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Evaluation of Virulence Factors and Detection of *vanA*, *vanB* and *esp* Genes from Clinical Isolates of Vancomycin - Resistant *Enterococcus faecalis*

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The great attention of scientific researches about vancomycin- resistant *Enterococcus faecalis* due to the great healthy problems in the world. Evaluation of *E. faecalis* virulence factors showed that all isolates (100%) were hemolysin, protease and aggregation substance producer, 30% were a gelatinase producer, and 40.7% were a lipase producer. The results of the tube method showed that all *E. faecalis* isolates (100%) were slime layer and biofilm producer but the amount of adherent layer were different among the isolates ranged from strong to moderate and weak. Antibiotics susceptibility test for 20 isolates was done against 11 antibiotics, eighteen *E. faecalis* isolates were multi drug resistance. Seventeen *E. faecalis* isolates were determined as Vancomycin-Resistant by the minimum inhibitory concentrations [MICs] using agar dilution method. The virulence factor Enterococcal Surface Protein (*esp*) which is chromosomal was amplified by Polymerase chain reaction (PCR) technique in a monoplex pattern, results of this investigation showed that 20 (100%) *E. faecalis* isolates gave the amplicon size 933 base pair for the *esp* gene. The genetic determinants of Vancomycin-Resistant *vanA* and *vanB* genes were amplified using monoplex and multiplex PCR techniques in order to identify vancomycin resistant (*van+*) and sensitive (lacking *van*) among (13) *E. faecalis*. The *vanA, vanB* genes were detected in 11 and 4 *E. faecalis* isolates, respectively. The results of monoplex and multiplex PCR revealed that the molecular weight of *vanA* and *vanB* genes were 550 and nearly 600 bp, respectively. The results revealed that the *vanA* and *vanB* amplicons have a genetic variation in their molecular weight during the electrophoresis of PCR product.

Keywords: Enterococcos faecalis, van genes, Antibiotic resisrant, Virulence factors, esp gene.

The Enterococci are a diverse and versatile group of bacteria with several intrinsic characteristics that allow them to survive and grow under a variety of conditions (Tellis and Muralidharan, 2012). Among the Enterococcal species described *Enterococcus faecalis* represented one of the most important causes of nosocomial infections, since it is responsible for 80 - 90% of human Enterococcal infections (Jett and Gilmore, 1990; Jones *et al*., 2004; Fernandes & Dhanashree, 2013).

The general interest for Enterococci and treatment of Enterococcal infections has increased due to the appearance of antibiotic multi resistant strains (Dahle´n *et al*., 2012). One of the major reasons why these organisms have survived in the 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 by receipt of foreign genetic material through the transfer of plasmids and transposons. Most Enterococci are tolerant to the bactericidal

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activity of antibiotics; bactericidal synergy between antibiotics is needed to treat most serious Enterococcal infections such as endocarditis and meningitis (Cetinkaya *et al*., 2000). There are at least three major reasons for the emergence of multidrug-resistant Enterococci: (i) baseline intrinsic resistance to several antimicrobial agents, (ii) acquired resistance via mobility of the resistance genes on plasmids and transposons, and chromosomal exchange, and (iii) the transferability of resistance (Mundy *et al*., 2000). Resistance to last-line drugs, such as vancomycin is common, and Enterococci are now disseminating this resistance to methicillin-resistant *Staphylococcus aureus* (MRSA) (Palmer and Gilmore, 2010).Among vancomycin-resistance phenotypes in Enterococci, VanA and VanB possess highest clinical importance (Sharifi *et al*., 2012). Glycopeptide resistance in Enterococci is mediated predominantly by mobile gene clusters that confer resistance to vancomycin and teicoplanin (Howden *et al*., 2013).

MATERIALS AND METHODS

One hundred thirty - five clinical specimens were collected from urine, blood, teeth root canal and burns of patients' suffering from urinary tract infection, bacteremia, endodontic infections and burns infections, respectively [Table 1].

The collected specimens were streaked on Pfizer Selective *Enterococcus*; and incubated at 37°C for 24 hr. VITEK system was employed for *E. faecalis* isolates confirmation.

Determination of minimum inhibitory concentrations [MICs] of Vancomycin

The MICs were determined to all *E. faecalis* isolates for the Vancomycin antibiotic. This test was achieved according to Morello *et al*. [12].

The MIC values were based on break point recommended by CLSI [13], for the estimation of response. For Vancomycin, 1-4µg was sensitive, more than 4µg isolate was considered as intermediate resistant and e" 32µg considered as resistant.

Detection of some Virulence factors phenotypically

Haemolysis activity was determined by streaking of pure isolates on blood agar plates, The Egg-Yolk agar and Skim milk agar were inoculated with the *E. faecalis* isolates for detection lipase and protease production; respectively. Gelatin liquefaction (Harley & Prescott, 2002) activity was determined by streaking of pure isolates on gelatin nutrient medium. Aggregation substance production (Creti *et al*., 2004) was determined by inoculate a pure *E. faecalis* isolates in tubes contained 5 ml of Trypticase soy broth with incubation 24 hr. at 37ºC with 5% CO2. Detection of the bacterial ability to produce biofilm was done according to Christensen *et al*., 1982.

- DNA Extraction from *E. faecalis* isolates by using Wizard® Genomic DNA Purification Kit.
- Plasmid DNA extraction from *E. faecalis* by using AccuPrep® Plasmid Mini Extraction Kit.
- Estimating of DNA concentration and purity: The DNA concentration and purity was determined by using Nano drop instrument from ACT gene.
- Polymerase Chain Reaction (PCR) Technique: PCR assay was performed in a monoplex and multiplex (for *vanA* and *vanB*) patterns in order to amplify genes under study for Vancomycin-Resistant *E. facalis*. The primers listed in table (2) were selected for this study; these primers were provided in lyophilized form.

The extracted DNA, primers and PCR premix, were thawed at 4ºC, PCR mixture was set up in a total volume of 25 µl included: 5 µl PCR premix, 2.5 µl of each primer (forward and reverse), 5 µl of DNA template and the rest volume was completed with sterile deionized distilled water, the negative control contained all material except template DNA, so instead that deionized distilled was added. The PCR reaction tubes were mixed briefly and placed into thermocycler PCR instrument where DNA was amplified according to the PCR program as indicating in Tables 3, 4, 5.

Determination of PCR Specificity The PCR products were analyzed and the result confirmed by using 1.5 % agarose gel electrophoresis, by dissolving 1.5 gm.

RESULTS AND DISCUSSION

Isolation and Identification of Vancomycin-Resistant *E. faecalis*

Twenty isolates of the genus *Enterococcus* were isolated from 135 clinical specimens have the

ability to grow on Pfizer selective *Enterococcus*. The highest numbers of isolates were distributed among urine specimens and the lowest one was observed among wound infection specimens, VITEK were employed to confirm the presence of *E. faecalis* isolates, regarding samples' type and isolated *Enterococcus* spp., there were a 20 isolates identified, they were demonstrated in table 7.

E. faecalis isolates from urine in this study was 46.6%, Alebouyeh *et al.* [19] showed that the percentage of *E. faecalis* isolates from urine was 75%.

For blood specimens, the percentage of *E. faecalis* isolates were 8.3%, this result was compatible with the results by Tellis and Muralidharan [2]; they showed that percentage of *E. faecalis* isolated from blood was 18%. While other studies reported by Al-Jarousha *et al.* [21], AL-khafaji *et al.* [22] and Mira *et al.* [23] revealed that the percentage of *E. faecalis* isolated from blood were 3%, 0% and 55.05%, respectively.

The percentage of *E. faecalis* isolated from root canal specimens was 14%, this result was higher than the result of local study obtained by Mahmoudpour *et al.* [24], who showed the percentage of *E. faecalis* isolated from root canal was 10%, another studiy by Zoletti *et al.* [25] and Preethee *et al.* [26], showed that the percentage of *E. faecalis* isolated from root canal was 80% and 46.87%, respectively. The percentage of *E. faecalis* isolated from wounds was 10%, this result was compatible with the result of Giacometti *et al.* [27] and Al-Jarousha *et al.* [21], who indicated that the percentage of *E. faecalis* isolated from wounds was 5.6%, 1.9%, respectively.

The differences between isolation percentages may be related to the number of specimens, the differences in the source of isolates, hospitals included in each study, their geographical regions and differences in the identification methods.

Determination of Vancomycin susceptibility

Vancomycin susceptibility was determined by the minimum inhibitory concentration [MIC] for all *E. faecalis* isolates, according to Clinical and Laboratory Standards Institute [CLSI], if the MIC d" 4 ¼g / ml then the isolate is sensitive, MIC 8–16 ¼g/mL the isolate have intermediate resistance and if the MIC e" 32 ¼g/ml the isolate is resistant to vancomycin.

The MICs result of vancomycin for *E. faecalis* isolates were indicted in the table 8.

Results of Vancomycin sensitivity test obtained by this study showed that from 20 isolates, 13 isolates [65%] were resistant to Vancomycin, 4 isolates [20%] were intermediate resistant and 3 isolates [15%] were sensitive.

In a study reported by Fatholahzadeh *et al.* [28] stated that 38% of *E. facalis* isolates were resistant to vancomycin. While Camargo *et al.* [29] demostrated that 20.8% of *E. faecalis* isolates were resistant to vancomycin and 79.1% of isolates were sensitive. The results of this study was close to the results of Chabuck *et al.* [30], Al-jmor [20] and Praharaj *et al.*[31], who showed that the percentages of vancomycin resistant were 71.43%, 50% and 90.6%, respectively.

The multidrug resistance (MDR) bacteria was defined as resistance to 3 or more types of antibiotics (Al-Jarousha *et al.* 2008), the emergence of multidrug-resistant (MDR) pathogens seriously threatens this classes of lifesaving drugs (Queenan and Bush, 2007), therefore; This study was showed the percentage of multidrug resistant *E. faecalis* isolates was 90% as shown in table (9). This result was adjacent to the results reported by Al-Jarousha *et al.* (2008) and Chabuck *et al.* (2011) were showed the percentage of MDR is 66.6% and 100%, respectively.

The unique *E. faecalis* (isolated from blood) was resistant to (Penicillin G, Ceftazidime, Gentamycin, Ciprofloxacin, Rifampin, Amoxiclav and Imipenem), therefore; this isolate was considered as Extended-resistant *E. faecalis* (Diekema *et al.*, 1999; Queenan and Bush, 2007; Papp-Wallace *et al.*, 2011).

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. 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 (Saifi *et al.*, 2008).

Iraq is one of the developing countries where all types of antibiotics are sold over the counter, an attitude that encourages self-medication.

From this study results, it can be conclude

that the resistance to multiple classes of antibiotics is common to *E. faecalis*, these findings were alarming the infections due the MDR and extended -resistant *E. facalis* isolates and difficult to treat (Al-Jmor , 2012).

Detection the Virulence factors of *E. faecalis*

Hemolysin production was detecting by culturing the isolates on blood agar base supplemented with 5% blood, and hemolytic activity was observed as clear zone (^{2}- hemolysis) surrounding the bacterial colonies. All the bacterial isolates showed ^{2}-hemolysis (Figure 1). This result was similar to the result reported by Al-Jmor (2012) that showed 100% of *E. faecalis* isolates have the ability to produce hemolysin. Jankoska *et al.* (2008) reported that 50% of isolates produce hemolysin. In local studies reported by AL-Khafaji *et al.* (2010) and Mousa (2012) that showed the percentage of hemolysin production by *E. faecalis* isolates was 15.1%, 40%. Tellis and Muralidharan (2012) showed the percentage of *E. faecalis* isolates which have ability to produce hemolysin is 44%.

Lipase production was detected on egg-yolk agar plates, the hydrolyzed clear zone around the colonies were considered a positive result. The results of this study showed that 25% of the *E. faecalis* isolates were lipase producer (Figure 1).

AL-Khafaji *et al.* (2010) showed that the percentage of lipase producer was 3%; while Al-Jmor (2012) has reported 2.94% of isolates were lipase producer, whereas; the result of this study was similar to the findings of Elsner *et al.* (2000), Dworniczek *et al.* (2005) were 35% and 33%, respectively.

The biological role of lipase in infection might be considered the most important step in bacterial infection, the most prominent role of microorganism extracellular lipases may be the digestion of host cellular lipids for nutrient acquisition, which results in sticking to host tissue and neighboring cell (Furumura *et al.*, 2006).

Protease production was detected on skim milk agar plates; the hydrolyzed clear zones around the colonies were indicating a positive result. The results showed 100% of isolates were protease producers (Figure 1). This result was close to Furumura *et al.* (2006) who demonstrated 75% of *E. faecalis* isolates was protease producer. Al-Jmor (2012) was reported a result similar to this study, showing that 100% of *E. faecalis* isolates was protease producing.

Gelatinase is extracellular metalloprotease secreted by *E. faecalis*, hydrolyzes gelatin, collagen, and casein, and has been implicated as a virulence factor in animal models. The ability of this enzyme to hydrolyze collagens and certain bioactive peptides suggests its participation in the initiation and propagation of inflammatory processes involving *E. faecalis* (Furumura *et al.*, 2006).

Table 1. Number and Nomenclature of bacterial isolates

Specimens	No. of specimens	*E. faecalis* isolate symbol
Urine	15	U
Blood	60	B
Root canal	50	R
Burns	10	W.i

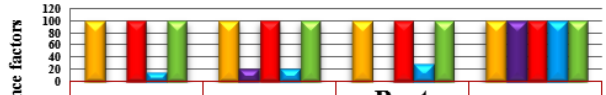
Table 2. The primers and their sequences used in conventional PCR

Gene	Primer name	Sequence 5'→3'	Size (bp)	References
vanA	VAF	GGGAAAACGACAATTGC	732	Satak *et al*., 1997; Depardieu *et al*., 2004;
vanA	VAR	GTACAATGCGGCCGTCGTTA	732	Fatholahzadeh *et al*., 2006; Biendo *et al*., 2010; Tellis and Muralidharan, 2012.
vanB	VBF	ATGGGAAGCCGATAGTC	635	Dutka-Malen *et al*., 1995; Jayaratne and
vanB	VBR	GATTTCGTTCCTCGACC	635	Rutherford, 1999; Biendo *et al*., 2010; Tellis and Muralidharan, 2012.
Esp	EF	TTGCTAATGCTAGTCCACGACC	933	Eaton and Gasson, 2001; Creti *et al*., 2004;
Esp	ER	GCGTCAACACTTGCATTGCCGAA	933	Jankoska *et al*., 2008.

Gelatinase production was detected on nutrient gelatin, liquefaction of gelatin after placed nutrient gelatin in refrigerator considered a positive result.

The results of this study showed that 10% of *E. faecalis* isolates were gelatinase producer (Figure 1). This result was in agreement to the result of Al-Jmor (2012) that represented the percentage of gelatinase producer was 11.76%, whereas Vergis *et al.* (2002) showed that 64% of *E. faecalis* isolated from patients with bacteremia, produced gelatinase, while Jankoska *et al.* (2008) was reported that 68% of *E. faecalis* isolates were gelatinase producer.

Aggregation substance (AS) of *E. faecalis* is a pheromone inducible surface protein encoded by pheromone plasmids that facilitates conjugative exchange, by mediating strong binding between cells and formation of cell aggregates (clumps). AS also contributes to pathogenicity by enhancing



	Urine isolates	Blood isolates	Root Canal isolates	wound isolate
Hemolysin	100	100	100	100
Gelatinase	0	20	0	100
Protease	100	100	100	100
Lipase	14.28	20	28.57	100
Aggregation Substance	100	100	100	100

Fig. 1. Percentages of Virulence factors produced by *E. faecalis* isolates

Table 3. Program was processed in PCR amplification of *vanA* gene according to Fatholahzadeh *et al*., 2006.

Stage	Temperature	Time	
Initial denaturation	94 ºC	5 min.	
Denaturation	94 ºC	45 sec.	30 cycles
Annealing	54 ºC	45 sec.	
Extension	72 ºC	45 sec.	
Final Extension	72 ºC	5 min.	

Table 4. Program was processed in PCR amplification of vanB gene according to Dutka-Malen *et al*., 1995

Stage	Temperature	Time	
Initial denaturation	94 ºC	2 min.	
Denaturation	94 ºC	1 min.	30 cycles
Annealing	54 ºC	1 min.	
Extension	72 ºC	1 min.	
Final Extension	72 ºC	10 min.	

Table 5. Program was processed in PCR amplification of esp gene according to Creti *et al*., 2004

Stage	Temperature	Time	
Initial denaturation	95 ºC	5 min.	
Denaturation	95 ºC	1 min.	30 cycles
Annealing	63 ºC	1 min.	
Extension	72 ºC	1 min.	
Final Extension	72 ºC	10 min.	

Table 6. Program was processed in Multiplex PCR amplification of vanA and vanB genes

Stage	Temperature	Time	
Initial denaturation	94 ºC	5 min	
Denaturation	94 ºC	1 min	30 Cycles
Annealing	54 ºC	1 min	
Extension	72 ºC	1 min	
Final Extension	72 ºC	10 min	

cell adhesion and internalization as well as favoring intracellular survival within macrophages (Glimore, 2002).

Aggregation substance was detected on trypticase soy broth, the presence of adherent dense cell growth in the bottom of the tube was considered a positive result (Figure 2).

The results showed that all tested isolates (100%) have produced a slime layer by this method but in different levels of adherent layer which ranged from weak to moderate and strong (Figure 3). The result of this study was similar with the result reported by Al-Jmor (2012), the result of this study was higher than the result reported by Necidova *et al.,* (2009) and Oli *et al.,* (2012) who found that 28% and 85% of *E. faecalis* isolates were produce slime layer, respectively.

The results in figure (1) , showed that all *E. faecalis* isolates (100%) were AS producer. The result of this study was similar to Paoletti *et al.* (2007) was showed that 100% of *E. faecalis* isolates were AS producer, While Tomita and Ike (2008) were reported (33%) of *E. faecalis* isolates have the ability to produce AS. Alebouyeh *et al.* (2005) was showed 23.3% of *E. faecalis* isolates were AS producer.

Table 7. Numbers and percentages of E. *faecalis* isolates from clinical specimens

Sample type	No. of samples	*E. faecalis*	% of *E. faecalis* from each source
Urine	15	7	46.6%
Blood	60	5	8.3%
Root canal	50	7	14%
Burns	10	1	10%
Total	135	20	78.9%

Table 9. Multi-drug resistant pattern of *E. faecalis* isolates

Number of Antibiotic typeswere resistant by isolates	Number of isolates	Percentage of isolates
2 or less	2	10%
3	3	15% MDR
4	2	10% MDR
5 or more	13	65% MDR

A positive correlation between the presence of Esp and the ability of an Enterococcal strain to form biofilms *in vitro* has been reported. None of the *esp*-deficient isolates tested in that study were capable of forming biofilms. However, it was also observed that insertional inactivation of *esp* did not cause a loss of the biofilm phenotype in every mutant tested (Toledo-Arana *et al*., 2001).

The involvement of Enterococcal surface protein in biofilm formation in the presence of a higher glucose concentration has been reported

Table 8. The Minimum Inhibitory Concentrations [MICs] of Vancomycin for E. faecalis isolates

Id	Isolates	Specimen	MIC [µg/ml]	Susceptibility
1	1U	urine	32	R
2	2U	urine	4	S
3	3U	urine	64	R
4	4U	urine	32	R
5	5U	urine	64	R
6	6U	urine	64	R
7	7U	urine	32	R
8	3B	Blood	64	R
9	4B	Blood	4	S
10	6B	Blood	16	IR
11	7B	Blood	128	R
12	8B	Blood	16	IR
13	1w.i	Wound	4	S
14	1R	Root canal	16	IR
15	2R	Root canal	32	R
16	3R	Root canal	64	R
17	4R	Root canal	128	R
18	5R	Root canal	32	R
19	6R	Root canal	64	R
20	7R	Root canal	16	IR

S: Sensitive, IR: Intermediate Resistant, R: Resistant.

by Tendolkar *et al*., (2004). Two *E. faecalis esp*-positive strains and produce significantly more bio volume and thickness of biofilm than their controls, esp-negative strains. In the same study, the presence of 0.5% glucose in the growth medium influenced the biofilm production by *E. faecalis* (Tendolkar *et al*., 2006).

Di Rosa *et al*., (2006) have explained that neither *esp* seemed to be required for biofilm formation: both *E. faecalis* and did not show a correlation between the presence of *esp* and biofilm

formation. The presence of *esp* and biofilm together was only found in strains from clinical settings, suggesting that there exists a synergy between this factor which serves as an advantage for the process of infection.

DNA and plasmid Extraction from *E. faecalis* isolates

The concentration results of DNA were ranged between 100-1800 ng/ml, while the purity of Genomic DNA was ranged between 1.8-1.9 in wavelength 260/280. This indicates the method was efficient in extraction and purification of DNA as shown in figure 4. The purpose of DNA extraction is for detection the virulence factor Enterococcal surface protein *esp* gene.

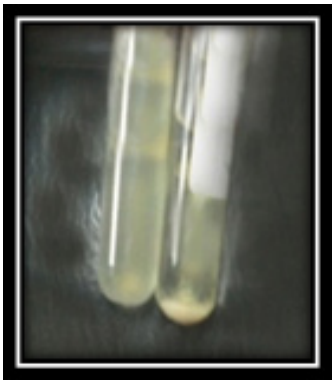


Fig. 2. Cell aggregation of *E. faecalis* strains in Trypticase soy broth (A) Negative AS, (B) Positive AS

Gel electrophoresis: agarose (0.8%), TBE buffer (1x), 70 volt for 1 hr., stained with ethidium bromide. 1: DNA extracted from the isolate 3B; 2: DNA extracted from the isolate 7B; 3: DNA extracted from the isolate 6U; 4: DNA extracted from the isolate 6B; 5: DNA extracted from the isolate 4B; 6: DNA extracted from the isolate 1R; 7: DNA extracted from the isolate 8B; 8: DNA extracted from the isolate 5R; 9: DNA extracted from the isolate 1U; 10: DNA extracted from the isolate 7U; 11: DNA extracted from the isolate 1W.i; 12: DNA extracted from the isolate 2U; 13: DNA extracted from the isolate 4R; 14: DNA extracted from the isolate 4U; 15: DNA extracted from the isolate 3U; 16: DNA extracted from the isolate 2R; 17: DNA extracted from the isolate 7R; 18: DNA extracted from the isolate 5U; 19: DNA

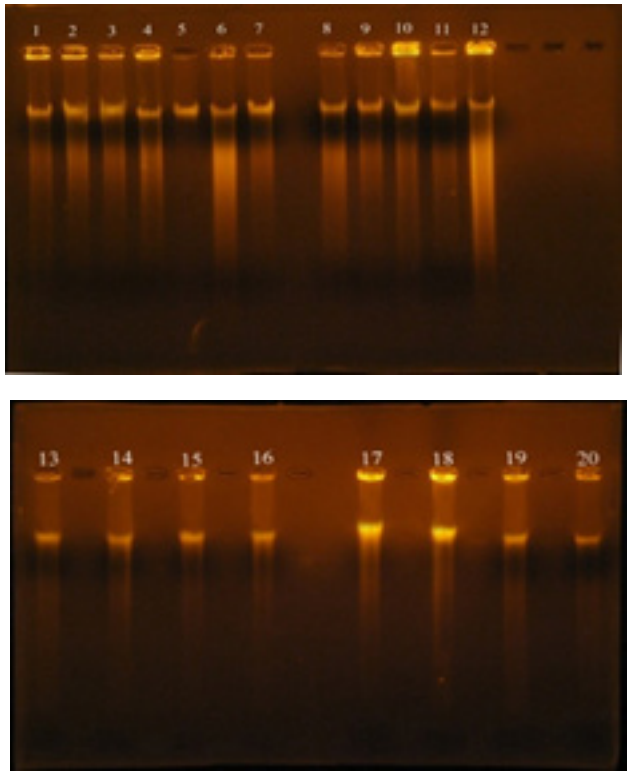
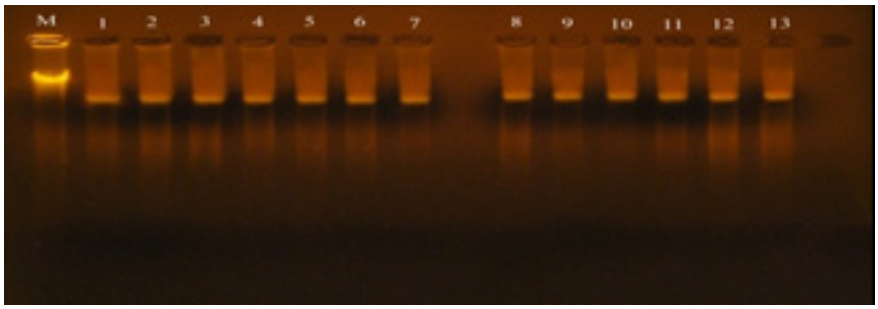


Fig. 3. DNA bands extracted from 20 *E. faecalis* isolates

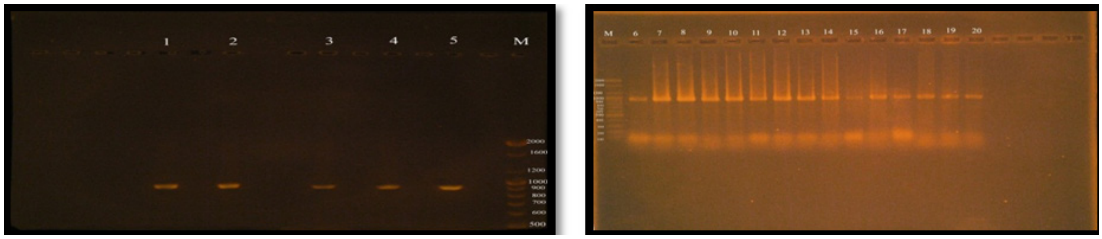
extracted from the isolate 6R; 20: DNA extracted from the isolate 3R.

The plasmid DNA was extracted from 13 isolates of *E. faecalis* using Accuprep ® PCR plasmid mini extraction kit, the isolates were (1w.i) isolate from wound infection was sensitive to vancomycin (MIC 4µg/ml), (1R) isolate from root canal was intermediate resistant to vancomycin (MIC 16 µg/ml), (1U, 7U, 5R) isolates from Urine and Root canal were resistant to vancomycin (MIC 32 µg/ml), (3B, 5U, 3U, 6U, 3R, 6R) isolates from Blood, Urine and Root canal, respectively (MIC 64 µg/ml) and (4R , 7B) isolated from root canal and blood (MIC 128 µg/ml). The result of agarose gel electrophoresis (figure 5), showed that all thirteen isolates have one large (Mega) plasmid, this result was similar to study reported by Kandela (2006) showed that *E. faecalis* isolates contain one large (Mega) plasmid. Flannagan *et al.* (2003) were showed that Vancomycin-resistant *E. faecalis* was found to contain two plasmids, designated pAM830 (45 kb) and pAM831 (95 kb). The pMG1-like plasmids were include the pHT plasmids (pHT^2 (65.9 kbp), pHT^2 (63.7 kbp), and pHT^2 (66.5 kbp) are highly conjugative pheromone-independent (Tomita and Ike, 2005).



Gel electrophoresis: agarose (0.8%), TBE buffer (1x), 60 volt for 2 hr., stained with ethidium bromide. M: chromosomal DNA as ladder; 1: plasmid DNA extracted from the isolate 6R; 2: plasmid DNA extracted from the isolate 1R; 3: plasmid DNA extracted from the isolate 5R; 4: plasmid DNA extracted from the isolate 5U; 5: plasmid DNA extracted from the isolate 4R; 6: plasmid DNA extracted from the isolate 3U; 7: plasmid DNA extracted from the isolate 6U; 8: plasmid DNA extracted from the isolate 7U; 9: plasmid DNA extracted from the isolate 7B; 10: plasmid DNA extracted from the isolate 3B; 11: plasmid DNA extracted from the isolate 2U; 12: plasmid DNA extracted from the isolate 3R; 13: plasmid DNA extracted from the isolate 1w.i.

Fig. 4. Plasmid DNA bands extracted from 13 E. faecalis isolates

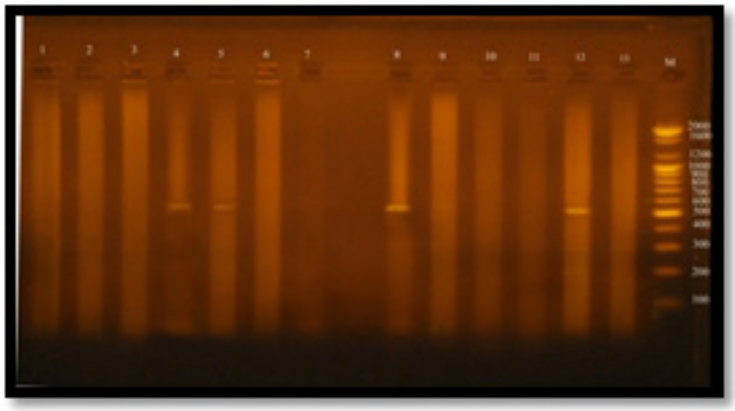


Gel electrophoresis (figure 6): agarose (1.5%), TBE buffer (1x), 70 volt for 1.5 hrs. stained with ethidium bromide. M: DNA ladder (100 bp); 1: Positive PCR amplification of the isolate 7R; 2: Positive PCR amplification of the isolate 6R; 3: Positive PCR amplification of the isolate 6B; 4: Positive PCR amplification of the isolate 7U; 5: Positive PCR amplification of the isolate 3U; 6: Positive PCR amplification of the isolate 4R; 7: Positive PCR amplification of the isolate 7B; 8: Positive PCR amplification of the isolate 2R; 9: Positive PCR amplification of the isolate 4U; 10: Positive PCR amplification of the isolate 6U; 11: Positive PCR amplification of the isolate 8B; 12: Positive PCR amplification of the isolate 1w.i; 13: Positive PCR amplification of the isolate 1U; 14: Positive PCR amplification of the isolate 5R; 15: Positive PCR amplification of the isolate 3B; 16: Positive PCR amplification of the isolate 2U; 17: Positive PCR amplification of the isolate 3R; 18: Positive PCR amplification of the isolate 5U; 19: Positive PCR amplification of the isolate 1R; 20: Positive PCR amplification of the isolate 4B.

Fig. 5. Gel electrophoresis of amplified PCR product of virulence factor Enterococcal surface protein (esp gene) in monoplex pattern

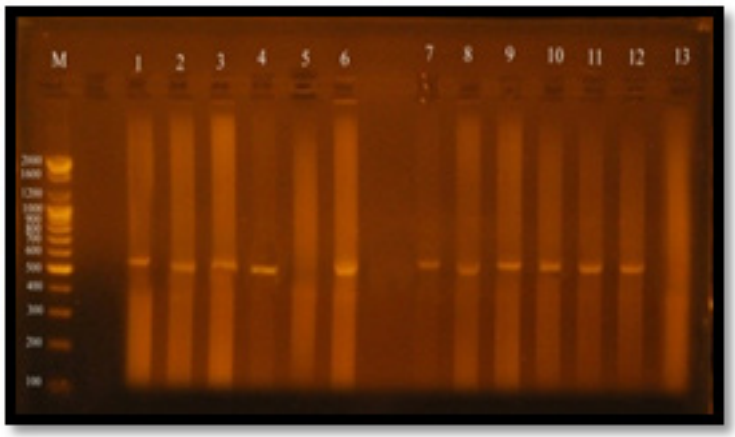
Enterococcal plasmids have been classified into 3 groups by a mixture of these methods; the Inc 18 group of plasmids (rep group 1, represented by pIP501 and partially rep family 2, represented by pRE25), the rolling circle replication plasmids (mostly rep family 4 and 6, represented by pMBB1 and pS86, respectively) and the pheromone responsive plasmids of *E. faecalis* (rep family 8 and 9, represented by pAM373 and pCF10) (Rosvoll, 2012).

The results obtained by Al-Jmor (2012) as result of plasmid extraction, the vancomycin resistant *E. faecalis* isolates have two large plasmids with molecular weight higher than (1 Kb) of DNA ladder, while the vancomycin sensitive isolates have one large plasmid.



Gel electrophoresis (figure 3-11): agarose (1.5%), TBE buffer (1x), 65 volt for 1 hrs. stained with ethidium bromide. M: DNA ladder (100 bp); 1: Positive PCR amplification of the isolate 6R; 2: Positive PCR amplification of the isolate 1R; 3: Positive PCR amplification of the isolate 5R; 4: Positive PCR amplification of the isolate 5U; 5: negative PCR amplification of the isolate 4R; 6: Positive PCR amplification of the isolate 3U; 7: Positive PCR amplification of the isolate 6U; 8: Positive PCR amplification of the isolate 7U; 9: Positive PCR amplification of the isolate 7B; 10: Positive PCR amplification of the isolate 3B; 11: Positive PCR amplification of the isolate 1U; 12: Positive PCR amplification of the isolate 3R; 13: negative PCR amplification of the isolate 1w.i.

Fig. 6. Gel electrophoresis of amplified PCR product of *vanA* gene (550 bp) of *E. faecalis isolates* in monoplex pattern



Gel electrophoresis (figure 3-12): agarose (1.5%), TBE buffer (1x), 65 volt for 1 hrs. stained with ethidium bromide. M: DNA ladder (100 bp); 4: Positive PCR amplification of the isolate 5U; 5: positive PCR amplification of the isolate 4R; 8: Positive PCR amplification of the isolate 7U; 12: Positive PCR amplification of the isolate 3R.

Fig. 7. Gel electrophoresis of amplified PCR product of *vanB* gene (nearly 600 bp) of *E. faecalis* isolates in monoplex pattern

vanA and *vanB* genes are carried on *Tn-1546* like transposon on the pHT² plasmid which have molecular weight(63.7 kbp) (Tomita and Ike, 2005, Tomita and Ike, 2008).

Gel electrophoresis: agarose (0.8%), TBE buffer (1x), 60 volt for 2 hr., stained with ethidium bromide. M: chromosomal DNA as ladder; 1: plasmid DNA extracted from the isolate 6R; 2: plasmid DNA extracted from the isolate 1R; 3: plasmid DNA extracted from the isolate 5R; 4: plasmid DNA extracted from the isolate 5U; 5: plasmid DNA extracted from the isolate 4R; 6: plasmid DNA extracted from the isolate 3U; 7: plasmid DNA extracted from the isolate 6U; 8: plasmid DNA extracted from the isolate 7U; 9: plasmid DNA extracted from the isolate 7B; 10: plasmid DNA extracted from the isolate 3B; 11: plasmid DNA extracted from the isolate 2U; 12: plasmid DNA extracted from the isolate 3R; 13: plasmid DNA extracted from the isolate 1w.i.

Gel electrophoresis (figure 3-13): agarose (1.5%), TBE buffer (1x), 65 volt for 1 hrs. stained with ethidium bromide. M: DNA ladder (100 bp); 1: Positive PCR amplification of *vanA* for the isolate 6R; 2: Positive PCR amplification of *vanA* for the isolate 1R; 3: Positive PCR amplification of *vanA* for the isolate 5R; 4: Positive PCR amplification of *vanA,vanB* for the isolate 5U; 5: positive PCR amplification of *vanB* for the isolate 4R; 6: Positive PCR amplification of *vanA* for the isolate 3U; 7: Positive PCR amplification *vanA* for the isolate 6U; 8: Positive PCR amplification of *vanA,vanB* for the isolate 7U; 9: Positive PCR amplification of *vanA* for the isolate 7B; 10: Positive PCR amplification of *vanA* for the isolate 3B; 11: Positive PCR amplification of *vanA* for the isolate 1U; 12: Positive PCR amplification of *vanA,vanB* for the isolate 3R; 13: negative PCR amplification of the isolate 1w.i.

Fig. 8. Gel electrophoresis of amplified PCR product of *vanA, vanB* genes of *E. faecalis isolates* in multiplex pattern

esp gene amplification by monoplex PCR technique

To detect the virulence factor Enterococcal surface protein *esp* gene in *E. faecalis*, Twenty *E. faecalis* isolates was subjected to PCR technique in a monoplex pattern.

The Positive result of *esp* gene was confirmed by 1.5% agarose gel electrophoresis stained with ethidium bromide, electrophoresed in 70 volt for 1.5 hrs and photographed under ultraviolet (UV) trans illuminator (figure 3-10). The results of present study showed that *esp* gene band detected at 933 bp region, all *E. faecalis* isolates have *esp* gene as virulence factor.

Figure (6) demonstrated that the twenty (100%) of *E. faecalis* isolates were obtained from clinical samples found it has *esp* gene, Shankar *et al.* (1999) was reported that 26.3% of *E. faecalis* isolates having the virulence factor ESP. Creti *et al.* (2004) and Jankoska *et al.* (2008) were reported that *E. faecalis* which have the *esp* gene was represented to 44.6% and 76%, respectively.

The results of this study were similar to results reported by; Jankoska *et al.* (2008) Eaton and Gasson (2001), who showed that the molecular weight of PCR product *(esp)* bands in gel electrophoresis is 933bp.

The results in this study have stated all *E. faecalis* isolates were produced *esp* gene regardless

Table 3-6. Comparison between the MIC values of *E. faecalis* ioslates and their *van* genotype

Id	isolates	MIC (µg/ml)	Susceptibility	*van* genotype
1	1U	32	R	*vanA*
3	3U	64	R	*vanA*
5	5U	64	R	*vanA, vanB*
6	6U	64	R	*vanA*
7	7U	32	R	*vanA, vanB*
8	3B	64	R	*vanA*
11	7B	128	R	*vanA*
13	1w.i	4	S	*ÜÜÜÜÜÜ*
14	1R	16	IR	*vanA*
16	3R	64	R	*vanA, vanB*
17	4R	128	R	*vanB*
18	5R	32	R	*vanA*
19	6R	64	R	*vanA*

of their susceptibility to vancomycin (resistant or sensitive).

Sharifi *et al.* (2012) and Comerlato *et al.* (2013) have stated there is no association between the virulence factor ESP and any other virulence factor or antibiotic resistance.

In this study all *E. faecalis* isolates which is *esp* producer have produced biofilm which was ranged between strong to moderate and weak. This explains a strong positive correlation between the presence of *esp* and biofilm formation, and *E. faecalis* biofilm productivity dependent on *esp* expression.

Chuang-Smith *et al.* (2010) speculated that Esp may mediate the interaction with primary surfaces and participate in the formation of this phenotype. In addition, Heinkens *et al.* (2007) showed that Esp is involved in biofilm formation.

There are diverging opinions concerning the role of Esp in biofilm production (Garth *et al.* 2008). Dworniczek *et al.* (2012) concluded that there is no clear relationship between the expression of *esp* or *gelE* and biofilm formation. Indeed, an analysis by Sillanpaa *et al.* (2010) showed efficient biofilm production in the absence of Esp.

Comerlato *et al.* (2013) was showed there is no association between the presence of *esp* and biofilm production, they were assumed that other factors are associated with this phenotype.

***vanA, vanB* Genes Amplification by Monoplex PCR Technique**

The accurate and rapid diagnosis of antibiotic resistance genes in the treatment of *E. faecalis* infections is extremely important in preventing the spread of infections. PCR-based molecular methods are often preferred for determination of antibiotics resistant genes. Using PCR technique, the genetic determinants of vancomycin resistant *vanA* and *vanB* were amplified to identify susceptible (lacking *vanA or vanB*) and resistant (*vanA or vanB*) for 13 *E. faecalis* isolates.

The results of the present study showed that, *vanA* gene bands were detected at 550 bp regions, Eleven *E. faecalis* isolates (84.6%) were produced 550 bp band (Figure 7), Four *E. faecalis* isolates (30.7%) produced *vanB* gene bands were nearly detected at 600 bp region, (Figure 8).

Regarding *vanA* gene bands, the sensitive isolate (1w.i) did not have *vanA* gene, since it is specific marker for vancomycin resistance.

While the resistant isolates (6R, 1R, 5R, 5U, 3U, 6U, 7U, 7B, 3B, 1U, 3R) have shown *vanA* gene bands except the resistant isolate (4R) haven't *vanA* band (it showed *vanB* genotype), the molecular weight of *vanA* bands was 550 bp.

vanA resistance is usually inducible by sub inhibitory concentrations of vancomycin and teicoplanin and it associated with production of a 38-40 kD membrane protein called VanA and encoded by *vanA* gene (Nicas *et al.*, 1989).

The deduced amino acid sequence of *vanA* indicates that it is related to the bacterial D-Ala:D-Ala ligases, which mediate the D-alanyl-D-alanine (D-Ala-D-Ala) linkages of the growing cell wall peptidoglycan (Malen *et al.*, 1990). VanA appears to be a D-Ala:D-X ligase of relaxed substrate specificity that can condense D-alanine with other amino acids, fatty acids, or hydroxy acids (Arthur *et al.*, 1992). In resistant isolates, D-Ala-D-Ala is replaced with D-Ala-D-lactate (D-Lac), which cannot bind glycopeptides, The production of D-Ala-D-Lac depends on the cooperative activity of three enzymes, VanA, VanH, and VanX (Arthur *et al.*, 1991). In vancomycin-resistant *E. faecalis,* the genes encoding these proteins are located on a plasmid and arranged sequentially within an operon. Induction of resistance is mediated by the transcription activator *vanR* and the membrane sensor *vanS,* located upstream of the other members of the gene cluster (Arthur *et al.,* 1992; French, 1998).

The results showed by Satak *et al.* (1997); Depardieu *et al.* (2004); Fatholahzadeh *et al.* (2006); Biendo *et al.* (2010); Tellis and Muralidharan, (2012), they were detected *vanA* gene in *E. faecalis* isolates and the molecular weight was 732 bp. While Clark *et al.* (1993); Kariyama *et al.* (2000) were reported the molecular weight of *vanA* from PCR product was 1030 bp. Miele *et al.* (1995); Patel *et al.* (1997); Lim *et al.* (2006); were reported that molecular weight of *vanA* gene from PCR product was 1029bp, 885 bp, 1114 bp, respectively. These results indicate that *vanA* gene have variation in molecular weight.

The results *vanA* in this study revealed that the gene have a genetic variation as showed in their molecular weight during electrophoresis of vancomycin resistant *E. faecalis* strains.

Figure 3-12shows that the molecular weight of *vanB* gene bands were near 600 bp region, Vancomycin resistant *E. facalis* strains and their MIC were (5U and 3R=64 µg/ml, 4R=128 µg/ml and 7U=32 µg/ml).

The *vanB* operon contains genes encoding a dehydrogenase, a ligase, and a dipeptidase, all of which have a high level of sequence identity (67%–76% identity) with the corresponding deduced proteins of the *vanA* operon, the function of the additional vanW protein found only in the *vanB* cluster is unknown (Patel *et al.*, 1998; Dahl *et al.,* 1999)

The regulatory system in class B strains appears insensitive to induction by teicoplanin. Teicoplanin induces the synthesis of VanA-related proteins but does not induce the production of VanB-related proteins. On the other hand, vancomycin induces the synthesis of the resistance proteins of both systems, and in fact, if a teicoplanin-susceptible *Enterococcus* with the *vanB* gene cluster is preexposed to vancomycin, the strain then tests teicoplanin resistant as well (Cetinkaya *et al.*, 2000).

Regarding to genetic variation, the results of this study showed that *vanB* gene bands have molecular weight closely to 600 bp, while the results reported by Satak *et al.* (1997); Depardieu *et al*. (2004); Biendo *et al.* (2010); Tellis and Muralidharan, (2012), they were detected *vanB* gene in *E. faecalis* isolates and the molecular weight was 635 bp. While Clark *et al.* (1993); Kariyama *et al.* (2000) were reported the molecular weight of *vanA* gene from PCR product was 433 bp. Miele *et al.* (1995); Patel *et al.* (1997) were reported that molecular weight of *vanA* gene from PCR product was 457bp, 885 bp, respectively.

vanA and *vanB* genes carried on *Tn-1546* transposon that it is ubiquity, this gene could move (transpose) from one plasmid to other in the same or different bacterial strain, this explained by the conjugation experiment. The complete genome sequence of *E. faecalis*, a vancomycin-resistant clinical isolate, revealed that more than a quarter of the genome consists of probable mobile or foreign DNA. One of the predicted mobile elements is a previously unknown *vanB* vancomycin-resistance conjugative transposon (Paulsen *et al.*, 2003).

In addition to transcriptional and translational regulatory mechanisms, many bacteria have evolved additional mechanisms to enable them to respond to changing environments. In these bacteria a genetically divers population is generated by reversible genetic changes known as phase variation, therefore; these isolates to survive in their environment, it must gain a new characters that enable them for the survivor, they are gained these characters with missing some of unimportant sequences from genes that still activated in absence these sequences (Dale and park, 2003).

Detection of *vanA, vanB* genes using Multiplex PCR Technique

The multiplex PCR assay in our hands is a convenient and rapid method for determining glycopeptide resistance genotypes for *Enterococcus* spp. in the clinical microbiology laboratory. The assay provides a more specific and rapid alternative to classical phenotypic methods for the detection of low level glycopeptide resistance (MIC range, 4 to 8 mg/ml) (Patel *et al*. 1997).

Figure 3-13 demonstrated that Twelve (95%) *E. faecalis* isolates out of thirteen isolates were obtained from clinical samples found to be vancomycin resistant isolates, Eleven *E. facalis* isolates (84.6%) have *vanA* gene bands were detected at 550bp regions, Four *E. faecalis* isolates (30.7%) have *vanB* gene bands were detected nearly at 600bp region. Three of *E. faecalis* isolates (5U, 7U and 3R) were having *vanA*-*vanB* cluster.

All *E. faecalis* isolates were showing *vanA* or *vanB* genotype in PCR assay, no other genotype were showed because of only the extracted pHT² plasmids were amplified in PCR for detection *vanA* or *vanB* genes or both, the other *van* genes are chromosomally.

The results of MIC showed a perfect similarity with the results of PCR, this reflect the efficiency of MIC method. Vancomycin-sensitive *E. faecalis* isolates were have MIC of vancomycin 4 µg/ml and they were havn't any *van* genes in gel electrophoresis of multiplex PCR product, While the Intermediate-resistant *E. faecalis* isolates showed *vanA* genotype and its MIC was 16 µg/ml (Table 3-6). The resistant *E. faecalis* isolates have shown identical results between MICs and PCR which was varied in its *van* genes, these isolates showed *vanA*, *vanB* or *vanAvanB* cluster genotypes. While it's MIC was e"32.

Resistance to glycopeptides is disseminating rapidly and has recently spread to

methicillin-resistant *S. aureus* (Sievert *et al.* 2002). Rapid and accurate methods are thus essential for the detection of such clinical isolates and the prevention of their transmission. In addition, MIC determination is time-consuming and does not detect GRE with low-level glycopeptide resistance and misidentification of *E. faecalis* can occur with commercial systems (Willey *et al,* 1999; Depardieu *et al.* 2004).

One of the limitations of the method proposed, or of other similar assays, could be the sequence variability among *van* genes that has occasionally been observed (Perichon *et al.* 1997).

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