Transfer of plasmids with resistance to antibiotics in Enterococcus species

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Abstract

In the governorate of Salah Al-din, the sea coast suffers a strong impact of anthropic origin due to the large discharges from the drains, without prior treatment. In this way, these effluents transport intestinal microorganisms, such as those belonging to the genus Enterococcus, towards the marine environment, carrying genetic information of resistance to antibiotics, and these can be transferred horizontally to the autochthonous and alien flora of the coastal areas, allowing this This information is maintained in the environment, which can then be introduced to the human population through the consumption of hydro biological products. For this reason, and because Enterococcus is considered an ideal indicator of faecal contamination in marine environments, the study of this microorganism was important. The results of the present work allow us to affirm that Enterococcus spp. are found in the coastal waters of Salah Al-din governorate associated with human diseases, which have plasmids and possibly also other transferable elements with genetic information for antibiotic resistance, which can be transmitted horizontally to other microorganisms with which they share the habitat. The resistance observed for Enterococcus spp from marine environments allows us to presume the importance of this opportunistic pathogen in the ecosystem, especially if we consider that the species studied are associated with human infections and due to the enormous capacity reported for the genetic transfer of this information, mainly due to conjugation to other organisms. This article carried out a study of 31 strains of the Enterococcus genus obtained from Salah Al-din Coastal Areas. Using the disc diffusion method, antimicrobial resistance to 12 different clinical antibiotics was shown for the majority, and streptomycin was observed for most strains. Whether these residues in plasmid were tested using a 0.003 percent SDS cure test. Plasmide resistant to erythromycin, streptomycin, ciprofloxacin and norfloxacin strains were selected in a standard strain by conjugation and conversion tests where eight Enterococcous strains were transported to erythromycin (one strain) and streptomycin (7 strains) with frequency by their resistance plasmids.

Keywords: Salah Al-din governorate, Antibiotic resistance, Plasmids, Enterococci

Introduction

The study of Enterococcus continues to be a necessity because it is the cause, in an increasing way, of opportunistic intra-hospital infections, many of them caused by multi-resistant strains, whose transfer can be carried out very easily in intra-hospital environments or can also involve the participation of environmental reservoirs that can be disseminated in an in apparent way in nature. A more in-depth study of the antimicrobial resistance of Enterococcus, and a broad evaluation of its potential in the transfer of these markers in nature, would allow us to understand the epidemiology of the transmission of these traits in our environment. It is necessary to determine the origin of the Enterococci present in water columns and to clearly establish the prevalence of Enterococcus spp. from animals that inhabit coastal areas. Despite the importance that this bacterium has been gaining in recent years, in our environment there is little study that determines the diversity of this genus in marine environments and even less its relationships with pathogenic species. It is important to highlight that when investigating the specific diversity of Enterococcus in these environments, it would also be determining which species are the ones that prevail in our environment (of human or animal origin) and if they maintain characteristics that pose a risk to human health. The present study analyzed the mechanisms of conjugative and transformation gene transfer of selected strains of marine origin, determining the frequencies at which antibiotic resistance markers are transferred to standard strains. This persistence of Enterococcus spp in the natural environment leads to the need to establish differences between the environmental lineages and those that are disseminated in clinical environments using molecular methods, which would allow us to understand the mechanisms of adaptation of the microorganism under unfavorable conditions. Coastal marine environments are home to various microorganisms, both autochthonous and alien, the latter being of very diverse origin. Within this diversity is the genus Enterococcus, and in a study carried out by Abbs et al., (2020) from water samples from the Salah Al-din City coastline, the presence of Enterococcus species of both human and animal origin was reported. While Enterococcus is a microorganism that has been introduced to the marine ecosystem with a highly proven presence of organic waste, studies in Enterococcus usually have implications for its clinical appearance and antibiotic resistance and some have an environmental context in which the methods of recognition are assessed for recreational use in waters.

In this context, it is necessary to know if those Enterococcus species present in Iraq coastal city Salah Al-din have antimicrobial resistance, and if this resistance can be transmitted and remain so in this marine environment. Current research is developed to evaluate the transfer of plasmids with resistance to antibiotics for clinical use in Enterococcus species isolated from waters of the Arabian Gulf in Salah Al-din governorate. To determine the resistance to macrolides, aminoglycosides and fluoroquinolones in four species of Enterocccus. Determine the nature of antimicrobial resistance and select those strains that have plasmids for this type of resistance. Determine the frequency of transfer, of the resistance plasmids under study, to reference Enterococcus strains.

Materials and Methods

3.1 Materials

3.1.1 Bacterial Strains and study Location: 31 Enterococcus strains isolated from the four bays that make up the coast of Salah Al-din governorate (Salah Al-din city, Iraq) and that

were previously identified as: E. faecalis, E. faecium, E. hirae and E. durans, belonging to the strain of the Molecular Microbiology Laboratory, Faculty of Biological Sciences, Iraq. In antibiotic resistance tests, Staphylococcus aureus ATCC 29213 was used as a control strain. In the genetic transfer tests by conjugation and transformation, the strain of Enterococcus faecalis-plasmid and with chromosomal resistance to rifampicin was used.

3.2 Methods: It began with the reactivation of all available Enterococcus strains, subsequently a susceptibility test to different antibiotics was performed, followed by a cure test for strains resistant to certain antibiotics, and finally conjugation and transformation tests were performed, to transfer analysis of antimicrobial resistance.

3.2.1 Reactivation of strains: The 31 Enterococcus strains were seeded in tubes containing 3mL of Brain Heart Infusion (BHI) broth and incubated at 37ºC for 24 h. Then, an inoculum was taken from the broths and streaked onto Petri dishes with BHI agar and incubated at 37ºC for 24 h. After this time, the best characteristic Enterococcus colonies were taken, they were seeded on selective bile esculent agar and incubated at 37ºC for 24-48 h. Afterwards, characteristic colonies of Enterococcus were taken and subjected to Gram staining to later be seeded in Luria-Bertoni broth (LB), the tubes were incubated at 37ºC for 24 h. Subsequently, for their maintenance, the strains were seeded in vials containing Luria Semisolid agar and in cry vials containing glycerine (50%) for their conservation at room temperature and at -30ºC, respectively, for subsequent studies.

3.2.2 Antimicrobial resistance testing

3.2.2.1 Disk Diffusion Test: First, an antibiotic sensitivity test was performed by the disk diffusion method (Bauer et al., 1966) on the 31 Enterococcus strains isolated from the sea and the reference Staphylococcus strains. 12 antibiotics for clinical use were used: erythromycin (Ery), penicillin (Pen), streptomycin (Str), ciprofloxacin (Cipr), levofloxacin (Levf), norfloxacin (Norf), tetracycline (Tetra), rifampicin (Rf), chloramphenicol (Clp), vancomycin (Van), azithromycin (Aztr) and clindamycin (Clnd). The susceptibility test was developed according to the Procedures Manual for antimicrobial susceptibility testing by the diffusion disk method of the Clinical and Laboratory Standards Institute (CLSI, 2004). The inoculum preparation was carried out through the previous development method, where the strains were previously seeded in BHI broth for 24 h at 37ºC and later they were seeded in BHI agar for the selection of the suitable colonies of Enterococcus. The best colonies were taken and seeded in BHI broth at 37°C until the concentration of 0.5 on the MacFarland scale. From this, with the help of sterile swabs, an inoculum was taken and spread on Mueller-Hinton agar contained in a Petri dish, trying to spread it over the entire surface of the agar and not leave spaces. Then, with the help of sterile forceps, 6 to 7 antibiotic discs were placed per plate. The incubation time varied depending on the antibiotic and the measurement of the diameter of the inhibition halos was established in millimetres, all this based on the standards provided by the CLSI manual (2004).

3.2.2.2 Minimum Inhibitory Concentration (MIC): Depending on the previous results of the sensitivity test, the Minimum Inhibitory Concentration (MIC) was evaluated for the strains that were resistant or showed intermediate sensitivity to streptomycin, erythromycin, ciprofloxacin and levofloxacin. The MIC was performed on all strains to determine and confirm whether resistance was high or low to the antibiotics considered in the study. The MIC test performed on selected antibiotics resistant and/or intermediate strains was based on

the Procedures Manual for testing antimicrobial susceptibility by the diffusion disk method (CLSI, 2004).

3.2.3 Curing the strains with Sodium Dodecyl Sulphate (SDS): In order to determine whether the resistance present in the Enterococus strains is due to the presence of plasmid, the strains resistant to the different antibiotics were cured. Strains resistant to norfloxacin, ciprofloxacin, erythromycin and streptomycin that do not show resistance to rifampicin were selected. For the curing process, a 10% Sodium Dodecyl Sulphate (SDS) stock solution was used and the concentration was determined by the procedure described below.

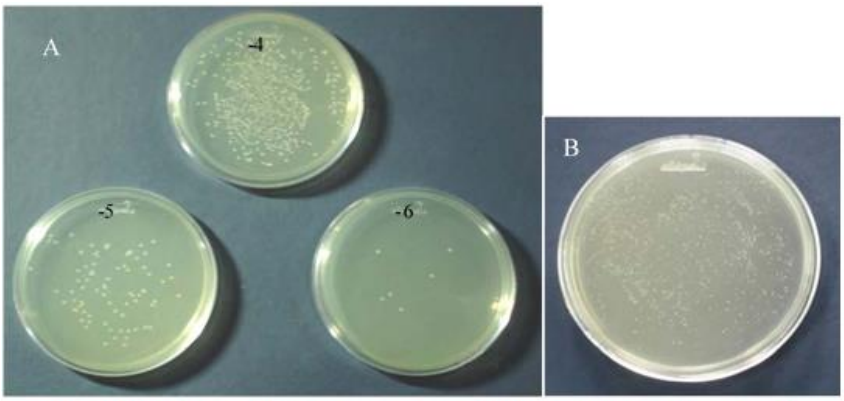
3.2.3.1 Determination of SDS concentration for curing: To determine the appropriate concentration of SDS for healing, four strains of different Enterococcus species (E. faecalis 43, E. faecium 70, E. hirae 15 and E. durans 10) were seeded in 3 mL of LB broth containing 19 different SDS concentrations, from 0.0002% to 1%, the tubes were left incubating at 37 ° C for 24 h. After this time, the maximum concentrations in which each of the strains grew were taken, which were between 0.002% - 0.004%, and a sensitivity test was carried out by the diffusion disk method, observing the susceptibility of the strains against the different antibiotics to which they were previously resistant (Morroni, 2016).

3.2.3.2 Strain cure: With what was previously obtained, the Enterococcus strains resistant to the four different antimicrobials were cured, for which 0.003% SDS was used. For this process, each of the strains were seeded in 3 mL of LB broth containing SDS and left to incubate at 37ºC for 24 h. After this time, a sensitivity test was performed using the diffusion disk method on each of the strains to verify whether the resistance was lost or maintained.

3.2.4 Conjugation test: It is necessary to indicate that in the horizontal transfer studies three criteria were taken into account for the selection of resistant strains: (1) that present a high frequency of resistance to a certain antibiotic, (2) that did not correspond to the resistance of the recipient strain, and (3) that is not reported as a natural resistance. For this test, the Enterococcus strains that were not sensitive to rifampicin and that presented plasmid resistance to erythromycin, streptomycin, ciprofloxacin or norfloxacin were used as donor strains; that is, those strains that have shown sensitivity after curing. Plasmid-free strain JH2-2 with chromosomal resistance to rifampicin was used as the recipient strain. The recommendations of Kurekci, (2016) and Sfaxi, (2011) were followed.

3.2.4.1 Conjugation test of antibiotic resistant strains: For this test, the donor strains, resistant to selected antibiotics, were seeded in 3 mL of BHI broth containing 10 µg / mL of ciprofloxacin and the recipient strain was seeded in 3 mL of BHI broth containing 72 µg / mL of rifampicin and were incubated at 37º C overnight and without aeration (Figure 1-3). After this time, an inoculum of each of the donor strains was taken and seeded in different tubes containing 4 mL of BHI broth with 10 µg / mL of ciprofloxacin. The recipient strain was seeded in a tube with 8 mL of BHI broth containing rifampicin (72 µg / mL), left at room temperature and with aeration until reaching a concentration of 0.5 on the Mac Farland scale (Rams, 2012). When the desired concentration was reached, 4 ml of the culture of the recipient strain was taken with 2 ml of the donor and these amounts were mixed in a sterile empty tube and kept at room temperature and without shaking overnight. In addition, to know the approximate number of donor cells that will participate in the conjugation, a donor count is made, for which six serial dilutions of the donor strain culture were made, with saline solution, and the last 3 dilutions (10^{-4}, 10^{-5} and 10^{-6}) of which 100 uL was taken and spread

on BHI Agar with ciprofloxacin. It was incubated at 37° C overnight. After this time, the conjugation was interrupted by vortexing for 30s and the transconjugants were counted, for which 100 µL of each of the mixed cultures was taken and spread on BHI Agar containing 10 µg / mL of ciprofloxacin and 72 µg / mL of rifampicin. It was kept at room temperature for 24 hours. Control sowings (Figure 2) were also carried out, where the cultures of the donor and recipient strains were streaked on plates with BHI agar containing ciprofloxacin, and also on plates with BHI agar with rifampicin. They were incubated at 37º C for 24 h.



A: Plates containing BHI agar with erythromycin (22 µg / mL) Count of donors of the Enterococcus faecalis 43 strain in the –4, -5 and –6 dilutions. B: Plate containing BHI agar with streptomycin and rifampin (72 µg / mL). Count of transconjugant colonies at dilution -1

Figure 1: Count of donor strains and transconjugants resistant to erythromycin

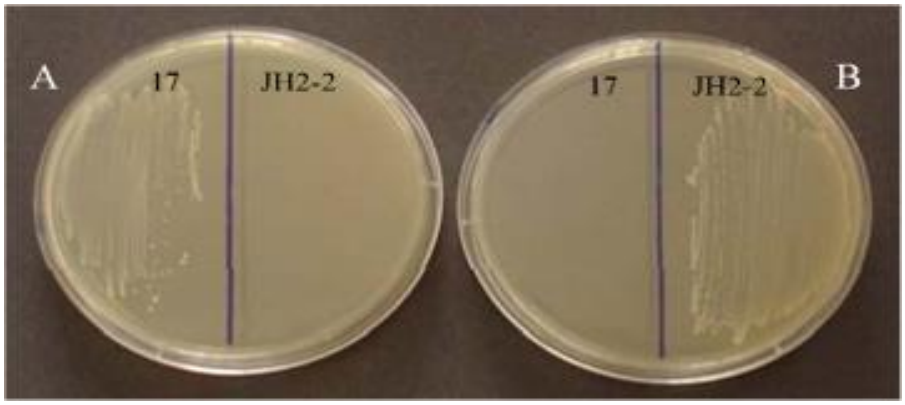


Figure 2: Control of donor and receptor in streptomycin and rifampicin; (A): Plate with BHI agar containing streptomycin (1000 µg / mL). (B): Plate with BHI agar containing rifampicin (72 µg / mL). The growth of the donor strain (17) is observed in A and not in B, otherwise the donor strain JH2-2 does not grow in A but in B.

It is important to mention that before conjugation control sowings were made, where the donor and recipient strains cultures were streaked on BHI agar plates containing streptomycin, and also on BHI agar plates with rifampicin. They were incubated at 37º C for 24 h.

.2.5 Extraction of plasmid DNA: The extraction of the plasmids used in the transformation test, described later, was carried out using the kit of ®Wizar Plus Minipreps DNA Purification System, the first step is the cell lysate, for which each of the transconjugants was seeded in flasks with 10 mL of BHI broth and incubated at 37 ° C overnight. The following day the content was centrifuged at 10,000 rpm for 10 minutes and the pellet was resuspended

in 300 µL of Cell Resuspension Solution. Then, 300 µL of Cell Lysis Solution was added and mixed by inversion (4 times), when it was well mixed, it was left at room temperature for 5 minutes and then 300 µL of Neutralization Solution was added and mixed by inversion (4 times). The lysate was centrifuged at 10,000 rpm for 5 minutes. The supernatant containing the plasmid was decanted. The next step is the purification of the plasmid DNA. For this, a minicolumn was taken from the kit and it was fixed in its upper part to a 3 mL syringe, and in the lower part it was attached to a 1.5 mL sterile empty tube. 1 mL of the Resin was taken from the kit and added to the syringe assembled to the minicolumn, then the supernatant obtained previously containing the plasmid was added. With the aid of a plunger, the resin was first passed through the minicolumn, then the supernatant was passed. Since the plasmid is now fixed on the minicolumn, a washing step was done. It was changed to a new 1.5 mL tube and 2 mL of the Wash Solution was added to the 3 mL syringe, and with the help of the plunger it was passed through the minicolumn. The syringe was detached from the minicolumn, but the tube was left, and it was centrifuged at 10,000 rpm for two minutes. For the last step, which was the elution of the plasmid, the minicolumn was transferred to a new 1.5 mL tube and 50 µL of Nuclease-Free Water (pretreated at 70 ° C) was added to the minicolumn, and it was allowed to wait for one minute. Then it was centrifuged at 10,000 rpm for twenty seconds and then the minicolumn was removed to finally store the plasmid DNA at -20 ° C.

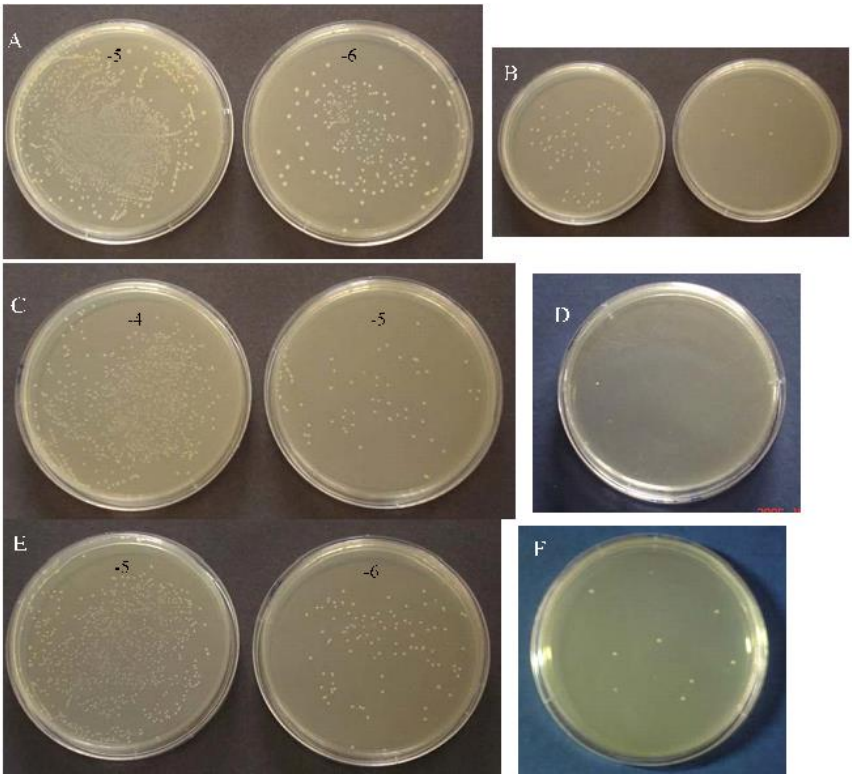


Figure 3: Count of donor strains and transconjugants resistant to streptomycin; (A): Plates containing BHI agar with streptomycin (1000 µg / mL) Count of donors of the strain Enterococcus durans 32 at dilutions -5 and -6; (B): Plate containing BHI agar with streptomycin and rifampicin (75 µg / mL). Count of transconjugant colonies at dilution -1; (C): Plates containing BHI agar with streptomycin (1000 µg / mL) Count of donors of the strain Enterococcus faecalis 42 in the –4 and -5 dilutions; (D): Plate containing BHI agar with streptomycin and rifampicin (75 µg / mL). Count of transconjugant colonies at dilution -1;

(E): Plates containing BHI agar with streptomycin (1000 µg / mL) Count of donors of the strain Enterococcus faecium 89 in dilutions -5 and -6; (F): Plate containing BHI agar with streptomycin and rifampicin (75 µg / mL). Count of transconjugant colonies at dilution -1

33.2.6 Electrophoretic run of plasmids: To demonstrate the presence of plasmids and observe their size, the plasmids extracted in the previous step were mixed with loading buffer in a 1: 1 ratio and were run by 1% agarose gel electrophoresis, in 1X TAE buffer, at 80mV. Then, the gel was stained in ethidium bromide and finally observed in a transilluminator with UV radiation (BIOMETRA TI1).

3.2.7 Transformation Test: For this test, we worked with the strains that carried out conjugation and two transformation techniques were used. The first technique used was a modification of the method of Pozzi et al (1996), carried out in species of the genus Streptococcus. First, the competition pheromone was obtained. For this, the JH2-2 receptor strain was seeded in two tubes containing 3 mL of TSB broth and incubated at 37 ° C overnight. The next day one of the tubes was taken, it was centrifuged at 12000 rpm for three minutes. The pellet was resuspended in TSB broth until a concentration of 0.5 was obtained on the Mac Farland scale. It was incubated at 37 ° C for 4h. After this time, the culture was centrifuged at 7000 rpm for 20 minutes and the supernatant containing the pheromone in crude form (peptide inducing competition) was decanted. For the transformation test, 450 µL was taken from the other culture of the JH2-2 strain seeded the day before, it was seeded in a tube containing 450 µL of competition medium (TSB [pH-8], 10% glycerol, 0, 16% bovine serum albumin, 0.01% $CaCl_2$) and 100 µL of crude pheromone and 5 µg / mL of plasmid DNA were added. It was incubated at 37 ° C for 180 minutes. The plasmids used came from each of the transconjugant colonies obtained in the conjugation test. For this, the donor strains that performed conjugation were cured with 0.003% SDS, and were used as recipients in the transformation test. An inoculum was taken from the cured strains to be plated on plates containing LB agar with antibiotic and on LB agar plates without antibiotic, as a control test. After confirming the cure, each of the cured strains was seeded in 3 mL of LB broth, left at 37 ° C overnight. After this time, 50 µL of this culture was taken and streaked on Luria agar without antibiotic and incubated for 48 h at 37 ° C. They were then subjected to 4 ° C for one hour and then 5 µL of the plasmid was added. It was left incubating for 6 h at 37 ° C. These colonies were then taken and resuspended in 0.5 mL of BHI broth and incubated at 37 ° C until obtaining 0.5 on the Mac Farland scale. The plasmids used were extracted from each of the transconjugant colonies obtained in the conjugation test.

Results

4.1 Antimicrobial resistance by diffusion disk

Sensitivity halos values were obtained in millimetres (mm), showing us that 83.9% of the 31 strains analyzed exhibited some type of resistance to the antibiotics used. 6.5% of the strains showed resistance to tetracycline, 9.7% to norfloxacin, 22.6% to ciprofloxacin, 19.3% to erythromycin, 9.7% to penicillin, 35.5% to rifampicin, 61.3% to streptomycin, 35.5% to azithromycin, and 61.3% to clindamycin. For the antibiotics vancomycin, levofloxacin, and chloramphenicol, only intermediate sensitivity was observed. The species that showed the highest resistance was Enterococcus faecium (100%), where all the strains with which we worked showed resistance to at least one antibiotic. 90.3% of the strains studied were

susceptible to penicillin and vancomycin. The studied strains express 16 different antibiotypes, finding multi-resistance in strains of the four species that have been considered in this study. 55% of the 31 strains studied expressed resistance to at least three different antibiotics, finding multiple resistances of up to 6 antibiotics. Only 16.1% of the strains did not show any type of resistance.

4.2 Minimum Inhibition Concentration (MIC)

With this test, several of the strains that showed intermediate sensitivity, expressed resistance to the four antibiotics used. Of the twelve strains with intermediate sensitivity observed in the diffusion disk test for ciprofloxacin, five of them were found resistant by the MIC test (Figure 4): E.hirae 31, E.durans 10, E.durans 11, E.faecium 7 and E.faecium 14. Regarding norfloxacin, of the seven strains with intermediate sensitivity to this antibiotic, five strains were found resistant: E.durans 5, E.hirae 29, E.durans30, E.durans32 and E.faecalis 43. Likewise, of the seven strains that showed intermediate sensitivity to streptomycin, three were found resistant by the MIC method: E.faecium 65, E.hirae 15 and E.faecalis 17. Finally, of the fifteen strains that presented intermediate sensitivity to erythromycin in the disk test, three of them expressed resistance in the Minimum Inhibitory Concentration test: E.faecium 65, E.hirae 15 and E.faecium 90. Consequently, the number of strains resistant to ciprofloxacin, norfloxacin, erythromycin and streptomycin, increased from 7, 3, 6 and 19 strains to 12, 8, 9 and 22 strains respectively. Similarly, the percentages of resistance that had previously been obtained with the diffusion disk test varied. Of the 31 strains analyzed, resistance to the following antibiotics was reported in the following order: streptomycin 71%; clindamycin 61%; ciprofloxacin, rifampicin, and azithromycin 35% each; norfloxacin and erythromycin 29%; penicillin 10% and tetracycline 6.5%. It is important to note that the MIC test showed very high resistance values for streptomycin (> 2400 µg / mL) and for erythromycin (> 240 µg / mL) in some of the strains studied. According to the results obtained, the strains were selected for the horizontal gene transfer studies, taking into account the absence of resistance to rifampicin because the recipient strain used in this study (E. faecalis JH2-2) carries this kind of resistance. The selected strains were subjected to the plasmid cure test.

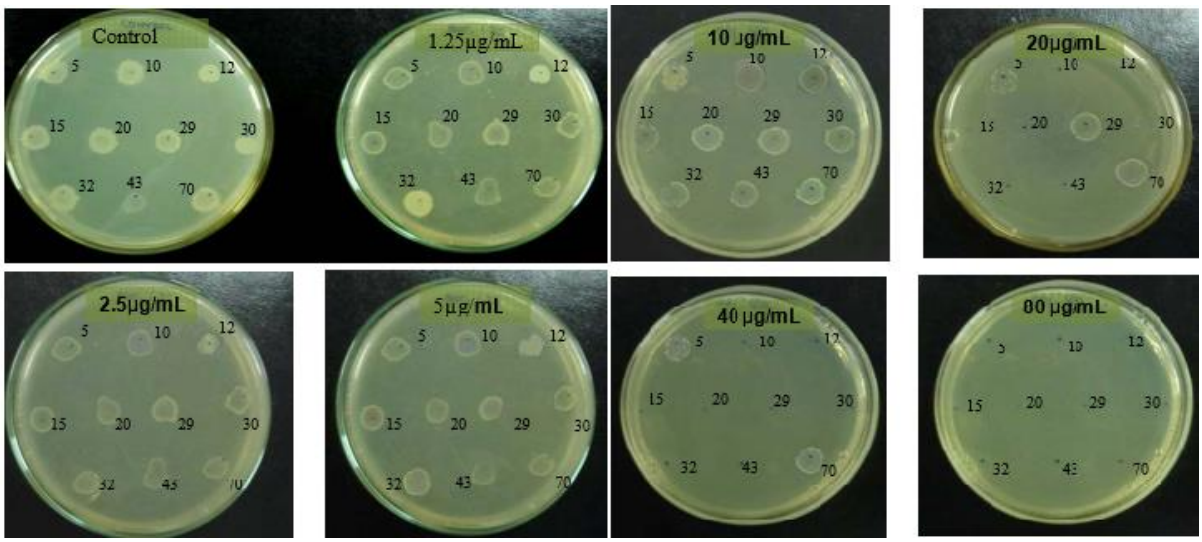


Figure 4: minimum inhibitory concentration (MIC) test (Plates with Muller Hinton agar containing seven concentrations of norfloxacin, and a control plate without antibiotics. Each

of the Enterococcus strains were seeded by spotting. The numbers indicate the registration number of each strain.)

4.3 Plasmid healing: Table 18 establishes the percentage of SDS that was used in the plasmid cure test: 0.003% was the highest concentration in which the loss of resistance to the antibiotic was manifested. All resistant strains under study, after being cured with 0.003% SDS, exhibited susceptibility towards antibiotics to which they previously showed resistance.

4.4 MIC in strains with intermediate sensitivity to rifampicin: The MIC performed on those cured strains that presented intermediate sensitivity to rifampin, shows us that these strains are not resistant to this antibiotic, thus being able to select the strains that would be used in the conjugation test.

4.5 Counting of transconjugants: With respect to the control that was made to the donor strains, these grew on the plates with BHI agar containing the antibiotic to which they showed resistance, but they did not grow on the plate with agar containing rifampicin. In contrast, the recipient strain JH2-2 did not grow on any of the plates containing BHI agar with one of the four antibiotics, but did grow on rifampicin agar. Of the two strains selected for norfloxacin resistance gene transfer (E. hirae 29 and E. hirae 15), neither showed transconjugant colonies. The same result was obtained for those species (E. hirae 23, E. durans 12 and E. faecium 90) selected for the conjugation of resistance to ciprofloxacin, where no transconjugant colonies were obtained. In Table 22, it can be seen that for the count of the number of donors, in the strains: E. faecalis 17, E. faecalis, 18, E. hirae 22 (resistant to streptomycin) and E. faecalis 43 (resistant to erythromycin) the last three dilutions were used, obtaining: 45 x 10^6, 16 x 10^7, 11 x 10^7 and 73 x 10^6 colonies respectively. For the other three strains: E. hirae 29, E. durans 32 and E. faecium 89 (with resistance to streptomycin) the last two dilutions were used and the following were obtained: 82 x 10^7, 2 x 10^8 and 84 x 10^7 respectively. For the species E. faecalis 42 (resistance to streptomycin), two dilutions were counted, having 79 x 10 6 colonies. The number of donors was obtained from the average of the colonies present in the dilutions. Of the two species chosen for the conjugation of resistance to erythromycin, only one (E.faecalis 43) obtained transconjugant colonies. In relation to streptomycin resistant strains, it is necessary to indicate that seven more strains were incorporated for the study due to the high resistance also shown to this antibiotic. Of the 14 streptomycin-resistant strains that were chosen for conjugation, transconjugants were obtained in seven strains: E. faecalis 17, E. faecalis 18, E. hirae 22, E. hirae 29, E. durans 32, and E. faecium 89 (Table 22). Transfers, by conjugation, of resistance to streptomycin and erythromycin to a standard strain F- (E. faecalis JH2-2) showed transconjugation frequencies ranging from 8 x 10^{-5} to 2 x 10^{-1}, the latter value being higher than expected

4.6 Electrophoretic run of plasmids: As can be seen in fig.5, the size of the plasmids with respect to the marker is greater. The largest band in the marker used is 10Kb, thus informing us that the plasmids are larger.

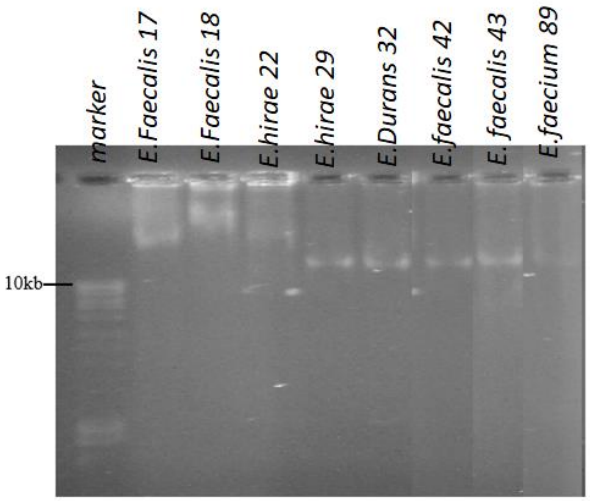


Figure 5: electrophoretic running of plasmids

4.7 Transformation Test: To carry out this test, plasmid DNA was previously obtained from the transconjugant strains of the study. No transforming colonies were observed for any of the two transformation tests carried out with the plasmids of the eight Enterococcus strains that did show conjugation: E. faecalis 17, E. faecalis 18, E. faecalis 43, E. hirae 22, E. hirae 29, E. durans 32, and E. faecium 89

Discussion

According to Sarra, et al, (2013), the 31 strains used in this study have participated in human clinical conditions. All of them were isolated from the governorate of Salah Al-din in the Arabian Gulf and were previously identified in the laboratory using phenotypic methods (Vignaroli, et al 2018). To avoid influencing the frequency of resistance expressed in each of the 31 strains, an attempt was made to choose a similar number of strains for each species. The twelve antibiotics were chosen based on prior research indicating high resistance to these antibiotics in different Enterococcus species from clinical and environmental isolates (Al-Dahmoshi, et al., 2020).

According to the results of the disk diffusion test, it has been possible to find 19 different antibiotic resistance patterns, observing patterns of up to 6 resistance markers and where 55% of the strains expressed resistance to at least three of the 12 antibiotics that were used in the experience, revealing that these microorganisms can not only have resistance to different antibiotics, but can maintain it even under adverse conditions such as the marine environment (Khalid, 2016).

In the present work, the resistance that these Enterococcus species mainly presented was towards streptomycin, and several studies previously reported show that different species of this genus; E. faecium and E.faecalis of clinical origin are characterized by being resistant to this antibiotic (Ruzauskas, et al., 2009). Similarly, despite the fact that E. faecalis is the species with the highest number of isolates in clinical samples, it has been repeatedly reported that the percentage of resistance of this species, coming from clinical and environmental isolates, is lower than that expressed by E. faecium against some antibiotics such as streptomycin (Sfaxi, et al, 2011); Other studies even report higher levels of resistance to this antibiotic in strains of E. faecium from environmental samples (Kurekci, et al, 2016).

It is also stated that resistance to glycopeptides occurs more frequently in this particular species of Enterococcus (Kurekci, et al, 2016). Similarly, according to other reports, Enterococcus durans and E. hirae frequently have a lower prevalence in clinical or environmental isolates and a higher prevalence and resistance of E. hirae has generally been reported over E. durans (Sfaxi, et al, 2011). In our study we see similar levels of resistance between the two species, including E. durans presents a higher percentage of resistance than E. hirae, and even more interesting is to observe that these two species express multi-resistance at higher frequencies than those expressed by E. faecalis. The high resistance to clindamycin observed in this study coincides with other results obtained in previous works, and may be mainly due to the fact that Enterococcus have a natural or intrinsic resistance to this antibiotic; although plasmid-mediated inactivation of the antibiotic has also been reported (Rams, et al, 2012).

The MIC test was performed for ciprofloxacin, norfloxacin and erythromycin, due to their location in plasmids previously reported, and streptomycin, also considering the high frequency of resistance found among the Enterococcus strains in the study. The results made it possible to confirm the resistance values found for these antibiotics with the disk diffusion test. Sensitivity was found in many of the strains reported as intermediates by the aforementioned test, but high resistance values of up to 80 µg / mL were also found for ciprofloxacin> 2400 µg / mL for streptomycin, 40 µg / mL for norfloxacin and> 240 µg / mL for erythromycin. These are close to or greater than the values previously reported in Enterococcus species where resistances greater than 64 µg/mL were found for ciprofloxacin,> 2000 µg / mL for streptomycin; > 32 µg / mL for norfloxacin and > 256 µg / mL for erythromycin; although it was found in a report that a strain of Enterococcus isolated from an environmental sample, presented resistance to streptomycin > 16000 µg / mL (Kurekci, et al, 2016). Through the MIC tests, which were performed against the four antibiotics, it was found that 25 strains presented resistance to each of the antimicrobials tested. With these data, those species that presented resistance to at least one of the four antibiotics to be tested were chosen, trying not to select those that present resistance to rifampicin since it is a chromosomal resistance present in the receptor strain that was used for the conjugation test. Thus, it was determined that of the 25 resistant strains, 11 showed resistance to rifampicin.

For the treatment of the 14 remaining strains, SDS (sodium dodecyl sulphate) was used because it has been shown to be very efficient in curing plasmids of Gram-positive bacteria (Wamel, et al, 2007), and according to works reported in Enterococcus faecalis it was shown that these microorganisms are very sensitive to this detergent, which is considered the choice for curing plasmids in Enterococcus strains. Initially, SDS concentration values were unknown for the cure of Enterococcus, but it was known that for S. aureus it was 0.002% (Oh, et al., 2006) so a range of concentrations was established in the initial standardization test.

The fourteen strains that lost resistance were interpreted as carriers of plasmid resistance to certain antibiotics and were selected for conjugation transfer tests, but within this group, we also indicated that there were four strains that initially presented intermediate sensitivity to rifampicin, for what was necessary to do a MIC for this antibiotic, ruling out that resistance (Table 1).

Table 1: MIC for strains with intermediate sensitivity to rifampicin

Species (n)	Control	Rifampicin concentration (µg / mL)					
		1.875	3.75	7.5	15	30	60
Enterococcus durans (11)	G	G	G	PG	NG	NG	NG
Enterococcus hirae (23)	G	G	PG	PG	-	NG	NG
Enterococcus faecalis (43)	G	G	G	PG	PG	NG	NG

Note: Those that grow at a concentration of 7.5 µg / mL are considered resistant.
Control: 0 µg / mL rifampicin. Growth (G), poor growth (PG), there was no growth (-).

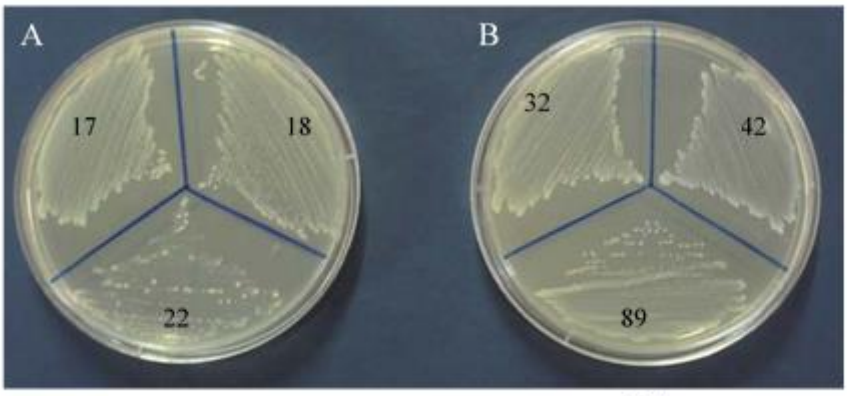
Once the resistance of the 14 strains had been observed, a group with different strains was taken for each antibiotic. The choice of these strains was made according to the degree of resistance they showed to a certain antibiotic in the diffusion disk test and the minimum inhibition concentration. Thus, two strains (Enterococcus hirae (15) and Enterococcus.hirae (29)) were used for norfloxacin; three strains (Enterococcus durans (12), Enterococcus faecium (90) and Enterococcus hirae (23)) for ciprofloxacin; two strains (Enterococcus faecalis 42 and Enterococcus faecalis 43) for erythromycin and seven strains (Enterococcus durans 11, Enterococcus durans 28, Enterococcus durans 32, Enterococcus faecium 89, Enterococcus faecalis 17, Enterococcus faecalis 18 and Enterococcus hirae 22) for streptomycin. As previously mentioned in the results, there was no conjugation of the strains resistant to norfloxacin and ciprofloxacin, but there was conjugation of a strain resistant to erythromycin (Enterococcus faecalis 43), which showed a high conjugation index. Of the seven strains chosen for streptomycin, only five conjugated: Enterococcus durans 32, Enterococcus faecium 89, Enterococcus faecalis 17, Enterococcus faecalis 18 and Enterococcus hirae 22, observing high conjugation indices that reach 2 $x10^{-2}$ (Table 2).

Table 2: Count of Transconjugants.

SPECIES	COUNT			NUMBER OF DONORS	NUMBER OF TRANSCONJUGANTS	CONJUGAC INDEX.
	-4	-5	-6			
Enterococcus faecalis 17	539	43	4	44×10^{5}	1506 (-1)	$2x10^{-2}$
Enterococcus faecalis 18	1380	149	16	15×10^{6}	2	5×10^{-6}
Enterococcus hirae 22	1066	109	10	10×10^{6}	1	$3x10^{-5}$
Enterococcus hirae 29	NA	817	80	81×10^{6}	6	8×10^{-6}
Enterococcus durans 32	NA	1568	197	1.8×10^{7}	62	2×10^{-5}
Enterococcus faecalis 42	770	77	0	78×10^{5}	2	$2x10^{-6}$
Enterococcus faecalis 43	800	74	6	74×10^{5}	798 (-1)	$2x10^{-1}$
Enterococcus faecium 89	NA	676	82	83×10^{6}	7	8×10^{-2}

The transconjugants of strains 17, 18, 22, 29, 32, 42 and 89 are observed for streptomycin. Strain 43 showed a transconjugant for erythromycin. (NA) uncountable number of colonies, (-1) additional dilution.

As it was observed that the other strains (used for conjugation with the other antibiotics) were also resistant to streptomycin and the resistance and cure values for this antibiotic were very well known, the remaining seven strains were used for a second conjugation test. Resistance to streptomycin. Of these 7 strains, only two strains performed conjugation viz. E. hirae 29 and E. faecalis 42. So we finally have seven streptomycin resistant strains, and one erythromycin resistant strain that completed conjugation. The frequencies of transfer of plasmids with resistance to erythromycin that have been reported in conjugation studies in Enterococcus have been 2.1 x 10^{-5} to 3 x 10^{-4} transconjugants per donor and 8 x 10^{-5} to 5 x 10^{-3} for streptomycin resistance plasmids (Figure 6) (Lü,et al, 2007). In this work, the conjugation index for plasmids with resistance to erythromycin was 2 x 10^{-1}, and from 8 x 10^{-6} to 3 x 10^{-3} for resistance to streptomycin. The results obtained allow to affirm the high capacity of transference of genetic information by conjugation of Enterococcus spp. marine.



Growth of the transconjugant colonies in BHI medium with streptomycin (1000 µg / mL). A: Transconjugants of donor strains 17, 18, 22 and B: 32, 42 and 89 resistant to streptomycin.

Figure 6: Transconjugants with streptomycin resistance

Regarding the size of the plasmids, these are greater than 10kb. This had already been observed in previous studies (De Boever et al, 2000) where conjugative plasmids with 45kb size resistance to erythromycin and another with 95kb size resistance to erythromycin and streptomycin were found. The failure to obtain transconjugants in the strains resistant to ciprofloxacin, norfloxacin and some with resistance to erythromycin and streptomycin, is possibly due to the large size of the plasmids, but it must be taken into account that the plasmids with resistance to antibiotics mentioned above are highly size but they are conjugative plasmids. Therefore, the reason that there has been no conjugation in certain strains, in the present study, may be due to the fact that they present resistance plasmids but not conjugatives. The failure to obtain transforming colonies is possibly due to the fact that these microorganisms do not easily transfer their plasmids by transformation. At the same time, despite the fact that it is asserted that these microorganisms possess natural competence, there are very few reports of a natural transformation of Enterococcus where stimulators or other methods of transformation far removed from the almost natural conditions that were intended have not been used to Enterococci in this study. Likewise, in a study carried out by Pienaar, et al (2016) it was observed that Enterococcus strains exposed to aquatic

environments adopted a viable non-colourable state (VBNC) and adapted to particular environmental conditions through changes in the composition of their cell wall, these microorganisms thickened its wall and thanks to this they remained alive under these conditions. This data is very important and must be considered since if Enterococcus found in the sea maintain this state for their survival, transformation would not be the horizontal gene transfer mode used to transfer resistance plasmids in the marine environment.

Conclusions

The four species tested in the current investigation, Enterococcus faecium, Enterococcus faecalis, Enterococcus hirae, and Enterococcus durans, show resistance to most of the 12 antimicrobials used, primarily streptomycin and clindamycin, in samples taken from the Basra city sea coast. Many factors contribute to antibiotic resistance in Enterococcus strains, one of which is the presence of plasmids, as demonstrated in this study by curing resistant strains with SDS transformation, which was not a horizontal transfer mode of antibiotic resistance genes in the Enterococcus strains used in this study. It is concluded that the Enterococcus species of marine origin employed in this study, which are considered harmful for humans and animals, have antibiotic resistance that can be transferred through conjugation under laboratory settings.

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