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Al-Quds University

Detection of Biofilm formation Among Clinical Isolates of *Enterococcus* spp. and their Antibiotic Susceptibility Pattern

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Detection of Biofilm Formation Among Clinical Isolates of *Enterococcus* spp. and Their Antibiotic Susceptibility Pattern

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Jerusalem – Palestine

1439/2018

Dedication:

I dedicate my work to my beloved husband (Saied), my sons (Majd, Mohammed), and my parents (Salim, Laila) who supported me all the way long, and to my lovely sisters and brothers (Mohammed, Walaa, Ahlam and Qusai).

All expressions of thanks and praise will not be fulfilled by your continued support.

Declaration:

I certify that this thesis submitted for the degree of Master, is the result of my own research, except where otherwise acknowledge, and that this study (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signed:

Dua'a Salim Abdullah Ja'fari

Date: 12-05-2018

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Abstract:

Background:

Enterococci, considered as normal commensal flora in oral cavity, intestines and genital tract of humans and animals have emerged as major opportunistic pathogens. Biofilm formation is an important virulence factor of enterococci species. Bacteria within biofilm are more resistant than their free-living (i.e., planktonic) counterpart. Urinary tract infection (UTI) is one of the most frequent types of nosocomial and community acquired infections in humans, and biofilm contribute significantly to their virulence and antibiotic resistance. The role of bacterial biofilms and antimicrobial resistance has great importance for public health. Hence this study was conducted to detect the biofilm formation by enterococci isolates from clinical settings and to compare the antibiotic resistance pattern of biofilm forming and non- biofilm forming species and to determine what role enterococcal surface protein *(esp*) gene plays in enterococcal biofilm formation.

Methodology:

A total of 50 isolates of enterococci were collected from patients, enterococcus species were identified using conventional microbiological methods. The antibiotic susceptibility patterns of the isolates were determined by the disc diffusion-method. The Microtiter plate method used to assess the ability of biofilm formation. All enterococcal isolates were examined for determination of *esp* gene, by polymerase chain reaction (PCR).

Results:

Of 50 enterococcal isolates, 70 % were recognized as *E. faecalis* and 30% of them were *E. faecium*. According to our results, overall, 69% of *E. faecalis* and 40% of *E. faecium* isolates were biofilm producers. Resistance to some antibiotics including ampicillin (36% P=0.001), tetracycline (54 % P=0.003), erythromycin (52% P=.004) and vancomycin (24% P=.002) was significantly higher among biofilm producers than non-biofilm producers enterococci. The *esp* gene was present in 50% isolates. In this study, there was not a significant relationship between presence of *esp* gene and biofilm formation.

Conclusion:

Biofilm formation is considered an important virulence factor in causing infections by enterococci. Our findings reinforce the role of biofilm formation in resistance to antimicrobial agents. Biofilm producing isolates were less sensitive to antibiotics than biofilm non-formers. biofilm formation decreases their susceptibility to antibiotics. Our results suggest that biofilm formation is complex and depends on various factors but not just *esp* gene in *Enterococcus* strains. Hence screening, timely detection and control of biofilm formation is necessary to increase patient outcome.

Table of Contents:

List of Tables

List of Figures:

List of Appendices

List of Abbreviations

Abbreviation	Word
Ace	***Enterococcus faecalis*** **adhesion to collagen**
Asa	**Aggregation substance**
Atn	***Enterococcus faecalis*** **antigen A**
CDC	**Centers for disease control and prevention**
CLSI	**Clinical Laboratory Standards Institute**
Efa A	***Enterococcus faecalis*** **antigen A**
Epa	**Enterococcal polysaccharide antigen**
Epb	**Endocarditis and biofilm-associated pili**
ESP	**Enterococcal surface protein**
Fsr	***Enterococcus faecalis*** **regulator**
Gel E	**Gelatinase**
LPS	**Lipopolysaccharide**
PBP	**Penicillin binding protein**
PCR	**Polymerase Chain Reaction**
rRNA	**Ribosomal ribonucleic acid**
TAE	**Buffer solution: Tris/acetic acid/EDTA**
TSB	**Tryptic soy broth**
UTI	**Urinary tract infection**
VRE	**Vanccomycin resistant** ***enterococcus***

Chapter One:

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1. Introduction:

Enterococci are Gram-positive, catalase-negative, non-spore-forming, facultative anaerobic bacteria, enterococci are often simply described as lactic-acid–producing bacteria, which usually inhabit the alimentary tract of humans in addition to being isolated from environmental and animal sources (Rahkila, Johansson et al. 2011). Enterococci are highly tolerant to desiccation and can persist for months on dried surfaces. Enterococci also tolerate extremes of pH, ionizing radiation, osmotic and oxidative stresses (Ramsey, Hartke et al. 2014)

Enterococci survive or grow over a wide range of temperatures for mesophilic bacteria, from 10 to 45°C (Moreno, Sarantinopoulos et al. 2006).

Enterococci were classified as group D Streptococci (Sherman 1938) until 1984, when *Streptococcus faecalis* and *Streptococcus faecium* were reclassified as *Enterococcus faecalis* and *Enterococcus faecium*, respectively (Schleifer and Kilpper-Bälz 1984).

Both species, *Enterococcus faecalis* and *Enterococcus faecium* cause human diseases, most commonly in the form of urinary tract and wound infections. Other infections, including those of the blood stream (bacteremia), heart valves (endocarditis), and the brain (meningitis) can occur in immunocompromised patients.

Enterococci also often colonize open wounds and skin ulcers, and are among the most common antibiotic-resistant bacteria (Van Tyne and Gilmore 2014).

Understanding the ecology, epidemiology and virulence of *Enterococcus* species is important for limiting urinary tract infections, hepatobiliary sepsis, endocarditis (Dahl and Bruun 2013), surgical wound infection, bacteremia and neonatal sepsis, to reduce the further development of antibiotic resistance (Moreno, Sarantinopoulos et al. 2006).

Enterococcaceae considered the second most common cause of nosocomial urinary tract infection next to *E. coli* and third most common cause of bacteremia (Winn and Koneman 2006).

The most common enterococci species are *Enterococcus faecalis* causing about 80 -90% of human infection followed by *Enterococcus faecium* (Stuart, Schwartz et al. 2006).

1.1. Virulence factor:

The virulence factors converged in *E. faecalis* and *E. faecium* which have been isolated in nosocomial infections, include antibiotic resistance, extracellular proteins (toxins), extra chromosome and mobile genetic elements, cell wall components, biofilm formation, adherence factors, and colonization factor such as bacteriocin, (Hancock and Gilmore 2006).

Other virulence factor such as Extracellular toxins such as cytolysin can induce tissue damage, increase mortality in combination with aggregation substance, and cause systemic toxicity. And Cell-surface associated factors like Adhesin to collagen of E. faecalis (*ace*) (Vidana 2015), Aggregation substance *(asa)*, Capsular polysaccharide, *E. faecalis* antigen A (*efaA*), *esp* gene, serine protease, capsule, and superoxide (Upadhyaya, Ravikumar et al. 2009).

1.1.1. Biofilm:

biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, it contains viable and

nonviable microorganism that adhere to the surface (proteins, glycoproteins, and carbohydrates) (Arampatzi, Giannoglou et al. 2011).

Bacteria in biofilm are resistant to phagocytosis, making biofilm extremely difficult to eradicate (O'Toole 2011).

According to the National Institutes of Health, biofilm account for over 80% of microbial infections (Lewis 2001), Biofilm associated infections are recurrent, chronic and highly resistant to antibiotics, about 10 to 1000 folds of more antibiotic concentrations required to kill the bacteria in biofilm than the free living forms (Mohamed and Huang 2007),(Upadhyaya, Ravikumar et al. 2009).

1.1.2. Biofilm formation

The five stages of biofilm development:

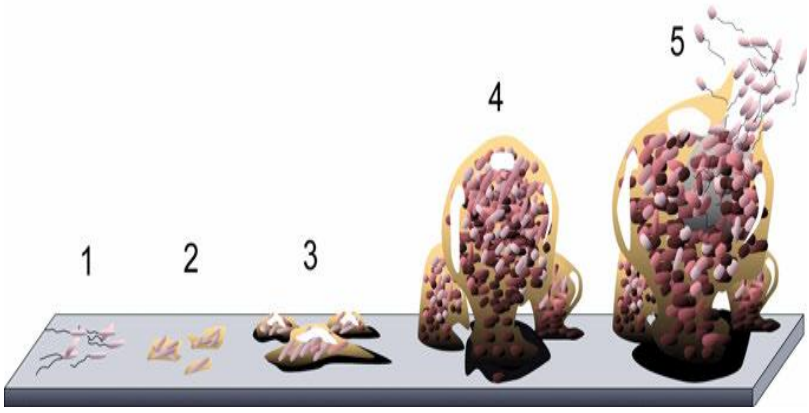
. Stage 1: Planktonic (free floating) bacteria adhere to the biomaterial surface.

Stage 2: Cells aggregate, form micro colonies and excrete extracellular polymeric substances (EPS), i.e. slime. The attachment becomes irreversible.

Stage 3: A biofilm is formed. It matures and cells form multi-layered clusters.

Stage 4: Three-dimensional growth and further maturation of the biofilm, providing protection against host defense mechanisms and antibiotics.

Stage 5: The biofilm reaches a critical mass and disperses planktonic bacteria ready to colonize other surfaces.(Unosson 2015)



(Unosson 2015)

Figure 1.1: **The five stages of biofilm development.**

1.1.3. Factors influencing biofilm production:

Nutrient contents of the growth medium, such as glucose, serum, availability of iron, CO_2, osmolarity, pH, and temperature influence biofilm production among different bacteria (Lim, Jana et al. 2004). Carbohydrate metabolism regulates biofilm production among various Gram-positive bacteria, including *E. faecalis* (Pillai, Sakoulas et al. 2004). One study has shown that tryptic soy broth (TSB) medium with 1% glucose supplementation enhances biofilm production in *E. faecalis* compared to TSB without glucose (Baldassarri, Cecchini et al. 2001).

Esp is a cell wall associated protein that has been implicated as a significant factor contributing to colonization, persistence of bacteria in the urinary tract and biofilm formation. *Esp*, was initially identified in a virulent gentamicin-resistant *Enterococcus faecalis* isolate (Strain No.MMH594) (Toledo-Arana, Valle et al. 2001).

1.2. Antibiotic resistant in Enterococci:

Enterococci can survive harsh environmental conditions such as long periods on environmental surfaces, including medical equipment, bed rails and doorknobs. They are tolerant to heat, chlorine and some alcohol preparations, which may help explain why these organisms are widely disseminated in the hospital setting. There are several risk factors for developing a nosocomial VRE (vancomycin-resistant *Enterococcus*) infection: close physical proximity to patients infected or colonized with VRE; a long period of hospitalization; multiple courses of antimicrobials; hospitalization in long-term facilities, surgical units or intensive-care units; solid organ and bone marrow transplantation; co-morbidities such as diabetes, renal failure or haemodialysis; and the presence of a urinary catheter (Arias and Murray 2012).

Enterococci are intrinsically resistant to many commonly used antimicrobial agents. All enterococci exhibit decreased susceptibility to penicillin and ampicillin, as well as high-level resistance to most cephalosporins and all semi-synthetic penicillin, as the result of

expression of low-affinity penicillin-binding proteins (PBP). For many strains, their level of resistance to ampicillin does not preclude the clinical use of this agent. Enterococci are also intrinsically resistant to clindamycin, enterococci also have a native resistance to clinically achievable concentrations of aminoglycosides, which prevent their use as single agents. Although *E. faecalis* is naturally resistant to quinupristin-dalfopristin, this combination is highly active against *E. faecium* strains that lack specific resistance determinants (Yang, Li et al. 2015).

Intrinsic resistance, which is encoded within the core genome of all members of the species differs from acquired resistance, in that the latter is present in only some members of the species and is obtained via the horizontal exchange of mobile genetic elements or via selection upon antibiotic exposure (Huycke, Sahm et al. 1998).

Moderate species-specific intrinsic resistance to aminoglycosides in *E. faecium* is enhanced by a chromosomally encoded rRNA methyltransferase known as EfmM that uses S-adenosyl methionine as a methyl donor to methylate a specific residue on 16S rRNA, in the context of the 30S ribosomal subunit (Shepard and Gilmore 2002).

Modifications in Pbp5 are associated with increased resistance to beta-lactams, such as ampicillin. For example, the Pbp5-encoding gene found in hospital-associated, ampicillin-resistant strains of *E. faecium* differs by ~5% from the corresponding gene in community-associated, ampicillin-susceptible strains (Garnier, Taourit et al. 2000).

Most studies that report an association between mutations in Pbp5 and enhanced ampicillin resistance have been performed on non-isogenic clinical isolates, in which unknown factors other than Pbp5 may influence resistance (Rice, Messer et al. 1995).

Since its been discovered in 1986, vancomycin-resistant *Enterococcus* has increasingly become a major nosocomial pathogen (O'Driscoll and Crank 2015).

Screening the enterococci isolates for antibiotic resistance is a important tool to obtain information about the prevalence of VRE and is crucial for controlling the spread of bacterial resistance (Olawale, Fadiora et al. 2011) . The *Esp* gene acts as an adhesion factor and is involved in biofilm synthesis. In addition, the fact that it can be transmitted with the *vanA* resistance gene is believed to contribute toward the wide spread of pathogenic VRE (Cho, Sung et al. 2011).

VRE can survive in the environment for prolonged periods (>1 week), can contaminate almost any surface, and can be passed from one patient to another by health care workers (Uttley, Collins et al. 1988). Whether VRE colonization leads to infection depends on the health status of the patient. Whereas immunocompetent patients colonized with VRE are at low risk for infection, immunocompromised host have an increased likelihood of developing infection following colonization (Zirakzadeh and Patel 2006).

1.3. Problem statement:

Biofilms pose a serious problem for public health because of the increased resistance of biofilm-associated organisms to antimicrobial agents and the potential for these organisms to cause infections in patients with indwelling medical devices (Donlan 2002, Upadhyaya, Ravikumar et al. 2009). Bacteria in biofilms are resistant to phagocytosis, making biofilms extremely difficult to eradicate from living hosts (Lewis 2001).

Detection of biofilm produced by enterococci and correlating them with their antibiotic resistant pattern and an understanding of the role of genetic and environmental factors in the development of biofilm may lead to improved strategies for biofilm control among enterococci (Stewart 2002).

1.4. Aims and Objectives:

- To detect biofilm production by enterococci clinical isolates.
- To determine what role *esp* gene plays in Enterococcal biofilm formation.
- To determine the antibiotic susceptibility pattern of enterococci to commonly used antibiotics.
- To compare biofilm and non-biofilm enterococci producers to their antibiotic susceptibility pattern.

1.5. Literature Review:

Enterococcus has been recognized since 1899, when Thiercelin identified it as an intestinal organism(Thiercelin 1899); its taxonomy and ecology were reviewed by Klein (Klein 2003). Many attempts have been made to `distinguish *Enterococcus* species from *Streptococcu*s species.

In 1937, Sherman classified *Streptococcus* species into four subgroups: faecal streptococci (enterococci), dairy streptococci, viridans group and pyogenous streptococci, Sherman noted that the enterococci subgroup included the Lancefield group *D streptococci* and suggested that the latter could be differentiated by hemolytic and proteolytic reactions (Sherman 1937).

In 1994, through the use of DNA hybridization and 16S rRNA sequencing, it was established that the species *Streptococcus faecalis* were distinct from the other streptococci to be designated another genus: *Enterococcus* (Jett, Huycke et al. 1994).

Traditional methods such as biotyping, serotyping and phage typing left questions as to which of the *Streptococcus* species actually belonged to the genus *Enterococcus* (Karatan and Watnick 2009).

Fisher and Colleagues classified *Enterococcus faecalis* as part of the group D *Streptococcus* system is a Gram-positive, commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals. Like other species in the genus *Enterococcus*, *E. faecalis* can cause life-threatening infections in humans, especially in the nosocomial environment, where the naturally high levels of antibiotic resistance found in *E. faecalis* contribute to its pathogenicity (Fisher and Phillips 2009).

E. faecalis can cause endocarditis and septicemia, urinary tract infections, meningitis, and other infections in humans. Several virulence factors are thought to contribute to *E. faecalis* infections. A plasmid-encoded hemolysin, called the cytolysin, is important for pathogenesis in animal models of infection, and the cytolysin in combination with high-level gentamicin resistance is associated with a five-fold increase in risk of death in human bacteremia patients. A plasmid-encoded factor called aggregation substance *(asa*) is also important for virulence in animal models of infection (Jett, Huycke et al. 1994).

Toole and Colleagues defined a biofilm as communities of microorganisms attached to a surface, and occur in response to a variety of environmental signals (O'Toole, Kaplan et al. 2000).

Costerton defined biofilm as a population of cells attached irreversibly on various biotic and abiotic surfaces, and encased in a hydrated matrix of exopolymeric substances, proteins, polysaccharides and nucleic acids (Costerton 2001).

The new definition of a biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription. This definition is useful, because some bacterial populations that fulfill the earlier criteria of a biofilm, which involves matrix formation and growth at a surface, do not actually assume the biofilm phenotype (Arampatzi, Giannoglou et al. 2011).

Microbes form a biofilm in response to many factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or in some cases, by exposure of planktonic cells to sub-inhibitory concentrations of antibiotics (Karatan and Watnick 2009).

In 2001 Baldassarri has shown that tryptic soy broth (TSB) medium with 1 % glucose supplementation enhances biofilm formation in *E. faecalis* compared to TSB without glucose (Baldassarri, Cecchini et al. 2001).

Kristich in 2004 observed that biofilm formation was eliminated by exposure to a medium to high osmolarity (2–3 % sodium chloride) without affecting the growth of the bacteria, suggesting that *E. faecalis* monitors the environment and modulates biofilm formation in response to specific conditions(Kristich, Li et al. 2004).

Gallardo noted in his study the effect of human serum on *E. faecalis* adhesion has been examined (Gallardo-Moreno, González-Martín et al. 2002).

In 2001 Shankar reported Role of *esp* protein in adherence and colonization. The *esp* protein of *E. faecalis* is emerged on the cell surface. This localization and enrichment among UTI-derived isolates suggested a role for *esp* in adherence and colonization (Shankar, Lockatell et al. 2001).

Toledo et al (2001) found a relationship between the presence of the *esp*-encoding gene and the biofilm formation by *E. faecalis*, since 93.5% of the *E. faecalis esp*-positive isolates were capable of forming a biofilm. Moreover, none of the *E. faecalis esp*-deficient

isolates were biofilm producers, and *esp* promotes primary attachment and biofilm production of *E. faecalis* on abiotic surfaces (Toledo-Arana, Valle et al. 2001).
Another study was shown that esp plays a role in *esp*-mediated biofilm enhancement in *Enterococcus faecalis* (Tendolkar, Baghdayan et al. 2005).
Another study showed the quantity of biofilm was significantly higher in *esp*-positive compared with *esp*-negative clinical *E. faecium* isolates ($P < 0.0001$). (Tsikrikonis, Maniatis et al. 2012)

In 2010 Upadhyaya concluded in his study that biofilm production has an important role in causing nosocomial infection. Although detection of the *esp* gene correlates with biofilm production, it may not be the only factor determining the formation of biofilm since few isolates produced biofilm without the *esp* gene (Upadhyaya, Lingadevaru et al. 2010).
On the other hand, some studies did not observe any relationship between *esp* gene and biofilm formation, and show that *E. faecalis* forms complex biofilms and does not require the *esp* surface protein (Kristich, Li et al. 2004), and no association between the presence of *esp* and the biofilm formation (Sandoe, Witherden et al. 2003).
The initial adhesion and production of biofilm are independent of the existence of *esp*. An *esp*-negative isolate was found to produce biofilm, and two *esp*-positive isolates did not form biofilm (van Merode, van der Mei et al. 2006).
Rosa (2006) have also shown that *E. faecalis* (36 out of 83) and *E. faecium* (9 out of 45) *esp*-positive isolates were not associated with biofilm formation. However, the same authors reported that some *esp*-positive strains produced thicker biofilms than *esp*-negative biofilm producers (Rosa, Creti et al. 2006). The exact factors, including *esp*, and mechanisms involved in biofilm production by enterococci are still unknown and are an area of active investigation.

Although *esp* did not show strong correlation for biofilm formation, its presence showed a significant association with the degree of biofilm production (Mohamed, Huang et al. 2004).
Several studies have attempted to identify additional factors that may influence the process of biofilm formation in *E. faecalis.* such as *fsr*, *gelE*, *epa*, *atn* genes (Mohamed, Huang et al. 2004).
Polysaccharides have been implicated in biofilm formation. These molecules are associated with the cell surface as a capsular polysaccharide or secreted as an exo

polysaccharide into the environment. An enterococcal polysaccharide antigen *(epa*) gene cluster mutant of *E. faecalis*, showed a 73% reduction in biofilm formation, suggesting that this gene encodes a putative glycosyltransferase that is involved in polysaccharide synthesis and biofilm production. An *E. faecalis* autolysin (*atn*) mutant showed a 30% reduction in biofilm formation. The two-component regulatory system mutant (*etaR)* showed a small reduction in biofilm formation (Mohamed, Huang et al. 2004).

Another study identified phenotypes linked to the strong biofilm formation of *E. faecalis* E99 by transposon mutagenesis. The gene cluster involved was named biofilm enhancer in *enterococcus* (Tendolkar, Baghdayan et al. 2006).

Kafil et al (2015) investigated the presence of virulence factors in enterococci strains isolated from clinical samples in Iranian Educational hospitals, endocarditis and biofilm-associated pili (*ebp*) was present in almost all of *E. faecalis,* this operon encodes pilus components shown to be important for pathogenesis in the endocarditis and UTI (Kafil and Mobarez 2015). This operon is also important for biofilm formation. High presence of this gene may indicate the importance of biofilm formation for pathogenesis of enterococci in clinical isolates, Results revealed that colonization factors were more prevalent in *E. faecalis* isolates; almost all isolates of *E. faecalis* contained *ace* (adhesion of collagen from *E. faecalis)*, *ebp* , *efaA* genes (*Enterococcus faecalis* antigen A) and *asa* (Kafil, Mobarez et al. 2013, Kafil and Mobarez 2015)).

In 2014 a study was carried out by Soares et al reported the correlation between biofilm formation and gelE (gelatinase) , *esp,* and *asa* genes in Enterococcus spp. clinical isolates, more biofilm formation shown by *E. faecalis* isolates that contain *gelE, esp* and *agg* than *E. faecalis* isolates did not contain these virulence genes (Soares, Fedi et al. 2014).
Hossein et al (2015) found a relationship between virulence profiles and biofilm production in clinical UTI isolates. By comparing isolate absorbance, *asa1* positive isolates had significantly higher biofilm formation than *asa1* negative isolates, as well as *efaA* positive isolates had higher biofilm formation than *efaA* negative isolates, no significant differences were found when comparing *ace* (Adhesion of collagen from *E. faecalis*) positive and -negative isolates. Also *esp* positive and negative isolates had no significant difference in biofilm formation, hyaluronidase *(hyl*) positive isolates had lower biofilm formation tendency, this confirm that the presence of *efaA* and *asa1* correlates with increased biofilm formation of urinary tract isolates (Kafil and Mobarez 2015).

In 2014 study showed *Enterococcus faecium* increased resistance to multiple antibiotic than *Enterococcus faecalis.* Significant relationship was found between biofilm production with antibiotic resistance to amoxicillin, co-trimoxazole, ciprofloxacin, gentamycin, cefotaxime, and cefuroxime, and demonstrated a high propensity among the isolates of Enterococci to form biofilm and a significant association of biofilms with multiple drug resistance (Akhter, Ahmed et al. 2014).

Biofilm forming isolates were significantly more resistant to antibiotics like, Erythromycin, Penicillin, Norfloxacin, Tetracycline, Ciprofloxacin, Amoxycillin/clavulanic acid, Gentamicin, Nitrofurantoin, than biofilm non-forming isolates. Resistant pattern of other antibiotics like Amikacin and Vancomycin were insignificantly higher for biofilm forming enterococci species. Overall biofilm forming enterococcal isolates showed higher resistance patterns than their counterpart. This observation is also noted in other studies (Chakraborty, Pal et al. 2015), (Hasan, Al-Duliami et al.). Other study reported the resistance pattern of biofilm forming Gram positive organisms as 100% to Penicillin, 70% to Rifampicin, 40% to Ciprofloxacin, 40% to Erythromycin and 30% to Cotrimoxazole and biofilm non-formers were more sensitive to these antibiotics, they also observed significant higher resistant of biofilm producing enterococci species in their study (Sindhanai, Avanthiga et al. 2016).

Also another study concluded that the high antibiotic resistance may indicate the intrinsic resistance of enterococci is due to circulation of transposable elements carrying resistant genes in clinical isolates, indiscriminate and uncontrolled usage of antibiotics and the presence of biofilm among the patient isolates (Talebi, Moghadam et al. 2015).

Chapter Two:

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2. Materials and Methods:

2.1. Bacterial collection and Identification:

A total of Fifty non-repetitive clinical isolates of enterococci obtained from two tertiary care centers in West Bank, Palestine (Al-Istishari Hospital and Ramallah Medical complex) were tested in this study. The isolates were of wound, rectal, and urine specimens (Fig3.1).

Initial identification of the isolates was done at the two hospitals by gram staining and culturing them on bile esculin agar. Further confirmation and speciation of enterococci was done at Al-Quds University Laboratory. All the isolates were Gram positive cocci, positive bile esculin, positive 6.5% NaCl tests and Catalase negative.

Enterococcus faecalis was presumptively distinguished from *Enterococcus faecium* via growth and fermentation characteristics on mannitol salt agar, arabinose, sucrose, sorbitol and pyruvate fermentation (Sindhanai, Avanthiga et al. 2016).

The isolates were then stored at -20 ^{0}C, 50% v/v sterile glycerol/trypticase soy agar.

2.2. Antimicrobial Susceptibility

Antimicrobial susceptibility testing was performed at Al-Quds University Laboratory on Muller Hinton agar (Hi Media, Mumbai) plates by the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) (Square 2012).

Antibiotics used in our study were Gentamycin, Ciprofloxacin, ampicillin, Erythromycin, Tetracycline and Vancomycin (Mast Diagnostics Ltd, UK).

2.3. Biofilm Formation Assay by Microtiter plate assay:

Quantitative determination of biofilm production was performed by using a modified Microtiter plate method (Seno, Kariyama et al. 2005) Briefly, *Enterococcus* isolates were grown overnight at 37°C in tryptic soy broth supplemented with 0.5% glucose. The culture was diluted1:100 in TSB medium, and 200 µl of this cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtiter plates (Corning Inc., Corning, NY, USA). The negative control wells contained TSB-0.5% glucose only, after 24 h at 37°C without shaking, wells were gently washed three times with 300µl of distilled water, dried in an inverted position, and stained with 300 µl crystal violet solution in water for 45 min. After staining, plates were washed 3 times with distilled water. Quantitative analysis of biofilm production was performed by adding 300 µl of ethanol-acetic acid (95:5, vol/vol) to destain the wells. One hundred microtiters from each well was transferred to a new microtiter plate and the level (optical density; OD) of crystal violet present in the destaining solution was measured at 450 nm using a microtiter plate reader (Seikagaku Co., Tokyo, Japan). Optical density of strains was assessed for biofilm formation as follows:

Weak 0.05-0.1 , Moderate 0.10-0.20, High >0.20 and all were considered positive for biofilm production. When the optical density are < 0.05 considered negative (Sindhanai, Avanthiga et al. 2016).

2.4. DNA Extraction:

Boiling method was used for DNA extraction, using a sterile loop, one colony resuspended into 50 µl of ultra-pure water then boiling for 10 minutes at 90℃, then it was centrifuged at 14.000 rpm for 1 minute (Yost and Nattress 2000).

2.4.1. Polymerase chain reaction (PCR) assay:

PCRs were performed in a final volume of 25 µl containing 12.5 µl from the Taq ready Mix (taq ready mix. hy labs) containing Taq DNA Polymerase, dNTPs, Mg2+ and Reaction Buffer at optimal concentrations for efficient amplification of DNA templates,1 µl from each forward and reverse primer, 8.5 µl ultra-pure water and 2 µl from the supernatant of the extracted DNA. The reaction mixtures were amplified in a PCR Thermal Cycler (ThermoHybaid) with an initial denaturation at 94 ^{0}C for 10 min, followed by 35 cycles as follows: Denaturation at 94 ^{0}C for 1 min, annealing at a temperature 60 ^{0}C for 1 min, and extension at 72 ^{0}C for 1 min, and a final extension step at 72 ^{0}C for 10 min. Negative control was included in each PCR reaction. Positive control strains *Enterococcus faecalis* ATCC 29212 (Marra, Dib-Hajj et al. 2007).

The amplicons were analysed by electrophoresis in 2 % agarose gels using TAE buffer (pH 8.0). The gels were stained with ethidium bromide and visualized on a UV trans illuminator (Hoefer UV-25; Pharmacia, Biotech). The size of amplified products was estimated by comparison with a 100 bp DNA ladder (Amersham; Pharmacia, Biotech) (Vankerckhoven, Van Autgaerden et al. 2004), The amplicon size of *esp* gene with the set of primers used was 933 bp.

The PCR primers used in this study for amplification of *esp* gene were as follows:

Esp11: 5′-TTGCTAATGCTAGTCCACGACC-3′(Eaton and Gasson 2001)

Esp12: 5′-GCGTCAACACTTGCATTGCCGAA-3′

2.5. Statistical analysis:

The correlation between the antibiotic resistance, biofilm formation ability and presence of the biofilm-related gene among Enterococci isolates was evaluated by the Pearson Chi-Square test using SPSS version 22. P values less than 0.05 were regarded as significant.

Chapter Three:

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3. Results:

3.1. Identification of Enterococcus spp:

Of the 50 clinical isolates of enterococci, 35 (70%) were *E. faecalis* and 15 (30%) were *E. faecium*. All the isolated strains were identifiable to the species level. Maximum number of the isolates were from urine (56%) followed by rectum (40%) and wound (4%) as shown in (fig.3.1). all data about the isolates were shown in Table 3.1.

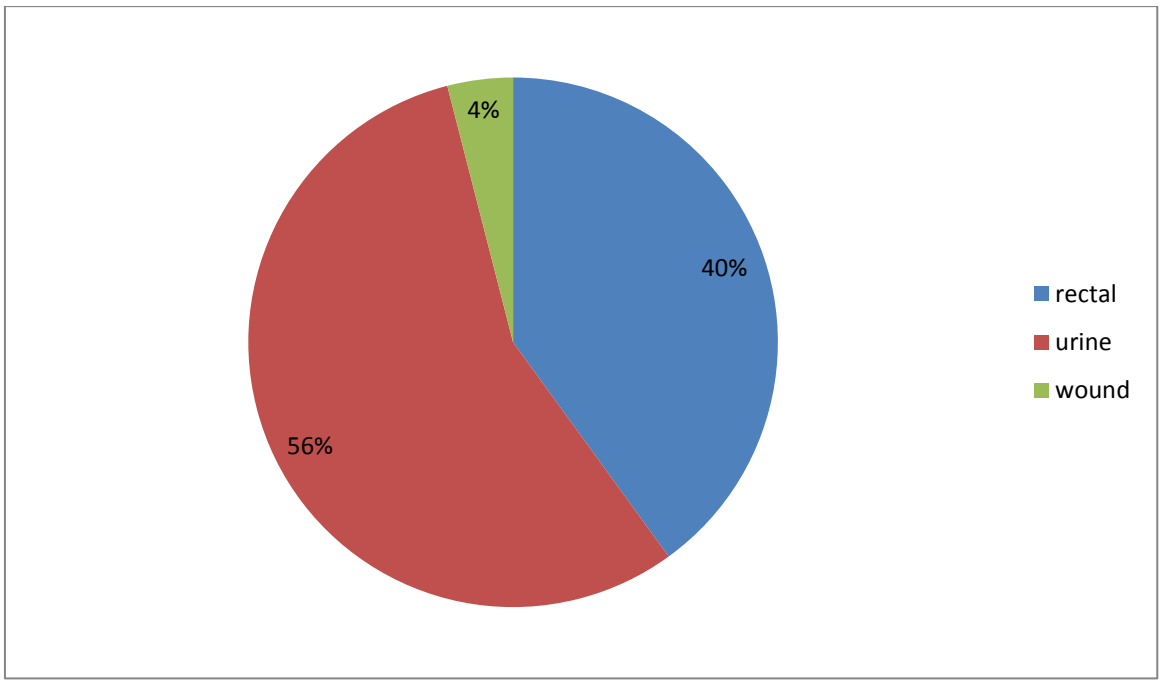

Figure 3.1: - Distribution of Enterococci among clinical specimens

Table 3.1. Relationship between *Enterococcus* spp, source, biofilm formation, *esp* gene and VRE

Isolate No.	species	Source of isolates	Biofilm formation	Vancomycin resistant *enterococcus* (VRE)	ESP gene
1	*E. faecalis*	Urine	Producer	VRE	Positive
2	*E. faecalis*	Rectal	Non-producer	VRE	Negative
3	*E. faecalis*	Urine	Producer	_	Negative
4	*E. faecalis*	Urine	Non-producer	_	Positive
5	*E. faecalis*	Urine	Non-producer	VRE	Positive
6	*E. faecalis*	Rectal	Non-Producer	_	Negative
7	*E. faecalis*	Urine	Producer	_	Positive
8	*E. faecalis*	Rectal	Producer	VRE	Positive
9	*E. faecalis*	Rectal	Producer	VRE	Positive
10	*E. faecalis*	Rectal	Non-Producer	_	Negative
11	*E. faecalis*	Rectal	Producer	VRE	Positive
12	*E. faecalis*	Wound	Producer	_	Negative
13	*E. faecalis*	Urine	Producer	_	Negative
14	*E. faecalis*	Urine	Producer	VRE	Negative
15	*E. faecalis*	Urine	Producer	_	Positive
16	*E. faecalis*	Urine	Producer	_	Negative
17	*E. faecalis*	Wound	Producer	VRE	Positive
18	*E. faecalis*	Rectal	Producer	_	Positive
19	*E. faecalis*	Urine	Producer	_	Negative
20	*E. faecalis*	Urine	Producer	_	Positive
21	*E. faecalis*	Urine	Producer	_	Positive
22	*E. faecalis*	Rectal	Producer	_	Negative
23	*E. faecalis*	Rectal	Non-producer	_	Positive
24	*E. faecalis*	Urine	Producer	_	Positive
25	*E. faecalis*	Urine	Producer	_	Positive
26	*E. faecalis*	Rectal	Producer	VRE	Positive
27	*E. faecalis*	Rectal	Producer	_	Positive
28	*E. faecalis*	Rectal	Producer	_	Negative

29	*E. faecalis*	Urine	Producer	_	Negative
30	*E .faecalis*	Urine	Non-producer	VRE	Negative
31	*E. faecalis*	Urine	Non-producer	_	Negative
32	*E. faecalis*	Rectal	Non-producer	_	Positive
33	*E. faecalis*	Urine	Producer	VRE	Negative
34	*E. faecalis*	Urine	Non-producer	_	Positive
35	*E. faecalis*	Rectal	Non-producer	_	Negative
36	*E. faecium*	Urine	Producer	_	Negative
37	*E. faecium*	Urine	Non-producer	_	Positive
38	*E. faecium*	Urine	Non-producer	VRE	Negative
39	*E. faecium*	Urine	Non-producer	_	Negative
40	*E. faecium*	Rectal	Producer	_	Negative
41	*E. faecium*	Rectal	Producer	VRE	Positive
42	*E. faecium*	Urine	Non-producer	_	Positive
43	*E. faecium*	Rectal	Non-producer	_	Negative
44	*E. faecium*	Rectal	Non-Producer	_	Negative
45	*E. faecium*	Urine	Non-producer	_	Negative
46	*E. faecium*	Rectal	producer	VRE	Positive
47	*E. faecium*	Urine	Producer	_	Positive
48	*E. faecium*	Urine	Non-producer	_	Positive
49	*E. faecium*	Urine	Non-producer	_	Negative
50	*E. faecium*	Rectal	Producer	VRE	Negative

3.2. Antibiotic susceptibility testing

The result for antibiotic testing showed that *E. faecium* in general was more resistant to antibiotics than *E. faecalis* without significant association P > 0.05 (fig3.2). In case of *E. Faecalis* resistance to the following antibiotics tetracycline, erythromycin, and ciprofloxacin, were 71%, 66%, 63% respectively, whereas in case of *E. faeciu*m tetracycline was 73%, erythromycin and ciprofloxacin 80%. For two antibiotic gentamycin and vancomycin, *E. faecalis* was slightly higher in resistance than *E. faecium* as shown in (fig.3.2).

The result showed about 11 of isolates were resistant to more than two antibiotic and considered multi drug resistant.

The results showed that vancomycin, ampicillin, and gentamycin were the most effective antibiotics against *Enterococcus* species.

None of the antibiotics used in the study were 100% effective to all enterococci strains.

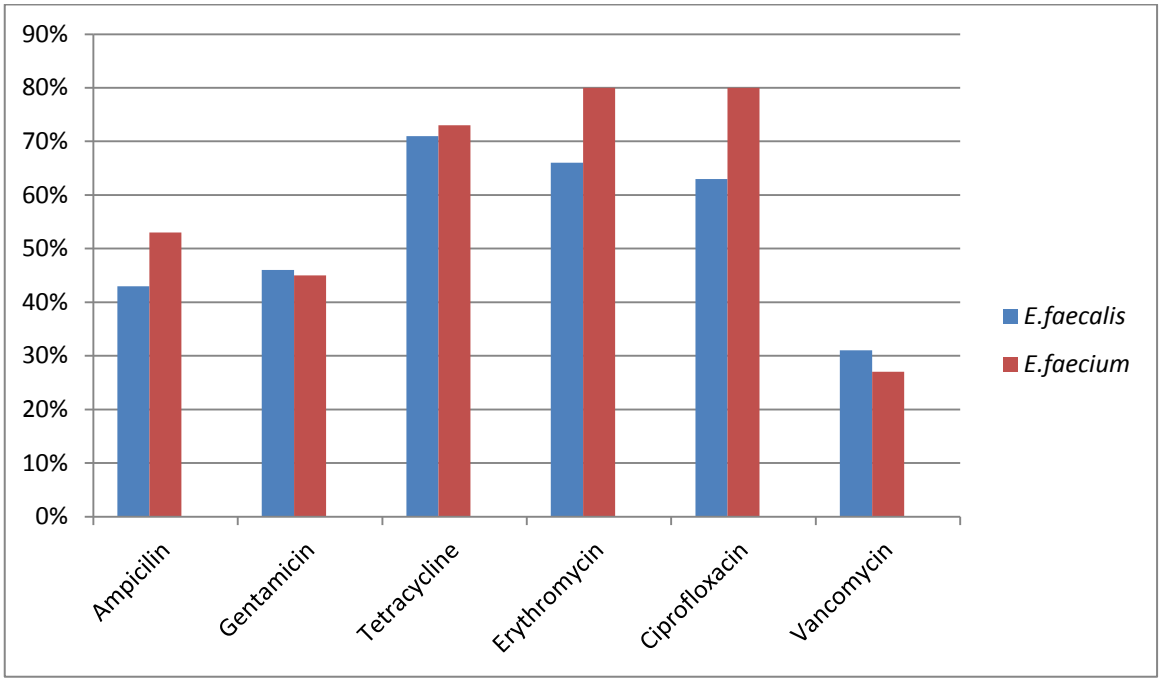

Fig.3.2. Percentage of antibiotic resistant pattern of all *Enterococcus* spp. to different antibiotics

3.3. Biofilm production:

30 isolates were biofilm producers (Table 3.2). Of the 35 *E. faecalis* isolates, 24 (69%) were biofilm producers, whereas 15 *E. faecium* isolates 6 (40%) were biofilm producers (Table 3.1).

Among the *E. faecalis* isolates 14.3% were strong, 42.8% moderate and 11.4% of them were found to be weakly adherent. Whereas among the *E. faecium* isolates 26.6% moderate and 13.3% were found weakly adherent (Table 3.3).

Antibiotic susceptibility pattern of enterococcal isolates are shown in (Table 3.4).

Statistical analysis indicated that there was a significant association between biofilm formation of enterococcal isolates and some antibiotic resistance such as ampicillin (36%P=0.001), tetracycline (54 %, P=0.003), erythromycin (50%, p= 0.004), and vancomycin (30%, p= 0.002) (Table 3.5).

Table 3.2: Prevalence of biofilm forming and non-biofilm forming Enterococci

Total no. of Enterococci isolates [%]	Biofilm producers	Biofilm Non-formers
50 [100%]	30[60%]	20[40%]

Table 3. 3. Biofilm formation ability of Enterococcus species in Microtiter plate method

Method	Biofilm formation	*E. faecalis* n=35	Percent	*E. faecium* n=15	percent	Total (%)
Microtiter plate assay	Strong	5	14.3%	0	0%	5(10%)
	Moderate	15	42.8%	4	26.6%	19(38%)
	Weak	4	11.4%	2	13.3%	6(12%)
	None	11	31.4%	9	60%	20(40%)

Table 3.4: Antibiotic susceptibility pattern of enterococcal isolates

	E. faecalis (n=35)			*E. faecium* (n=15)					
Antibiotics	Sensitive isolates	Intermediate isolates	Resistant isolates	Sensitive isolates	Intermediate isolates	Resistant isolates	Total No. of sensitive isolates	Total No. of intermediate isolates	Total No. of resistant isolates
Ampicillin	20 (57%)	0	15 (43%)	7 (47%)	0	8 (53%)	27 (54%)	0	23 (46%)
Gentamicin	17 (49%)	2 (6%)	16 (46%)	8 (53%)	1 (3%)	6 (40%)	25 (50%)	3	22 (44%)
Tetracycline	10 (29%)	0	25 (71%)	4 (27%)	0	11 (73%)	14 (28%)	0	36 (72%)
Erythromycin	7 (20%)	5 (14%)	23 (66%)	3 (20%)	0	12 (80%)	10 (20%)	5	35 (70%)
Ciprofloxacin	13 (37%)	0	22 (63%)	3 (20%)	0	12 (80%)	16 (32%)	0	34 (78%)
Vancomycin	24 (69%)	0	11 (31%)	11 (73%)	0	4 (27%)	35 (70%)	0	15 (30%)

Table 3.5: Antibiotic susceptibility according to biofilm formation

Antibiotics	Sensitive isolates		Intermediate isolates		Resistant isolates		Pearson Chi-Square (fisher's exact test)
	Biofilm former	Biofilm non-former	Biofilm former	Biofilm non-former	Biofilm former	Biofilm non-former	
Ampicillin	8	19	0	0	18	5	(.001) Significant
Gentamicin	17	8	2	1	10	12	(.603) In Significant
Tetracycline	4	10	0	0	27	9	(.003) Significant
Erythromycin	2	8	1	4	25	10	(.004) Significant
Ciprofloxacin	6	10	0	0	20	14	(.186) In Significant
Vancomycin	11	24	0	0	12	3	(.002) Significant

3.4. PCR detection of esp gene

Of the 50 enterococci isolates, 25 (50%) were positive for *esp* gene by PCR as shown in (table 3.1). out of which 19 were *E. faecalis* and 6 for *E. faecium*. The amplicon size of *esp* gene with the set of primers used was 933 bp. Statistical analysis indicated that there was no significant relationship between presence of *esp* gene and biofilm formation among enterococci (P = 0.377) (Table 3.6).

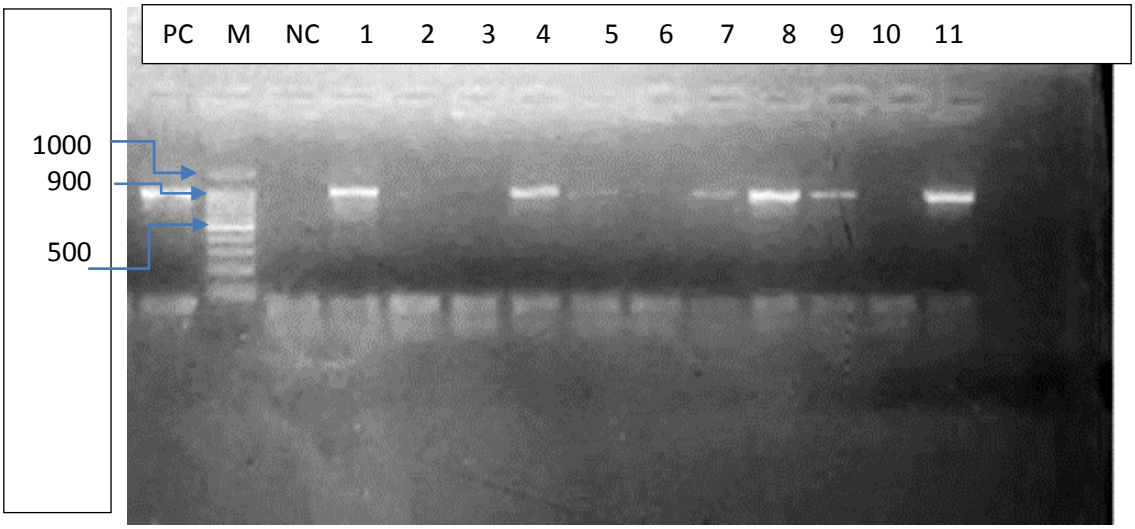

Figure 3.3.: PCR amplification of esp gene of *positive as well as negative E. faecalis* (1-11). M: 100bp marker: PC: Positive control of *E. faecalis*. NC: Negative control

Table 3. 6: Relationship between biofilm-forming and presence of *esp* gene in enterococcal isolates.

Gene	Biofilm producer (%)	Non-biofilm producer (%)	Total (%)	p-value person chi-square
Esp positive	17	8	25	.377
Esp negative	13	12	25	

Chapter Four:

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4. **Discussion**:

Enterococci have become an important cause of a variety of infections during the last decades, that mainly affect weak and immunocompromised patients and are basically hospital-acquired or healthcare associated infections. Often, the ability of enterococci to both establish and maintain these infections is directly related to biofilm formation on indwelling devices or within the urinary tract itself (Hatt and Rather 2008). The most encountered species are *E. faecalis* and *E. faecium*.

12In our study, of the 50 isolates, *E. faecalis* was the most encountered with 70% (35 isolates) and *E. faecium* 30% (15 isolates) (Table 3.1). Our finding is in line with earlier study in which *E. faecalis* accounted 74% and *E. faecium* 26% (Sindhanai, Avanthiga et al. 2016). In another study 92% of the isolates were *E. faecalis* and 8% were *E. faecium*. Higher rates of *E. faecalis* could be due to its greater inherent virulence (Srivastava, Mehta et al. 2013).

Enterococci are one of the main agents causing urinary tract infection and Catheter-associated urinary tract infections apart from Gram-negative pathogens (Tenke, Kovacs et al. 2006). In our study greater number of enterococci species were isolated from urine specimens (56%) followed by rectal (40%) and wound (4%) (Fig 3.1). Comparable observation is seen in the studies conducted by Nautiyalet.al and Jada et.al. (Nautiyal, Jauhari et al. , Jada and Jayakumar 2012) They reported that 62.13% and 59% of the specimens were urine respectively.

Examining of antimicrobial susceptibility can lead appropriate antibiotics prescription and curbing of emergence of drug resistance (Lakshminarayana, Chavan et al. 2015). In the present study *E. faecium* showed in general higher resistant to the antibiotics used in comparison to *E. faecalis* without significant association (Fig 3.2) as reported previously. (d'Azevedo, Dias et al. 2006, Sharifi, Hasani et al. 2013). In our study highest resistance of enterococci (*E. faecium*) was found to Erythromycin and ciprofloxacin (80%) which was in

concordance with other studies (Sreeja, Sreenivasa Babu et al. 2012, Kafil, Mobarez et al. 2013). The lowest resistance was to vancomycin since 69% (24 isolates) of *E. faecalis* and 73% (11 isolates) of *E. faecium* were sensitive which was in agreement with previous study (Hayakawa, Marchaim et al. 2012).

The isolation of VRE has steadily increased worldwide. A study from the CDC indicates that 80% *E. faecium* isolates were resistant to vancomycin; on the contrary, about 7% of *E. faecalis* isolates were vancomycin-resistant (Hidron, Edwards et al. 2008).

In our study we noted high resistance to several antibiotic tested by enterococci species. Many of our isolates showed multiple drug resistance which is similar to other studies (Rams, Feik et al. 2013, Komiyama, Lepesqueur et al. 2016). The high resistance in our study could be explained due to the continuous inappropriate and misuse of antibiotics in Palestine.

Bacterial attachment on surfaces and the development of bacterial communities is an important step in infection and biofilm-formers are antimicrobial resistance (Soto 2014). In this study the results indicated that 69% of *E. faecalis* and 40% of *E. faecium* isolates were biofilm producers. Similar studies reported that *E. faecalis* isolates produce a biofilm more often than *E. faecium* (Mohamed and Huang 2007),(Rosa, Creti et al. 2006).

In the present study, resistance to some antibiotics including Ampicillin, Tetracycline, Erythromycin, and vancomycin, was significantly higher among biofilm than non-biofilm producers with p value of (.001), (.003), (.004) and (.002) respectively whereas, resistance to gentamycin and ciprofloxacin was insignificant (Table3.5). Several studies showed significant relationship between biofilm productions in *Enterococci* spp. with antibiotic resistance (Sandoe, Wysome et al. 2006, White 2007, Akhter, Ahmed et al. 2014).

Biofilm formation and virulence genes have been investigated for enterococci in several studies. However, their pathogenicity mechanisms are not well understood (Kafil and Mobarez 2015).

In the present study 50% of enterococcal isolates were found positive for *esp* gene, there was no significant relationship between presence of *esp* gene and biofilm formation with a p value (0.377). Other studies showed that *esp* promotes biofilm formation in enterococci (Moniri, Ghasemi et al. 2013) , (Soares, Fedi et al. 2014) While other studies have suggested that *esp* gene are not required for enterococci biofilm formation (Dworniczek, Wojciech et al. 2005),(Maestre, Aguilar et al. 2012), Several studies pointed out that a

variety of virulence factors and environmental conditions can effect biofilm formation among enterococcus species.

4.1. Conclusion:

Enterococci are recognized as a major cause of nosocomial infections and form biofilms that are dependent on multiple genetic factors. Microbial biofilms may pose a public health problem. The microorganisms in biofilms are difficult or impossible to treat with antimicrobial agents; detachment from the device may result in infection. The results suggest that in vitro biofilm formation of enterococci is very complex and the presence of *esp* genes does not appear to be sufficient for the production of biofilm. Further investigation is required to understand the biofilm formation. High percentage of resistance to the antibiotics used in the study in general and to vancomycin in particular is an alarming which require an intervention to control the misuse of antibiotics, this has posed a serious problem not only in the treatment of enterococcal infections but also because the organism can horizontally transfer this resistant determinant to other Vancomycin-susceptible species like *Staphylococcus aureus*.

Our study indicated that overall there was a significant relationship between biofilm formation of enterococcal isolates and the emergence of antibiotic resistance. The results reinforce the role of biofilm formation in resistance to antimicrobial agents in enterococci. An understanding of the role of genetic and environmental factors in the development of biofilm may lead to improve strategies for biofilm control among enterococci.

Appendices

Bacterial culture media

Table 1: Bacterial culture media used during this study

Media	Manufacture
Blood agar	Himedia (India)
Muller Hinton agar	Himedia (India)
TSB media (tryptic soy broth)	Himedia (India)
Bile Esculin agar	Himedia (India)
Mannitol Salt agar	Himedia (India)

Reagent

Table 2:

Reagent	Manufacture
Gram stain reagents	Sigma (USA)
DNA molecular weight marker (100) bp ladder	Promega (USA)
Taq ready Mix	Hy.labs
Primers	TIB MOLBIOL (Germany)
Antibiotic disks	Himedia (India), Oxoid (UK)
Ethidium bromide	Sigma (USA)

Equipment

Table 3: Apparatus and special media that were used in the study

Thermal cycler	Eppendorf
Research pipettes	Eppendorf
PCR microfuge tube, 0.2ml	Eppendorf
Microwave oven	LG
Elisa reader	
Hoefer shortwave UV light table (trans illuminator	Hoefer (USA)
Micro-Centrifuge	Sanyo (UK)
Electrophoresis set –up	Bio-Rad (USA)

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الكشف عن تكوين طبقة البيوفيلم في عينات الانتيروكوكس ومقارنة فحص حساسية المضادات الحيوية

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الملخص:

المكورات المعوية، التي تعتبر طبيعية في تجويف الفم والأمعاء والمسالك التناسلية للإنسان والحيوان برزت المكورات المعوية كممرضات انتهازية رئيسية. تشكيل بيوفيلم هو عامل مهم لأنواع المكورات المعوية. البكتيريا داخل البيوفيلم أكثر مقاومة من نظيرتها الحية الحرة.

عدوى المسالك البولية (UTI) هي واحدة من أكثر أنواع العدوى المكتسبة في المستشفيات والمجتمعات المكتسبة في البشر، والتي تساهم في مقاومتها للمضادات الحيوية. إن دور الأغشية الحيوية البكتيريّة ومقاومة المضادات الميكروبية له أهمية كبيرة في الصحة العامة. ومن هنا أجريت هذه الدراسة للكشف عن تشكيل البيوفيلم بواسطة عينات من المكورات المعوية جمعت من ا لمستشفى الاستشاري ومجمع رام الله الطبي وقمنا بمقارنة نمط مقاومة المضادات الحيوية بين البكتيريا المكونة للبيوفيلم وغير المكونة ولتحديد ما هو الدور الذي يلعبه جين ال esp في تكوين البيوفيلم.

تم جمع 50 عينة من المكورات المعوية من المرضى، وتم تحديد الأنواع المعوية باستخدام طرق ميكروبيولوجية تقليدية. تم تحديد أنماط الحساسية للمضادات الحيوية من العينات بواسطة قرص ديفيوجن ميثود بالنسبة الى معهد المعايير السريرية والمخبرية.

طريقة لوحة Microtiter تستخدم لتقييم قدرة تشكيل البيوفيلم. تم فحص جميع العينات للمكورات المعوية لتحديد الجين المرتبط بالأغشية الحيوية ، esp ، بواسطة تفاعل البلمرة المتسلسل (PCR).

النتائج:

من 50 عينة، تم التعرف على 70٪ منها على أنها فيكاليس، و30٪ منها كانت فيشيام. وفقا لنتائجنا، وبشكل عام، كان 69٪ من الفيكاليس و40٪ من الفيشيام مكونات للبيوفيلم. كانت مقاومة بعض المضادات الحيوية بما في ذلك الأمبيسلين (36٪ P = 0.001)، التتراسيكلين (54٪، P = 0.003) ، والإريثروميسين, والفانكومايسين (24%)002.=p) أعلى بشكل ملحوظ بين منتجين البيوفيلم من غيرالمنتجين. كان الجين esp موجودًا في عزلات بنسبة 50٪. في هذه الدراسة، لم تكن هناك علاقة ذات دلالة إحصائية بين وجود هذا الجين وتشكيل بيوفيلم.

استنتاج :

النتائج التي توصلنا إليها تعزز دور تشكيل البيوفيلم في مقاومة العوامل المضادة للميكروبات ،كانت العينات المنتجة للبيوفيلم أقل حساسية للمضادات الحيوية مقارنة مع العينات الغير منتجة للبيوفيلم. تشكيل البيوفيلم يقلل من قابلية البكتيريا للمضادات الحيوية. نتائجنا تشير إلى أن تكوين البيوفيلم يعتمد على عوامل مختلفة ولكن ليس فقط على وجود جين ال esp.

قد ازداد عدد العوامل الوراثية المعروفة بدورها في إنتاج البيوفيلم في السنوات الأخيرة، بسبب توافرالمقاربات الجينومية والبروتيومية، ولكن من الواضح أن هناك حاجة إلى المزيد من الأبحاث للسماح بفهم أفضل لتنظيم إنتاج طبقة البيوفيلم، قد يؤدي الفهم الكامل لدورالعوامل الجينية والبيئية في تطويرالبيوفيلم إلى تحسين استراتيجيات التحكم في البيوفيلم بين المكورات المعوية.