

ORIGINAL ARTICLE

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

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ABSTRACT

Key words:

Immunocompromised; Enterococci; VRE; *vanA*; *asa1*

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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 $\geq 16\mu\text{g/ml}$. 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 ¹. 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 ²⁻⁴.

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* ⁵. 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 ^{6,7}.

Treatment of enterococcal infections is challenging as *Enterococci* are inherently resistant to several classes of antimicrobial agents such as cephalosporins, monobactams, sulphonamides and clindamycin ⁸. 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-alanine group of the peptidoglycan chains ⁹. There are nine phenotypes of acquired vancomycin resistance in enterococci including *Van A*, *B*, *C*, *D*, *E*, *G*, *L*, *M*, and *vanN*. 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* ¹⁰. 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.

METHODOLOGY

This study was carried out at Beni-Sueif 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 epindorpha 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/disc), Levofloxacin (5µg/disc), Erythromycin (15µg/disc), Vancomycin (30mcg/disc), Teicoplanin (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 $\geq 32\mu\text{g/mL}$ were considered as resistant; MIC value of 8–16µg/mL as intermediately resistant; and MIC of $\leq 4\mu\text{g/mL}$ were considered susceptible to vancomycin. Any *Enterococcus* was considered *VRE* if the MIC was $\geq 16\mu\text{g/ml}$ 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- GCA 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 (Biometra 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

	asa1	van A
Initial denaturation	95°C for 30 s	95°C for 30 s
Annealing	49°C for 90 s	50°C for 60 s
Extension	72°C for 60s,	72°C for 60s,
Final extension	72°C for 5 min	72°C for 5 min

PCR products were analyzed by gel electrophoresis and the amplified genes were visualized by placing on UV transilluminator (Whatman, Biometra, Germany) at 220 nm 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

Departments	Number (n=80)	Percentage (%)	Enterococci (N=36)
ICU	39	48.8%	13
Internal medicine	16	20%	10
Oncology	6	7.5%	1
Rheumatology	7	8.7%	2
Nephrology	12	15%	10

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.

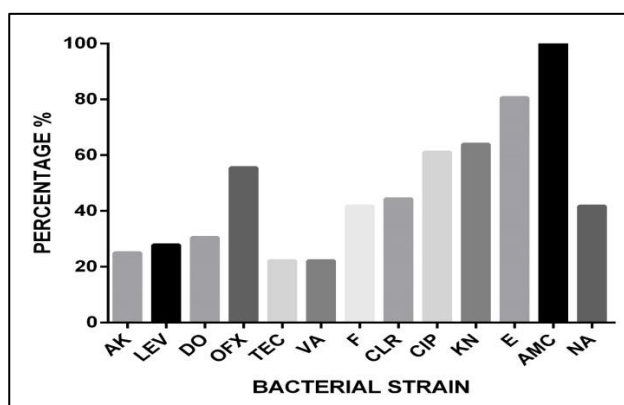


Fig. 1: Frequency of antibiotic resistance among the 36 enterococcal isolates

Percentage of *asaI* and *vanA* genes among the enterococcal isolates:

Of the 36 enterococcal isolates, 23(63.3%) were *asaI* gene positive, and 12(33.3%) were *vanA* gene positive as shown in Figures 2 & 3.

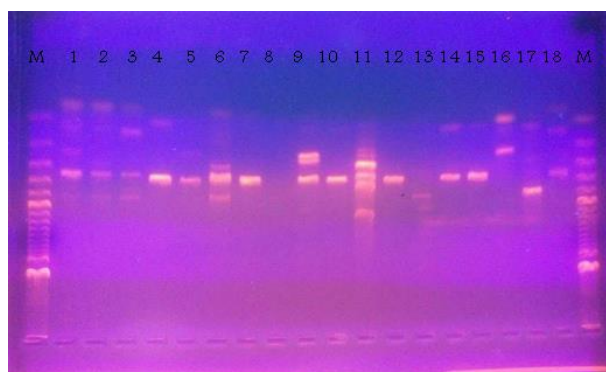


Fig. 2: Gel electrophoresis of PCR amplified *asaI* gene (378bp) in enterococcal isolates. Lane M was 100bp DNA ladder. Lane 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 14, 15, 17, 18 considered as positive for *asaI* gene . Lane 8, 13, 16 considered as negative for *asaI* gene.

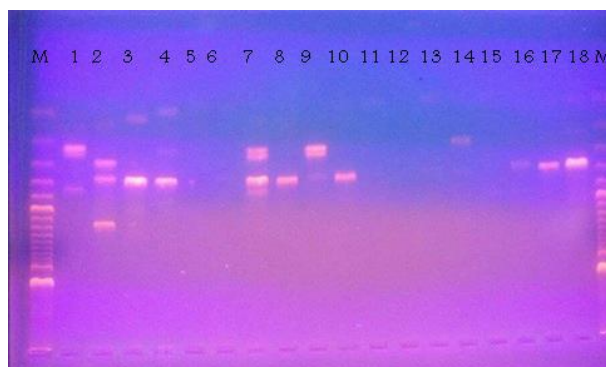


Fig. 3: Gel electrophoresis of PCR amplified *van A* gene (732bp) in enterococcal isolates. Lane M was 100 bp DNA ladder. Lane 2 considered as positive for *van A* gene. Lane 3,4,5,6,7,8,9,10, 11,12,13,14,15,16, 17, 18 considered as negative for *van A* gene

Relation between *asaI* and *vanA* genes and vancomycin resistance phenotype:

Our study revealed that *asaI* 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 *asaI* gene and different risk factors among the 36 enterococcal isolates:

There was no statistically significant difference (p-value >0.05) between positive and negative *asaI* 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 *asaI* 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 organisms¹⁹. 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 respectively²⁰. 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 respectively²¹.

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 Ciprofloxacin²². 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 isolates²³. In contrast Prahraj et al study revealed that 34 (9.26%) out of 367 isolates of *Enterococcus* species were Vancomycin resistant²⁴. The study conducted by Shenawy, et al reported that Vancomycin resistance was as high as 35.2% among their enterococcal clinical isolates²¹. 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 resistance²⁵. 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* genes²⁶ and was lower than Prahraj et al study who reported that *vanA* gene was found in 87.5 % of their *VRE* isolates²⁴.

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 *asaI* gene in 63.8% of enterococcal isolates which was to some extent comparable to the study conducted by Elsner et al in which *asaI* gene was detected in 40% of their enterococcal isolates²⁷. In other studies by Cafini et al and Archimbaud et al *asaI* gene was detected in 78.5% of their enterococcal isolates^{28, 29}.

Our study revealed also a high percentage of *asaI* 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.

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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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