ORIGINAL ARTICLE

Detection of Enterococcal asa1 and vanA Genes in Clinical Samples from Adult Immunocompromised Patients

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ABSTRACT

Background: Vancomycin resistant Enterococci (VRE) is important opportunistic nosocomial pathogens due to rapid spread, and limited treatment options. Objectives: The aims of this study were to assess incidence of enterococcal infections and VRE in adult immunocompromised patients and detection of asa1 and vanA genes among these isolates. Methodology: The study included 80 adult immunocompromised patients admitted to Beni-Suef University Hospital. Antimicrobial susceptibility was done by disc diffusion method then MIC was done for vancomycin resistant strains and Enterococcus was considered VRE if the MIC was ≥ 16μg/ml. PCR was done for detection of vanA and asa1 genes. Results: Enterococci were detected in 36 samples, 22.2% of isolates were vancomycin resistant. vanA and asa1 were detected in 33.3% and 63.8% of isolates respectively. VanA gene was detected in 4 (50%) of VRE whereas vanA gene was detected in 4 VSE isolates. Conclusion: Further studies are needed for detection of other virulence and resistance genes and their role in bacterial pathogenicity.

INTRODUCTION

Enterococcus is a genus of widespread facultative anaerobic, non-spore forming Gram-positive cocci arranged singly, in pairs or short chains. In the past, Enterococci were classified as an important member of the genus streptococcus. They are an essential component of alimentary tract microbiota in man and warm-blooded animals. Although considered to be harmless bacterial flora, Enterococci have emerged as one of the leading causes of hospital acquired infections. Enterococci are associated with urinary tract infection, intra-abdominal or pelvic wound infections, bacteraemia, infective endocarditis and rarely meningitis. Enterococcus faecium and Enterococcus faecalis are the species that are incriminated in major part of enterococcal infections.

Several genes encoding for virulence factors have been described in E. faecium and E. faecalis and related to bacterial pathogenicity including asa1, esp, hyl, gelE, and cyl. Aggregation substance (AS) is a surface protein adhesion encoded by asa1 gene and mediates contact of the donor and recipient bacterial cells through binding to Enterococcal binding substance allowing efficient transfer of transmissible conjugative plasmids. Moreover, AS promotes bacterial adhesion to extracellular matrix helping bacterial colonization and also helps Enterococci to resist phagocytosis through inhibition of reactive oxygen species production inside macrophages.

Treatment of enterococcal infections is challenging as Enterococci are inherently resistant to several classes of antimicrobial agents such as cephalosporins, monobactams, sulphonamides and clindamycin. Moreover, overuse of broad spectrums antimicrobial agents in hospital particularly in intensive care units exert a selective pressure on the organism leading to mutation. Enterococcal resistance to vancomycin or teichoplanin is of critical concern due to their importance in treatment of multi-drug resistant strains. The mechanism of enterococcal resistance to vancomycin involves interference with cell wall synthesis by interacting with D-alanyl-D-alamin group of the peptidoglycan chains. There are nine phenotypes of acquired vancomycin resistance in enterococci including Van A, B, C, D, E, G, L, M, and vanV. The most predominant types worldwide are VanA and VanB, in which the genes encoding resistance are associated with mobile genetic elements. vanA display a high degree of resistance to vancomycin and teicoplanin and mainly associated Enterococcus faecium. This study aims to detect incidence of enterococcal infections and VRE in adult immunocompromised patients with detection of vanA and asa1 genes among these enterococcal isolates.

Key words: Immunocompromised; Enterococci; VRE; vanA; asa1

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METHODOLOGY

This study was carried out at Beni-Suef University Hospital over a period of one year from June 2016 to June 2017. The study was conducted on 36 enterococcal isolates collected from a total 80 immunocompromised adult patients admitted into different wards including Intensive Care Unit, Rheumatology Unit and Dialysis Unit. These 80 clinical specimens were as follow: 42 urine, 31 blood and 7 sputum samples. Samples were sent to the Clinical Microbiology Laboratory for further investigations.

The study was approved by Ethics Committee, Faculty of Medicine, Beni-Suef University.

Bacterial identification:

Enterococcal isolates were identified by the standard laboratory methods including colony morphology on blood agar, Gram stained smear, catalase negative test, growth on bile esculin producing blackening of agar (Oxoid Co, England). Every enterococcal sample was collected on broth glycerol in 2 epindorphs and stored at-70°C for subsequent PCR analysis.

Antimicrobial susceptibility testing:

Agar disc diffusion method was performed, and interpreted according to the Clinical Laboratory Standards Institute guideline (CLSI) 11. The following antimicrobials were tested: Ciprofloxacin (5 µg/disk), Levofoxacin (5µg/disc), Erythromycin (15µg/disc), Vancomycin (30mcg/disc), Telcoplanin (30 mcg/disc), Amoxicillin-Clavulanic acid (30µg/disc), Kanamycin, Ofloxacin (5µg/disc), Doxycycline (30 µg/disc) and finally Nitrofurantoin (300 µg/disc) only for urine samples (Oxoid Co. England) 12.

Minimum inhibitory concentration (MIC):

Minimum inhibitory concentration (MIC) for VRE was determined using agar dilution method according to CLSI 2016 using Brain-heart infusion agar (Oxoid Co. England). Enterococci which had MIC value ≥32µg/mL were considered as resistant; MIC value of 8–16µg/mL as intermediately resistant; and MIC of ≤4 µg/mL were considered susceptible to vancomycin. Any Enterococcus was considered VRE if the MIC was ≥ 16µg/ml 13-15.

Conventional Polymerase chain reaction (PCR) for detection of vanA and asa1 genes:

All enterococcal isolates were taken for detection of vanA gene and asa1 gene using PCR in Microbiology Department for Research and postgraduate studies in Faculty of Pharmacy and Biochemistry Department in Faculty of Medicine, Beni-Suef University. DNA templates were prepared by boiling protocol method using heat block (Fisher scientific, England) and purified with phenol chloroform protocol 16. Polymerase chain reaction was performed in a total volume of 25 µl consisting of 1µl of each primer, 12.5 µl Dream Taq Green PCR Master Mix (2X), 250 ng of genomic DNA and then the volume was completed to 25 µl with nuclease-free water. The negative control contained all the reagents except DNA template. PCR amplification for VanA and Asa1 was performed by using the following sets of primers synthesized by Invitrogen Company, UK. Primers for vanA gene Forward 5-GGG AAA ACG ACA ATT GC-3 and Reverse 5-GTA CAA TGC GGC CGT TA-3 (732pb) and for asa1 gene is Forward 5- CAG CGC TAT TAC GAA CTA TGA -3 and for Reverse 5- TAA GAA AGA ACA TCA CCA CGA -3 (375pb) 5,17

Programmable thermal controller PCR machines DNA thermal cycler (Biometa An Analytic Jena Company, Germany and Sensquest labcycler, Germany) were used. Thermal cycler was used under the conditions as shown in table 1 18.

Table 1: Thermal cycler conditions used for amplification of asa1 and van A genes

<table>
<thead>
<tr>
<th></th>
<th>asa1</th>
<th>van A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C for 30 s</td>
<td>95°C for 30 s</td>
</tr>
<tr>
<td>Annealing</td>
<td>49°C for 90 s</td>
<td>50°C for 60 s</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C for 60 s</td>
<td>72°C for 60 s</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C for 5 min</td>
<td>72°C for 5 min</td>
</tr>
</tbody>
</table>

PCR products were analyzed by gel electrophoresis and the amplified genes were visualized by placing on UV transluminator (Whatman, Biometra, Germany) at 220 nl wave length and were photographed directly.

Data management and statistical analysis:

All collected data and results were revised for completeness and consistency. Pre-coded data were entered into the computer using "Microsoft Office Excel Software" program (2010) for windows. Data were then transferred to the Statistical package of Social Science Program (SPSS), Version 22.0 (IBM Corp., New York, USA). All data were expressed by a count of patients and were analyzed using chi-square test at p-values<0.05 to be significant.

RESULTS

This study was conducted on 80 immunocompromised patients admitted to different hospital wards over the period of one year. Enterococci were isolated from 36 (45%) of the total collected 80 samples. Most of our enterococcal infections were detected in patients admitted to ICU department (48.8%) as shown in table (2). The majority of our enterococcal isolates were recovered from urine samples (61.2%) followed by blood (33.3%) and sputum (5.5%).
Among our patients, 25% had liver failure, 17.5% had renal failure, 6.25% had malignancy, and 12.5% had SLE. As regards diabetes mellitus (DM) 42.5% of patients had underlying DM.

Table 2: Frequency of enterococci among immunocompromised patients and distribution of patients in different departments

<table>
<thead>
<tr>
<th>Departments</th>
<th>Number (n=80)</th>
<th>Percentage (%)</th>
<th>Enterococci (N=36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICU</td>
<td>39</td>
<td>48.8%</td>
<td>13</td>
</tr>
<tr>
<td>Internal medicine</td>
<td>16</td>
<td>20%</td>
<td>10</td>
</tr>
<tr>
<td>Oncology</td>
<td>6</td>
<td>7.5%</td>
<td>1</td>
</tr>
<tr>
<td>Rheumatology</td>
<td>7</td>
<td>8.7%</td>
<td>2</td>
</tr>
<tr>
<td>Nephrology</td>
<td>12</td>
<td>15%</td>
<td>10</td>
</tr>
</tbody>
</table>

Frequency of antibiotic resistance among the 36 Enterococcal isolates:

100% of our enterococcal isolates were resistant to Amoxicillin Clavulanic and 72.5% were resistant to Erythromycin. Meanwhile, about 50% of isolates were resistant to Kanamycin, Ciprofloxacin, and Ofloxacin. Resistance to Vancomycin, and Teicoplanin were 22.2%. Figure 1.

Fig. 2: Gel electrophoresis of PCR amplified asa1 gene (378bp) in enterococcal isolates. Lane M was 100bp DNA ladder. Lane 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 14, 15, 17, 18 considered as positive for asa1 gene. Lane 8, 13, 16 considered as negative for asa1 gene.

Fig. 3: Gel electrophoresis of PCR amplified van A gene (732bp) in enterococcal isolates. Lane M was 100bp DNA ladder. Lane 2 considered as positive for van A gene. Lane3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18 considered as negative for van A gene.

Relation between asa1 and vanA genes and vancomycin resistance phenotype:

Our study revealed that asa1 gene was positive in 50% of VRE isolates and 67.8% of VSE isolates while van A gene was positive in 50% of VRE isolates and 28.5% of VSE isolates.

Relation between asa1 gene and different risk factors among the 36 enterococcal isolates:

There was no statistically significant difference (p-value >0.05) between positive and negative asa1 gene, as regards usage of antibiotic, and distribution in different departments. On the other hand, there was a statistically significant difference (p-value <0.05) as regards presence of DM and presence of underlying disease with high percentage of positive asa1 among liver failure patients (60.9%).

There was no statistically significant difference (p-value >0.05) between positive and negative van A, as regards presence of diabetes mellitus disease, usage of antibiotic, and distribution in different departments. On the other hand, there was a statistically significant
difference (p-value <0.05) between positive and negative van A, as regards presence of underlying disease with high percentage of positive van A, among renal failure patients (66.7%).

**DISCUSSION**

In the last few decades, enterococci have emerged as important nosocomial pathogens and the most important reason for this is the trend of increasing antimicrobial resistance seen in these organisms\(^{19}\). The majority of our enterococcal isolates were recovered from urine specimens followed by blood and sputum respectively. Our result coincides with Hasani et al as they reported that highest number of their enterococcal isolates were recovered from the urine and blood samples respectively \(^{20}\). Also, in Shenawy et al studies isolation of Enterococci and it was significantly higher from urine followed by endotracheal aspirate, sputum and wound swab specimens respectively \(^{21}\).

All our enterococcal isolates were resistant to Amoxicillin- Clavulanic followed by resistance to Erythromycin while Vancomycin and Teicoplanin were the most effective antibiotics. Batistão et al reported that resistance to Erythromycin was the most frequent among all antibiotics they used followed by Tetracycline and Ciprofloxacin\(^{22}\). Regarding Vancomycin resistance our study showed that 8 (22.2%) isolates of enterococcal isolates were resistant to Vancomycin (VRE), this was in correspondence with Shokry et al reported that the VRE percentage was 14.9% of overall isolates\(^{23}\). In contrast Praharaj et al study revealed that 34 (9.26%) out of 367 isolates of Enterococcus species were Vancomycin resistant 24. The study conducted by Shenawy et al reported that Vancomycin resistance was as high as 35.2% among their enterococcal clinical isolates\(^{21}\). The high percentage of VRE among our isolates probably reflects the increased use of Vancomycin in our hospital over the past few years. This fact shows the importance of strict enforcement of antibiotic policies with greater adherence to infection control measures in order to prevent emergence and spread of antibiotic resistant bacteria.

Several studies reported that vanA genotype is of a serious concern due to the risk of transmission of this gene to other organisms. From an epidemiological point of view, the most dangerous VRE are vanA and vanB genotypes as they are responsible for the majority of acquired transferable resistance \(^{25}\). In the present study, vanA was detected in 33.3% (12 out of 36) of enterococcal isolates. VanA genotype was detected in 50% (4 out of 8 isolates) of VRE similarly Zadeh et al study revealed that 59.09% of VRE contained VanA genes 26 and was lower than Praharaj et al study who reported that vanA gene was found in 87.5 % of their VRE isolates \(^{24}\).

In the present study, there were 4 vanA gene negative isolates resistant to both vancomycin and teicoplanin and this phenotypic pattern may be mediated by other van genes so, we recommend performance of further PCR analysis for detection of other vancomycin resistance genes. Our study detected vanA gene in 8 of 28 VSE which suggest that these genes were nonfunctioning.

Our study detected asa1 gene in 63.8% of enterococcal isolates which was to some extent comparable to the study conducted by Elsner et al in which asa1 gene was detected in 40% of their enterococcal isolates \(^{27}\). In other studies by Cafini et al and Archimbaud et al asa1 gene was detected in 78.5% of their enterococcal isolates \(^{28,29}\).

Our study revealed also a high percentage of asa1 among liver failure patients (60.9%) while vanA gene was high among renal failure patients (66.7%). This may be attributed to their overuse of antibiotics as they are usually undergo frequent hemodialysis sessions and often have complicated illnesses, which may places them at greater risk for VRE infection or colonization.

In conclusion VRE has become a critical nosocomial pathogen because of its rapid spread, limited options for treatment therefore a strict adherence to infection control measures with avoidance of abuse of antimicrobial chemotherapeutic agents are key factors to stop the circle of antimicrobial resistance. Further studies are needed including phenotypic identification of enterococcal species, biofilm detection and molecular studying of other virulence and vancomycin resistance genes.

**Ethics approval and consent to participate:**

The study was approved from clinical pathology department, Faculty of Medicine, Beni-Suef University. A signed informed consent was obtained from patients. The ethical committee signed on the work approval.

**Acknowledgement:**

I would like to express my gratitude and appreciation to my professor Mona El-Khlyous, Professor of Clinical Pathology, Faculty of medicine Beni-Suef University for providing a good scientific atmosphere and for all her support and help.

**Conflicts of interest:**

- The authors declare that they have no financial or non-financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.
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