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Detection of high level aminoglycoside resistance genes among clinical isolates of *Enterococcus* species

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Abstract

Background: Enterococci are intrinsically resistant to clinically achievable concentrations of aminoglycosides. However, high-level resistance to aminoglycosides (HLAR) is primarily due to the acquisition of genes encoding aminoglycoside-modifying enzymes (AMEs). Aminoglycosides along with cell wall inhibitors are given clinically for treating enterococcal infections. The current study was conducted to investigate the rate of HLAR and to determine aminoglycoside resistance encoding genes profile in enterococcal isolates from different clinical specimens.

Results: From 120 *Enterococcus* species, 50 (41.7%) enterococcal isolates were proven to have HLAR, 78% (39/50) have high-level gentamicin resistance (HLGR), and 74% (37/50) were high-level streptomycin-resistant (HLSR). HLGR isolates carried aminoglycoside modifying gene *aac (6′)-Ie-aph (2′)-Ia* in 26/39 (66.7%) of isolates, whereas 32/37 (86.5%) of HLSR carried *aph (3′)-IIIa* gene and were observed in *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. casseliflavus*. The *aph (2′)-Ib*, *aph (2′)-Ic*, and *aph (2′)-Id* that encode HLGR could not be detected.

Conclusions: The high detection rate of HLAR among the studied *Enterococcus* species and the coexistence of HLGR and HLSR strains provide crucial insights to the necessity of routine testing for HLAR in the microbiology lab. The main AME genes among HLGR and HLSR enterococci were *aac (6′)-Ie-aph (2″)-Ia* and *aph (3′)-IIIa*, respectively.

Keywords: *Enterococcus* species, High-level aminoglycoside resistance, Aminoglycoside-modifying enzyme gene

Background

Enterococci have emerged as an important source of hospital-acquired infections, including those related to the surgical site, respiratory tract, urinary tract, skin and soft tissue infections, and bacteremia. Control and treatment of enterococcal infections are problematic due to their intrinsic resistance to various antimicrobials, their capabilities to develop new resistance and to survive in the external environment for a long time [1, 2].

Enterococci acquire resistance to a wider range of antimicrobial agents particularly, aminoglycosides, glycopeptides, and beta-lactams. This poses a therapeutic challenge to clinicians as they are left with very few treatment options [3, 4]. A common regimen for the treatment of serious enterococcal infections is the synergistic combination of cell wall inhibitors as vancomycin with aminoglycosides [5].

Although enterococci are intrinsically resistant to low levels of aminoglycosides, high-level resistance to aminoglycosides (HLAR) is mediated by acquisition of genes encoding aminoglycoside-modifying enzymes (AME). High-level gentamicin resistance (HLGR) in enterococci is predominantly mediated by *aac (6′)-Ie-aph(2′)-Ia* gene, which encodes the bifunctional aminoglycoside modifying enzyme AAC (6′)-APH (2′). The action of such enzyme in enterococci eliminates the synergistic activity of gentamicin when combined with a cell wall active agent, such as ampicillin or vancomycin. Other AME genes conferring gentamicin resistance such as *aph (2′)-Ib*, *aph (2′)-Ic*, and *aph (2″)-Id* have been also detected in enterococci. Furthermore, high-level streptomycin and kanamycin resistance in enterococci are mediated by *aph (3′)-IIIa* [6].

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To our knowledge, limited studies on AME genes profile were done in Egypt. The current study was conducted to investigate the rate of HLAR and to determine aminoglycoside resistance-encoding genes profile in enterococcal isolates from different clinical specimens.

Methods

Bacterial isolates and species identification

A prospective study was conducted from November 2016 to March 2018. A total number of 120 non-repetitive enterococcal isolates were collected from different clinical samples from outpatient clinic and hospitalized patients. The clinical specimens were initially cultured on MacConkey agar (HiMedia, India) and Cysteine-Lactose-Electrolyte-Deficient (CLED) media (Bio-Rad, USA). Isolates of enterococci were identified by Gram staining, colony morphology, catalase test, and growth on Bile Esculin agar (Oxoid, England). All isolates were identified to species level using Vitek2 automated system (bioMérieux, France).

Detection of HLAR in enterococcal isolates

Enterococcus species isolates were screened for HLAR by Kirby-Bauer disc diffusion method using streptomycin (300 μg) and gentamicin (120 μg) discs (Bio-Rad, France), and results were interpreted according to the Clinical and Laboratory Standards Institute [7]. Isolates that revealed HLAR by disc-diffusion method were further tested for determining the minimum inhibitory concentrations (MICs) of streptomycin and gentamicin using the E-test (bioMérieux Mercy, France). Overnight bacterial suspensions of test isolates were adjusted to 0.5 MacFarland turbidity and were plated on Muller Hinton agar (MHA) (HIMEDIA) plates and E-test strips (Bio-Merieux France) were applied. The plates were incubated at 35 °C for 15–18 h. Results were interpreted according to The European Committee on Antimicrobial Susceptibility Testing [8].

Bacterial isolates were stored at − 70 °C in the form of glycerol stock until processed for DNA extraction and molecular analysis of AME [9].

Molecular analysis of aminoglycoside modifying genes by PCR assay

Genomic DNA was extracted from Enterococcus strains by cell lysis using a simple boiling technique [10]. The quality and quantity of DNA were assessed with NanoDrop 1000 (Thermo Scientific, USA). PCR assay for AME genes: aac (6′)-Ie-aph (2″)-Ia, aph (2″)-Ib, aph (2″)-Ic, aph (2″)-Id, and aph (3′)-IIIa, was carried out using 4 μL of bacterial DNA extract, 25 pmol of each primer (Table 1), 200 mM of each dNTP (Promega, Inc., USA), 10 mM KCl PCR buffer, 1.5 mM $MgCl_2$, and 1.5 U Taq polymerase (Gotaq Flexi DNA, M8305, Promega, Inc., USA). Amplification was carried out on a Bio-Rad thermal cycler using standard PCR protocol. The cycling conditions were as follows: 5 min of initial denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and strand extension at 72 °C for 1 min and a final extension for 10 min [11]. A negative control (lacking DNA) was included in each PCR assay. PCR products were analyzed on a 2% agarose gel stained with ethidium bromide. DNA amplicons were visualized using a gel documentation system (cleaver scientific, UK).

Results

A total of 120 *Enterococcus* species isolates were revealed from different clinical samples. The predominant species comprised *E. faecalis* 68 (56.7%), *E. faecium* 36 (30%), *E. gallinarum* 12 (10%), and *E. casseliflavus* 4 (3.3%). Among the studied 120 *Enterococcus* species isolates, 50 (41.7%) were HLAR. The highest rate of isolation was from urine specimens 72% (36/50), followed by blood 14% (7/50), pus 6% (3/50), wound swabs 4% (2/50), and both of ascetic fluid and sputum revealed only 1 strain (1/50) for each of them representing 2%.

The 50 HLAR isolates included 78% (39/50) HLGR and 74% (37/50) HLSR isolates by disc-diffusion method. HLAR was confirmed in all 50 *Enterococcus* species isolates by E test and had MIC values of > 512 μg/ml and > 128 μg/ml for gentamycin and streptomycin respectively.

Table 1 Primer sequences for amplification of aminoglycoside resistance genes used in PCR assay

Target genes	Primer sequences (5′-3′)	Amplicon bp	References
aac(′)-Ie-aph(2″)-Ia	5′-CAGGAATTTATCGAAAATGGTAGAAAAG-3′ 5′-CACAATCGACTAAAGAGTACCAATC-3′	369	[11]
aph(2″)-Ib	5′-CTTGGACGCTGAGATATATGAGCAC-3′ 5′-GTTTGTAGCAATTCAGAAACACCCTT-3′	867	
aph(2″)-Ic	5′-CCACAATGATAATGACTCAGTTCCC-3′ 5′-CCACAGCTTCCGATAGCAAGAG-3′	444	
aph(2″)-Id	5′-GTGGTTTTTACAGGAATGCCATC-3′ 5′-CCCTCTTCATACCAATCCATATAACC-3′	641	
aph(3″)-IIIa	5′ -GGCTAAAATGAGAATATCACCGG-3′ 5′-CTTTAAAAAATCATACAGCTCGCG-3′	523	

HLAR was predominant in *E. faecalis* 60% (30/50) followed by *E.faecium* 30% (15/50), *E. gallinarum* 8% (4/50), and *E. casseliflavus* 2% (1/50) by Vitek2 compact system. The species distribution and specimen source of HLAR *Enterococcus* strains were listed in Table 2.

Detection of AME encoding genes was performed for the 50 HLAR *Enterococcus* species isolates and showed that 39 (78%) were HLGR of which 26/39 (66.7%) of the isolates carried the *aac (6′)-Ie-aph (2″)-Ia* gene (Table 3, Fig. 1), while from the HLSR 37(74%) *Enterococcus* species strains, 32/37 (86.5%) carried *aph (3′)-IIIa* gene (Table 3, Fig. 2). Aminoglycoside resistance genes as *aph (2″)-Ib*, *aph (2′)-Ic*, and *aph (2′)-Id* that encode HLGR could not be detected among the studied isolates.

E. faecalis and *E. faecium* were the predominant *Enterococcus* species isolates of the present study. They were found to carry the bifunctional enzyme encoding gene *aac (6′)-Ie-aph (2″)-Ia* in 17/30 (56.7%) and 8/15 (53.3%) strains respectively and *aph (3′)-IIIa* gene in 18/30 (60%) and 11/15 (73.3%) strains respectively (Table 3).

The coexistence of *aac (6′)-Ie-aph (2″)-Ia* and *aph (3′)-IIIa* genes were revealed in *E. faecalis*, *E. faecium*, and *E .casseliflavus* strains. The two *E. gallinarum strains* were found to carry *aph (3′)-IIIa* gene only (Table 4).

Discussion

Enterococci have long been considered as one of the most common causes of nosocomial infections. The rise of drug-resistant strains presents a serious problem to control in enterococcal infections. Several resistant *Enterococcus* species have been reported, including *E. faecalis*, *E. faecium E. avium*, *E. durans*, *E. gallinarum*, *E. casseliflavus*, *E. raffinosus*, *E. mundtii*, *E. malodoratus*, and *E. hirae* [12]. As previously found in Egypt [5, 13], *E. faecalis* and *E. faecium* are the predominant strains revealed in all clinical specimens in the present study.

Aminoglycosides are considered efficient in treating serious infections caused by both Gram-positive and Gram-negative organisms. However, the acquisition of extrinsic resistance to high-level aminoglycoside antibiotics in enterococci renders these strains a serious challenge in clinical settings [14].

Table 2 Distribution of HLAR *Enterococcus* species from various clinical specimens

Clinical specimens	Distribution of *Enterococcus* species (*n* = 50)				
	E. faecalis	*E. faecium*	E. *gallinarum*	E. *casseliflavus*	Total
Urine	19	13	3	1	36
Blood	7	0	0	0	7
Wound	2	0	0	0	2
Ascitic fluid	0	0	1	0	1
Pus	2	1	0	0	3
Sputum	0	1	0	0	1
Total	30	15	4	1	50

Data are presented as numbers (*N*)

Table 3 Distribution of aminoglycoside modifying enzyme-encoding genes among *Enterococcus* species with high-level aminoglycoside resistance

HLAR MIC and detection of genes by PCR	Distribution of HLAR in *Enterococcus* spp. (*N* = 50)				
	E. faecalis (30)	*E. faecium* (15)	*E. gallinarum* (4)	E. *casseliflavus* (1)	Total (*N*)
HLGR (MIC)	21	15	2	1	39
HLGR (gene detection)					
aac(6′)-Ie-aph(2″)-Ia	17	8	0	1	26
aph(2″)-Ib	0	0	0	0	0
aph(2″)-Ic	0	0	0	0	0
aph(2″)-Id	0	0	0	0	0
HLSR (MIC)	23	10	3	1	37
HLSR (gene detection)					
aph(3′)-IIIa	18	11	2	1	32

• Data are presented as numbers (*N*)
HLAR high-level aminoglycoside resistance, *HLGR* high-level gentamycin resistance, *HLSR* high-level streptomycin resistance

Distribution of HLAR in enterococcal isolates varied in different reports in the world. The current study results (41.7%) were comparable to those obtained by El-Ghazawy et al. [5] (35.3%) that studied the prevalence of HLAR in 133 enterococcal strains obtained from different clinical samples at Alexandria Main University Hospital, Egypt. However, higher rates of HLAR enterococci (72%) were obtained by Padmasini et al. [11] in India. Another study by El Mahdy et al. [15] at Mansoura University Hospitals in Egypt showed also higher HLAR results (66.3%) in 80 enterococcal isolates recovered from urine samples. The present study revealed a high prevalence of HLSR strains (74%), although the clinical use of streptomycin for infections caused by enterococci has long been restricted due to intrinsic low-level resistance of the organism [11].

Previous studies by Padmasini et al. [11], Bhatt et al. [16], and Niu et al. [17] have reported that HLGR (42.7%, 65%, and 42.7%) was more common than HLSR (29.8%, 45%, and 27.4%) in all species of isolated enterococci respectively. On the contrary, there was nearly no difference between the prevalence rates of HLGR and HLSR among our studied *Enterococcus* species isolates. Similar finding was found in Turkey by Kurtgoz et al. [18].

HLAR was found to be more common in *E. faecalis* and *E. faecium* [19], on the other hand, Abamecha et al. [20] and Bhatt et al. [16] reported that HLAR was a common problem among *E. faecium* isolates only. A

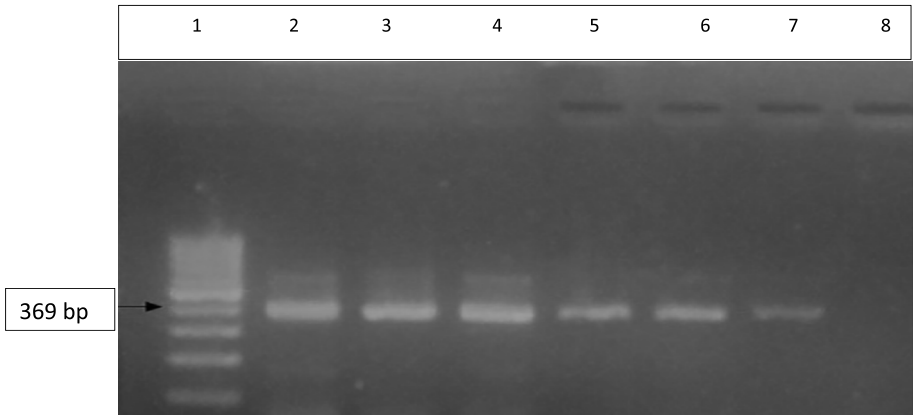

Fig. 1 Agarose gel electrophoresis of amplified product AME gene generated by PCR assay. Lane 1: molecular weight marker (ladder 100 bp); lane (2–7): aac (6′)-Ie-aph(2″)-Ia positive (369 bp). Lane 8: negative control

surveillance study that was conducted in 20 European countries had reported 32% and 22% HLGR and 41% and 49% HLSR among HLAR *E. faecalis* and *E. faecium*, respectively [19]. In the current study, HLGR is 70% and 100%, while HLSR is 76% and 67% among *E. faecalis* and *E. faecium* respectively. This emphasizes susceptibility differences within different enterococcal species.

In accordance with previous studies [5, 11, 17], the *aac (6′)-Ie-aph (2′)-Ia* gene (66.7%) and *aph (3′)-IIIa* (86.5%) were identified as the most common AME genes among HLGR and HLSR strains, respectively. Nevertheless, the prevalence of the previous two genes was variable among these former studies. Padmasini et al. [11] stated that the *aac (6′)-Ie-aph (2′)-Ia* was found in 68.4% of their HLAR enterococcal isolates which is comparable to our results, while 77.4% carried *aph (3′)-IIIa* gene. Niu et al. [17] and El Ghazawy et al. [5] revealed also higher rates of *aac (6′)-Ie-aph (2″)-Ia* gene being 89.3% and 95.7%, respectively, which reflects their finding of higher pervasiveness of HLGR in their isolates.

It is noteworthy that the newer aminoglycoside modifying genes *aph (2′)-Ib*, *aph (2′)-Ic*, and *aph (2′)-Id* that encode HLGR were not detected among the studied strains. This was in agreement with Padmasini et al. [11]. In contrast to our results, *aph (2′)-Ic* gene was detected by El-Ghazawi et al. [5].

Both *aac (6′)-Ie-aph (2″)-Ia* and *aph (3′)-IIIa* genes co-existed in isolated *E. faecalis*, *E. faecium*, and *E. casseliflavus* strains, which was in accordance with results obtained by Padmasini et al. [11] and El Mahdy et al. [15]. However, 9 out of the 50 of HLAR enterococcal strains did not carry any of the formerly tested genes. This may be due to the expression of genes other than genes analyzed in this study.

Conclusion

In conclusion, the high detection rate of HLAR among the studied *Enterococcus* species and the coexistence of HLGR and HLSR strains provide crucial insights into the necessity of HLAR testing as a routine microbiology procedure. The main AME genes among HLGR and

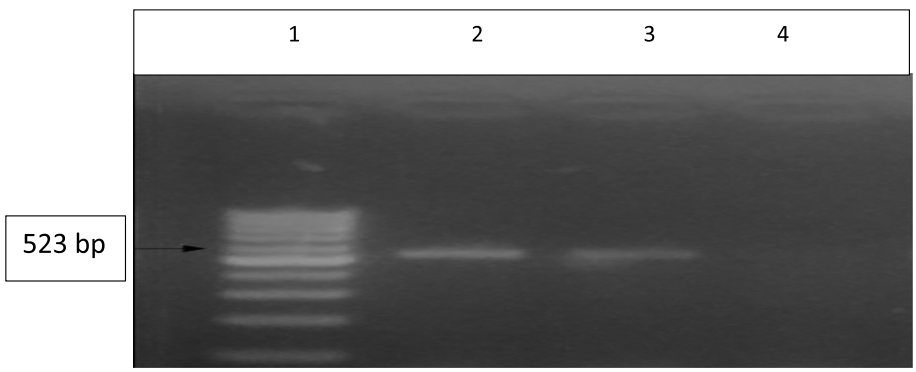

Fig. 2 Agarose gel electrophoresis of amplified product AME gene generated by PCR assay. Lane 1: molecular weight marker (ladder 100 bp); lanes (2 and 3): aph (3′)-IIIa positive (523 bp); lane (4): negative control

Table 4 Aminoglycoside modifying enzyme-encoding genes profile among the 50 high-level aminoglycoside resistance *Enterococcus* species strains

Enterococcus species	Aminoglycoside modifying enzyme-encoding genes profile			
	aac(6′)-Ie-aph(2″)-Ia + ve	*aph(3′)-IIIa* + ve	*aac(6′)-Ie-aph(2″)-Ia* + ve and *aph(3′)- IIIa* + *ve*	*aac(6′)-Ie-aph(2″)-Ia* ve and *aph(3′)- IIIa*- ve
E. faecalis (*N* = 30)	7	8	10	5
E. faecium (*N* = 15)	2	5	6	2
E. gallinarum (*N* = 4)	0	2	0	2
E. casseliflavus (*N* = 1)	0	0	1	0

Data are presented as numbers (*N*)

HLSR enterococci were *(6′)-Ie-aph (2″)-Ia* and *aph (3′)-IIIa*, respectively. The limited AME-encoding genes among the studied HLAR *Enterococcus* species highlight the restricted gene distribution and transfer of resistant genes within a geographical region. The implementation of an efficient infection control program and regular surveillance of antimicrobial resistance of enterococci is essential in order to establish a rational antibiotic policy for the better management of enterococcal infections.

Abbreviations

AMEs: Aminoglycoside-modifying enzymes; CLED: Cysteine-Lactose-Electrolyte-Deficient; CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing; HLAR: High-level resistance to aminoglycosides; HLGR: High-level gentamicin resistance; HLSR: High-level streptomycin resistance; MHA: Muller Hinton agar; MICs: Minimum inhibitory concentrations

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Authors' contributions

MD was responsible for the idea and concept of the research and revised the whole manuscript. DS analyzed, interpreted the data, and revised the manuscript. AE-S performed the microbiological identification and interpreted the results and was a contributor in writing the manuscript. AEF analyzed, interpreted the data and was a major contributor in writing and revising the manuscript. AA performed the molecular detection. ARA and IED revised the manuscript. MS performed the molecular detection and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data and material are available with the corresponding author upon reasonable request.

Ethics approval and consent to participate

All specimens included in the study were archived, and codes were used instead of patient names. The protocol of the study was approved by TBRI institutional review board under Federal Wide Assurance (FWA00010609) and the work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for Experiments in Humans and its later amendments (GCP guidelines) or comparable ethical standards.

Consent for publication

Not applicable as the specimens were coded and no patient data was used.

Competing interests

The authors declare that they have no competing interests.

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References

1. Murray BE (1990) The life and times of the enterococcus. Clin Microbiol Rev 3:46–65
2. Baldir G, Engin DO, Küçükercan M, Inan A, Akçay S, Özyürek S et al (2013) High-level resistance to aminoglycoside, vancomycin, and linezolid in *Enterococci* strains. J Microbiol Infect Dis 3(3):100–103
3. Papaparaskevas J, Vatoupoulos A, Tassios PT, Tassios A, Avalmani N, Legakis NJ, Kalapothaki V (2000) Diversity among high –level aminoglycoside – resistant enterococci. J Antimicrob Chemo 45:277–283
4. Bhatt P, Shete V, Sahni AK, Grover N, Chaudhari CN, Dudhat VL et al (2014) Prevalence of high-level aminoglycoside resistance in enterococci at a tertiary care centre. Int J Recent Sci Res 5(8):1515–1517
5. El-Ghazawy I, Okasha H, Mazloum S (2016) A study of high level aminoglycoside resistant enterococci. Afr J Microbiol Res 10(16):572–577
6. Shete V, Grover N, Kumar M (2017) Analysis of aminoglycoside modifying enzyme genes responsible for high-level aminoglycoside resistance among *Enterococcal* isolates. J Pathog 2017;(Article ID 3256952):1–5
7. CLSI (2017) *Performance Standards for Antimicrobial Susceptibilty Testing*, 27th ed. CLSI supplement M100 Wayne,PA: Clinical and LaboratoryStandards Institute.
8. European Committee on Antimicrobial Susceptibility Testing. 2016 Breakpoint tables for interpretation of MICs and zone diameters, version 6.0
9. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (Glycerol shock), vol 16, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 32–35
10. Englen, Kelley LC (2008) A Rapid DNA isolation procedure for the identification of *Campylobacter jejuni* by the polymerase chain reaction. Appl Microbiol 31:421–426
11. Padmasini E, Padmaraj R, Ramesh S (2014) High level aminoglycoside resistance and distribution of aminoglycoside resistant genes among clinical isolates of *Enterococcus* species in Chennai, India. Sci World J 4:1–5
12. Tsiodras S, Gold HS, Coakley EP, Wennersten C, Moellering RC Jr, Eliopoulos GM (2000) Diversity of domain V of 23srRNA gene sequence in different *Enterococcus* species. J Clin Microbiol 38:3991–3993
13. Diab M, Fam N, El-Baz A (2000) A study on uropathogenic *Enterococcus* species. EJMM 9(4):745–751
14. Krause K, Serio A, Connolly L (2016) Aminoglycosides: an overview. Cold Spring Harb Perspect Med 6(6):a027029
15. El-Mahdy R, Mostafa A, El-Kannishy G (2018) High level aminoglycoside resistant enterococci in hospital-acquired urinary tract infections in Mansoura, Egypt. GERMS 8(4):186–190
16. Bhatt MP, Patel A, Sahni B, Praharaj SC, Grover CN, Chaudhari CN et al (2015) Emergence of multidrug resistant enterococci at a tertiary care Centre. Med J Armed Forces India 71(2):139–144

17. Niu H, Yu H, Hu T, Tian G, Zhang L, Guo X et al (2016) The prevalence of aminoglycoside- modifying enzyme and virulence genes among enterococci with high-level aminoglycosides resistance in Inner Mongolia, China. Braz J Microbiol 47:691–696
18. Kurtgoz SO, Ozer B, Inci M, Duran N, Yula E (2016) Vancomycin and high-level aminoglycoside resistance in *Enterococcus* species. Microbiol Res 7:6441
19. Schmitz EJ, Verhoef J, Fluit AC (1999) Prevalence of aminoglycoside resistance in 20 European University hospitals participating in the European SENTRY antimicrobial surveillance programme. Eur J Clin Microbiol Infec Dis 18(6):414–421
20. Abamecha A, Wondafrash B, Abdissa A (2015) Antimicrobial resistance profile of *Enterococcus* species isolated from intestinal tracts of hospitalized patients in Jimma, Ethiopia. BMC Res Notes 8:213

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