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RESEARCH ARTICLE

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EMERGENCE OF VANA AND VANB GENE AMONG VANCOMYCIN-RESISTANT ENTEROCOCCI IN FAECAL SAMPLES FROM ICU PATIENTS.

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Abstract

Background & objectives: Enterococci have emerged as important nosocomial pathogens and appearance of resistance to many of the antimicrobials used for Gram-positive organisms has made the management of infections due to *Enterococcus* species difficult. Resistance to glycopeptide antibiotics, especially vancomycin is of special concern. This study was undertaken to determine the prevalence of stool colonization with vancomycin resistant Enterococcus (VRE) and also evaluate the risk factors for colonization with vancomycin resistant Enterococcus among Hospitalized ICU patients isolates obtained from stool samples at MMIMSR, mullana

Methods: Test was performed for VRE isolates collected over a period of one year (Feb2015- March2016).Total 50 Faecal samples were collected by using rectal swab for hospitalized ICUs patients then subjected to Cultures were done on MacConkey and Blood agar. After presumptive diagnosis as an enterococcus spp. using different biochemical tests. Again cultures on special VRE screen agar media to identify vancomycin resistant Enterococcus. The results were further supported by modified Kirby-bauer disk diffusion method with vancomycin (30µg) as per CLSI guideline. The VRE isolates were analyzed by PCR for vanA and vanB gene.

Results: A total of 34 (68%) *Enterococcus faecalis* and 16 (32%) *Enterococcus faecium* were detected among the faecal isolates and 4 (8%) were VRE. The VRE isolates were multidrug resistant and linezolid resistance was also found to be seen. According to CLSI guideline isolates showing diameter of zone of inhibition ≤16mm were considered among the VRE. The vanB gene was found in one of 4 VRE isolates and three vanA genes were found. All identified VRE patients were in age group within 21-40 years. Prolonged hospital stay (more than 15 days) was found to be significant risk factor for ICU patients.

Interpretation & conclusions: In current practice, vancomycin resistance enterococci are emerging as nosocomial infection

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particularly in ICUs patients because of long time exposure in hospital. The following recommendations should be considered as hygiene maintains is the only way to prevent VRE. Prolonged use of vancomycin drug should not be recommended by the physician. We should take another major precaution including active surveillance cultures when patients admitted to hospital and after weekly culture for VRE till discharge. Future potential assembly studies are needed to comprehend better the epidemiology of VRE broadcast.

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Introduction:-

Enterococci have emerged as an upgrading important cause of nosocomial infections in the last decades. Though, earlier it was a harmless commensal of GIT. But during the past decade, there has been a worldwide trend in increasing occurrence of nosocomial infection (hospital acquired).as well as bacteremia, endocarditis, UTI's, SSI and other device associated infections. Enterococci were reported as the second most common cause of nosocomial infections.[1] Urinary tract infections (UTIs) are most frequent infections caused by enterococci sp. intra abdominal and intra pelvic abscesses or post surgery wound infections have been generally the second most frequent cause of enterococcal infections.[2] Previously vancomycin was the reserved drug for the recent enterococcal infection but the appearance of vancomycin resistance strains has been increasing reported. Those patients are treating with Glycopeptide like antibiotics are more at risk of picking up VRE. The most likely modes of transmission proximity from patient to patient are colonized with VRE especially those with diarrhoea. Either by direct contact through transient carriage of VRE on the hands of personnel or indirectly by infected environmental surfaces and other equipment for patient care. Most infections with these micro-organisms are aspectable to the patient's own flora. VRE are capable of prolonged survival on hands, gloves and environmental surfaces such as over-bed tables and call bells, door handles and stethoscopes.[3] the emergence and spread of vancomycin resistance as well as other glycopeptide agents like teicoplanin among Enterococcus spp. significantly reduces the number of treatment options. In addition, increase of vancomycin-resistant Enterococci represents an immediate threat to public health.[4, 5] the prevalence and incidence of VRE colonisation vary broadly among hospitals and studies have suggested that such VRE rates are elevated among critically ill patients, particularly those admitted to different ICUs, limiting the therapeutic options accessible.[6] The evolutions of multidrug and vancomycin-resistant variants which do not respond to the standard antibiotic regimes are posing a great challenge. *E. Faecium* considered as the source of acquired vancomycin resistance is in the species. Amongst the eight types of acquired vancomycin resistance genotypes vanA-vanN which are known in Enterococci, the worldwide most prevalent genotype is VanA and followed by vanB.[21]VanB type resistance is expressed by resistance to vancomycin and susceptibility to glycopeptides like teicoplanin. This means that the MICs against vancomycin are several dilution steps lower in VanB strains (4–64 mg/L) than vanA (commonly 16–512 mg/L). This may make difficult performance of analytical assays, assessing the resistance of phenotype and predicting the corresponding genotype.[7]. Linezolid, Daptomycin are commonly choice able drug against VRE. Until recent times, the VRE strains were found sensitive to Linezolid. Resistance to Linezolid is gradually developing, pretense several questions on the virulence factors and their survival mechanisms.[8]

Material & Methods:-

This study was carried out in the department of Microbiology, Maharishi Markandeshwar institute of medical science and research (MMIMSR), Mullana, India, from February 2015 to March 2016. All isolates of *Enterococcus* species obtained from faecal samples those were admitted in ICUs. The study protocol was approved by the institutional ethics committee.

Faecal sample were collected by using culture swab then subjected to culture on blood agar or MacConkey agar. On MacConkey agar dark magenta color bacterial colony seen. Identification of enterococcus spp. done by different biochemical reaction like catalase, bile-esculine, PYR test & salt tolerance test. After presumptive diagnosis of *Enterococcus spp*. further strain was cultured on VRE screen agar Media[9] containing 6µg/lit Vancomycin drug.

After incubation of 24 hours at 37°c growth seen on special media. The results were further supported by modified Kirby-bauer disk diffusion method with vancomycin (30µg) as per CLSI guideline. The isolates showing diameter of zone of inhibition ≤16 were considered as vancomycin resistant. The VRE isolates were analyzed by PCR for vanA and vanB gene

Polymerase chain reaction (PCR) for vanA and vanB gene:-
The isolates resistant to vancomycin were taken for plasmid DNA isolation and amplification. A single colony was picked from a freshly streaked blood agar plate and inoculated in 3 ml L-B broth where it was grown at 37°C for 12-16 h with constant shaking. The culture was incubated for 1 h at 37°C with lysozyme (5 mg/ml). The bacterial cells were harvested by centrifugation at 6000 x *g* for 15 min at 4°C. The pellet was taken and resuspendend in tris-acetate-EDTA (TAE) buffer and heated in a heat block at 95°C for 5 min. It was further centrifuged at in 'g' 15000×*g* for 5 min at 4°C and the supernatant containing DNA was used as a source of template for amplification.

The PCR amplification for *vanA* was performed[10] with some modifications. The reaction mixture with a final volume of 50 µl contained 3 µl of purified plasmid DNA, 1 × PCR buffer (20 mM Tris-HCl/50 mM KCl,*p*H 8.4), 7 mM MgCl2, 0.2 mM each deoxynucleoside triphosphate, 0.5 nM each primer, and 2.5 units of Taq polymerase (Qiagen, Mumbai). The PCR conditions
were 95° C for 5 min for the first cycle; 95°C for 1 min, 50°C for 1 min and 720C for 1 min for the next 30 cycles and final extension at 72°C for 10 min.

The *vanA* gene primer sequence (5'-3') used was: forward primer - A1 GGGAAAACGACAA TTGC and reverse primer -A2 GTACAATGCGGCCGTTA[11]. Amplification of gene was carried out by DNA thermal cycler with the help of specific primers for *vanA* gene. *E. faecalis* ATCC 51299 was used as positive control for *vanA* and the negative control consisted of all reagents but no DNA template. PCR product was analyzed by electrophoresis with 1.5 per cent agarose gel and 0.5 mg ethidium bromide added separately. The *vanB* operon encompassing the *vanB* ligase gene was generated with primers 5_-TGCTTCCAATGAGACGGGCG-3_ and 5_-CTTTGTGCCGATGATGCGAT-3_ by PCR with the Expand Long Template PCR system. PCR elongation times and temperatures were adjusted according to the expected size of the product and the nucleotide sequences of the primers, respectively, as recommended by the manufacturer.

The PCR products to be sequenced were purified with a QIAquick PCR purification kit (Qiagen Pty. Ltd.). The sequences of the PCR products were determined by cycle sequencing with the same primers used for the PCR and the ABI Prism BigDye Terminator (version 3.0) Ready Reaction Cycle Sequencing kit (Applied Biosystems) with an ABI Prism 3100 genetic analyzer. The chromatograms were read and the contiguous sequences were constructed with Vector NTI Advance software (version 8.0; Informax Inc., Bethesda, Md.). The sequences were compared for homology with sequences in the GenBank, EMBL, DDBJ, and PDB databases by using the
BLASTN local alignment search tool (1) and the National Center for Biotechnology Information website (www. ncbi.nlm.nih.gov). The sequences of the *vanB* ligase gene were aligned by using the AlignX module of Vector NTI Advance software (version 8.0) and the CLUSTAL W algorithm[12].

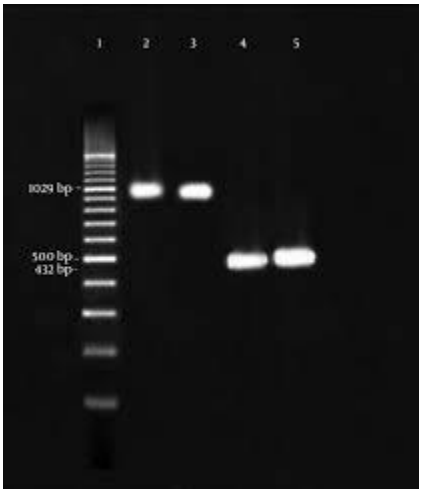

Fig 4:- Agarose gel electrophoresis showing amplification of 432

bp fragment for vanA and 500bp fragment for vanB of VRE from the Faecal samples
. Lanes 1-3, 5-6: VRE strains represents the vanA amplified 732 bp fragment,
Lane 7 - *E. faecium* ATCC 29212 (negative control), Lane 8-100bp ladder marker.

Result:-

A total of 50 isolates of *Enterococcus* species were isolated over the one year period from faecal samples. Out of total only 8% VRE were found. Out of total 34 (68%) *Enterococcus faecalis* and 16 (32%) *Enterococcus faecium* were detected among the faecal isolates of ICU patients and 4 (8%) were VRE. The vanB gene was found in one of 4 VRE isolates and three vanA gene was found by PCR. According to age wise distribution we showed that 8% faecal carriage of VRE isolated within 21-40 year among those 3(6%) were male and 1(2%) was female patient and in distribution of different ICU, 1(2%) VRE were found from MICU out of 11 patients and similarly out of 7 patients 1(2%) VRE were seen from ICCU. Very high significantly associated result found in both MICU [P value= 0.0001*] and ICCU [P value= 0.0076*]. In duration of hospital stay more than 15 days 4(8%) VRE were identified from 36 patients. The association is considered to be very statistically [p value= 0.006*] significant. In case of hospital acquired infection 3(6%) VRE were isolated from 43 patients. According to ward transfer in ICU patients 3(6%) VRE were found among 29 patients. Those were taking invasive procedure in hospitalized patients, 4(8%) VRE were isolated. In the base of continue used of medical equipment, 8% VRE were isolated from 19 patients. This association is considered to be very statistically [P value= 0.008*] significant. Highest number of VRE 8% was isolated from 11 patients specially treated with glycopeptide like drug. It considered to be statistically [P value= 0.017*] significant. Out of 50 patients 15 were using vancomycin, among which 6% VRE were found. It considered to be statistically [P value= 0.041*] significant with VRE.

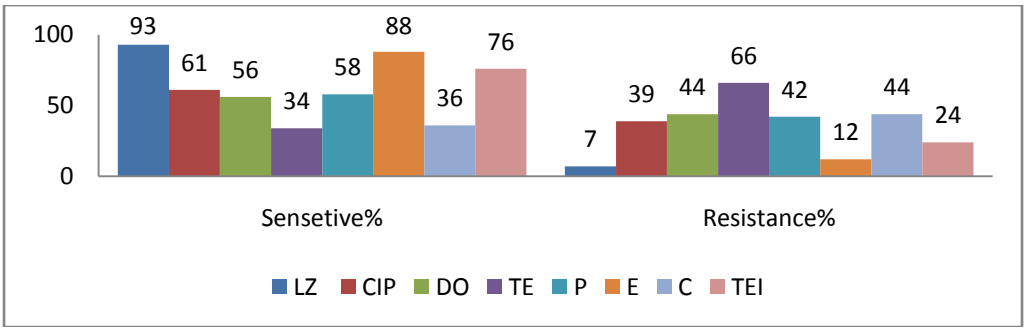

Fig. 1:- Antibiogram sensitivity of VRE isolates. TEI, teicoplanin; LZ, linezolid; CIP, ciprofloxacin; DO, doxycycline; TE, tetracycline; P, penicillin; E, erythromycin; C, chloramphenicol.

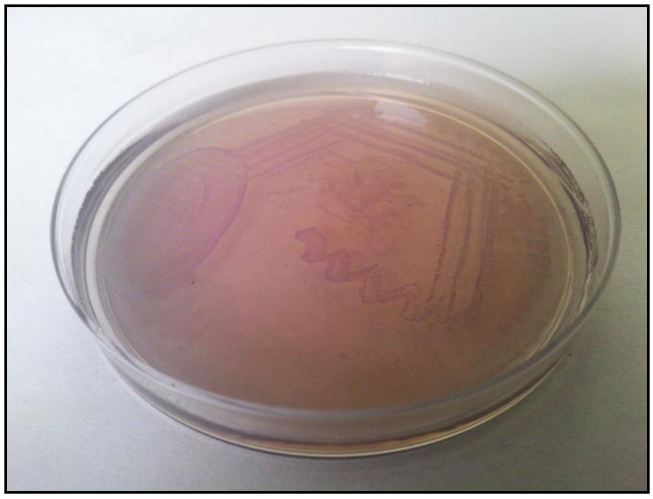

Figure 2:- Enterococcus Growth On Vre Screen Agar Media.

Rate of VRE isolates in faecal sample collected from different ICU.

Type of ICU	MICU	SICU	NICU	PICU	TICU	BICU	ICCU	P Value
No. of Patient	11	5	5	15	4	3	7	
No. of VRE found	1 (2%)	0	0	1 (2%)	0	1 (2%)	1 (2%)	**0.070**

***Significance $P \leq 0.05$**

Rate of VRE isolates in ICU patient in respect to duration of hospital stay

LENGTH OF HOSPITAL STAY	DAYS				P VLUE
	1-5	6-10	11-15	>15	
NO. OF PATIENTS	6(12%)	14(28%)	17(54%)	13(26%)	
NO. OF VRE FOUND	0	0	0	4(8%)	0.006*

***Significance $P \leq 0.05$**

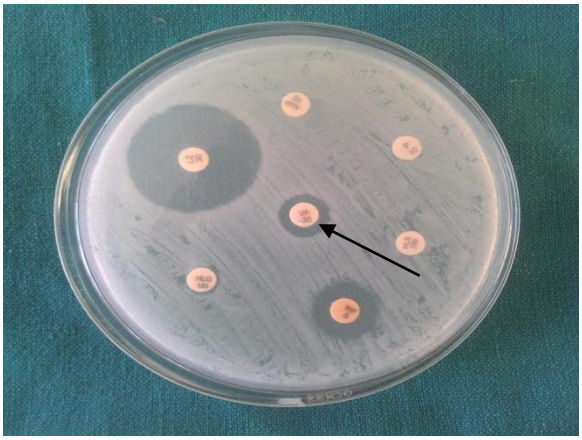

Figure 3:- Kirby-Bauer Disk Diffusion Method Showing Vre.

Risk factors for acquiring Vancomycin resistant Enterococcus among hospitalized patients

Risk Factor	VRE		VSE		TOTAL	P VALUE
	N	%	n	%		
Gender						
male	3	10.3	26	89.6	29	**0.568**
female	1	4.7	20	95.2	21	
Age						
< 10 years	0	00	2	100	2	
10-20 years	0	00	9	100	9	**0.318**
20-40 years	4	16.6	20	83.3	24	
41-60 years	0	00	11	100	11	
>60 years	0	00	4	100	4	
ICU admission						
yes	4	8	46	92	50	**0.000**
no	0	0	00	00	00	
Length of hospital stay						
1-5 days	0	0	6	100	6	
6-10 days	0	0	14	100	14	**0.006***
11-15 days	0	0	17	100	17	
>15 days	4	30.7	9	69.2	13	

Hospital infection						
yes	1	14.2	6	85.7	7	**0.509**
no	3	6.9	40	93.02	43	
Ward transfer						
yes	3	10.3	26	89.6	29	**0.473**
no	1	4.76	20	95.2	21	
Invasive procedure						
yes	4	10.8	33	89.1	37	**0.216**
no	0	00	13	100	13	
Medical equipment						
yes	2	10.5	17	89.4	19	**0.616**
no	2	6.4	29	93.5	31	
Antibiotic consumption						
yes	4	8.0	46	92	50	**0.000**
no	0	00	00	00	00	
Antibiotic type						
Aminoglycoside	0	00	7	100	7	
Cephalosporin	0	00	5	100	5	
Macrolide	0	00	4	100	4	**0.017***
Quinolone	0	00	6	100	6	
Tetracycline	0	00	8	100	8	
Glycopeptide	4	36.3	7	63.6	11	
Penicilin	0	00	9	100	9	
Vancomycin consumption						
yes	3	20.0	12	80.0	15	**0.041***
no	1	2.8	34	97.1	35	

***Significance $P \leq 0.05$**

Discussions:-

The rapid emergence of resistance in Enterococci and the increasing incidence of colonization and infection with VRE have become health care issues that have caused serious concern to physicians and health authorities' alike.[13] The emergence of VRE has reported in an enhance in the incidence of infections that are caused by these organisms and that cannot be treated with currently available antimicrobial agents.[14] Although the prevalence of VRE infections in India is much lower than in the western world, it has been increasing in the past one decade. **Mathur *et al***[15] from New Delhi were the first to report VRE from India in 1999. Another study from north India reported vancomycin resistance in only 1 per cent of the *Enterococcus* species strains.[16] followed by a study from Chandigarh in which 5.5 per cent of 144 *Enterococcus* isolates from urine specimens were identified as VRE.[17] According to our study, vancomycin resistance was found 8 per cent among all isolated *Enterococcus spp*. from faecal samples. During this study it was found high rate of VRE were seen among individuals who were admitted in MICU (2%) and comparable to 7% in ICU[18] and 5.3% VRE in SICU.[19] Another risk factor for VRE nosocomial transmission is prolonged hospitalization, we observed that 8% patients with VRE had hospital stay of more than 15 days. This may be due to in prolonged hospitalization. The patients got frequently with counter with health care worker, medical equipment, common bed pan and other hospital items and this finding in accordance with in Australia.[20] According to current study on VRE associated in hospital infection 2% VRE were isolate from 14% patient those were got hospital infection during hospital stay. No statistically significant result seen in case of hospital infection. However, a much higher frequency has been reported in United States.[21] Patient transfer from one ward to different ward, it might be the possibility of acquisition of VRE. In our findings 3(6%) were isolated as VRE those had been transferred from ICU to different ward and compared to higher percent (83.3) in Gaza.[22] VRE is a nosocomialy acquired pathogen, the persons who have undergone invasive procedure are more liable to attain it. Out of the, 4(8%) hospitalized patient found to have VRE has history of invasive procedure and comparable to 13.6% VRE by ***Tornieporth NG***[23] et al and ***Slaughter S***[24] It was also observed in the present study that out of total hospitalized patients 2(4%) were presumed as VRE from 19(38%) medical equipment used patients. This finding is very similar to ***Wong AH***[25] et al in America and by ***Boyce J***[26] et al. Antibiotic use has been identified as one of the most imperative possibility factors for VRE acquisition. In case control studies, colonisation and infection with VRE have

been associated with exposure to third-generation cephalosporins, antibiotics active against anaerobes, ciprofloxacin, and aminoglycosides.[27, 28, 29] Another risk factor for VRE is the consumption of vancomycin which belongs to the class glycopeptide, similar result have been shown by the present study where all the 4 patients with VRE gave history of consumption of vancomycin. This result was significantly associated (P = 0.017*) with VRE. Similarly comparable to 6. 2% by ***Tacconelli E***[30] in America and ***Assadian O***[31] et al in Iran. The total volume of antibiotic agents and the duration of antibiotic treatment or prophylaxis seem to be important risk factors for the acquisition of VRE.[32,33,34] In this study 3(6%) of patients with VRE were previously exposed to continuous vancomycin use, this results means that vancomycin appear to influence selection for VRE in fecal colonization. This is in agreement with other findings ***Husni R***[35] et al (2002) they concluded that use of oral vancomycin was associated with VRE infection among cancer patient.

Conclusion:-

The prevalence of VRE in faeces of hospitalized patients at MMIMSR was 8%. Faecal colonization, prolonged hospital stay and long time antibiotic treatment has an important role in development of resistant to vancomycin. The following recommendations should be considered as our collective experiment opinion. Hygiene recommendations for VRE must be practical, effective, and feasible, and should take into account current infection epidemiological findings. This is the only way that patients can be guaranteed effective care and safety despite limited resources. Education of Health care workers with implementation and observation of hand-washing practices constitutes a very effective step in preventing the spread. Keeping a check over visitors, regarding their entry or exit in the ICUs further helps in controlling VRE. active surveillance cultures we should take (cultures at hospital admission, weekly cultures, and cultures of high-risk patients) and the subsequent prompt isolation of VRE-positive patients and preventive isolation of high-risk patients. In future prospective group studies are needed to understand better the epidemiology of VRE transmission. In particular, the temporal acquisition of vancomycin resistance in enterococcus in patients after starting antibiotic therapy is still to be defined.

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