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Distribution of virulence genes among *Enterococcus spp.* strains isolated from recreational water samples in Mosul city and evidence for horizontal gene transfer of antibiotic resistance genes

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Abstract
Ten water samples were collected from different recreation sites in Tigris river and some swimming pools in Mosul city. The isolation percentage of *Enterococcus spp.* was 30% using Hicrome *Enterococcus faecium* agar for initial isolation and 16S rRNA gene sequencing for final identification. The aim of this study was to determine the incidence of eight virulence genes (*gelE*, *efaAfs*, *ace*, *esp, cyl, asa1, eep,* and *agg*) in *Enterococcus spp*. and study the possibility of gene transfer for virulence and antibiotic resistance genes between pathogenic enterococci and those isolated from recreation sites via horizontal gene transfer. Our results detected the presence of virulence genes in 0.0 - 66.7% of environmental isolates and in 66.7- 100% of pathogenic isolates. The virulence genes detected in environmental enterococci were *gelE* (66.6%), *ace* (66.6%), *efaAfs* (66.6%) and *agg* (33.3%). Even though the percentages for virulence gene detected in environmental enterococci were lower to what was found in pathogenic enterococci, such results show the existence of virulence genes in environmental enterococci that may lead to serious health issues. *Enterococcus* isolates from water were tested for their ability to resist 8 antibiotics and results showed that they were 100% resistant to gentamycin, 66.6% resistant to streptomycin, ciprofloxacin and kanamycin, and 33.3% resistant to ampicillin, tetracycline, and chloramphenicol, while none of the isolates were resistant to vancomycin. Conjugation experiments showed the possibility of antibiotic resistance genes to transfer between pathogenic enterococci but not to our environmentally isolated enterococci. However, identifying self-transmissible plasmids in *Enterococcus* may still lead to the spread of antibiotic resistance genes to other compatible recipient enterococci in nature.

Keywords: *Enterococcus*, conjugation, virulence genes

Introduction

Enterococcus spp. are Gram positive bacteria that mainly inhabit the intestine of healthy humans and animals therefore can reach water surface through many actions such as leaking septic systems, domestic animal waste, or runoff from contaminated areas etc. (Mbanga *et al*.,2021) [22]. This reflects its importance from an environmental point of view by using them as an evidence of fecal contamination especially the two species *E. faecalis* and *E. faecium. Enterococcus* are opportunistic pathogens that have been associated with nosocomial infections and plays an important role in the distribution of antibiotic resistance, because they exhibit resistance to a wide variety of antimicrobial drugs and have the ability to exchange genetic information through conjugation (Jackson *et al*., 2004) [7]. Many virulence factors have been identified in the enterococcal genome which usually plays an important role in the pathogenesis at different stages including, initial attachment, destroying tissues, acquiring resistance to commonly used drugs as well as in avoiding immune system response. The pathogenesis of *Enterococcus* occurs in multiple steps, the major step is the adherence to host tissues and this is accomplished via two main groups of virulence factors known as collagen binding proteins (Ace) and aggregation substances such as Agg and Asa1. Ace is a surface protein that participates in binding to the collagen type I and IV and also plays an important role in colonization by binding to proteins of the extracellular matrix. Agg is a protein that responses to pheromone induction that mediates the adherence of *Enterococcus* (Hashem *et al*., 2021) [13]. while *asa1* is another aggregation factor used during conjugation to aggregate enterococcus mating strain on one another (Vankerckhoven *et al*., 2004) [30]. Another important virulence factor in *Enterococcus* is its gelatinase enzyme (GelE) which functions to hydrolyze gelatin and casein thus damage host tissues and plays an important role in the spread of enterococci pathogens in their host (Franz *et al*., 2011). The purpose of this study was to identify virulence genes in environmentally isolated *Enterococcus* and detect horizontal gene transfer among *Enterococcus* species through the transfer of antimicrobial resistance genes.

Materials and methods

Sample collection

Ten water samples were collected from swimming pools and recreation areas from Tigris river in Mosul city during the period October to December, 2020. In addition, three previously identified strains of *E. feacalis* were

taken from Al-Salam hospital for the purpose of comparison with our environmental samples. Sterile (1L) glass bottles were used to collect water samples from pools and recreation areas in Tigris river. Water samples were collected at a depth of 30cm below water surface. All water samples were labeled and transported in a cooled container directly to the laboratory for further analysis (Lata *et al*., 2009).

Isolation of *Enterococcus spp.* from water

Membrane filtration method was used for the isolation of *Enterococcus spp.* from water samples. One hundred milliliter of pool or river water was filtered through 0.45µm pore membrane filter paper. The flow of water was facilitated by using an air vacuum pump attached to a conical flask to collect the unwanted filtered water. The filter paper was placed on the surface of a selective chromogenic Hicrome *Enterococcus faecium* agar media. Plates were incubated at 37ºC for 24h and colonies were selected based on their shape and color. According to Himedia company, *E. faecalis* and E. hirae should appear blue while E. faecium should appear green. Single colonies were streaked twice on Hicrome *Enterococcus faecium* agar to confirm purity of the bacterial isolate.

Identification of *Enterococcus*

Blue and green colonies on Hicrome *Enterococcus faecium* agar were microscopically examined, tested for their ability to hydrolyze esculin on bile esculin agar, produce catalase, and to grow at 45Cº. Colonies that were expected to belong to *Enterococcus spp.* were further identified by 16S rRNA gene sequencing. PCR products were run on 1% agarose and stained with Midori Green Advance DNA stain (Germany). Purified PCR products were sent for sequencing at Psomagen company (USA).

Antimicrobial susceptibility test

Screening for antibiotic resistance towards ampicillin (50µg/ml), vancomycin (30µg/ml), gentamycin (10µg/ml), streptomycin (20µg/ml), ciprofloxacin (10µg/ml), tetracycline (30µg/ml), kanamycin (50µg/ml), chloramphenicol (50µg/ml) was performed by agar dilution method using Muller Hinton agar plates containing antibiotics at final concentrations mentioned above. Antibiotics were dissolved in appropriate solvents based on the type of antibiotic and the stock solutions were stored at-20 until needed. Muller Hinton agar media was sterilized by autoclaving at 121ºC for 20 minutes. Antibiotics were added from the prepared stock solution after the media was cooled down to 45ºC. After the media was solidified, isolates were streaked on antibiotic containing media and the plates were incubated at 37ºC for 24h. Results were recorded after incubation period was ended (wayne, 2011).

PCR and Genomic DNA isolation

DNA was extracted from *Enterococcus* strains using Geneaid DNA extraction kit following the steps recommended by the company. Primers used for ampification and estimated product size for each band are listed in table 1. PCR of *gel*E, *efaAfs*, *ace*, *esp*, *cyl*, *asa1*, *eep*, and *agg* virulence genes was performed in 20µl reaction using Promega master mix following recommended conditions and using the protocols listed in tables 2-4, respectively. Amplification of 16S rRNA gene was conducted as shown elsewhere (Abdulrazzaq and Faisal, 2022).

Table 1: List of primers used in this study.

Primer name	Sequence (5' - 3')	Product size (bp)	Reference
27F	AGAGTTTGATCMTGGCTCAG	1500	(Nagara *et al*., 2017)
1522R	AAGGAGGTGATCCARCCGCA		
efaAfs-F	GACAGACCCTCACGAATA	705	(Eaton *et al*., 2001)
efaAfs-R	AGTTCATCATGCTGTAGTA		
*gel*E-F	ACCCCGTATCATTGGTTT	419	
*gel*E-R	ACGCATTGCTTTTCCATC		
agg-F	AAGAAAAAGAAGTAGACCAAC	1,553	
agg-R	AAACGGCAAGACAAGTAAATA		
ace-F	GGAATGACCGAGAACGATGGC	616	
ace-R	GCTTGATGTTGGCCTGCTTCCG		
esp-F	TTGCTAATGCTAGTCCACGACC	933	
esp-R	GCGTCAACACTTGCATTGCCGAA		
eep-F	GAGCGGGTATTTTAGTTCGT	956	
eep-R	TACTCCAGCATTGGATGCT		
asa1-F	GCACGCTATTACGAACTATGA	375	(Vankerckhoven *et al*, 2004)
asa1-R	TAAGAAAGAACATCACCACGA		
*cyl*A-F	ACTCGGGGATTGATAGGC	688	
*cyl*A-R	GCTGCTAAAGCTGCGCTT		

Table 2: Program for ampilification of *gelE*, *efaAfs*, *agg, esp* gene (Eaton *et al*., 2001).

Segment	No. of cycles	Temperature ºC	Time (minutes)
Initial Denaturation	1	95	3:00
Denaturation	30	95	00:30
Annealing		46	00: 30
Extention		72	1 :00
Final extention	1	72	3:00

Table 3: Program for amplification of *ace* gene (Eaton *et al*., 2001).

Segment	No.of cycles	Temperature ºC	Time (minutes)
Initial Denaturation	1	95	3:00
Denaturation	30	95	00:45
Annealing		60	00:45
Extention		72	1:00
Final extention	1	72	3:00

Table 4: Program for amplification of *eep, cylA,* and *asa1* virulence genes (Vankerckhoven *et al*., 2004) [30].

Segment	No.of cycles	Temperature ºC	Time (minutes)
Initial Denaturation	1	95	3:00
Denaturation	30	95	00:45
Annealing		50	00:45
Extention		72	1:30
Final extention	1	72	3:00

Conjugation experiment

Conjugation was conducted between pathogenic *Enterococcus faecalis* as a donor strain and two different recipients (pathogenic and environmental *Enterococcus*) putting into consideration the presence of two antibiotic resistance marker differences between donor and recipient enterococci as described elsewhere (M Faisal, 2012). Transconjugants were examined for their ability to resist antibiotics and compared to the antibiotic resistance profile of the recipient to identify the antibiotic resistance genes that transfered between enterococci. Plasmid DNA was isolated from transconjugant cells then PCR was performed using primers for virulence genes that were present in the donor cells and absent in the recipient to determine the virulence genes that posess the ability to transfer between enterococci.

Results and discussion

***Enterococcus* isolation and identification**

Ten water samples were analyzed for the presence of *Enterococcus spp.* depending on the chromogenic properties of Hicrome *Enterococcus faecium* agar. Blue and green colonies were selected and further identified by their ability to produce a black precipitate from asculin hydrolysis, grow on 45ºC, and produce catalase. Suspected colonies were further diagnosed by 16S rRNA gene sequencing and results detected the presence of *Enterococcus spp.* in 30% of water samples. Three species of *Enterococcus* were diagnosed and submitted to genbank (NCBI) and were given specific accession numbers. Sequence blast of these isolates showed that they belong to *E. gallinarum* (RH1) (OM844022) and *E. faecium* (RH2 (OM844027) and RH3 (OM844028)).

Antibiotic susceptibility

The three environmentally isolated *Enterococcus* isolates exhibited variations in patterns of antibiotic resistance as seen in table 5. Our results showed that they were 100% resistant to gentamycin, 66.6% resistant to streptomycin, ciprofloxacin and kanamycin, and 33.3% resistant to ampicillin, tetracycline, and chloramphenicol, while none of the isolates were resistant to vancomycin. Our results were similar to the findings of Enayati et.al., (2015) as none of the their *E. faecium* isolates were resistant to vancomycin (Enayati et.al.,2015). The reasons behind the spread of antibiotic resistance in environmental bacteria is because such antibiotics reach the environment via human excretions (urine and feces) and domestic animals, improper handling and disposal of unused drugs, and by direct environmental contamination in aquaculture, Environment contamination with fecal bacteria increases opportunities for gene exchange between resident environmental bacteria and bacteria adapted to the intestinal tract of human or domestic animals. A great proportion of these bacteria may carry multiple genetic elements (plasmids, integrative conjugative elements, insertion sequences, transposons or integrons) that can facilitate the transfer of genes to closely related species (Larsson & Flach 2021).

When comparing the antibiotic resistance pattern of our isolates to pathogenic *E. faecium*, our results showed that *E. faecium* isolates were relatively more resistant to antibiotics compared to *E. gallainrum* as this isolate was sensitive to all antibiotics except gentamycin and tetracycline. The resistance of *E.. gallinarum* to tetracycline can be attributed to the acquisition of several genes encoding gene *tet*M, *tet*k, and most importantly *tet*S which

was found to be present in most tetracycline resistant *Enterococcus* isolates as reported elsewhere (Cho *et al*., 2020). In addition, *E. faecium* isolates had a similar antibiotic resistance pattern to pathogenic enterococci which makes us believe that such strains originated in water samples from a pathogenic source through fecal contamination of water.

Results of antibiotic resistance of the most pathogenically known species of enterococci, *E. faecium* and *E. faecalis,* shows that these isolates were totally resistant to streptomycin, gentamycin and ciprofloxacin. While the most effective antibiotics were tetracycline (0% resistance) and vancomycin (20% resistance). Emaneini *et al*., (2016) tested 27 strains of *Enterococcus* to high levels of gentamycin and found that 96.2% of the isolates were resistant to this antibiotic which agrees with our results (Emaneini *et al*.,2016). Resistance to ciprofloxacin in *E. faecium* and *E. faecalis* can be attributed to the presence of a mutated copy of *gyr*A (Adeniji *et al*., 2021). However, resistance to chloramphenicol is often caused by the *cat* gene, which encodes chloramphenicol acetyl transferase (Schwarz *et al*., 2004).

Enterococci isolated from clinical samples had a higher frequency of antimicrobial resistance than those obtained from water. This result is not surprising because exposure to antibiotics is more common in pathogenic bacteria compared to environmentally isolated bacteria (Castillo- Rojas *et al*., 2013).

Table 5: Antibiotic resistance pattern of *Enterococcus* isolates. R: resistance; S: sensitive; Ap: ampicillin; Sm: streptomycin: Gm: Gentamycin; VA: vancomycin; Cipro: ciprofloxacin; TE: tetracycline; Cm: chloramphenicol; Kan: Kanamycin

Isolate No.	Source of isolation	Antibiotics (µg/ml)							
		AP(50)	Sm(20)	Gm(10)	VA(30)	Cipro(10)	TE(30)	Cm(30)	Kan(50)
E. casseliflavus (RH1)	Water	S	S	R	S	S	R	S	S
E. faecium (RH2)	Water	R	R	R	S	R	S	R	R
E. faceium (RH3)	Water	S	R	R	S	R	S	S	R
E. faecalis (RH4)	Pathogenic	S	R	R	R	R	S	R	R
E. faecalis (RH5)	Pathogenic	S	R	R	S	R	S	R	S
E. faecalis (RH6)	pathogenic	R	R	R	S	R	S	S	R

Enterococcus virulence factors

E. faecium and *E. casseliflavus* isolated from swimming pools and recreation areas from Tigris river and the three *E. faecalis* isolates from hospitals were analyzed for the presence of the following eight enterococcal virulence genes: *gelE*, *efaAfs*, *ace*, *esp, cyl, asa1, eep,* and *agg*. Our results showed the presence of these virulence genes in 0.0 - 66.7% of the environmental isolates and in 66.7- 100% of pathogenic isolates as shown in table 6. Out of all virulence genes tested, only four were detected in environmental enterococci while all virulence genes were detected in pathogenic enterococci. Figure (1-a) shows that *gelE* virulence genes were identified in two environmental *E. faecium* isolates (66.7%) while all pathogenic isolates of *E. faecalis* possessed this virulence gene (Figure 1-b). Similar results were previously reported by Waar *et al*. (2002) and Archimbaud *et al*. (2002) as they found that GelE -positive were significantly more frequently distributed among clinical isolates (75–86%) than in commensal strains (40 %). Also *gel*E presence was not strictly correlated with its expression, as only 36.4% of *gel*E-positive strains produced gelatinase. Generally, the proportion was found to be higher in invasive strains (45.5%) compared to non-invasive strains (33.3%). Around half the commensal strains expressed *gel*E although the majority of environmental isolates possessed this gene (creti *et al*., 2004).

Similar results were obtained from *efaAfs* as this gene was identified in *E. faecium* strains (66.6%) and not in *E.gallinarum*, however all pathogenic isolates contained this gene (Figure 2 A,B). These results were in accordance with a previous study in which *efaAfs* was always present in strains of *E. faecalis* isolates (Creti *et al*., 2004). This study also mentions that *efa*A genes display allelic sequence variation and that these variations can influence the ability of surface characteristics between strains from different sources. Due to variability in sequence, these genes have recently been used as possible markers for multilocus sequence typing of *E. faecalis.*

The only virulence gene identified in *E.gallinarum* and not in *E. faecium* was *agg*, however this gene was identified in two *E. faecalis* pathogenic strain (66.7%) as shown in figure 3a and b, some stuides have shown that *agg* gene was only present in *E. faecalis* isolated from food, clincal and enviroment origin and not from other enterococci strains (Eaton *et al*., 2001, Franz *et al*., 2001, Adeniji *et al*., 2021) [12]. While other studies show the *agg* was present in *E. facealis*, *E. faceium* and *E. durans* isolated from food, clincal and enviroment sources (Semedo etal., 2003, Abriouel *et al*., 2008, Lanthier *et al*., 2011) [28, 2, 16]. Agg is a surface protein which is located on pheromone-responsive *E. faecalis* plasmids. It is produced in response to pheromones secreted by recipient *E. faecalis* cells and causes aggregation of donor and recipient cells, thus facilitating the transfer of plasmids that may carry virulence and antibiotic resistance genes (Barbosa *et al*., 2010) [4].

Table 6: Percentages of *Enterococcus* virulence genes in environmental and pathogenic isolates.

Virulence genes	Detection in Environmental strains	Detection in Pathogenic strains
afaAfs	2 (66.7%)	3(100%)

gelE	2 (66.7%)	3(100%)
ace	2 (66.7%)	2 (66.7%)
agg	1 (33.3%)	2 (66.7%)
esp	0 (0.0%)	3 (100%)
cylA	0 (0.0%)	3 (100%)
asa1	0 (0.0%)	3 (100%)
eep	0 (0.0%)	3 (100%)

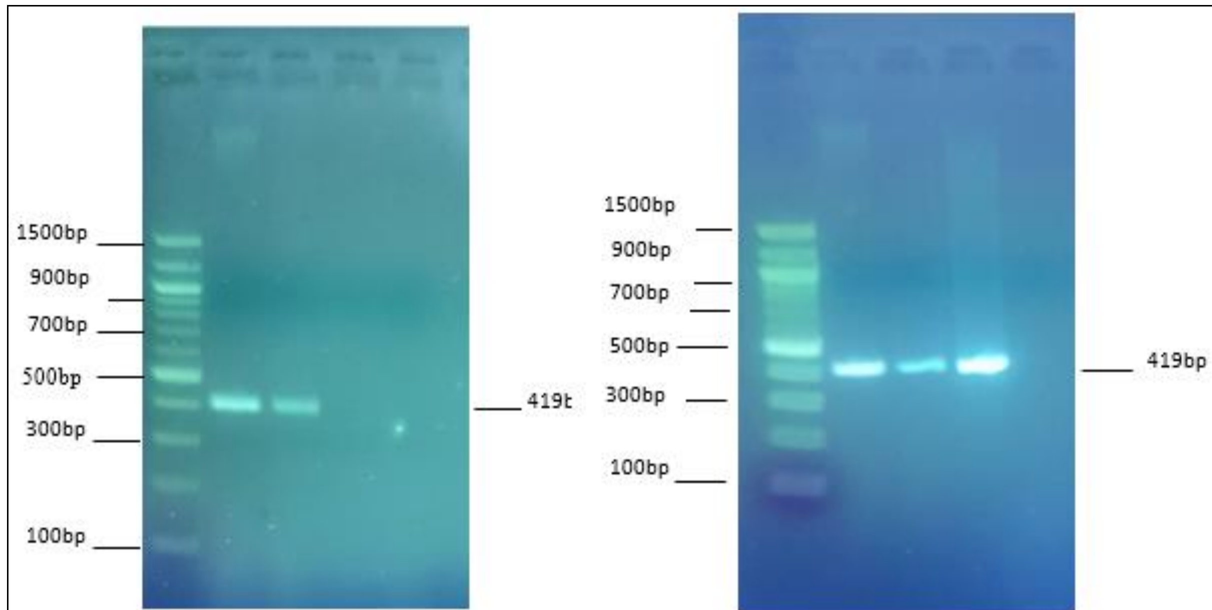

Fig 1: Prevalence of *gelE* virulence gene in environmental (A) and pathogenic (B) enterococci.

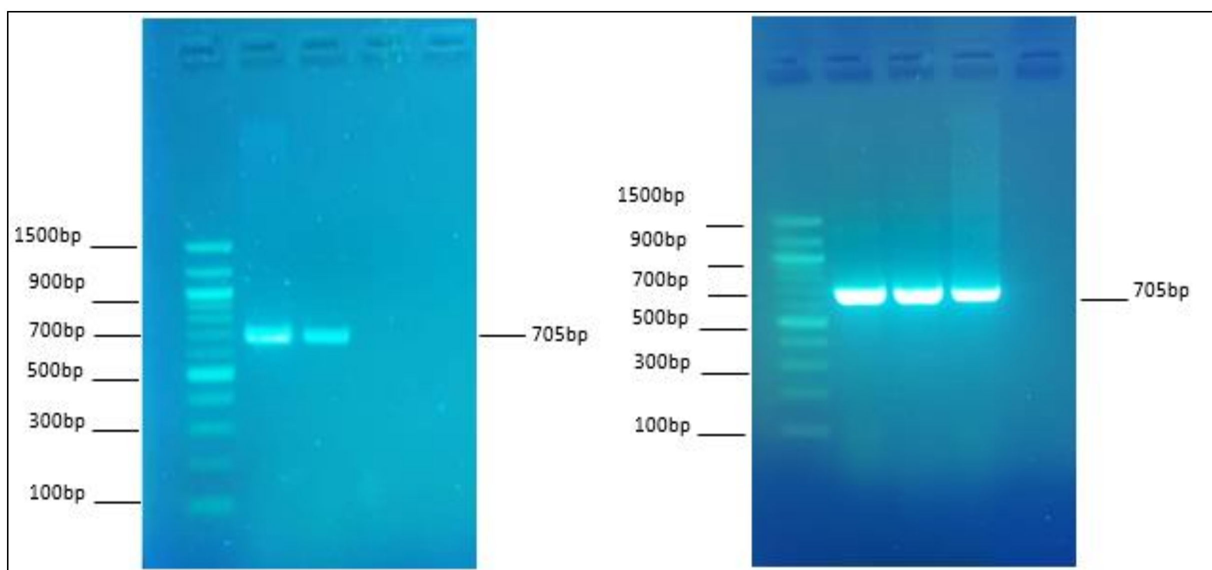

Fig 2: Prevalence of *efaAfs* virulence gene in environmental (A) and pathogenic (B) enterococci.

Finally, *ace* virulence gene was identified in two *E. faecium* environmental strains (66.7%), and also in two isolates (66.7%) of pathogenic *E. faecalis* (66.6%) as shown in figure 4a and b. Several previous studies have detected ace in *Enterococcus* (Lysakowska *et al*., 2012, Medeiros *et al*., 2014). Singh *et al*. (2010) observed that the deletion of *ace* in *E. faecalis* leads to significant attenuation of the ability of *Enterococcus* to colonize and to cause aortic valve endocarditis. Their knockout data showed that *ace* gene plays an important role in early stage colonization possibly through mediating *Enterococcus* adherence to collagen exposed at the site of infection (Singh *et al*. 2010). These results show the importance of this gene in pathogenecity and therefore indicates that environmentally isolated enterocci may carry a relatively high percentages of ace gene thus may cause severe infections.

Our results indicates the occurrence of virulence genes at higher ratios in pathogenic compared to environmentally isolated enterococci, however, such ratios are still high and can lead to serious infections.

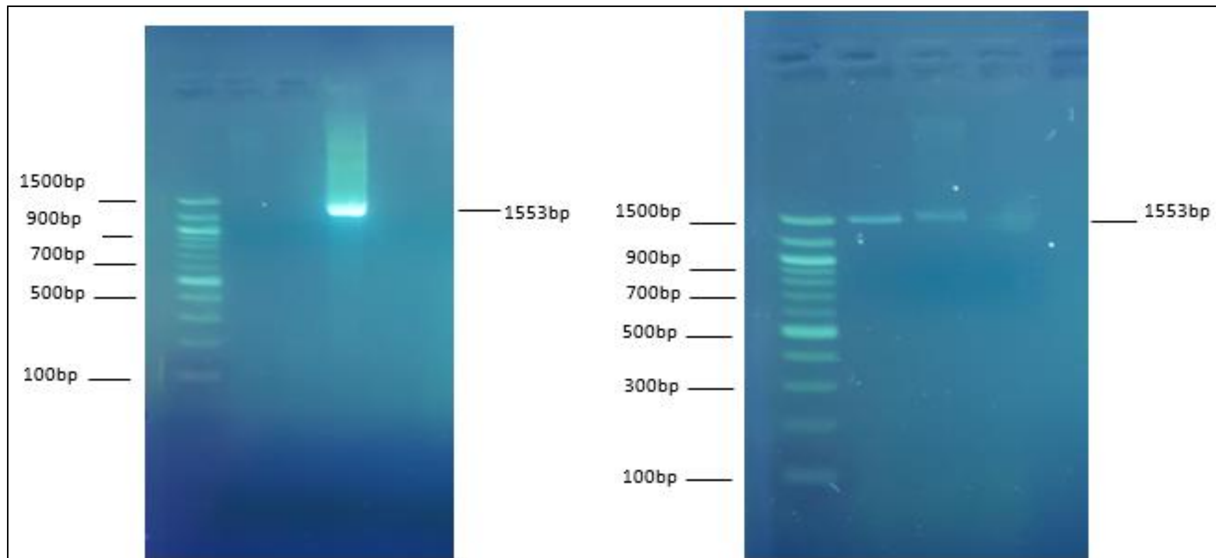

Fig 3: Prevalence of *agg* virulence gene in environmental (A) and pathogenic (B) enterococci.

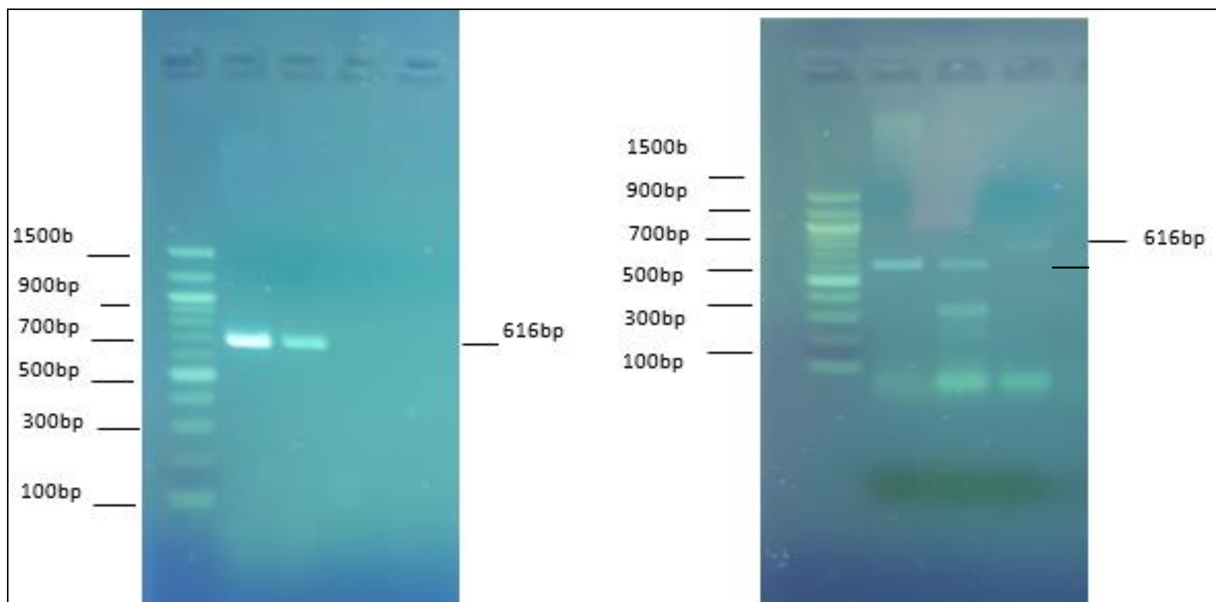

Fig 4: Prevalence of *ace* virulence gene in environmental (A) and pathogenic (B) enterococci.

Horizontal gene transfer in *Enterococcus*

Two conjugation experiments were conducted, the first was between *E. faecalis* RH5 and *E. faceium* RH3 and the second between *E. faecalis* RH5 and *E. faecalis* RH6 benefiting from the antibiotic resistance differences in chloramphenicol and kanamycin. Results shown in table 7 shows the successful transfer of antibiotic resistance for chloramphenicol between RH5 and RH6 as appeared in the transconjugant cells. The frequency of conjugation was $1.25\times10^{-}7$ which was similar to what was observed by Lund *et al*. (2006) as they found that donor strains with *esp* genes had higher frequency for conjugation than those lacking *esp* (Lund *et al*. 2006). The frequency of conjugation in *Enterococcus* is affected by many factors such as the efficiency of the donor, the efficiency of the recipient, and the presence of pheromone induction system in matting strains. Depending on these factors, it was shown that frequency for vancomycin resistance transfer by conjugation varied between 1.09×10^{-1} to 9.74×10^{-5} (McCarron *et al*., 2019) [23]. Such results shows that antibiotic resistant genes can be transferred between enterococci. Even though conjugation occurred successfully between RH5 and RH6, no transconjugant colonies were observed between RH5 and RH3 the reason behind this result could be that both strains are F plasmid containing strains.

Table 7: Conjugation frequency between donor and recipient *Enterococcus*

***E. faecalis* RH5 genetic characteristics**	***E. faecalis* RH6 genetic characteristics**	**Transconjugant genetic characteristics**	**Conjugation frequency**
Sm^R, Gm^R, $Cipro^R$, $Kana^S$, Cm^R, *eep*$^+$	Sm^R, Gm^R, $Cipro^R$, $Kana^R$, Cm^S, *eep*$^-$	Sm^R, Gm^R, $Cipro^R$, $Kana^R$, Cm^R, *eep*$^-$	$1.25\times10^{-}7$

Analysis of transconjugant cells for their acquisition of *eep* indicated that this virulence gene is more likely chromosomally located as it was incapable of transferring to transconjugants. However, our results show that chloramphenicol resistance is carried on self-transmissible plasmids that can be transferred between enterococci species and lead to the spread of antibiotic resistance. In a study conducted by Oancea *et al*., (2004) [26], conjugation between both *E. faecalis* and *E. faecium* led to the transfer of the virulence gene *esp* coupled with the transfer of antibiotic resistance determinants to the recipient (Oancea *et al*.,2004) [26]. Their results for antibiotic resistance transfer come in agreement with our results, however due to low number of strains tested (had differences in two antibiotic resistant markers) we were unable to study conjugation transfer of virulence genes in more strains to obtain more precisely results. Nevertheless, the presence of fertility plasmids in *Enterococcus* that carry antibiotic resistance genes may lead to the spread of antibiotic resistance and the spread of other virulence genes when present on self-transmissible plasmids. A recent study was capable of isolating *E. faecium* that harbored 5 different plasmids. The plasmid pELF1 (approximately 143 kb) was found to carry vancomycin resistance and was able to transfer to *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. hirae*. This transfer not only transferred the resistance to vancomycin but streptomycin, erythromycin, and kanamycin (Hashimoto *et al*., 2019) [14].

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