Prevalence of vancomycin resistant enterococci in Hilla City, Iraq

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Abstract:
Objectives: To identify the prevalence of enterococci species in hospitalized patients with urinary tract infection in Hilla City, to determine their susceptibilities to vancomycin and other antibiotics.

Materials and methods: Two hundred urine samples were obtained from patients who were admitted to three main hospitals in Hilla city during a period of three months (from November 2010 to February 2011). After identification of enterococcal species by biochemical tests, the antibiotic susceptibility of isolates was determined by standard disk diffusion test according to CLSI. MIC tests for vancomycin and teicoplanin were also carried out for VRE isolates by methods of agar-dilution and HiComb test.

Results: This study revealed that out of 200 samples, *Enterococcous* species was isolated only from 7 samples, which were identified to genus level. 5/7 isolates were catheter-related infection. According to susceptibility data obtained, Five isolates were resistant to vancomycin (71.43%) with an MIC ranging from 8-32 µg/ml but they were sensitive to teicoplanin (MIC < 0.25 µg/ml), thus exhibited VanB phenotype, and the majority of isolates (86%) showed multi-drug resistance pattern. All isolates were highly resistant (85.72%) for each of chloramphenicol, rifampicin and erythromycin. On the other hand, 71.43% of isolates were resistant to each of penicillin and tetracycline while they were moderately resistant to ampicillin (57.14%) and ciprofloxacin (42.86%), in comparison to relatively low resistance to nitrofurantoin 14.29%.

Conclusion: Finding of this study shows an emergence of VRE along with increased rate of multi-drug resistant enterococci in the area of the study.

Key words: Vancomycin-resistant enterococci, Iraq.

Introduction:

Enterococci are gram-positive, opportunistic bacteria that inhabit the gastrointestinal tracts of humans and many animals. Enterococci are the second most frequently reported cause of surgical wound infections and nosocomial urinary tract infections and the third most frequently reported cause of bacteremia (McCormick *et al.*, 2000; Courvalin, 2006). Resistance to environmental conditions such as heat or desiccation allow prolonged survival and poor compliance with hand-washing procedures by health care workers results in the rapid spread of

enterococci in hospitals (Zirakzadeh and Patel, 2006; Ott and Wirick, 2008). Moreover, strains of enterococci have acquired resistance to essentially most of the antimicrobial agents over the past three decades. Nosocomial infections with entrococci are major concern at many hospitals and have rapidly increased in many countries worldwide (Kacmaz and Aksoy, 2005). Vancomycin-resistant enterococci (VRE) belong nowadays to the most important nosocomial pathogens worldwide, and they usually cause infections in severely debilitated, immunocompromised patients who undergo prolonged and intensive antibiotic therapy (Moellering, 1992).

The prevalence of vancomycin resistance in enterococci (VRE) has dramatically increased (Schouten *et al.*, 2000). The National Nosocomial Infection Surveillance (NNIS) system in the USA has revealed a significant increase in the percentage of invasive nosocomial *Enterococcus* strains displaying high-level vancomycin resistance. The first VRE strains, *Enterococcus faecium* and *Enterococcus faecalis*, were isolated in 1986 in France (Leclercq *et al.*, 1988) and in the United Kingdom (Uttley *et al.*, 1989), and since then they have been identified in many other countries. The frequency of colonization varies widely, being 2 to 5% in France (Boisivon *et al.*, 1997), 12% in the Netherlands (Van den Bogard *et al.*, 1997) and 20% in Korea (Seong *et al.*, 2004) mostly involving patients in intensive care units (ICU).

Six types of vancomycin resistance have been reported in enterococci (*VanA, VanB, VanC, VanD, VanE and VanG*). The resistance to vancomycin is inducible and encoded by the *vanA* gene cluster, which is carried on transposons (Courvalin, 2006). Transfer of resistance can occur via conjugative plasmids. Enterococci as reservoirs of antibiotic resistance genes tend to transfer their resistance genes to the other bacteria, among them methicillin- resistant *Staphylococcus aureus* (Chang *et al.*, 2003). Monitoring the antibiotic resistance of enterococci isolated from clinical specimens is a useful tool to get information about prevalence of VRE and will be essential for controlling the spread of bacterial resistance.

The aim of this study was to determine the distribution of clinical enterococci isolates and their drug susceptibility patterns at three hospitals in Hilla city, Iraq.

Materials and methods:

Samples collection: This study included (200) patients suffering from signs and symptoms of urinary tracts infection (UTI) who diagnosed as having UTI by a urologist. They were admitted to three main hospitals in Hilla city, into different wards including Intensive Care Unit, Coronary Care Unit, Burns, Oncology, Surgical and Dialysis units, during a period of three months (from November 2010 to February 2011). The patients' residence in hospital ranged from (4 days-6 weeks). Urine specimens were collected from each patient and were plated onto different culture media (Azide-Crystal-Blood agar medium, MacConkey agar and Chromo agar) and incubated aerobically at 37 °C overnight. The bacterial isolates were preserved in brain heart infusion broth supplemented with 5% glycerol at -20 °C for 6-8 months.

Identification of isolates: Isolates were identified to the genus level based on the standard biochemical and microbiological methods such as: morphologic appearance on Gram-stain (gram positive cocci forming short chains), catalase negative, ability to hydrolyze esculin in the presence of bile, growth in the presence of 6.5% NaCl at 45 ºC, pyrrolidonylarylamidase +ve (PYR), Hippurate hydrolysis +ve and Lancefield group D (Domig *et al.*, 2003).

Antimicrobial susceptibility testing: Susceptibility to vancomycin and some other antimicrobial agents for all enterococci isolates was determined by the standard disk diffusion method and interpreted according to the CLSI (2010), on Muller-Hinton agar incubated for 18-24 hour at 37 ºC. The following antibiotic disks (Bioanalyse/ Turkey) were used, penicillin (10 IU), ampicillin (10µg), erythromycin (15µg), Tetracycline (30µg), vancomycin (30µg), Teicoplanin (30µg), ciprofloxacin (5µg), Rifampin (5µg) chloramphenicol (30µg) Linezolide (30µg) and nitrofurantoin (300µg). The value of minimum inhibitory concentration (MIC) of each VRE isolate for vancomycin and teicoplanin included the two-fold agar dilution susceptibility method and HiComb MIC commercial test (Himedia/India) according to the NCCLS (2003a). Susceptibility test results were assessed after 24-48 hr incubation at 37ºC. The MIC values were based on break point recommended by CLSI (2010) for estimation of the response. For Vancomycin 1-4µg (more than 4µg isolate is considered resistant), and for teicoplanin 0.25-1µg.

Results:

Results of morphological and biochemical characterization tests revealed that out of 200 samples, *Enterococcous* species was isolated only from 7 samples (3.5%) (Figure-1), which were identified to genus level from which 5/7 (71.4%) of enterococcal isolates were obtained from catheterized patients and the remaining 2/7 (28.57%) were obtained from non-catheterized patients.

The Susceptibility data obtained *in vitro* for 7 isolates with 11 antibiotic substances are shown in Table-1. Multidrug-resistant isolates were found in all of isolates. Of 7 enterococci only five (71.43%) isolates were resistant to vancomycin (MICs =8-32µg/ml) but still sensitive to teicoplanin (MICs ≥0.25µg/ml) and exhibited VanB phenotype Table-2.

In addition to vancomycin, all of the enterococcal isolates (n= 7) demonstrated resistance to a wide variety of other antimicrobial agents such as penicillin, ampicillin, ciprofloxacin, chloramphenicol, rifampicin, tetracycline, nitrofurantoin and erythromycin, while they were susceptible to linezolid (Figure-2).
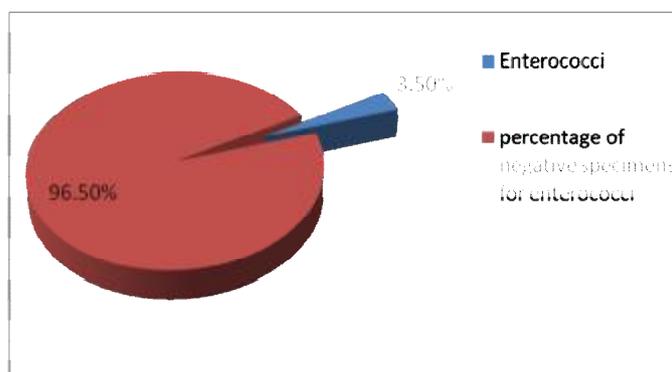
Figure (1) Percentage of enterococcal isolates recovered from urine specimens

Table (1) Antibiotic resistance among Enterococcal isolates

Isolates No.	Antibiotics										
	P	**A**	**VA**	**Te**	**C**	**Lin**	**Cf**	**E**	**T**	**N**	**Rad**
1	R	R	R	S	S	S	R	R	R	S	R
2	S	S	R	S	R	S	R	R	S	S	R
3	R	R	R	S	R	S	S	R	S	R	S
4	R	S	R	S	R	S	S	R	R	S	R
5	R	R	R	S	R	S	R	S	R	S	R
6	R	R	S	S	R	S	S	R	R	S	R
7	S	S	S	S	R	S	S	R	R	S	R
% of resistance	71.4	57.1	71.4	0	85.7	0	42.86	85.7	71.4	14.29	85.7

Abbreviations: **A**=Ampicillin, **P**=Penicillin, **VA**=Vancomycin, **Te**=Teicoplanin, **C**=Chloramphenicol, **Lin**=Linizolid, **Cf**=Ciprofloxacin, **E**=Erythromycin, **T**=Tetracyclin, **N**=Nitrofurantoin, **Rad**=Rifampicin.
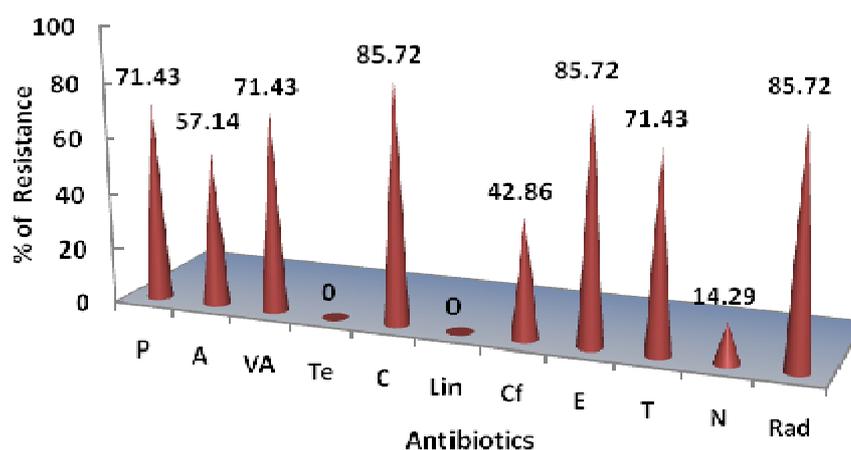

Figure (2) Antibiotic resistance among Enterococcal isolates

A=Ampicillin, **P**=Penicillin, **VA**=Vancomycin, **Te**=Teicoplanin, **C**=Chloramphenicol, **Lin**=Linizolid, **Cf**=Ciprofloxacin, **E**=Erythromycin, **T**=Tetracyclin, **N**=Nitrofurantoin, **Rad**=Rifampicin

Table (2): MICs of vancomycin and teicoplanin against enterococci isolates

Isolates No.	MIC (µg/ml) of:	
	VA (≥ 4 µg/ml)	Te (≥ 0.5 µg/ml)
1	32	0.25
2	8	0.125
3	16	0.125
4	16	0.25
5	8	0.25
6	4	0.25
7	1	0.06

*Numbers between the brackets refer to breakpoints recommended by CLSI (2010).
VA=Vancomycin, Te=Teicoplanin, MIC=Minimum inhibitory concentrations.

Discussion:

Enterococci, an original flora of the intestinal tract, oral cavity, and the genitourinary tract of the humans and animals, are known to be relatively avirulent in healthy individuals, but have become important opportunistic pathogens, especially in hospitalized patients (Murray, 1990). They belong to group D streptococci as characterized by Lancefield in 1938, whose taxonomy has changed considerably in the last few years.

Resistance is known to arise where use of antimicrobial agent is high and spread of these resistance bacteria is easy. Because of the limited therapeutic options and lack of enough information and programs to control rapid spread of Enterococci species, the mortality of the enterococcal infection is on the rise (Akhi *et al.*, 2009).

A major reason for the survival of *Enterococcus* in hospital environment is their intrinsic resistance to several commonly used antibiotics and, perhaps more important, their ability to acquire resistance to all currently available antibiotics, either by mutation or through the transfer of plasmids and transposons (Cetinkaya *et al.*, 2000).

These low isolation rate (3.5%) observed in current study was in agreement with that of Orenstein and Wong (1999) in Virginia, who found that enterococci account for approximately 5% of UTIs and most of these are nosocomial. In another study was done in Brazil by Barros *et al.* (2009), who found that enterococcal isolation rate from urine specimens was (4%).

Although *Enterococcus* spp prevalence is not common, it has been recognized as an important uropathogen and urinary tract infections constitute the most common type of disease produced by *Enterococcus* spp. (Patterson *et al.*, 1995). In renal transplant recipients, was reported *Enterococcus* as an emergent and important cause of urinary tract infections, although *E. coli* remains the most common pathogen in these patients (Alangaden, 2007). The presence of enterococcal species among catheterized individuals was also reported by other investigators (Lloyds *et al.* 1998), who showed that enterococcal species accounted for 35% of urinary tract isolates. Cornia *et al.* (2006) investigated the microbiology of bacteriuria in a large cohort study of elderly male inpatients and outpatients and identified *Enterococcus* as the most frequent uropathogen, this pathogen was isolated in 22.5% of the patients and approximately 45% of the infections were catheter-related.

The *Enterococcus* species, especially *Enterococcus faecalis* and *Enterococcus faecium*, account for 15% to 30% of CAUTIs (Maki and Tambyah, 2001) and are now considered the third leading cause of hospital-acquired UTIs (Stickler, 2008). The ability of many enterococcal isolates to produce biofilms, which is a major component of the patho-physiology of CAUTIs, correlated with persistent infections (Mohamed *et al.*, 2004). Enterococcal factors involved in UTI pathogenesis, including the enterococcal surface protein Esp, the pilus-associated sortase C (SrtC), and the endocarditis and biofilm-associated pilus (Ebp). Moreover, well-characterized adhesins and biofilm determinants often associated with enterococcal UTI isolates, like aggregation substance (AS) and the housekeeping sortase A (SrtA), were reported to be dispensable for virulence in the urinary tract (Guiton *et al.*, 2010).

The multi-drug resistance pattern (MDR) shown in this study, were matched with that of Al Jarousha *et al.* (2008) who found that isolated enterococci showed MDR pattern (resistance to equal or more than 3 drugs) to a number of antibiotics including penicillin, vancomycin, methicillin and tetracycline, while they showed moderate resistance to nitrofurantoin and ciprofloxacin.

Resistance to multiple classes of antibiotics is common in enterococci as had seen in this study, this finding was alarming as infection due to MDR enterococcal isolates are difficult to treat (Saifi *et al.*, 2008). Enterococci showed a great resistance to most of the commonly used antibiotics in hospitals where development of antibiotic resistance is often related to the overuse, and misuse of the antibiotics prescribed. Iraq is one of the developing countries where all types of antibiotics are sold over the counter, an attitude that encourages self-medication.

The high incidence of vancomycin-resistant enterococci (VRE) isolated in hospitals throughout the world was reported as 100% in Poland and Korea (Kawalec *et al.*, 2001; Jung *et al.*, 2006). Furthermore, VRE incidence rate was 30.5% in Greece (Simeon *et al.*, 2006). This high incidence has been also observed in our study (Hilla/ Iraq) 71.43%. The prevalence of vancomycin resistance in enterococci (VRE) has dramatically increased in few years (Schouten *et al.*, 2000).

The source of resistant organisms in the study population could be food, water, and/or person-to-person transfer. Lack of proper hand hygiene and overcrowding in health care facilities may enhance the spread of the antibiotic resistant organisms. Uncontrolled consumption of antimicrobial agents is likely to play a major role in the development of antibiotic resistant bacteria as suggested by Ruppe *et al.* (2009).

In addition to that, all the isolates were sensitive to teicoplanin, and this revealed that all the VRE isolates in this study showed Van B phenotype, this result was accordant with result of Bell *et al.* in Australia (1998) who showed high prevalence of Van B phenotype among his enterococcal isolates. Moreover, most of the reported VRE in India were of *VanB* or *VanC* phenotype (Karmarkar *et al*, 2004; Taneja *et al,* 2004).

Vancomycin-resistant enterococci (VRE) were first reported in 1980s, nearly 30 years after vancomycin was clinically introduced. The primary inciting factor was

likely the use of orally administered vancomycin for treating antibiotic-associated diarrhea in hospitals (Arthur *et al.*, 1996).

Five phenotypes of vancomycin resistance; termed van A, van B, van C, van D, and van E are known. The van A and van B phenotypes are clinically significant as these phenotypes can be induced by vancomycin use. Van A enterococcal isolates exhibit high-level resistance to both vancomycin and teicoplanin, while van B isolates have variable resistance to vancomycin and remain susceptible to teicoplanin. VanA and VanB phenotypes can be found in both *E. faecalis* and *E. faecium* (Zubaidah *et al.*, 2006). VanA and VanB, encoded by two distinct gene clusters, the *vanA* and *vanB* clusters, respectively, which are carried on transposons Tn*1546* and Tn*1547*, respectively. VanC confers low-level vancomycin resistance and is not associated with resistance to teicoplanin. VanD phenotype is manifested by moderate resistance to vancomycin and low level resistance or susceptibility to teicoplanin. VanE and VanG phenotypes confer low- and moderate-level of resistance to vancomycin, respectively, while retaining susceptibility to teicoplanin (Mohanty *et al.,* 2006).

Glycopeptide resistance in enterococci involves alteration in the cell wall composition in the peptidoglycan precurser D-Ala-D-Ala (vancomycin-susceptible) to D-Ala-D-lactate (D-Lac). The latter has 1000 times less affinity for Vancomycin (Gilmore, 2002).

The rapid emergence of VRE in the United States has been attributed to the intensive clinical use of vancomycin in both parenteral and oral forms in that country. In addition high-level usage of cephalosporins, which promote enterococcal superinfection (Leclercq and Courvalin, 1997). In Europe, investigators have postulated an additional role for the use of the glycopeptide avoparcin as a growth promoter in intensive animal industries, resulting in colonization with VanA *E. faecium* and subsequent transmission to humans via the food chain (Aarestrup *et al.*, 1996).

The resistance rate to ampicillin 57.14% in enterococci isolates in this study was close to the resistance rate to ampicillin recorded in Ireland 51% and Tabriz/ Iran 57.66% (Lavery *et al.*, 1997; Akhi *et al.*, 2009). Moreover, resistance rate to ampicillin reported by Mathur and his colleagues in India 66% was higher than that in this study (Mathur *et al.*, 2003). Furthermore, in this study 71.43% of isolates were penicillin resistant.

Since ampicillin and other penicillin derivatives are the drug of choice in the treatment of enterococcal infections and frequently prescribed for patients, usually predispose them to colonization with enterococci, which are naturally resistant to these antibiotics. The relatively high resistance of isolates in this study to ampicillin and penicillin is of great concern especially in clinically serious condition.

Enterococci exhibit intrinsic resistance to penicillinase-susceptible penicillin (low level), penicillinase-resistant penicillin, cephalosporin, nalidixic acid, aminoglycoside and clindamycin. Enterococci are resistant to most β-lactam antibiotics because of low affinity penicillin binding proteins (PBP), which enable them to synthesize cell wall components even in the presence of modest concentration of most β-lactam antibiotics; due to the over production of low affinity

PBP-5 (a protein that can take over the function of all PBPs), higher level of resistance is observed, increased PBP-5 production was associated with a deletion within an upstream open reading frame that was characterized as a penicillin-binding protein synthesis repressor (psr) (Lollier *et al.*, 1998). A more recent study suggests that psr may serve as a global regulator of cell-wall synthesis genes in enterococci (Massidda *et al.*, 1996). Ampicillin resistance resulting from changes in PBP-5 is primarily a clinical problem in *Enterococcous* spp. making the success of β-lactam monotherapy unpredictable for severe enterococcal infections. Moreover, the relatively high ratio of resistance to these antibiotics was not attributed only to production of low affinity PBP; also, it could be due to the production of β-lactamase enzyme (Murray and Samoraj, 1983), as many sporadic outbreaks of nosocomial *E. faecalis* and *E. faecium* infection appeared with penicillin resistance due to β-lactamase production (Murray *et al.*, 1991).

Rifampicin resistance in this study was high and accounts 85.72% from all *Enterococcus* isolates (5/7). Resistance to rifampin by *Enterococcus* isolates was recorded by several authors with different percentages; it was 42% according to Arias *et al.* (2003) and 27.2% according to Al-Khafaji (2006). This high resistant rate in this study may be due to increase rifampicin usage in the treatment of tuberculosis (TB) as it is an important chemotherapeutic agent for use against tuberculosis, which is endemic in our country and its prevalence was increased due to poor health services after recurrent wars.

Rifampicin inhibits DNA-dependent-RNA polymerase in bacterial cells by binding its beta-subunit, thus preventing transcription to mRNA and subsequent translation to proteins, resistant to it is due to mutations in the *rpo*B gene encoding the β-subunit of RNA polymerase, that replace phenylalanine, tryptophan, and tyrosine with non-aromatic amino acids result in poor bonding between rifampicin and the RNA polymerase. Rifampicin-resistant bacteria produce RNA Polymerases with different β subunit structures, which are not readily inhibited by the drug (Christopher, 1994; Enne *et al.*, 2004).

Chloramphenicol resistance in this study was 85.72%. In other studies it was recorded in different percentages; 100%, 27.2%, 4.3% and 53% in Poland, Iraq, Brazil and Iran respectively (Kawalec *et al.*, 2001; Al-Khafaji, 2006; Fracalanzza *et al.*, 2007; Rahimi, 2007).

Chloramphenicol resistance is most commonly associated with the chloramphenicol acetyl-transferase gene, *cat*, and this will turn the drug ineffective by the acetylation enzymes (Schwarz *et al.*, 2004). The inactivation of chloramphenicol can also be performed by xenobiotic acetyltransferases. A third mechanism of chloramphenicol inactivation is performed by chloramphenicol phosphotransferases. Finally, chloramphenicol resistance may be due to mutations in 23S rRNA, thereby changing the binding site of chloramphenicol in the cells or due to efflux pumps (Aakra *et al.,* 2010).

In this study, 85.72% of isolates were resistant to erythromycin, which was close to the resistance rate in India (Mathur *et al.*, 2003) who found that 85% of enterococcal isolates were resistant to erythromycin. However, this percentage of erythromycin resistance by *Enterococcous* spp was higher than that reported from

Lebanon 59% (Zouain and Araj, 2001), and it was lower than resistance percentage reported in Tabriz, Iran 91.24% (Akhi *et al.*, 2009).

Resistance to erythromycin in enterococci is typically mediated by the *erm*B rRNA methylase gene (methylation enzymes add methyl group to rRNA) (Roberts *et al.*, 1999).

Furthermore, 71.43% of isolates were resistant to tetracycline. This result was in accordance with that of Chopra and Roberts (2001) and Hayes *et al.* (2004) who revealed that tetracycline resistance among MDR enterococci were 70% and 68% respectively.

In enterococci, two major groups of tetracycline resistance genes have been identified. The first group include *tet* (M), *tet* (O) and *tet* (S) genes that encoding ribosomal protection proteins, and the second group encodes tetracycline efflux pumps proteins by the *tet* (L) and *tet* (K) genes (Kobashi *et al.* 2007). Increase in tetracycline resistance rate may be due to its use as growth promoter in animals (Chopra and Roberts, 2001).

The high prevalence of tetracycline resistance and the occurrence of multiresistance against tetracycline and erythromycin are associated with the presence of the Tn*916*-Tn*1545* family, which are conjugative transposons carrying *tet*(M) and/or *erm*(B). Tn*916*-Tn*1545* was originally isolated from *E. faecalis* and has been shown to be present in many bacterial species (Sun *et al.*, 2009).

In this study, ciprofloxacin resistant was 42.86%, while it was reported to be 10%, 3.14%, 34% and 60.58% in Japanese, French, Lebanon and Iranian studies respectively (Nakanishi *et al.*, 1991; Guirguitzova *et al.*, 1998; Zouain and Araj, 2001; Akhi *et al.*, 2009). These results indicated diverse geographic distribution of ciprofloxacin resistant enterococci.

Fluoroquinolones including ciprofloxacin act by forming complexes with DNA gyrase and topoisomerase IV, thereby blocking DNA replication and triggering events leading to bacterial death, resistance is mediated chiefly through mutations in quinolone resistance-determining regions (QRDRs) of GyrA (subunit of DNA gyrase) and/or ParC (subunit of topoisomerase IV). Additionally, the development of different efflux systems is another major way by which bacteria can decrease the intracellular accumulation of fluoroquinolones (Kuo *et al.*, 2009).

Nitrofurantoin is one of the effective antibiotics on enterococci species, but the results of this study, showed 14.29% resistance to nitrofurantoin. The low resistance rate to nitrofurantoin may be due to little prescription of this drug for urinary tract infections in Hilla city/ Iraq, as compared to high resistance rate in India 22.76%, and in Tabriz and Tahran in Iran 36.5%, due to common prescription of this drug in UTI treatment (Rahangdale *et al.*, 2008; Akhi *et al.*, 2009).

No resistance to linezolid was detected in *Enterococcus* isolates. This result was accordant with that of Cetinkaya *et al.* (2000), Rahangdale *et al.* (2008) and Saifi *et al.* (2008) who showed that all the enterococci isolates in their study were sensitive to linezolid, which was recently launched for the treatment of gram-positive infections.

Linezolid is an oxazolidinone antibiotic that inhibits bacterial ribosomal protein synthesis at the 50s subunit; it is effective against enterococci regardless of their

resistance to other antimicrobial agents, it has potent in vitro activity against vancomycin-resistant *E. faecalis* and *E. faecium*, as well as good therapeutic efficacy for VRE bacteremia and endocarditis (Moellering, 2003), but first linezolid-resistant strains have already been described in linezolid treated patients which is mediated by mutations as a single nucleotide transvertion at position 2576 in 23S rRNA, which appears to alter the interaction of the antibiotic with its target (Kauffman, 2003; Arias *et al.*, 2008). Therefore, its use should be restricted in order to reduce an increase in linezolid-resistant organisms. Furthermore, linezolid has some limitations due to its predominantly bacteriostatic activity and lack of a bactericidal effect.

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