Antibiotic susceptibility pattern of enterococci isolated from clinical samples.

Soamyaa Srivastava*

Department of Microbiology and Fermentation technology, Jocob school of Biotechnology and Bioengineering, Sam higginbottom institute of Agriculture, Technology and Sciences, Allahabad, 211001.

Enterococcus **species are ubiquitous in nature and the primary habitation is considered to be gastrointestinal tract of human and animals. Urine and blood samples were collected from 100 patients showing symptoms of nosocomial infection. Females were found to have significantly elevated incidence of urinary tract infection. The isolates were further identified on the basis of cultural, morphological, biochemical and physiological characteristics which revealed that isolates were *Enterococcus gallinarum,* they showed antibiotic susceptibility pattern towards 14 commercially used antibiotic. The data in the report showed glycopeptide resistance in *E. Gallinarum (10%).* *Enterococcus gallinarum* were found to be moderately resistant to vancomycin. Minimum inhibitory concentration (MIC) Minimum bactericidal concentration (MBC) was carried out to find out the smallest concentration of vancomycin in a series that inhibited the growth of the bacteria. To become VRE they obtain a new DNA in the form of plasmids or transposons which encode genes that confer vancomycin resistance. The fact that these strains are only moderately resistant to vancomycin (MIC, 32µg/ml) suggests that resistance mechanism might not be very efficient but properties of the glycopeptides may play a role in their differential activities. Vancomycin is the least able to reach the target site or that it has lower affinity for its target compared with the other antimicrobial agents.**

[1]Enterococci is a genus of lactic acid bacteria and belong to phylum firmicutes. They are gram -ve bacteria which often occurs in pairs or in short chains. They are facultative anaerobic organism. Enterococci are primarily opportunistic pathogen; certain species of enterococci are common commensal org in the intestines of humans i.e. E. faecalis, E. faecium, E. gallinarium. As we know that Vancomycin resistant enterococci (VRE) has emerged as a nosocomial pathogen and increasing antibiotic resistance of this bacterial pathogen presents a global growing threat to human health. The very first reports of VRE predicted that resistance may arise due to four possible routes those may be (i) inactivation of antibiotic (ii) suequestration of antibiotic in outer cell wall layer by specific and non specific binding (iii) increased production of those intermediates to which vancomycin binds (iv) change in the target site. Risk factors for the development of vancomycin resistant enterococci (VRE) include blood stream infection, cancer,

*Corresponding Author: Soamyaa Srivastava, c/o surendra Prasad, 1810/4, srivastava colony, kajarahwa pokhra, near police lines,mirzapur (U.P) India. Phone: (+91)8853029817. Email: charu_1122@yahoo.co.in.

endocarditis, urinary tract infection, diabetes, wounds, bacteraemia, mucosis, meningitis. Factors of VRE colonization includes host charecteristics as immunosuppression, hospital factors include location of ICU, extended length of stay, use of antimicrobial agent. Various samples that are used for the detection of VRE include pre/post operational wounds, urine samples, peri-anal or peri- rectal samples, stool and blood specimen. Urinary tract infection (UTI) remains a significant cause of morbidity in all age groups. In several population they have high risk of reoccurance and have many predisposing factors like diabetes, weak immune system, pregnancy, catheterization etc. Moreover the pathogens are developing resistance against antibiotics, these are the compelling reasons for the ineffective treatment. Effective treatment guidelines for managing UTI, microbiological detection of uropathogen and study of their antibiotic susceptibility can be of great help in determining risk stratification criteria for predicting treatment failure for nosocomial pathogens. The main objectives include isolation and identification of vancomycin resistant enterococci isolated from clinical samples, to evaluate potential risk factors associated with development of vancomycin resistant enterococci, to determine the antibiotic susceptibility pattern of the VRE, and to determine Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of vancomycin resistant enterococci.

Material and Methods

The study was conducted at the post graduate laboratory of Depertment of Microbiology and fertmentation technology, Jacob school of biotechnology and bioengineering, SHIATS. Total of 100 samples were collected from the infected patients for the detection of VRE colonization with the help of sterile cotton swabs, the samples included urine samples and blood samples. Blood samples were transported by adding anticoagulant agent (1% citrate) and urine samples from infected patients were collected separately in sterilized vials

Isolation of Vancomycin resistant enterococci

Vancomycin resistant enterococci were isolated **(VanHorn *et al.,* 1996).** urine and blood samples were diluted and swabbed or inoculated on the media (M-enterococcus agar or nutrients agar) and was supplemented with 6µg of vancomycin/ml and was allowed to incubate in ambient air at 37°C ± 0.2 for 24 - 48 hours and was further analysed.

Identification of Vancomycin resistant enterococci

Isolate were identified on the basis of cultural, morphological, biochemical and physiological characteristics. ***Cultural characteristics****:* colony colour shape, margin, texture were observed for all the isolates. ***Morphological characteristics****:* suspected colonies were picked from plates and smear preparation was done, further gram staining was carried out and was observed under 100x, purple coloured non motile gram +ve cocci in pairs or in short chains. ***Biochemical characteristics:*** The Vancomycin-Resistant Enterococci was acknowledged with a series of biochemical tests (**Facklam and Collins** (**1988))** aiming to identify the strains on genus and species level. ***Physiological characteristics***: The physiological characteristics included growth at different temperature, pH and salt concentration.

Determination of antibiotic susceptibility pattern

The Kirby-Bauer or disk diffusion tests (Bauer *et a*l., 1966) were used to determine whether the organism is susceptible or resistant to a selection of antimicrobial agents. Mueller Hinton or Nutrient agar plates were used for this test. A nutrient agar plate was poured to a depth of 4 millimetres. After solidifying, the plates were swabbed/inoculated for confluent growth. A variety of antibiotic disks were added, and plates were then incubated for 18 – 20 hours at 37°C. After incubation, there were "bacteria-free" circles of varying sizes around some of the disks

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration is referred as the concentration of the antimicrobial agent that inhibits the growth of microorganism after incubation. A pure culture of the micro-organism was grown in nutrient agar for 24h. Further the antimicrobial agent was diluted a number of times, usually 1:1 through a sterile diluents (nutrient agar). After that a volume of standardised inoculums equal to the volume of diluted antimicrobial agent was added to each dilution vessel. Later the inoculated, serially diluted antimicrobial agent was incubated at an appropriate temperature (35°) for 18 – 20h. After incubation, the series of dilution vessel were observed for the microbial growth which was indicated by the presence of turbidity. As a result in the dilution series the tubes which did not demonstrate the growth of micro-organism corresponds with the MIC of the antimicrobial agents (Hassan *et al*., 2011).

Determination of Minimum Bactericidal Concentration (MBC)

Minimum Bactericidal Concentration is defined as the lowest concentration of antimicrobials that will prevent the growth of an organism after subculture on to antibiotic free media. A pure culture of the micro-organism was diluted in nutrient broth and a stock dilution (1:1) of the antibiotics was made. The antimicrobial dilution was incubated with the organism at 37°C for 18-20h.Turbidity indicated the growth of the organism and MIC was reported. In order to determine MBC, the dilutions representing the MIC and at least two of the more concentrated dilutions were plated and incubated for 24h. After incubation the plates were enumerated to determine viable CFU/ml. As a result MBC was considered to be the lowest concentration that demonstrates a pre-determined reduction (99%) in CFU/ml when compared to the MIC dilutions (Mazurova *et al*., 2007)

Statistical analysis:

Chi square statistical test was used to analyse data. Chi square is the sum of the squared difference between observed (O) and the expected (E), divided by the expected data in all possible categories. The formula for calculating chi square (X^2)

O= observed value
E= expected value

Result

Table 1: Incidence of E. gallinarium in clinical samples

No. of samples examined	No. of samples positive for *E.gallinarium*
100	4

Table 2: Incidence of E.gallinarium in different types of clinical samples

S.No	Type of samples examined	No. of samples positive for *E.gallinarium*
1	Urine samples	3
2	Blood samples	1

Analysis of risk factors associated with incidences of E.gallinarium

Table 1: Incidence of E. gallinarium w.r.t age groups of patients

S.No	Age Groups (years)	No of samples tested	No of +ve samples
1.	**0-30**	12	**2** (16.6%)
2.	**31-60**	8	1(12.5%)
3.	**61-90**	5	1(20%)

X^2 (cal) =17.015>X^2 (tab) =16.268; Significant, ($p<0.05$)

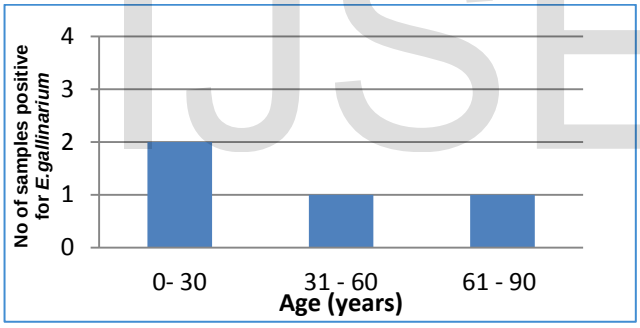


Table 2: Incidence of E. gallinarium sp. w.r.t sex of patients

S.No	Sex	No of samples tested	No of +ve Samples
1.	**Male**	10	1(10%)
2.	**Female**	15	**3**(20.0%)

X^2 (cal) =16.57>X^2 (tab) =16.268 ; Significant, ($p<0.05$)

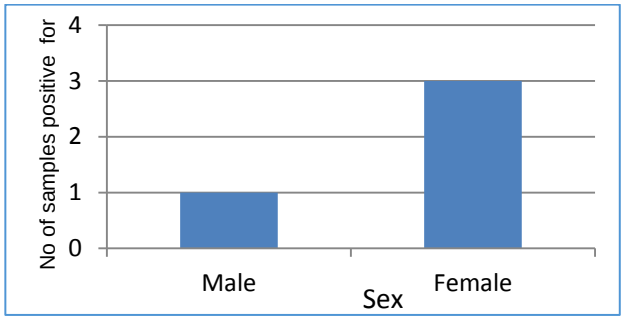


Table 3: Incidence of E. gallinarium w.r.t disease associated with patients

S.No	Disease Associated	No of samples tested	No of samples +ve
1.	Urinary tract infection	15	**3**(20%)
2.	Bacteraemia	7	1(14%)
3.	Diabetes mellitus	3	0

X^2 (cal) =17.64>X^2 (tab) =16.268; Significant, (p<0.05)

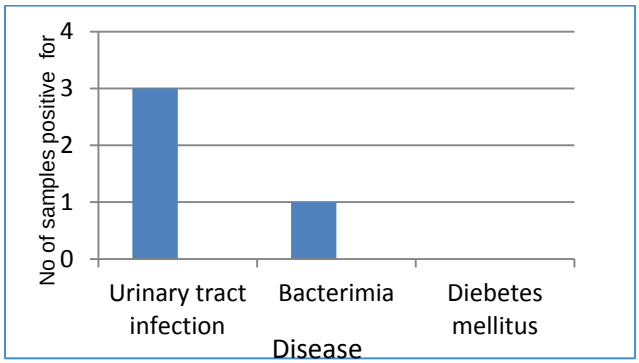
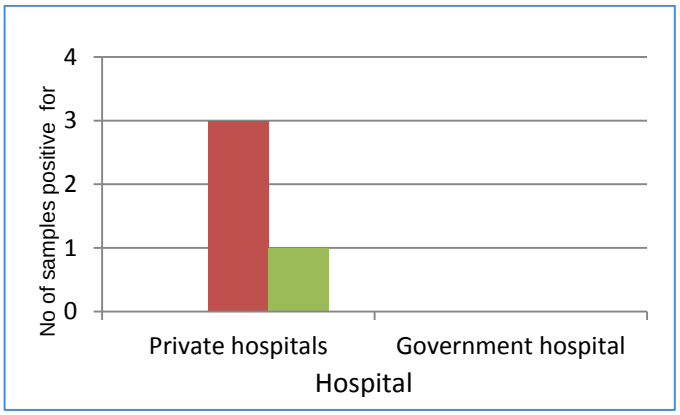


Table 4: Incidence of E. gallinarium w.r.t hospital category of patients

S.No	Hospital category		No of samples tested	No of samples +ve
1.	Private	Hays memorial	2	0
		Jivan Jyoti	20	**3**(20%)
		Swaroop Rani	3	1(14%)
2.	Government	None		

X^2 (cal) =17.8>X^2 (tab) =16.268; Significant, (p<0.05)



Antibiotic susceptibility testing

The antibiotic susceptibility pattern of *Enterococcus gallinarium* from different clinical samples was performed. *E.gallinarium* was found to be susceptible against Imipenum, Ampicilline, Amoxicillin, Tiecoplanine, Gentamicin, Streptomycin, Erythromycin, Methicillin, Tetracycline,

Ciprofloxin, Rifampicine. Intermediate against Vancomycin, Penicillin, and resistant towards Ceftazidime. Among the four isolates of *E.gallinarium*, none of them was reported as multi drug resistant strain (MDR).

Table (3): Antibiotic susceptibility testing

S.No	Antibiotic(s)	Abb	Concentration	Zone of inhibition in (mm)			
			(µg)	Isolate 1	Isolate 2	Isolate 3	Isolate 4
1.	**Vancomycin**	**va**	30	I 12	**I** 10	**I** 12	**I** 10
2.	**Ceftazidime**	**Ca**	30	**R** 0	**R** 0	**R** 0	**R** 0
3.	**Penicillin**	**P**	10	**I** 20	**I** 15	**I** 6	**R** 0
4.	**Imipenum**	**I**	10	**S** 8	**S** 12	**S** 10	**S** 12
5.	**Cefepime**	**C**	30	**S** 10	**S** 15	**S** 12	**S** 10
6.	**Ampicillin**	**Amp**	10	**S** 15	**S** 15	**S** 17	**I** 15
7.	**Methecillin**	**M**	30	**S** 7	**S** 6	**R** 0	**S** 6
8.	**Amoxicillin**	**AM**	15	**S** 20	**S** 20	**S** 20	**S** 20
9.	**Tiecoplanin**	**TIE**	30	**S** 14	**S** 16	**S** 15	**S** 12
10.	**Gentamycin**	**G**	10	**S** 20	**S** 18	**S** 19	**S** 20
11.	**Streptomycin**	**S**	10	**S** 17	**S** 20	**S** 20	**S** 20
12.	**Erythromycin**	**E**	15	**S** 19	**S** 20	**S** 19	**S** 19

13.	**Tetracycline**	**TE**	30	**S** 20	**S** 15	**S** 15	**S** 15
14.	**Ciprofloxin**	**CF**	5	**S** 18	**S** 15	**S** 20	**S** 18
15.	**Rifampicine**	**R**	5	**S** 20	**S** 15	**S** 20	**S** 20

S= Sensitive, I= Intermediate, R= Resistant

Table (4): Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Organism	**MIC and MBC of *E. gallinarium* isolates**		
Enterococcus gallinarium	**Initial MIC at 24h (µg/ml)**	**Final MIC at 48h (µg/ml)**	**MBC (µg/ml)**
E.gal 1	32	32	32
E.gal 2	16	32	32
E.gal 3	32	32	32
E.gal 4	32	32	32

Table (5): Identification of *E. gallinarium*: Morphological, cultural and physiological characteristics.

Entrerococcus gallinarium	**Organism**		
Translucent colonies	**Nutrient agar**	**Growth on**	**Morphological characteristics**
Mauve colour colonies	**M-Enterococcus agar**		
Gram +ve	**Gram reaction**		**Cultural characteristics**
Diplococci	**Shape**		

9.6		**pH**	**Physiological characteristics**
6.5%		**NaCl conc.** (%)	
+ve	10°C	**Growth temp**	
+ve	45°C		

Table (6): Biochemical characteristics of *E.gallinarium* isolates.

S.No	Biochemical test	*E.gallinarium*
1.	Catalase test	-ve
2.	Bile Esculine Hydrolysis	+ve
3.	Mannitol Fermentation	+ve
4.	Pigment production	-ve
5.	Amino Acid Decarboxylation	+ve
6.	Citrate Utilization	+ve
7.	Vogas-Proskaur test	-ve
8.	Gelatinase test	-ve
9.	Oxidase test	+ve
10.	Nitrate Reduction test	-ve
11.	Indole test	-ve
12.	Urease test	-ve
13.	Phenylalanine Deaminase	+ve
14.	Hydrogen Sulphide Production	-ve
15.	Motility agar	+ve
16.	Arginine Dehydrolase	+ve
17.	Leucine Arylamidase	+ve
18	**Carbohydrate Fermentation Test**	
	• Dulcitol	-ve
	• D- Fructose	+ve
	• Galactose	+ve
	• Mannitol	+ve
	• Melibiose	+ve
	• D- Raffinose	+ve
	• Sorbitol	-ve
	• Sucrose	+ve
	• D- Xylose	+ve
	• Rhamnose	-ve

Result and discussions

This study provides antibiotic susceptibility analysis of enterococci isolated from clinical samples. In the present study, the incidence of *E.gallinarium* isolated from urine and blood samples is only 4% where as the reported incidence is 1-5%. The evaluation of different samples (urine and blood samples) for the isolation of *E.gallinarium* reported that blood samples showed only 1% where as urine samples showed 3% incidence. Therefore perhaps the significant route for the development of Vancomycin resistant enterococci is via urine. In the

analysis of risk factors associated with incidences of *E.gallinarium.*For the analysis of age groups. The patients were grouped in 3 groups according to the age. Total of 12 samples were tested from age group in which two were positive for *Enterococcus gallinarium*, 8 and 5 samples were tested for age groups 31-60 and 61- 90 respectively in which 1 from each age groups were positive.In the following study, the analysis of incidence of *E.gallinarium* with respect to age group of patients, the patients were grouped under different age groups(0-30, 31-60, 61-90), In the chi square distribution test for the incidence of *E.gallinarium* w.r.t age group of patients, the calculated value (17.015) was greater than the tabulated value (16.268), thus the data was found to be significant and the null hypothesis was rejected and it was reported that 0-30 years are more likely to develop the VRE infections. Considering the sex of the patients, 10 and 15 samples were collected from male and female respectively among which 1 and 3 were respectively positive for *Enterococcus gallinarium.* In the chi square distribution for the incidence of *E.gallinarium* w.r.t to sex of the patient, the calculated value (16.57) was greater than the tabulated value (16.268), thus the data was found to be significant and the null hypothesis was rejected, in this study the analysis of incidence *of E. gallinarium* with respect to sex of patients showed that as compared to men (10%), women are more likely to be infected with vancomycin resistant enterococci (20%). While analysisng the disease associated with the patients, Patients were categorised according to their associated disease, 15 samples of UTI, 7 of bacteraemia, 3 of diabetes mellitus were tested in which 3, 1 and 0 were respectively positive for *Enterococcus gallinarium*. In the chi square distribution for the incidence of *E.gallinarium* w.r.t to the disease associated with patients, the calculated value (17.64) was greater than the tabulated value (16.268), thus the data was found to be significant and the null hypothesis was rejected. The analysis of incidence of *E.gallinarium* with respect to disease associated with patients, the most significant disease responsible for the development of VRE is perhaps urinary tract infection(UTI), followed by bacterimia. In the study conducted by **Huycke *et al, 2011*** *and* **Zaas *et al, 2011*** the risk factors involved for the development of VRE infections includes blood stream infection, bacterimia, endocarditis, cancer, catheterization, meningitis etc. considering the hospital category of the patients, The hospitals were categorised as private and government, 4 samples from private and none from government hospitals were positive for *Enterococcus gallinarium*. In the chi square distribution test for the incidence of *E.gallinarium* w.r.t to the category of hospitals, the calculated value (17.8) was greater than the tabulated value (16.268), thus the data was found to be significant and the null hypothesis was rejected. In the conducted study of analysis of incidence of E.gallinarium with respect to the hospital category showed that among different private hospitals subjected under study, jivan jyoti (Allahabad) was more prone for the development of VRE. Studies showed that initial and final Minimum inhibitory concentration (MIC) of *E.gallinarium* isolate 1 was 32 µg/ml and 32 µg/ml respectively, *E.gallinarium* isolate 2 was 16 µg/ml and 32 µg/ml respectively, E.gallinarium isolate 3 was 32 µg/ml and 32 µg/ml respectively, E.gallinarium isolate 4 was 32 µg/ml and 32 µg/ml respectively. Minimum bactericidal concentration was 32 µg/ml for all the four isolates.

Conclusions

From the present study, it was concluded that:

- In the isolation of Enterococci, 100 samples of urine and blood were examined, among which 4 samples were positive for *E.gallinarium*, showing 4% incidence. Among the clinical samples of blood (30) and urine (70), blood sample showed 1% incidence where

as urine sample showed 3% incidence. Enterococcoi isolated from urine and blood samples

- *E. gallinarium* was found to be susceptible against Imipenum, Ampicilline, Amoxicillin, Tiecoplanine, Gentamicin, Streptomycin, Erythromycin, Methicillin, Tetracycline, Ciprofloxin, and Rifampicine in different clinical samples. Intermediate or moderate against Vancomycin, Penicillin, and resistant towards Ceftazidime. Among the four isolates of *E.gallinarium*, none of them was reported as multi drug resistant strain (MDR).

- The study of risk factors involved in the development of Vancomycin resistant enterococci with respect to different age groups were reported (0-30 years) , with respect to sex of patients, females were found to be more susceptible to the VRE infection, with respect to disease associated, the significant disease involved were urinary tract infection(UTI), bacteremia, diabetes mellitus, with respect to the location of hospitals, among private hospitals, jivan jyoti showed maximum incidence of *E.gallinarium* infections.

- This Study showed that initial and final Minimum inhibitory concentration (MIC) of *E.gallinarium* was 16-32µg/ml. Minimum bactericidal concentration was 32 µg/ml for all the four isolates of *E.gallinarium.*

. In conclusion to the present investigation, it's concluded that there is 4% incidence of *Enterococcus gallinarium* isolated from clinical samples which showed intermediate or moderate vancomycin resistance and a possible threat towards vancomycin resistance enterococci. Age groups (0-30), sex of the patients (M/F), associated diseases like urinary tract infection, bacteraemia, and more exposure to private hospitals are the potent risk factors towards the increasing threat towards vancomycin resistant enterococci.

The present work was carried out to find the compelling reasons such as the improper use of the antibiotic for the treatment that triggers the development of resistance in enterococci against vancomycin which predicts the most common cause for the treatment failure. Antibiotic susceptibility pattern analysis was of great help in determining the risk and effective treatment guidelines against the pathogen. Thus in order to stop the further development of resistance in *Enterococcus* sp. proper concentration of vancomycin should be admistered which inhibits the bacteria completely or it should be replaced by some newer antibiotics for more effective treatment.

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