A study of high level aminoglycoside resistant enterococci

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Enterococci are a common cause of nosocomial infection and prevalence of antibiotic resistance among them is increasing. This study aimed to identify the prevalence of high level aminoglycoside resistant enterococci at Alexandria Main University Hospital. A total of 133 enterococci strains isolated from clinical specimens were all subjected to Bauer Kirby disc diffusion to detect antibiotic susceptibility pattern. High level aminoglycoside resistance (HLAR) and vancomycin resistance were confirmed by minimum inhibitory concentration (MIC). The HLAR enterococci were further identified by API 20 STREP to species level and nitrocefin test was used to detect beta lactamase production. Furthermore, polymerase chain reaction (PCR) for detection of gentamycin resistance was done to all HLGR enterococcal strains and for detection of vancomycin resistance genes. Among the 133 enterococcal isolates, 47 (35.3%) were found to be HLAR (31 Enterococcus faecalis, 13 Enterococcus faecium and 3 Enterococcus avium). They were all negative for beta lactamase production, 78.7% were erythromycin resistant, 63.8% resistant to doxycyclines, 51.06% to chloramphenicol, 46.8% to penicillin, 42.5% to rifampicin, and 40.4% to ampicillin. All HLAR enterococcal isolates were sensitive to Teigycyciln and Linezolid except one strain was resistant to linezolid. Urinary enterococcal isolates were also found to be 88.4, 84.6, 80.7 and 15.3% resistant to ciprofloxacin, levofloxacin, norfloxacin, and nitrofurantoin, respectively. Regarding PCR, all HLGR strains had Aac 6’-Ie-aph (2’)-Ia gene except for 2 strains. It was found also that 3 HLAR enterococcal strains were vancomycin resistant, all of which were E. faecium with Van A genotype. HLAR enterococci constituted 35.3% from the total enterococci isolated during the period of study denoting the importance of these isolates as nosocomial pathogens. This situation obligates the clinical microbiologist to try to identify the most useful active antibiotic for treatment. On the other hand, physicians should use antibiotics appropriately and comply with the infection-control policies in an effort to prevent further spread of high level aminoglycoside resistant enterococci.

Key words: Alexandria Egypt, enterococci, high level aminoglycoside resistance, aminoglycosides, gentamycin, antibiotic resistance, vancomycin.

INTRODUCTION

Enterococci have constituted a unique taxonomic entity since the mid-1980s when results of DNA–DNA hybridization experiments suggested their separation into the new bacterial genus, Enterococcus species from the former genus Streptococcus species (Werner, 2013). It has emerged as a super nosocomial infecting pathogen not only due to their inherent resistance to multiple antimicrobial agents (as, clindamycin, cephalosporins...
and aminoglycosides), but also because they have the capacity to acquire and disseminate determinants of antibiotic resistance (as vancomycin resistance gene clusters). Moreover, the increasing number of predisposed patients who are hospitalized and are immunosuppressed, catheterized and receiving multiple antimicrobial agents has associated this organism with hospital acquired infections. (Arias and Murray, 2012).

A common regimen for treatment of serious enterococcal infections such as septicemia and endocarditis is the synergistic combination of cell wall inhibitors as penicillin, ampicillin or vancomycin with aminoglycosides such as streptomycin or gentamycin (Levison and Mallela, 2000).

Unfortunately, this synergy is lost in enterococci exhibiting high level aminoglycoside resistance (HLAR) due to production of aminoglycoside modifying enzymes which inactivate aminoglycoside by adenylation and phosphorolysis or through ribosomally mediated resistance (Gaindo et al., 2005). Making accurate detection of HLAR enterococci and rapid implementation of antibiotic policy an important issue. Also, identification at the species level of enterococci isolated from clinical specimens is considered necessary, as is quantitative evaluation of their resistance to penicillin, ampicillin, vancomycin, teicoplanin and high-level resistance to gentamicin and streptomycin (Facklam et al., 1999).

High level gentamicin resistance (minimum inhibitory concentration [MIC]≥500 µg/ml) in enterococci is predominantly mediated by aac (6')-le-aph(2')-Ia, which encodes the bifunctional aminoglycoside modifying enzyme (AME) AAC(6')-APH(2'). Recently, newer AME genes such as aph(2')-Ib, aph(2')-Ic and aph(2')-Id have been detected as also conferring gentamicin resistance in enterococci (Padmasini et al., 2014).

The aim of the present work was to study the antibiotic susceptibility pattern of HLAR enterococci among enterococcal isolates in Alexandria Main University Hospital.

MATERIALS AND METHODS

The study was carried out on 133 enterococcal strains that were isolated from different clinical samples referred to routine microbiology laboratory over a period of six months.

Strains that were suspected to be enterococci from their colonial morphology on blood agar were further subjected to Gram staining, Catalase test (negative) and growth on bile Esculin agar (grew as black colonies with black halo) (Wade, 1997). Those strains were subjected to antimicrobial susceptibility testing by the Bauer Kirby method as recommended by CLSI (2014), including testing sensitivity to discs of gentamycin (120 µg) and streptomycin (300 µg) to identify those strains of enterococci that possessed high level aminoglycoside resistance (HLAR). Also, strains proved to be vancomycin (30 µg) resistant (diameter zone ≤14 mm) and teicoplanin (30 µg) resistant (diameter zone ≤10 mm) by Bauer Kirby technique were subjected to motility testing using soft agar to exclude Enterococcus gallinarum or Enterococcus casseliflavus which are the only motile enterococci which have intrinsic vancomycin resistance mechanism (CLSI, 2014). All HLAR were further studied by identifying their species level using analytical profile index API 20 STREP according to the manufacturer instructions (Biomerioux, Marcy L’ Étoile France) and they were also tested for beta lactamase production using nitrocefin discs (Oxoid).

MIC broth micro dilution method

All strains which were previously identified as HLGR by disc diffusion (zone diameters were 6 mm) for gentamycin (120 µg), were confirmed by MIC using broth microdilution to gentamycin. The results were read for turbidity, any growth at 512 µg was considered HLGR enterococci. MIC was also done for vancomycin and teicoplanin resistant strains among HLAR strains using the CLSI recommended breakpoints shown in Table 1. Enterococcus faecalis ATCC 29212 was used as a negative control (CLSI, 2014).

PCR

PCR was done for detecting gentamycin resistance genes and Van A and Van B resistant enterococcal genotypes among HLAR isolates using the primers shown in Table 2 (Padmasini et al., 2014; Biendo et al., 2010).

DNA extraction

DNA extraction was done by suspending 3 to 5 colonies of enterococci grown overnight on blood agar in 25 ul of a 0.25% sodium dodecyl sulfate 0.05 N NaOH solution and boiled for 15 min. Then, 200 µl of H2O was added to the mixture, then centrifugation at 4000 rpm for 1 min was done and DNA was obtained from the supernatant for PCR reactions (Mounir, 2011).

PCR for detection of HLAR

PCR was carried out in 2 separate reaction tubes one for Aac (6')-le-aph (2'/)-Ia gene and the second for the other 3 genes (Aph (2'/)-Ib, Aph (2'/)-Ic, Aph (2'/)-Id) as multiplex PCR according to Padmasini et al. (2014) method with the following amplification conditions: initial denaturation (95°C for 5 min), followed by 32 cycles each of: Denaturation (95°C for 1 min), annealing (58°C for 1 min), extension (72°C for 1 min), final extension (72°C for 5 min) (Padmasini et al., 2014).

Conventional PCR for identification of vancomycin resistance genes done according to Biendo method

The amplification of DNA was done by the following cycling

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program that consisted of initial denaturation (94°C for 2 min). Thirty cycles each consists of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min (Biendo et al., 2010).

Statistical analysis used in this study

Qualitative data were described using number and percent. Comparison between different groups regarding categorical variables was tested using Chi-square test. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

RESULTS AND DISCUSSION

Since early 1970s, enterococci were considered as nosocomial pathogens which coincided with increased expression of antimicrobial resistance by members of the genus and this contributed to extensive administration and misuse of antimicrobial agents (Dadfarma et al., 2013).

In our study, a total of 133 (6.3%) enterococcal strains were isolated from 2100 clinical specimens during our 6 months study period, with the highest rate of isolation being from urine; 86 strains (64.6%), followed by pus 21 strains (15.7%), blood 15 (11.2%), then sputum 10 (7.5%) and only 1 (2.1%) strain was isolated from peritoneal aspirate. These results were comparable to others, who found that maximum number of enterococci isolates were from urine samples. Which is consistent with enterococci being one of the leading causes of UTI and associated with the increased usage of in dwelling urinary catheter in our hospital (Preeti et al., 2013; Adhikari, 2010).

Antimicrobial susceptibility pattern and HLAR among our isolates were detected using disc diffusion method according to CLSI guidelines; where 47 strains (35.3%) were HLAR and the remaining 86 (64.6%) strains were non-HLAR among which 72, 43, 41.9, 26.7, 20, 6.9, 6.9 and 3.4% were resistant to erythromycin, penicillin, rifampicin, doxycycline's, chloramphenicol, vancomycin, teicoplanin and linezolid. Regarding HLAR enterococci (47 strains), the highest resistance was also to erythromycin (78.7%) followed by doxycycline's (63.8%), chloramphenicol (51.06%), penicillin (46.8%), rifampicin (42.5%) and ampicillin (40.4%). The resistance to vancomycin and teicoplanin was 6.3% for each and only 2.1% were resistant to linezolid. Doxycycline, chloramphenicol, and ampicillin resistance were significantly higher among HLAR enterococci than non HLAR enterococci with P value 0.021, 0.030 and 0.001, respectively (Table 3). As mentioned earlier, the greatest percentage of resistance was recorded for Erythromycin (74.4%), this was in agreement with Mounir et al. (2011), while Jain et al. (2011) reported that 100% of their enterococcal isolates were erythromycin resistant, which may be attributed to frequent use of Macrolides for empirical treatment of many infections.

In the current study, 6.8% of our enterococcal isolates were glycopeptide resistant, which although is higher than some rates reported in literature (Mounir et al., 2011; Asha Peter et al., 2013), but it is still relatively reassuring that 93% of our isolates are glycopeptide susceptible. As this situation is contrary to the situation in most hospitals in the USA (Perlada et al., 1997) and Europe (Schouten et al., 2000) where high prevalence of vancomycin resistance reached >20% in Ireland, Greece, Portugal as reported by The European Antimicrobial Resistance Surveillance System (EARSS).

Linezolid and tigecycline are the alternative option for treatment of vancomycin resistant enterococci (VRE) (Tsai et al., 2012). In this study, the resistance pattern to linezolid among our isolates whether HLAR or non HLAR was very low and none of them were tigecycline resistant.

Table 1. CLSI MIC interpretative criteria break points (µg/ml) to Vancomycin and Teicoplanin.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin</td>
<td>≥32</td>
<td>16-8</td>
<td>≤4</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>≥32</td>
<td>16</td>
<td>≤8</td>
</tr>
</tbody>
</table>

Table 2. Oligonucleotide primers used in PCR assay for detection of HLAR and Vancomycin resistant genotype Enterococci.

<table>
<thead>
<tr>
<th>Gene (6')-le-aph (2/)-la</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aac (6')-le-aph (2/)-la</td>
<td>CAGGAAATTATCGAAAAATGTTAGAAAAG</td>
<td>CACAATCGACTAAAGAGTACCAATC</td>
<td>369</td>
</tr>
<tr>
<td>Aph (2/)-lb</td>
<td>CTTGGACGCTGAGATATGAGCACC</td>
<td>GTTTGTAGCAATTGAGAAACCCCTT</td>
<td>867</td>
</tr>
<tr>
<td>Aph (2/)-lc</td>
<td>CCACATGATAATGACTCGTTCCC</td>
<td>CCACAGCTTCCGATAGCAAGAG</td>
<td>444</td>
</tr>
<tr>
<td>Aph (2/)-Id</td>
<td>GTGTTTTTACAGGAATGCCATC</td>
<td>CCCCTCTCATACCAATCCCATATAAC</td>
<td>641</td>
</tr>
<tr>
<td>Van A</td>
<td>GGG-AAA-ACG-ACA-ATT-GC</td>
<td>GTA-CAA-TGC-GGC-CGT-TA</td>
<td>732</td>
</tr>
<tr>
<td>Van B</td>
<td>ATG-GGA-AGC-CGA-TAG-TC</td>
<td>GAT-TTC-GTT-CTT-CCA-CC</td>
<td>635</td>
</tr>
</tbody>
</table>
No substantial difference in our result and the results of multiple studies that reported no or minimal resistance among their isolates (Vaibhav et al., 2013; Asha Peter et al., 2013; Sieńko et al., 2014). So tigecycline and linezolid are up till now the drugs of choice for infections caused by VRE. However, the emergence of any linezolid and or tigecycline resistant enterococci is an alarming problem in the treatment of VRE infections.

As regard urinary antibiotic resistance in the present study, 63.9, 61.6 and 56.9% of the urinary enterococcal isolates were resistant to ciprofloxacin, levofloxacin and norfloxacin, respectively, while 91.7% were sensitive to nitrofurantoin as shown in Table 4. This high resistance rate to ciprofloxacin can be attributed to its frequent use in empirical treatment of UTIs (Preeti et al., 2013; Vaibhav et al., 2013). Our high percentage of sensitive urinary enterococci to nitrofurantoin was in agreement with Preeti et al. (2013) who reported that 88.5% of urinary enterococcal isolates were sensitive to nitrofurantoin. Lower percentage was reported by Butcu et al. (2011) who had 60% of their urinary enterococcal isolates sensitive to nitrofurantoin. Looking at the 26 HLAR urinary enterococcal isolates we found that The percentage of resistance to ciprofloxacin, levofloxacin and norfloxacin were significantly higher than non HLAR enterococci with P value 0.013, 0.016 and 0.021, and one of our HLAR urinary isolates was vancomycin resistant and nitrofurantoin sensitive, putting this together with the high resistance rate among our enterococci to the previously mentioned antibiotics, a consideration should be made to discourage the irrational use of nitrofurantoin and keep it as a possible drug of choice for the treatment of resistant urinary enterococcal isolates (Butt et al., 2004).

Multidrug resistant (MDR) strains were 84.2% (112) of the 133 enterococcal isolates, including all 47 HLAR enterococci and 65 were non HLAR enterococci. Multidrug resistance was also reported by Jain et al. (2011) as 71% of his enterococcal isolates were multidrug resistant. On the other hand, Dadfarma et al. (2013) reported that 45.7% were MDR and 31.7% among them were HLGR.

We further studied our 47 (35.3%) HLAR Enterococcus isolates; they were all resistant to gentamicin and among them 27.07% showed combined resistance to both high level gentamicin and streptomycin. These results were confirmed by performing MIC testing and all 47 strains were found resistant to gentamicin concentration of up to 512 µg/ml, making HLGR testing an accurate marker for detecting HLAR enterococci (Ira et al., 2013; Bhatt et al., 2015).

The species distribution among our HLAR enterococci was 65.9% E. faecalis, 27.6% Enterococcus faecium and 6.3% Enterococcus avium. This was on the contrary to some reports indicating that HLAR is a more common problem among E. faecium isolates (Abamecha et al.,

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### Table 3. Resistance pattern to Enterococci isolated in the study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non HLAR (86)</th>
<th>HLAR (47)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin (120 µg)</td>
<td>0</td>
<td>47 (100)</td>
<td>47 (35.3)</td>
</tr>
<tr>
<td>Sulfamethoxazole (200 µg)</td>
<td>0</td>
<td>36 (76.6)</td>
<td>36 (27.1)</td>
</tr>
<tr>
<td>Erythromycin (25 µg)</td>
<td>26 (30.7)</td>
<td>37 (78.5)</td>
<td>63 (47.9)</td>
</tr>
<tr>
<td>Penicillin (5.1 µg)</td>
<td>37 (43.0)</td>
<td>22 (46.8)</td>
<td>59 (44.4)</td>
</tr>
<tr>
<td>Rifampicin (15 µg)</td>
<td>36 (41.9)</td>
<td>20 (42.5)</td>
<td>56 (42.1)</td>
</tr>
<tr>
<td>Doxycycline's</td>
<td>30 (35.8)</td>
<td>48 (62.7)</td>
<td>78 (58.3)</td>
</tr>
<tr>
<td>Chloramphenicol (30 µg)</td>
<td>18 (20.9)</td>
<td>24 (51.0)</td>
<td>42 (31.6)</td>
</tr>
<tr>
<td>Amoxicillin (10 µg)</td>
<td>6 (7.1)</td>
<td>19 (40.4)</td>
<td>25 (18.7)</td>
</tr>
<tr>
<td>Vancomycin (0.06 µg)</td>
<td>6 (7.1)</td>
<td>1 (2.1)</td>
<td>7 (5.2)</td>
</tr>
<tr>
<td>Teicoplanin (0.06 µg)</td>
<td>6 (7.1)</td>
<td>3 (6.3)</td>
<td>9 (6.8)</td>
</tr>
<tr>
<td>Linezolid (0.005 µg)</td>
<td>3 (3.5)</td>
<td>1 (2.1)</td>
<td>4 (3.1)</td>
</tr>
<tr>
<td>Teigycyclin (0.005 µg)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>86 (64.6)</td>
<td>47 (35.3)</td>
<td>133 (100)</td>
</tr>
</tbody>
</table>

### Table 4. Urinary isolates resistance pattern of the Enterococci to the 4 antibiotics used in UTI.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non HLAR (%)</th>
<th>HLAR (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>32 (53.3)</td>
<td>23 (88.4)</td>
<td>55 (63.9)</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>31 (51.6)</td>
<td>22 (84.6)</td>
<td>53 (61.6)</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>28 (46.7)</td>
<td>21 (80.7)</td>
<td>49 (56.9)</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>7 (11.6)</td>
<td>4 (15.3)</td>
<td>11 (12.7)</td>
</tr>
<tr>
<td>Total</td>
<td>60 (69.7)</td>
<td>26 (55.3)</td>
<td>86 (100)</td>
</tr>
</tbody>
</table>
2015; Bhatt et al., 2015), while others stated that HLR was equally distributed among both species (Fernandes and Dhanashree, 2013). However, species identification has gained much importance because of the naturally occurring differences in the susceptibility of these species (Arias and Murray, 2012).

Among HLR enterococci, aminoglycoside modifying enzymes genes (Aac (6/)-le-aph (2//)-la) was detected in 45 (95.7%) of HLR enterococci (Figure 1); constituting 96.5% of E. faecalis and 91.6% of E. faecium. While only 2 (4.3%) were found to have Aph (2//)-Ic gene; constituting 3.4% of E. faecalis and 8.3% of E. faecium. On the other hand, Aph (2//)-Ib and Aph (2//)-Id were not detected in any isolates. This was in agreement with Wanxiang et al. (2015) and Li et al. (2015) who stated that 94.4% of HLR enterococci were Aac (6/)-le-aph (2//)-la positive and in contrast to our results he detected Aph (2//)-Id in 1.3% of his HLR enterococcal isolates and none of their strains were positive to Aph (2//)-Ic or Aph (2//)-Ib. Padmasini et al. (2014) stated that Aac (6/)-le-aph (2//)-la found in 68.4% of their HLR enterococcal isolates, while none of other genes were detected and attributed. The HLR among the rest of enterococcal isolates to other mechanisms or other genes not discovered till now.

VRE among HLR enterococci constituted 3 of the 47 strains. They were all E. faecium constituting 23.07% (3 out of the 13 E. faecium strains). This high distribution of VRE among E. faecium may be attributed to Inc. 18 plasmid which is a broad spectrum plasmid that helps enterococci to get vancomycin resistance gene from vancomycin resistant Staphylococci (Zhu et al., 2008). In contrast to many studies (Abdulhakim et al., 2014; Hasani et al., 2012; Ira et al., 2013), Adhikari et al. (2010) did not report any VRE.

In this study all the VRE among the HLR enterococci were confirmed by MIC and found not to be only resistant to vancomycin and teicoplanin (≥32 µg/ml), but also showed high level Vancomycin and Teicoplanin resistance (256 µg/ml) and were all proved to be Van A genotype explaining the presence of high level resistance to both vancomycin and teicoplanin. These results were in agreement with other multiple studies (Vaibhav et al., 2013; Lee et al., 2013). On the other hand Ira et al. (2013) reported that 96.9% were Van A genotype and were E. faecalis except one isolate which was Enterococcus gallinarum in combination with intrinsic Van C genotype, 2 isolates were Van B, both were Enterococcus munditii.

Glycopeptide resistance in enterococci is one of the most important challenges. VRE takes place among the important nosocomial pathogens, in that the treatment options are limited, it easily spreads in the hospital setting through contaminated hands and surfaces and it is likely to transfer vancomycin resistance to other pathogens. As VRE is known to spread in the hospital setting. Centers for Disease Control and Prevention (1995) suggests that aggressive infection control be implemented and that hospital staff conform to the isolation precautions in order to control and prevent VRE infection.

None of the HLR Enterococcus isolates were beta lactamase producers by nitrocefene test as was the situation with Mounir et al. (2011) and Asha Peter et al. (2013), while Jain et al. (2011) found only one out of 66 HLR enterococcal isolate to be Beta lactamase producer. This explains that the resistance to beta lactam antibiotics in our HLRG enterococci is not due to beta lactamase enzyme and may be attributed to accumulation of point mutations in the penicillin binding region of PBP5.

To conclude, the current study highlighted the importance of HLR enterococci as nosocomial pathogens in our setting. Detecting HLR is an important task; it should be adopted as a part of the routine microbiology work. Prevention of growing resistance to linezolid and tigecycline among vancomycin resistant enterococcal isolates should be our rational in fighting antibiotic resistant enterococci. This could be achieved by

Figure 1. Lanes 1, 2, 3, 4, 6, 8, 9 show the presence of Aac (6/)-le-aph (2//)-la (369 bp).
careful monitoring of their resistance pattern and adherence to an antibiotic policy created by the infection control team. Another point to be emphasized is the importance of nitrofurantoin as a therapeutic option for resistant urinary enterococcal infections.

**Conflict of interests**

The authors have not declared any conflict of interests.

**REFERENCES**


