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RESEARCH PAPER

Molecular and Bacteriological Study of *Enterococcus faecalis* Isolated from Different Clinical Sources

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A B S T R A C T:
Enterococci are Gram-positive bacteria that cause serious nosocomial infections, including urinary tract, bloodstream infections and endocarditis. During the period of September 2018 to February 2019, forty four isolates of *E. faecalis* were isolated from 826 clinical specimens including; 35(5.07%) isolates of *E. faecalis* from urine, 7(7.60%) isolates from high vaginal swab and 2(8.69%) isolates from blood patients in different hospitals. All isolates that described above were identified depending on cultural criteria, morphological criteria, biochemical tests and further confirmed by Vitek 2 compact systems. The results of eleven antimicrobial against obtained isolates revealed that 100% of *E. faecalis* were resistant to cefotaxime, vancomycin, amoxicillin and erythromycin. While, it were 100% sensitive to doxycycline, imipenem, and nitrofurantion. Whereas, most isolates were differ in their susceptibility to amikacin, gentamycin, tetracycline and azithromycin. On the other hand, the results of biofilm found that 13.63% of isolates were produce strong biofilm, 54.54% were produce moderate biofilm and 31.81% were produce weak biofilm. The results of molecular analysis by using PCR showed that isolated *E. faecalis* were carried 97.72%, 90.90%, 63.63% of *ebpR*, *asa1*, *esp* genes respectively.

KEY WORDS: Antimicrobial resistant, Biofilm, *Enterococcus faecalis*, Virulence genes.

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1. INTRODUCTION

Enterococcus faecalis (*E. faecalis*) is one of the most widely recognized types of enterococcus which causes 85 to 90% of enterococcal infections. Gram-positive bacteria were before classified as group D Streptococcus, because of specific antigen which is teichoic acid. The vast majority of these microbes are non-hemolytic and occasionally are alpha-hemolytic which can be catalase and oxidase enzymes, non- motile, facultative anaerobic bacteria (Van Tyne and Gilmore, 2014; Anagnostopoulos et al., 2018). Enterococci are mesophilic bacteria that could develop from 10 ^{o}C to 45 ^{o}C with ideal temperature included between 30 ^{o}C and 35^{o}C (García-Solache and Rice, 2019). *E. faecalis* develop in salt 6.5% (sodium chloride), bile salts 40% and pH=9.6 (Higuita and Huycke, 2014). It can survive in a temperature of 60 °C for 30 minutes which differentiate them from other Enterococcus species (Alipour et al., 2014). Moreover, *E. faecalis* is known to be opportunistic pathogen and is a common reason for nosocomial infections and cause some diseases in humans and these bacteria have been associated in bacteremia, endocarditis, urinary tract diseases or different infections. Lately, a role of *E. faecalis* in pancreatic and colorectal cancers has also been

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recommended but this remains questionable(O'Driscoll and Crank, 2015; de Almeida et al., 2018). The enterococci have an amazing capacity to adjust to various conditions and have an affinity to obtain antibiotic resistance, which has led to the appearance of multi-drug resistant variants, across the genus**.** The *E. faecalis* is naturally resistant to many antibiotics, for example, penicillin, ampicillin, piperacillin and vancomycin which have just bacteriostatic before bactericidal effects (Kristich et al., 2014). Furthermore, Opportunistic diseases has been related with the creation of virulence factors, adherence to cells, ability for biofilm creation and protection from antimicrobials (Bhatty et al., 2015). Many virulence factors have been recognized that are related with a wide range of *E. faecalis* diseases; in particular, aggregation substance (AS), gelatinase (GelE) and biofilm-associated Pili (Ebp), and biofilm creation (Singh et al., 2010). The purposes of this study were isolation and identification of *E. faecalis* from various clinical sources from various hospitals patients, antimicrobial sensitivity, detection of biofilm producer bacteria and detection of some virulence genes by using PCR technique.

Fritillaria zagrica Stapf. is a species very closely allied to *F. tulipifolia* and *F. armena*.

2. MATERIALS AND METHODS

2.1. Isolation of *E. faecalis* bacteria

Forty four isolates of *E. faecalis* were obtained from 826 specimens were collected from 555 female and 271 male patients in Rizgary (Erbil province) and Shahidan Qaladze teaching hospitals (Sulaimani province) within five months (September 2018 to February 2019) and from different clinical sources including; 690 specimens from urine, 92 from high vaginal swab, 23 from blood and 21 from stool. All specimens transferred to laboratory for microbiological examinations. All isolates were identified depending on cultural, morphological, and some biochemical tests, in addition to Vitek 2 Compact system (Navas et al., 2014). Also the bacterial isolates were screened for catalase, oxidase, coagulase , gelatinase , lipase, DNase, protease, urease , beta lactamase and hemolysin tests (Sharma, 2007; Cappuccino and Sherman, 2008; Stratev et al., 2015).

2.2 Antimicrobial Resistant Test

Antimicrobial resistant test was used to known the resistant of isolated *E. faecalis* against 11 antimicrobials by using Kirby Bauer method (Clinical and Institute, 2009), including amikacin (10 µg), amoxicillin (25 µg), azithromycin (15 µg), cefotaxime (30 µg), doxycycline (10 µg), erythromycin (10 µg), gentamycin (10 µg), imipenem (10 µg), nitrofurantion (100 µg), tetracycline (10 µg), and vancomycin (30 µg).

2.3 Biofilm Assay

All obtained *E. faecalis* were tested for biofilm production by using microtiter plate assay according to (Mathur et al., 2006).

2.4. Molecular Study

2.4.1. Isolation of DNA from *E. faecalis*

The way that used for isolation of DNA from bacterial cells was done by using Presto™ Mini gDNA bacteria kit. The steps includ; Sample preparation, lysis, DNA binding, washing and elution.

2.4.2. Primer

The studied primers were provided by macrogen (Table 1). Macrogen made the primer as lyophilized powder, thus the concentration defines as pmol. Primers were prepared by adding 300µl of deionized water to each lyophilized primers (forward and reverse) for each gene separately. Then 20µl from each primer put in 180µl deionized distilled water as work stock, and then used in PCR reaction and the volume of each primer stock solution was 100 pmol/µl.

2.4.3. Detection of *E. Faecalis* Genes

DNA extraction was done by the tissue buffer boiling method as defined by (Aghdam et al., 2017). Extracted DNA assisted as a template for the enlargement of virulence genes particular for *E. faecalis* includes *asa1and ebpR*. Conventional PCR was performed in 25 µl volumes reactions that contained 20–200 ng DNA, 0.5 µM of 1 µl of each specific primers for each gene, 1.5 mM MgCl2, and 200 µM of each dNTP, 1× PCR buffer and 2 U DNA Taq-polymerase (Cinnage, Tehran, Iran) as described by (Asgharzadeh et al., 2015). An initial denaturation at 94 °C for 10 min was followed by 35 cycles of 1 min denaturation at 94 °C, annealing at 58 °C for (*esp*) /52 °C (for *ebpR* and *asa1*) for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min (Aghdam et al., 2017). PCR products were analyzed in 1.5% agarose gels and visualized under UV after staining with 0.5 µg × ml-1 DNA

safe stains. AccuPower PCR PreMix is the great technology and easy to do DNA amplification. It comprises DNA polymerase, dNTPs, a tracking dye and reaction buffer in a premixed format, freeze-dried into a pellet. Primer (1.3 µl) of each forward and reverse, (2.5µl) of DNA template were added to AccuPower PCR tube then 20µl of distilled water added to AccuPower PCR tubes. After that, lyophilized blue pellet dissolved by vortexing. PCR done for samples, process in the thermal cycler for 30 cycles (Table 2).

2.4.4. Detection of DNA Content by Agarose Gel Electrophoresis

The most public way to isolate DNA molecules according to size is electrophoresis technique in 1.5% agarose gel (Igeltjørn, 2009; Mishra et al., 2010). The DNA concentration and purity was determined by using Nanodrop spectrophotometer (Thermo Scientific/ United States) in agarose gel (Philippe and Deborah, 2010)

3. RESULTS AND DISCUSSIONS

3.1. Frequencies of *E. faecalis* isolates

In the present study 44(5.33%) isolates of *E. faecalis* were isolated from 826 specimens (555 females and 271 males) including (Urine, stool, High vaginal swab and Blood) during five months (September 2018 to February 2019) as illustrated in table (3). In the present study, the results showed that 35(5.07%) isolates of *E. faecalis* were obtained from urine samples, the current results similar with

Table 1. Primers sequences and their product size.

Primers	Primer sequences	Product size	References
Forward primer(*asa1*)	5'-GCA CGC TAT TAC GAA CTA TAT GA-3'	375bp	(Kafil and Mobarez, 2015).
Reverse primer(*asa1*)	5'-TAA GAA AGA ACA TCA CCA CGA-3'		
Forward primer(*ebpR*)	5'-AAA AAT GAT TCG GCT CCA GAA-3'	101bp	(Bourgogne et al., 2007).
Reverse primer (*ebpR*)	5'-TGC CAG ATT CGC TCT CAA AG-3'		
Forward primer(*esp*)	5'-GGA ACG CCT TGG TAT GCT AAC-3'	95bp	(Shankar et al., 1999).
Reverse primer (*esp*)	5'-GCC ACT TTA TCA GCC TGA ACC-3'		

Table 2. PCR protocol and thermo cycling conditions.

Gene name	Initial denaturation	Cycles	Denaturation	Annealing	Elongation	Final elongation
asa1	94°C/10min	35	94°C/1min	52°C/1min	72°C/1min	72°C/10min then 4°C→∞
ebpR	94°C/10min	35	94°C/1min	52°C/1min	72°C/1min	72°C/10min then 4°C→∞
Esp	94°C/10min	35	94°C/1min	58°C/1min	72°C/1min	72°C/10min then 4°C→∞

Table 3. Frequency of *E. faecalis* in different clinical sources.

Sources	No. of samples	No. of isolates	Percentages
Urine	690	35	5.07%
Stool	21	0	0%
High Vaginal Swab	92	7	7.60%
Blood	23	2	8.69%
Total	826	44	5.3%

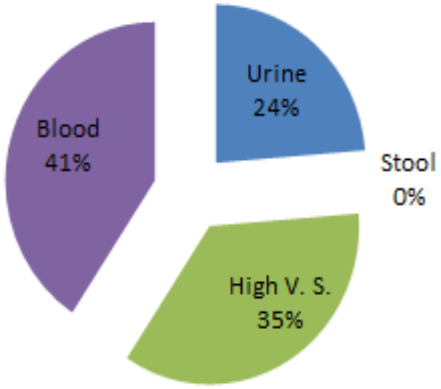

Figure 1. Distribution of E. faecalis isolates in different clinical sources.

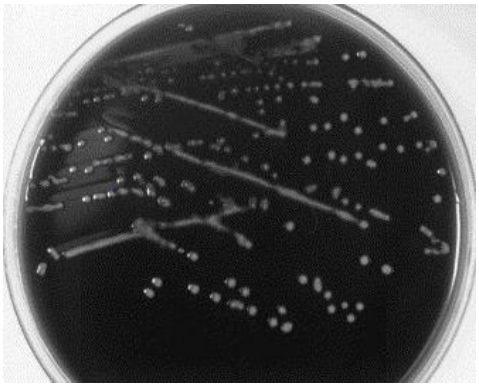

Figure 2. *E. faecalis* colonies on Bile Esculin Agar.

results of Daza et al. in (2001) and Al Sweih et al. in (2005) which they isolated (6%) and (4.2%) of *E. faecalis* from urine samples, respectively. while no isolates was isolated from stool specimens, On the other hand 7(7.60%) isolates of *E. faecalis* were isolated from high vaginal swab, this result was near with recent studies of Udo *et al.* in (2003) and Kudo et al. in (2014) which they isolated (9%) and (9.7%) of *E. faecalis* from high vaginal swab, respectively; whereas, 2(8.69%) of *E. faecalis* were isolated from the blood. These results were different with recent studies of Al-Husseiny, in (2008) and Kehinde et al. in (2013) which they isolated (16.27%) and (5%) of *E. faecalis* from blood, respectively (Figure 1). The percentages of isolation may be different and may be related to the time of collecting specimens, the number of specimens, the differences in the sources, hospital included and differences in the identification methods. All isolates identified as *E. faecalis* on bile esculin agar (Figure 2) depend on creamy color of colony which conversion of media to black, it comprise of 40% bile salt assistance in inhibition growth of Streptococci belong to group D antigen made this media valuable in determination of enterococcus from other non-enterococcus bacteria that belong to group D antigen (Hemraj et al., 2013). Additionally, the *E. faecalis* are Gram positive, do not form spore, not motile, do not produce oxidase, catalase, DNase, and urease, and ferment glucose without gas generation (Arias et al., 2010; Jayasundara, 2014; Subramanian et al., 2015; Sangeetha et al., 2016). They are facultative anaerobic bacteria, round, single, pairs or may be short chains (Silva et al., 2012).

The results of lipase showed that 4(9.09%) isolates were given positive results and 40(90.90%) isolates were gave negative and these results was close with results of (AL-Khafaji et al., 2010; Biswas et al., 2014). Moreover, 26(59.09%) of the isolates were positive for protease test and 18(40.90%) of isolates were negative and these results similar to Fuka et al. in (2017). Furthermore, out of all isolates of *E. faecalis* just 24(54.54%) hydrolyzed gelatin and the other isolates do not and these results agreed with the consequences of Mohamed and Murray, in (2005) and Zoletti et al. in (2011). However,

39(88.63%) were coagulase negative and 5(11.36%) were coagulase positive and these outcome is close that result which described by Kent in (2013) which they detected Coagulase-negative *Enterococci* represented 93%. All 44 isolates of *E. faecalis* from different sources were β-hemolysin .The current study was an endeavor to distinguish β-lactamase creation between isolated bacteria and the results found that all *E. faecais* were positive for β- lactamase creation. These results was similar with results obtained by Al-Duliami et al. in (2011) which they distinguished 76.7% of β-lactamase makers of *E. faecalis*.

3.3. Antibiotics Susceptibility Test

Antibiotic sensitivity test was conducted for 44 *E. faecalis* isolates using 11 types of antibiotics with different action, the percentage of resistance (Figure 3). The results found that 100% of *E. faecalis* were resistant to cefotaxime, vancomycin, amoxicillin and erythromycin. While, 95.45%, 86.36%, 81.81% and 68.18% of isolates were resistant to each of tetracycline, amikacin, gentamycin and azithromycin respectively.

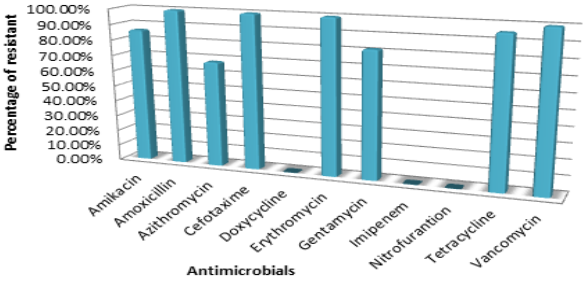

Figure 3. Percentage of antimicrobial resistant in *E. Faecalis.*

Moreover, 100% of obtained *E. feacalis* were susceptible for doxycycline, imipenem and nitrofurantion. These results similar with those results reported by Ullah et al. in (2015). and Yang and Juett, in (2016) where they detected that 100% of isolates were resisting to cefotaxime and vancomycin. Also, these results was near with results reported by AL-Gheethi et al. in (2013) where they detected that 95.23% of isolates were resistant to amoxicillin. As well as, Stępień-Pyśniak et al. in (2016) demonstrated that *E. faecalis* isolates absolute resistance 100% against erythromycin. Furthermore, Endo et al. in (2014); Talebi et al. in (2015); Samadi et al. in (2015) and Pourcel et al. in (2017) detected that 66.7% of isolates were resisting to azithromycin, 86% of isolates were resisting to tetracycline, 80% of isolates were resisting to amikacin and 77.2% of isolates were resisting to gentamicin, respectively; Also results of doxycycline, imipenem and nitrofurantion were agreed with the results reported by Yüceer and Özden Tuncer, in (2015); Sarah *et al.* in (2015) and El-Kersh *et al.* in (2016) which they detected that 100% of isolates were sensitive to doxycycline, 100% of isolates were sensitive to imipenem and 88% of isolates were sensitive to nitrofurantion, respectively.

3.4. Detection of Biofilm Producer *E. faecalis*

The results of this test showed that 6 (13.63%) of *E. faecalis* were strong biofilm producer, While, 24(54.54%) were moderate biofilm producer and 14(31.81%) isolates of *E. faecalis* were weak biofilm producer. These results was a little bit differ from results of Al-Hashimy and Alhalaby in (2016) where they detected that 20(40%) of *E. faecalis* were strong biofilm producer, 26(52%) of *E. faecalis* were moderate biofilm producer and 4(8%) were strong biofilm producer. The most dependable technique was microtiter plate method. The biofilm is a community of bacteria living with each other in an organised structure as microcolonies and it is encased in a matrix composed of an extracellular polymeric substance.

The above method was the better screening test for biofilm production than other methods for Gram positive bacteria because microtiter assay was easy to perform and assess both qualitatively and quantitatively (Abdullah and barzani, 2016). Also, Mathur et al., (2006) reported that the microtiter assay (TCP) method was an accurate and reproducible method than other methods for screening and this method can serve as a reliable quantitative tool for determining biofilm formation by clinical isolates of microorganism. However, the bacterial biofilm make the bacteria to adhere to inert materials and increased antibiotic resistance (Davies, 2003; Høiby et al., 2010).

3.4. Molecular Study

3.4.1. DNA Extraction

DNA of all isolated Gram positive bacteria effectively extracted by utilizing genomic DNA mini kit (Presto ^{TM} Mini gDNA bacteria kit), Then DNA concentration was estimated by Nanodrop spectrophotometer, and DNA concentration was among 50-90ng/µl. Proportion of tests absorbance at 260/280nm were 1.8–1.85. The proportion of tests absorbance at 260 / 230nm was between1.8-2 and commonly accepted a pure for DNA (Philippe

and Deborah, 2010). Also, DNA bands were affirmed and examined by agarose gel electrophoresis. The results revealed that all DNA successfully extracted and all isolates gave bands with molecular weight 1500bp (Yang et al., 2018) as shown in figure (4).

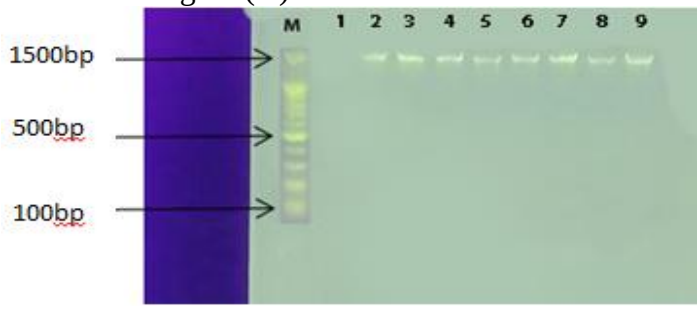

Figure 4. Genome profile of *E. faecalis* isolates performed with agarose gel electrophoresis. M: DNA ladder (100bp). Lane 1: Negative control; Lanes 2, 3, 4, 5, 6, 7, 8 and 9: Are genomes of *E. faecalis* isolates.

3.4.2. Detection of *E. faecalis* Virulence Genes

3.4.2.1. Detection of *ebpR* gene by PCR

In present study, PCR method showed that 43(97.72%) of *E. faecalis* isolates were harboring *ebpR* gene with molecular weight of 101bp as showed in figure (5). This result in agreement with Aghdam et al. in (2017) which they isolated 100 isolates of *E. faecalis* from patient's dental root canals and they examined for the occurrence of virulence genes and their results showed that 91(91%) had *ebpR* gene.

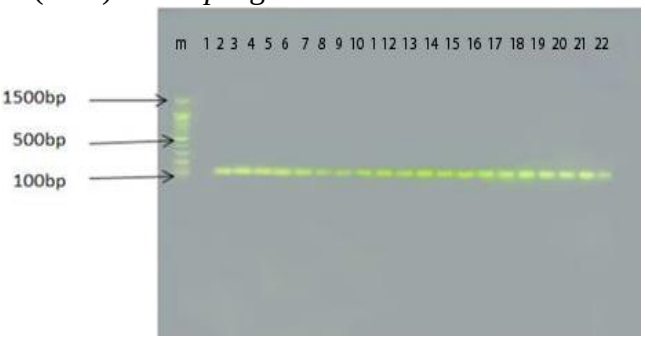

Figure 5. Polymerase chain reaction products on gel electrophoresis for *ebpR* gene. M: DNA ladder (100bp). Lane 1: Negative control; Lanes 2, 3, 4, 5 , 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 and 22: Amplified PCR product of *ebpR* gene *(*101bp) for *E. faecalis* isolates.

On the other hand Kafil and Mobarez in (2015) isolated 196 isolate of *E. faecalis* from urinary tract infection of patients in various wards and they reported that 183 (93.36%) of isolates were harbor *ebpR* gene. Like many bacteria, *E. faecalis* encodes various adhesions involved in colonization or infection of various niches. Two well-considered *E. faecalis* adhesions, aggregation substance (AS) and endocarditis- and biofilm-related pili (Ebp), both add to biofilm development on abiotic surfaces and in endocarditis, suggesting that they may be communicated at the same time. If they are co-communicated on the same cells and what is the practical effect of co-expression on single cells and inside a population. When Ebp and AS are communicated on the same cells, pili interfere with AS-mediated clumping and impede AS-mediated conjugative plasmid move during planktonic development (Afonina et al., 2018).

3.4.2.2. Detection of *asa1*gene by PCR

In present study, PCR method indicated that 40 (90.90%) of *E. faecalis* isolates which isolated from various sources were carried *asa1* gene with molecular weight 375bp as showed in figure (6), This result in agreement with near result of various studies are available for the prevalence of *asa1* in enterococcal isolates obtained from various sources. In a study 8 out of 10(80%) of *E. faecalis* harbored *asa1* gene and majority (62.5%) of them were being isolated from urine specimen. Additionally, in other study the scientist found that 76(38.77%) isolates had *asa1* gene from 196 isolates of enterococcus species which isolated from patients with urinary tract infections. In various wards including serious care units, women particular ward, pediatrics, nephrology and internist (Kafil and Mobarez, 2015). Aggregation substance (*asa1*) is a sex pheromone plasmid-encoded surface protein, which promotes the conjugative exchange of sex pheromone plasmids by arrangement of mating aggregates among donor and recipient cells (Aspri et al., 2017) there is considerable evidence for a functional role of collection substance in the collaboration of *E. faecalis* with its mammalian host. Surface expression of aggregation substance rises adherence to host tissues and furthermore regulates the natural resistant reaction to enterococcal disease, thus increasing virulence in various models of opportunistic infection (Chuang et al., 2009). The development of biofilms *in vitro* is also greatly upgraded by aggregation substance (Bhatty et al., 2015).

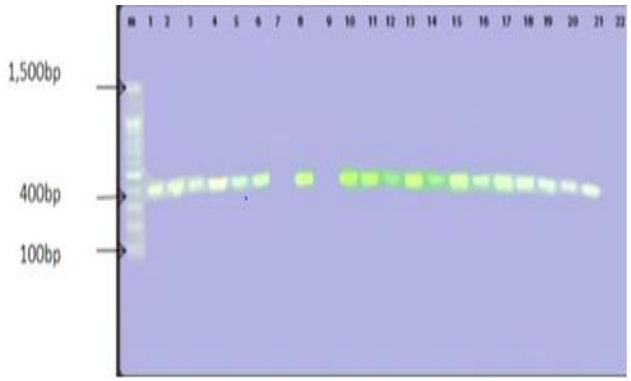

Figure 6. Polymerase chain reaction products on gel electrophoresis for *asa1* gene. M: DNA ladder (100bp). Lane 22: is negative control; Lanes 1, 2, 3, 4, 5, 6, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 and 21 Amplified PCR product of *asa1* gene (375bp) for *E. faecalis* isolates; Lanes 7,9: negative for *asa1* gene.

4.5.2.3. Detection of *esp* gene by PCR

In current study, the results indicated that 28(63.63%) of *E. faecalis* isolates were carried *esp* gene with molecular weight 95bp (Figure 7). This result in agreement with the results of (Strateva et al., 2016) which they found in their study that 60% of *E. faecalis* isolates carried *esp* gene. Enterococcus surface protein (esp) is a virulence factor that helps in the adhesion, but its role in biofilm formation is still opposing (Shridhar and Dhanashree, 2019). Esp is encoded on a pathogenicity island in *E. faecalis* and is involved in biofilm formation and binding to epithelial cells (Zou and Shankar, 2016). *E. faecalis* surface protein (Esp) is known to help surface attachment leading to biofilm development. It has been presented that Esp insertion-deletion mutants form unstructured and weak biofilms (Heikens et al., 2007). Percentage of positive and negative results for virulence genes showed in table (4).

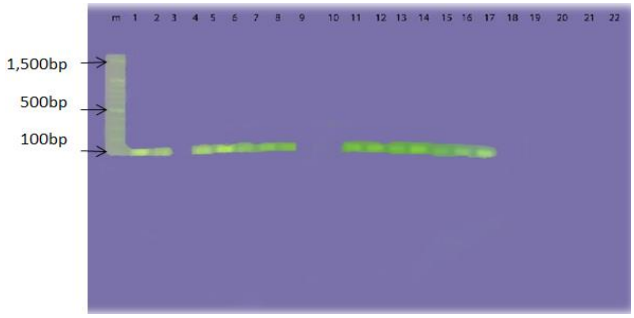

Figure 7. Polymerase chain reaction products on gel electrophoresis for *esp* gene. M: DNA ladder (500bp). Lanes 1, 2, 4, 5, 7, 6, 8, 11, 12, 13, 14, 15, 16 and 17: Amplified PCR product of *esp* gene (95bp) for *E. faecalis* isolates. Lanes 3, 9, 10, 18, 19, 20, 21 and 22: Negative for *esp* gene.

Table 4. Prevalence of virulence genes.

Virulence Genes	% Positive	% Negative
ebpR	(97.72%)	(2.27%)
asa1	(90.90%)	(9.09%)
Esp	(63.63%)	(36.36%)

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