Identification of Enterococcal Species by Polymerase Chain Reaction Technique, with Study of Some Immunological Features

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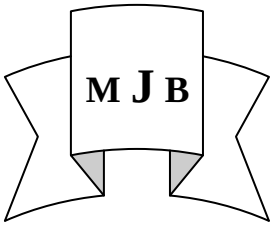


Abstract

Objectives: Detection of genes responsible for identification of enterococci to the species level using PCR, determination of enterococcal serum resistance and phagocytic index.

Materials and methods: seven enterococcal isolates were obtained from two hundred urine samples. After identification of enterococcal isolates by biochemical tests, all of them were subjected to PCR technique using species-specific PCR primers for *E. faecalis* and *E. faecium*, enterococcal serum resistance and phagocytic index were detected.

Result: This study revealed that five isolates (71.43%) reacted with *ddlE. faecalis*; whereas the remaining (28.57%) isolates reacted with *ddlE. faecium*. Also, revealed that phagocytic index of two *E. faecalis* and one *E. faecium* isolates were 27%, 25% and 19%, respectively, while one *E. faecium* isolate showed phagocytic index as 8%. Moreover, enterococcal serum resistance showed that *E. faecalis* was more sensitive to the serum as compared to *E. faecium*.

Conclusion: Enterococcal isolates can't be easily identified to the species level by conventional or traditional methods but it can be easily identified by species-specific PCR primers *ddlE. faecalis* and *ddlE. faecium. Enterococcous faecalis* tends to be more sensitive to innate immune parameters as compared to *E. faecium.*

Key words: *E. faecalis* and *E. faecium* species-specific PCR primers, *Enterococcous* spp.

تشخيص المسبحيات المعوية الى مستوى النوع باستخدام تقنية Polymerase chain reaction مع دراسة بعض صفاتها المناعية

الخلاصة

تضمن البحث دراسة بكتريولوجية وجينية وبعض الصفات المناعية لجنس *Enterococcous* إذ تم الحصول على ٧ عزلات من ضمن ٢٠٠ عينة ادرار من المرضى.

لم يتم تشخيص العزلات إلى مستوى النوع بإستخدام الفحوصات الكيموحيوية، على خلاف الفحص الدقيق والسريع لعزلات *Enterococcous* spp باستخدام Polymerase chain reaction (PCR) مع البوادئ المتخصصة لتحديد أنواع *E. faecalis,* *E. faccium*، وقد أظهرت نتائج الPCR عائديه ٥ عزلات لنوع *E. faecalis* وعزلتين لنوع *E. faccium*.

من خلال دراسة بعض الخصائص المناعية لعزلات *Enterococcous* بما في ذلك البلعمة والمقاومة لمصل الدم، أظهرت النتائج إن عزلتين من *E. faecalis* كانت حساسة للبلعمة والمصل، بينما كانت عزلة واحدة من النوع *E. faccium* مقاومة لكلا الاختبارين المذكورين.

Introduction

Enterococci, an indigenous flora of the intestinal tract, oral cavity, and the genitourinary tract of the humans and animals, known to be relatively avirulent in healthy individuals [1], but have become an important cause of a wide

variety of opportunistic and invasive infections. Correct identification is necessary to monitor which species are causing disease, for treatment purposes and No single system of classification suffices for the differentiation of this heterogeneous group of organisms [2]. A variety of molecular methods has been developed for the identification of enterococci and streptococci to species level. The targets used for molecular diagnoses include genes encoding rRNA, RNA polymerase, D-alanine-D-alanine ligase, manganese-dependent superoxide dismutase (*sodA*$_{int}$) and tRNA gene intergenic spacer region. Recently, optimal identification of non-haemolytic streptococci was performed by phylogenetic sequence analysis of four housekeeping genes (*ddl*, *gdh*, *rpoB* and *soda*) [3]

The production of capsular polysaccharide is a known virulence factor, as it aids in avoidance of the host innate immune response. *E. faecalis* is known to produce capsular polysaccharide serotypes A-D that contribute to pathogenesis and evasion of the host innate immune response [4]. An additional cell wall polysaccharide in *E. faecalis* and *E. faecium* called enterococcal polysaccharide antigen (Epa) has also been shown to contribute to resistance to phagocytic killing and may explain why the protective effect of the capsule is not more substantial in *E. faecalis*. Unlike the capsule, the Epa polymer and its genetic locus appear to be highly conserved in *E. faecalis* [5].

A common feature of the invasive isolates of many species is the ability to avoid the bactericidal effects of serum. A bacterium that can survive in human blood has the potential to spread to different organs, escaping the killing mechanism of complement- and antibody-mediated opsonization. In species comprising pathogens and commensals, serum resistance is often attributed to the pathogens as an acquired trait that allowed them to cause disease in their host [6].

Materials and Methods

Identification of isolates: Seven enterococcal isolates out of 200 urine samples were obtained from hospitalized patients. Isolates were identified to the genus level based on the standard biochemical and microbiological methods [7].

DNA extraction and PCR: DNA of the seven enterococcal isolates were extracted by the use of DNA Purification Kit according to the instructions of the manufacturer. PCR was performed in a total volume of 25 µl then DNA amplification was carried out in the thermal cycler at a condition listed at Table (1), with primers listed in Table (2). Later on PCR product were assessed by 1% agarose gel electrophoresis with electric current 60 volt for half to 1hrs.

Table 1 PCR condition of primers used in this study

PCR condition		
Stage	**Temp.(ºC)**	**Time(min.)**
Initial denaturation	94	5
Denaturation	94	1
Annealing	54	1
Initial extension	72	1
		for 30 cycles
Final extension	72	7

Table 2 Primers used in this study with their sequences

Primer		Sequence	Product size(bp)
ddl E. faecalis	F	5'-ATCAAGTACAGTTAGTCTTTATTAG-3'	941
	R	5'-ACGATTCAAAGCTAACTGAATCAGT-3'	
ddl E. faecium	F	5'-TTGAGGCAGACCAGATTGACG-3'	658
	R	5'-TATGACAGCGACTCCGATTCC-3'	

Phagocytic Index: Four enterococcal isolates (2 *E. faecalis* and 2 *E. faecium*) were examined for detection of PMNs-mediated killing of enterococci and phagocytic index. Separation of neutrophils was carried out according to the method described by [8]. Clinical isolate of *Stap. aureus* was used as an indicator for phagocytic index. This was performed according to the procedure outlined [9]. Two hundred PMNs were counted and the percentages of active phagocytic cells were determined.

Serum bactericidal assay:2 isolates (*E. faecalis, E. faecium*) were used as a test system and *E. coli* was used as a control. Enterococcal isolates were grown in nutrient broth for 2-3 hrs at 37 ℃, were harvested, and adjusted to a concentration of 2 x 10^6 bacterial cells/ml with physiological saline. Bacterial suspension 25 µl was mixed with 75µl of pooled normal human serum in sterilized tubes.

Viable counts of bacteria were determined according to MacFaddin [7] after incubation for 1,2 and 3hrs. Each isolate was tested at least three times. Isolates were considered serum resistant or serum sensitive if the grading was the same in all experiments. *E.coli* and a mixture of bacterial suspension and normal saline was used as controls [10].

Results

Enterococcal species identification: All enterococcal isolates in present study were subjected to PCR technique using species-specific PCR primers for *E. faecalis* and *E. faecium* which based on specific detection of genes encoding D-alanine:D-alanine (D-Ala:D-Ala) ligases. Five isolates 5/7 (71.43%), of the enterococcal isolates, reacted positively with the *E. faecalis* species-specific primer (*ddlE. faecalis*) with one band for each isolate at about 900-950 bp; whereas the remaining 2/7 (28.57%) isolates reacted with the *E. faecium* species-specific primer (*ddlE. faecium*) with one band for each isolate at about 600-700 bp Figure (1; 2), respectively.

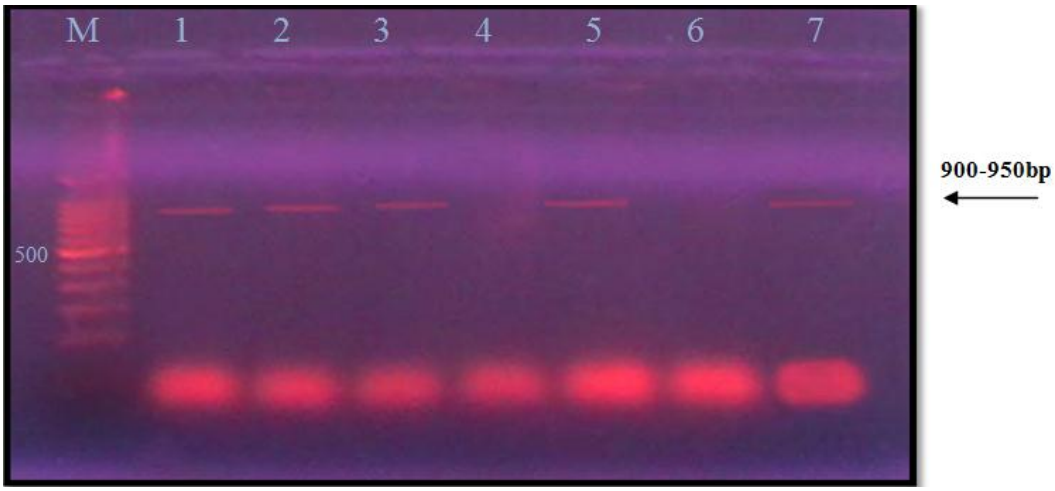


Figure 1 Agarose Gel Electrophoresis of PCR products for detection of *ddl E. faecalis* gene in enterococcal isolates. Lane M=100 bp DNA Ladder (molecular marker), lanes 1-7 refer to the enterococcal isolates' number

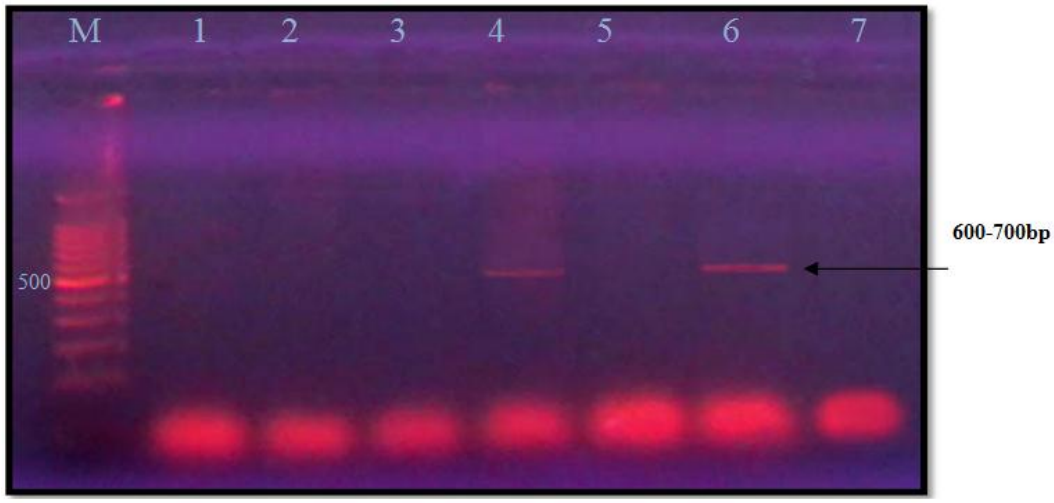


Figure 2 Agarose Gel Electrophoresis of PCR products for detection of *ddlE. faecium* gene in enterococcal isolates. Lane M=100 bp DNA Ladder (molecular marker), lanes 1-7 refer to the enterococcal isolates' number.

Phagocytic index (PI): Phagocytosis was made at 37°C in the presence of normal fresh human PMNs. Results revealed that PI of two *E. faecalis* and one *E. faecium* isolates were 27%, 25% and 19%, respectively which considered higher than that of *Staph. aureus* 14%. The PI value of the remaining *E. faecium* isolate was 8% Figure (3).

Enterococcal serum resistance: Figure (4 a, b) showed the effect of fresh human serum on the growth of *E. faecalis*, *E. faecium* and *E. coli* after one and two hours of incubation on nutrient agar respectively. *E. faecalis* was the most sensitive to the effect of serum when compared to the other isolates. While, *E. faecium* was the least affected.

On the other hand, the result expressed in Figure (4 c), which showed the effect of fresh human serum on the growth of *E. faecalis*, *E. faecium* and *E. coli* after three hours of incubation, *E. faecalis* was the most sensitive, which showed more than 85% reduction in viability, while, *E. faecium* was not affected.

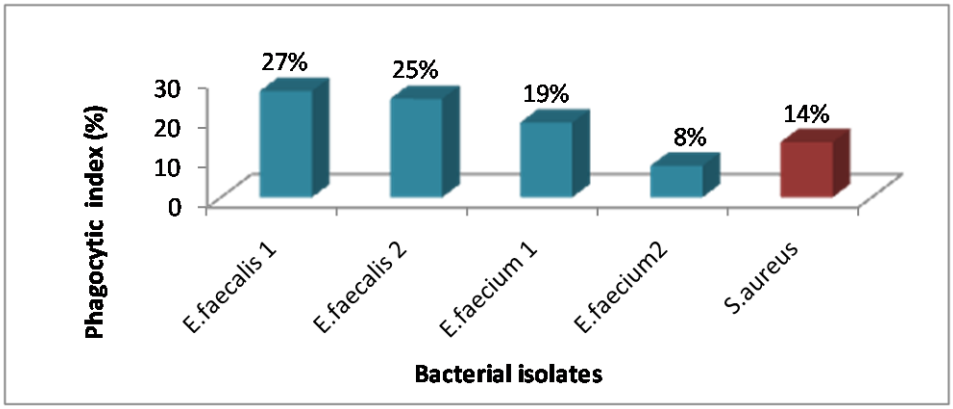
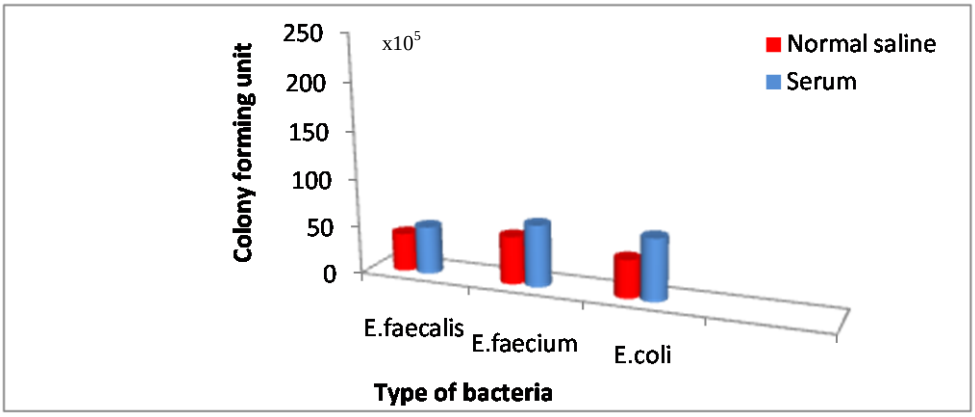
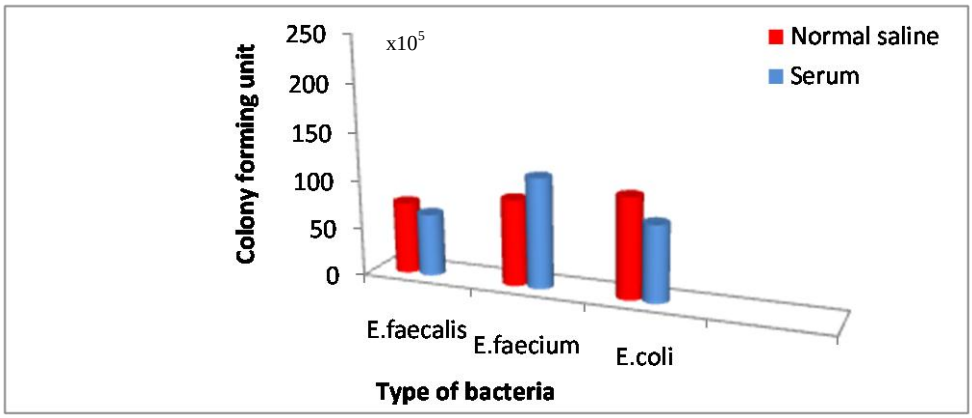


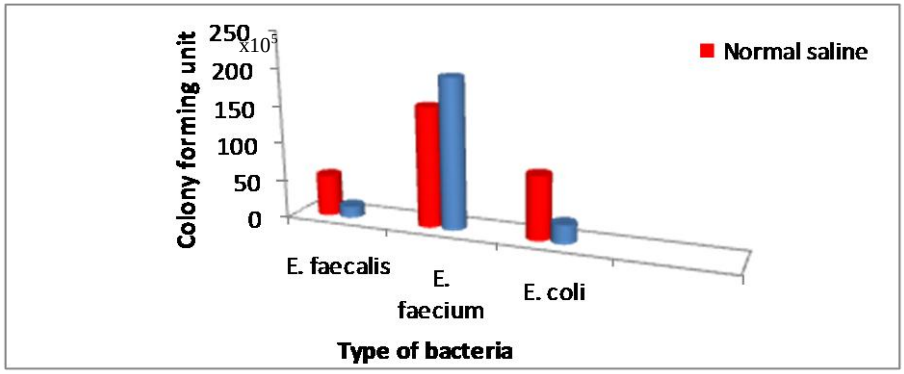
Figure 3 Phagocytic index of enterococcal isolates in comparison to *Staph. aureus*



a - Anti- serum activity for *E. faecalis, E. faecium* and *E.coli* after **l** hr of incubation.



b - Anti- serum activity for *E. faecalis, E. faecium* and *E.coli* after **2**hr of incubation.



c- Anti- serum activity for *E. faecalis, E. faecium and E.coli* after 3hr of incubation.
Figure 4 a, b, c Anti - serum activity for *E. faecalis, E. faecium* and *E.coli* after 1, 2 and 3 hours of incubation.

Discussion

The results of this study indicated that *E. faecalis* among other isolates of enterococci, was the dominant species of *Enterococcus* in hospitalized patients in Hilla/ Iraq. These results were strongly agreed with other different studies reported that *E. faecalis* accounts for 80 to 90% of infections, followed by *E. faecium*, which accounts for 5 to 10% of infections, the other enterococcal species account for the remainder [11]. In another study carried out in Brazil using PCR and primers targeted at specific sequences in the *ddl* (D-Ala–D-Ala ligase) chromosomal genes of the two species (*E. faecalis* and *E. faecium*); similar finding were obtained which identified *E. faecium* as 48.8% and *E. faecalis* as 51.2% [12].

On the other hand, Biavasco *et al*. [13] in Genoa, Italy identified species prevalence and reported *E. faecium* as the predominant species, using PCR and species-specific genes *E. faecium* was 66% and *E. faecalis* 33%. Species-specific PCR primers *ddlE. faecalis* and *ddlE. faecium* were documented as competent for enterococcal species identification by different studies, and the use of a PCR with primers for *ddl E. faecalis* and *ddl E. faecium* may be the most simple molecular approach for both rapid and precise identification of enterococci, while avoiding the drawbacks of commercial kits [14,15].

Accurate species identification of enterococci has become important with the wide prevalence of acquired-vancomycin resistance and the presence of less epidemiologically important, inherently vancomycin resistant enterococci. Conventional identification methods, which are based on culturing, require 2 to 3 days to provide results. PCR has provided means for the culture-independent detection of enterococci in a variety of clinical specimens and is capable of yielding results in just a few hours [12].

About the results of phagocytic index, these results mean that the first three isolates were sensitive to phagocytosis and the last one was resistant. These results were approximately similar to the result obtained by Arduino *et al.* [10)] who reported that eight *E. faecalis* and two *E. faecium* strains were studied, of which two *E. faecium* isolates were more resistant to PMN-mediated killing than the *E. faecalis* isolates. Moreover they proved that all strains of *E. faecalis* are readily phagocytosed by PMNs in the presence of complement, and demonstrated that 50% of clinical isolates of *E. faecium* are resistant to PMN-mediated

phagocytosis and killing, and that a bacterial surface carbohydrate was most likely responsible for this effect. *Enterococcous* species have different virulence factors, which aid in resistance against defense mechanisms of the host; one of them is the aggregation substance. Aggregation substance (AS) is a surface-bound protein encoded by pheromone-responsive plasmids of *E. faecalis* and expressed in response to pheromone induction. (AS) has been reported to promote direct, opsonin-independent binding of *E. faecalis* to human neutrophils via a complement receptor-mediated mechanism. As a consequence of this special type of binding, *E. faecalis*-bearing AS was shown to be resistant to killing by human neutrophils, despite marked phagocytosis and neutrophil activation [16].

Another virulence factor of enterococcal immune evasion is the capsular polysaccharide and it has shown that serotypes C and D strains are more resistant to opsono-phagoctyosis by neutrophils than unencapsulated serotypes A and B, and the presence of the capsule is associated with pathogenic lineages of *E. faecalis*. As the presence of capsule prevents detection of lipoteichoic acid on the surface of serotypes C and D strains but not on unencapsulated strains [17].

Certain strains of *E. faecium* were found to be resistant to phagocytosis by neutrophils, probably because of a carbohydrate-containing moiety expressed by these strains, specific antibodies could overcome this resistance most likely by promoting deposition of complement [18]. So, although enterococci rarely cause diseases in healthy individuals, they can become pathogenic in patients in intensive care units, in hospitalized patients with severe underlying diseases or an impaired immune system, and in elderly people. Severely ill patients with hematologic malignancies and deep neutropenia are especially at an increased risk of developing enterococcal bacteremia. Phagocytic susceptibility may be associated with bacterial factors as well as host factors. The susceptibility profile may vary with in the same genus and species [19].

Finally, about the serum resistance; its result was agreed with Arduino *et al.* [10] who showed that after the addition of pooled normal fresh human serum, *E. faecalis* showed a reduction in viability of >99% after 2-3 hours of incubation, while viable counts of two strains of *E. faecium* were unchanged.

Also, results showed that serum resistance profile of *E. faecalis* and *E. coli* after one hour of incubation tend to be similar to that after three hours. In addition, both these isolates appeared to be more sensitive as the time exceeding the 2 hours when they compared with the relative rate of growth in the control (normal saline). This may be due to the fact that serum components may need time to exhibit their effect on bacterial growth which was clearly observed after 3 hours. The pathogenicity of bacteria that invade blood stream is partly a function of their ability to evade the bactericidal effect of serum, which is mediated by the complement cascade [20]. Commensal bacteria are generally vulnerable to bactericidal effect of serum, while nosocomial bacteria tend to be much serum resistant [21]. For enterococci, the complement system appears to be of primary importance, and activation of the alternative pathway of complement is sufficient. Microorganisms use a wide variety of mechanisms to resist the alternative pathway of complement [10]. It was demonstrated that serum may modulate

bacterial surface antigen expression [22].

Guzman *et al.* [23] suggested that adherence of *E. faecalis* to eukaryotic cells (including PMNs) could be mediated by carbohydrate residues present on the bacterial cell surface. *E. faecalis* isolates from UTIs express D-galactose and L-fucose ligands when grown in serum, whereas the isolates normally did not express these ligands when they were grown in brain heart infusion broth. Growth in serum raised the adherence of *E. faecalis* isolated from UTI to eukaryotic cells by at least 1.5- to three-folds.

The thick cell wall of Gram-positive bacteria (such as *E. faecium*) prevents complement-mediated lysis; therefore, complement primarily plays an opsonic role in the host defense against Gram-positive bacteria [24]. In addition, extracellular gelatinase expressed by *E. faecalis* and *E. faecium* destroys complement in human serum, by hydrolyzing C3a and degenerating C3b. This, along with the expression of capsular polysaccharides may also contribute to the resistance of *E. faecium* to phagocytosis and result in serum resistance [25,26].

From all these results, we can recommend that it is better to use molecular techniques that allow a rapid, accurate detection of these bacteria since phenotypic methods are not biased enough. Also, evaluation of the immune response of patient and applied the strategies of immune enhancement.

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