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Original Article

Speciation of enterococcal isolates and antibiotic susceptibility test including high level aminoglycoside resistance and minimum inhibitory concentration for vancomycin

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ABSTRACT

Introduction: Enterococci have been considered as relatively low virulence but they are known to cause various clinical infections like urinary tract infections, endocarditis, intra abdominal and pelvic infections. The emergence of vancomycin resistant Enterococci (VRE) in addition to the increasing incidence of high level aminoglycoside resistance (HLAR), presents a serious challenge for clinicians treating the patients with infections due to Enterococci. Methods: A total of 54 Enterococcal isolates from various clinical samples were included and processed according to standard protocol and speciation was based on Facklams conventional method. Antibacterial susceptibility pattern was determined by Kirby Bauer disc diffusion method with recommended drugs including high level aminoglycoside resistance. Minimum inhibitory concentration (MIC) for vancomycin was done by agar dilution method. Results: *E.faecalis* was the predominant species isolated among various clinical samples. Among 25 urinary isolates 24 (96%) were *E.faecalis* and one (4%) was *E.faecium*. Similarly among 9 blood isolates 7 (77.78%) were *E.faecalis* and two (22.22%) were *E.faecium*. Out of 54 strains, 46.29% of isolates were resistant to ciprofloxacin, 31.48% for ampicillin and 29.62% for penicillin. E.faecium showed more resistance than *E.faecalis* for gentamicin and *E.faecalis* showed more resistance than *E.faecium* for streptomycin. All strains were susceptible to vancomycin and MIC between1 to4 µg/ml. Conclusion: *E.faecal* is most common than E.faecium. Enterococcus species were susceptible to vancomycin with MIC ≤ 4 µg/ml. Key Messages: Among the Enterococcus species, *E.faecalis* is the most common species, Maximum isolates were obtained from urine samples, and all the clinical isolates were susceptible to vancomycin.

1. Introduction

Enterococci are the most common aerobic and facultative anaerobic, gram positive cocci [1]. Enterococci have been considered as relatively low virulence but they are known to cause various clinical infections like urinary tract infections, endocarditis, intra abdominal and pelvic infections [2]. Enterococcus *faecalis* and Enterococcus *faecium* are most common species, both accounts up to 90% of clinical isolates [1]. Enterococci have emerged as an important cause of nosocomial infections and antibiotic resistance among Enterococci is a major obstacle for treatment [3]. The high level resistance to animoglycosides has made the therapeutic combination of penicillin and gentamicin ineffective [4]. The emergence of vancomycin resistant Enterococci (VRE) in addition to the increasing incidence of high level aminoglycoside resistance (HLAR), presents a serious challenge for clinicians treating the patients with infections due to Enterococci [5]. Resistance to glycopeptide antibiotics has been transferred between Enterococcus species and from Enterococci to other gram-positive organisms, including Staphylococci, Streptococci and Listeria by exchange of resistance encoding genes by conjugation [6]. Resistance to vancomycin and the emergence of VRE need to be carefully monitored especially in tertiary care hospitals [6-7].

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2.Materials and Methods

Objectives to speciate the Enterococcal isolates from various clinical samples. To determine antimicrobial susceptibility pattern of Enterococcal isolates by Kirby Bauer disc diffusion method. To study the high level aminoglycoside resistance (HLAR) among Enterococcal isolates by Kirby Bauer disc diffusion method. Minimum Inhibitory Concentration (MIC) of Vancomycin among the isolated strains of Enterococci by agar dilution method.

The present study was carried out in a tertiary care hospital at Belgaum. A total of 54 Entrococcal isolates were included in the study over a period of one year.

The genus Enterococcus was confirmed by Grams stain, catalase test, Bile - Esculin hydrolosis (Fig-1), Heat tolerance 60^{0}C for 30 minutes in water bath and salt tolerance (6.5% NaCl) 1,3,4,6 ,7. Speciation was based on Facklams conventional method [8] (Fig-2) and Potassium tellurite (0.04%) reduction for E.faecalis, fermentation of arabinose, mannitol and sorbitol for E.faecium, [1,3,5] Deamination of arginine tested in Mollers decarboxylation broth and Voges Proskauer test (Coblentz method) [6]. Motility hanging drop, pigment production was observed after over night growth on tryptic soya agar [9]. Antimicrobial susceptibility to ampicillin, penicillin, vancomycin, teicoplanin, linezolid, ciprofloxacin and nitrofurantion was determined by Kirby Bauer disk diffusion [10]. High level amninoglycoside resistance of gentamicin (120µg) and streptomycin (300µg) by Kirby Bauer disk diffusion method. MIC of vancomycin was determined for entrococcal isolates by agar dilution method (Fig-3). Minimum Inhibitory Concentration (MIC) by agar dilution method is described under following headings [11-14].

Figure 1. Bile Esculin hydrolysis

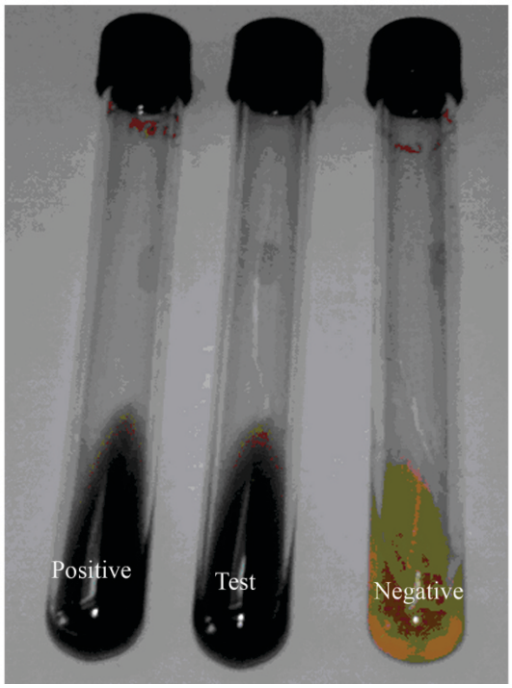
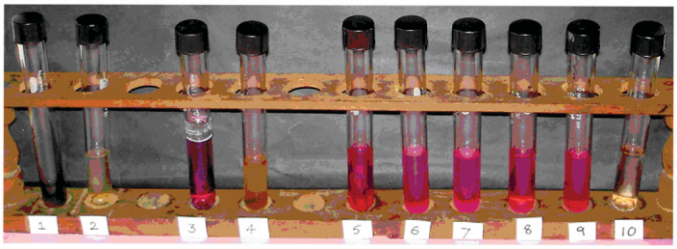


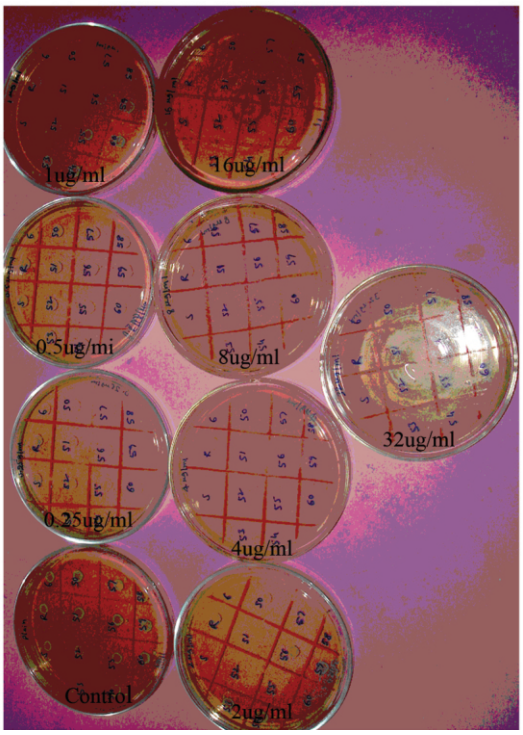
Figure 2. Biochemical tests for *E.faecalis*



1.Bile Esculin hydrolysis +

2.Salt tolerance +, 3.Voges Proskaur _. 4.Sugar fermentation (Glucose +, Lactose +, Sucrose +, Mannitol +, Sorbitol +, Arabinose _) from left to right.

Figure 3. MIC for Vancomycin by agar dilution method



Procedure: 1) Antibiotic dilution.
2) Preparation of inoculums.

1) Antibiotic dilution.

a) Dilution range made according National Committee for Clinical Laboratory Standards (NCCLS) guide lines, take two dilutions above and below the decided range.

For Vancomycin to Enterococci (1- 128 µg/ml).

Sensitive ≤ 4 µg/ml, Intermediate 8-16µg/ml, Resistant ≥ 32µg/ml. 18 ml of Mueller Hinton agar with 2ml of antibiotic. 1 ml should contain antibiotic for 10ml of media highest concentration, so needed is 128 µg/ml. Thus for 10ml it is 1280 µg/10ml.

b) Calculation of Stock Solution

$$\text{Volume (ml)} = \frac{\text{wt of antibiotic (mg) x Potency } (\frac{\mu g}{mg})}{\text{concentration } (\frac{\mu g}{ml})}$$

for 20ml, stock solution, $20 = \frac{x \text{ mg x } 1000}{1280}$

x=25.6mg

So, 25.6 mg of vancomycin was dissolved in 20ml of distilled water. Stock solution can be kept 8^0C for one week and pure drug in deep freezer at - 20^0C.

c) Do serial double dilution with 2ml volume. In this study dilution range (32µg/ml→ 0.25µg/ml) Then cool the Mueller Hinton agar to 45 to 50^0C after autoclave (121^0C 15lb for 15 minutes) Add 18ml of Mueller Hinton agar for every dilution, mix and pour in respective Petri dishes. All the plates along with control plate are incubated at 35^0C for 18hrs to check sterility.

2) Preparation of inoculums:
Inoculate 5-6 colonies into 3ml of peptone water; incubate for 3 hours adjust the turbidity to 0.5 Mc Farland standards ($1.5x10^8$ CFU/ml).

Later 1:10, 1:100 dilutions to get 10^6 CFU/ml, finally 10µl of diluted growth carries 10^4 CFU per spot inoculation to respective plates. Inoculated all test strains with susceptible and resistant strains into various concentration of agar plates and drug free (control) plate. Incubate at 37^0C for 18-20 hours.

Vancomycin susceptible →E.faecalis ATCC 29212
Vancomycin resistant →E.faecalis ATCC 51299.

Reporting
1. Confirm all the test strains along with control strains have grown on control plate (plate without the drug)
2. Are controls within the normal range? If above points are confirmed then only reporting to be done and MIC is validated.

Reporting of MIC:

The lowest concentration of drug which could inhibit the growth of the strain is taken as minimum inhibitory concentration of the drug for the strain.

Reading plates:
a. Examine control quadrant for adequate growth. If growth is poor or absent (eg a few isolated colonies or a faint haze), test is uninterruptable.
b. Examine drug quadrants for absence or presence of growth (consider any growth is significant)
c. If growth is equivocal (cannot determine if colonies are present or not) Incubate plates for an additional 24 hrs before determining final results.

3. Results

The total of 54 Enterococcus species were isolated from various clinical samples and 46 isolates were pure culture of Enterococci, where as remaining Eight isolates were associated with Staphyloccus *aureus* (2), Pseudomonas *aeruginosa* (3) and Escherichia coli (3).

Among Enterococcal isolates *E.faecalis* was the predominant species (51) followed *E.faecium* (3).

Among 25 urinary isolates 24 (96%) were E.*faecalis* and one (4%) was E.*faecium*. Similarly among 9 blood isolates 7 (77.78%) were E.*faecalis* and two (22.22%) were E.*faecium*. Among 51 E.*faecalis* isolates, 27 (52.94%) were sensitive to ampicillin, 28(54.90%) to penicillin and ciprofloxacin. 51 (100%) sensitive to vancomycin, teicoplanin and linezolid. 24(47.05%) were resistant to ampicillin, 23 (45.09%) were resistant to pencillin and ciprofloxacin. Among three E.*faecium* isolates, two (66.67%) isolates were sensitive to ampicillin, one (33.33%) for penicillin and ciprofloxacin. Three (100%) isolates were sensitive to vancomycin, teicoplanin and linezolid. One (33.33%) isolate was resistant to ampicillin, two (66.67%) for penicillin and ciprofloxacin.

22 isolates of E.*faecalis* showed high level resistance to gentamicin (43.14%) and two isolates of E.*faecium* showed resistance to gentamicin (66.67%). Similarly, high level resistance to streptomycin was observed among 21 isolates (41.17%) and one isolate (33.33%) of E.*faecalis* and E.*faecium* strains respectively. All our isolates have MIC ranged between 1to 4 µg/ml for vancomycin.

4.Discussion

Enterococci are considered as a part of the normal flora of gastrointestinal and genitaltract of human. These are relatively low virulence organism but can cause urinary tract infections, wound infections, intra abdominal infections, bacteremia, septicemia and endocarditis particularly in hospitalized patients [15]. Species identification of Enterococci has gained importance in the last decade. Enterococcus species have ability to acquire new antibiotic resistance determinants including vancomycin resistance [16]. *E.faecalis* is the predominant species followed by *E.faecium* in various studies conducted [17-20].

In our study *E.faecalis* was predominant species followed by *E.faecium* and this study is similar to previous studies as mentioned above. Next common species *E.gallinarum, E.casseliflavus, E.durans, E.hirae, E.mundtii* and *E.raffinosu*s were isolated in different studies [21-23], in our study no other species have been isolated. In most of the studies on Enterococcus, the maximum number of isolates was from urine [18,21-23]. In our study also most of the isolates (46.29%) are from urine (Table-1). The bladder, prostate and kidney are commonly infected by Enterococci, especially in patients with structural abnormalities of the urinary tract, indwelling catheters or following instrumentation.

Table-1. Enterococcus species among various clinical samples

Sample	Number	Species		Percentage
		E.faecalis	*E.faecium*	
Urine	25	24	01	03
Exudates				
a)Pus	14	14	–	25.93
b)Endotracheal tube tip	03	03	–	5.56
c) Foley's catheter tip	01	01	–	1.85
Blood	09	07	02	16.67
Body fluids	02	02	–	3.7
Total	54	51	03	100

More than 50% of *E.faecalis* isolates were resistant to ampicillin and ciprofloxacin, 1.9% of isolates for vancomycin and teicoplanin by Kirby Bauer disc diffusion method [22]. In another study the 40% of *E.faecalis* isolates were resistant to ampicillin, penicillin and ciprofloxacin [23]. In our study more than 50% of *E.faecalis* isolates were sensitive to ampicillin, penicillin and ciprofloxacin (Table -2).

Table-2. Antibiotic susceptibility pattern of *E.faecalis*

Antibiotic	Sensitive		Resistant	
	No.	%	No.	%
Ampicillin (10µg)	27	52.94	24	47.05
Penicillin (10U)	28	54.90	23	45.09
Ciprofloxacin (5µg)	28	54.90	23	45.09
Nitrofurantoin (30µg)	24	100	–	-
Vancomycin (30µg)	51	100	–	-
Teicoplanin (30µg)	51	100	–	-
Linezolid (30µg)	51	100	--	-

Nitrofurantoin was used for urinary isolates. Urinary isolates – 24

Emergence of vancomycin resistant strains 5% in one study [2] and another study resistance was 1.9% of strains for vancomycin and teicoplanin [22]. No emergence of vancomycin and teicoplanin resistant strains in recent study [23]. In present study also all strains are susceptible to vancomycin and teicoplanin.

About 40% of *E.faecium* isolates were resistant to ampicillin and ciprofloxacin, 12.5% of *E.faecium* isolates were resistant to vancomycin and teicoplanin by Kirby Bauer disc diffusion method [22]. In another study more than 50% of *E.faecium* isolates were resistant to ampicillin and ciprofloxacin and more than 60% for penicillin [23]. In our study, most of the *E.faecium* isolates 70% were sensitive to ampicillin, more than 60% of *E.faecium* isolates were resistant to penicillin and ciprofloxacin (Table-3) which is similar to above mentioned studies [22,23].

Table-3: Antibiotic susceptibility pattern of *E.faecium*

Antibiotic	Sensitive		Resistant	
	No.	%	No.	%
Ampicillin (10µg)	02	66.67	01	33.33
Penicillin (10U)	01	33.33	02	66.67
Ciprofloxacin (5µg)	01	33.33	02	66.67
Nitrofurantoin (30µg)	01	100	–	-
Vancomycin (30µg)	03	100	–	-
Teicoplanin (30µg)	03	100	–	-
Linezolid (30µg)	03	100	--	-

Nitrofurantoin was used for urinary isolate. Urinary isolate – 01.

Emergence of vancomycin resistant *E.faecium* strains 2.2% in one study [19] and 12.5% in another study [22]. Emergence of teicoplanin resistant *E.faecium* strains 12.5% [22]. In recent study vancomycin and teicoplanin resistant strains were not emerged [23]. In our study also *E.faecium* strains were susceptible to vancomycin and teicoplanin.

High level aminoglycoside resistance (HLAR) pattern of Enterococcus species by Kirby Bauer disc diffusion method is seen in our study (Table-4). This correlates to the observation of many similar type of studies [21,23]. In our study among *E.faecalis* isolates resistant pattern was more for gentamicin than streptomycin, which is similar to previous studies [21, 23]. Among *E.faecium* isolates resistant pattern was more for gentamicin than streptomycin which is similar to previous study [21] but it was more for streptomycin than gentamicin in another study [23].

Table-4: High level aminoglycoside resistance (HLAR) pattern

Antibiotic (µg)	Species	Number	Sensitive		Resistant		Total
			No.	%	No.	%	No.
Gentamicin (120µg)	*E.faecalis*	51	29	56.86	22	43.14	54
	E.faecium	03	01	33.33	02	66.67	
Streptomycin (300µg)	*E.faecalis*	51	30	58.82	21	41.17	54
	E.faecium	03	02	66.67	01	33.33	

Minimum Inhibitory Concentration (MIC) for vancomycin by agar dilution method has done in our study. Even though there were studies suggestive of arising resistance pattern in Enterococci to vancomycin [2,19,21]. No such pattern is observed in our isolates and isolates have MIC range between 1 to 4µg/ml for vancomycin (Table-5).

Table-5: Minimum Inhibitory Concentration (MIC) for Vancomycin by Agar dilution

Enterococcus species	Number	1µg/ml	2µg/ml	4µg/ml
E.faecalis	51	13	13	25
E.faecium	03	–	02	01
Total	**54**	**13**	**15**	**26**

***Sensitive ≤ 4 µg/ml Intermediate sensitive 8 to 16 µg/ml, Resistant ≥ 32 µg/ml**

5. Conclusion

Among the Enterococcus species, E.*faecalis* is most common than E.*faecium*. Maximum isolates were obtained from urine samples followed by exudates and blood. Antibacterial susceptibility pattern reveals that E.*faecium* is more resistant than E.*faecalis*. HLAR pattern among Enterococcus species, E.*faecium* showed more resistance than E.*faecalis* for gentamicin and E.faecalis showed more resistance than E.*faecium* for streptomycin. All clinical isolates were susceptible to vancomycin with MIC ≤4 µg/ml.

6.References

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