

## Occurrence and distribution of enlA gene in clinical isolates of *Enterococcus faecalis* according to isolates sources

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### Abstract

Enterolysin A, is a cell wall-degrading bacteriocin (metalloendopeptidase) secreted by some *E. faecalis* strains which can kill a wide range of bacteria and on of virulence factors of this bacterium. The prevalence of enlA gene in *E. faecalis* isolated from clinical sources in our country is not studied. So the present study was performed to determine the prevalence of enlA gene in *E. faecalis* isolated from different types of infections and the distribution of this gene among these isolates according to infection site. The present study was conducted on Forty two clinical isolates of *Enterococcus faecalis* isolated in a previous study from patients attending Tikrit Teaching Hospital in Tikrit city, Iraq, with GIT , UTI ,vaginal, wound and burn infections which were re-identified according to microscopic, macroscopic and biochemical tests. Enterolysin A structural gene was determined in *E. faecalis* isolates by specific primer (EN1EF1:TTC TTC TTA TTC TGT CAA CGC AGC , ENIER1:GAC TGT GAA ATA CCT ATT TGC AAGC) using PCR technique. The results of PCR for enlA gene (the gene responsible for cell wall hydrolyzing activity) showed that (16.6%) of *E. faecalis* isolates possess this gene ,since gel electrophoresis results showed DNA bands with molecular size 960bp in comparison with DNA marker. According to the distribution of enlA gene of *E. faecalis* isolates in respect to isolates sources, the results showed that this gene was detected in 1(14.3%) of vaginal isolates, 1(10%) of urine isolates, 5(35.7%) of stool isolates, and no of wound and burn isolates were harbor this gene. No literatures concerning the distribution of enlA gene according to sources of infections were available for comparison. So from our results we can concluded that the distribution of this gene is low in clinical isolates of *E. faecalis*, while it is important in stool isolates for competition of this bacterium with intestinal normal flora and colonization.

**وجود وتوزيع جين enlA في العزلات السريرية لبكتريا *Enterococcus***

***faecalis* اعتمادا على مصادر العزل**

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### المستخلص

Enterolysin A من البكتريوسينات المحطمة للجدار الخلوي (metalloendopeptidase) التي تفرز من قبل بعض سلالات بكتريا *Enterococcus faecalis* والتي تستطيع قتل مدى واسع من البكتريا وهو احد عوامل ضراوة هذه البكتريا. ان انتشار جين enlA في بكتريا *E. faecalis* المعزولة من مصادر سريرية لم يدرس على المستوى المحلي لهذا تم تصميم الدراسة الحالية لتحديد مستوى انتشار هذا الجين في عزلات *E. faecalis* من مصادر الاصابات المختلفة وتوزيع هذا الجين بين هذه العزلات اعتمادا على مصادر الاصابة. اجريت هذه الدراسة على 42 عزلة سريرية من بكتريا *E. faecalis* تم عزلها في دراسة سابقة من المرضى المراجعين لمستشفى تكريت التعليمي في مدينة تكريت ، العراق ، والمصابين باخماج الحروق و الجروح والتهاب المهبل والمسالك البولية واخماج الجهاز

الهضمي والتي تم تأكيد تشخيصها بالاعتماد على الاختبارات المظهرية والمجهريّة والكيموحيوية . تم تحديد هذا الجين في عزلات بكتريا *E. faecalis* بواسطة بادئ مختصص (EN1EF1:TTC TTC TTA TTC TGT CAA CGC AGC , ENIER1:GAC TGT GAA ATA CCT ATT TGC AAGC)

باستخدام تقنية الـ PCR . اظهرت نتائج الـ PCR لجين *enla* (المسؤول عن فعالية تحليل الجدار الخلوي ) امتلاك عزلات *E. faecalis* لهذا الجين بنسبة (16.6%) مقارنة مع الدليل الحجمي للـ DNA . فيما يخص توزيع جين *enla* في عزلات بكتريا *E. faecalis* اعتمادا على مصادر هذه العزلات ، اظهرت النتائج وجود هذا الجين في عزلة واحدة من عزلات المهبل وبنسبة (14.3%) وعزلة واحدة من عزلات الاذراع أي بنسبة (10%) وفي خمس عزلات من عزلات الخروج وبنسبة (35.7%) . بينما لم تظهر النتائج وجود هذا الجين في عزلات الحروق والجروح. لم تشر المصادر لانتشار هذا الجين اعتمادا على مصادر الاصابة لغرض المقارنة لذلك فمن خلال النتائج يمكن الاستنتاج بان انتشار هذا الجين منخفضا " في العزلات السريرية لبكتريا *E. faecalis* بينما يعتبر هذا الجين مهما" في عزلات الخروج لاهميته في تنافس هذه البكتريا مع النبيت الطبيعي في الامعاء لغرض الاستعمار .

## Introduction

Enterococci are a component of the normal intestinal flora. In recent years, they have been reported as a major cause of nosocomial infections, and they are among the most common pathogens isolated from infected surgical sites and blood-stream and urinary tract infections. Enterococcus faecalis is responsible for about 80–90 % of all enterococcal infections and Enterococcus faecium accounts for most others (Jett et al., 1994; Johnson, 1994).

Enterococci are natural inhabitants of the gastrointestinal tract of humans and animals, but are also found in other anatomical sites including the vagina and oral cavity, and in plants and insects (Devriese et al., 1992; Muller et al., 2001; Pabich et al., 2003). Several reports have documented that Enterococcus faecalis is among the leading causes of several human infections, including bacteraemia, septicaemia, endocarditis, urinary tract infections, wound infections, neonatal sepsis and meningitis (Lautenbach et al., 1999; Giacometti et al., 2000; Higaki et al., 2002). And ranks among the top

three nosocomial bacterial pathogen(Devriese et al., 2006)

*E. faecalis* strains possesses several putative virulence determinants, including haemolysin/bacteriocin (also called cytolsin) (Ike et al., 1990), aggregation substance (AS) (Galli et al., 1990), gelatinase (Su et al., 1991), enterococcal surface protein (Esp) (Shankar et al., 1999), adhesion-associated protein EfaA (*E. faecalis* endocarditis antigen A) (Lowe et al., 1995), enhanced expression of pheromone (Eep) (An et al., 1999) and other factors.

Nilsen *et al.*, (2003) found other virulence determinant produced by Enterococcus faecalis LMG 2333, a novel type III bacteriocin. This bacteriocin, designated enterolysin A, which is a heat-labile protein with a broad inhibitory spectrum, which breaks down the cell walls of sensitive bacteria. It is a large(with molecular weight of 34501 Da),heat labile bacteriocin. It showed activity towards Lactobacillus, Lactococcus, Pediococcus, Enterococcus ,Listeria, Bacillus, and staphylococcus spp..The sequence analysis of enterolysin A suggested that this

bacteriocin consists of two separate domains, an N-terminal catalytic domain and a C-terminal substrate recognition domain. This bacteriocin is encoded as a preprotein consisting of a 343-amino-acid polypeptide bearing a 27-amino-acid-signal peptide. The N-terminal part of enterolysin A is linked by a threonine-proline-rich region to a putative C-terminal recognition domain (Hickey et al., 2003; Nilsen et al., 2003). Genes related to enterolysin A production are probably chromosome encoded (Javorsky & Vanat, 1992). A gene was found immediately downstream of the *enlA* gene that could be a putative transcription regulator (Hickey et al., 2003). Nilsen et al., (2003) showed that enterolysin A possess cell and posed cell-wall hydrolyzing activity.

PCR procedures are used to identify the virulence factors of *E. faecalis* (Creti et al., 2004; McGowan-Spicer et al., 2008; Han et al., 2011).

No information is available on the prevalence of *enlA* gene in *E. faecalis* isolated from clinical sources in our country, So the present study was performed to determine the prevalence of *enlA* gene in *E. faecalis* isolated from different types of infections and the distribution of this gene among these isolates according to isolates sources.

## **Materials and Methods**

### **- Bacterial isolates:**

Forty two clinical isolates of *Enterococcus faecalis* isolated in previous study from patients attending Tikrit Teaching Hospital in Tikrit city, Iraq, with GIT, UTI, vaginal, wound

and burn infections were re-identified according to microscopic, macroscopic and biochemical tests like: catalase, oxidase, voges-proskauer, starch hydrolysis, growth in 6.5% NaCl broth, sodium hippurate hydrolysis, growth at 100C and 45C, growth at pH 9.6, pyruvate fermentation, arginine hydrolysis, growth in 0.04% potassium tellurite, growth in 0.1% methylene blue, sugars fermentation and motility test (Facklam (1972); Cruickshank et al., 1975; Kelley & Post, 1980; Collee et al., 1996; Macfaddin, 2000).

### **- Genomic DNA Extraction:**

Genomic DNA extraction was done according to the method described by Roeder and Broda, (1987) and Thottappilly et al., (1999) as follows: Colonies of *E. faecalis* isolates were transferred to 10ml of nutrient broth and incubated for 24hrs. at 370C. The bacterial cells were collected by centrifugation, then washed with 0.1mM TE buffer. The washed bacterial cell was suspended into 200µl of 2xCTAB buffer. Washed pellet was resuspended by the addition of 100µl of (20%) SDS and incubated at 650C for 20 min. After incubation, 200µl of phenol:chloroform:isoamyl alcohol solution was added with gentle mixing then centrifuged at 10000 rpm for 10 min. The aqueous phase (upper layer) was transferred to sterile eppendorff tubes and an equal volume of phenol-chloroform-isoamyl alcohol solution was added. Tubes were mixed gently and centrifuged at 10000 rpm for 10 min. Then the aqueous phase was transferred to new sterile eppendorff tubes and precipitated with -200C absolute ethanol.

The tubes were mixed by inversion and centrifuged at 10000 rpm for 10 min. After that ethanol poured off and the pellet was washed with 70% ethanol. Then washed with (70%) ethanol, DNA was dried and resuspended in 100µl of TE buffer and stored at -200C until use.

#### **-Agarose gel electrophoresis for genomic DNA:**

Agarose gel electrophoresis was conducted according to Sambrook et al.,(1989) using Agarose gel in a concentration (0.8%) . DNA samples were mixed with 3/10 volume of loading buffer and were loaded carefully into the wells of the submerged gel. Then the gel was ran into two cycles, the first cycle at 45 volt,60mA,15 min then the second cycle at 60 volt,90mA,90min. DNA bands were visualized and photographed using UV transilluminator.

#### **-DNA concentration:**

The concentration of the isolated DNA was calculated using the spectrophotometric method by UV-visible scanning spectrophotometer according to Sambrook et al.,(1989) as follows : 20µl of DNA was transferred to 980µl TE buffer (pH7.5) in a 1ml quartz cuvette. The content was mixed well and the absorbance was recorded at 260nm on spectrophotometer. The quantity of DNA was calculated, using the guide that 1ml of a solution with an A<sub>260</sub> of 1.0 is equivalent to 50mg/ml of double stranded DNA. A ratio of absorbance at 260nm to that at 280nm of approximately 1.8-2.0 indicates that the sample is free from protein

contamination which is absorbed strongly at 280nm.

#### **- Detection of *enlA* gene *E.faecalis* isolates using PCR technique:**

Method described by De Marques & Suzart,( 2004) was used in the present study which was carried as follows; Five microliters of PCR buffer,0.5µl of dNTPs,1µl of each forward and reverse primer (table 1), and 0.4µl of Taq DNA polymerase was collected in sterile eppendorff tube. Then mixed by vortex for 30sec. centrifuged for 30 sec. in order to precipitate all solutions. The volume of previous mixture was completed to 20µl by adding 12.1µl of sterile deionized D.W., then mixed and centrifuged for 30 sec. 5 µl of template DNA of was added to the mixture tube to give a final volume of 25µl .Table(2)illustrated the volumes and concentrations of PCR mixture used in the present study.

Then the reaction mixture were amplified in a PCR Thermal Cycler (Applied Biosystem ,Singapore) according the following program:

An initial denaturation at 940C for 5 min, followed by 30 cycles of 940C for 45 sec., annealing at a temperature specific for each primer pair ranged from [52 to 62 0C;Table (3-6)] for 1 min,72 0C for 1 min, and a final extension step at 72 0C for 3 min.

#### **- Agarose gel electrophoresis for PCR product:**

All steps in agarose gel electrophoresis for genomic DNA were done for PCR product except the following steps:

-Agarose gel was prepared with a concentration(1%) by dissolving 1.5gm of agarose in 150ml of 1X TBE.

-The gel was run into two cycles,the first cycle at 45volt,60mA,15min and the second cycle at 60 volt, 90mA, 120min.

## **Results and Discussion**

Bacteriocins are antimicrobial peptides or proteins that inhibit growth of bacteria closely related to the producing organism (Tagg et al., 1976).Members of lactic acid bacteria(LAB)genera,including lactobacilli, lactococci, pediococci, leconostocs, streptococci and enterococci, produce bacteriocins which are currently classified into three different major classes including class I,II,III bacteriocins(Nes et al., 1996).

Mature enterolysin A consists of 316 amino acids and has a calculated molecular weight of 34,501, and the theoretical pI is 9.24. The N terminus of enterolysin A is homologous to the catalytic domains of different cell wall-degrading proteins with modular structures. These include lysostaphin, ALE-1, zoocin A, and LytM, which are all endopeptidases belonging to the M37 protease family. The N-terminal part of enterolysin A is

linked by a threonine-proline-rich region to a putative C-terminal recognition domain.

The results of PCR for enlA gene (the gene responsible for cell wall hydrolyzing activity) showed that (16.6%) of *E. faecalis* isolates possess this gene ,since gel electrophoresis results showed DNA bands with molecular size 960bp in comparison with DNA marker as shown in figure (1).

De Marques and Suzart, (2004) were firstly studied on the distribution of this virulence gene in clinical isolates of *E. faecalis* and they determined this gene at low rate (9.5%) among clinical studied isolates which is in agreement with our result .

Another study conducted by Nigutova K. (2007) showed that Enterolysin A structural genes were detected in approximately one-sixth of the Gram-positive ruminal cocci examined by PCR using primers targeting the enterolysin A structural gene.

Enterolysin A represent a bacteriocin produced by different strains of *E. faecalis* of ruminal origin, clinical isolate or faecal samples ( Tomita et al., 1996; Nilsen et al., 2003; Ananou et al., 2005a,b; Nigutová et al., 2007; Kang et al., 2009).

*Enterococcus faecalis* isolates from chourico (traditional Portugal fermented sausage)

were studied by Lauková et al., (2011) and the results showed that antibiotic sensitive strains were bacteriocin active.

Enterolysin A is the first bacteriocin from an Enterococcus belonging to class III, the large and heat-labile bacteriocins. To our knowledge, only three class III bacteriocins from LAB have been characterized previously at the genetic level; these are helveticin J from *L. helveticus* 481 (Joerger et al., 1990), zoocin A from *S. zooepidemicus* 4881 (Simmonds et al., 1997), and millericin B from *S. milleri* NMSCC 061 (Beukes et al., 2000 ; Beukes et al., 2001). Whereas helveticin J exhibits no sequence similarity to enterolysin A, zoocin A and millericin B do. The latter two bacteriocins exert their bactericidal activities by hydrolyzing peptide bonds in the peptidoglycan of susceptible cells. Since EnlA degrades cell walls of sensitive bacteria and exhibits sequence similarity to these bacteriocins, it is likely that EnlA has a similar mode of action.

No common denominator has been identified in the interpeptide bridges in the peptidoglycan chains of bacteria sensitive to enterolysin A. However, all the sensitive bacteria have the following stem peptide sequence in their peptidoglycan: l-Ala-d-Glu-l-

Lys-d-Ala. This suggests that it is most likely that enterolysin A exerts its activity by hydrolyzing a peptide bond in the stem peptide, suggesting that its activity is similar to that of millericin B (Beukes et al., 2000 ; Beukes et al., 2001).

The distribution of enlA gene for *E. faecalis* isolates according to isolates sources is shown in table (3). The results showed that this gene was detected in 1(14.3%) of vaginal isolates, 1(10%) of urine isolates, 5(35.7%) of stool isolates , and no of wound and burn isolates were harbor this gene.

No literatures concerning the distribution of enlA gene according to sources of infections were available for comparison.

So from our results we can concluded that this gene is low distributed in clinical isolates of *E. faecalis* in general. According to samples sources, it was more common in stool isolates in comparison with other samples sources which reflect the importance of this factor in competition of this bacterium with intestinal normal flora and colonization, and the low importance of this factor in other body sites, since there is no need for competition in these sites.

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**Table (1)** Oligonucleotide primers used to amplify *enlA* marker gene in *E. faecalis* by PCR

Virulence gene*	Nucleotide sequence (5' - 3')	Ta (°C)
<i>enlA</i>	EN1EF1:TTC TTC TTA TTC TGT CAA CGC AGC ENIER1:GAC TGT GAA ATA CCT ATT TGC AAGC	59

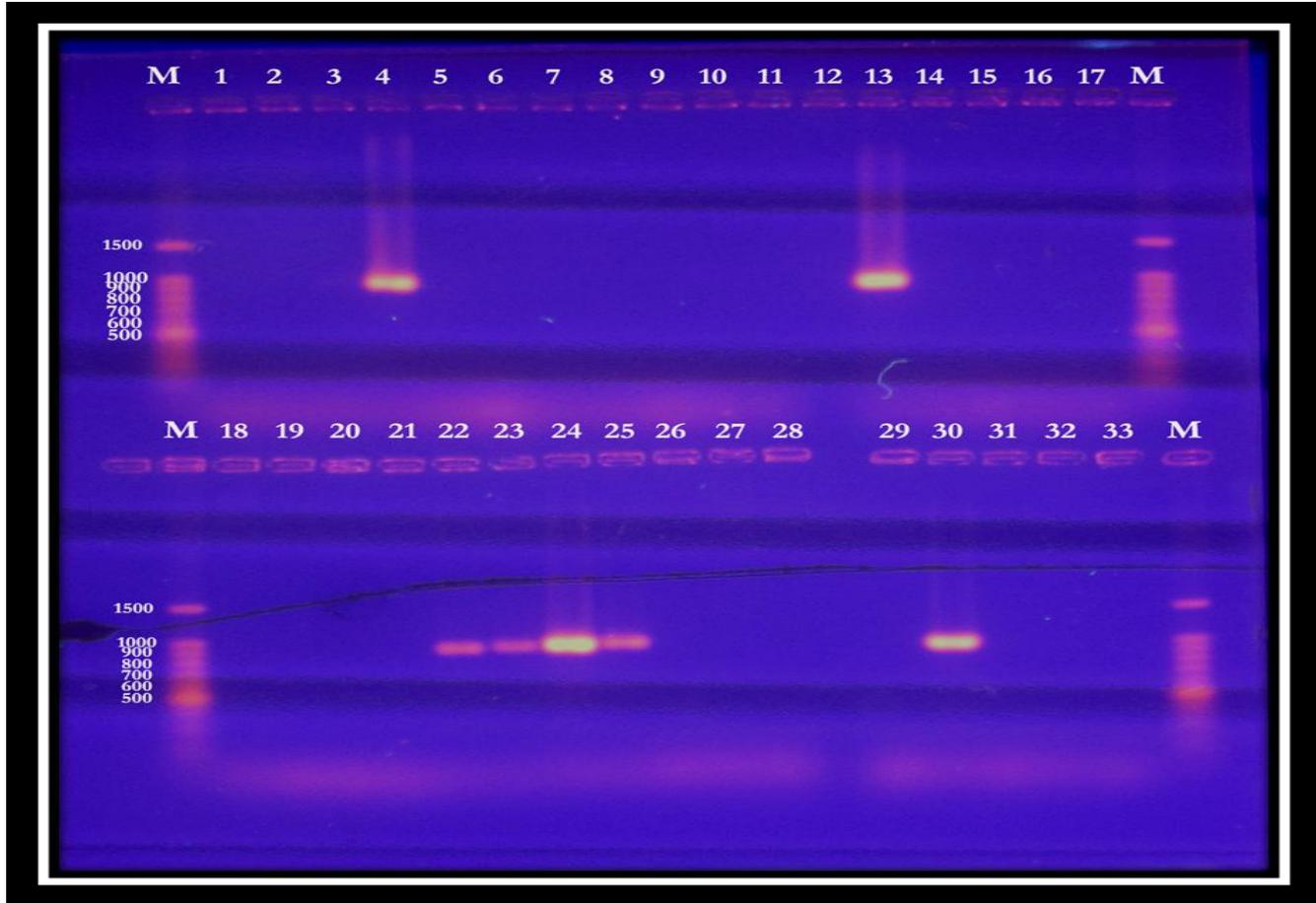
❖ *enlA*, enterolysin A belonging to class III bacteriocins which exhibits identity with cell wall-degrading enzymes produced by Gram-positive bacteria., Ta, Annealing temperature.

**Table (2)** Volumes and concentrations of PCR mixture used in present study.

Component	Stock concentration	Final concentration	Volume For each sample
PCR buffer	5X	1X	5µl
dNTPs	10mM	200 µM	0.5 µl
Forward Primer	50pmol	50 pmol	1 µl
Reverse			1 µl
<i>Taq</i> DNA polymerase	5U per µl	2U	0.4 µl
Sterile deionized D.W			12.1 µl
DNA sample		30ng/ µl	5 µl
Total volume			25µl

**Table (3)** Distribution of *enlA* gene in *E. faecalis* according to samples sources

Virulence genes	<i>Efaecalis</i> Isolates harboring virulence genes											
	Stool n=14		Urine n=10		Wounds n=10		Vaginal swabs n=7		Burns n=1		Total n=42	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
<i>enlA</i>	5	35.7	1	10	0	0	1	14.3	0	0	7	16.6



**Figure(1):gel electrophoresis of PCR product for *enlA* gene.**

**M:100 bp DNA ladder marker, Lanes(1-7):Vaginal isolates, Lanes(8-17):Urine isolates**

**Lanes(18-31):Stool isolates, Lanes(32,33):Wound isolates**

**Note: other wound and burn sample were also negative ,since they didn't showed any PCR product**