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Brief Original Article

First detection of vanB phenotype-*vanA* genotype vancomycin-resistant enterococci in Egypt

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Abstract

Introduction: Enterococci have emerged in last two decades as serious hospital acquired pathogens particularly vancomycin resistant strains (VRE). The study aimed to identify the prevalence of enterococcal isolation from hospital infections and colonization as well as determine vancomycin resistance phenotypes and genotypes. Methods: Sixty *enterococcus* isolates were isolated from patients, health care workers and hospital environment, identified and tested for antimicrobial susceptibility. *Enterococcus* species were identified by Real-time PCR and vancomycin resistance was assessed by agar dilution method and Real-time PCR.

Results; out of 300 samples (20%) were enterococci (53.3% were *E. faecium*, 31.7% *E. faecalis* and 10% other *enterococci)*. Among of them 40/60 (66, 6%) were isolated from infections and 33.3% were isolated from colonization. multiple drug resistance was reported in (100%) of isolates, while (95%) and (45%) of isolates were resistant to vancomycin and ticoplanin respectively. VanA phenotype, *vanA* genotype was identified in (47.4%) of isolates, while vanB phenotype, *vanA* genotype was identified in (33.3%) of vancomycin resistant isolates.

Conclusion; VanB phenotype-*vanA* genotype was identified in (33.3%) of vancomycin resistant enterococcal isolates. To our knowledge it is the first identified incidence of such strains in Egypt and Africa.

Key words: *E. faecium*; *E. faecalis*; real-time PCR; VRE; vanB phenotype-*vanA* genotype.

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Introduction

Enterococci are normal intestinal flora of both animals and humans, however the importance of enterococci as pathogenic bacteria has increased with the development of their resistance to multiple drugs [1,2]. Two species of enterococci have been recognized as major hospital acquired pathogens (*Enterococcus faecium,* "*E. faecium"* and *Enterococcus faecalis, "E. faecalis"*), particularly vancomycin resistant strains (VRE) [1,3]. VRE are multi drug resistant bacteria which can spread in hospitals through medical staff, environmental surfaces and instruments causing healthcare-associated infections that are associated with increasing mortality rates and length of hospital stay [2,4]. Resistance to vancomycin is developed through acquiring the vancomycin resistance genes clusters (*vanA-M)* [5]. Several phenotypes of vancomycin resistance were recognized such as vanA, vanB, vanC, vanD and vanE phenotypes [6,7]. While vanA phenotype resistant strains are non- susceptible to both vancomycin (MICs > 64 µg/ mL) and teicoplanin (MICs >16 µg/ mL), the vanB phenotype resistant strains are non-susceptible to vancomycin but susceptible to teicoplanin [6]. *vanA* genotype of VRE (strains carry *vanA* gene) with low or moderate levels of teicoplanin resistance has emerged recently in different regions like China [8], Japan [9], Korea [10], Bulgaria [11] and later in Mexico [12] and termed by some authors as vanB phenotype-*vanA* genotype VRE [8,10]. The cause of heteroresistance to teicoplanin in *enterococcus* isolates carrying the *vanA* gene is not well understood. Some authors explained such heterogeneity by the occurrence of mutations, either in the *vanA* gene cluster or in other regulatory elements [13,14]. Vancomycin resistance among *enterococcus* isolates is an increasing burden on medical care resources in most of the developing countries, so the use of fast and accurate techniques to assess the antimicrobial resistance is critical to control the spread of resistance and to collect data about the VRE prevalence [15]. While data about enterococcal infections in Egypt are still scarce, this study aims to estimate the extent of spread of *E. faecalis* and *E. faecium* and to determine

VRE phenotypes and genotypes in patients and hospital reservoirs at Minia University Hospital, south Egypt.

Methodology

A total of 300 samples were collected from Surgery departments in Minia university hospitals, south Egypt (teaching hospitals provide care to adult and pediatric patients in 35 wards including 800 beds) in the period from October 2014 to January 2015. The samples were collected from patients (150) who developed clinical signs of infections at least 72 hours after hospital admission over the period of study (100 wound swabs from septic wounds and 50 blood cultures from patients with suspected bacteremia). Only one isolate per patient was included in the study. Moreover 75 samples from hands of healthcare workers (doctors and nurses) and 75 environmental samples (floors, walls, bed linens, water taps, toilet seats, etc.) were collected from the same departments. The study protocol was approved by the local institutional review board at the authors' affiliated institution and consent was obtained from all the study participants.

Isolation and biochemical identification of enterococci

All samples were cultured on brain heart infusion with 5% blood and bile aesculin azide agar (Oxoid). Identification of the isolated enterococci to the genus level was performed by gram staining, blackening of bile aesculin azide agar, catalase reaction; tolerance to cultivation at 10°C, 45°C and with 6.5% NaCl. Identification to the species level was performed by motility test, sugar fermentation tests (arabinose, glycerol, lactose, mannitol, maltose, sorbitol, sucrose and ribose) and pyruvate utilization test.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the isolates was determined by disc diffusion method according to the clinical laboratory standard institute guidelines [16,17] for the following antimicrobial agents; gentamicin (10 µg), vancomycin (30µg), teicoplanin (30µg), linezolid (30µg), tetracycline (30µg), ciprofloxacin (5µg), ampicillin (10µg), amoxicillin-clavulanic (30µg), erythromycin (15µg), Imipenem (10µg), Cefepime (30µg) and rifampin (5µg) (Bioanalyse, Ankara, Turkey). Minimum inhibitory concentrations (MICs) of vancomycin and teicoplanin were determined by the agar dilution method to identify the phenotypes of VRE isolates [17]. Resistant isolates to at least one member of three different antimicrobial groups were considered MDR [18].

Molecular identification of enterococcus species and detection of van A gene using real-time PCR

Enterococcal DNA was extracted using Gene JET Genomic DNA purification kit (Thermo Scientific, New York, USA) according to the manufacturer's instructions. PCR reactions were performed using real time thermal cycler (Applied Biosystem 7500 fast, New York, USA). The amplification reactions (25µL) were performed using a universal ready-to-use solution for quantitative real-time PCR (Thermo Scientific, New York, USA). Each reaction include 12.5µL Maxima SYBR green qPCR Master Mix (2X), the master mix includes Maxima Hot Start *Taq* DNA polymerase, SYBR Green I dye and dNTPs in an optimized PCR buffer. The reaction includes also, 0.3µM forward primer, 0.3µM reverse primer (Macrogen, Seoul, Korea), 0.05µL ROX solution, and 2.5µL of DNA then completed to 25µL with nuclease-free water. The genes were amplified using the following primers; F; ATCAAGTACAGTTAGTCT [19] and R; ACGATTCAAAGCTAACTG [20] for *ddl E. Faecalis,* F; TAGAGACATTGAATATGCC [19] and R; TCGAATGTGCTACAATC [20] for *ddl E. faecium,* and F GGGAAAACGACAATTGC [19] and R; GTACAATGCGGCCGTTA [19] for *vanA* gene.

Data analysis and statistics

Statistical analysis was done on SPSS package version 17.0 (SPSS Inc., Chicago, IL, USA). Chi-squared tests were performed for categorical data, while student's *t*-test (or Mann Whitney U test when appropriate) was performed for comparison of continuous data.

Results

Isolation and identification of enterococcus species

Out of the 300 collected samples; 60 isolates (20%) were identified as enterococci among of them 40/60 (66.6%) were isolated from wound samples and blood cultures while 20 (33.3%) were colonized health care workers and hospital environment. The rates of isolation from different sources were 35% (wound samples), 10% (blood samples), 14.7% (healthcare workers samples) and 12% (environmental samples) According to biochemical identification of species there were; 63.3% *E. faecium*, 10% *E. faecalis*, and 26.7% other enterococci. However, molecular identification using PCR showed different distribution of species; (32/60, 53.3%) *E. faecium*, 19 isolates (31.7%) were *E. faecalis*, while 9/60 (10%) were other species. The sources of these isolates are summarized in Table 1.

Table 1. The frequency of *Enterococci* isolation according to the source of sample.

Source	Total no. of samples	Positive cases	*E. faecium*	*E .faecalis*	others	P value
Wound	100	35 (35%)	17 (17%)	12 (12%)	6 (6%)	
Blood	50	5 (10%)	2 (4%)	1 (2%)	2 (4%)	0.001*
HCWs	75	11 (14.7%)	9 (12%)	2 (2.7%)	(0%)	
Environment	75	9 (12%)	4 (5.3%)	4 (5.3%)	1 (1.3%)	
Total	**300**	**60 (20%)**	**32 (10.6%)**	**19 (6.3%)**	**9 (3%)**	

*significant.

Antimicrobial Susceptibility of enterococcus isolates

Among the 60 tested e*nterococcal* isolates, (100%) isolates were resistant to cefepime, 57 (95%) to ampicillin, 52 (86.6%) to rifampin, 50 (83.3%) to erythromycin, 48(80%) to amoxicillin-clavulanic, 48 (80%) to gentamicin, 47 (78.3%) to tetracycline, 41 (68.3%) to ciprofloxacin, 13 (21.7%) to linezolid. 5, 11 (45%) to teicoplanin and (8.3%) to imipenem. only 3 (5%) of isolates were sensitive to vancomycin. Multidrug resistance (MDR) was detected in all isolates (100%) (Table 2).

Phenotyping and genotyping of VRE isolates

The MIC was detected to distinguish intermediate from resistant strains to vancomycin and teicoplanin that revealed, 57 isolates (95%) had MIC ≥ 128 µg/mL to vancomycin and 27 isolates (33.3%) had MIC ≥ 16 µg/mL to teicoplanin. Out of 57 VRE isolates, 27 isolates (47.3%) were vancomycin-resistant with MICs ≥ 256 µg/mL and teicoplanin resistant with MICs ≥ 16 µg/mL, that represents the vanA phenotype and they were also van A genotype (positive to *van A* gene). They were isolated from different sources, 13 *E. faecium* isolates (11 isolates from wound samples and 2 from environmental samples) and 14 *E. faecalis* (12 isolates from wound samples, 1 isolate from blood cultures and 1isolate from health care workers samples). However 19/57 (33.3%) of isolates were resistant to vancomycin with MICs > 256 µg/ mL and sensitive to teicoplanin with MICs ≤ 2µg/ mL that called vanB phenotype. In addition, they were positive to *vanA* gene representing the term known recently by some authors *"vanA genotype*- vanB phenotype". They were 15 *E. faecium* isolates (2 from blood samples, 6 from wound samples 2 from environmental samples and 5 from health care workers samples) and 4 *E. faecalis* (environmental samples). Only 3 isolates were vancomycin sensitive, all of them were isolated from health care workers hands (Table 3).

Discussion

In this study the prevalence of *enterococcus* isolates was 20% among 300 samples collected from patients and environmental samples in Minia university hospitals, Egypt that was comparable to the prevalence in previous studies in Egypt; 20.5% [21] and 16.9% [22]. However other researches in Egypt reported different results; 5.6% for vancomycin sensitive (VSE) and 3.2% for VRE [23]. In the current study, the frequency of isolation from wound samples was the higher (35%), followed by healthcare workers (14.7%), then hospital environment (12%) and finally from blood cultures (10%). Ghonaim *et al*. 2009 [21] reported 25% of wound samples and 17.2% of blood samples were enterococci, however they did not isolate enterococci from their HCWs and environmental samples at all. Our

Table 2. Antimicrobial susceptibility patterns of *enterococcal* isolates.

Antibiotic	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Vancomycin (Va30)	3	5%	0	0%	57	95%
Teicoplanin (TEC30)	32	53.3%	1	1.7%	27	45%
Ampicillin (AM10)	3	5%	0	0	57	95%
Ciprofloxacin (CIP5)	12	20%	7	11.7%	41	68.3
Erythromycin (E15)	2	3.3%	8	13.4%	50	83.3%
Cefipime (FEP30)	0	0	0	0	60	100%
Tetracycline (TE30)	8	13.3%	5	8.4%	47	78.3%
Gentamicin (CN10)	10	16.7%	2	3.3%	48	80%
Rifampin (RD5)	7	11.7%	1	1.7%	52	86.6%
Linezolid (LNZ30)	38	63.3%	9	15%	13	21.7%
Imipenem (IPM10)	55	91.7%	0	0	5	8.3%
Amoxicillin-Clavulanic (AMC30)	12	20%	0	0	48	80%

Table 3. Phenotypic and genotyping characterization of VRE isolates.

Entero. species	N	Van DD	MIC µg/ mL	TEC DD	MIC µg/ mL	phenotype	VanA gene	Source
E. faecium	11	R	>1024	R	>32	Van A	+	W
E. faecium	8	R	>1024	S	>2	Van B	+	2B,6W
E. faecium	2	R	>512	R	>16	Van A	+	E
E. faecium	7	R	>256	S	>2	Van B	+	2E, 5H
E. faecium	2	R	>128	S	>2	Van B	-	H
E. faecium	2	S	>2	S	> 2	-	-	H
E. faecalis	6	R	>1024	R	>32	Van A	+	1B, 5W
E. faecalis	5	R	>1024	R	>16	Van A	+	1H, 4W
E. faecalis	3	R	>256	R	>16	Van A	+	W
E. faecalis	4	R	>256	S	>2	Van B	+	E
E. faecalis	1	S	>2	S	>2	-	_	H
Other species	7	R	>1024	S	>2		-	W, 1B
Other species	2	R	>1024	S,I	>4		-	1B, 1E

Blood (B); health care workers (H); wound (W); environment (E); vancomycin (V); Teicoplanin (TEC); minimum inhibitory concentration (MIC); disk diffusion (DD); resistant (R); intermediate (I); sensitive (S).

study disagrees with other studies that reported enterococci were isolated mainly from blood cultures [23,24]. Previously, the ratio of *E. faecalis* infections to infections caused by all other species of enterococci was about 10:1 [25]. In the recent years, *E. faecium* is reported as the most common *enterococcus* species associated with hospital acquired infections [22,26,27]. The cause of this shift may be the increasing rate of vancomycin resistance particularly in *E. faecium* [28]. The current study agrees with these recent reports, where *E. faecium* is the most prevalent (53.3% *E. faecium,* 31.7% *E. faecalis* and 10% other enterococci). Regarding antimicrobial susceptibility, the current study reported high resistance rates among *enterococcus* isolates to a wide range of antimicrobials. These findings agree with many reports from Egypt [21,23,29] and other countries [3]. This is a serious medical challenge, as it reduces the chance of treatment, prolongs the hospital stay and increases the cost of medical care. Different reports show an increasing rate of VRE infections in Egypt; El Kholy *et al*. 2003 [30] reported that <5% of *enterococcus* isolates were vancomycin resistant, after that Ghonaim *et al.* 2009 [21] reported that VRE isolates constituted 20.9% of hospital associated enterococcal infections, then, Labib *et al*., 2013 [22] reported a percentage of (62.5%). The current study reported that 95% of isolates were resistant to vancomycin. This is a very alarming and requires a high level of infection control instructions for patients, medical staff and hospital environment to avoid VRE infection. Antibiotic use guidelines in many developing countries particularly Egypt may be insufficient, so the drug resistance rates are so high. The majority of the VRE isolates (47.3%) were vanA phenotype and *vanA* genotype (high level of resistance to both vancomycin and teicoplanin and also positive *vanA* gene). However 19/57 (33.3%) of VRE isolates were resistant to vancomycin and sensitive to teicoplanin (vanB phenotype), however they carry *vanA* gene representing the term known recently by some authors "vanA genotype-vanB phenotype" [8-10]. Such strains were reported previously in Bulgaria [11] and in Mexico [12]. This type was reported also for the first time in Middle East in Saudi Arabia, where 33% of their isolates were *vanA* genotype-vanB phenotype [31] that was similar to our result. However such strains have not yet been detected in Africa particularly Egypt. In our knowledge, the current study has reported such strains for the first time in Egypt and Africa so this type may be emergent all over the world in the near future. This heteroresistance to teicoplanin in *enterococcus* strains that have the *vanA* gene is not yet completely understood. Some authors attributed that to mutations either in the *vanA* gene cluster or the *vanS* gene [13,14] or may be due to the presence of an insertion sequence IS1216V in the coding region of the *vanS* regulatory element [10].

Conclusion

The study has reported that, 33.3% of isolates were vanB phenotype and positive for *vanA* gene representing strains known by *vanA* genotype-vanB phenotype that is detected for the 1st time in Egypt and Africa so molecular typing and MIC estimation are recommended in the study area during teicoplanin therapy to avoid hospital acquired outbreaks. The study also focused on the potential of hospital workers and environment to act as reservoirs for hospital-acquired infections caused by VRE.

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