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Prevalence of vancomycin resistant genes among *Enterococcus faecium* isolated from patients with urinary tract infection

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Abstract

So far, the treatment of urinary tract infection remains a challenge around the world, especially in developing countries because the resistance to antibiotics is highly prevalent among bacterial isolates. *Enterococcus faecium* considered one of the most important causes this infection in recent years, this study was conducted to investigate the extent of the prevalence vancomycin resistant genes in this bacteria. 64 clinical isolates of *Enterococcus faecium* were collected from patients with urinary tract infection in several hospitals in Diyala and Baghdad. In addtion of phenotypic method, all isolates diagnosed as *E. faecium* by genotypic method was done by Polymerase Chain Reaction (PCR) technique to detection *ddlE. faecium* gene. Multiplex PCR technique has been used to investigate *vanA* and *vanB* genes by using specific primers which showed the prevalence in percentage (84% and 16%) respectively. The minimum inhibitory concentration of vancomycin was determined for resistant isolates at 32-512 µg /ml.

Keywords: *Enterococcus faecium*; vancomycin; *vanA* and *vanB* genes; urinary tract infection.

INTRODUCTION

Enterococci are Gram-positive cocci facultative anaerobic organisms ,normal flora and saprophyte of the human gastrointestinal tract, acts as opportunistic pathogens (Hollenbeck and Rice, 2012). Recent surveillance data indicates that the Enterococcus is the third most commonly isolated nosocomial pathogen Where it reached about 12% of all hospital infections(Venubabu et al., 2011). DNA homology studies suggested that they are a distinct genus; *Enterococcus faecalis* and *Enterococcus faecium* are the most common species in patient infections (Furtado et al., 2014, Hollenbeck and Rice, 2012). Enterococci have the potential for resistance to all clinically useful antibiotics. Their emergence as important nosocomial pathogens has coincided with increased expression of antimicrobial resistance by members of the genus. The mechanisms underlying antibiotic resistance in enterococci may be intrinsic to the species or acquired through mutation of intrinsic genes or horizontal exchange of genetic material encoding resistance determinants (Culyba et al., 2015, Hollenbeck and Rice, 2012). The rise in prevalence of enterococcal infections in humans is influenced to some degree by the ability of enterococci to escape the action of our most commonly used antibiotics (Hollenbeck and Rice, 2012). Epidemiological information also suggest that enterococci are important reservoirs for transmission of antibiotic resistance genes among different species of bacteria. Thus, the occurrence of antimicrobial resistant enterococci, especially VRE is a persisting clinical problem worldwide (Sanal et al., 2013).

VRE is fast becoming a major cause of health care-associated urinary tract infections (UTIs). Enterococci account for 15% of all catheter-associated urinary tract infections (CAUTIs), ranking second overall in the US (Sievert et al., 2013), which is an increase from previous years when it was ranked third11,49 They are more common in men and are usually associated with recurrent UTIs (Arias and Murray, 2015).

Over the last two decades, vancomycin-resistant *E. faecium* (VREF) has emerged worldwide as an important cause of nosocomial infections, especially in immune compromised patients. The most common nosocomial infections produced by these phathogenes are urinary tract infections which associated with instrumentation and antimicrobial resistance (Top et al., 2008, Raad et al., 2005)]. In addition, high-level aminoglycoside resistance and glycopeptide resistance are usually much higher among this species when compared to *E. faecalis* (Cereda et al., 1997).

Glycopeptides, such as vancomycin and teicoplanin, act by blocking cell wall formation. Resistance to this class of antibiotics, detected first in 1986, is due to synthesis of altered peptidoglycan precursor ending in D-alanine-D-lactate or D-alanine-D-serine in place of D-alanine-D-alanine and by the removal of precursors terminating in D-alanine. Resistance can be acquired or intrinsic and strains may be resistant to vancomycin and teicoplanin, or to vancomycin only(Depardieu and Courvalin, 2017). Vancomycin resistance (*van*) gene clusters are found in human pathogens such as *E. faecalis*,*E. faecium* and *S. aureus*. Expression of the van genes is activated by the Van S and Van R two-component system in response to extracellular glycopeptides antibiotic and consider two major types of inducible vancomycin resistance are found in pathogenic bacteria (Hong et al., 2008, Viera, 2001).

Resistance to vancomycin has been increasing during recent years due to increase in the presence of plasmid bearing resistance genes in these organisms and it is an important problem because this antibiotic is very effective against gram-positive bacteria (Hughes et al., 2017). The resistance inducing gene for vancomycin is called van gene and is subtyped to A, B,D, C, and E. Type A and B are located on transposon Tn1546 (Daghighi et al., 2014). These two genes cause high-grade resistance to vancomycin can be potentially introduced to conjugative plasmid transferred within enterococcal strains as well as to the other organisms such as Staphylococcus and can

increase the potential risk of vancomycin-resistant in the community (Kang et al., 2014, Daghighi et al., 2014). While genes type D, C, and E cause low-grade resistance to vancomycin and are located on chromosome (Daghighi et al., 2014). *vanB* confers a high degree of vancomycin but susceptibility to other glycopeptides like teicoplanin since only the former antibiotic is capable of inducing the *vanB* resistance type (Kafil and Asgharzadh, 2014, Werner et al., 2012).

Vancomycin-resistant enterococci (VRE) have become important nosocomial pathogens causing outbreaks worldwide, (VRE) can cause morbidity and mortality in patients that have long period hospitalization therefore, this study was conducted.

METHODS AND MATERIALS

Sample collection and identification

A total of 135 Enterococci isolates were collected from obtained during 2017 from different hospitals in Diyala governorate and Baghdad, Iraq. They were isolated clinically from midstream urine from patient suffering urinary tract infections.

All isolates were identified as Enterococci by colonial morphology, Gram stain, oxidase and catalase reaction,growth in Azid Maltose Agar Medium, ability to grow at 10ºC and 45ºC, growth on bile-esculin agar, with esculin hydrolysis and tolerance to 6.5% sodium chloride. *E. faecium* was diagnosed depending on its ability to ferment arabinose on Cephalexin-Aztreonam-Arabinose Agar (Betty et al., 2007).

Susceptibility testing

Screening for vancomycin resistance was performed by disk diffusion method on Mueller-Hinton agar according to "Clinical and Laboratory Standard Institute" guidelines (CLSI, 2011). It has also been determined the minimum inhibitory concentration (MIC) for vancomycin to *E. faecium* isolates which showed resistant against vancomycin .This test was done according to Morello et al (2006).

DNA extraction

ZR fungal/Bacterial DNA MiniprepTm kit (Zymo Research) was used for the extraction of DNA from bacteria isolates. DNA extraction procedures were performed by following the manufacturer's instructions. Extracted samples were stored at-20°C.

Amplification of $ddl_{E. faecium}$

Primers for identifying and classifying bacteria $ddl_{E. faecium}$ was used, giving products of 658 bp Table(1). Uniplex PCR was performed in a 25-µl volume that was prepared by adding 12.5µl of GoTaq®Green master Mix (2X) promega, 3µl template DNA, 1.5µl from each forward and reverse primers with final concentration 1 poml /µl, finally volume was completed to 25µl by adding nuclease free water. PCR condition illustrated in Table (2) and PCR products were detected in 1 % agarose gel for 1 hr. at 75 V, stained with ethidium bromide and visualized by transilluminator.

Detection of *vanA and vanB* genes

The prevalence of vancomycin genes (*vanA* and *vanB*) among *E. faecium* local isolates in this study was detect by using multiplex PCR with specific primers and amplicon size Table (1). PCR mixture was prepared by adding 12.5µl of GoTaq®Green master Mix (2X) promega, 3µl template DNA, 1.5µl from each forward and reverse primers with final concentration 1 poml/µl, finally volume was completed to 25µl by adding nuclease free water. PCR condition illustrated in Table (2) and PCR products were detected in 1 % agarose gel for 1 hr. at 75 V, stained with ethidium bromide and visualized by transilluminator.

RESULTS AND DISCUSSIONS

Identification of *E.faecium*

Bacteria isolates have been diagnosed in two ways: the first is phenotypic by microscopic and cultural characteristics, in addition of biochemical tests, the second is genotypic by polymerase chain reaction (PCR).

The preliminary identification of Enterococcus species dependent in large on appearance of these bacteria on differentiation type of culture media with addition to microscopic of Enterococcus members. Enterococcus species appearance on blood agar aziad medium as small glassing gray colony. All members of Enterococcus isolates in this study were hydrolysis esculin, hydrolysis of esculin was absorbed as dark brown around colony. Cephalexin-Aztreonam-Arabinose Agar was used for differentiate between *E.faecalis* and *E.faecium* based on arabinose fermentation, on this medium *E.faecium* appearance as yellow colonies due to fermentation arabinose while *E.faecalis* appearance as pinkish colonies.

Table (1): Primers and amplified PCR products used in study

Gene	Primer sequences (5'→3')	size (bp)	Origin	Reference
$ddl_{E. faecium}$	F: TTGAGGCAGACCAGATTGACG R: TATGACAGCGACTCCGATTCC	658	Alpha	Sharifi et al., 2012
vanA	F: GGGAAAACGACAATTGC R: GTACAATGCGGCCGTTA	733	Alpha	Kariyama et al., 2000
vanB	F: AAGCTATGCAAGAAGCCATG R: CCGACAATCAAATCATCCTC	420	Alpha	Kariyama et al., 2000

Table (2): PCR condition to genes used in study

Amplified gene	Initial denaturation	No. of cycles	Denaturation	Annealing	Elongation	Final extension
$ddl_{E. faecium}$	95°C/ 5 min	30	95°C/1 min	56°C/1 min	72°C/1 min	72°C/7 min
vanA,vanB	95°C/ 5 min	30	95°C/1 min	54°C/1 min	72°C/1 min	72°C/7 min

All (135) Enterococcus isolates were tested for $ddl_{E.\ faecium}$ gene by PCR and this gene are specific for *E. faecium*. The results revealed that only (64) isolates were yielded this gene by PCR and it showed the ability to arabinose fermentation, so it were diagnosed as *E.faecium*. Figure (1) shows the genetic detection of $ddl_{E.\ faecium}$ gene to different isolates of the bacteria under study and this gene were approximately equal to 658bp.

Molecular techniques like (PCR) have become most important technique for more rapid and precise for detection bacteria that causes diseases in species level (Saleem, 2017).

Screening of vancomycin resistant

A screening test for ability of *E.faecium* isolates to resistant the action of vancomycin antibiotic was detected grossly by used the disc diffusion method. In this method diameters of inhibition zones were compared to CLSI record (2011). Accordingly the ability to resistance, the result of this experiment have been shown that (23%) 15 isolates of *E.faecium* were resistance to vancomycin, (16 %) 10 isolates was intermediated and (61 %) 39 isolates were sensitive.

Vancomycin minimum inhibitory concentration

Determination of minimum inhibitory concentration for vancomycin resistant isolates of *E.faecium* was done, the isolates incorporated in this test were those have high or intermediate resistant to vancomycin. These isolates have measurement of resistance to vancomycin greater than the break point of search resistance clearly by CLSI (2011). According the resistance 25 *E.faecium* isolates showed a value of MIC ranging from 32-512 µg /ml. Taneja et al., (2003) indicted that the MIC for resistance to VanA range from 64 µg\ml to up. and for VanB ranging 32-64 µg\ml.

Based on MIC results, could be indicates to the presence of both type VanA and VanB.

Screening of vancomycin genes

Multiplex PCR amplification process was carry out on all resistance isolates of *E. faecium*, the results of amplified process showed that 21(84%) isolates contain *vanA* gene and 4(16%) isolates contain *vanB* gene. Figure (2) shows positive results to amplified fragment of *vanA* and *vanB* genes with amplicon size equal 733bp and 420bp respectively. Figure (2) shows positive results to amplified fragment of *vanA* and *vanB* genes.

There are many studies done around the world to detect prevailing vancomycin genes of *E. faecium* in those countries. In a study perform by Salem-Bekhit et al., (2012), demonstrated that *vanA* predominant in *E. faecium* that isolated from KSA more than *vanB* gene. The results were obtained from Iranian study which done by Kafil and Asgharzadh (2014), studying 43 *E. faecium* isolates obtained from variety of clinical specimens in Iran, which found 24 isolates had *vanA* gene and 19 isolates had *vanB* gene. While in other study perform by Talebi et al., (2008), who found that resistant *vanA* gene was the dominant resistance of vancomycin among Enterococcus species in Iran. Also Gozalan et al., (2015) show that resistant *vanA* gene was dominant resistant of vancomycin among *E.faecium* in Turky.

Sievert et al., (2013) suggested that *E. faecalis* is more pathogenic than *E. faecium*, but the latter exhibits more resistance, composing the majority of VRE infections. Also O'Driscoll and Crank (2015) indicated that VanA is responsible for most of the human cases of VRE around the world, and is mostly carried by *E. faecium*, a change in the precursor to d-Ala-d-Lac (VanA, VanB) causes a 1,000-fold decrease in affinity for vancomycin (Fisher and Phillips, 2009, Werner et al., 2008).

Although *vanA* is responsible for most of the human cases of VRE around the world, and is mostly carried by *E. faecium* (O'Driscoll and Crank, 2015) however, the results of this study confirmed that some *E. faecium* isolates carry *vanB* gene. This is logically possible because this *vanB* gene carry on transposons (Tn 1546) and can acquired through the mechanism to transfer of resistance gene between these members of bacteria through horizontal movement of resistance gene from other Enterococcus species to *E. faecium*.

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