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Original Research Article

Determination of putative virulence factors among clinical isolates of enterococci isolated from a military hospital in the eastern province of Saudi Arabia

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ABSTRACT

Background: The pathogenic potential of enterococci to produce life-threatening infections is well-documented. The scientific community has, of late, evinced a renewed interest in the putative virulence factors of enterococci. Objective of the study was to determine the putative virulence factors of clinically isolated Enterococcus species from a military hospital and to describe the association between virulence factors and vancomycin susceptibility.
Methods: A total of 245 enterococci were isolated from clinical samples collected from KFMMC, a leading military hospital in the eastern province of Saudi Arabia. Following species identification and antimicrobial susceptibility testing using the Vitek 2 system; the isolates were tested for the production of caseinase, gelatinase, biofilm, and presence of haemolysin.
Results: Among the enterococcal isolates, 36.7% produced caseinase, 38% produced gelatinase, 24.1% exhibited biofilm formation, and 30.6% were positive for haemolytic activity. A significant association between vancomycin susceptibility patterns and the virulence factors, gelatinase and haemolytic activity, were noted. No significant associations were observed between vancomycin susceptibility patterns and the presence of caseinase or the formation of biofilms.
Conclusions: Virulence factors are invariably produced by several clinical isolates of enterococci in our hospital, and some virulence factors are associated with vancomycin susceptibility.

Keywords: Biofilm, Caseinase, Enterococci, Gelatinase, Haemolysin, Vancomycin resistance

INTRODUCTION

Over the years, the Gram-positive *Enterococcus* has progressed from being innocuous intestinal commensals with uncertain clinical significance to multidrug-resistant bacteria triggering disturbing clinical consequences.[1] This increasing clinical significance, coupled with the isolation of strains resistant to all therapeutic options, has shifted the focus of interest on the possible factors associated with the colonisation and pathogenesis. Among the more than 50 species identified so far, *E. faecalis* remains the most

common clinically isolated enterococcal species both in the hospital and community settings.[1,2]

Gram-positive cocci like *Staphylococcus aureus* and *Streptococcus pyogenes* produce potent toxins such as Toxic Shock Syndrome Toxin (TSST) or the erythrogenic toxin. Enterococci do not possess a comparable toxin. However, over the years several putative virulence factors such as gelatinase, caseinase, haemolysin, enterococcal surface protein (Esp), aggregation substance (AS) biofilm formation, accessory colonisation factor (Ace), serine proteases, capsule, cell wall polysaccharide and superoxide have all been identified, and animal models have proven their association with infections.[3-5] Along with inherent and acquired antimicrobial resistance determinants, these factors replenish the repertoire of strategies used by this organism to initiate and sustain infections such as urinary tract infections (UTI), bacteremia, endocarditis and severe wound infections in humans.[6,7] Studies have established the associations between biofilm formation and urinary tract infection, adhesion and haemolysin with bloodstream infections and also between gelatinase and caseinase with wound infections.[8]

Haemolysin or cytolysin is a bacterial toxin, usually encoded by pheromone-responsive plasmids, that has regular membrane lytic capability efficiently lysing both eukaryotic cells (hemolysin) and prokaryotic cells (bacteriocin). This exotoxin demonstrates bacteriocidal properties towards Gram-negative bacteria and toxic properties (haemolysis) towards erythrocytes, leukocytes and macrophages.[9] The incidence of death as a result of infection due to a cytolysin-producing *Enterococcus* has been reported to be five times higher than that ascertained in a non-cytolysin-producing *Enterococcal* infection.[10]

Gelatinase and caseinase are extracellular proteases secreted by *E. faecalis* that hydrolyse gelatin, collagen, and casein. They are traditionally considered to be elements that augment an organism's ability to amass nutrients by proteolysis but during that process cripple host tissues by direct or indirect methods such as degeneration of host connective tissues, alteration of immunoglobulins or complement molecules and enhancement of biofilm formation.[3,5,11]

Awareness of the virulence characteristics of commonly encountered enterococcal strains might aid in decoding the complex pathogenic progression of these adaptable microorganisms.[12] Records regarding virulence factors of *Enterococcus* species among clinical enterococcal strains in eastern regions of Saudi Arabia are still scarce and mostly at the molecular level.[13] Molecular methods are powerful tools in microbial diagnosis, but the fact remains that the presence of a gene encoding virulence or resistance does not provide information on the control gene necessary for the expression of this particular trait.

The gene, though present, maybe silent or non-functional rendering the organism incapable of expression of the trait encoded by the detected gene.[14] These findings reinforce the complexity of the processes involved in *Enterococcus* virulence. Taking into consideration these possibilities and throw some light upon pathogenic armamentarium of enterococci retrieved from this part of Saudi Arabia, phenotypic detections of four such determinants namely, caseinase, gelatinase, haemolysin and biofilm production were undertaken for the study. Additionally, the relationship of these virulence factors with vancomycin resistance was also investigated.

METHODS

This cross-sectional analytical study was done on 245 *Enterococcus* isolates collected from various clinical specimens at the microbiology laboratory of the King Fahd Military Medical Complex (KFMMC), a modern tertiary care military hospital in Saudi Arabia, over 18 months from June 2018 to December 2019. The isolates collected were transferred to the microbiology laboratory at Prince Sultan Military College of Health Sciences (PSMCHS) situated in the same compound for analysis. Patient information anonymisation was a mandatory first step before processing, and repetitive positive cultures of the same patient were eliminated.

In this study, the identification tests and antimicrobial susceptibility tests were carried out using the automated bioMérieux VITEK® 2 compact system (bioMérieux, Marcy l'Etoile, France). Isolate identification and susceptibility were accomplished using the Vitek Gram-positive GP cards and AST GP67 cards, respectively. Following identification and susceptibility testing, the isolates were analysed for the virulence factors - caseinase, gelatinase, haemolysin and biofilm production. *E. faecalis* ATCC 29212, *E. faecium* ATCC 700221 were used as controls.

Caseinase production

The production of caseinase was tested by using Mueller-Hinton agar containing 3% skimmed milk. The plates were streaked with 10ul of suspension followed by incubation at 37o C for 24 hours. The presence of a clear zone around the colonies indicated caseinase activity.[15]

Gelatinase production

Gelatinase production was ascertained on Tryptic soy agar containing 3% gelatin. Small wells were punched on the agar using sterile Pasteur pipette, and these were inoculated with 10 µL suspension of the isolate. The appearance of turbid zones around the well after incubation at 37ºC for 24 hours indicated gelatinase production. Brief incubation of the plates at 4ºC ensured better visualisation of the zones.[16]

Haemolysin production

Haemolysin production was assessed by culturing isolates on blood agar plates prepared from Brain Heart Infusion agar containing 5% human blood. Observation of hemolytic zones around the colonies after incubation for 24 hours at 37o C indicated haemolysin production.[17,18]

Biofilm production

Biofilm production was performed according to the method described by O'Toole with minor modifications.[19] The isolates were grown on Luria broth for 24 hours. Following incubation, 1/100 dilution of the suspension was made using Tryptic soy broth. 100 µl of this suspension was inoculated onto a 96 well polystyrene microtiter plate. Replicates of each isolate were tested in eight wells. The wells were incubated for 24 hours. Upon completion of incubation, the plates were washed two times in distilled water. The plates were blotted dry, and 125 ul of 0.1% crystal violet was added to stain the biofilm formed. After 15 minutes, the wells were washed and allowed to dry overnight. Following drying, 30% acetic acid in water was added to each well of the microtiter plate to solubilise the crystal violet. The eluted biofilms were then transferred onto a new plate, and optical density was assessed at 550 nm using 30% acetic acid in water as the blank. Biofilm production was determined using the formula described by Stepanovic et al.[20]

Analysis of the results obtained after detection of the virulence factors and their association with vancomycin susceptibility were analysed by Pearson Chi-Square test using IBM SPSS Statistics for Windows, version 21(IBM Corp., Armonk, NY, USA). The level of significance was set up at $p<0.05$.

RESULTS

The current study was done on 245 Enterococcus isolates. Of these, 220 (89.80%) were *Enterococcus faecalis*, and 25 (10.20%) were *Enterococcus faecium.* Fourteen isolates of *Enterococcus faecium* were vancomycin-resistant enterococci (VRE) with a MIC≥256 µg/ml. Among the 245 isolates, 137 (55.9%) were recovered from urine followed by 102 (41.6%) samples from pus.

Table 1: Percentage incidence of virulence factors in isolates.

Species type	Caseinase	Gelatinase	Hemolysis	Biofilm
	Positive (%)	Positive (%)	Positive (%)	Positive (%)
Enterococcus faecalis	84 (38.2)	89 (40.5)	75 (34.1)	55 (25)
Enterococcus faecium	6 (24)	4 (16)	0 (0)	4 (16)

Table 2: Cross-tabulation of the frequency of virulence factor-caseinase production and vancomycin susceptibility status.

Vancomycin susceptibility testing status	Virulence factor-caseinase			Chi-square tests		
	Negative	Positive	Total	Pearson chi-square	Df	P value
Susceptible	143 (61.90)	88 (38.10)	231 (100)	3.22	1	0.073
Resistant	12 (85.70)	2 (14.30)	14 (100)			
Total	155 (63.30)	90 (36.70)	245 (100)			

Table 3: Cross-tabulation of the frequency of virulence factor-gelatinase production and vancomycin susceptibility status.

Vancomycin susceptibility	Virulence factor-gelatinase			Chi-square tests		
	Negative	Positive	Total	Pearson chi-square	Df	P value
Susceptible	139 (60.2)	92 (39.8)	231 (100)	5.999	1	0.01
Resistant	13 (92.9)	1 (7.1)	14 (100)			
Total	152 (62)	93 (38)	245 (100)			

Table 4: Cross-tabulation of the frequency of virulence factor- haemolysin production and vancomycin susceptibility testing status.

Vancomycin susceptibility	Virulence factor- haemolysin			Chi-square tests		
	Negative	Positive	Total	Pearson chi-square	Df	P value
Susceptible	156 (67.5)	75 (32.5)	231 (100)	6.551	1	0.01
Resistant	14 (100)	0 (0)	14 (100)			
Total	170 (69.4)	75 (30.6)	245 (100)			

Table 5: Cross-tabulation of the frequency of virulence factor-biofilm formation and vancomycin susceptibility testing status.

Vancomycin Susceptibility testing status	Virulence factor-biofilm			Chi-square tests		
	Negative	Positive	Total	Pearson chi-square	Df	P value
Susceptible	174 (75.3)	57 (24.7)	231 (100)	0.779	1	0.377
Resistant	12 (85.7)	2 (14.3)	14 (100)			
Total	186 (75.9)	59 (24.1)	245 (100)			

Other specimens, with far fewer percentages, included those from rectal swabs and blood. The distribution of frequency and the percentage of virulence factor production among the 245 enterococcal isolates are summarised in Table 1. 93 (38%) enterococcal isolates produced gelatinase followed by 90 (36.7%) that produced caseinase. Haemolysin production was observed in 75 isolates (30.6%) while 59 (24.1%) exhibited biofilm formation.

Caseinase production and vancomycin susceptibility

Cross-tabulation of the frequency of caseinase production with vancomycin susceptibility testing showed that 88 (38.10%) isolates were vancomycin susceptible while only two (14.30%) caseinase producing isolates were resistant to vancomycin (Table 2). Chi-square test reported no significant association between vancomycin susceptibility status and caseinase production.

Gelatinase production and vancomycin susceptibility

A comparative analysis of gelatinase production with vancomycin susceptibility testing indicated that 92 (39.8%) gelatinase producing isolates were vancomycin susceptible while only 1 (7.1%) isolate was resistant to vancomycin (Table 3). Chi-square test reported a significant association between vancomycin susceptibility status and gelatinase production.

Haemolysin production and vancomycin susceptibility

A cross-tabulation evaluation of the frequency of haemolytic activity and vancomycin susceptibility testing showed that 75 (32.5%) vancomycin susceptible isolates exhibited haemolysin production while none of the isolates that were vancomycin-resistant presented with a haemolytic activity (Table 4). Chi-square test reported a significant association between vancomycin susceptibility status and haemolytic activity.

Biofilm formation and vancomycin susceptibility

A comparison of the frequency of biofilm formation with vancomycin susceptibility demonstrated 57(24.7%) isolates that produced biofilms exhibited susceptibility while only 2 (14.30%) isolates were resistant to vancomycin (Table 5). Chi-square test reported no significant association between vancomycin susceptibility status and biofilm formation.

DISCUSSION

The ability of a commensal organism to acquire determinants to gain a competitive edge over other microorganisms and work around the host immune defence is the established route for pathogenesis.[6,21] This aptitude of enterococci, to utilise both intrinsic and acquired antibiotic resistance, has opened the possibility of effectively manipulating putative virulence factors to impede pathogenic progression.[16,22-25] The current study was done on 245 clinical enterococcal isolates comprising of 220 (89.8%) *E faecalis* and 25 (10.2%) *E faecium*. These were evaluated for the presence of four virulence factors, namely biofilm formation, caseinase production, gelatinase production and hemolytic activity. Efforts were also, contemporaneously, made to determine whether there was any relationship between expressed virulence factors with vancomycin resistance.

Gelatinase and caseinase can cleave fibrin. This property negatively affects host tissue facilitating bacterial migration and dissemination as exhibited notably in *E. faecalis*.[26] Association of gelatinase and caseinase with enterococcal wound infections has been documented.[8] Even though they are both proteases, some data does suggest that the gelatin hydrolysing activity is different from caseinase activity.[15] In this study, we found that 38% produced gelatinase, while 36.7% produced caseinase. This finding is in agreement with a study done in India that reported 39% of clinical isolates of enterococci produced gelatinase.[27] Our study found that gelatinase and caseinase production was similar, and this was in contrast to a report from Brazil.[15] This observation may be because our sample size was much more extensive (n=245) than that of the study in Brazil (n=32).

Haemolysin or cytolysin is a toxin produced by enterococci and is virulent in animal models and human infections associated with increased infection severity.[28] It is produced by ~30 % of *E. faecalis* strains and can lyse human red blood cells as well as white blood cells.[29] Our study is in agreement as 34.1% of *E. faecalis* produced haemolysin whereas none of the *E. faecium* isolated produced haemolysin.

The ability to adhere to biomaterials by biofilm formation is an essential aspect of enterococcal virulence. This property has abetted the evasion of several host defence mechanisms and has proven to be detrimental to antibiotic activity. The net result has been an increase in the persistence of infections, particularly those due to

indwelling catheters.[30,31] Enterococci cause 25% of all catheter-associated urinary tract infections.[32] They also are significant pathogens in wound infections and infective endocarditis, both of which are associated with biofilm formation.[3] Our study found that 24.1% of enterococcal isolates produced biofilms and is similar to a study from India that found biofilm formation in 26.1%.[33] It has to be emphasised that very few studies on virulence factors have been reported from Saudi Arabia, and the ones available are genotypic studies.[13,34] Consequently, a practicable comparison with other Saudi studies cannot be conducted at this stage.

In the past, a few studies have explored the relationship between vancomycin resistance and the prevalence of virulence determinants. Most studies were inconclusive, but a few displayed significant direct correlation. [15,27,35,36] We report a significant association between vancomycin susceptibility patterns and two virulence factors, namely gelatinase and haemolysin production ($p<0.05$). A significant association of vancomycin susceptibility with caseinase and biofilm production could not be established.

Our study focused on a few virulence factors and described the phenotypic expression of these virulence factors. As multiple genes code these virulence factors, the possible associations between the genes and virulence factors need further evaluation. Majority of studies on enterococcal virulence are genotypic though some reports suggest that the relationship of virulence markers with specific virulence genes might not be significant.[37] Prospective studies should, therefore, contemporaneously evaluate both genotypic and phenotypic factors.

CONCLUSION

This study found a substantial prevalence of virulence factors among clinical isolates of enterococci in our hospital and was able to determine an association of two important putative virulence factors with vancomycin susceptibility. Further studies on the virulence properties of *E. faecalis* are necessary to evaluate pathogenicity fully. Our study has provided valuable new information on the incidence of these significant virulence properties in eastern Saudi Arabia. A gamut of virulence factors, coupled with progressing antibiotic resistance, can pose a significant challenge to a proper treatment regimen.

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