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INVESTIGATION OF GENETIC DIVERSITY OF CLINICAL ENTEROCOCCUS SPECIES IN UTI PATIENTS BY USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS IN IRAQ

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By Walaa Abdulla Majeed MAJEED

January 2024

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science

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ABSTRACT

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Master of Science in Biology

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January 2024

The emergence of *Enterococcus* bacteria as important nosocomial pathogens in recent years has led to the need to investigate the factors involved in the pathogenesis of infections caused by these microorganisms. The aim of this study was to determine the presence, virulence characteristics and genetic diversity of *Enterococcus* genus bacteria, which are highly prevalent in hospitals in Salahaldeen, Iraq. Therefore, within the framework of this thesis study, the highest rate of *E. faecalis* species was detected, followed by *E. faecium*, *E. avium* and *E. gallinarum* species. The most frequently isolated clinical sources were urinary tract infections (UTI), gastrointestinal tract (GIT) and blood samples. All isolates identified as *E. faecium* and *E. gallinorum* from UTI and hospital colonised isolates were resistant to antibiotics, while *E. avium* isolates were resistant to vancomycin. The highest resistance rates were found in erythromycin, quinupristin/dalfopristin, tetracycline, norfloxacin, ciprofloxacin, levofloxacin and vancomycin. In addition, 83 (78.3%) of the study samples were classified as multidrug resistant. The most common virulence genetic determinant among *E. faecalis* samples was gelE, followed by asa1, cylA and esp genes. The frequency of esp gene was higher among *E. faecium* samples, followed by hyl gene. Three clonal groups were predominant among the study samples, one of which was *E. faecalis*, defined by the majority of samples resistant to vancomycin and high-level aminoglycosides and carrying the virulence genetic profile formed by the asa1, cylA and gelE genes; the other two included *E. faecium* samples, which were mostly resistant to vancomycin and

carrying virulence genetic markers, especially hyl. In addition, the presence of a single sample of *E. avium* resistant to vancomycin and carrying the esp virulence gene is noteworthy. In addition, two clones of vancomycin-resistant *E. gallinarum* were isolated from colonisation of the gastrointestinal tract (GIT) of patients, which is not a common occurrence. Most of the high multidrug resistance rates were isolated from patients hospitalised in intensive care units. This study highlights the growing concern about *Enterococcus* infections in hospitals, particularly multidrug resistance and vancomycin resistance. Surveillance and infection control measures are essential to prevent the spread of resistant bacteria. Alternative treatment strategies and antibiotics need to be developed to combat these difficult-to-treat infections.

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Keywords: Analytical profile index, *Enterococcus*, RAPD, Urinary tract infections

ÖZET

IRAK'TA İDRAR YOLU ENFEKSİYONU HASTALARINDA KLİNİK ENTEROKOK TÜRLERİNİN GENETİK ÇEŞİTLİLİĞİNİN RASTGELE ÇIKARTILMIŞ POLİMORFİK DNA (RAPD) ANALİZİ İLE İNCELENMESİ

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Enterococcus cinsi bakterilerin son yıllarda önemli nozokomiyal patojenler olarak ortaya çıkması, bu mikroorganizmaların neden olduğu enfeksiyonların patogenezinde rol oynayan faktörlerin araştırılması ihtiyacını ortaya çıkarmıştır. Bu çalışmanın amacı, Irak'ın Salahaldeen kentindeki hastanelerde yüksek oranda görülen *Enterococcus* cinsi bakterilerin varlığını, virülans özelliklerini ve genetik çeşitliliğini belirlemektir. Dolayısıyla bu tez çalışması çerçevesinde en yüksek oranda *E. faecalis* türü tespit edilmiş, bunu *E. faecium, E. avium* ve *E. gallinarum* türleri izlemiştir. En sık izole edilen klinik kaynaklar idrar yolu enfeksiyonları (İYE), gastrointestinal sistem (GİS) ve kan örnekleridir. İYE ve hastanede kolonize izolatlardan *E. faecium* ve *E. gallinorum* olarak tanımlanan tüm izolatlar antibiyotiklere dirençliyken, *E. avium* izolatları vankomisine dirençli olarak tespit edilmiştir. En yüksek direnç oranları eritromisin, kinupristin/dalfopristin, tetrasiklin, norfloksasin, siprofloksasin, levofloksasin ve vankomisinde belirlenmiştir. Ayrıca, çalışma örneklerinin 83'ü (%78,3) çoklu ilaca dirençli olarak sınıflandırılmıştır. *E. faecalis* örnekleri arasında en yaygın virülans genetik belirleyicisi gelE iken, bunu asa1, cylA ve esp genleri takip etmiştir. *E. faecium* örnekleri arasında esp geninin sıklığı daha yüksek olup bunu hyl geni izlemiştir. Çalışma örnekleri arasında üç klonal grup baskın bulunmuştur; bunlardan biri vankomisin ve yüksek düzeyde aminoglikozidlere dirençli örneklerin çoğunluğu ile tanımlanan ve asa1, cylA ve gelE genlerinin oluşturduğu virülans genetik profilini taşıyan *E. faecalis*; diğer ikisi ise çoğunlukla vankomisine dirençli olan ve virülans

genetik belirteçlerini, özellikle de hyl'i taşıyan *E. faecium* örneklerini içermektedir. Buna ek olarak, vankomisine dirençli ve esp virülans genini taşıyan tek bir *E. avium* örneğinin varlığı dikkat çekicidir. Ayrıca hastaların gastrointestinal sisteminin (GİS) kolonizasyonundan iki vankomisin dirençli *E. gallinarum* klonu izole edilmiştir ki, bu da çok sık karşılaşılan bir durum değildir. Yüksek çoklu ilaç direnci oranlarının çoğunluğu yoğun bakım ünitelerinde yatan hastalardan izole edilmiştir. Bu çalışma, hastanelerde E*nterococcus* enfeksiyonları, özellikle de çoklu ilaç direnci ve vankomisin direnci konusunda artan endişeleri vurgulamaktadır. Dirençli bakterilerin yayılmasını önlemek için sürveyans ve enfeksiyon kontrol önlemleri şarttır. Tedavisi zor olan bu enfeksiyonlarla mücadele etmek için alternatif tedavi stratejileri ve antibiyotiklerin geliştirilmesi önem arz etmektedir.

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Anahtar Kelimeler: Analitik profil indeksi, *Enterococcus,* RAPD, İdrar yolu enfeksiyonları

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CONTENTS

LIST OF SYMBOLS

dL	Deciliter
°C	Degrees Celsius
g	Gram
L	Liter
µmol	Micromole
mg	Milligram
mL	Milliliters
mM	Millimoles
ng	Nanogram
nmol	Nanomoles
%	Percent
±	Plus-minus
m2	Square-meters

LIST OF ABBREVIATIONS

EARSS	European antimicrobial sesistance surveillance system
ESP	Enterococcal surface protein
GIT	Gastrointestinal tract
ICU	Intensive care unit
LAPASE	Leucine aminopeptidase
MLEE	Multilocus enzyme electrophoresis
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
REA	Restriction enzyme analysis
UTI	Urinary tract infection

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1. INTRODUCTION

1.1 Urinary Tract Infection

Urinary tract infection (UTI) is the invasion, multiplication and colonization of part of the urinary system by bacteria. It is characterized by being a frequent pathology, reaching an audience of all ages, but it has a higher incidence among risk groups such as women and pregnant women, diabetic patients, children, the elderly and patients with coronary artery disease (Vasudevan *et al.* 1995). Regarding the predominance of females, this is mainly due to the anatomy of the urethra, sexual intercourse, previous episodes of cystitis, poor hygiene and pregnancy, with a higher prevalence in patients with low immunity, obese and with low socioeconomic conditions. UTI is considered serious when it is related to factors that facilitate the persistence of infection, such as metabolic conditions, stenosis, tumors, foreign bodies and catheters. But it can be a simple UTI when only the bladder and urethra are attached (Heyns 2012).

UTI is commonly common in pregnant women, due to anatomical and physiological changes that occur in their body, in which about 17 to 20% of Brazilian pregnant women will have a UTI episode, especially in the second trimester of pregnancy, which increases the chances of maternal-fecal complications. This is considered the third most frequent clinical complication during pregnancy, only behind gastrointestinal and respiratory infections (Menezes *et al.* 2020).

From this perspective, UTI is the second bacterial infection that occurs mostly in humans, second only to respiratory infection. In Iraq country, this infection is classified as the most frequent of bacterial infections, since, of 1,000 clinical consultations, 80 are for UTI. It is important to note that the occurrence of its causative microorganism varies from region to region, thus, the susceptibility profile requires monitoring to provide guidance and updated information on therapeutic options (Santos *et al.* 2019). An infectious condition usually occurs due to ascending bacteria, originating from fecal debris or from the microbiota of the vaginal or perianal skin and from the intestinal flora

itself. Gram-negative bacteria, in general the Enterobacteriaceae group, responsible for a large portion of urethritis, cystitis and pyelonephritis, mainly *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus miriabili*, *Enterobacter spp* and *Pseudomonas aeruginosa* are also known as important uropathogens. Gram-positive bacteria are less frequent as etiological agents of UTI, and the species with the greatest epidemiological value are *Staphylococcus saprophyticus*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Enterococcus faecalis* (Barreto *et al.* 2018).

The term enterococci (derived from the French enterocoque) was initially used in 1899 to designate Gram-positive diplococci of intestinal origin. In the same year, a case of infective endocarditis caused by bacteria organized in short chains, most in pairs, was reported, similar to representatives of the genus *Streptococcus*, being described as a new species called *Micrococcus zymogenes*. In 1908, it was recognized that this species actually belonged to the genus *Streptococcus* (Lebreton *et al.* 2014).

In 1906, the term *Streptococcus faecalis* was used to name a microorganism isolated from a patient with endocarditis, however, it was considered very characteristic of the human intestine, which explains its nomenclature (Švec and Franz 2014). After the name given by Andrewes and Horder in 1919, Orla-Jensen began to use different terminologies when describing similar samples. *Streptococcus glycerinaceus* and *Streptococcus faecium*.

In 1937, an attempt was made to standardize the term enterococcus, which until then had been used ambiguously to designate both different species of *Streptococcus* of intestinal origin, and samples strictly identical to *S. faecalis*. Sherman proposed a classification scheme that divided members of the genus *Streptococcus* into four groups: pyogenic, viridans, lactic and enterococci. The Sherman classification correlated with the serological classification proposed by Rebecca Lancefield in 1933 for *Streptococcus*, in which enterococci were described as belonging to group D based on their antigenic characteristics (Schleifer and Kilpper-Bälz 1984).

In his new organizational criteria, Sherman described as belonging to the enterococci group all the species already known as enterococci until then, namely: *S. faecalis* also called *S. faecium* or *S. glycerinaceus* until now *Streptococcus zymogenes, Streptococcus liquefaciens* and *Streptococcus durans*. Later studies revealed that the species described as *S. faecium* had different biochemical characteristics from those observed in *S. faecalis*. In 1970, there was a proposal for the creation of the Enterococcus genus from the Lancefield group D *Streptococcus* species, based on cell arrangement and phenotypic characteristics. The new genus would harbor two species: *S. faecalis* and *S. faecium*, each including their respective subspecies. However, the genetic evidence necessary for the validation of the Enterococcus genus was only provided in 1984 through the DNA-DNA and DNA-RNA hybridization experiments performed by Schleifer and Kilpper-Balz. The results of this study finally showed that these microorganisms shared a low genetic correlation with the other members of the *Streptococcus* genus, justifying the creation of the Enterococcus genus (Schleifer and Kilpper-Bälz 1984).

The rapid development of molecular microbiological methods in recent years has brought many changes in the classification of microorganisms. One of the bacterial genera whose taxonomies underwent major changes was *Streptococcus* genus. Taking into account the changes in the genetic structures of the bacteria, Lancefield D group *streptococci* were classified as Enterococcus genus, Lancefield N group *streptococci* have also been studied in different genera as Lactococcus. Today, molecular methods such as DNA-DNA reassociation experiments and sequencing of 16S rRNA genes are used in the identification and systematization of species belonging to the genus Enterococcus isolated from different environments. The determination of the fatty acid composition of bacteria by gas-liquid chromatography, which has been used for a long time, has important diagnostic value (Byappanahalli *et al.* 2012).

1.2 Objectives of study

The aim of the study was to determine, in samples collected from patients at Shirqat General Hospital and Salahaldeen Teaching Hospital in Salahaldeen, Iraq, from March

2021 to February 2022, how common and different are the genetic factors that make Enterococcus viruses more or less dangerous. To identify the species of Enterococcus samples and the molecular diagnosis of Enterococcus species associated with infectious processes (UTI) or colonization in patients hospitalized in the two hospitals. To identify genes associated with the expression of the main virulence factors of enterococci and genetic diversity. To compare the presence of virulence genes between susceptible and resistant samples to vancomycin. To evaluate the genetic diversity of the samples using the Random Amplified Polymorphic DNA (RAPD) technique.

2. LITERATURE REVIEW

2.1 Urinary Tract Infections (UTIs)

In a study of (Allami *et al.* 2022) participants at Medical City Hospital in Baghdad, Iraq are being surveyed to ascertain the incidence of urinary tract infections (UTIs), the microorganisms r*esp*onsible for them, and the risk factors connected with them. Standard procedures were used to diagnose 237 mid-stream morning pee samples that were obtained aseptically. Questionnaire data were used to learn about the causes of urinary tract infections. Patients with urinary tract infections at Baghdad's Medical City Hospital were 63 out of 237. Among the 63 bacterial uropathogens found, *Escherichia coli* accounted for 21, *Klebsiella pneumoniae* for 13, *Staphylococcus aureus* for 10, *Enterococcus faecalis* for 8, Enterobacter cloacae for 4, *Pseudomonas aeruginosa* for 3. Lower stomach or back pain in conjunction with a urinary tract infection was found to be significantly related to hospital (department), sex, and age ($p<0.05$). There was a strong connection between hospital (department), sex, age, and lower abdomen or back pain and UTI, with the prevalence of bacterial UTIs being highest in those aged 30-39 at 16/63. Studying 100 patients in Al-Kut City, Iraq, (Taher *et al.* 2021) reported that *Staphylococcus aureus* (48%), *Escherichia coli* (24%), *Klebsiella pneumoniae* (17%), *Enterococcus* species (5%) and *Pseudomonas aeruginosa* (2%) were the most commonly diagnosed bacteria. The chance of a diabetes diagnosis varies greatly by patient age and gender ($p<0.001$ and $p<0.038$, r*esp*ectively). This association was shown to be non-significant after looking at the different types of bacteria in the environment.

Women in the city of Kirkuk were found to have urinary tract infections by Al-Samarrai and Korshid (2018). The mean age of females (28 years) infected with *E. coli* (37.84%), *Staphylococcus aureus* (31.97%), *Klebsiella pneumonia* (28.76%), and *Proteus mirabilis* (25.24%). According to Iranian researchers, *E. coli* is responsible for more than half of all urinary tract infections. In addition, 86% of the sample group was 25 years or older, suggesting that older women are more likely to develop a UTI caused by *E. coli*. Additional evidence that *E. coli* causes urinary tract infections in women (78%

of whom were less than 30 years old) was identified in a study conducted in Diyala, Iraq (Salman *et al.* 2021).

2.2 Epidemiological Typing Methods

Because enterococci are important causes of nosocomial infections and often show multi-drug resistance, there is an increasing need for epidemiological studies and infection control to identify their types and subtypes. Furthermore, typing studies are required to support the idea of exogenous acquisition of enterococcal infections. Especially due to the increasing frequency of VREs, investigation of nosocomial outbreaks caused by the spread of drug-resistant enterococci strains is of major interest. In addition to epidemic research, the methods used in epidemiological studies are also used to monitor the spread of enterococci in different environments and hosts, and to monitor multidrug resistant strains. Previously, epidemiological studies of enterococcal infections were carried out according to their phenotypic characteristics. However, although these approaches sometimes provide useful information, their use in epidemiological studies is limited due to their time consuming, reproducibility and difficult to interpret, and inability to differentiate between strains. The use of phenotypic methods together with the molecular techniques developed in recent years can provide useful information (Udo *et al.* 2004).

The development of various molecular techniques, which are quite successful in the differentiation of enterococci, has brought an important understanding from the epidemiological point of view. As a result of the use of typing techniques with higher discriminatory power, it is possible to distinguish between patients with strains that can be acquired exogenously by direct and indirect contact. It has also been reported that resistant enterococci spread within and between hospitals. The first molecular techniques used were plasmid profile analysis and restriction enzyme analysis (REA). These techniques can be useful in some situations, but there are some problems with their use. Determination of DNA restriction endonuclease profiling with PFGE provides extraordinary benefits in differentiating enterococcal strains. Multilocus Enzyme Electrophoresis (MLEE), ribotyping and PCR-based typing methods such as RAPD-

PCR or REP-PCR are also used to investigate the genetic relationship between enterococci strains. Sequencing or examining PCR products with Restriction Fragment Length Polymorphism (RFLP) are also methods that can be used to show and monitor the differences between specific resistance genes in enterococci. According to the results of many studies, PFGE, in which genomic DNA is cut with the Sma1 enzyme, is a very useful method that provides significant advantages in the differentiation of strains. Currently, PFGE is the most useful and reliable standardization method and is considered the "gold standard" for the epidemiological examination of enterococcal infections (Papaparaskevas *et al.* 2000).

Although various protocols have been published for the examination of enterococci with PFGE, standardized protocol development is needed to make interlaboratory comparisons. Although PFGE is more discriminatory than other methods, the epidemiological interpretation of PFGE profiles is not always conclusive. Differences in the PFGE profile are obtained associated with the appearance of genetic changes, leading to problems in the evaluation of clonality. More appropriate interpretation remains controversial, as in some cases it is difficult to define the level of polymorphism. As a result, there is no complete, uniform typing technique for enterococci, Combining the results of various typing techniques, *esp*ecially based on genomic polymorphism, can be used as an indicator of close relationship. The use of PFGE using different restriction enzymes or in combination with an additional typing technique is recommended to help clarify epidemiological interpretations. The general principle in interpreting molecular typing information is to examine the band differences in the PFGE profile (Papaparaskevas *et al.* 2000).

2.3 Protein Fingerprinting by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Due to the time consuming and low discrimination power of numerical biotyping based on physiological and biochemical properties, alternative typing methods based on phenotypic features that can make typing faster, more reliable and with high discrimination power, cell wall or all cell proteins in a mobile matrix under electrical

current. The SDS-PAGE method, which is based on their separation by migrating according to their weight, has been used frequently, it has provided rapid screening of many strains with this method and reliable results can be obtained at the species and/or subspecies level. This method, in which the protein profiles obtained in the polyacrylamide gel are considered as fingerprints for the proteins expressed by the bacteria, can be used in combination with other classification schemes to identify unknown isolates. The results obtained in recent studies have shown that there is a high correlation between the protein fingerprint of microorganisms and DNA-DNA homology values. (Pacini *et al.* 2006) Used SDS-PAGE as a reference method for the identification of enterococcal species in Italian cheeses and obtained results consistent with RAPD-PCR (Güler 2007).

Showed that the differentiation of p*hyl*ogenetically closely related *E.durans, E.hirae and E.villorum* species can be done by SDS-PAGE as well as genomic methods75. Klare *et al.* on the other hand, suggested that a cytoplasmic protein of 39 kDa was associated with resistance in glycopeptide-resistant *E.faecium* strains they isolated between 1993-1995. They also showed that SDS-PAGE patterns of penicillin-binding proteins (PBPs) in enterococci were correlated with DNA-DNA hybridization results. They emphasized that these patterns can be considered as a definitive method for the identification and differentiation of new species (Devriese *et al.* 2002).

2.4 Multi-Locus Enzyme Electrophoresis (MLEE)

The multilocus enzyme electrophoresis method is a standardized method that has been used for a long time in population genetics and classification of eukaryotic organisms. In the last 20 years, it has been used increasingly to understand bacterial evolution and classification. The difference of MLEE from other phenotypic methods for classification and epidemiological relationship detection is that it can give all allele profiles as electrophoretic type. This method is based on the characterization of intracellular enzymes according to their electrophoretic action ability and distinguishes organisms from others according to the electromorpho types they form. An objective database can be constructed for population characterization from these electrophoretic

types. Generally, MLEE targets loci that naturally show high variability in up to 20 housekeeping enzymes, so the technique has a much higher discrimination power than monolocus enzyme electrophoresis (Udo *et al.* 2003).

Enterococci produce more than 1600 enzymes, most of which have structural functions that regulate vital functions such as division, proliferation, and energy transfer. These isoenzymes show different catalytic properties and therefore different electrophoretic action ability. Enzyme diversity depends on direct genetic factors such as primary isoenzymes and alloenzymes, or indirect genetic factors such as secondary isoenzymes formed by post-translational modification. Isoenzymes can be distributed in different cell components and are encoded by at least two different genes. Multiple loci encoding enzymes with similar substrate specificity allow for gene duplications. As a result of point mutations, the composition of amino acids encoded by duplicate genes changes and electrophoresis detects enzymes with different action abilities. Differences in electrophoretic motion are caused by difference in charge and/or size. At least 50% of putative enterococcal enzymes have been investigated by the two-way isoelectrofocus method. In the phenotypic identification and typing of enterococci, besides these methods, many researchers have used long-chain fatty acids, met*hyl* ester analysis of cell membrane fatty acids, enterocin analysis, mass spectro analysis of cell membrane pyrolysis extraction, Fourier infrared spectroscopy (FTIR) and proton magnetic resonance imaging. Various methods such as spectroscopy (1H MRS) have been tried, and it has been reported that some of these methods, which have limited use, show a discrimination power close to PFGE (Green *et al.* 1995).

2.5 Genotypic Typing Methods

Recent research has focused on molecular biology techniques for rapid identification and typing of enterococcal isolates. Molecular typing methods used in epidemic epidemiology due to enterococci were compiled by Willey *et al.* in 1994. Main techniques in bacterial classification, such as the G+C content of chromosomal DNA and DNA-DNA hybridization experiments, are often used in the characterization of newly isolated and identified strains. The amplification of specific regions in the

genome under in-vitro conditions, the polymorphism of the bands formed by cutting specific mutations in the amplified products with restriction enzymes, and finally the analysis of the polymorphism of the band patterns formed at the end of digestion of the total genome with a restriction enzyme under specific conditions were used for the clonal identification of epidemic strains in the analysis of epidemics caused by enterococci (Karayasheva and Radeva 2017).

2.6 Restriction Enzyme Analysis (REA) Based Methods

In the chromosomal restriction analysis of enterococci, DNA fragments obtained as a result of cleavage of DNA isolated from microorganisms with one or more of the appropriate restriction enzymes are separated according to band sizes by agarose gel electrophoresis. Typing is done according to the polymorphism of the obtained band patterns. E. faeciums isolated from clinical specimens were examined by REA and PFGE methods, but they reported that both methods were not ideal (Iwanejko *et al.* 1989).

2.7 Plasmid DNA Profile Analysis

After the plasmid DNA is extracted and hydrolyzed with a specific enzyme such as REA or PFGE, it is separated according to their molecular weight on the agarose gel. Band polymorphism is used for typing purposes. However, the method has significant disadvantages. For example, plasmids can be lost during fermentation and other technological processes or rearrangement by conjugative transfer (Mayer *et al.* 1988). Used plasmid analysis together with plasmid restriction analysis and PFGE for typing *E. faecium* strains. With the combination of plasmid profile analysis and PFGE, it was possible to identify closely related strains isolated from homemade Pecorino Sardo cheese.

Son *et al.* (1999) Also reported that resistance patterns and plasmid profile analysis can be used as a complementary technique to RAPD-PCR in the typing of E. faecium strains85.

2.8 Pulsed Field Gel Electrophoresis (PFGE)

With the pulsed field gel electrophoresis method, band polymorphism, which is the result of digestion of the total genome with a restriction enzyme, is evaluated, not the relatively short amplicons obtained as a result of amplification of a known specific region in the genome, as in REA. Starting from the assumption that some of the bands to be obtained from the large genome will be quite large, electrophoresis conditions different from PCR-RFLP are used in this method. DNA molecules larger than 50 kb show the same mobility in conventional gel electrophoresis, where DNA fragments are separated according to their molecular size under unidirectional regular electric current. For this reason, DNA fragments larger than 50 Kb appear as broad, undifferentiated bands in the gel and are impossible to distinguish by standard gel electrophoresis. In the PFGE method, even DNA molecules larger than 50 kb can be separated easily, and it is possible to evaluate the entire genome, as a result of periodically changing the direction of the electric current and sending a direct current on the DNA fragments in the gel at specified intervals. In order to protect the bacterial enzymes from spontaneous degradation in the PFGE method, the DNA isolated from the bacteria embedded in the agarose gel without losing its integrity is cut by hydrolyzing a restriction enzyme with a known 5-6 base long recognition region while still in the gel. DNA fragments obtained in agarose gel are subjected to electrophoresis by changing the current direction and intensity at regular intervals. After electrophoresis, after staining the gel with Ethidium bromide, the DNA fragments in the gel are visualized in UV light and the band polymorphism obtained is evaluated visually or using computer programs. According to the band patterns obtained with PFGE, the size of the chromosomal DNA can be estimated and it is possible to associate the strains clonally. Since PFGE is a highly discriminatory, reproducible and easily interpretable method, it is accepted as a reference method in genotypic identification, *esp*ecially in the surveillance of nosocomial infections. The most important disadvantages of the method are that it

requires expensive equipment, is time consuming, and standard protocols for many species still have not been established. Pulsed field gel electrophoresis method has also been successfully used to determine the clonal relationships of VRE / GREs isolated from hospital-acquired infections and environmental samples (Van Belkum *et al.* 2007).

2.9 Nucleic Acid Amplification Based (NAAB) Techniques

Techniques for the detection of mutations are based on amplification of specific gene regions of microorganisms and known gene regions such as resistance genes (Domig *et al*. 2003).

2.9.1 PCR based techniques

As in all microorganisms, it has been successfully used in the determination of species, strains and genotypes of enterococci. For this purpose, primers targeting the elongation factor *tuf* gene (EF-Tu) in enterococci were used to detect the D-alanine/D-ligas gene (ddl) and groESL gene at the genus level. Primers targeting have also been used for species-level diagnostic purposes (Domig *et al*. 2003). Species-specific primers targeting the ddl gene were approved by Wittenberg *et al.* (2001) for *E.durans and E.hirae*. PCR-based methods have also been applied for the detection of antibiotic resistance, so that while rapid susceptibility testing is performed, on the other hand, it can be shown in clonal relationship between the isolated strains due to vertically spreading resistance genes or gene mutations. (Dutka-Malen *et al.* 1995) Reported that they could detect both species-level diagnosis and glycopeptide resistance with primers targeting the ddl gene. Miele *et al.* (1995) developed a primer set for the van genes, the same year Klare *et al.* (1995) used the PCR method for the vanA gene. New primers targeting the vanA and vanB gene were also developed, and the same primers were used by Giraffa *et al.* (1998) used the fluorescence-based PCR system LightCycler. Multiplex PCR protocols for the detection of glycopeptide resistance using primers that capture the vanA, vanB, vanC1, C2 and C3 genes simultaneously with the identification with primers targeting 16S rRNA of enterococci, to be used later in the diagnosis and

epidemiological surveillance of enterococci developed. In addition, PCR techniques targeting virulence genes of enterococci have been developed (Johnson *et al.* 1998).

2.9.2 Random amplified polymorphic DNA-PCR (RAPD-PCR)/ arbitrarily primed-PCR (AP-PCR)

It is a method based on the demonstration of DNA polymorphism using random primers. It is a PCR-based method targeting the polymorphism of DNA fragments resulting from the amplification of primer-DNA hybrids formed by the random binding of randomly arranged short primers at high Mg+ concentration at low annealing temperature. The RAPD method differs from conventional PCR in that it uses a single primer instead of two and has a low heat of attachment. It has been suggested that RAPD-PCR is a reliable technique for the identification and differentiation of enterococci isolated from clinical specimens and food specimens70. It has been reported that it is a fast and reliable technique for the epidemiological surveillance of hospital-acquired VREs, but its reproducibility is low for typing enterococci with high levels of aminoglycoside resistance (HLAR). In some studies, it has been shown that the results of RAPD and PFGE methods are quite compatible. Devriese et al. (2002), using AP-PCR, he grouped all strains of *E. durans, E. hirae and E. villorum* according to differences in band patterns (Devriese *et al.* 2002).

2.9.3 Specific and random amplification-PCR: SARA-PCR

It is a method that enables the identification of Enterococci species and display of the vanA gene in a single reaction. The technique is based on the use of the vanA primer set. In strains with the vanA gene, complex patterns occur with a band of about 700bp (Devriese *et al.* 2002).

2.9.4 Amplified fragment length polymorphism (AFLP)

This method is a method that targets the ligation of DNA fragments, which are released after the open-ended cutting of total genomic DNA with restriction enzymes, with the help of ligase enzymes with open-end compatible oligonucleotide adapters and their amplification using primers that target oligonucleotide adapters inserted by ligation. As with conventional PCR methods, polymorphism of amplicons is identified on agarose gel or by capillary electrophoresis. Examined enterococci with AFLP and PFGE in their study and reported that these two techniques showed good compatibility. However, in some studies, the AFLP method for VREs gave discordant results compared to PFGE (Jureen *et al.* 2003).

2.9.5 Repetitive polymerase chain reaction (Rep-PCR)

The repetitive-PCR method is based on PCR representation of copies of repetitive DNA sequences scattered throughout the bacterial genome. These repetitive DNA elements are located at different distances in the genome, depending on the genus and species. As a result of amplification using primers targeting these repetitive elements, DNA fragments of different lengths are formed. The polymorphism of these fragments is considered as specific DNA fingerprinting. In comparative studies, it has been suggested that the interpretation of polymorphic DNA patterns formed by Rep-PCR is more difficult than the patterns obtained by PFGE, and there is incompatibility between them (Bedendo and Pignatari 2000)

2.9.6 Intergenic rRNA spacer regions-PCR (ITS-PCR)

It is an amplification method that is specific for enterococcal species, using primers targeting the intergenic spacer region located between 16S and 23S rRNA70. Amplicons were analyzed by high resolution 6% non-denatured acrylamide-bisacrylamide gel electrophoresis. *E. avium, E. raffinosus, E. malodoratus, E. pseudoavium, E. faecalis and E. hirae* examined by this method showed similar

profiles. In order to obtain more distinctive results, the amplicon was cut with the Sau3A1 restriction enzyme and the resulting fragment polymorphism could distinguish between E. faecium, *E. casseliflavus, E. mundtii, E. durs, and E. hirae,* but no specific polymorphism for *E. gallinarum* (Poyart *et al.* 2000).

2.9.7 Other amplification based methods

Various amplification-based modifications targeting specific regions in the bacterial genome have been used to identify enterococci isolated from various clinical and environmental specimens at the genus, species, and strain level. Among them, The "Amplified rDNA Restriction Analysis – ARDRA" method targeting the fragments surrounding the 16S rDNA gene, the "PCR-amplified 16S rDNA-RFLP" method, which is the combination of PCR-Ribotyping, SDS-PAGE and 16S rDNA sequencing analysis, the amplification of the groESL gene with specific primers. The "Broad range PCR-RFLP" method, which targets the band polymorphism resulting from the digestion of the amplicon with *Hae*III and *RsaI*, is the "Temporal temperature gradient gel electrophoresis", which is claimed to be *esp*ecially useful for the identification of clinically important enterococci species and reveals the single nucleotide difference in the DNA sequence with the Tm temperature difference. -TGGE method, "Denaturating Gradient gel electrophoresis -DGGE" method, which has been successfully applied in the differentiation of E.*casseliflavus, E.durans, E.faecalis* and E.faecium strains, and "tRNA-intergenic length polymorphism" which has been tried in the differentiation of enterococcal species of human and animal origin. analysis -tDNA-PCR" method, ITS regions, *esp*ecially 16S rRNA and 23S rRNA genes, Chaperonin ("Nucleic acid hybridization" method using probes targeting cpn60) gene, groES and groEL genes, vanA, vanB, vanC and vanD gene and finally HLGR genes, Reverse Transcriptase-PCR (RT) showing specific mRNA using primers targeting vanA and vanB genes -PCR) method is an important method that finds use (Domig *et al.* 2003).

3. MATERIALS AND METHODS

3.1 Population

A total of 500 women aged over 16 years attended at the two hospitals (Shirqat General Hospital and Salahaldeen Teaching Hospital) in the city of Salahaldeen, Iraq outpatient clinic and women in the same age group who were hospitalized with a UTI from March 2021 and February 2022 were invited and accepted to participate in the research and accepted 100 members to participated in the current research.

3.2 Sampling

For this cross-sectional descriptive study, 100 samples of bacteria of the *Enterococcus* genus were analysed from the bacteriomes of the Biotechnology Support Laboratory, located at the Shirqat General Hospital and Salahaldeen Teaching Hospital. These samples, in turn, were isolated from clinical specimens considered indicative of infection. The collections were made from patients hospitalized in intensive monitoring units - intensive care units (ICU) and high dependency care unit (HDCU) - and infirmary - emergency room, maternity and oncology sector - from two hospitals in the city of Salahaldeen, Iraq. The study followed the pattern of a sample collected per patient, except in cases in which the same individual carried different species of *Enterococcus* or when samples of the same species showed divergent results in antimicrobial susceptibility tests. The samples were stored at -20ºC in the form of a suspension containing 10% (w/v) of Skim Milk and 10% (v/v) of glycerol. In order to carry out the experiments, the samples were previously reactivated through seeding by exhaustion in a 5% sheep blood agar of commercial origin and then incubated for 18h - 24h at a temperature of 36°C ± 1. The present study offered little risk to the patients included here, since the collection of biological material was included in the routine of hospital institutions, as part of the necessary diagnostic procedures. Thus, there was a waiver of the Free and Informed Consent Term (FICT). In order to preserve patient data, when available, this study was approved by the ethics committee of Shirqat General Hospital and Salahaldeen Teaching Hospital.

3.3 Inclusion/Exclusion Criteria

Women aged 16 years and over were selected for this study with symptoms of UTI, both outpatients and inpatients. Women who did not accept to participate in the research, under 16 years of age, and men with UTI of any age group were excluded. In this study, recurrent UTIs were considered to be one or more previous episodes of UTI reported by the patient and/or the finding of previous infection in the analysis of the medical records, and non-recurrent, the absence of previous infection.

3.4 Data Collection Instrument

Outpatients were invited to participate in the research and, in case of agreement, they signed the Free and Informed Consent Form applying a questionnaire about personal data, background and current complaints, in the time of delivery of urine. Regarding hospitalized patients, with a positive laboratory diagnosis for UTI, the questionnaire was administered at the bedside.

3.5 Microbiological Analysis of Urine

The microbiological results of the positive urine cultures were obtained at the Microbiology Laboratory of the Shirqat General Hospital and Salahaldeen Teaching Hospital. The procedures performed from the collection of clinical specimens to the performance of the antibiogram are described below.

3.5.1 Collection of samples

Urine samples were obtained from outpatients and/or hospitalized at the Shirqat General Hospital and Salahaldeen Teaching Hospital. The collection was carried out in the different clinics of the hospital, in the case of inpatients or in the case of outpatients, at their home, according to the antisepsis care recommended by the hospital laboratory.

Immediately after collection, the material was immediately sent to the Shirqat General Hospital and Salahaldeen Teaching Hospital Microbiology Laboratory for processing.

3.5.2 Isolation of microorganisms

Urine samples were plated for colony counts on CLED agar (Cystine Lactose Electrolyte Deficient Agar) with a calibrated loop of 1/1000 for cloudy urine and 1/100 for clear urine, and the plates were incubated at 37°C for 24 hours. The reading was performed after this period and samples with a count above 100,000 CFU/mL were considered positive (Sanitária-Anvisa 2001).

3.6 Genetic Study of the Virulence of Enterococcus Samples

The search for genes encoding pathogenic factors in *Enterococcus* samples was performed by PCR, using primers described in previous studies (Abd-Alrazzaq and Faisal 2022). Positive and negative controls (mix without addition of DNA) were used in all reactions. For genes *gelE, cylA, cylM, efaAfs, efaAfm, cpd, cob, ccf* and *esp,* the amplification reaction was performed in a final volume of 25 µL, containing 60 ng of DNA, 0.4 µM of each primer, 200 µM of dNTP, 0.02 U/µL Taq DNA polymerase, 1.5 mM $MgCl_2$ and 1X buffer. Amplification was performed according to (Eaton and Gasson 2001) model.

For the *ace* gene, the amplification reaction was performed in a final volume of 25 µL, containing 60 ng of DNA, 0.4 µM of each primer, 800 µM of dNTP, 0.04 U/µL of Taq DNA polymerase, 1.5 mM $MgCl_2$ and 1X buffer. Amplification was performed under the following conditions: 30 cycles at 94 °C for 60 s, 56 °C for 60 s and 72 °C for 60s (Mannu *et al.* 2003). For the *hyl* gene, the amplification reaction was performed in a final volume of 25 µL, containing 60 ng of DNA, 0.1 µM of each primer, 200 µM of dNTP, 0.05 U/µL of Taq DNA polymerase, 1.5 mM $MgCl_2$ and 1X buffer (Vankerckhoven *et al.* 2004). Amplification was performed under the following conditions: 30 cycles at 94 °C for 60 s, 56 °C for 60 s and 72 °C for 60 s (Klare *et al.*

2005). For the *cylLl* and *cylLs* genes, the amplification reaction was performed in a final volume of 25 µL, containing 60 ng of DNA, 0.5 µM of each primer, 100 µM of dNTP, 0.05 U/µL of Taq DNA polymerase, 2.5 mM $MgCl_2$ and 1X buffer. Amplification was performed under the following conditions: initial denaturation at 94 °C for 3 min, 35 cycles at 94 °C for 60 s, 55 °C for 60 s and 72 °C for 2 min, followed by final extension at 72 °C for 7 min (Semedo *et al.* al., 2003) (Table 3.1).

Table 3.1 List of primers used in amplification reactions to search for genes associated with virulence in *Enterococcus* samples

Gene	GENE PRODUCT / FUNCTION	Sequence (5'→3')	AMPLICON (BP)
gelE	gelatinase	F: ACC CCG TAT CAT TGG TTT R: ACG CAT TGC TTT TCC ATC	419
cylM	Cytolysine (post-translational modifier)	F: CTG ATG GAA AGA AGA TAG TAT R: TGA GTT GGT CTG ATT ACA TTT	742
cylA	Cytolysine (activating component)	F: TGG ATG ATA GTG ATA GGA AGT R: TCT ACA GTA AAT CTT TCG TCA	517
esp	surface protein	F: TTG CTA ATG CTA GTC CAC GAC C R: GCG TCA ACA CTT GCA TTG CCG AA	933
efaAfs	Adhesion protein (*E. faecalis*)	F: GAC AGA CCC TCA CGA ATA R: AGT TCA TCA TGC TGT AGT A	705
efaAfm	adhesion protein (E. faecium)	F: AAC AGA TCC GCA TGA ATA R: CAT TTC ATC ATC TGA TAG TA	735
cpd	sex pheromone	F: TGG TGG GTT ATT TTT CAA TTC R: TAC GGC TCT GGC TTA CTA	782
cob	sex pheromone	F: AAC ATT CAG CAA ACA AAG C R: TTG TCA TAA AGA GTG GTC AT	1405
ccf	sex pheromone	F: GGG AAT TGA GTA GTG AAG AAG R: AGC CGC TAA AAT CGG TAA AAT	543
ace	Accessory colonization factor (collagen adhesin)	F: AAA GTA GAA TTA GAT CCA CAC R: TCT ATC ACA TTC GGT TGC G	320
hyl	Hyaluronidase	F: GAG TAG AGG AAT ATC TTA GC R: AGG CTC CAA TTC TGT	661
cylLl	Cytolysine (structural unit)	F: GAT GGA GGG TAA GAA TTA TGG R: GCT TCA CCT CAC TAA GTT TTA TAG	253
cylLs	Cytolysine (structural unit)	F: GAA GCA CAG TGC TAA ATA AGG R: GTA TAA GAG GGC TAG TTT CAC	240

3.7 Genetic Study of Antimicrobial Resistance of Enterococcus Samples

The search for genes encoding antimicrobial drug resistance and pathogenicity factors in *Enterococcus* samples was performed by PCR, using primers and conditions described in previous studies (Abd-Alrazzaq and Faisal 2022). Positive and negative controls (mix without addition of DNA) will be used in all reactions (Table 3.2).

Table 3.2 Amplification reactions to search for genes encoding antimicrobial drug resistance in *Enterococcus*

Target	Mix composition	Sequence (5'→3')	Reaction Conditions	AMPLICON (BP)
TetM	Primer erm(A):0.5µm erm(B):0.5µm mef(A/E):0.2 µm tet (M):0.4 µm tet(O):0.3 µm dNTP:0.2 mM MgCl2:1.5 mM Tag DNA polymerase : 2 U Primer:6.5 mg/ml dNTP:0.2 mM MgCl2:1.5 mM Tag DNA polymerase: 0.25 U/µL Primer:0.4 µM dNTP:0.2mM MgCl2:1.5 mM Tag DNA polymerase: 0.4 U	F: GTG GAC AAA GGT ACA ACG AG R: CGG TAA AGT TCG TCA CAC AC	93 °C 3 min	406
TetO		F: AAC TTA GGC ATT CTG GCT CAC R: TCC CAC TGT TCC ATA TCG TCA		515
TetL		F: TGG TGG AAT GAT AGC CCA TT R: CAG GAA TGA CAG CAC GCT AA	30x /93 °C, 1 min	229
ErmA		F: CCC GAA AAT ACG CAA AAT TTC AT R: CCC TTT TAC CCA TTT ATA AAC G	62 °C, 1 min 65 °C, 4 min	590
ErmB		F: TGG TAT TCC AAA TGC GTA ATG R: CTG TGG TAT GGC GGG TAA GT	65 °C, 3 min	745
mefA/E		F: CAA TAT TCC GGG CAG GGC AAG R: AAG CTG TTC CAA TGC TAC GG	35x / 94 °C, 1 min	317
Ant(4")-la		F: CTG CTA AAT CGG TAG AAG C R: CAG ACC AAT CAA CAT GGC ACC	55 °C, 1 min/ 72 °C, 1 min	294
VanA		F: GGG AAA ACG ACA ATT GC R: GTA CAA TGC GGC CGT TA	94 °C, 2 min /52 °C, 2 min / 72 °C , 2 min	732
vanB		F: ATG GGA AGC CGA TAG TC R: GAT TTC GTT CCT CGA CC	29x ,94°C ,25s/52 °C, 40s/72°C,50s	635

*Targets: tetM/O/L, tetracycline, ermA/B, erythromycin, mefA/E, erythromycin, ant(4')-Ia, aminoglycosides, vanA/B, vancomycin)

3.8 Evaluation of Gelatinase and Cytolysin/Hemolysin Production

To evaluate gelatinase production, the bacteria were inoculated, in streaks, on nutrient agar with 3% gelatin and the material was incubated at 37 ºC for 18 h. Then, the cultures were stored at 4 °C for 5 h, and then the reading was performed by evaluating the presence of clear zones around the colonies, indicative of gelatin hydrolysis, resulting from the action of gelatinase (Sousa *et al.* 2014).

To investigate the production of cytolysin/hemolysin, the samples were inoculated, also in streaks, in TSA medium plus 5% horse blood. After incubation at 37 ºC for 24 h, the reading was performed, by verifying the presence of clear zones around the colonies, indicative of hemolysis, resulting from the action of hemolysin (Sousa *et al.* 2014).

3.9 Evaluation of the Susceptibility Profile to Antimicrobial Drugs of the Samples

The evaluation of the antimicrobial susceptibility profile of the samples under study was performed using the agar diffusion method, according to (CLSI 2015) specifications. Colonies of *Enterococcus* obtained in TSA were transferred to tubes containing saline solution (0.85% NaCl) until turbidity corr*esp*onding to the McFarland scale 0.5. Then, the bacterial suspensions were seeded over the entire surface of the Mueller Hinton30 Agar with the aid of a sterile swab. After about 5 min, commercial discs impregnated with 30 Difco antimicrobial agents (Table 3.3) were deposited on the surface of the culture medium, keeping a minimum distance of 24 mm between the centers of the discs. The reading was performed after 16-18 h of incubation (24 h for vancomycin), at 37ºC, by measuring the diameters of the inhibition halos of the bacterial samples and comparing them with the classification recommended by the CLSI (Table 3.3). As a control, the sample *S. aureus* ATCC 25923 was used.

Table 3.3 Antimicrobials, concentrations and standard of interpretation for Enterococcus spp., according to the (CLSI 2015)

			HALO DIAMETER (mm)		
ANTIMICROBIAL	**Symbol**	**CLASS**	**SENSITIVE**	**INTERMEDIARY**	**RESISTANT**
Ampicillin 10 µg	APMC	penicillins	≥ 17	-	≤ 16
Ciprofloxacin 30 µg	CFX	fluoroquinolones	≥ 21	16-20	≤ 15
Chloramphenicol 30 µg	CLMP	Phenicol	≥ 18	13-17	≤ 12
Erythromycin 15 µg	ERYT	macrolides	≥ 23	14-22	≤ 13
Levofloxacin 5 µg	LFX	fluoroquinolones	≥ 17	14-16	≤ 13
Linezolid 30 µg	LNZ	oxazolidinones	≥ 23	21-22	≤ 20
Penicillin G 10 I.U.	PEN-G	penicillins	≥ 15	-	≤ 14
Tetracycline 30 µg	TETC	Tetracycline	≥ 19	15-18	≤ 14
Vancomycin 30 µg	VANC	glycopeptide	≥ 17	15-16	≤ 14

To be classified as multidrug-resistant (MDR), the sample must show resistance to at least three antimicrobial agents from at least three different classes, as defined by Abamecha *et al.* (2015).

3.10 Search for Integrons in Enterococcus Samples

For the investigation of integrons and gene cassettes associated with antimicrobial drug resistance, the primers described in Table 3.4 were used.

Table 3.4 List of primers used in the amplification reaction to search for intI1 and intI2 genes (genes that encode integrases)

Gene	SEQUENCE (5'→3')	**AMPLICON (BP)**
intI1	F: ACG GCA AGC AGG CGG TTT T	565
	R: GAA TCT AGG GGT ACA CAT TG	
intI2	F: TTG GTG CGC CAA ATT CAG G	403
	R: CGG CAA AGT GCA CAT GAT G	

The amplification reaction was performed in a final volume of 25 µL, containing 60 ng of DNA, primer (4 µM for intI1 primers, 3 µM for intI2 primers), 800 µM of dNTP, 0.025 U/µL of Taq DNA polymerase, 1.5 mM $MgCl_2$ and 1X buffer. Amplification was performed under the following conditions: an initial denaturation cycle at 94°C for 5

min, 30 cycles at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 2 min and a final extension cycle at 72 °C for 7 min. Positive and negative controls (reaction mix without the addition of DNA) were included in all reaction lots.

3.11 PCR/RAPD-PCR analysis and genetic sequencing

The stage of genetic identification of lactic acid bacteria was carried out using RAPD-PCR, species-specific PCR and genetic sequencing methods, in the laboratories Shirqat General Hospital and Salahaldeen Teaching Hospital in order to confirm the species and subspecies. The methodology for the isolation and morphological characterization followed the protocol used in the technique at 37°C.

3.11.1 Revitalization of strains

The strains were revitalized by the technique of striation of colonies in petri dishes with specific culture media (M17-BioLife for *Enterococcus* and MRS-BioLife® for Lactobacilli) and incubated at 44 °C for 48 hours under aerobic conditions for *Enterococcus*. After revitalization, the strains were stored in a glycerol solution (80%) and subsequently frozen at -80 °C according to the following steps:

- "Over-night" (o/n) of the strains: a colony from each plate, which had growth, was inoculated into test tubes with 10 mL of the specific broths, leaving them multiply under suitable temperatures overnight.

- After this period, 2 mL of each tube were centrifuged (13200 rpm for 5 minutes) in order to precipitate the cells.

- The supernatant was discarded and the cell mass was resuspended in 0.25 mL of the specific broth (MRS or M17) together with 0.25 mL of the "freezing mix" (20 mM $MgSO_4$, 80% glycerol), and 0.5 mL of 10% skimmed milk powder (BioLife), sterilized by autoclaving at 121 °C for 15 minutes. After homogenization, the strains

were stored at -80°C (stock culture) until the RAPD-PCR and PCR-Species-Specific analysis were performed.

3.11.2 Preparation of stock strains for molecular techniques

Sterilely, a portion of each stock culture was inoculated aseptically into 10 mL tubes containing the specific broths (MRS and M17) and incubated overnight at specific temperatures and conditions for each strain.

3.11.3 DNA extraction from culture plates

1. Purely obtained enterococci colonies were suspended in distilled water in Mc Farland 5 turbidity.

2. 500µl of the prepared bacterial suspension was taken into an Eppendorf tube and centrifuged at 8000 xg for 3 minutes at room temperature (HERAEUS Biofuge Primo R).

3. The supernatant was discarded and 500 µl of TE buffer (10mM TrisHCl, 0.1mM EDTA, pH7.5) was added to the pellet.

4. Then it was kept at 100oC for 10 minutes in a dry heat block (BİOSAN Termoblock TDB-120).

5. 150 µl beads (SIGMA) were placed in 500 µl bacterial suspension and mechanically lysed for 7 minutes at maximum speed (10) in a Mickle device (Mickle Tissue Disintegrator, Mickle Labratory Engineering Co. Ltd.).

6. Centrifuged at 12,000 xg for 10 minutes.

7. 200 µl of supernatant was taken and transferred to a new ependorf. DNA quantitation was done in a spectrophotometer (CHEBIOS s.r.l. Optimum-One UV-VIS Spectrophotometer) and stored at -20oC until used as a template in the PCR amplification process.

3.11.4 Amplification with S13 primer

DNA extracts from enterococci isolates were used for random amplification using primer S13 (5'-TTCGCCCCC T-3') prepared from short oligos. Amplification, 2.5 µl of 10x PCR buffer, 3.5 mM $MgCl_2$, 200 µM of each dNTP, 0.8 µl of primer (from 100pmol/µl stock solution), 3 U of Taq polymerase and 3 µl of template DNA (100ng/µl) and a total of 25 µl of sterile distilled water was performed in the PCR mix completed to the amplification steps were completed in the thermal cycler (APPLIED BIOSYSTEMS 2720 Thermal Cycler) in the following cycle order:

5 min at 94 ^{o}C. first denaturation

50 sec at 94 ^{o}C. denaturation

1 min at 37^{o}C. annealing

75 seconds extension at 72 ^{o}C

10 min at 72^{o}C. final elongation

45 cycles

3.11.5 Detection of fragments by agarose gel electrophoresis

In order to show the presence of fragments of different sizes formed after the amplification process, the amplicons were subjected to migration separation in agarose gel in an electrical environment. This process was done as follows,

1. A 2% agarose gel was prepared with 1xTBE solution to be used as an electrophoresis matrix.

a. 100 mL of 1xTBE was added to 2 g of agarose (Sigma Agarose A5093- 500G), which was weighed into a balloon.
b. The mixture was melted in the microwave oven until homogenization was achieved.
c. It was cooled by keeping it at room temperature not to fall below 60 °C.
d. A final concentration of 0.5 mg/mL was obtained by adding 5 µl of 10 mg/mL stock solution of ethidium bromide into molten agarose.
e. The prepared 2% agarose gel containing 0.5% ethidium bromide was slowly poured onto the gel mold tray, which was prepared in advance and the combs were placed accordingly.
f. The gel mold was allowed to solidify by keeping it at room temperature for 20-30 minutes.
g. The combs were slowly removed by placing the gel mold in the electrophoresis tank (OWL Separation Systems Model B2 Mini Gel Electrophoresis System).

2. 15 µl of each sample was taken and mixed with 3 µl of 6x concentration loading buffer prepared with 20% sucrose and 0.25% bromophenol blue on parafilm. first and DNA marker (Fermentas O'Range Ruler, 100bp Ladder, Lot: 0302) was loaded into well 7 and 18 µl of this mixture was loaded into the other wells as a sample for each.

3. The power supply of the tank (LABNET International Power Station 300) was started and 120 V current was given. Electrophoresis was stopped when the dye reached 2/3 of the gel by following the migration of bromophenol blue in the gel.

3.11.6 Analysis of imaging and band profiles

1. After electrophoresis, the gel was visualized under UV light. DNA band images were photographed using the Gel logic 1500 imaging system (Kodak Company, NY, USA). Pictures were saved in TIFF format.

2. Band profiles were analyzed using the GelCompar II software system (version 5.0, Applied Maths, Sint-Martens-Latem, Belgium). First of all, normalization was performed between the pictures with the help of 2 standards (executed in the 1st and 7th wells) in each picture. Using the "Unweighted pair group method with mathematical averaging (UPGMA)", the dendrogram of the band profiles was created and aggregation analysis was performed. The relationship between the strains was determined according to the "Dice" similarity coefficient depending on the bands. In the calculation of the similarity coefficient, the band and profile tolerance were taken as 1.5%.

3.11.7 Purification of the PCR product

Purification of the PCR product is necessary in order to eliminate primers and dNTPs (deoxynucleotide triphosphates) not used in the reaction. The ExoSAP-IT kit (GE Healthcare) was used, which contains exonuclease I + alkaline phosphatase enzymes. The process was performed in PCR microtubes by mixing 5 μL of the PCR reaction with 2 μL of the ExoSAP-IT kit solution. The microtubes were incubated in the thermocycler with the following cycle: 37 °C/15 minutes + 80 °C/15 minutes and then 8 °C. The purified product can be used immediately or stored at -20°C.

3.11.8 Purification of the sequencing product

The product obtained from the sequencing reaction was purified in order to eliminate BigDye dNTPs (deoxynucleotide triphosphates) not incorporated in the reaction. The methodology used was based on purification through the use of ethanol / EDTA / sodium acetate, according to the following steps:

a) 20 μL of each PCR microtube was transferred to 1.5 mL microtubes, keeping them in aluminum foil to protect from light.

b) 2 μl of 125 mM EDTA pH 8.0 and 2 μl of 3M Sodium Acetate/pH 5.6 were added.

c) 50 µL of absolute ethanol was added and the tubes were vortexed.

d) It was left at room temperature for 15 minutes.

e) Centrifuge at 13200 rpm x 15 minutes.

f) The supernatant was discarded by inversion of the tube, adding 100 µl of 75% ethanol.

g) Centrifuged at 13200 rpm x 10 minutes.

h) The supernatant was removed by inverting the tube and adding 70 µl of 75% ethanol.

i) Centrifuge at 13200 rpm x 10 minutes.

j) The supernatant was removed with a micropipette.

k) The sediment was allowed to dry under a laminar flow hood for at least 30 minutes.

l) Centrifuge at 13200 rpm x 10 seconds.

m)The pellet was resuspended in 15 µl of ultrapure water.

3.12 Ethical Considerations

This study was approved by the Ethics Committee in Human and Animal Medical Research of the Shirqat General Hospital and Salahaldeen Teaching Hospital in the city of Salahaldeen, Iraq.

3.13 Database and Statistical Analysis

The program used was Excel (Microsoft Office 2010) to set up a database obtained from the questionnaires, as well as the microbiological results (clinical specimen, isolated microorganism and susceptibility profile to antimicrobials). The associated factors for infection and the microbiological results, among individuals from the community and the hospital environment, were performed through the analysis of the X^2 test to compare the percentage values (qualitative variables). For values where $n \leq 5$, the Fisher's exact test was used. Statistical significance was defined as a p-value < 0.05.

4. RESULTS AND DISCUSSION

4.1 Epidemiological Data

Of the patients at the Hospitals who agreed to participate in the research, 100 women aged between 17 and 87 years were interviewed, resulting in 100 (100%) positive urine cultures, being 68 (68%) outpatients and 32 (32%) hospitalized.

4.2 Factors Associated with UTI

Using the questionnaire applied to the women and analysing the medical records, it was possible to verify some factors associated with UTI. It was shown that menopause (45%), hypertension (20%) and diabetes (6%) and haemodialysis (7%) were the most frequent (Table 4.1).

Table 4.1 Frequency and percentage of factors associated with Urinary Tract Infection in women treated at Shirqat General Hospital and Salahaldeen teaching Hospital

General features	**n**
Menopause	45
Hypertension	20
Diabetes	6
Hysterectomy	2
smoking	1
Pregnancy	2
Chemotherapy	3
hemodialysis	7
Rheumatism	2
Urinary incontinence	2
cholelithiasis	3
CRI	1
HPV	1
Syphilis	1
diabetic nephropathy	1
Bilateral Nephrostomy	1
Urinary retention	1
pyelonephritis	1
Total	100

n: number of samples, CRI: Chronic renal failure, HPV: Human Papilloma Virus among the variables analysed for patients with UTI infection or not, we found that there were no statistically significant differences for menopause, hypertension, hysterectomy, and smoking. The only risk factor for recurrent UTI in this population was the presence of diabetes in patients ≥ 50 years (Table 4.2).

Table 4.2 Analysis of factors associated with UTI in outpatient and hospitalized women over 16 years of age, treated at Shirqat general hospital and Salahaldeen teaching Hospital

Variables	Age Group	UTI (n= 100)
Menopause	<50 years	6
	≥50 years	39
Hypertension	<50 years	4
	≥50 years	16
Diabetes	<50 years	3
	≥50 years	3
Chemotherapy	<50 years	1
	≥50 years	2
HPV	<50 years	1
	≥50 years	0
Haemodialysis	<50 years	2
	≥50 years	5

4.3 Sampling

This study comprised 100 bacterial samples that were obtained from two hospitals in the city of Salahaldeen, 59% of these samples coming from Shirqat General Hospital and 41% from Salahaldeen Teaching Hospital. Among the 100 samples, 75% were collected from clinical specimen's indicative of infection, 39 from SGH and 36% from STH. 25% samples were indicative of colonization, among these, 20 were isolated from patients from Shirqat General Hospital and only 5 from Salahaldeen Teaching Hospital, as shown in Figure 4.1.

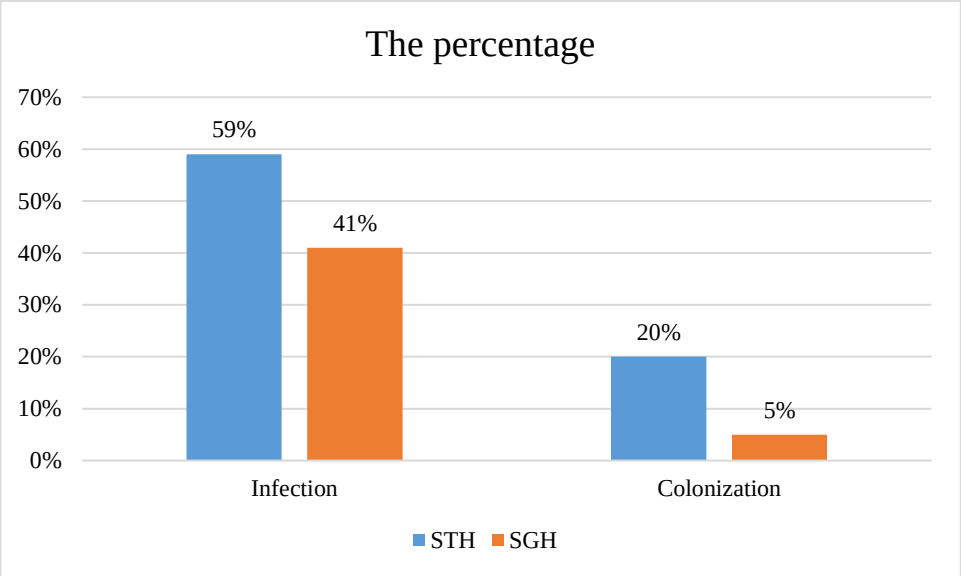

Figure 4.1 Distribution of *Enterococcus* samples according to source of isolation (infection and colonization) and institution of origin

Regarding the hospital sectors where the samples originated, 69% of them were isolated from intensive monitoring units in Shirqat General Hospital. In this same hospital, 18% of the samples were obtained from the ward and a portion of 13% have an unknown sectoral origin. Regarding the sectoral origin of the samples from STH 40% were obtained from intensive monitoring units, while 47% were isolated from ward patients and 14.0% had unknown origin.

The use of the MALDI-TOF MS technique allowed the identification of four species of the genus *Enterococcus* among the samples, with *E. faecalis* being the predominant species, representing 73% of all bacterial samples that make up this study. The second most frequent species in the study, in general, was E. faecium with 23% samples. In smaller amounts, three samples 2% of *E. avium* and 2% of *E. gallinarum* were isolated. Table 4.3 shows the distribution of samples by hospital unit of origin.

Table 4.3 Distribution of samples according to species and institution of origin

Species	Shirqat General Hospital	STH	Total samples by species
E. faecalis	40 (66.66%)	34(85%)	74(74%)
E. faecium	18 (30%)	4(10%)	22(22%)
E. avium	1(1.66%)	1 (2.55%)	2(2%)
E. gallinarum	1(1.66%)	1 (2.55%)	2(2%)
Total (n)	60 (100%)	40(100%)	100(100%)

Regarding the distribution of species according to the hospital sector of origin, it can be observed that most *E. faecalis* samples 50.6% were isolated from patients hospitalized in intensive monitoring units, while 31.2% samples of this species came from the infirmary. However, it should be noted that 18.2% of these have an unknown sectoral origin. Among the E. faecium samples, 79.2% were isolated from patients under intensive monitoring and 20.8% from the ward. Among the *E. avium* samples, 2.55% were obtained from the ward and one from intensive monitoring, while the 2.55% *E. gallinarum* samples were obtained from patients under intensive monitoring.

4.4 Susceptibility to Antimicrobials

The results of the 18 antimicrobial susceptibility tests performed with the 106 study samples, considering the CLSI (2018) interpretation criteria, are summarized in Figure 4.2, where the highest percentages of drug resistance can be observed in descending order.

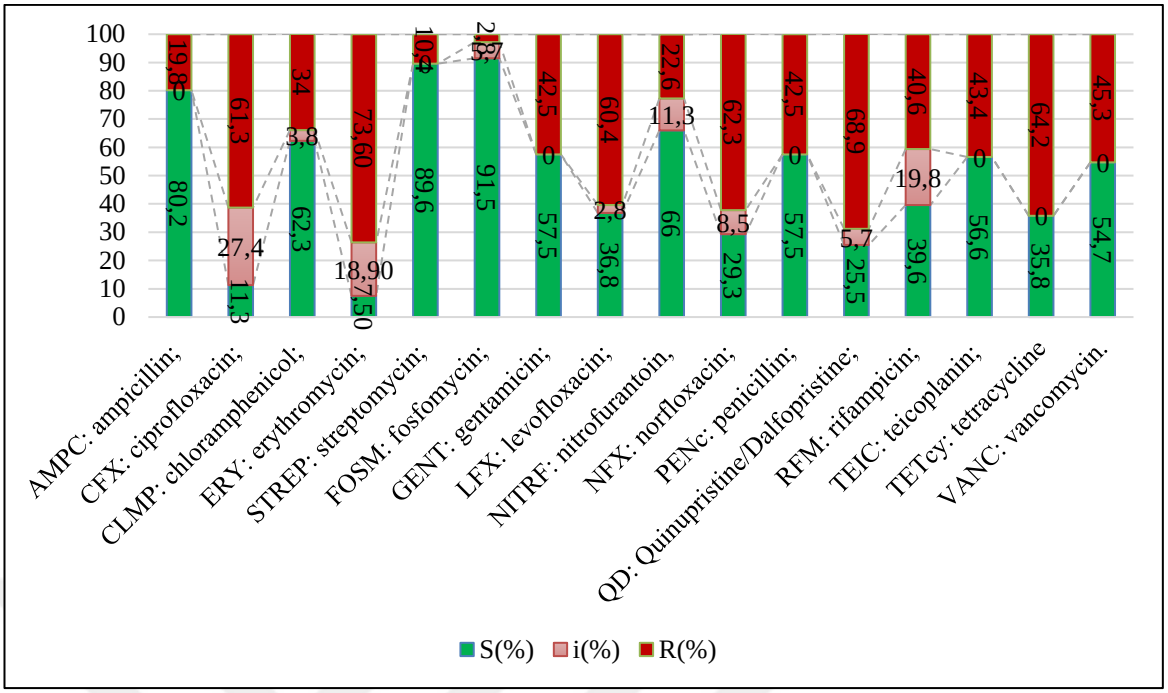

Figure 4.2 Distribution of susceptibility results of study samples to tested antimicrobials (S =sensitive, i=intermediatory, R=resistance)

It was found that the highest frequencies of resistance were obtained against antimicrobials: erythromycin (73.6%), quinupristin/dalfopristin (68.9%), tetracycline (64.2%), norfloxacin (62.2%), ciprofloxacin (61.3%) and levofloxacin (60.4%). The glycopeptides, vancomycin and teicoplanin, also reached high percentages of resistance, showing 45.3% and 43.4% of resistant samples, r*esp*ectively, to such drugs. On the other hand, the drugs linezolid and tigecycline inhibited the growth of all samples, regardless of the species, hospital sector of origin or isolation site, proving to be the most effective, followed by the drugs: fosfomycin (2.8%) and streptomycin at high levels (10.4%), with the lowest levels of resistance.

Among the 106 samples, there were samples classified as intermediate to the following drugs: ciprofloxacin (27.4%), rifampicin (19.8%), erythromycin (18.9%), nitrofurantoin (11.3%), norfloxacin (8.5%), Fosfomycin and quinupristin/dalfopristin (5.7%), chloramphenicol (3.8%) and levofloxacin (2.8%). The comparative analysis of the percentage of resistance between species (Table 4.3) revealed that *E. faecalis* samples presented higher frequencies than E. faecium, with regard to the following antimicrobials: quinupristin/dalfopristin (88.3% in *E. faecalis* versus 12.5% in E.

faecium), tetracycline (83.1% in *E. faecalis* versus 0.0% in E. faecium), gentamicin at high levels (57.1% in *E. faecalis* versus 4.2% in E. faecium) and chloramphenicol (45.5% in *E. faecalis* versus 4.2% in E. faecium).

Most *E. faecalis* samples also showed a high frequency of resistance to erythromycin (70.1%), norfloxacin (59.7%), ciprofloxacin (58.4%) and levofloxacin (57.1%), similar to those observed among the E. faecium samples, as can be seen in Table 4.4. In relation to E. faecium samples, a high frequency of resistance to ampicillin (87.5%), nitrofurantoin (83.3%), penicillin (95.8%), rifampicin (83.3%), vancomycin (70.8%) and teicoplanin (66.7%), higher than the percentages found among *E. faecalis* samples. Of the 17 (70.8%) vancomycin-resistant E. faecium samples, 16 (66.7%) were also resistant to teicoplanin. However, a single sample was sensitive to this drug, after MIC determination. Of the 21 ampicillin-resistant E. faecium samples (87.5%), all were also resistant to penicillin. *E. avium* samples showed susceptibility to the vast majority of antimicrobials tested, except for the following drugs: erythromycin (one sample), quinupristine/dalfopristine (one sample), tetracycline (two samples) and vancomycin (one sample). For the only vancomycin-resistant *E. avium* sample, according to the disk-diffusion technique, a continuous gradient diffusion test was performed with E-test® strips, in order to confirm this result, as well as the susceptibility to teicoplanin, also determined by the disk-diffusion test. The MIC data obtained for teicoplanin (8 μg/mL) confirmed its classification as sensitive to this drug and for vancomycin (≥ 256 μg/mL) as resistant. With regard to *E. gallinarum* isolates, the samples showed resistance to a variety of antimicrobials, highlighting the resistance of both to glycopeptides, to erythromycin and to tetracycline.

Table 4.4 Distribution of the percentages of resistance of the samples to antimicrobials according to the species

	RESISTANCE PERCENTAGE							
Antimicrobials	*E. faecalis (n=74)*	%	E. faecium *(n=22)*	%	*E. avium (n=2)*	%	*E. gallinarum(n=2)*	%
ampicillin	0	0.00	21	95.45	0	0.00	0	0.00
ciprofloxacin	45	60.81	19	86.36	0	0.00	1	50.00
chloramphenicol	35	47.30	1	4.55	0	0.00	0	0.00
Erythromycin	54	72.97	21	95.45	1	50.00	2	100.00
Streptomycin*	10	13.51	1	4.55	0	0.00	0	0.00
phosphomycin	0	0.00	2	9.09	0	0.00	1	50.00
Gentamicin*	44	59.46	1	4.55	0	0.00	0	0.00
levofloxacin	44	59.46	19	86.36	0	0.00	1	50.00
linezolid	0	0.00	0	0.00	0	0.00	1	50.00
nitrofurantoin	3	4.05	20	90.91	0	0.00	1	50.00
norfloxacin	46	62.16	19	86.36	0	0.00	1	50.00
Penicillin	21	28.38	23	104.55	0	0.00	1	50.00
quinupristin/dalfopristin	68	91.89	3	13.64	1	50.00	1	50.00
rifampicin	22	29.73	20	90.91	0	0.00	1	50.00
Teicoplanin	28	37.84	16	72.73	0	0.00	2	100.00
Tetracycline	64	86.49	0	0.00	2	100.00	2	100.00
tigecycline	0	0.00	0	0.00	0	0.00	0	0.00
vancomycin	28	37.84	17	77.27	1	50.00	2	100.00

**high levels*

Of the 100 specimens of the *Enterococcus* genus that compose the sample of this study, 78.3% presented multi-resistance to antimicrobials. Among the 22 E. faecium samples, 21 were classified as multidrug-resistant, distributed in 19 different non-susceptibility profiles, which can be seen in Table 4.5 ,4.6. Regarding the *E. faecalis* samples, 74.0% exhibited multi-resistance to the tested drugs, divided into 30 non-susceptibility profiles, one of which was characteristic of 18.2% samples of this species. Among the three samples of *E. avium*, only one showed to be MDR, while both samples of *E. gallinarum* showed this attribute.

Table 4.5 Distribution of antibiotics in the 80 samples classified as multidrug-resistant according to the Enterococcus species in the study

Antibiotics	n	Bacterial Species
AMPC(+), CFX(+), ERY(+), LFX(+), NITRF(+), NFX(+), PENc(+), RFM(+), TEIC(+), VANC(+)	5	*E. faecium*
AMPC(+), CIPI, ERY(+), NITRF(+), NORI, PENc(+), QD(+), RFM(+)	1	
CIPI, ERII, NITRF(+), PENc(+), QD(+), RFM(+)	1	
AMPC(+), CFX(+), ERY(+), STREP(+), LFX(+), NITRF(+), NFX(+), PENc(+), RFM(+), TEIC(+), VANC(+)	1	
AMPC(+), CFX(+), ERY(+), FOSM(+), LFX(+), NITRF(+), NFX(+), PENc(+), RFM(+), TEIC(+), VANC(+)	1	
CIPI, ERII, NITRF(+), NORI, PENc(+), QD(i), RFM(+)	1	
AMPC(+), CFX(+), CLOI, ERY(+), FOSM(i), LFX(+), NITRF(+), NFX(+), PENc(+), QD(+), RFM(+), TEIC(+), VANC(+)	1	
AMPC(+), CFX(+), CLMP(+), ERY(+), FOSM(+), GENT(+), LFX(+), NITRF(+), NFX(+), PENc(+), RFM(+), TEIC(+), VANC(+)	1	
AMPC(+), CFX(+), ERY(+), FOSM(+), LFX(+), NITRF(+), NFX(+), LFX(+), RFM(+), TEIC(+), VANC(+)	1	
AMPC(+), CFX(+), ERY(+), LFX(+), NITRF(i), NFX(+), PENc(+), RIFI, TEIC(+), VANC(+)	1	
AMPC(+), CFX(+), ERY(+), FOSM(+), LFX(+), NITRF(+), NFX(+), PENc(+), RFM(+)	1	
ERY(+), NITRF(+), RFM(+)	1	
AMPC(+), CFX(+), ERY(+), LFX(+), NITRF(+), NFX(+), PENc(+), RIFI, TEIC(+), VANC(+)	1	
AMPC(+), CFX(+), ERY(+), LFX(+), NITRF(+), NFX(+), PENc(+), TEIC(+), VANC(+)	1	
AMPC(+), CFX(+), ERY(+), FOSM(i), LFX(+), NITRF(+), NFX(+), PENc(+), RFM(+)	1	
AMPC(+), CFX(+), ERY(+), LFX(+), NFX(+), PENc(+), RFM(+), TEIC(+), VANC(+)	1	
AMPC(+), CFX(+), ERY(+), LFX(+), NITRF(i), NFX(+), PENc(+), QD(+), RFM(+), VANC(+)	1	
AMPC(+), CFX(+), ERY(+), LFX(+), NITRF(+), NFX(+), PENc(+), RFM(+), VANC(+)	**1**	

Table 4.6 Distribution of antibiotics in the 80 samples classified as multidrug-resistant according to the Enterococcus species in the study

Antibiotics	n	Bacterial Species
CFX(+), CLMP(+), ERY(+), GENT(+), LFX(+), NITRF(+), NFX(+), QD(+), RFM(+), TEIC(+), TETcy(+), VANC(+)	1	*E. faecalis*
CIPI, ERII, LFX(i), NITRF(+), NORI, RFM(+), TETcy(+)	1	
CLOI, ERY(+), GENT(+), NITRF(i), QD(+), RFM(+), TETcy(+)	1	
CFX(+), CLMP(+), ERY(+), GENT(+), LFX(+), NFX(+), PENc(+), QD(+), TEIC(+), TETcy(+), VANC(+)	13	
CFX(+), ERY(+), GENT(+), LFX(+), NFX(+), QD(+), RIFI, TETcy(+)	4	
CFX(+), CLMP(+), ERY(+), GENT(+), LFX(+), NITRF(i), NFX(+), PENc(+), QD(+), RFM(+), TETcy(+)	1	
CIPI, CLMP(+), ERY(+), STREP(+), NITRF(i), QD(+), RFM(+), TETcy(+)	1	
CIPI, CLOI, ERII, NITRF(+), QD(+), RFM(+), TETcy(+)	1	
CFX(+), ERY(+), GENT(+), LFX(+), NFX(+), PENc(+), QD(+), RFM(+), TETcy(+)	1	
CIPI, CLMP(+), ERY(+), STREP(+), NITRF(+), NORI, QD(+), RIFI, TETcy(+)	1	
CFX(+), ERY(+), QD(+), RFM(+)	1	
CFX(+), CLMP(+), ERY(+), GENT(+), LFX(+), NFX(+), QD(+), RIFI, TEIC(+), TETcy(+), VANC(+)	3	
CFX(+), CLMP(+), ERY(+), GENT(+), LFX(+), NFX(+), PENc(+), QD(+), RIFI, TEIC(+), TETcy(+), VANC(+)	3	
CFX(+), CLMP(+), ERY(+), GENT(+), LFX(+), NFX(+), QD(+), TEIC(+), TETcy(+), VANC(+)	4	
CFX(+), NFX(+), PENc(+), QD(+), TEIC(+), TETcy(+), VANC(+)	1	
CIPI, CLMP(+), ERY(+), STREP(+), QD(+), RFM(+), TETcy(+)	4	
CIPI, ERY(+), GENT(+), QD(+), RFM(+), TETcy(+)	1	
CIPI, ERY(+), STREP(+), NFX(+), QD(+), TETcy(+)	1	
CIPI, ERY(+), STREP(+), QD(+), RFM(+), TETcy(+)	1	
ERY(+), STREP(+), GENT(+), TETcy(+)	1	
CIPI, ERY(+), STREP(+), QD(+), TETcy(+)	1	
CFX(+), CLMP(+), ERY(+), LFX(+), NFX(+), QD(+), RFM(+), TETcy(+)	1	
CFX(+), ERY(+), STREP(+), LFX(+), NFX(+), QD(+), RFM(+), TETcy(+)	1	
ERY(+), GENT(+), QD(+), TETcy(+)	1	
CFX(+), CLMP(+), ERY(+), FOSM(i), GENT(+), LFX(+), NFX(+), PENc(+), QD(+), TEIC(+), TETcy(+), VANC(+)	1	
CFX(+), CLMP(+), ERY(+), GENT(+), NFX(+), QD(+), RIFI, TETcy(+)	1	
CFX(+), ERY(+), GENT(+), LFX(+), NFX(+), QD(+), RFM(+), TETcy(+)	2	
CFX(+), CLMP(+), ERY(+), GENT(+), LFX(+), NFX(+), QD(+), TETcy(+)	1	
CFX(+), ERII, GENT(+), LFX(+), NFX(+), QD(+), TETcy(+)	1	
CIPI, ERY(+), QD(+), TETcy(+), VANC(+)	1	*E. avium*
ERY(+), QD(+), TEIC(+), TETcy(+), VANC(+)	1	*E. gallinarum*
CFX(+), ERY(+), FOSM(+), LFX(+), NITRF(+), NFX(+), PENc(+), RFM(+), TEIC(+), TETcy(+), VANC(+)	1	

(+): resistant, (i): intermediate, AMPC: ampicillin, CFX: ciprofloxacin, CLMP: chloramphenicol, ERY: erythromycin, STREP: streptomycin, FOSM: fosfomycin,

GENT: gentamicin, LFX: levofloxacin, NITRF: nitrofurantoin, NFX: norfloxacin, PENc: penicillin, QD: Quinupristine/Dalfopristine, RFM: rifampicin, TEIC: teicoplanin, TETcy: tetracycline and VANC: vancomycin.

4.5 Characterization of Genotypes Associated with Virulence in Enterococcus

The 100 samples of the study were selected for genotypic research of the main virulence factors of *enterococci*, through the PCR-Multiplex technique, namely: cytolysin (*cylA*), gelatinase (*gelE*), aggregating substance (*asa1*), enterococcal surface protein (*esp*) and glycosyl hydrolase (*hyl*).

With the result, it was observed that the most frequent genetic determinant of virulence among the samples was *gelE*, found in 63.2% of the samples, followed by *asa1*, present in 61.3%, *cylA* and *esp* in 44.3% and, to a lesser extent, the *hyl* gene, detected in only 11.3% samples, as seen in Figure 4.3.

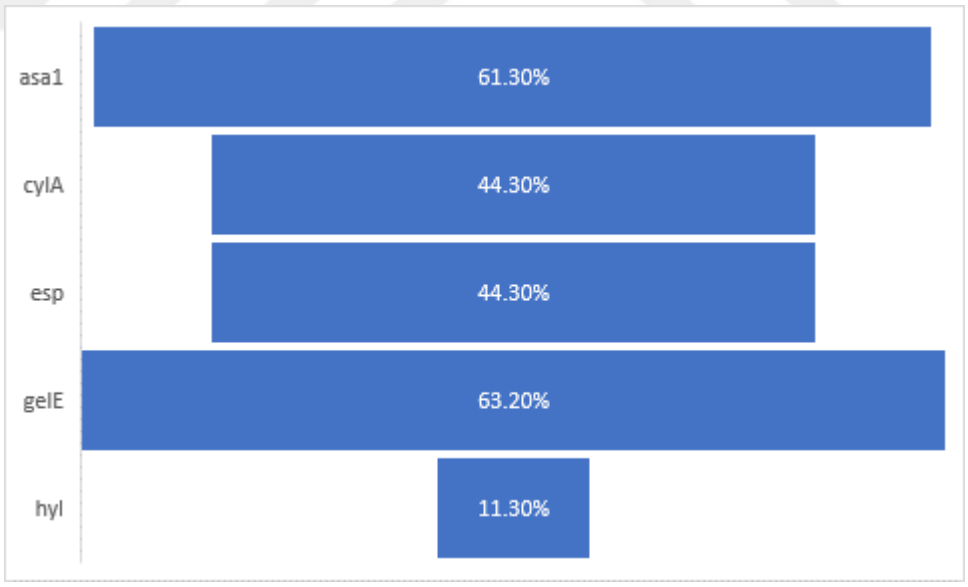

Figure 4.3 Distribution of genetic virulence determinants among study samples

Regarding the distribution of the main virulence genes among the species, and according to the institution of origin of the samples (Table 4.7), it was observed that the

genetic determinants asa, *cylA* and *gelE* were only present in samples of *E. faecalis*. The *esp* gene was present in different species, with 14 samples of *E. faecalis*, 10 samples of E. faecium and one sample of *E. avium*. While the *hyl* gene, on the other hand, was found exclusively among 6 E. faecium samples. Among the *E. gallinarum* samples, none of the investigated genetic determinants of virulence was detected.

Table 4.7 Distribution of genetic virulence determinants according to the species and institution of origin of the sample

	Shirqat General Hospital				**STH**		
Genetic determinants of virulence	E. faecalis (n/%)	E. faecium (n/%)	E. avium (n/%)	E. gallinarum	E. faecalis(n/%)	E. faecium(n/%)	E. avium(n/%)
	(n=40)	(n=16)	(n=2)	(n=2)	(n=35)	(n=4)	(n=1)
asa1	13	1	0	1	11	0	0
	43.33	6.25	0.00	50.00	31.43	0.00	0.00
cylA	11	1	0	0	9	0	0
	36.67	6.25	0.00	0.00	25.71	0.00	0.00
esp	7	8	1	0	7	2	0
	23.33	50.00	50.00	0.00	20.00	50.00	1.00
gelE	9	0	0	1	2	0	100
	30.00	0.00	0.00	50.00	5.71	0.00	0.00
hyl	0	4	1	0	6	2	0
	0.00	25.00	50.00	0.00	17.14	50.00	0.00

As for the hospital sector from which the samples originated, it was observed that most samples carrying the genes associated with virulence were isolated from patients hospitalized in intensive monitoring units (Table 4.8).

Table 4.8 Distribution of genetic virulence determinants according to the hospital sector of origin of the sample

	Hospital sector		
Genetic determinants of virulence	Ward (n=30)	Intensive monitoring (n=54)	Not available (n=16)
asa1	7(23.33%)	14 (25.93%)	3 (18.75%)
cylA	6 (20%)	9 (16.67%)	4 (25%)
esp	5 (16.67%)	10 (18.52%)	3 (18.75%)
gelE	7(23.33)	9 (16.67%)	4 (25%)
hyl	5 (16.67%)	12 (22.22%)	2(12.5%)

The analysis of the distribution of genetic determinants of virulence between MDR and non-MDR samples revealed that all virulence genes surveyed were more frequent among MDR samples, as seen in Table 4.9.

Table 4.9 Distribution of genetic virulence determinants between MDR and non-MDR samples

Genetic determinants of virulence	MDR samples (n=80)	%	Non-MDR samples (n=20)	%	Total (n)
asa1	19	23.75	10	50	29
cylA	16	20	4	20	
esp	19	23.75	2	10	
gelE	15	18.75	2	10	
hyl	11	13.75	2	10	

The samples were also organized by the combination of virulence genetic determinants, that is, by their r*esp*ective virulence genetic profiles. The analysis of the distribution of such profiles of *E. faecalis* among the institutions of origin allowed the perception of a predominant profile among the samples of this species, associated with both hospitals, constituted by the genes *asa1*, *cylA* and *gelE*, present in 22 (55%) from samples from SGH and 10 (28.57%) from samples from STH, totalling 35 samples from the total species (Other profiles could also be observed, although less frequent (Table 4.10).

Table 4.10 Distribution of the genetic virulence profiles of *Enterococcus faecalis* according to the institution of origin of the sample

Virulence profiles of E. faecalis samples	SGH n=40	%	STH n=35	%
asa1	1	2.50	1	2.86
asa1, cylA	1	2.50	3	8.57
asa1, cylA, esp, gelE	2	5.00	4	11.43
asa1, cylA, esp	1	2.50	1	2.86
asa1, cylA, gelE	22	55.00	10	28.57
asa1, esp	3	7.50	1	2.86
asa1, esp, gelE	1	2.50	3	8.57
asa1, gelE	2	5.00	6	17.14
esp	1	2.50	2	5.71
esp, gelE	2	5.00	3	8.57
gelE	4	10.00	1	2.86
Total	40	100.00	35	100.00

Regarding the virulence profiles of E. faecium samples, a lower diversity was observed, making it possible to detect the *esp* gene in four samples from SGH and two from STH, as well as the association of *esp* and *hyl* genes, present in 12 samples from SGH and two from STH. In addition, four samples, two from each hospital, previously identified as E. faecium, did not have the genotypic virulence markers investigated. Regarding the source of isolation, the profile *asa1*, *cylA*, *gelE*, belonging exclusively to *E. faecalis* samples, was the most frequent both among colonization samples, present in 15 (93.8%) of these, and in samples collected from sites of infection, detected in 20 (32.8%). Among the E. faecium samples associated with colonization of the intestinal tract (n=8), the profile constituted by the *esp* and *hyl* genes was present in seven (87.5%), while one (12.5%) had only the spec gene. Among the samples of the same species isolated from sites suggestive of infection (n=16), the *esp* and *hyl* profile was detected in seven (43.8%) of them, followed by the *esp* profile, present in 2 (6.9%) and 7 (11.86%) samples did not show any of the genes investigated (Table 4.11).

Table 4.11 Distribution of virulence genetic profiles according to the hospital sector of origin of the sample

	Hospital sector					
virulence profiles	Ward (n=29)	%	Intensive monitoring (n=59)	%	Not Available (n=12)	%
asa1	2	6.9	1	1.69	1	8.33
asa1, cylA	2	6.9	1	1.69	1	8.33
asa1, cylA, esp, gelE	3	10.34	2	3.39	0	0
asa1, cylA, esp	1	3.45	2	3.39	1	8.33
asa1, cylA, gelE	5	17.24	22	37.29	4	33.33
asa1, esp	3	10.34	1	1.69	0	0
asa1, esp, gelE	2	6.9	4	6.78	0	0
asa1, gelE	2	6.9	2	3.39	2	16.67
esp	2	6.9	7	11.86	1	8.33
esp, gelE	5	17.24	1	1.69	2	16.67
esp, hyl	1	3.45	12	20.34	0	0
gelE	1	3.45	4	6.78	0	0
Total	29	100	59	100	12	100

Regarding the association between the different virulence profiles and the hospital sectors of origin of the samples, it was observed that the *asa1*, *cylA* and *gelE* profile (the most common among the *E. faecalis* samples) and the profile composed of the *esp* and

hyl genes (the most common among E. faecium samples) were the most frequent among samples from patients hospitalized in intensive monitoring units, being present in 22 (37.29%) and 5 (17.24%) of them, r*esp*ectively.

The disposition of the virulence genetic profiles between VRE and VSE samples was verified, noting that the vancomycin-resistant samples presented more specific profiles, with more samples carrying the *asa1*, *cylA* and *gelE* profile (detected in 51.11% of the samples). VRE samples) and the *esp* and *hyl* profile (found in 24.44% of these samples). Regarding vancomycin-susceptible samples, greater diversity was observed between such profiles (Table 4.12).

Table 4.12 Distribution of virulence genetic profiles between vancomycin-resistant (VRE) and susceptible (VSE) Enterococcus samples

Virulence profiles	VRE(n=45)	%	VSE(n=55)	%
asa1	1	2.22	3	5.45
asa1, cylA	2	4.44	1	1.82
asa1, cylA, esp, gelE	0	0.00	7	12.73
asa1, cylA, esp		0.00	1	1.82
asa1, cylA, gelE	23	51.11	8	14.55
asa1, esp	0	0.00	2	3.64
asa1, esp, gelE	1	2.22	5	9.09
asa1, gelE	1	2.22	7	12.73
esp	4	8.89	3	5.45
esp, gelE	1	2.22	3	5.45
esp, hyl	11	24.44	3	5.45
gelE	0	0.00	5	9.09
no bookmarks	1	2.22	7	12.73
Total	45	100.00	55	100.00

The comparative analysis of the distribution of genetic virulence profiles of MDR and non-MDR samples shows that the most frequent profiles among the former were *asa1*,

cylA and *gelE* [33 (41.25%)] and *esp* and *hyl* [11(13.75%)], while among the non-MDR the most frequent profile was *asa1* and *gelE* [4 (5%%)] (Table 4.13).

Table 4.13 Distribution of virulence genetic profiles between MDR and non-MDR samples

	MDR samples		**Non-MDR samples**	**%**
Virulence profiles	n=80	%	n=20	
asa1	1	1.25	2	10.00
asa1, cylA	3	3.75	1	5.00
asa1, cylA, esp, gelE	5	6.25	2	10.00
asa1, cylA, esp	2	2.50	1	5.00
asa1, cylA, gelE	33	41.25	1	5.00
asa1, esp	2	2.50	1	5.00
asa1, esp, gelE	3	3.75	2	10.00
asa1, gelE	1	1.25	3	15.00
esp	7	8.75	2	10.00
esp, gelE	3	3.75	2	10.00
esp, hyl	11	13.75	1	5.00
gelE	4	5.00	1	5.00
no bookmarks	5	6.25	1	5.00
Total	80	100	20	100

4.6 Evaluation of the Genetic Diversity of the Samples Through the Technique of PCR-RAPD

4.6.1 PCR-RAPD analysis

As a result of amplification of genomic DNA samples of strains isolated from clinical materials of patients with UTI, by RAPD method, which is another molecular epidemiological method used to detect clonal relationship, 4-8 polymorphic DNA bands with molecular weights ranging from approximately 50-1000 bp were obtained (Figure 4.4). Among the 12 different gene clusters called “a-l”, it was observed that 8 isolates were included in the “a” cluster with the highest number of members, followed by the “e” cluster consisting of 6 isolates (Figure 4.5).

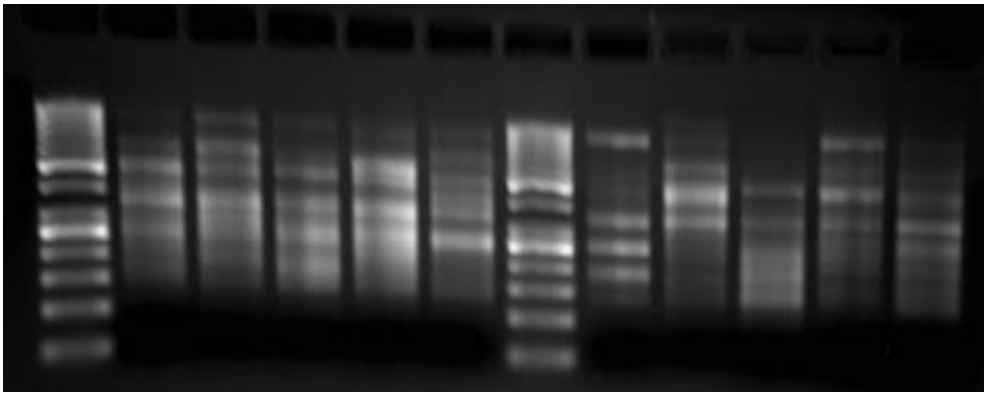

Figure 4.4 RAPD-derived gel image of VRE isolates leading to UT infection

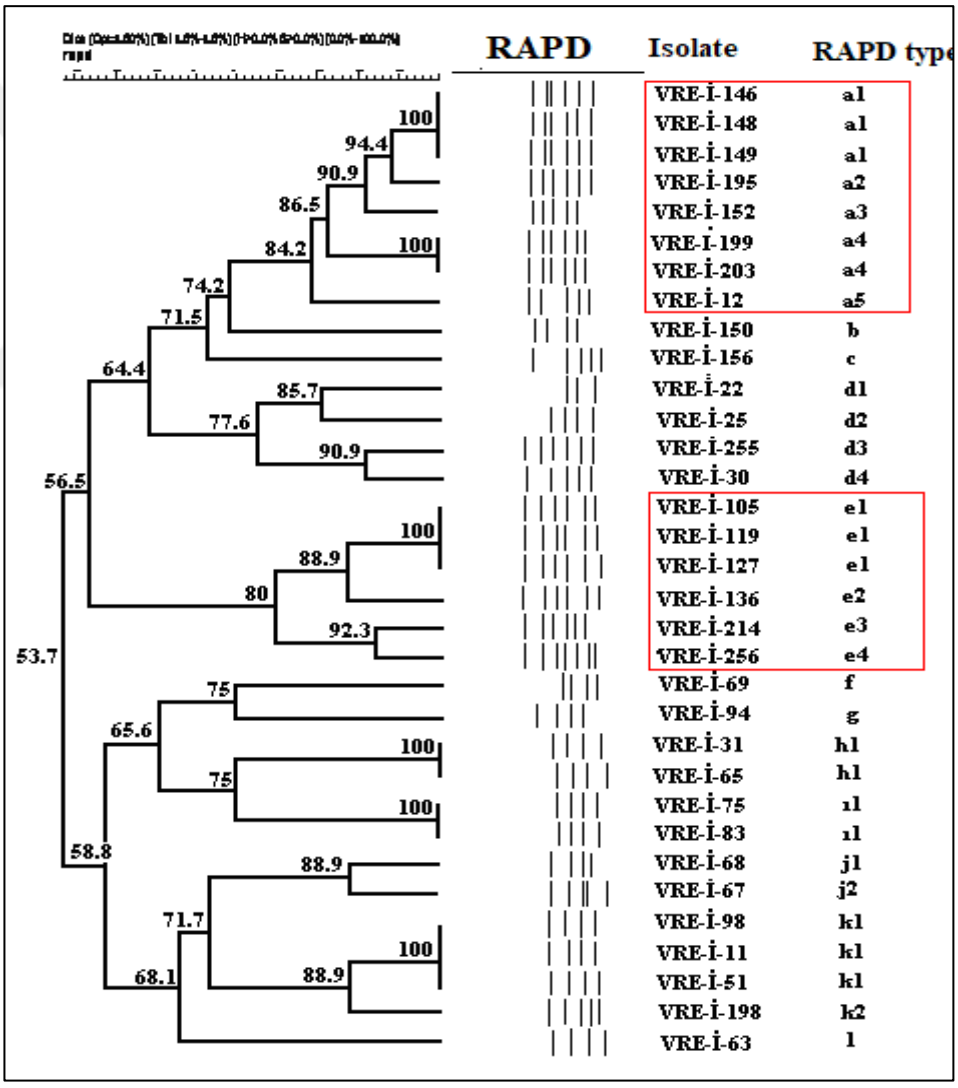

Figure 4.5 RAPD-derived dendrogram of VRE isolates leading to UTI

As a result of genotyping of Enterococci isolates leading to colonization with RAPD, at least 4-10 polymorphic DNA bands with molecular weights varying between approximately 50-1000 bp were obtained (Figure 4.6), and according to the band polymorphism, the isolates were divided into 14 different gene clusters called “a-n”. were found to be located. It was determined that the largest cluster was the one with 4 members and called “a” cluster, followed by “b, h, l and j” clusters with 3 members (Figure 4.7).

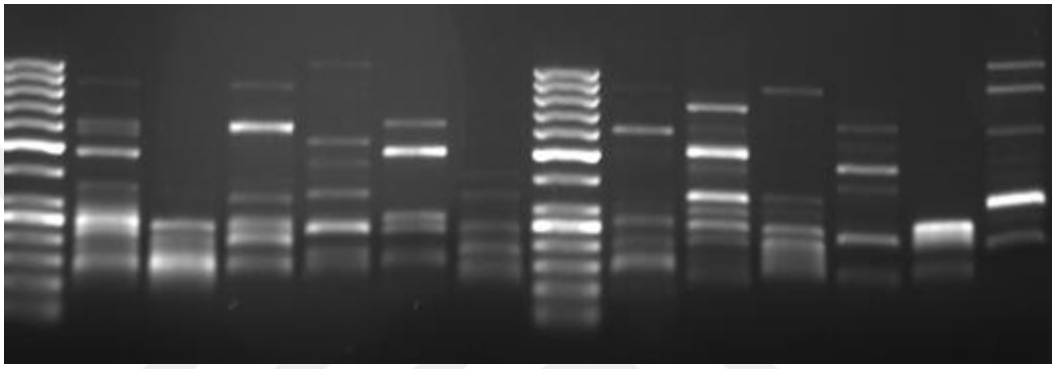

Figure 4.6 Gel image of colonization-leading isolates obtained by RAPD

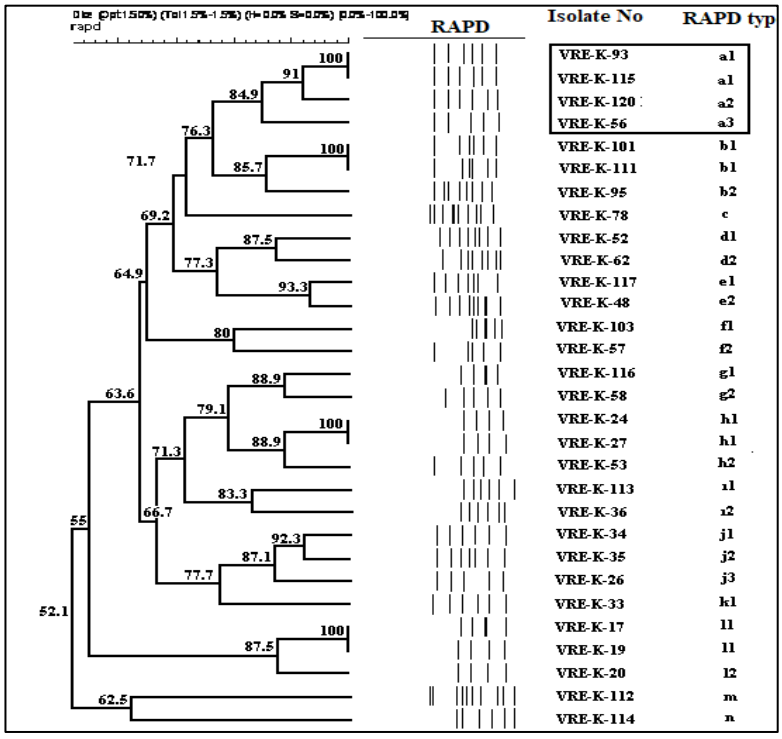

Figure 4.7 RAPD-derived dendrogram of colonization-leading isolates

At least 5-10 polymorphic DNA bands with molecular weights ranging from approximately 50-1000 bp were obtained from 16 VSE strains examined by Randomly Amplified Polymorphic DNA method (Figure 4.8), 11 RAPD clusters named “a-k” according to the dendrogram obtained with GelComparII program. were observed (Figure 4.9). It was observed that there were 3 isolates each in the "a" and "h" clusters, followed by the 2-membered "e" cluster, and one member each in the other 8 clusters.

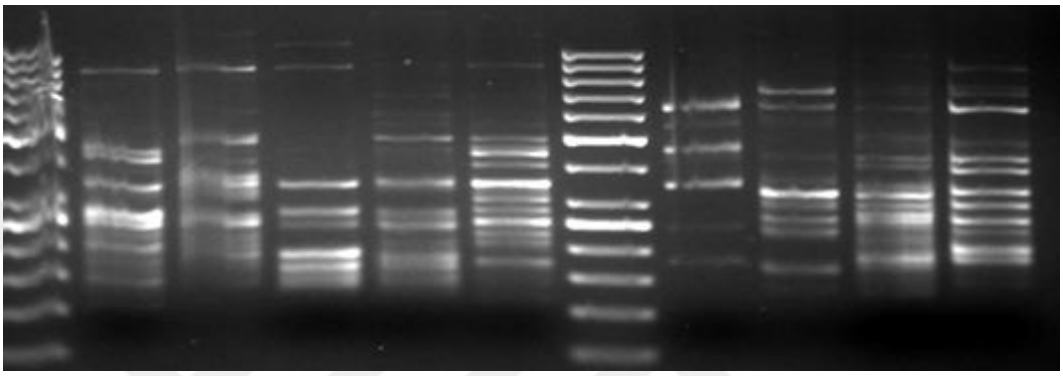

Figure 4.8 Gel image of VSE isolates obtained by RAPD

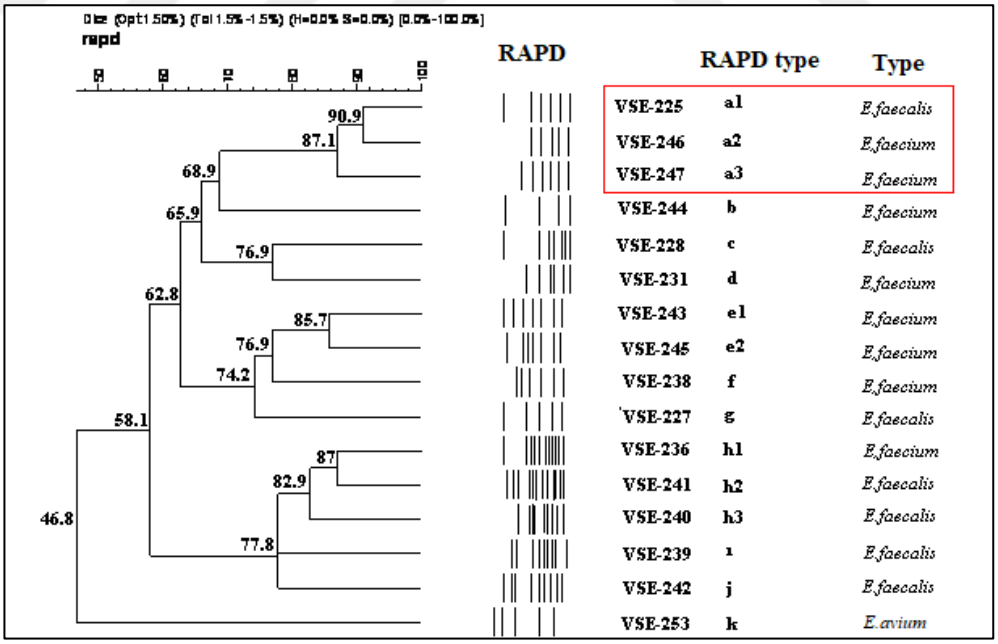

Figure 4.9 Dendrogram of VSE isolates obtained by RAPD

4.6.2 Genetic diversity of E. avium samples

Regarding the analysis of the genetic diversity of *E. avium* samples using the PCR-RAPD technique, it was observed that the three samples exhibited different pulse types, not composing any clonal group. However, pulse types *AvII* and *AvIII* showed greater similarity to each other, and despite coming from different institutions, both samples are susceptible to vancomycin and were isolated from tissue fragments, in addition to not having any of the genetic determinants of virulence evaluated. On the other hand, the pulse type *AvI*, which represents a colonization sample from a patient admitted to SGH, in addition to having exhibited resistance to vancomycin, presented the genetic determinant of virulence *esp* (Figure 4.10).

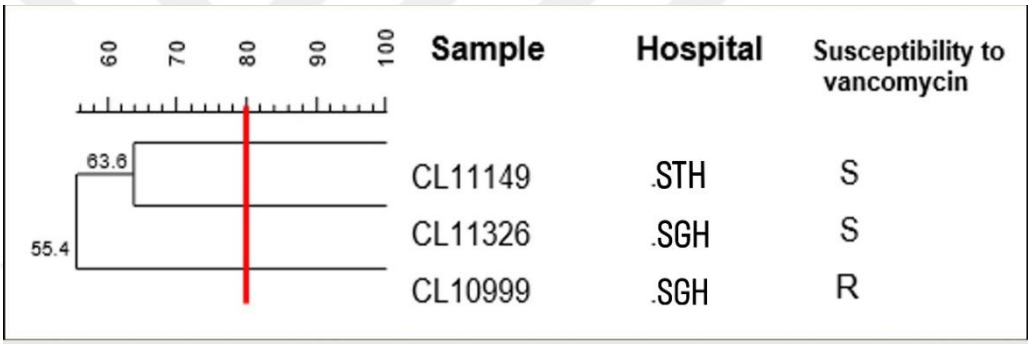

Figure 4.10 Dendrogram resulting from computerized analysis of the chromosomal DNA fragmentation profiles of the three Enterococcus avium samples. R: resistant, S: likely

4.6.3 Genetic diversity of *E. gallinarum* samples

The two samples of *E. gallinarum* exhibited the same electrophoretic profile, being, therefore, considered clones. Both were associated with the colonization of patients admitted to SGH and showed resistance to vancomycin, in addition to not showing any of the genetic determinants of virulence investigated in this study.

5. DISCUSSION

In this study, 100 urine cultures of women aged between 15 and 87 years were analysed. Of these participants, 45% were menopausal. Outpatients were 68% and hospitalized 32%. By analysing the medical records, it was not possible to determine whether the hospitalized patients with UTI acquired the infection during the period of hospitalization.

The *Enterococcus* genus has emerged as an important cause of infections in hospitalized patients in recent decades, which can be attributed to different factors, such as the selective advantage provided by antimicrobial resistance and the expression of virulence factors, both involved in the adaptation of these microorganisms to the environment. hospital environment, as well as its persistence in the host (Miller *et al.* 2016).

In the present study, most *Enterococcus* samples were collected from clinical specimens suggestive of infectious processes (Figure 4.1), with a higher frequency of isolated urine samples being observed in both hospitals (Table 4.1), represented by 28 (36.4%) from the *E. faecalis* samples and 12 (50.0%) from the E. faecium samples (Table 4.3). The initial colonization of the urinary tract by *Enterococcus* can be commonly made possible by the use of devices such as urinary catheters (Miller *et al.* 2016). Urinary tract infections are among the most common types of infections caused by *Enterococcus* in adults, which corroborates what was observed in our study (Barros 2009, Swaminathan 2010).

D*esp*ite the lack of information on the clinical conditions of the patients from which the samples indicative of infection was isolated in this study, we know that most samples of *E. faecalis* [50.6%], of E. faecium [79.2%], one of the *E. avium* samples and both *E. gallinarum* samples were isolated from patients hospitalized in intensive monitoring sectors, such as ICU, ICU and UADC, which comprise patients in more fragile health states and, therefore, more vulnerable to acquiring infections. It has already been described that the incidence of nosocomial infections in intensive care units is two to

five times higher than in the general hospital population of inpatients (Arias and Murray 2012, Dasgupta *et al.* 2015, Ziakas *et al.* 2013).

Of the 27 samples suggestive of colonization of the gastrointestinal tract, 25 (92.6%) were isolated from patients hospitalized in the SGH and only two (7.4%) from patients in the STH, this difference can be explained by the VRE screening regimen of these patients. hospitals, since in the SGH the collection of specimens by rectal swab is a routine practice, while in the STH it is only performed with a medical prescription. However, all 27 samples were isolated from patients hospitalized in intensive monitoring units, harbouring samples of the four identified *Enterococcus* species, among which 26 (96.3%) were classified as VRE. In view of the opportunistic characteristic of *Enterococcus*, the colonization of patients in such circumstances by bacteria of this genus, mainly by VRE, represents a serious threat to public health, since colonization is the first step for the installation of an enterococcal infection, in addition to that these patients are more prone to the development of serious infections, which are difficult to treat and have a high potential for death (Goh *et al.* 2017, Van Tyne and Gilmore 2014).

In addition, dissemination of these microorganisms occurs easily through health professionals and contamination of hospital devices (Arias and Murray 2012, Chatterjee *et al.* 2007). A longitudinal study that followed 752 patients of hematopoietic stem cell transplantation between 2004 and 2008 found that 93 (12.4%) of patients developed VRE bacteremia in the first year after transplantation. And among patients who were already colonized by VRE before transplantation, 14.0% developed bacteremia, while among those who were not colonized this incidence was only 4.0%, which statistically proves that patients colonized by VRE are at greater risk of developing bacteremia after this type of procedure than non-colonized patients. Considering that the VanA phenotype is characterized by simultaneous resistance to the antimicrobial's vancomycin and teicoplanin, among the VRE samples, 45 [28 (36.4%) of *E. faecalis*, 16 (66.7%) of E. faecium and one (50.0%) of *E. gallinarum*] were associated with this phenotype, being also resistant to teicoplanin, while only two samples (one of E. faecium and one of *E. avium*) were associated with the VanB phenotype, characterized

by resistance to vancomycin and teicoplanin susceptibility. The prevalence of the VanA phenotype over the VanB phenotype corroborates the literature data (Table 4.3) (Marchi *et al.* 2018).

The frequency of resistance to vancomycin and ampicillin in E. faecium was higher than that observed in *E. faecalis*, which, in relation to ampicillin, has been described since the late 1970s, when an increase in the MIC of ampicillin from 4 to 64 μg/mL in E. faecium isolates, and in the following decade, a phenotype resistant to high levels of this antimicrobial emerged, with a MIC greater than 128 μg/mL. This also corroborates the high frequency of penicillin-resistant E. faecium samples [23 (95.8%)], which is a common phenotype in this species (Miller *et al.* 2016). already in regarding the higher frequency of vancomycin resistance in E. faecium [17 (70.8%)], this was noticed in the 1990s in several countries, due to the wid*esp*read use of vancomycin and broad-spectrum antimicrobials in hospitals in the United States. United States and the use of avoparcin in animal husbandry in Europe, which also supports the high frequency of resistance to teicoplanin in samples of this species [16 (66.7%)], as it also belongs to the glycopeptide class (Cattoir and Leclercq 2013, Gao *et al.* 2018).

High frequency of nitrofurantoin resistance was also observed among E. faecium samples [20 (83.3%)]. This antimicrobial is often prescribed for the treatment of lower urinary tract infections, and until recently it was considered an effective alternative for the treatment of VRE infections at this site (Zhanel *et al.* 2013). However, the results of this work show the opposite, of the 17 samples of E. faecium resistant to vancomycin, none showed susceptibility to nitrofurantoin, being 15 resistants and two intermediates to this drug. This indicates that nitrofurantoin may no longer be a guarantee of therapeutic success for infections of this nature.

The increase in the number of infections caused by E. faecium in recent years represents a great threat to public health, in view of the high rates of resistance to different classes of antimicrobials presented by this species, which are much more difficult to treat than those caused by E. faecium. *E. faecalis* (Arias and Murray 2012, Gao *et al.* 2018).

The high frequency of *E. faecalis* samples resistant to tetracycline [64 (83.1%)] and to high concentrations of gentamicin [44 (57.1%)] observed in this study is common, and is known to be due to transfer processes resistance genes. A high frequency of resistance was also observed for the combination of quinupristine/dalfopristine antimicrobials [68 (88.3%)], which was already expected considering that resistance to the streptogramin class is an intrinsic characteristic of this species (Miller *et al.* 2014). However, the presence of five (6.5%) samples of *E. faecalis* susceptible to quinupristine/dalfopristine and four (5.2%) intermediate samples was also found. The susceptibility of *E. faecalis* to streptogramins has also been described in previous studies and can be attributed to mutations in the Isa locus, r*esp*onsible for the expression of resistance to this class of antimicrobials. In general, both E. faecium and *E. faecalis* samples showed high percentages of resistance to erythromycin, norfloxacin, ciprofloxacin and levofloxacin (Table 4.3). Resistance to erythromycin, an antimicrobial of the macrolide class, was the most frequent in the study, comprising a total of 78 (73.6%) samples (Figure 4.2). This feature is wid*esp*read among *Enterococcus* and is related to the acquisition of resistance genes. A high frequency of resistance to norfloxacin [66 (62.2%)], ciprofloxacin [65 (61.3%)] and levofloxacin [64 (60.4%)] was observed. of mutations in genes encoding enzymes involved in the processes of replication, transcription and nucleic acid synthesis among members of this genus, promoting an alteration in the target site of fluoroquinolones (Miller *et al.* 2014).

The ability of the antimicrobial's linezolid and tigecycline to inhibit the growth of all *Enterococcus* samples in the study (Figure 4.2) reaffirms the choice of these drugs as still promising alternatives for the treatment of infections caused by VRE. However, the prescription of such drugs should be done with caution and in a controlled manner, since cases of bacteria resistant to both have been reported as agents of severe infections. Therefore, the wid*esp*read use of these drugs could favor the spread of resistant samples and further limit the few therapeutic strategies available for the treatment of VRE infections (Arias and Murray 2012).

Regarding the distribution of the genetic determinants of virulence investigated in this study, it was observed that the genes *asa1*, *cylA* and *gelE* were present exclusively in

samples of *E. faecalis*, being also the most frequent among the samples of this species in both hospitals, which corroborates the findings of other authors. The *gelE* gene, detected in 67 samples, was the most frequent determinant in the study (Figure 4.3), which is consistent with the results of other researchers (Heidari *et al.* 2016). The gene *esp* was detected more frequently among *E. faecalis* samples, but also present in E. faecium and *E. avium* samples (Table 4.5). The frequency of genes encoding adhesins, such as *asa1* and *esp*, and gelatinase (*gelE*) among *E. faecalis* samples was also observed in a study carried out in Bulgaria between June 2013 and June 2015, involving 510 *Enterococcus* samples (370 E. faecalis and 140 of E. faecium), being significantly higher when compared to the frequency of these genes among E. faecium samples. The detection of the genetic determinant of *hyl* virulence only among E. faecium samples in this study is consistent with reports in the literature, which describe this gene with an almost exclusive frequency among bacteria of this species (Arias and Murray 2012, Gao *et al.* 2018).

An interesting finding of our research was the detection of the *esp* gene in one of the *E. avium* samples. The presence of this genetic determinant of virulence among samples not belonging to the *E. faecalis* and E. faecium species is rare, and it is important to note that this is a sample isolated by rectal swab from a patient hospitalized in intensive care at the HRT. The expression of the *Enterococcus* surface protein encoded by this gene in *E. avium* may confer several advantages to the pathogenicity of this species (usually associated only with *E. faecalis* and E. faecium) such as its adhesin function and its ability to form biofilms., which favor the colonization process, as well as the persistence of the bacteria on abiotic surfaces, in addition to being able to escape the host's immune system defenses through the expression of different protein phenotypes (Zheng *et al.* 2017).

Furthermore, the high frequency of these virulence genes associated with samples isolated from patients in intensive care (Table 4.6) may potentiate the pathogenesis of infectious processes, taking into account that these patients are often immunosuppressed (Chatterjee *et al.* 2007). The greater distribution of the *asa1*, *esp* and *gelE* genes among samples susceptible to vancomycin and the greater frequency of the *cylA* and *hyl* genes

in VRE samples was observed in the present study. In a study carried out with 115 *E. faecalis* samples from different regions of Brazil in 2008, 24 VRE and 91 VSE, the presence of cylLL, cylLS, *cylA*, *esp* and *asa1* genes was investigated, with no statistically significant difference being reported. between VRE and VSE samples in the distribution of these genes. In another study carried out in Porto Alegre in 2013, where 50 samples of *Enterococcus* (30 of *E. faecalis* and 20 of E. faecium), with 20 samples of *E. faecalis* VRE as well as all of E. faecium, the presence of the genetic determinants *asa1*, *gelE* and *esp* was investigated, and no significant difference was alleged in the distribution of these genes according to resistance to vancomycin (Comerlato *et al.* 2013).

However, a 2016 study conducted in India with 500 *Enterococcus* samples (440 VSE and 60 VRE) reported that the presence of *gelE*, *esp* and *hyl* among VRE samples of clinical origin was higher than that observed among VSE samples of the same origin. And in relation to samples of fecal origin, the frequency of *asa1*, *esp* and *gelE* in VRE was also significantly higher than that of VSE, which differs from the findings of our study (Biswas *et al.* 2016). The higher frequency of all genetic determinants of virulence analyzed in the study among MDR samples (Table 4.7) can be explained by the coexistence of virulence and resistance genes in the same mobile genetic element or island of pathogenicity. The co-selection of these genes provides a great evolutionary advantage to bacteria of the genus *Enterococcus*, since the presence of virulence factors can favor the colonization of the host organism and the evasion of the defenses of its immune system, as resistance to the antimicrobial agents allows the bacterium to circumvent therapeutic strategies, contributing to the increase in its pathogenicity and, consequently, to the development of more persistent and severe infections (Wurster *et al.* 2016).

Regarding the genetic virulence profiles and their distributions among the samples, it was observed that the combination of the determinants *asa1*, *cylA* and *gelE* was the most frequent among *E. faecalis* samples in both hospitals (Table 4.8). In the study carried out by (Camargo *et al.* 2008) with 115 samples of *E. faecalis* from different Brazilian regions, the set of factors: aggregative substance (*asa1*), gelatinase (*gelE*) and

surface protein of *Enterococcus* (*esp*) was more common among the samples, being present in 12 (75.0%) of 16 samples of the predominant clone, differing from the findings of our study by the presence of *esp* instead of *cylA* among the most frequent virulence genetic profiles. The same pattern was observed in a study carried out with 132 *Enterococcus* samples (108 of which were identified as *E. faecalis*) from cancer patients in Rio de Janeiro, among which the genetic profile of virulence composed of the genes *asa1*, *esp* and *gelE* also was the most frequent. The *cylA* gene, despite being less frequent in the study by Santos *et al.* (2017), was constantly found in association with the *asa1*, *esp* and *gelE* genes, which can be attributed to the presence of these determinants in the same mobile genetic element or in the same pathogenicity island.

However, in the study by Camargo *et al.* (2008), a single sample closely related to the predominant clone expressed cytolysin rather than Esp, suggesting that this sample may have acquired the operon involved in cytolysin expression and lost the *esp* gene. This theory may provide an explanation for the origin of *E. faecalis* samples carrying the *asa1*, *cylA* and *gelE* profile that are predominant in the present study. Similarly, a characteristic genetic profile was more frequent among samples of E. faecium, composed of the *esp* and *hyl* genes, predominant both among samples associated with the process of colonization of the gastrointestinal tract and with suggestive cases of infection. The combination of virulence factors encoded by these genes can provide important attributes to samples of this species, such as biofilm formation, evasion of host immune system defenses (since the *esp* gene can encode alternative forms of the protein), adhesion to cells epithelial cells of the urinary tract, in addition to facilitating intestinal colonization and invasion of the peritoneum (Miller *et al.* 2016, Zheng *et al.* 2017).

The virulence profiles formed by the *asa1*, *cylA* and *gelE* genes of *E. faecalis* and *esp* and *hyl* of E. faecium were also the most frequent among samples isolated from patients hospitalized in intensive care (n=61), representing 24 (39.3 %) and 13 (21.3%) of these samples, r*esp*ectively (Table 4.9). The highest frequency of both profiles was also observed among VRE samples [26 (54.2%) and 12 (25.0%), respectively] (Table 4.10) and between MDR samples [35 (42.2%) and 13 (15.7%), respectively] (Table 4.11).

Van Den Braak *et al.* (2000) compared RAPD and PFGE methods in the genotyping of VRE clinical isolates and found that E. faecium, *E. faecalis, E. gallinorum, E. avium* were isolated in the Netherlands and 50 in England. In their study where they evaluated 100 VRE isolates consisting of species, noting that the results of RAPD and PFGE are similar, they emphasized that both methods are a good alternative to each other.

However, Vancanneyt *et al.* (2002) identified 78 vancomycin-susceptible and resistant E.faecium isolates isolated from various sources such as humans, animals, and food in different European countries such as Belgium, Germany, Greece, Ireland and the Netherlands, and in a few non-European countries, such as RAPD, AFLP and In their studies that they examined with the PFGE technique, They reported that the reproducibility of the RAPD-PCR technique was 0.91, and the reproducibility of the PFGE technique was 0.93.

In addition, Werner *et al.* (2003), in their study in which they compared RAPD, AFLP and PFGE methods in order to determine the genetic relationships of 24 enterococcal isolates, they identified 22 of the 24 transconjugates with PFGE as clonally related and 2 as possibly related strains that differed by more than 3 bands. In their evaluation with the RAPD method, they worked with 3 different primers and reported that all of the transconjugates were similar with the 2nd and 3rd primers, and that they obtained 2 different types with the RAPD 1 primer. The group that found all transconjugates to be associated with AFLP majorly stated that the RAPD method could not show minor changes in the genome, and because of its low reproducibility and low discrimination power, this method could not be used as PFGE or PFGE.

We also evaluated *Enterococci* isolates from two hospitals, which we evaluated with the RAPD method. When we analysed VRE isolates associated with UTI infections with the RAPD method, we obtained 12 different gene clusters called “a-l” (Figure 4.5). We saw that there are 8 isolates in the "a" cluster, which has the highest number of members, followed by the "e" cluster, which consists of 6 isolates. The 12 gene clusters we obtained with RAPD showed that this method had higher discriminatory power, which gave only 6 gene clusters. However, the distributions of the members in the

clusters formed by RAPD method are not similar in terms of clonal relationship. Also, the VRE-I-31 isolate, which was defined as E. avium by phenotypic methods and formed the special single-membered "E" cluster in PFGE typing (Werner *et al.* 2008), was classified as "C5" with PFGE in the gene clustering made by RAPD method in the current study. It was found that it was 100% similar to the identified VRE-I-65 E. faecium isolate. On the other hand, when we repeated the RAPD by applying the same primers and test conditions, we obtained different band numbers and dendrograms. This result showed that RAPD had high discriminatory power but low specificity and sensitivity.

As a result of typing of VRE isolates causing colonization with RAPD, it was found that they were included in 14 different clusters (a-n) and the largest cluster was “a” cluster with 4 members, followed by 3-membered b, h, l, j and 2-membered d, e each. We found that, *f, g, ı* clusters come from and there are 4 clusters with 1 member (Figure 4.6, 4.7). As a result of comparing the profiles obtained with RAPD with PFGE (Wijetunge *et al.* 2012). It was determined that the isolates with 100% similarity with RAPD were in different clusters with PFGE in the work done by (Muñoz-Atienza *et al.* 2016), and isolates with 100% band similarity with PFGE were found in different clusters with the RAPD method, and the two methods were not compatible with each other in this group.

On the other hand, it was observed that VSE strains examined with RAPD formed 11 RAPD clusters called “a-k”. 3-membered “a” and “h” clusters, 2-membered “e” cluster and 1-membered 8 clusters were obtained (Figure 4.8, 4.9). As a result, the number and member distributions of the clusters formed based on the RAPD results in all samples were not similar, the RAPD results of the same samples were inconsistent with each other, that is, the repeatability of RAPD was low. It has been concluded that it is a reliable, high discriminatory, standardizable molecular epidemiological method for surveillance.

In addition to the *E. faecalis* and E. faecium samples, it is worth mentioning the presence of an *E. avium* type characterized by vancomycin resistance and detection of the virulence genetic determinant *esp*, in addition to both *E. gallinarum*, which despite

not having displayed any virulence gene, are also resistant to vancomycin and MDR, belonging to the same type. Results indication of the dissemination of highly pathogenic *Enterococcus* samples between hospitals in the city of Salahaldeen, Iraq (Santos *et al.* 2017). Thus, the present study provides evidence supporting the need to control high-risk clones associated with nosocomial infections of the *Enterococcus* genus in this region.

6. CONCLUSIONS AND RECOMMENDATION

The emergence of bacteria of the Enterococcus genus as important nosocomial pathogens in recent decades reflects the need to investigate the factors involved in the pathogenesis of infections caused by these microorganisms. Among these factors are the increasing resistance to antimicrobials that accompanies these infections over the years and the presence of virulence factors that contribute to their development.

The lack of information on the virulence attributes and genetic diversity of Enterococcus that circulate in hospitals in the city of Salahaldeen, Iraq motivated this research. Of the samples collected for this purpose, the vast majority were identified as belonging to the species E. faecalis, followed by the species *E. faecium*, *E. avium* and *E. gallinarum.* The most frequent clinical sources of isolation of these samples were urine, GIT and blood, indicating the presence of these microorganisms both in association with colonization and infectious processes.

Among the isolates associated with UTI and isolates that colonize the hospital, all of the isolates defined as *E. faecium* and *E. gallinorum* and *E. avium* isolates are resistant to vancomycin, and teicoplanin resistance among these strains has reached such high rates (93.5%-80%) in the world. Averages and exceeds the US data, but no resistance has yet developed to any of the glycopeptide group antibiotics in our community-acquired isolates.

The highest percentages of resistance in the study samples were to antimicrobials: erythromycin, quinupristine/dalfopristine, tetracycline and to members of the fluoroquinolone class (norfloxacin, ciprofloxacin and levofloxacin) and vancomycin. In addition, 83 (78.3%) of the study samples were classified as MDR.

Among E. faecalis samples, the most frequently found virulence genetic determinant was gelE, followed by asa1, cylA and esp genes, while among E. faecium samples the frequency was higher for the esp gene, followed by the hyl gene.

Three clonal groups of greater clinical importance were found to be predominant among the study samples, one of which was E. faecalis, defined, in the majority, by samples resistant to vancomycin and high levels of aminoglycosides and carrying the genetic profile of virulence formed by the asa1 genes., cylA and gelE, and the other two encompassing samples of E. faecium, mostly resistant to vancomycin and carrying the genetic determinants of esp and hyl virulence. In addition to the circulating clonal groups of E. faecalis and E. faecium, the presence of a single sample of E. avium resistant to vancomycin and carrying the esp virulence gene stands out, which is a rarity among samples of this species, being associated with colonization of the GI tract of an SGH patient, Two vancomycin-resistant E. gallinarum clones were also isolated from the GIT of patients admitted to the SGH, which also represents an unusual event. Among these samples, there was a high frequency of multidrug resistance and the vast majority were isolated from patients hospitalized in intensive monitoring units.

Although infections originating from the endogenous flora of the hospitalized patient and colonizations with small outbreaks are seen from time to time, nosocomial VRE infections are caused by an epidemic strain that dominates the hospital, but some mutations occur in the genome due to the persistence of this strain in the hospital for a long time, and colonization-associated strains also have RAPD. With the method, they generally form large clonal clusters within themselves and a large clonal cluster associated with infection strains, therefore, the PCR-RAPD method can be used as a method with high discriminatory power in epidemic surveillance.

The applicability of PCR-RAPD methods used in the epidemiological surveillance of VRE strains that cause UTI infections is to indicate colonization from clinical materials. Since the RAPD method produces results with low reproducibility and sensitivity, it has been concluded that it cannot be an alternative to the PFGE method. Based on these results, the following recommendations were made.

- For hospital-acquired VRE infections, new preventive guides should be prepared according to hospital conditions targeting healthcare professionals, patients and their relatives, and training activities should be accelerated.

- Active surveillance should be carried out for all UT infections, especially VRE, and microbiology laboratories should be used more effectively in this surveillance.

Resistance to all glycopeptide group antibiotic derivatives should be questioned in c-VRE isolates and antibiotic use should be controlled.

The identification of virulence factors associated with the invasion and severity of enterococcal infections reveals the possibility of developing alternatives to control these diseases, such as the prevention of biofilm formation by these species or the inhibition of other virulence factors, which is relevant, considering that antimicrobial therapeutic treatment options are limited for this genus. In addition, this is the first study on Enterococcus virulence in the city of Salahaldeen, Iraq, contributing with epidemiological data of an important nosocomial pathogen of national and international importance.

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APPENDICES

APPENDIX 1. Ethical approvals

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