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Antibiotic Resistance, Molecular typing, Biofilm Formation in *Enterococcai* Isolates Causing Urinary Tract Infection

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Vancomycin resistant *Enterococci* (VRE) are becoming a major emergence problem concern in urinary tract infection (UTI) at Shpin Elkom Teaching Hospitals, Menofia University. The aim of study to estimate the extent of spread of *Enterococcai* infection as well as vancomycin-resistant. *Enterococci* were isolated from 200 UTI patients and identified *as E. faecalis* by biochemical tests using the API test kit. VRE was determined by agar disc diffusion. The results showed that, 34 (26.5%) *Enterococci* were isolated from UTI patients. *E. faecalis* (64.7%) was the most common isolate followed by *E. faecium* (35.3%) out of 34 UTI patients. Among the UTI 64.7% *Enterococci* were phenotypically resistant to Vancomycin. Vancomycin resistance genes (*vanA*, *vanB*) and biofilm formation (*esp* gene) were detected by amplifying the respective genes by PCR. This study shows increase prevalence of *Enterococci* and VRE isolates as a cause of urinary tract infection in our hospitals. It should be still careful of appropriate use of antibiotics such as vancomycin. Detection and containment of VRE, a more targeted, systematic approach is needed among patients.

Key words: *Enterococci*, Urinary Tract Infection, Vancomycin Resistance, PCR, API test kit.

Infections caused by vancomycin-resistant *Enterococcus* (VRE) have emerged as a significant problem among hospitalized patients, being increasingly associated with urinary tract infections (*Raad et al.,* 2005). The Center for Disease Control and Prevention's National Nosocomial Surveillance Survey listed *Enterococci* as the second most common cause of *Nosocomial* UTI *(Wavare et al., 2015*). One of the main reasons why *Enterococci* can survive in the hospital environment is their resistance to a variety of antimicrobials. In fact, in addition to their intrinsic resistance to low levels of aminoglycosides, cephalosporins, lincosamydes and many β-lactams, *Enterococci* are also able to acquire resistance to many antibiotics, either by mutation of existing chromosomal genes or by transfer of resistance determinants*(Aberna et al., 2011).* The past two decades have therefore witnessed the rapid emergence of multidrug resistant *Enterococci*. In addition to antimicrobial resistance, several putative factors that may contribute to enhanced virulence have been described in *E.faecalis* although the molecular mechanism of virulence is still not completely understood. Adherence to host cells is considered to be a crucial step in the establishment of many bacterial infections, and a number of adhesion factors have been identified so far, such as the aggregation substance (AS), the endocarditis-associated antigen (EfaA), the enterococcal surface adhesin (Ace), the *Enterococcal* surface protein (Esp) (Aberna *et al*., 2011). It has been hypothesized that the presence of specific genes associated with virulence or invasiveness might enhance the ability of nosocomial enterococci to colonise hospitalized patients, but conflicting observations have been reported (Soto *et al.*, 2014). The *esp* gene has been associated with the ability of *Enterococcus* to form biofilm and adhere to

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plastic surfaces the presence of the *esp* gene in the organisms, the organisms' abilities to produce and colonize biofilm, and the organisms' susceptibilities to antibiotics in the biofilm environment. Biofilms are currently estimated to be responsible for over 65% of nosocomial infections and 80% of all microbial infections *(soto et al., 2014)*. The aim of study was to estimate the extent of spread of *Enterococcal* infection as well as vancomycin-resistant *Enterococci* (VRE) as a cause of urinary tract infection at Menofia University and Shpin Elkom Teaching Hospitals. And also to know species prevalence, characterize VRE phenotypes and genotypes by multiplex PCR, phenotypic detection of biofilm formation and *esp* gene detection.

MATERIALS AND METHODS

Collection of samples

This study was carried out during the period of February 2013 to December 2014, patients were from Shpin Elkom Teaching Hospitals Menofia University. The mid stream urine samples were collected in sterile container from 200 patients suspected to have urinary tract infection and transported immediately to the laboratory. The samples were inoculated onto blood agar and MacConkey agar media (Oxoid, Hampshire, UK) and incubated aerobically at 37°C for 24 h. Colonies were identified according to the standard microbiological methods.

Identification of *Enterococci*

Selective culture was performed on bile esculine agar for all colonies suspected to be *Enterococci. Enterococci* were identified on the basis of cultural characteristic, morphology, and biochemical tests using the API test kit (Cheesbrough *et al*., 2004).

Test of VRE isolates and susceptibility

All *Enterococcal* isolates were tested for Vancomycin susceptibility using the agar disc diffusion method and confirmed by the broth dilution method, which determined the minimum inhibitory concentration (MIC) according to the method described by Clinical Laboratory Standards Institution *(CLSI., 2011)* . Phenotypic classification of VRE was performed according to vancomycin and teicoplanin MICs (Cetinkaya *et al.*, 2000)

Detection of vanA and vanB genes : Vancomycin resistance genotypes (*vanA* and *vanB*) were detected by amplifying the respective genes by multiplex PCR. The oligonucleotide primers chosen for amplification of the *vanA* and *vanB* genes are shown in Table (1) (Clark *et al.*, 1993).

Rapid DNA extraction method : DNA extraction was performed *(Cho et al., 2011)*.

Amplification of *vanA* and *vanB* genes DNA : PCR reaction mixture (50 µl) consisted of 10 µl 5× Taq Master Mix, 0.2-1 µmol/l each primer, 2-50 ng template DNA, and was then filled up to 50 µl PCR-grade H2O. The samples were subjected to predenaturation of the reaction mixture for 4 min at 95°C; A 30 cycles amplification consists of Denaturation 94°C for 1 min, 45°C for 45 s, and 72°C for 1 min, and a final elongation for 7 min at 72°C; these reactions were performed in a Gene Amp PCR System 9600 (Perkin-Elmer Cetus Corp., Norwalk, Connecticut, USA) (Cho *et al.*, 2011). The amplified PCR products were detected by agarose-gel electrophoresis according to the method described by Cho *et al.* (Cho *et al., 2011)*.

Detection of Biofilm Formation : In micro plate titer assay, all Enterococcal strains (10^9cfu/30µL) were cultured in BHI in 96 well microtiter plates at 37 ºC for 48 h. After incubation broth was aspirated and wells were washed with PBS. 0.5% crystal violet stain was added for 5min. The plates were then washed with tap water and 200µL of 95% ethanol was added. The biofilm formation was considered positive when an optical density at 570nm was equal or more than 0.2 (Wakimoto *et al.*, 2004).

Congo Red Agar (CRA) : The medium i.e. Congo Red Agar was prepared as a concentrated aqueous solution and poured in the Petri plates. The culture of Enterococci from each sample was streaked on these plates. Colonies were observed after incubation for 48 hrs at 37°C. Black bacterial colonies with a rough, dry and crystalline consistency are biofilm producers. Red or pink bacterial colonies are classified as weak or non biofilm producers (Taj *et al*., 2012).

Detection of the *esp gene* using PCR :Rapid DNA extraction method : DNA extraction was performed (Cho *et al.*, 2011). PCR amplification of the *esp* gene was performed using primers shown in table (2) (Clark *et al.*, 1993).

The PCR reaction mixture consisted of 250 ng of DNA; 0.2 µl each of 2-deoxyadenosine 5-triphosphate, 2-deoxycytosine 5-triphosphate, 2-deoxyguanosine 5-triphosphate, and 2-deoxythymidine 5-triphosphate; 2.5 mmol/l MgCl2; and 2.5 U of AmpliTaq DNA polymerase in 1×reaction buffer. The samples were subjected to initial denaturation at 95°C for 2 min, A 30 cycles of amplification consists of Denaturation (94°C for 45 s) Annealing (63°C for 45 s) Extension (72°C for 1 min)(Giridhara *et al.*, 2009). The amplified PCR products were detected by agarose-gel electrophoresis according to the method described by Cho *et al. (Cho et al., 2011)* . The PCR product bands (515 bp) were visualized by ethidium bromide staining.

RESULTS

Isolated Enterococcus spp

This study included 200 inpatients from Shpin Elkom Teaching Hospitals Menofia University. Out of 200 urine samples processed in the present study, 120 (60%) were culture positive and remaining 80 (40 %) were culture negative. The results illusterated in figure (1) shows that the most common isolated organism causing urinary tract infection was *Escherichia coli* (37.5%) followed by *Enterococcus* spp (26.5%) . On the other hand , it was found other bacteria isolates such as, Coagulase negative *Staphylococcus; Klebsiella pneumonia ; Pseudomonas aeruginosa* and *Staphyococcus aureus* while with low percentage .

Table 1. Primers of PCR amplification of *vanA* and *vanB* genes

Gene	Position	Primers
Van A	130	CAT GAA TAG AAT AAA AGT TGC AAT A
	1136	CCC CTT TAA CGC TAA TAC GAT CAA
Van B	138	GTG ACA AAC CGG AGG CGA GGA
	570	CCG CCA TCC TCC TGC AAA AAA

Table 2. Primers of PCR amplification of *esp gene*

Gene	Position	Primers
Esp	515 bp	5-TTGCTAATGCTAGTCCACGACC-3 5-GCGTCAACACTTGCATTGCCGAA-3

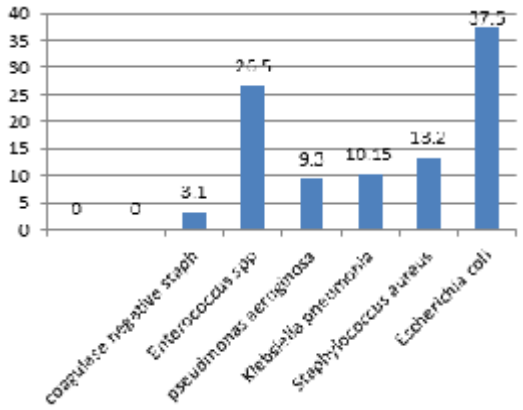


Fig. 1. Histogram showing the percentage of bacterial isolates obtained from urine samples , Shpin Elkom Teaching Hospitals Menofia University.

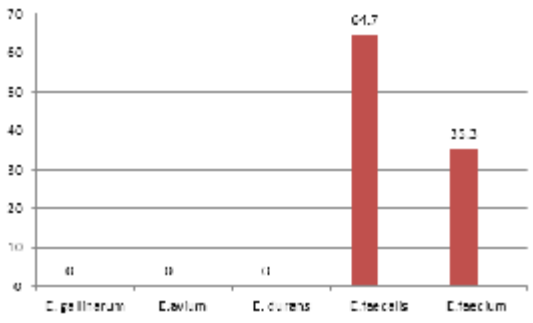


Fig. 2. Histogram showing the percentage of bacterial species identification of enterococcai clinical isolates by API system isolates obtained from urine samples , Shpin Elkom Teaching Hospitals Menofia Univ

Regarding Species identification of *Enterococcai* clinical isolates by API system, 35.3% were *E faecium* and 64.7% were *E faecalis* (table , 3 and fig. 2). While *E. durans*; *E. avium* and *E. gallinarum* were not detected.

Vancomycin resistant *Enterococci* (VRE)

Phenotypic identification of *Enterococcai* isolates showed that, 22(64.7%) were resistant (table 6).

Molecular detection

With primer pairs of *van A* and *van B* genes adopted for detection *Enterococcal* isolates . Expected fragments, 433 bp (amplification product of *van B* gene), and 1030 bp (amplification product of *van A* gene), were amplified by multiplex PCR from DNA extracted of 3 and 7 isolates . Other isolates (2, 4, 6, 8) show negative results. Data in table, (7) and fig.(3) revealed the number of *Enterococcus* species carring *van A* and *van B* genes were 16 (47.05%) out of 34 Enterococcal isolates carring Vancomycin resistant gene, 10(29.4%) carring Vancomycin resistant Van A gene and 6(17.6%) carry *Van B* resistant gene (table, 7) .

Table 3. Species identification of *Enterococcal* clinical isolates by API system

Specimen	Total Enterococal isolates	*Efaecium*		*Efaecalis*		*E. durans*		*E.Avium*		*E. gallinarum*	
	No	No	%	No	%	No	%	No	%	No	%
Urine(n= 200)	34	12	35.3	22	64.7	0	0	0	0	0	0

Vancomycin resistant, 12 (54.5%) were Van A resistant phenotype, 6(27.3%) were Van B, 2(9.09%) were Van C and 2 (9.09%) were Van D (table 4).

The distribution of Enterococci isolates and VRE among 200 urine specimen , it was found the total isolates were 128 with percentage 64 % out of 200 urine specimen . The Enterococci isolates were 34 (26.56 %) out of 128 total isolates and VRE were 22 (64.7 %) out of 34 Enterococci isolates (table 5).

Regarding the distribution of different *Enterococcus* species among VSE and VRE, 8 (66.7%) of *E faecium* were vancomycin resistant. As regard *E. faecalis*, 14(63.6%) were vancomycin

Biofilm Formation

All *Enterococcal* strains (10^9cfu/30µL) were formed the biofilm formation and considered positive which an optical density at 570nm was equal or more than 0.2 . Enterococci isolates streaked on Congo Red Agar (CRA) medium . Black colonies were observed with a rough, dry and crystalline consistency are biofilm producers. Red

Table. 4. Distribution of different VRE phenotypes according to *Enterococcus* species

VRE species (n=22)	Resistant phenotype								Total	
	Van A		Van B		Van C		Van D			
	No	%	No	%	No	%	No	%	No	%
E faecium (n=8)	4/12	(33.3)	2/6	(33.3)	2/2	(100)	0	(0)	8/22	36.36
E. faecalis(n=14)	8/12	(66.6)	4/6	(66.6)	0	(0)	2/2	(100)	14/20	63.63
Total(n=22)	12/22	(54.5)	6/12	(27.3)	2/2	(9.09)	2/2	(9.09)	22	64.7

Table 5. Distribution of enterococci and VRE according to urine sepecimen

Urine specimen (n=200)	Total isolates		Enterococcal isolates		VRE	
	No	(%)	No	(%)	No	(%)
Urine(n=200)	128	(64)	34/128	(26.56)	22/34	(64.7)

or pink bacterial colonies are classified as weak or non biofilm producers. The table (8) showed that 18 (52.9%) of all *Enterococcal* isolates were biofilm producers, 3(25%) were *E. faecium* and 15(68.2%) were *E. faecalis* .

Regarding the distribution of biofilm production among vancomycin resistant and vancomycin sensitive enterococci , 18(59.9%) of biofilm producing isolates were vancomycin resistant, 5(41.67%) were vancomycin sensitive (table,9).

Expected fragments , 480 bp (amplification product of *ESP* gene, biofilm formation), was amplified by PCR from DNA extracted of 16 *Enterococcus* species. other 18 species showed negative results. Data in table, (10) and fig. (4) revealed that,the number of *Enterococcus* species carrying *ESP* gene was 16 (47.05%) out of 34 Enterococcal isolates, whereas 2 (54.54 %) and 14 (63.63 %) for *E. faecium* and *E. faecalis* respectively .

DISCUSSION

Enterococci are a common cause of urinary tract infections (UTIs) among hospitalized patients. The rising prevalence of vancomycin-resistant enterococci (VRE) is of particular concern within many institutions because of its association with increased mortality and health care costs, as well as limited treatment options (Brett *et al.,* 2010).

The natural ability of enterococci to acquire, accumulate, and share extra chromosomal elements encoding virulence traits or antibiotic resistance genes , in part, explains their increasing importance as nosocomial pathogens. Acquired resistance to various antimicrobial agents and available antibiotics currently limits the therapeutic options. It is believed that nosocomial enterococci might have virulence elements that increase their ability to colonize hospitalized patients (Brett *et al.,* 2010).

In this study, *E coli* was the most common isolated organism from urinary tract

Table 6. Distribution of different *Enterococcus* species among VSE and VRE

Enterococcus species (n=34)	VSE(n=12)		VRE (n=22)	
	No	(%)	No	(%)
E faecium(n=12)	4/ 12	(33.3)	8/12	(66.7)
E. faecalis(n=22)	8	(36.4)	14	(63.6)
Total	12	35.3	22	64.7

Table 7. Distribution of *van A* and *van B* genes among *Enterococcus* species

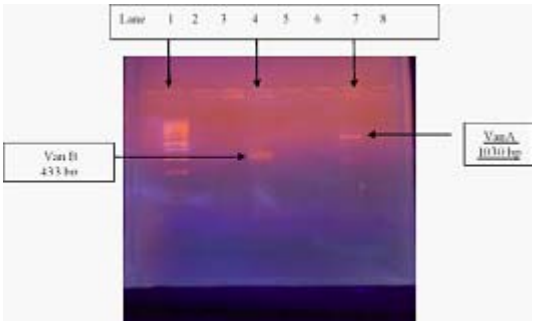
Enterococcus species	Total No. isolates	VRE	Vancomycin-resistance gene(n=16) VanA		VanB	
			No	(%)	No	(%)
E. faecium	12	5	4/12	(33.3)	1/12	(8.3)
E. faecalis	22	11	6/22	(27.2)	5/22	(22.7)
Total	34	16(47.05)	10/34	(29.4)	6/34	(17.6)

Table 8. Distribution of biofilm producing enterococcal isolates

Enterococcal isolates	Biofilm producer		Non biofilm producer	
	No	%	No	%
E. faecium(n=12)	3	25	9	75
E. faecalis(n=22)	15	68.2	7	31.8
Total (34)	18	52.9	16	47.1

infection(37.5%) followed by *Enterococcus* spp (27.5%). Phenotypic identification of enterococcal isolates showed that 22(64.7%) were Vancomycin resistant, 66.7% were *E. faecium* and 63.6% were *E. faecalis* .

Regarding the distribution of enterococcal species as a cause of urinary tract infection, *E. faecalis* was the common isolated specie (64.7%) followed by *E. faecium* (35.3%). *Sharifi et al., 2013* reported that (73.4%) Enterococcus faecalis and (26.6%) *E. faecium* isolates were isolated from urine samples respectively. Results of *Wavare et al., 2015* reported that 4.2% enterococci were isolated from UTI patients. *E. faecalis* (78%) is the most common isolate followed by *E. faecium* (15%). The rare species (9%) like *E. durans, E. avium, E. gallinarum* and *E. hirae* were also isolated. The rising prevalence of vancomycin-resistant enterococci (VRE) is of particular concern within many institutions because of its association with increased mortality and health care costs, as well as limited treatment options. Vancomycin resistance has been classified into five phenotypes, VanA to VanE. Of these, only the pheno-type VanC is intrinsically present in two species (*E. gallinarum* and *E. casseliflavus*). All the others are acquired in the two principal species (*E. faecalis* and *E. faecium)(Schouten et al., 2000).*



PCR, Lane 1 shows the DNA ladder, lanes 3 and 5 show positive bands at 433 bp (amplification product of *van B* gene), lane 7 shows positive band at 1030 bp (amplification product of *van A* gene), Other lanes (2, 4, 6, 8) show negative results

Fig. 3. Stained agarose gel showing the amplification products by multiplex

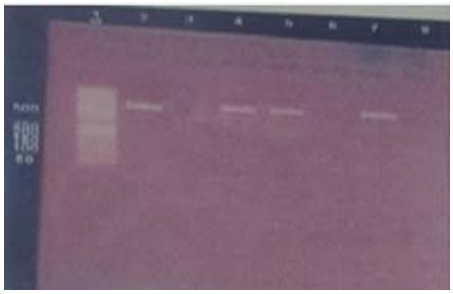


Fig. 4. Stained agarose gel showing the amplification products of *ESP* gene by PCR, Lane 1 shows the DNA ladder, lanes 2,4 and 5 show positive fragment at 433 bp, lane 3 and 6 shows negative results . lane 7 positave control and lane 8 negative control

Table 9. Distribution of biofilm production among vancomycin resistant and vancomycin sensitive enterococci

VRE	Biofilm production		Non Biofilm production		Total	
	No	%	No	%	No	%
VRE +ve	13/22	59.09	9/22	40.9	22/34	64.7
VRE –ve	5/12	41.67	7/12	58.3	12/34	35.3
Total	18/34	52.9	16/34	47.1	34	100

Table 10. Occurrence of *esp* gene among different enterococcal isolates

Enterococcus species	Total No. isolates	esp +ve		esp-ve	
		No	(%)	No	%
E. faecium	12	2	54.54	10	45.45
E. faecalis	22	14	63.63	8	36.36
Total	34	16	47.05	18	52.94

Van A phenotype is more widely distributed (54.5%) and thus the predominant type of resistance reported, Moreover, vancomycin resistance has appeared preferably in *E. faecalis*(66.6%), making therapy extremely problematic. *Coombs et aL., (2010*) reported that Strains of *E. faecium* predominate among VRE, with an average of 50% showing resistance to vancomycin. *(Hossein et al., 2014)* also reported that overall prevalence of VRE was (23.65%) which Vancomycin resistant *E. faecalis* were (16.03%) and in *E. faecium* were (33.75%) .

Genotypic detection of van genes among enterococcal isolates showed that 16 (47.05%) carrying van genes , 10(29.4%) carry van A and 6(17.6%) carry van B resistant gene Most *vanA* VRE were identified as *Enterococcus faecium* (33.3%). Also *(Hossein et al., 2014)* reported that *vanA* and *vanB* genes prevalence was significantly different between *E. faecium* and *E. faecalis* isolates, *vanA* was dominant resistance gene in *E. faecium* and *vanB* was dominant in *E. faecalis*.

Detection of multidrug resistance *Enterococci*, particularly VRE is an alarming situation, since these organisms limit the number of therapeutic options available to the clinician. Antibiotic resistance alone cannot explain the virulence of enterococci. The pathogenesis of most infections follows a common sequence of events involving colonization of and adhesion to host tissues, invasion of the tissue and resistance to defense mechanisms of the host. The pathogen must produce pathological changes either directly by toxin production or indirectly by inflammation *(Johnson, 1994).* However, each of virulence traits may be associated with one or more of the stages of infection . It was reported that biofilm bacteria are up to a 1,000 times more resistant to phagocytosis, antibodies and antibodies *(Costerton et al., 1999).* Among the associated explanations is the delayed penetration of antimicrobial agents through the exo-polysaccharide matrix, suppression of growth rate within the biofilm and production of a subpopulation of microorganisms in the biofilm that can develop into a phenotypic state that is highly protected *(Stewart & Costerton, 2001).*

Our study showed that 18(52.9%) of all enterococcal isolates were biofilm producers. 15(68.2%) were *E. faecalis*. Regarding the distribution of biofilm production among vancomycin resistant and vancomycin sensitive enterococci , 13(59.09%) of biofilm producing isolates were vancomycin resistant , 5(41.67%) were vancomycin sensitivity.

Our study also showed that 47.05% of all enterococcal isolates were esp gene carriers. In a previous study, the capacity to form biofilms was found to be common among clinical *E. faecalis* isolates particularly within the subpopulation carrying the *esp* gene which is believed to promote primary attachment of and biofilm formation by *E. faecalis* on abiotic surfaces *(Toledo-Arana et al., 2001).* On the other hand, however, *Dworniczek et al. (2003)* and *Mohamed et al. (2004)* reported in their studies that the *esp* gene was not required for biofilm formation. In addition to the presence of *esp*, another recent study presented data that supports the hypothesis of in vitro biofilm production by *E. faecalis* in the absence of the whole pathogenicity island harbouring the *esp* coding sequence *(Kristich et al., 2004).* Results *Ramadhan, Hegedus , (2004)* indicate that possession of the esp gene is neither necessary nor sufficient for production of biofilms in *Enterococci.*

CONCLUSION

There is dramatic increase in vancomycin resistance among enterococci. They also have an ability to transfer the *vanA* and *vanB* gene to self-transferable (with in genus-to other enterococci) .

The present study concludes that the overall incidence of enterococci among urinary tract infections is 26.5% . Among the genus *Enterococcus, E. faecalis* is most common isolate (64.7%) followed by *E. faecium*(35.3%). Vancomycin resistance is high (64.7%) in our hospital. Linezolid, fosfomycin or nitrofurantoin may be considered to treat the patients with VRE. The use of vancomycin is acceptable only for life threatening illnesses unless there is no other choice.

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