0	0	0	0	0	0
E. malodoratus, n=20	0	0	0	0	0	0	0	0	0
E. durans, n=13	0	0	0	0	0	0	0	0	0
E.gallinarum,n=14	0	2[14.3%]	2[14.3%]	1[7.1%]	0	0	1[7.1%]	0	0
TOTAL= 214	**38 [17.8%]**	**18 [8.4%]**	**18 [8.4%]**	**30 [15.0%]**	**20 [9.4%]**	**20 [9.4%]**	**30 [15.0%]**	**20 [9.4%]**	**20 [9.4%]**
Fecal VSE	Rabbit RBC	Human "O"RBC	Human "B"RBC	Rabbit RBC	Human "O"RBC	Human "B"RBC	Rabbit RBC	Human "O"RBC	Human "B"RBC
E. faecalis,n=70	9[12.9%]	5[7.1%]	5[7.1%]	5[7.1%]	5[7.1%]	5[7.1%]	5[7.1%]	5[7.1%]	5[7.1%]
E. faecium,n=50	4[8.0%]	3[6.0%]	3[6.0%]	3[6.0%]	3[6.0%]	3[6.0%]	3[6.0%]	3[6.0%]	3[6.0%]
E.gallinarum, n=72	2[2.8%]	1[1.4%]	1[1.4%]	1[1.4%]	1[1.4%]	1[1.4%]	1[1.4%]	1[1.4%]	1[1.4%]
E. raffinosus,n=7	1[14.3%]	1[14.3%]	1[14.3%]	1[14.3%]	1[14.3%]	1[14.3%]	1[14.3%]	1[14.3%]	1[14.3%]
E. hirae,n=17	0	0	0	0	0	0	0	0	0
E. dispar,n=10	0	0	0	0	0	0	0	0	0
TOTAL=226	**16[7.0%]**	**10[4.4%]**	**10[4.4%]**	**10[4.4%]**	**10[4.4%]**	**10[4.4%]**	**10[4.4%]**	**10[4.4%]**	**10[4.4%]**
Clinical VRE	**Hemagglutination test of trypsin treated bacterial cells**			**Hemagglutination test of pepsin treated bacterial cells**			**Hemagglutination test of protease treated bacterial cells**		
	Rabbit RBC	Human "O" RBC	Human "B" RBC	Rabbit RBC	Human "O" RBC	Human "B" RBC	Rabbit RBC	Human "O" RBC	Human "B" RBC
E. faecalis, n = 14	3 [21.4%]	3[21.4%]	3[21.4%]	4[28.5%]	4 [28.5%]	4[28.5%]	4[28.5%]	4[28.5%]	4[28.5%]
E. faecium,n=17	3 [17.6%]	3 [17.6%]	3 [17.6%]	4 [23.5%]	3 [17.6%]	3 [17.6%]	4 [23.5%]	3 [17.6%]	3 [17.6%]
E. gallinarum, n=5	2 [40.0%]	0	0	1	0	0	1[20.0%]	0	0
TOTAL= 36	**8[22.2%]**	**6[16.6%]**	**6[16.6%]**	**9[25.0%]**	**7[19.4%]**	**7[19.4% %]**	**9[25.0%]**	**7[19.4%]**	**7[19.4%]**
Fecal VRE	Rabbit RBC	Human "O"RBC	Human "B"RBC	Rabbit RBC	Human "O"RBC	Human "B"RBC	Rabbit RBC	Human "O"RBC	Human "B"RBC
E. faecalis,n=12	2 [16.6%]	3 [25.0%]	3 [25.0%]	3 [25.0%]	2 [16.6%]	2 [16.6%]	3 [25.0%]	2 [16.6%]	2 [16.6%]
E. faecium,n=8	2 [25.0%]	2 [25.0%]	2 [25.0%]	2 [25.0%]	2[25.0%]	2 [25.0%]	2 [25.0%]	2 [25.0%]	2 [25.0%]
E. gallinarum,n=4	0	0	0	0	0	0	0	0	0
TOTAL= 24	**4[16.6%]**	**5[20.8%]**	**5[20.8%]**	**5[20.8%]**	**3[12.5%]**	**3[12.5%]**	**5[20.8%]**	**3[12.5%]**	**3[12.5%]**

The rate of HA activity of enzyme treated bacterial cell suspension was further reduced in case of fecal VSE. Trypsin treated bacterial cell suspension agglutinated 7.0% rabbit RBC followed by 4.4% of human "O" and human "B" RBCs each. The rate of agglutination with pepsin and protease K treated bacterial cells was even much lower as compared to trypsin treated HA test results.

Of the fecal VRE, 20.8% strains agglutinated rabbit RBC after pepsin and protease K treatment whereas 16.6% trypsin treated strains agglutinated rabbit RBC. Cell suspensions treated with 20.8% trypsin, and 12.5% each of pepsin & protease K were found to agglutinate human "O" and human "B" RBCs [**Table 4]**.

According to the result of reduced HA activity of enzyme treated bacterial cell suspensions [including clinical VRE], 22.2% trypsin treated bacterial cell suspensions were found to agglutinate rabbit RBC in comparison to 25.0% each of pepsin and protease treated bacterial cell suspensions that agglutinated rabbit RBC. 16.6% of trypsin treated cell suspension was found to agglutinate human "O" and human "B" RBCs each whereas 19.4% of pepsin and protease K treated cell suspension agglutinated human "O" and human "B" RBCs each. The rate of agglutination with enzyme treated cell suspensions were further reduced in case of fecal VRE being 16.6% on trypsin treatment. [**Table 4]**.

Discussion

Bacterial adherence to host cells appears to be a multifactorial phenomenon involving specific and non-specific interactions. The ability of bacteria to attach to and agglutinate erythrocytes may be used as an *in vitro* model for studying host bacterium interaction and the mechanism of attachment. Few reports are available regarding the use of human RBC for detecting HA, and the studies available do not elaborate the specific group of RBCs used[11].

A total of 2614 strains of various organisms were isolated from both outdoor and indoor patient departments. Enterococcus species grew in 774 samples, out of which 250 strains were randomly taken up for further study. Out of the 250 isolates, 239 [95.6%] were isolated in pure culture while the remaining 11 [4.4%] enterococcal infections were polymicrobial in combination with gram negative bacteria. Usually enterococci are a part of mixed flora commonly found in the gastrointestinal tract and it is difficult to differentiate colonization from true infection. Interactions among various bacteria have been demonstrated, and several studies suggest that enterococci can act synergistically with other intestinal bacteria to enhance the rate of infection.

In our study hemagglutination [HA] activity was more common with rabbit RBC in both clinical and fecal isolates, in VRE as well as VSE. However, none of the fecal isolates agglutinated with sheep RBC. Enzyme treatment of bacterial cell suspension was furthur found to reduce HA activity. Enzyme treatment of bacterial cells with pepsin, trypsin or protease had an inhibitory effect on the hemagglutinating activity of some strains of clinical and fecal VSE/VRE. Of the VSE, trypsin treated bacterial cells agglutinated 17.8% rabbit RBC, followed by 8.4% each of human "O" and human "B" RBCs. However the rate of agglutination of pepsin and protease K treated bacterial cells [which included clinical VSE] was much lower with rabbit RBCs as compared to trypsin treated HA test results. The rate of HA activity of all types of RBCs with three different enzyme treated bacterial cell suspension was further reduced in case of fecal VSE.Of the clinical VRE, only 22.2% trypsin treated bacterial cell suspensions were found to agglutinate rabbit RBC in comparison to 25.0% each of pepsin and protease treated bacterial cell suspensions that agglutinated rabbit RBC. Likewise, 16.6% of trypsin treated cell suspension was found to agglutinate human "O" and human "B" RBCs each whereas 19.4% of pepsin and protease K treated cell suspension agglutinated human "O" and human "B" RBCs each. The rate of agglutination with enzyme treated cell suspensions were further reduced in case of fecal VRE being 16.6% with rabbit RBC on trypsin treatment. 20.0% each of trypsin treated followed by 12.5% each of pepsin and protease K treated cell suspensions were found to agglutinate human "O" and human "B" RBCs. This observation helps us to conclude that enterococcal hemagglutinins were proteins in nature. Similar observation was seen in other studies where trypsin treatment of bacterial cell suspension leads to partial or total loss of HA activity[7].It is evident from our study that all the VR *E. faecalis* and *E. faecium* showed 100% resistance to ampicillin and penicillin. *E. faecium* 71.4% [10/14 each] showed resistance to piperacillin,

tetracycline and erythromycin and ciprofloxacin. Our results were concordant with many Indian studies showing a gradual increase in the resistance rates of penicillins and ampicillin over the years. Multidrug resistance was observed in unusual species besides *E. faecalis* and *E. faecium*. Non complaint medication in the community may predispose for emergence of multidrug resistant pathogens like multi drug resistant enterococci (MDRE)[12,13].

In the present study, of the VSE, *E faecium* exhibited maximum resistance to gentamicin 46.5% [20/43] and streptomycin 67.4% [29/43]. However, combined resistance was highest in *E. gallinarum*, being 35.7% [5/14]. Likewise, of the VRE, 41.1% [7/17] strains of *E. faecium* were gentamicin resistant while streptomycin resistance was maximum in *E. faecalis* 42.8% [6/14]. Again combined resistance was highest in *E. faecium* [20.0%].The reason for higher prevalence could be because of the set up where chronic cases are prevalent and there is a wider usage of broad spectrum antibiotics. More enterococcal strains were resistant individually to streptomycin, than to gentamicin, or to both the drugs at a time. Since enterococcal resistance to gentamicin and streptomycin occurs by different mechanisms and if strains show resistance to both the drugs, then no aminoglycoside combination would prove to be effectively bactericidal.

Conclusion

Our study reveals the prevalence of unusual species of enterococci to be as high as 50.4% in eastern part of Bihar.In conclusion, it can be said that the current study has helped to identify different properties of hemagglutinins in Enterococcus and has made several observations to explain the study outcome. Further studies are required to elaborate upon these explanations.

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