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Molecular study of virulence genes to distinguish between the *Enterococcus* Sp. Isolates

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Abstract :

The study aimed to use specific primer genes to identify and differenated the *Enterococcus* spp. Isolated from different clinical specimens by detected the virulence factors encoding genes such as *efaA* (endocarditis-associated antigen), *esp* (enterococcal surface protein) , *eep* (stimulating of pheromones expression) and *enlA* (enterolysin A) genes by PCR techniques.

Regard to genotypic study the outcome showed that 21(84%) isolates of *E.faecalis* ,4(40%) of *E.faecium* and 5(100%) of *E.avium* were carrying *efaA* gene and 17(68%) of *E.faecalis* , 5(50%) of *E.faecium* , 5(100%) of *E.avium* were carrying *esp* gene while *eep* gene was carrying only by *E.faecalis* and *E.faecium* ; 12(48%) and 3(30%) respectively . Also the results revealed that only 1(4%) isolates of *E.faecalis* have *enlA* gene .

Introduction

The virulence of enterococci is associated with several genes, including *ace* (collagen binding cell wall protein), *acm* (surface-exposed antigen), *agg* (aggregative pheromone-inducing adherence to extra-matrix protein), *agrBEfs* (AgrB protein of *E. faecalis*), *esp* (enterococcal surface protein), *hyl* (hyaluronidase), *cadI* (pheromone cAD1 precursor lipoprotein),

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the *cAM373* gene (sex pheromone cAM373 precursor), *cylABLM* (hemolysin), *efaAEfs* (endocarditis-specific antigen), *sagA* (secreted secreted antigen), and *gelE* (gelatinase) (1Hancock and Gilmore,2006)These virulence factors have been reported in enterococci isolated from food of animal origin Plasmid-encoded aggregation substance was found to contribute to *E. faecalis* adhesion to renal epithelial cells in vitro (2Valenzuela *et al.*,2009).

A novel feature of the esp protein is the presence of identical, large (82- and 84-amino-acid) repeat motifs encoded by nearly identical tandem repeating units within the structural of *esp* gene. Homologous recombination within these repeat units at the genetic level leads to addition or deletion of repeat units, resulting in an alteration in the size of the encoded protein (3Shankar *et al.*,1999). The esp protein might be involved in adhesion functions during the initial stages of infection, facilitating interaction with host receptors and may be detrimental to survival and persistence, favoring expression of a less- extended form of esp to evade the immune response, analogous to the phase variation observed for uropathogenic (4Klibi *et al.*,2007) .

Several factors important for virulence such as pheromone-inducible multifunctional aggregation substance AS (*asa1*, *prgB*, *asp1* gene variants and *asc10* on pathogenicity island), extracellular surface protein esp (encoded by *esp* gene on pathogenicity island), a group of hydrolytic enzymes including hyaluronidase and gelatinase (encoded by the plasmidic *hylEfm* gene and chromosomal *gelE* gene), secreted virulence factor such as cytolysin (encoded by *cylLL cylLs* genes on pathogenicity island or on the

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plasmid) and fsr locus, which regulates other genes important for virulence (5,6,7Kreft *et al.*, 1992; Rice *et al.*, 2003; Thurlow *et al.*, 2010).

Methodology:

Specimens Collection :

A total of (50) clinical specimens were collected from patients suffering from various clinical signs. All specimens were cultured on the MacConkey agar plates and incubated at 37°C under aerobic condition for 18 - 24 hour and confirmed by vitek system 8.

Molecular Study of *Enterococcus* species

1. Isolation of Bacterial Chromosomal DNA

Total DNA was extracted from colonies grown on agar plates by boiling method according to Yi *et al.*,(9) with some modifications.One bacterial colony was scraped using sterile toothpick from surface of agar plates and suspended in 40 µl Tris-EDTA buffer. The suspension was heated for 15 min at 100 °C followed by 5 min on ice rapidly. The suspension containing DNA was stored at -20 °C until used as template for PCR.

Concentration of DNA was determined spectrophotometrically by measuring its optical density at 260 nm (Extinction coefficient of dsDNA is 50 µg/ml at 260 nm) the purity of DNA solution is indicated by ratio of OD260/OD280 which is in the range of 1.8± 0.2 for pure DNA (10Stephenson ,2003).

2. PCR Assay :

The PCR assay was performed to detect the (efaA- esp-eep and enlA) genes for confirmation the identification of Enterococcus species and to detect the virulence factors encoded genes , these primers synthesized by Bioneer (Korea) .

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The PCR assay was performed to detect the(**efaA**) gene for confirmation the identification of *entrococcus sp*, These primers synthesized by Alpha DNA.F **efaA** - 5- GAC AGACCCTCACGAATA -3 Reverse **efaA** 5- AGTTCATCATGCTGCTGTAGTA -3. F - **esp** 5- TTGCTAATGCTAGTCCACGACC -3 Reverse **esp** 5- GCGTCAACACTTGCAT TGCCGAA -3. F - **eep** 5- GAGCGGGTATTTTAGTTCGT -3 Reverse **eep** 5- TACTCC AGCATTGGATGCT -3 F -*B* **enlA** 5- TTCTTCTTATTCTGTCAACGCAGC -3 Reverse **enlA** 5- GACTGTGAAATACCTATTTGCAAGC -3.

Optimization of pcr was accomplished after several trials, thus the following mixture was according to information of (Bioneer company /Korea) , Final volume 20 µL . The PCR products and the ladder marker are resolved by electrophoresis on 1.0 % agarose gel. The resolved band is indicative of the corresponding of (*efaA-esp-eep* and *enlA*) genes. The molecular weight identification of resolved band is based on their correspondence to the ladder bands (11Oho *et al.*,2000).

Gel electrophoresis was used for detection of DNA by UV transilluminator (12Sambrook and Russell , 2001).

Results and discussion

-Identification of *Enterococcus* spp. by VITEK-2 Compact System

The final identification was performed with the automated VITEK-2 compact system using GP-ID cards which contained 47 biochemical tests and one negative control .The results demonstrated that only (42) isolates were confirmed as *Enterococcus* spp. with ID message confidence level best (Probability percentage from 92 to 99).

Detection of Virulence Factors Encoding Genes

A collection of 40 *Enterococcus* spp. isolates recovered from different clinical specimens was molecular screened for the presence of virulence factors encoding genes, such as endocarditis-associated antigen (*efaA*), enterococcal surface protein (*esp*), stimulating of pheromones

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expression (*eep*) and enterolycin A (*enlA*) .The presence of genes that encode *efaA*, *esp*, *eep* and *enlA* was investigated by PCR technique as in the table (1).

The examined isolates of *Enterococcus* spp. showed varying presences of virulence factors encoding genes . The gene that encode *efaA* was found 21(84%) in *E .faecalis* ,4(40%) in *E.faecium* and 5(100%) in *E.avium* figure (1). This is in accordance with a previous study in which the gene was present in medical *E. faecalis* isolates (13Eaton and Gasson , 2001). The present data indicated that the presence of this virulence factor was slightly higher than those reported by other authors (14,15Franz *et al.*, 2001; Creti *et al.*, 2004).

Also, the gene that encode *esp* was found 17(68%) in *E.faecalis*, 5(50%) in *E.faecium* and 5(100%) in *E.avium* figure (2) while the gene that encode *eep* was found 12(48%) in *E .faecalis* and 3(30%) in *E.faecium* figure 3). The results showed that the gene encode *enlA* was found in 1(4%) in *E .faecalis* figure 4). The findings were identical with Champagne *et al.*, (162011) who fonud all 31 *E. faecalis* isolates were positive for the cell wall adhesin (*efaAEfs*), the 11 *E. faecium* isolates were positive for the *E. faecium*-specific cell wall adhesin *efmAEfm* (the *efmA* gene of *E. faecium*).

The present isolates of *Enterococcus* spp. showed that *efaA* and *esp* genes found in *E .faecalis* , *E.faecium* and *E.avium*. The *esp* gene is normally found in *E. faecalis*, but more recently has been identified in hospital-acquired strains of *E. faecium* (17Sava *et al.*,2010). The virulence

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gene *esp* has been shown to reside on a large pathogenicity islands (PAI) of *E. faecium* and *E. faecalis* (18McBride *et al.*,2009).

Table (1): Distribution of genotypic virulence determinants in *Enterococcus* species isolates .

primers / *Enterococcus* species	percentage of positive isolates %			
	efaA	*esp*	*eep*	*entA*
E .faecalis (no. 25)	21(84%)	17(68%)	12(48%)	1(4%)
E .faecium (no. 10)	4 (40%)	5(50%)	3(30%)	0%
E .avium (no. 5)	5(100%)	5(100%)	0%	0%

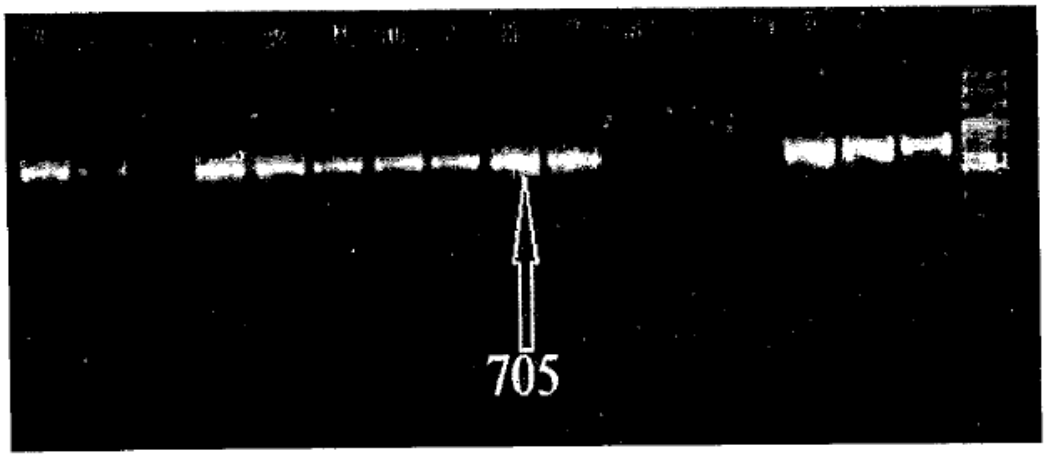

Figure (1): Ethidium bromide-stained agarose gel electrophoresis of PCR amplification products of *Enterococcus* spp. isolates that amplified with *efaA* gene primers with product 705 bp for 1 hr. at 80volt/cm.
Lanes (1-5) represent *E. avium* , Lanes (6-10) *E. faecalis* and Lanes (11-16) *E. faecium*.

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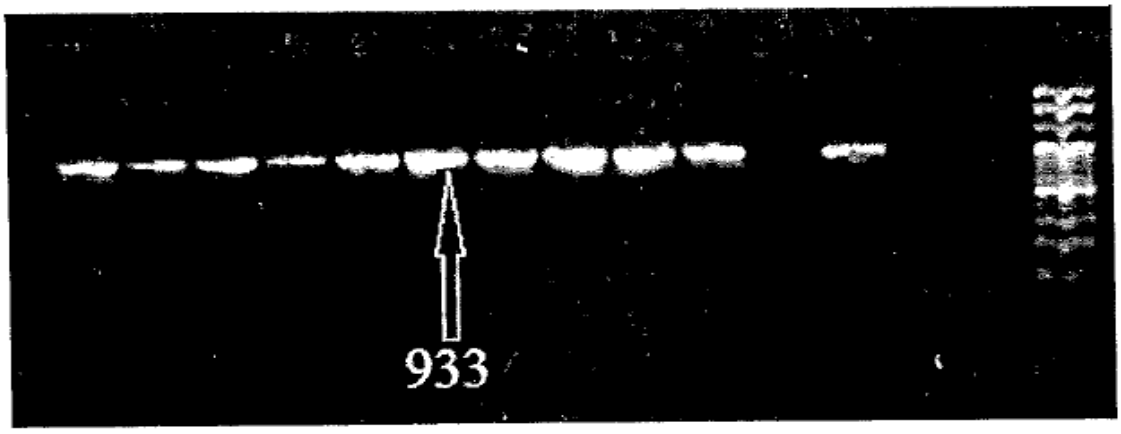

Figure (2):Ethidium bromide-stained agarose gel electrophoresis of PCR amplification products of Enterococcus spp. isolates that amplified with esp gene primers with product 933 bp for 1 hr. at 80volt/cm. Lanes (1-5) represent E.avium , Lanes (6-10) E. faecalis and Lanes (11-13) E. faecium.

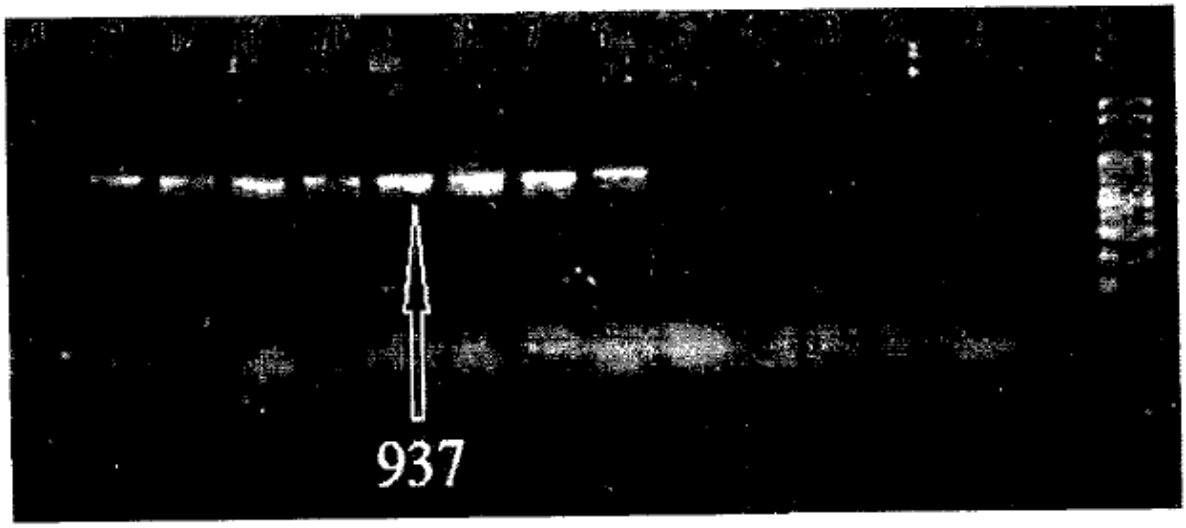

Figure (3): Ethidium bromide-stained agarose gel electrophoresis of PCR amplification products of *Enterococcus* spp. isolates that amplified with *eep* gene primers with product 937 bp for 1 hr. at 80volt/cm .
Lanes (1-5) represent *E.avium* , Lanes (6 -10) *E. faecalis* and Lanes (11-14) *E.faecium*.

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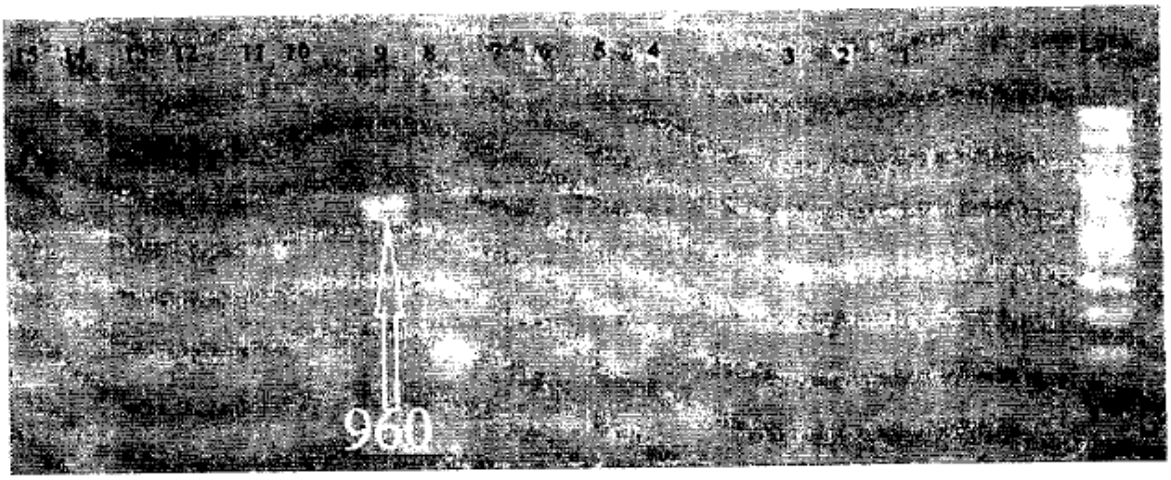

Figure (4): Ethidium bromide-stained agarose gel electrophoresis of PCR amplification products of *Enterococcus* spp. isolates that amplified with *enlA* gene primers with product 960 bp for 1 hr. at 80volt/cm . Lanes (1-5) represent *E. avium* , Lanes (6-10) *E. faecalis* and Lanes (11-15) *E. faecium.*

These observations seemed to agree with the findings of Elisa *et al.*, (2004) which was found that this virulence determinant was found at a low rate (9.5%) among clinical strains, with a tendency for it to be present more often among urinary strains than in purulent exudates and rectal swab strains, also, the results disaggrement with the findings of Maysa *et al* . (2012) who reported that *enlA* was not found in all isolates of *E.faecalis* ,the reasons may be returned to the difference in geographic location , enviroment factors encountered the patients ,the methods of isolation and decrease in the number of specimens.

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