Molecular Characterization of Antibiotic Resistant Enterococci

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Abstract: Infections caused by antibiotic-resistant Enterococci are increasing and are becoming difficult to manage due to the diminishing number of available antibiotics to treat them. This study was conducted to investigate the species prevalence, antimicrobial resistance pattern and β-lactamase production among clinical isolates of Enterococci. The molecular basis of carriage of resistance genes to high level aminoglycosides and vancomycin and the genetic relatedness between the strains were also studied. A total of 120 isolates of antibiotic resistant Enterococci were investigated. Minimum inhibitory concentrations (MIC) to high-level aminoglycoside and glycopeptide antibiotics were determined by agar dilution and E-test methods respectively. Genes encoding aminoglycoside modifying enzymes (AMEs) and vancomycin-resistance determinants were detected by polymerase chain reaction (PCR). They were typed by pulsed field gel electrophoresis (PFGE). The clinical isolates of Enterococci were cultured from urines (70%), wounds (15%), blood (7%), ears (5%) and fluids (3%). They comprised E. faecalis (92.5%), E. faecium (6.7%) and E. casseliflavus (0.8%). None of the Enterococci produced penicillinase but 9.2% were resistant to ampicillin. Resistance to erythromycin (62.5%), tetracycline (60%), ciprofloxacin (40%) and chloramphenicol (28.3%) were more prevalent. High-level resistance to aminoglycosides; amikacin, gentamicin, kanamycin, tobramycin and streptomycin were detected in 26.7%, 20.8%, 21.6%, 20.8%, 23.3% of the isolates respectively. Most of the high-level aminoglycoside-resistant isolates contained genes coding the bifunctional aminoglycoside modifying enzymes AAC (6’)-APH (2’), APH (3’) and ANT (6’) but not ANT (4’) enzyme. The results demonstrated a low prevalence of vancomycin resistance (4.2%) among Enterococci. All of them gave positive results for the presence of the vanA genotype. PFGE revealed heterogenous patterns with no dominant clone suggesting that the strains acquired resistance independently. It is concluded that although the prevalence of glycopeptide resistance was low among the studied isolates, their presence together with high-level aminoglycoside resistance calls for regular surveillance of antibacterial susceptibilities to detect emerging resistance and prevent the establishment and spread of multiply antibacterial-resistant strains.

Keywords: Enterococci, resistance, aminoglycosides, vancomycin

INTRODUCTION

Enterococci are commensals of the gastrointestinal tract of most human beings. They have gained increasing clinical importance through the 1990s due to changes in hospital patients and antimicrobial use patterns\(^\text{[1,2]}\). They have been associated with infections of the urinary tract, post-surgical wounds, septicaemia, endocarditis and meningitis\(^\text{[3,4]}\).

Infections caused by antibiotic-resistant Enterococci are increasing and are becoming difficult to manage due to the diminishing number of available antibiotics to treat them\(^\text{[5,6]}\). They have a remarkable ability to adapt to exposure to antibiotics maintaining intrinsic resistance to penicillins and low-level resistance to aminoglycosides. In addition, they have demonstrated a capacity to acquire resistance to other antibiotics including high-level resistance to aminoglycosides and glycopeptides\(^\text{[7,9]}\).

High-level aminoglycoside resistance among Enterococci is increasingly being reported worldwide\(^\text{[6,11,12]}\). The presence of high-level aminoglycoside resistance results in the loss of synergy between cell wall synthesis-inhibiting antibiotics (penicillins and glycopeptides) and aminoglycosides (gentamicin, tobramycin, netilmicin, kanamycin and amikacin), making the treatment of serious infections difficult\(^\text{[13]}\).

High-level aminoglycoside resistance in Enterococci is mediated by aminoglycoside-modifying enzymes (AMEs)\(^\text{[14]}\). Numerous AMEs encoded by different genes have been reported worldwide\(^\text{[15]}\). The most common AMEs in Gram-positive cocci are the AAC (6’)-APH (2’) which inactivates gentamicin, kanamycin, tobramycin, neomycin and amikacin; APH(3’) which inactivates kanamycin and amikacin; ANT (4’) which inactivates kanamycin, neomycin and tobramycin; and ANT (6’) which inactivates streptomycin\(^\text{[16]}\).

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High-level gentamicin resistance (MIC > 500 mg/L) is encoded by the aac (6')-le-aph (2')-la gene which encodes the bifunctional enzyme AAC (6')-APH (2'). In recent years, novel aminoglycoside modifying enzymes, APH (2')-Id(19), APH (2')-Ic(18), APH (2')-Ib(17), and AAC (6')-Ii(20,21) have been described in Enterococci.

The recent emergence of glycopeptide resistance in Enterococci has further limited the therapeutic alternatives and has increased mortality sometimes up to 60-70%[22]. Vancomycin-resistant enterococci (VRE) have caused outbreaks in many centers in the USA and is recognized as an important resistant pathogen in Europe highlighting their growing importance as hospital pathogens[23]. Infections caused by VRE are associated with severe adverse outcomes such as extended length of hospital stay, increased cost and increased mortality due to the reduction in the choices of antibacterials available to treat such infections[6,24].

The aim of this study is to determine the species prevalence and antimicrobial resistance patterns of Enterococcus clinical isolates in general, with respect to the prevalence of resistance to high level aminoglycoside and vancomycin and B-lactamase production. The aim was also to investigate the molecular basis of carriage of resistance to high level aminoglycosides and vancomycin and the genetic relatedness between clinical isolates.

MATERIAL AND METHODS

Enterococci Isolates: A total of 120 strains were obtained from different clinical samples (between January 2004 and December 2006) at the clinical microbiology laboratory in Theodor Bilharz Research Institute (TBRI), Cairo, Egypt. They were cultured from urine, wound swabs, blood, endotracheal secretions and ear swabs. Isolates studied were selected on the basis of their sensitivity patterns. Only those that were resistant to antibiotics were included.

Identification of Enterococcal Isolates: The isolates were identified to the genus and species level by cultural characteristics, Gram’s stain, catalase test, bile aesculin reactions and by biochemical tests using API Strep (bioMerieux, France). Streptococcus group antigens were also detected using group D antisera (SlideX Strepto-kit; bioMerieux, France).

Susceptibility Testing: Susceptibility to tetracycline, erythromycin, chloramphenicol, ampicillin, ciprofloxacin, vancomycin and teicoplanin was performed on all isolates by disk diffusion method on Mueller–Hinton agar[25]. Detection of high-level aminoglycoside resistance was performed with disks containing gentamicin (120 µg), kanamycin (200µg) and streptomycin (300 µg). Isolates showing zone sizes of < 10 mm for the three antibiotics were selected for the determination of the minimum inhibitory concentration (MIC) of gentamicin, kanamycin, streptomycin, tobramycin, amikacin. This was performed by the agar dilution method with antibiotic dilutions ranging from 8 to 4000 µg/ml. E. faecalis strain ATCC 51299 was used as a resistance control for detecting high-level aminoglycoside resistance while E. faecalis strain ATCC 29212 was used as susceptibility control. The MICs of ampicillin, vancomycin and teicoplanin were determined for all isolates using E-test strips (AB Biodisk, Sweden) according to the manufacturer’s instructions. The results were read after incubation at 35 °C for 24 h. Isolates with MIC ≥ 16 µg/ml for ampicillin and ≥ 32 µg/ml for vancomycin and teicoplanin were considered to be resistant for these agents.

Detection of B-lactamase Production: All 120 isolates were tested for B-lactamase production with nitrocefin (Oxoid, England) according to the manufacturer’s instructions. Nitrocefin solution (5 µl) was dropped onto a loopful of pure overnight growth placed on a filter membrane. The development of a red colour within 60 sec indicated a positive result. Staphylococcus aureus strain ATCC 29213 was used as a positive control.

Detection of Resistance Genes by PCR:

Detection of Vancomycin-resistance Determinants: Genes encoding the vancomycin-resistance determinants vanA and vanB were investigated by PCR using specific primers[26,27]. The primers were VanA (VA1, 59-GGG AAAACGACAAAT GC-39 and VA2, 59-GTACAAATGC GGC CGT TA-39), VanB (VA3, 59-CCC GAA TTT CAA ATG ATT GAA AA-39, and VA4, 59-CGC CAT CCT CCT GCA AAA-39). E. faecalis E206 (vanA) and E. faecium E2781 (vanB) were used as controls.

Detection of Aminoglycoside Modifying Enzymes (AMEs): PCR was used to detect genes encoding the AMEs AAC (6')-APH (2'), APH (3'), ANT (4'), ANT (6'). The oligonucleotide primers[28] used are listed in Table 1.

DNA for PCR was isolated as described previously except that lysozyme (1 mg/ml) was used instead of lysozyme in the lysis solution[24]. Amplification was performed with a Perkin Elmer 9600 thermocycler (Perkin Elmer, Cetus, Norwalk, CT) using a kit from Gibco BRL. The reaction mixture consisted of 45 µl of Supermix (22 mM Tris HCl pH 8.4, 55 mM KCl, 1.65 mM MgCl2, 220 µM dGPT, 220 µM dATP, 220 µM

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were resistant to ampicillin. Resistance to erythromycin faecium results show that only 11 (9.2%) isolates (six according to species is presented in Table 3. The antibacterial resistance by disk diffusion method according to species is presented in Table 3. The results show that only 11 (9.2%) isolates (six E. faecium, four E. faecalis and one E. casseliflavus) were resistant to ampicillin. Resistance to erythromycin (62.5%), tetracycline (60%), ciprofloxacin (40%) and chloramphenicol (28.3%) were more prevalent. High-level resistance to aminoglycosides, amikacin, gentamicin, kanamycin, tobramycin and streptomycin were detected in 26.7%, 20.8%, 21.6%, 20.8%, 23.3% of the isolates respectively. Vancomycin and teicoplanin resistance were detected in 5 (4.2%) isolates (three E. faecium and two E. faecalis).

The 25 isolates that were resistant to gentamicin by disk diffusion had gentamicin MICs of 256 to > 4000 µg/ml. Five isolates (three E. faecalis and two E. faecium) had gentamicin MIC values of 256 µg/ml (Table 4). The MIC range for kanamycin and streptomycin were 2000 to > 4000 µg/ml and 256 to 2048 µg/ml for amikacin and tobramycin. Twenty of the 25 gentamicin resistant isolates were recovered from urine, 3 were from wound, one from blood and one from endotracheal secretions. The high-level resistant isolates were also multiply resistant to other antibiotics. Excellent correlation was observed between the high-level disk tests and the agar screening test in the detection of high-level aminoglycoside resistance.

All vancomycin-resistant isolates (2 E. faecalis and 3 E. faecium) had MIC values > 256 µg/ml for vancomycin (Table 5). One E. faecalis and two E. faecium had MIC values > 256 µg/ml for teicoplanin. Four of the VRE were isolated from urine while one was isolated from wound swab. Three of these isolates were also resistant to ampicillin while all were resistant to high-level gentamicin.

Detection of B-lactamase Production: All strains tested for production of B-lactamase enzyme gave a negative test reaction.

Detection of Resistance Genes by PCR: Detection of Van Genotypes: The 5 vancomycin-resistant Enterococci were investigated for their vancomycin-resistance genotypes by PCR. All isolates gave positive results for the presence of the vanA genotype (Fig. 1a). A732 bp PCR product was obtained in all the positive isolates. No vanB products were detected in any of the isolates (Fig. 1b).

Detection of Genes Encoding High-level Aminoglycoside Resistance: The isolates expressing high-level resistance to amikacin, gentamicin, kanamycin, tobramycin and streptomycin were investigated for the presence of genes encoding the AAC (6')-APH (2''), APH (3''), ANT (4') and ANT (6') enzymes. The results are summarized in Table 4. All isolates with gentamicin MIC > 500 µg/ml gave positive results for the aac(6')-aph(2'') genes encoding the AAC (6')-APH (2'') enzymes. In addition, five isolates, three E. faecalis and two E. faecium, with

| Table 1: Primers For The Amplification Of Aminoglycoside Resistance Genes |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Genes           | Primer pairs (5' – 3') | Product size (kb) |
| aac (6') - aph (2') | CCA AGA GCA ATA AGG GCA TA | 222 |
| ant (4')          | GCA AGG ACC GAC AAC ATT TC | 174 |
| aph (3')          | GCC GAT GTG TGC GAA AA | 269 |
| ant (6')          | GCC TTT CCG CCA CCT CAC CG | 597 |

Pulsed Field Gel Electrophoresis (PFGE): Cells were prepared for PFGE as described previously. Each block was digested with 20 U of SmaI restriction endonuclease (New England Biolabs, MA) and incubated at 25 °C for 4 hr. Electrophoresis was performed with the CHEF DR-III system (Bio-Rad) with the following running parameters: initial pulse, 5 sec; final pulse, 40 sec; voltage, 6 V/cm; time, 20 hr; and temperature, 12 °C. The gel was stained in ethidium bromide solution (0.5 µg/ml) and detected with UV transillumination. DNA banding patterns were compared visually and isolates that had the same number of fragments of the same size were considered to be the same.

RESULTS AND DISCUSSIONS

Results:

Enterococci Isolates: A total of 120 antibiotic-resistant Enterococci were isolated from 84 urine cultures (70%); 18 wound swabs (15%); eight blood cultures (7%); six ear swabs (5%) and four body fluids (3%) including ascetic fluid, endotracheal secretions and peritoneal dialysis fluids. The isolates consisted of 111 (92.5%) E. faecalis, eight (6.7%) E. faecium and one E. casseliflavus (0.8%). The distribution of species and sources are shown in Table 2.

Antibacterial Resistance: The distribution of antibacterial resistance by disk diffusion method according to species is presented in Table 3. The results show that only 11 (9.2%) isolates (six E. faecium, four E. faecalis and one E. casseliflavus) were resistant to ampicillin. Resistance to erythromycin...
gentamicin MIC of 256 µg/ml also gave positive results for the genes encoding AAC (6')-APH (2'') enzymes. The ant(6) genes were detected only in isolates with streptomycin MIC >1000µg/ml. The aph(3') genes encoding the APH (3') enzymes were detected in 33 isolates. None of the isolates contained the ant(4') genes. Nineteen of the 35 high-level aminoglycoside resistant isolates contained genes for all three enzymes, AAC (6')-APH (2''), APH (3'), and ANT (6') and 6 contained both AAC (6')-APH (2'')
Fig. 1: PCR amplification of (a) vanA and (b) vanB genes. (a) Lanes: 1, molecular size markers; 2-3; vancomycin resistant E. faecalis isolates and 4-6; vancomycin resistant E. faecium isolates. 7; E. faecalis E206, vanA positive control strain; 8; E. faecalis ATCC 29212, vancomycin-susceptible strain. All of the vancomycin-resistant isolates generated a 732 bp PCR product. (b) Lanes: 1, molecular size marker; 2-6; representatives of vancomycin-resistant strains; 7, E. faecium E2781, vanB positive control strains.

and APH (3'). Eight isolates contained genes for both APH (3') and ANT (6') and one isolate contained only the ANT (6') enzymes. One E. faecalis isolate that was resistant to kanamycin (MIC >4000µg/ml), tobramycin (MIC 2048 µg/ml) and amikacin (MIC 512 µg/ml) gave negative results for all the AME genes tested.

Pulsed Field Gel Electrophoresis (PFGE): The antibiotic resistant isolates were typed by PFGE to ascertain their genetic relatedness. Two E. faecalis of the vancomycin-resistant isolates had identical PFGE patterns as shown in Fig. 2. These two isolates were also resistant to high level gentamicin and were obtained from different patients. One isolate was from a urine sample and the other was from a diabetic foot wound swab. The other isolates were not related.

Discussion: Enterococci are considered to be a part of the normal flora of the bowel, genital tract and anterior urethra of humans[10]. Enterococci cause mostly urinary tract and intra-abdominal or pelvic wound infections. They can cause bacteraemia with high mortality[31]. They are also responsible for a variety of community-acquired infections[4].

This study investigated the species prevalence and antibacterial resistance patterns of Enterococci isolated from clinical samples. The majority of isolates were recovered from urine (70%), followed by wound (15%) and blood (7%) samples which is consistent with reports that Enterococci have become the leading cause of urinary tract, surgical wound infections and bacteremia[8,33,34]. Most of the Enterococci were E. faecalis (92.5%) followed by E. faecium (6.7%), while E. casseliflavus accounted for only 0.8% of the isolates. This was comparable to the distribution of enterococcal species in some studies[4,33,38] but contrasted with others where E. faecium was the most common isolated species[5,24,40,41].

Fig. 2: PFGE patterns of antibiotic resistant Enterococci. Lane O contains S. aureus NCTC 8325 digested with Smal as size markers. Sizes given are in kilobases. Apart from isolates in lanes a and c that show related patterns, the other isolates are not related.

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Of the 120 isolates, 62.5%, 60% and 28.3% were resistant to erythromycin, tetracycline and chloramphenicol respectively which were similar to levels reported for these antibiotics among Enterococci isolated in UK[4] and Germany[5].

It is reassuring that 98.2 and 96.4% of the E. faecalis in the current work were vancomycin and ampicillin susceptible respectively contrary to the situation in most hospitals in the USA[6,48,49] and Europe[50,51] where high prevalence of vancomycin and ampicillin resistance are common. Low prevalence of vancomycin resistance has also been reported in Enterococci isolated in Mexico City[52] and Turkey[53]. This finding indicates that vancomycin retains its therapeutic efficacy against most E. faecalis infections. On the other hand, three out of eight E. faecium isolates were vancomycin resistant consistent with other findings that E. faecium are usually more resistant than E. faecalis[54,55].

Although 79.2 and 76.7% of the isolates were susceptible to gentamicin and streptomycin, suggesting that gentamicin should maintain a synergistic effect when combined with wall-active agents such as vancomycin and ampicillin in the treatment of enterococcal infections. The detection of high-level gentamicin resistance in 19.8% of E. faecalis and 25% of E. faecium isolates is cause for concern, as it may signify the beginning of a major resistance problem. These isolates are significant because they were also multiply resistant to other antibiotics indicating that the use of these antibiotics should be done after susceptibility testing.

Vancomycin-resistance phenotypes in Enterococci have been classified as van A, van B, van C, van D and van E based on levels of resistance, cross-resistance to teicoplanin and inducible or constitutive nature of the resistance. Two of these, van A (vancomycin MIC<64µg/ml; teicoplanin MIC>16µg/ml) and van B (vancomycin MIC 4-1024µg/ml; teicoplanin MIC ≤ 0.5µg/ml) determinants have been described primarily in E.faecalis and E.faecium[18]. All VRE in the present study expressed vancomycin-resistance patterns compatible with the van A phenotype and all gave positive results in PCR experiments for the van A genotype.

The current study investigated the prevalence of genes encoding aminoglycoside-modifying enzymes in high-level aminoglycoside-resistant Enterococci isolated from clinical samples.

The aac(6')-aph(2'') gene was the most common AME gene in gentamicin resistant Enterococci in other countries[23,48,49,50]. Some investigators[49,51] have also detected the aac(6')-aph(2'') in Enterococci with gentamicin MIC of 256 µg/ml and 128µg/ml respectively, supporting the the presence of the aac(6')-aph(2'') in low-level gentamicin-resistant Enterococci. However, it will be important to exclude the presence of the aph(2'') -Ib, aph(2'') -Ic, aph(2'') -Id genes by Southern blot hybridization and/or PCR amplification. This will help to select more appropriate antibiotics.

The second AME that was detected in this study was APH (3'). This enzyme confers resistance to amikacin, but not gentamicin[52]. Importantly, it was found among enterococcal isolates with low level of resistance to gentamicin. The majority of isolates in this group were also resistant to amikacin. In such cases, combination of ampicillin plus tobramycin is likely to be clinically more effective.

High-level streptomycin resistance (MIC>1000 mg/L) in Enterococci can be due to a single mutation of a ribosomal protein or enzymatic inactivation by modifying enzymes encoded by the ant(6') or aph(3') genes[53]. In the present study, the streptomycin-resistant isolates contained either the ant(6) or aph(3') genes or both which is comparable to reports from Spain[54], Greece[55] and Japan[56]. None of the isolates contained the ant(4') genes, which have been reported previously in Enterococci[48,49,53].

The results further demonstrated that the majority of the isolates contained multiple genes for AMEs. Similar findings have been reported in other studies[40,48,50]. The presence of these multiple genes for AMEs in the isolates implies that neither streptomycin nor gentamicin can be used to obtain synergy with a glycopeptide or B-lactam for the treatment of enterococcal infections[52,57].

The study also sought to determine the genetic relatedness of the isolates. PFGE revealed that only two E. faecalis isolates were related (Fig.2). These were resistant to vancomycin and high level gentamicin. The rest were unrelated suggesting that the strains acquired resistance independently, possibly by horizontal transfer of the resistance determinants. The absence of a dominant clone among the isolates is consistent with the fact that there has been no evidence of an outbreak.

Although the prevalence of glycopeptide resistance was low among the studied isolates, their presence together with high-level aminoglycoside resistance calls for regular surveillance of antibacterial susceptibilities to detect emerging resistance and prevent the establishment and spread of multiply antibacterial-resistant strains.
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