MOLECULAR DETECTION OF VIRULENCE FACTORS OF *ENTEROCOCCUS FAECALIS* ISOLATED FROM URINE SAMPLES IN DUHOK CITY, KURDISTAN REGION/IRAQ.

Haval Mohammed Khalid
Dept. of Biology, Faculty of Science, University of Zakho, Kurdistan Region-Iraq.
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ABSTRACT:

Enterococcus faecalis **is one of the leading causes of many infections and mainly urinary tract infections. This pathogen developed high resistance to multiple antibiotics and it harbor many virulence factors genes. This study aimed to determine the antibiotic resistance patterns and screening for some virulence factor genes of *E. faecalis* isolated from urinary tract infection. Urine samples were collected from 788 outpatient's clinic having clinical signs of UTI that visited Azadi Teaching Hospital in Duhok city. Urine samples were cultured on bacteriological media and isolated colonies identified using standard bacteriological methods. Antibiotic susceptibility was performed by Kirby Bauer test. All isolates were subjected to species-specific PCR assay for confirmatory identification followed by targeting virulence genes. Twenty five isolates of *E. faecalis* were detected and confirmed by species-specific PCR assay that expressed high antibiotic-resistance to many selected drugs except norfloxacin, penicillin and ampicillin. The most prevalent genes among all isolates were *cpd* genes followed by *asa1*, *ace, esp,* and *gelE*. Bearing of virulence genes combination were more frequent among multiple-antibiotic resistant strains. This study highlighted on *E. faecalis* as causes of UTI in Duhok city that showed multiple resistances to common antibiotics and harboring more than one virulence gene.**

Keywords: *Enterococcus faecalis*, Urinary tract infection, PCR, Virulence genes.

INTRODUCTION

Enterococci are one of the most dominant bacterial groups inhabiting the intestinal tract of human and animals, it is considered as a causative agent for many serious infections, such as endocarditis, septicemia and urinary tract infections (Murray, 1990). Previous studies indicated that enterococci represent the second leading cause of urinary tract infections (UTI) and is a significant nosocomial pathogen (Schouten *et al*. 2000; Kaçmaz and Aksoy 2005). It has been reported that many factors are associated with a greater risk of acquiring enterococcal infections, such as, antimicrobial resistance and expression of virulence factors which may account for the establishment and maintenance of this opportunistic pathogen as a major community-acquired and nosocomial pathogens. Many studies revealed an increasing resistant of enterococci to many antibiotics, such as β-lactams, aminoglycosides, and more recently to glycopeptides. This could be attributed to the use of broad-spectrum antibiotics or multi-antibiotic regimes, which permit enterococcal overgrowth and superinfection (Kaçmaz and Aksoy 2005).

Enterococcus faecalis strains possess numerous putative virulence determinants, including gelatinase production, *Enterococcus* surface protein (*esp*), aggregation substance (*asa1*) and biofilm formation (Chuang *et al.* 2009). Gelatinase is a zinc metalloprotease, encoded by *gelE*, with hydrolytic capacity (Lindenstrau *et al.* 2011). *asa1*, encoded by a plasmid gene that mediates binding to the host epithelium and it appears to mediate bacterial aggregation during conjugation and facilitating plasmid exchange (Schlievert *et al.* 2010). The *esp* protein, is encoded by the *esp* gene, that seems to contribute to the colonization and persistence of *E. faecalis* strains in ascending infections of the urinary tract. Furthermore, *esp* may mediate the interaction with primary surfaces and participate in biofilm formation which substantially increases bacterial survival in biopolymers and may also be involved in antimicrobial resistance (Ballering *et al.,* 2009 and Chuang-Smith *et al.* 2010). Knowledge of the virulence characteristics of circulating *Enterococcus* strains may help to understand the complex pathogenic process of these opportunistic pathogens (Sharifi *et al.,* 2012).Therefore, this study aimed to screening for genes encoding pathogenicity-associated factors for isolates of *E. faecalis* from UTI in Duhok city, in addition to investigate the antibiotics susceptibility patterns of the isolated strains.

MATERIALS AND METHODS

Settings: This study conducted on 25 samples isolated from788 examined urine samples from out-patients, having clinical signs of urinary tract infections visited Azadi Teaching Hospital in Duhok city, Kurdistan region/Iraq, from June 2015 to December 2015.

Sample Collections: From each patient a mid-stream urine sample was collected using a clean sterile container.The collected samples were transferred to the laboratory unit at Azadi teaching Hospital within one to two hours for processing.

Culture and Identification: All samples were cultured on blood and selective agar media, then they were phenotypically identified to the species level using conventional bacteriological and biochemical methods (Manero and Blanch, 1999).

Antimicrobial Susceptibility: The antimicrobial susceptibility of the strains was determined using the disk diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI 2006) guidelines for the following antimicrobial agents: Gentamicin (10µg), Cefazolin (10µg), Cefoxitin (10µg), Ampicillin (25µg), Pencillin G (10µg), Oxacillin (5µg), Daptomycin (10µg), Trimethoprim-sulfamethoxazole (1.225/23.75µg),Vancomycin (10µg), Clindamycin (10µg), Erythromycin (15µg), Linezolid (5µg), Nitrofurantoin (100µg), Levofloxacin (5µg), Norfloxacin (10µg), Rifampin (5µg), and Tetracycline (10µg).

Molecular Characterization

DNA extraction

DNA was extracted from purified and identified colonies using the genomic DNA purification kit supplied by Jena Bioscience (GmbH,
Germany).

Detection of *E. faecalis* using species specific PCR

The detection of *E. faecalis* isolates was performed using universal primer (D-Ala:D-Ala) ligases as shown in Table 1 identities were later confirmed by species specific primer (Kariyamaet *al.* 2000), primer sequences shows in Table 1.

Detection of virulence genes by Polymerase Chain Reaction

The primers sequences used to amplify genes encoding virulence genes are listed in Table (1). Each 25µl of PCR reaction contained 2.0µl (10pmol) of each primer, 14 µl of free nuclease water, 2 µl of DNA template and 5µl of 5x master mix (Jena Bioscience GmbH, Germany). The sequence of each primer is shown in Table 1.

The PCR amplification products were visualized by electrophoresis on 1.5% agarose gel for 45mints at70v. The size of the amplicon was determined by comparison with molecular marker 100 bp (Jena Bioscience GmbH, Germany).

Table 1: Primers sequences used for detection of *E. faecalis* and its virulence genes.

Primer name	Gene	Sequence (5' - 3')	Product size (bp)	References
ddl E. faecalis	(D-Ala:D-Ala) ligases	F/ ATCAAGTACAGTTAGTCTTTATTAG R/ ACGATTCAAAGCTAACTGAATCAGT	**941**	Kariyama *et al.*, 2000
asa1	Aggregation substance	F/ GCACGCTATTACGAACTATGA R/ TAAGAAAGAACATCACCACGA	**375**	Vankerckhoven *et al.* 2004
gelE	Gelatinase	F/ TATGACAATGCTTTTTGGGAT R/ AGATGCACCCGAAATAATATA	**213**	Vankerckhoven *et al.* 2004
esp	Enterococcal surface protein	F/ AGATTTCATCTTTGATTCTTGG R/ AATTGATTCTTTAGCATCTGG	**510**	Vankerckhoven *et al.* 2004
cpd	sex pheromones	F/ TGGTGGGTTATTTTTCAATTC R/ TACGGCTCTGGCTTACTA	**782**	Eaton and Gasson 2001
ace	collagen-binding protein	R/ GGAATGACCGAGAACGATGGC F/ GCTTGATGTTGGCCTGCTTCCG	**616**	Creti *et al.* 2004

RESULTS

Bacterial isolates and susceptibility testing

From a total of 788 urine samples cultured, 25(3.2%) isolates of *E. faecalis* were identified.

The results of antibiotic susceptibility test using the disk diffusion method; revealed that the isolated *E. faecalis* were 100% resistance to Gentamicin, Cefazolin, Cefoxitin, Oxacillin, Trimethoprim-Sulfamethoxazole, Clindamycin and Tetracycline. On the other hand, variable resistance rates were observed toward other antibiotics like Erythromycin (96%), Rifampin (72%), Ampicillin (20%) and Vancomycin (4%) as indicated in Table(2).

All 25 isolates of *E. faecalis* isolates were confirmed by successfully amplification of 914 bp amplicon of *ddl* gene which used as species specific primer for detection of *E. faecalis* as shown in Figure 1.

Table 2: Resistance rates among *E. faecalis* isolates from urine samples.

Antibiotics	No of isolates (%)
Gentamicin	25 (100)
Cefazolin	25 (100)
Cefoxitin	25 (100)
Oxacillin	25 (100)
Trimethoprim-Sulfamethoxazole	25 (100)
Clindamycin	25 (100)
Tetracycline	25 (100)
Erythromycin	24 (96)
Rifampin	18 (72)
Norfloxacin	7 (28)
Pencillin G	7 (28)
Levofloxacin	6 (24)
Ampicillin	5 (20)
Vancomycin	1 (4)
Daptomycin	0
Linezolid	0
Nitrofurantoin	0

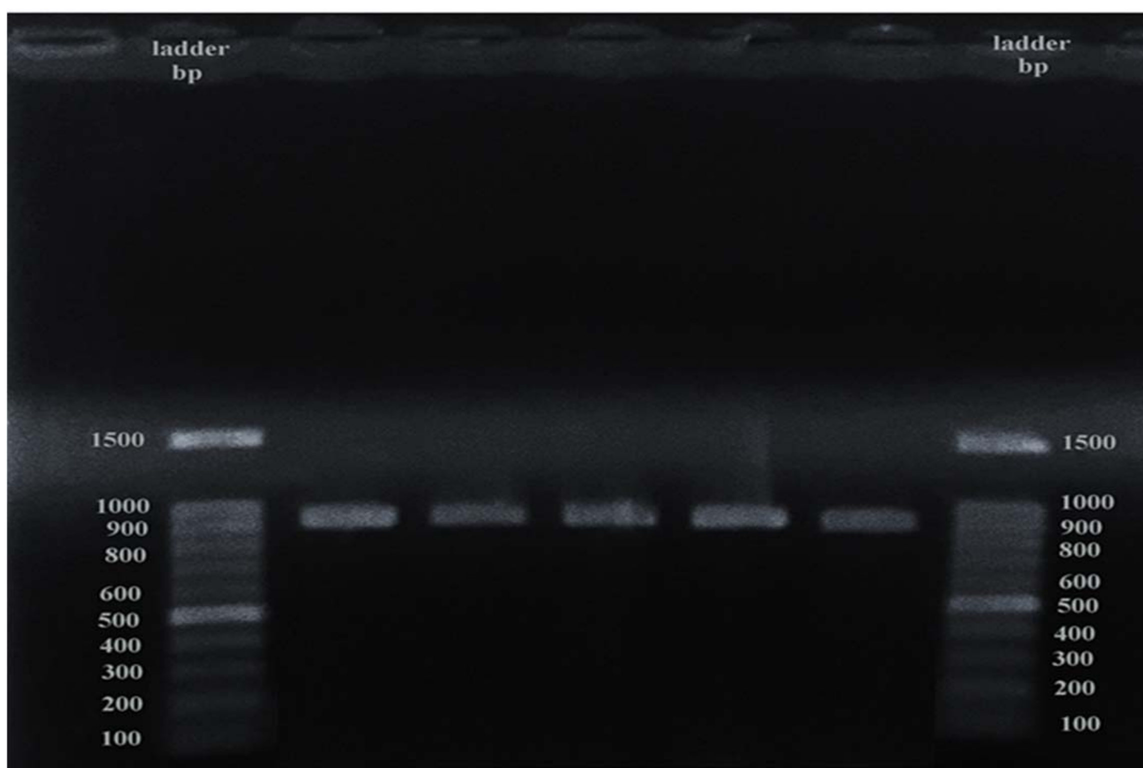

Figure 1: Species specific PCR amplification for *E. faecalis* produced with ddl amplicon with molecular weight 941 bp.

Table (3). Shows the frequency of five virulence factors among 25 isolates of *E. faecalis* using PCR assay; the highest number (96%) of the isolates harbored *cpd* gene followed by *asa1* gene and other virulence factors *ace*, *esp*, and *gelE*.

Table 3: Distribution of virulence factors among 25 isolates of *E. faecalis* Obtained from Urine samples

Virulence factor	No and (%) of isolates out of 25
cpd	24 (96)
asa1	22 (88)
ace	18 (72)
esp	17 (68)
gelE	15 (60)

Regarding the frequency of bearing a single and/or multiple virulence determinants by *E. faecalis* isolates, as indicated in Figure (2); and table(4) that 7(28%) out of 25 isolates, harbored all of the five used genes. Moreover, variable results observed with other genes and only one isolate (4%) harbored the gene *ace*.

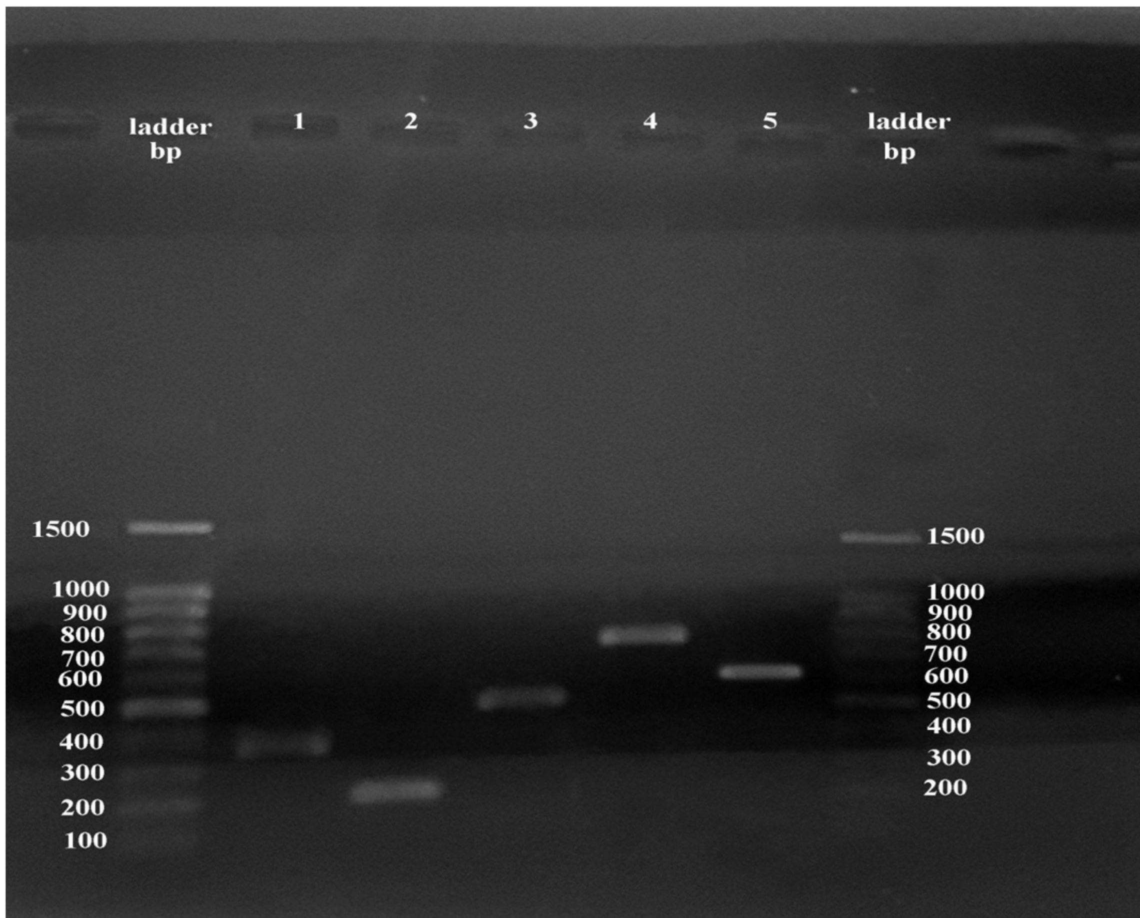

Figure2. Detection of PCR amplified products of virulence genes

Lane 1 asa1, lane 2 gelE , lane 3 esp, lane 4 cpd, lane 5 ace, Ladder molecular weight 100 bp.

Table4: Frequency of bearing of virulence gene combinations among *E. faecalis* isolates

Virulence factor	No. (%)
ace	1 (4)
asa1, gelE, cpd	4 (16)
asa1, cpd, ace	2 (8)
asa1, esp, cpd	2 (8)
asa1, esp, cpd, ace	5 (20)
gelE, esp, cpd, ace	2 (8)
asa1, gelE, esp, cpd	1 (4)
asa1, gelE, cpd, ace	1 (4)
asa1, gelE, esp, cpd, ace	7 (28)
Total	25 (100)

Table 5: Relationship between multiple-antibiotic resistance and bearing of Virulence genes among 25 isolates of *E.faecalis.*

Virulence genes type	Frequency among multiple- antibiotic resistant strains No. (%)
cpd	24 (96)
asa1	23 (92)
esp	18 (72)
ace	18 (72)
gelE	14 (56)

DISCUSSION

Infections in both community and hospital that caused by *E. faecalis* species are becoming more serious in our locality due to increased antibiotic resistance strains. In the present study 25 (3.2%) isolates of *E. faecalis* have been recovered from collected and cultured urine samples; and most (84 %) of these isolates were resistant to at least five of the tested antibiotics. This is an alarming sign, since these organisms limit the number of therapeutic options available to the clinician. This is in accordance to what has been reported by Sharifi *et al.* (2013) in Iran who stated that 79.3% of *E. faecalis* isolates were resistant to at least three antibiotics used.This study showed that most isolates exhibited high resistance rates to all used antibiotics; resistance to erythromycin was 96%and to Rifampin was 72%. Similar resistance rates were reported by Salah *et al.* (2008) and Sharifi *et al.* (2013) and they attributed it to the possibility of abusing of antibiotics and to the huge use of broad-spectrum antibiotics that exerted selected pressure to the emergence of multiple-resistant *Enterococcus* strains in our community.In the present study, *E. faecalis* isolates showed absolute resistance (100%) to gentamicin and a low resistance to norfloxacin (28%). In contrast, a study in Italy reported low resistance of *E. faecalis* isolates to both gentamicin and norfloxacin (Cosentino *et al.* 2010). On the other hand, Vankerckhoven *et al.* (2004) and Xia *et al.* (2013) documented high resistance level to both gentamicin and norfloaxcin.Vancomycin-resistant enterococci (VRE) probably represent the most serious challenge among many microbes with antibiotic resistance causing human infections (Al-Zu'bi *et al.* 2004). The present results showed that *E. faecalis* isolates exhibited extreme low-resistance to vancomycin (4%).This result is in accordance to Cosentino *et al.* (2010);Wierzchowska *et al.*(2012) and Xia *et al.* (2013) while they were inconsistence with Sharifi *et al.* (2013) as they found high (64%) resistance to vancomycin, probably this may be due to the disuse of vancomycin in our region and therefore, is considered to be the last line of treatment against *Enterococcus* infections.

The results of this study also showed that most of *E. faecalis* isolates were less resistance (20%) to ampicillin and penicillin (28%), similar rate of resistance have been observed by Mengeloğlu *et al.* (2011) and Bhardwaj *et al.*(2013).Fontana *et al.* (1983) attributedthis low resistance in enterococci to the production of high affinity penicillin–binding protein. On the other hand, higher resistances have been reported by Wierzchowska *et al.* (2012) and Xia *et al.* (2013). This might be due to the production of a low-affinity penicillin-binding protein while resistance to lactamase and amino-glycoside is conferred by plasmid-encoded enzymes which weaken the role of lactam therapy (Thouverez and Talon, 2004; Yazgi *et al.*, 2002).

Antibiotic resistance alone cannot explain the virulence of enterococci Pathogenesis to cause infection; many other events are included, such as colonization and adhesion to host tissues, invasion of the tissue and resistance to defense mechanisms of the host. However, each of virulence factors may be associated with one or more of the stages of the infection mentioned above. In this study, all of the *E. faecalis* strains tested harbored multiple virulence determinants; of them the gene *cpd* encoding for sex pheromone peptides which was the predominant and showed the highest incidence (96%) among all isolates. Abriouel *et al.* (2008) in Spain also showed higher frequency of this gene among clinical *E. faecalis* isolates The results of the present study is completely different from those

of Sharifi *et al.*(2013), who found lower incidence of this gene, moreover, *cpd*-positive *E. faecalis* strains in this study were more frequent among high resistant strains. This gene could facilitate the getting of the relevant sex pheromone plasmid and therefore, the associated virulence and resistance determinants (Klibi *et al.* 2007).

In the present investigation, the *asa1*gene, (which encodes aggregation substance), was found in high frequency (88%) among *E. faecalis* strains. A high incidence of this gene in *E. faecalis* was reported in previous studies (Waar *et al.* 2002; De Marques and Suzart, 2004; Dupont *et al.* 2008). Generally, the rate of *asa1* gene in this study indicated a significant association between the presences of *asa1* and both emergence of UTI and antibiotic resistance characterization.

The *ace* gene which codes for collagen-binding protein has been detected in high frequency (72%) in *E. faecalis isolated* strains. This is in agreement with a previous study (Cariolato *et al.* 2008). Singh *et al.* (2010) stated that the deletion of the *ace* gene resulted in a significant attenuation of the ability of *E. faecalis* to colonize host tissue and showed that *ace* plays an important role in the early stages of colonization, possibly by mediating the adherence of *E. faecalis* to collagen exposed at the site of tissue injury. Furthermore, Lebreton *et al*. (2009) mentioned that *ace* has a valuable drug target against human UTI.

The occurrence of *esp* in clinical isolates of this study was 68%; the same occurrence rate (68%) was recorded by *Medeiros et al.* (2014).Also high occurrence of this gene have been reported by Archimbaud *et al.*(2002); Arularasi Aberna and Prabakaran (2011) as they reported rates of 72.4 and 67.5%, respectively. Furthermore, *esp*-positive *E. faecalis* strains, showed high resistance (72%) to the most of tested antibiotics in the current study, this is consistence with Sharifi *et al.* (2013). The high prevalence of *esp* among isolates involved in UTI in the previous study, suggested their role in increased virulence, colonization and persistence of *E. faecalis* within the urinary tract (Shankar *et al.* 2001).

Among the five genes investigated in the present study, the gene *gelE* was least detected (60%), this gene codes for gelatinase which is an extracellular zinc metalloendopeptidase. Somewhat, similar isolation rates ranged from 45.3 to 70.9% were observed in other studies (De Marques and Suzart, 2004; Arularasi Aberna and Prabakaran, 2011).While higher percentage of this gene have been reported by Semedo *et al*. (2003) and Creti *et al.* (2004). Furthermore, in this study, the frequency of this gene among multiple antibiotic resistant isolates was lower (56%) as compared with the frequency of other genes. *esp* protein encoded by *esp* gene assumed to play a role in the primary surface attachment, contributing to the colonization and persistence on urinary tract (Shankar *et al.* 2001; Toledo-Arana *et al.* 2001).

CONCLUSION

The current study indicated a high prevalence of *E. faecalis* harboring high resistance rates to all of the tested antibiotics in our locality this is an alarming sign and more worrisome in hospital setting. The distribution of virulence genes was more common in *E. faecalis* strains and the high incidence of multiple virulence factors could potentially contribute to bacterial colonization and pathogenesis of *E. faecalis* in the urinary tract. They may act as reservoirs of virulence factors, enabling the dissemination to other bacterial pathogens. The higher prevalence of *cpd* determinant may explain the role of this gene in the severity of the infection and the emergence of resistance to the tested antibiotics. Additional investigations are needed to evaluate the expression of such factors, which may not be revealed by *in vitro* phenotypic tests during the course of infection.

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پوخته

بەکتریا *Enterococcus faecalis* ئێك ژ ئەگەرێن سەرەکییێن گەلەك هەودانانە ب تایبەتی هەودانێن میزرەوێ ،ئەڤ بەکتیریایە شیایە گەلەك گهورینکاریا ب سەر خوڤە بینیتن بو بەرگریا ژمارەکا زور یا ئەنتی بایوتیکا ، کو هەلگرا جینێن توندێن نەخوشییێن هەودانان، ئارمانج ژ ڤێ خوێندنێ ژبو دیارکرنا جورێن بەرگریا بو ئەنتی بایوتیکا هەروەسا لێرێنینەك بو نیاسینا جینێن هەلگرێن فاکتورێن توندیێ ل بەکتریا *E. faecalis* ئەوێن هاتینە جودا کرن ژ هەودانێن میزرەوێ ، بو ڤێ مەرەمێ 788 سامپلێن میزێ هاتنە کومکرن ژ وان نەخوشێن سەرەدانا کلینیکا دەرڤەی نەخوشخانا ئازادی ل باژێرێ دهوکێ کرین و هاتینە دەستنیشانکرن ب هەودانێن میزرەوێ، هەمی سامپلێن میزێ هاتنە چاندن ل سەر میدیاییێن بەکتریولوجییێن تایبەت و هەمی بەکتریاییێن جوداکەر هاتنە دەستنیشانکرن و نیاسین ب رێکێن ستاندەرێن بەکتریولوجی، و ب ریکا (Kirby Bauer) تێستا بەرگریا ئەنتی بایوتیکا بو هاتەکرن، هەروەسا هەمی بەکتریاییێن جوداکەر هاتنە پشکنینکرن بریکا (PCR) ژ بو لێرینینا جینێن توند ، ب تنێ 25 بەکتریاییێن جوداکەر هاتنە بەرنیاسین و دەستنیشان کرن ب ڤێ ریکێ کو بەرگریەکا مەزن هەبوو بو هەمی ئەنتی بایوتیکێن هاتینە بکار ئینان ژبلی Ampicillin, penicillin, Norfloxacin ، جینێ *cpd* ب رێژەکا بەرفرەهـ هاتە دیتن ل ناڤ هەمی بەکتریاییێن جوداکەر و ل دیفدا جینێن *asa1, ace, esp, gelE*. هەروەسا کومێن جینێن دیارکری پێکڤە گرێدای ب جورەکێ بەرفرەهـ هاتەدیتن لناڤ ڤان بەکتریاییێن جوداکەر، کو ئەڤ جینە هەلگرێن فاکتەرێن توندیێ و پێکهاتییێن بەرگریا ئەنتی بایوتیکا نە.

ئەڤ خوێندنە بو بەکتریا *E. faecalis* هاتە ئەنجام دان کو ئەگەرێ هەودانا میزرەوا بو ل دەف ئەو نەخوشێن سەرەدانا کلینیکا دەرڤەی نەخوشخانا ئازادی کرین ژ بەرکو بەرگرییەکا مەزن نیشان دای بەرامبەر ئەنتی بایوتیکێن بەرنیاس و هەلگرتنا وان ب جینێن توندێن هەودانا.

التشخيص الجزيئي لعوامل الضراوة في بكتريا *Enterococcus faecalis* المعزولة من عينات الادرار في مدينة دهوك–أقليم كوردستان العراق

الملخص:

Enterococcus faecalis تعد هذه البكتريا من احدى المسببات لمعضم الاصابات وبشكل رئيسي اصابات المجاري البولية. لقد طورت هذه البكتريا مقاومة عالية ومتعددة تجاه العديد من المضادات الحياتية وتحمل جينات لمعضم عوامل الضراوة والامراضية. الهدف من هذه الدراسة هوتحديد انماط المقاومة للمضادات الحياتية وكذلك التقصي لمعرفة الجينات الحامله لعوامل الضراوة في بكتريا *E. faecalis* المعزولة من اصابات المجاري البولية. جمعت عينات الادرار من المرضى ذوي الاعراض السريرية لاصابات المجاري البولية الزائرين لمستشفى ازادي التعليمي في مدينة دهوك. تم زرع عينات الادرار على اوساط بكتيريولوجية وتم تشخيص العزلات بالطرق البكتريولوجية القياسية.اجري فحص المضادات الحياتية حسب طريقة (Kirby Bauer). خضعت جميع العزلات لتقنية تفاعل متضاعف السلاسل(PCR) لتحري جينات الضراوة. تم تحديد وتاكيد تشخيص خمسة وعشرون عزلة باستخدام التقنية الاخيرة وابدت هذه العزلات مقاومة عالية ضد جميع المضادات الحياتية المستعمله باستثناء المضادات الحيويةAmpicillin, penicillin Norfloxacin. من اكثر الجينات الشائعه ضمن جميع العزلات كان الجين *cpd* تلاه الجينات *asa1, ace, esp, gelE*. كذلك كان شائعا في هذه البكتريا حمل مجموعة مترابطة من الجينات في ان واحد ضمن العزلات المتعددة المقاومة للمضادات الحياتية. هذه الدراسة سلطت الضوء على ان بكتريا *E. faecalis* كانت المسبب لاصابات المجارية البولية في مدينة دهوك وابدت مقاومة عالية للمضادت الحياتية وحملت الكثير من جينات عوامل الضراوة.