

Full Length Research Paper

# ***vanA* in vancomycin-resistant *Enterococcus faecalis* isolated in Baghdad**

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*Enterococcus faecalis* has emerged as an important nosocomial pathogen worldwide, infections and outbreaks of Vancomycin-resistant enterococci (VRE) still appear to be rare in Baghdad. In the present study, 20 isolates of *E. faecalis* were collected from 252 clinical samples (151 urine and 101 blood) from different hospitals in Baghdad and 3 isolates from 50 stool samples from healthy people during the period 1/10/2010 to 1/12/2010. All isolates were identified through morphological, cultural and biochemical tests using Rapid ID-32 strep. Vancomycin-resistance phenotype was determined by the agar diffusion method; results showed that five clinical isolates (25%) and two faecal isolates (66.7%) were resistant to vancomycin. Minimum inhibitory concentrations (MIC) of clinical VRE isolates ranged between 4 to 512 µg/ml and the MICs for isolates of faecal origin were 64 and 128 µg/ml. Seven vancomycin-resistant *E. faecalis* isolates were examined for their drug resistance and plasmid DNAs. Of the 5 clinical isolates, 2 isolates exhibited resistance to azlocillin (Azi) and ofloxacin (Ofx). All clinical and stool isolates were resistant to oxacillin (Ox), cefepime (Fep), rifampicin (Ra) and tetracycline (Te). Imipenem and ampicillin were found to be the most effective agents against the isolates. The detection of plasmid DNA by gel electrophoresis showed that some *E. faecalis* isolates carried a high molecular weight plasmid that was transferred to the recipient by filter mating. The putative presence of *vanA* gene was examined by PCR, using specific primers. Positive PCR amplifications were obtained in all 7 isolates for *vanA* gene. Vancomycin resistant *E. faecalis* increased gradually in Baghdad hospitals and high dissemination of *vanA* gene, which encoded high resistance level to vancomycin. Continued surveillance is required to prevent further spread of these serious resistances.

**Key words:** Bacteria, vancomycin, *Enterococcus faecalis*.

## **INTRODUCTION**

Enterococci are known to be opportunistic nosocomial pathogens capable of causing life-threatening infections, such as endocarditis and bacteremia, mostly in immunocompromised patients (Levison and Mallela, 2000) occasionally reported to cause urinary tract infections, septicemia, and endocarditis (Sun et al., 2009). Since *Enterococcus* spp. are resistant to multiple antibacterial drugs, there are only limited options for effective therapy and prophylaxis of serious infections (Kawalec et al., 2004). The emergence of vancomycin-resistant enterococci (VRE) followed a worst-case scenario for

nosocomial pathogens. The isolation of VRE (VanA type) was first reported in 1988 in the United Kingdom (Uttley et al., 1988) and France (Leclercq et al., 1988), and shortly thereafter, it was reported in the United States (Sahm et al., 1989). Since then, VRE have been identified in many countries.

Vancomycin resistance is conferred by one of two functionally similar operons, *vanA* or *vanB*. The *vanA* and *vanB* operons are highly sophisticated resistance determinant, probably evolved in other species and acquired by enterococci (Rice, 2001). In general,

enterococcal isolates with lowered susceptibility to vancomycin can be categorized as vanA, vanB, and vanC. vanA and vanB pose the greatest threat because they are the most resistant and the resistance genes are carried on a plasmid. Since the resistance genes are carried on a plasmid they are readily transferable through conjugation (Paulsen et al., 2003). In addition to the increase in VRE, the potential spread of vancomycin resistance to *Staphylococcus aureus* or *Staphylococcus epidermidis* is a serious public health concern. The genes for vancomycin resistance are frequently plasmid-borne and have been transferred *in vitro* from enterococci to staphylococci (Palazzo et al., 2005). Clinical strains of vancomycin-resistant *Staphylococcus haemolyticus* and *S. epidermidis* have already been reported. Spread of vancomycin resistance must be controlled to prevent the development of untreatable staphylococcal infections (Srinivasan et al., 2002).

In Asia, VRE have been isolated from hospitalized patients or food animals in China, Japan, South Korea, Taiwan, and Thailand (Zheng et al., 2007). In particular, they have frequently been isolated in South Korea (Yu et al., 2003) and Taiwan (Lauderdale et al., 2002). In Iraqi hospitals, very rare studies have investigated the incidence of *vanA* gene among Vancomycin resistant *Enterococcus faecalis*. The aims of this study were to determine the distribution of Vancomycin-resistant *E. faecalis* isolates, and studying the *vanA* gene in this bacteria which isolated from hospitals and healthy persons.

## MATERIALS AND METHODS

### Bacterial isolates

A total of twenty (20) *E. faecalis* isolates were collected from 252 clinical samples (151 urine and 101 blood) from different hospital in Baghdad and 3 Isolates from 50 stool samples from healthy people during the period 1st October to 1st December, 2010. For each isolate, the clinical source and limited demographic data were documented

### Bacterial identification

All isolates have been identified by morphological, cultural and biochemical tests such as API 20 STREP test (bioMérieux, France) and Rapid ID-32STREP system (bioMérieux, France). All isolates were identified using the agar supplemented by potassium tellurite reduction, motility, and pigment production tests (Macfaden, 2000; Forbes et al., 2002).

### Antimicrobial susceptibility testing

For susceptibility testing, the agar diffusion method was used, performed, and interpreted according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2009). The following antimicrobials were tested (obtained from Bioanalyse-Turkey):

ampicillin (AM) (10 µg), rifampin (RA) (5 µg), tetracycline (TE) (30 µg), streptomycin(S) (10 µg), ofloxacin(OFX) (5 µg); vancomycin (VA)(30 µg); Erythromycin(E) (15 µg), Azlocillin(AZL) (75 µg), Cefepime (FEP) (30 µg), Imipenem (IMP) (10 µg) and Oxacillin (OX) (1µg), *S. aureus* ATCC 29213 were used as reference strains. Minimum inhibitory concentrations of Vancomycin (Rospira-USA) was determined by the broth dilution method according to (Morello et al., 2003) an overnight pure culture of each isolates grown in Mueller-Hinton broth (Oxoid/England) was diluted 100-fold with fresh broth. An inoculum of approximately  $5 \times 10^5$  cells was plated on a series of Mueller-Hinton broth cultures containing a range of concentrations of the vancomycin. The tubes were incubated at 37°C/24 h. The results were in compliance with standards recommended by Clinical and Laboratory Standards Institutes (formerly NCCLS) (15).

### Plasmid DNA extraction

The plasmid DNA of the clinical and stool isolates was isolated by using alkaline method according to Klaenhammer (1984) and analysed on 0.8% agarose gel.

### Conjugation experiments

Filter mating was performed as described previously (Ike et al., 1983; Dahl et al., 2000). A 100 µL sample of mating mixture containing donor strains and the recipient strain was spread on a 45 µM nitrocellulose membrane filter (Millipore) placed on top of brain heart infusion (BHI) agar. After 18 h incubation at 37°C, cells were resuspended in 1 ml BHI broth and spread on BHI agar containing antibiotic.

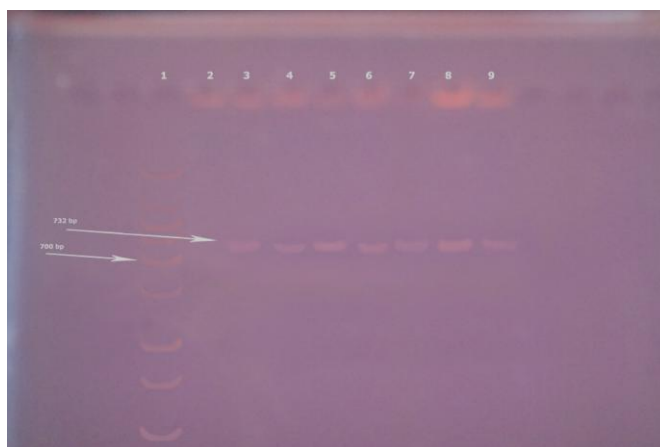
### PCR-based detection of vancomycin resistance genes

A PCR assay was used to detect genes coding for vancomycin resistance in enterococci (*van A* and *van B*). Primer pairs used to amplify fragments of the *van A* (F = GGGAAAACGACAATTGC and R=GTACAATGCGGCCGTTA). Primer pairs used to amplify fragments of the *vanB* (F=AAGCTATGCAAGAAGCCATG and R=CCGACAATCAAATCATCCTC). Four to five well-isolated colonies obtained after overnight growth on trypticase soy agar were resuspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 50 mM NaCl, 5 mM EDTA [pH 8.0]). Proteinase K was then added to a final concentration of 1 mg/ml, and the suspension was incubated at 50°C for 60 min, followed by incubation at 100°C for 10 min. Processed samples were used immediately for PCR or stored at 4°C until they were used.

The PCR amplification mixture consisted of reaction buffer (final concentration, 2 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM Tris-HCl [pH 8.3]), 400 mM (each) dATP, dCTP, dTTP, and dGTP, 0.1 mM *vanA* primer pair, 0.5 mM *vanB*, 50 U/ml of *Taq* polymerase (Promega/USA), and 1.0 ml of enterococcal DNA in a total volume of 10.0 ml. DNA amplification was carried with a Gradient PCR System (TechNet-500 /USA). The cycling conditions consisted of an initial denaturation step at 95°C for 4 min and then 30 cycles of 95°C for 45 s, 54°C for 1 min, and 72°C for 1 min. In the final cycle, the extension step was carried out at 72°C for 7 min. A reagent blank (containing all the components of the reaction mixture with water instead of target DNA), were run in every PCR procedure. Amplified PCR products were detected by agarose gel electrophoresis with 1.5%. A DNA marker (Promega/USA) was run with each gel, and the VRE genotype was determined by the size of the amplified product.

**Table 1.** Susceptibility of the 20 isolates of vancomycin-resistant *E. faecalis* to 10 antibiotics.

Antibiotic used	Resistant %	
	Clinical isolates	Faecal isolates
Tetracycline	100	100
Oxacillin	100	100
Cefepime	100	100
Rifampicin	100	100
Ampicillin	0	45
Azlocillin	40	100
Erythromycin	80	100
Streptomycin	80	45
Ofloxacin	40	0
Imipenem	0	0



**Figure 1.** Presence of *vanA* gene in VRE. *faecalis* isolates.

## RESULTS

Clinical isolates were obtained mostly from urine 17 isolates (11.25 %), followed by blood 3 isolates (2.97 %). Five clinical isolates (25%) and two stool isolate (66.7%) were resistant to vancomycin. Minimum inhibitory concentrations for Vancomycin were determined, VR *E.faecalis* clinical isolates that had concentrations between 4-512 µg/ml and the MICs for stool isolates (2 isolates) were 64 and 128 µg/ml. The resistance patterns for the VRE. *faecalis* isolates are shown in Table 1. All VRE. *faecalis* isolates were resistant to Oxacillin ,Cefepime ,Rifampicin and Tetracycline. In addition, four clinical isolates were resistant to streptomycin, two isolates were resistant to ofloxacin, and one stool isolate was resistant to Ampicillin. All VRE. *faecalis* isolates were susceptible to Imipenem. The detection of plasmid DNA by gel

electrophoresis showed that some *E. faecalis* isolates carried a high molecular weight plasmid that was transferred to the recipient by filter mating. The detection of *vanA* gene by PCR (Figure 1) showed the high dissemination of *vanA* gene which encoded to high resistance level to vancomycin between the local VRE. *faecalis* isolates in comparison to *vanB* gene .

## DISCUSSION

Infection or colonization with VRE results in a wide spectrum of possible deleterious effects for patients in health care settings. Studies have demonstrated that VRE can cause serious infections, and the attributable mortality of bacteremia caused by VRE has been estimated to be nearly 40% (Werner et al., 1999). The percentage of the clinical isolates in the present study was slightly in a good agreement with percentage results in other study in Baghdad (Alsaadi, 2007; Al Kafaji, 2005). Enterococci account for approximately 110 000 urinary tract infections, 25 000 cases of bacteremia, 40 000 wound infections, and 1 100 cases of endocarditis annually in the United States (Emori and Gaynes, 1993).

In our study, 25% of clinical isolate and 66.7% of stool isolate were resistant to vancomycin, these results agreed with those of other results (Al Kafaji, 2005), but in disagreement with that of (Alsaadi, 2007) which showed that all isolates of *E. faecalis* were sensitive to vancomycin but in the study of (Kandela, 2006) the resistance was higher the current study. Kühn et al., (2005) studied the occurrence of VRE in 2 580 samples collected in different geographical regions in Europe, high proportions of VRE-positive urban sewage samples were found in Sweden, Spain, and the United Kingdom (52 to 90%), apparently reflecting a human source rather than an animal source; despite the fact that the consumption of antibiotics in hospitals in Sweden (population, 9 million) is among the lowest in Europe. Thus, it is possible that antibiotic-resistant enterococci present in the human population are enriched in hospitals, released from there into municipal sewers to become established, and then further released via sewage treatment plants into recipient water and transferred back into humans. However, 7% of isolates from Germany and 16.7% of isolates from Switzerland and Greece were vancomycin-resistant enterococci (Bouchillon et al., 2004).

Vancomycin-resistant *E. faecalis* tend to be multidrug resistant against a large number of currently available antimicrobial agents, compromising treatment options and increasing the likelihood of inadequate antimicrobial therapy and increase in morbidity and mortality (Kawalec et al., 2004). In the present study, VRE. *faecalis* showed resistance to a wide range of antimicrobial agents. Our results illustrated that, Imipenem and Ampicillin has a good activity against both clinical and stool isolates.

Close to this result was reported in a study carried out by (Alsaadi, 2007). Our results detected that all vancomycin resistant *E. faecalis* isolates were resistant to Oxacillin, Cefepime, Rifampicin and Tetracycline. Multiple-drug-resistant enterococci and vancomycin-resistant enterococci (VRE), in particular, are opportunistic pathogens and major causes of nosocomial infections in immunocompromised patients (Zheng et al., 2009).

Since the first report of vancomycin-resistant enterococci (VRE) in 1988, VRE have emerged as an important cause of hospital-acquired infections, particularly in the United States. According to the National Nosocomial Infection Surveillance System of the Centers for Disease Control and Prevention, over a period of 10-year the percentage of VRE has increased from 0.4 to 25% among enterococcal isolates from patients in intensive care units (ICUs) (Patel, 2003). Regarding the origin of VRE, European studies demonstrated that the carriage of VRE in the community was probably caused by the use of antibiotics as growth promoters in animals (Bates, 1997). VRE are usually transmitted by contact. No skin found that VRE could survive for at least 1 h on gloved and ungloved fingertips and for 5 to 7 days on environmental surfaces (Noskin et al., 1995). Other investigators have shown that VRE can exist in stool specimens of a carrier for up to 2 years, providing a source for environmental spread (Roghamann et al., 1997). Results of PCR detection showed that *vanA* gene was present in all the tested isolates regardless of their origin, if they are clinically or stool. Elsewhere in South Africa, four clinical isolates of *E. faecalis vanB* and one strain of *Enterococci gallinarum* were isolated in a hospital in Bloemfontein, Free State, in 1995 (Derby et al., 1998). In a hospital in Cape Town, an *E. faecium vanA* strain was isolated in 1993 from a clinical specimen (Derby et al., 1998).

The *vanA* gene results in high levels of vancomycin resistance, as well as, resistance to teicoplanin. The MICs of vancomycin for enterococci with the *vanB* gene may be from low to very high, but these enterococci remain susceptible to teicoplanin. Both these classes of vancomycin resistance (VANA and VANB VRE) have been detected mainly in *E. faecium* and *E. faecalis*. These types of resistance are acquired and encoded by genetic elements, which are transferable. To date, all published outbreaks of VRE have been restricted to these two classes of vancomycin resistance (Toye et al., 1997). In 1992, Handwerger et al. reported that the occurrence of glycopeptide-resistant enterococci with a *VanA* phenotype was rare in the United States. The *VanB* phenotype shows variable levels of vancomycin resistance but remains susceptible to teicoplanin and is inducible by vancomycin alone. The genes encoding the *VanB* resistance phenotype are more commonly chromosomal but can also be transferred by conjugation.

In conclusion, Vancomycin Resistant *E. faecalis* increased gradually in Baghdad Hospitals and high dissemination of *vanA* gene, which encoded high resistance level to vancomycin. Continued surveillance is required to prevent further spread of these serious resistances.

## REFERENCES

- Al Kafaji JK (2005). Bacteriological and genetic study of *Enterococcus faecalis* isolated from clinical and environmental sources in Babil. Ph.D thesis, college of science, Al-Mustanseriya University.
- Alsaadi FSA (2007). Study of the resistance of *Enterococcus faecalis* that caused urinary tract infection to some antibiotics and its production for  $\beta$ -Lactamase enzymes. M.Sc. thesis, college of science, Al-Mustanseriya University. p. 75.
- Bates J (1997). Epidemiology of vancomycin-resistant enterococci in the community and the relevance of farm animals to human infection. J. Hosp. Infect. 37:89-101.
- Bouchillon SK, Johnson BM, Hoban DJ, Johnson JL, Dowzicky MJ, Wu DH, Visalli MA, Bradford PA (2004). Determining incidence of extended spectrum  $\beta$ -lactamase producing Enterobacteriaceae, vancomycin-resistant *Enterococcus faecium*, and methicillin-resistant *Staphylococcus aureus* in 38 centres from 17 countries: the PEARLS study 2001–2. Int. J. Antimicrob. Agents 24:119-124.
- CLSI (2009). Performance standards for antimicrobial susceptibility testing, 19<sup>th</sup> supplement, CLSI document M 100-S19. 29(3). CLSI, Wayne, Pennsylvania, USA.
- Dahl KH, Lundblad EW, Rokens TP (2000). Genetic linkage of the *vanB2* gene cluster to *Tr5382* in vancomycin-resistant enterococci and characterization of two novel insertion sequences. J. Microbiol. 146:1469-1479.
- Derby P, Allan B, Lambrick M, Gay Elisha B (1998) Detection of glycopeptide-resistant enterococci using susceptibility testing and PCR. S. Afr. J. Epidemiol. Infect. 13:66-69.
- Emori TG, Gaynes RP (1993). An overview of nosocomial infections, including the role of the microbiology laboratory. Clin. Microbiol. Rev. 6:428-442.
- Forbes BA, Sahm DF, Weissfeld AS (2002). Baily and Scott's Diagnostic Microbiology. 11<sup>th</sup> edition, Mosby, Company Baltimore. USA. pp. 102-104.
- Handwerger S, Perlman DC, Altarac D, McAuliffe V (1992). Concomitant high-level vancomycin and penicillin resistance in clinical isolates of enterococci. Clin. Infect. Dis. 14:655-661.
- Ike Y, Craig RC, White BA, Yagi Y, Clewell DB (1983). Modification of *Streptococcus faecalis* sex pheromones after acquisition of plasmid DNA. Proc. Natl. Acad. Sci. USA. 8:5369-5373.
- Kandela NJN (2006). Production, purification and characterization of enterocin from *Enterococcus faecalis* local isolates from different clinical sources. Ph.D thesis, college of science, Al-Mustanseriya University. p. 108.
- Kawalec M, Ziolkowski R, Ozorowski T, Kędzierska J, Baran M, Hryniewicz W (2004). Abstr. 14th Eur. Congr. Clin. Microbiol. Infect. Dis., abstr. p. 812. Clin. Microbiol. Infect. 10(Suppl. 3):207.
- Klaenhammer TR (1984). A general method for plasmid isolation in lactobacilli. J. Curr. Microbiol. 10:23-28.
- Kühn I, Iversen A, Finn M, Greko C, Burman LG, Blanch AR, Vilanova X, Manero A, Taylor H, Caplin J, Domínguez L, Herrero IA, Moreno MA, Möllby R (2005). Occurrence and relatedness of vancomycin-resistant Enterococci in animals, humans, and the environment in different European regions. Appl. Environ. Microbiol. 71(9):5383-5390.
- Lauderdale TL, McDonald LC, Shiao YR, Chen PC, Wang HY, Lai JF (2002). Vancomycin-resistant enterococci from humans and retail chickens in Taiwan with unique *VanB* phenotype-*vanA* genotype incongruence. Antimicrob. Agents Chemother. 46:525-527.
- Leclercq R, Derlot E, Duval J, Courvalin P (1988). Plasmid-mediated

- resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N. Engl. J. Med. 319:157-161.
- Levison ME, Mallela M (2000). Increasing antimicrobial resistance: therapeutic implications for enterococcal infections. Curr. Infect. Dis. Rep. 2:417-423.
- Macfaden GF (2000). Biochemical test for identification of medical bacteria .3<sup>rd</sup> Ed. Lippincott Williams & Wilkins CO, London. pp. 57-76.
- Morello JA, Granato PA, Mizer HE (2003). Laboratory Manual and Workbook in Microbiology :Application to patient Care.17<sup>th</sup> ed. The McGraw-Hill Companies. pp. 97-99.
- Noskin GA, Stosor V, Cooper I, Peterson LR (1995). Recovery of vancomycin-resistant enterococci on fingertips and environmental surfaces. Infect. Control Hosp. Epidemiol. 16:577-581.
- Palazzo ICV, Araujo MLC, Darini ALC (2005). First report of vancomycin-resistant staphylococci isolated from healthy carriers in Brazil. J. Clin. Microbiol. 43(1):179-185.
- Patel R (2003). Clinical impact of vancomycin-resistant enterococci. J. Antimicrob. Chemother. 51:13-21.
- Paulsen IT, Banerjee L, Myers GS, Nelson AKE, Seshadri R, Read TD, Fouts DE, Eisen JA, Gill SR, Heidelberg JF, Tettelin H, Dodson L, Umayam L, Brinkac M, Beanan S, Daugherty RT, DeBoy S, Durkin J, Kolonay R, Madupu W, Nelson J, Vamathevan B, Tran J, Upton T, Hansen J, Shetty H, Khouri T, Utterback D, Radune KA, Ketchum BA, Dougherty CM (2003). Role of mobile DNA in the evolution of Vancomycin-Resistant *Enterococcus faecalis*. Science 299:2071-2074.
- Rice LB (2001). Emergence of vancomycin-resistant Enterococci. J. Emerg. Infect. Dis. 7(2):183-187.
- Roghmann MC, Qaiyumi S, Johnson JA, Schwalbe R, Morris JG (1997). Recurrent vancomycin-resistant *Enterococcus faecium* bacteremia in a leukemia patient who was persistently colonized with vancomycin-resistant enterococci for two years. Clin. Infect. Dis. 24:514-515.
- Sahm DF, Kissinger J, Gilmore MS, Murray PR, Mulder R, Solliday J, Clarke B (1989). *In vitro* susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. Antimicrob. Agents Chemother. 33:1588-1591.
- Srinivasan A, Dick J, Perl TM (2002). Vancomycin resistance in Staphylococci. Clin. Microbiol. Rev. 15(3):430-438.
- Sun J, Song X, Kristiansen BE, Kjæreng A, Willems RJL, Eriksen HM, Sundsfjord P, Sollid JE (2009). Occurrence, population structure, and antimicrobial resistance of Enterococci in marginal and apical periodontitis. J. Clin. Microbiol. 47(7):2218-2225.
- Toye BA, Shymanski J, Bobrowska M, Woods W, Ramotar K (1997). Clinical and epidemiologic significance of Enterococci intrinsically resistant to vancomycin (possessing the *vanC* genotype). J. Clin. Microbiol. 35(12):3166-3170.
- Uttley AH, Collins CH, Naidoo J, George RC (1988). Vancomycin-resistant enterococci. Lancet 1:57-58.
- Werner EB, Tammy MR, Gaye OH, Richard PW, Michael BE (1999). Molecular epidemiology of vancomycin-resistant *Enterococcus faecium* in a large urban hospital over a 5-year period. J. Clin. Microbiol. 37(12):3912-3916.
- Yu HS, Seol SY, Cho DT (2003). Diversity of Tn1546-like elements in vancomycin-resistant enterococci isolated from humans and poultry in Korea. J. Clin. Microbiol. 41:2641-2643.
- Zheng B, Tomita H, Takako IT, Ike Y (2009). Isolation of VanB-Type *Enterococcus faecalis* strains from nosocomial infections: First report of the isolation and identification of the pheromone-responsive plasmids pMG2200, encoding VanB-Type vancomycin resistance and a Bac41-Type Bacteriocin, and pMG2201, encoding erythromycin resistance and cytolysin (Hly/Bac). Antimicrob. Agents Chemother. 53(2):735-747.
- Zheng B, Tomita H, Xiao YH, Wang S, Li Y, Ike Y (2007). Molecular characterization of vancomycin-resistant *Enterococcus faecium* isolates from mainland China. J. Clin. Microbiol. 45:2813-2818.