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Different Variants of Coa Gene in Isolated Staphylococcus Aureus

Zahra gholami[1], Esmaeil Fattahi[2*]
1- Department of Biology of Plant Sciences, Payame Noor University (PNU), P.O. Box, 6519949854,Hamedan, Iran
2- *Faculty member of Biology. Amol Branch, Islamic Azad University, Amol, Iran.

Abstract
Staphylococcus aureus is a pathogen prevalent among humans and animals that causes a large number of diseases. Meanwhile, the coagulase enzyme is a major pathogen of this bacterium. Also, a study of the genetic diversity of the coding gene of this enzyme (Coa) is one of the molecular methods of determining the typing of Staphylococcus aureus isolates. The number of 130 samples, including joint liquid (7 samples), urine (57 samples), sputum (30 samples), blood (10 samples), ulcer (17 samples), abdominal mass liquid (3 samples), abscess (3 samples) and tracheal secretions (3 samples) were collected from patients presenting to Rasht's Medical Diagnosis Laboratories and then transferred to laboratories. To determine the coagulase typing, the highly variable region of the gene of this enzyme was amplified in the PCR reaction, and then subjected to enzymatic digestion using the restrictive Alu1 endonuclease enzyme. Meanwhile, the diversity of the enzymatic digestion fragment lengths was examined. In sum, in the PCR reaction, two products (with 680 and 750 bp lengths) and six different RFLP patterns (400+280, 340+280, 340+470bps, and the non-digestion of the 750bp fragment) were obtained. The results indicated the genetic diversity of the coagulase gene in staphylococcus aureus isolates in the region, as the 280+400 bp PCR-RFLP pattern was found to be the dominant pattern.

Keywords; Coa gene, staphylococcus aureus, PCR-RFLP pattern

Introduction

Since resistance to anti-microbial drugs is increasing and staphylococcus aureus shows more drug resistance than other bacteria, defense mechanisms of the immune system as anti-microbial strategies receive much attention (1, 2). On the other hand, staphylococcus aureus resists to or escapes from the body's defense mechanisms by different methods (3). The production of coagulase enzyme by staphylococcus aureus has drawn the attention of clinical microbiologists and is considered a major index in identifying this bacterium (4, 5). The coagulase enzyme in staphylococcus aureus is an extracellular enzyme that has the same functionality of the conversion of fibrinogen to fibrin and is catalyzed by thrombin. Coagulase-thrombin not only coagulates fibrinogen but also helps proteolytic and sterolytic activities (6, 7). Using white blood cells, coagulase creates a barrier of fibrin around the lesion site to protect staphylococcus against phagocytosis (8, 9). The Coa gene, which encodes coagulase protein, is highly polymorphic due to variable sequences in the ´3 coding region and can be used to distinguish the isolates of this bacterium. The variable region of the Coa gene is composed of repetitive sequences of 81 bp, as the number of repetitions of these sequences varies in various strains of staphylococcus aureus (10). In many countries, the typing of the staphylococcus aureus genotype has been a part of monitoring programs and served as a major tool to study the origins of strains, determine clonal relevance, and the epidemiology of outbreaks. Molecular typing plays a basic role in epidemiological studies of opportunistic infections caused by Staphylococcus aureus. The most effective molecular typing is the Pulsed Field Gel Electrophoresis method, which is a complicated, expensive, and time-consuming technique (11). For this, some measures have been taken to achieve simple, fast, and cheap techniques. Various studies indicated that the amplification of the highly variable genetic regions and the enzymatic digestion analysis of the product (PCT-RFLP) have been

effective methods in the molecular typing of staphylococcus aureus. The widespread prevalence and hygienic importance of infections caused by staphylococcus aureus have led to many studies across the world about pathogenic genes, and the identification of various biotypes and genotypes of this bacterium. Coagulase has been recognized as the most important pathogen in staphylococcus aureus (12). The importance of staphylococcus aureus in hospital-acquired infections has led to the growing knowledge of the genetic diversity of these bacteria in control, prevention, treatment, and prognosis of infection outcomes (13,14). As stated, the goal of the present study was to investigate the genetic patterns of the coagulase enzyme of staphylococcus aureus isolated from clinical samples and also to investigate the relationship between the genetic pattern of coagulase enzyme and the type of clinical infections caused by this bacterium.

Study Method

The number of 130 samples, including joint liquid (7 samples), urine (57 samples), sputum (30 samples), blood (10 samples), ulcer (17 samples), abdominal mass liquid (3 samples), abscess (3 samples) and tracheal secretions (3 samples) were collected from patients presenting to Rasht's Medical Diagnosis Laboratories and then transferred to laboratories. Urine samples were centrifuged at 1500 rpm for 10 minutes, and the resulting deposits were used for microbial culture. However, other samples were directly cultured. The prepared samples were cultured in a TBS enrichment medium for 24 hours at 37°C; after being enriched linearly, they were cultured in a blood agar medium (Germany's Merck), and were incubated at 37°C for 24 hours. After the microbial growth, gram staining and catalase tests were performed on grown colonies, and hemolytic colonies that had cluster-shaped gram-positive cocci and were catalase-positive were selected as the staphylococcus-suspected colonies and were again cultured in the blood agar medium.

After 24 hours of incubation at 37°C, the grown colonies were simultaneously cultured in a Baird-Parker medium (Germany's Merck, and Mannitol Salt) and in an agar medium (Germany's Merck), and the coagulase test was carried out on them. The colonies capable of coagulating the citrated plasma of rabbits created dark colonies with a transparent halo, and fermented mannitol sugar was selected as the staphylococcus aureus-suspected colonies, and was cultured in a liquid TSB medium (Germany's Merck) at 37°C for 24 hours for subsequent tests. To confirm the presence of staphylococcus aureus in the selected colonies, the PCR test was employed. To this aim, first, the genomic DNA of the isolates grown in the TSB medium was extracted using the boiling method. The PCR tests use a pair of primers that help the detection of the 16srDNA gene, introduced by Kumar et al. (2010) (15).

To confirm the diagnosis and purification of bacteria, this study used the catalase test, the DNase test, gram staining, DNA extraction, PCR reaction, PCR-RFLP reaction, and the antibiogram test. The standard strain No. ATCC 23235 of staphylococcus aureus was used as the positive control sample, and distilled water as the negative control sample in this test.

As for the genetic classification of staphylococcus aureus isolates from hospital-acquired infections, the PCR test was used to detect the coagulase gene. In this stage, the isolates from the previous stage were selected and tested by using primer pairs introduced by Javid et al. (2018) (16).

COAG3: ATAGAGATGCTGGTACAGG

COAG2: GCTTCCGATTGTTCGATGC

To extract the DNA of gram-positive bacteria, Cinnagen Company's kit (Cat. No. PR881614) was used. According to the kit instruction, first, 10 to 20 mg of the 24-hour culture of the bacterium in a nutrient agar medium was dissolved in a microtube containing 200 µL of sterile distilled water, and then the microtubes were centrifuged at 4500 rpm for 10 minutes. The resulting deposit was collected and genomic DNA extraction stages were performed.

The studied bacterium was cultured in a loop form in a DNase Test Agar medium, and then the plates were incubated at 37°C for 18-24 hours. Following the incubation, the 0.1% toluidine blue reagent was poured onto the simple DNase Test Agar plates, which coated the plate surface. In positive samples, transparent halos were noted around the colony or the inoculation site in the culture medium

(8,12).

Results

In this survey, 30 isolates from mannitol-fermenting gram-positive cocci bacteria in the mannitol salt agar medium that were capable of producing catalase, coagulase, hemolysin, and DNase enzymes were identified and studied as staphylococcus aureus. The frequency percentage of the bacteria isolated from different clinical samples is given in Diagram 1.

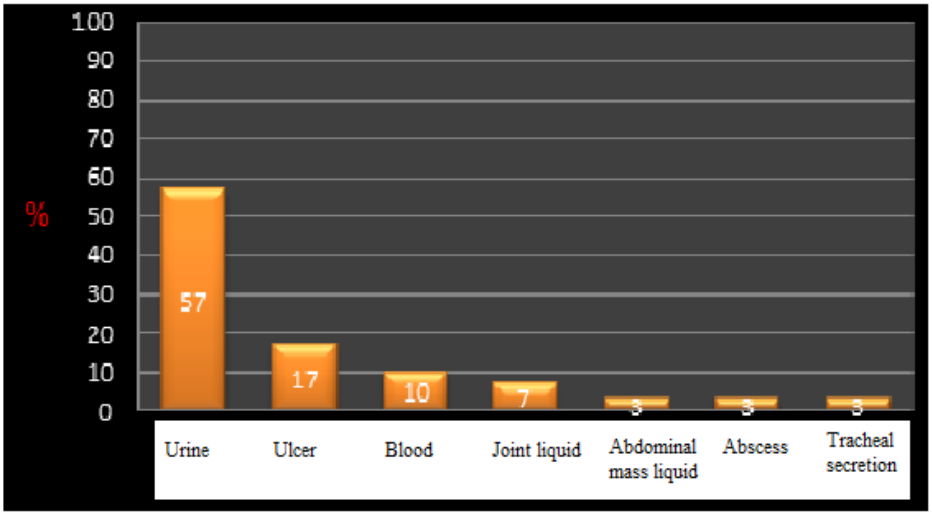

Diagram 1: Percentage of bacteria isolated from clinical samples

Catalase test results of the clinical isolates of staphylococcus aureus are given in Image 1.

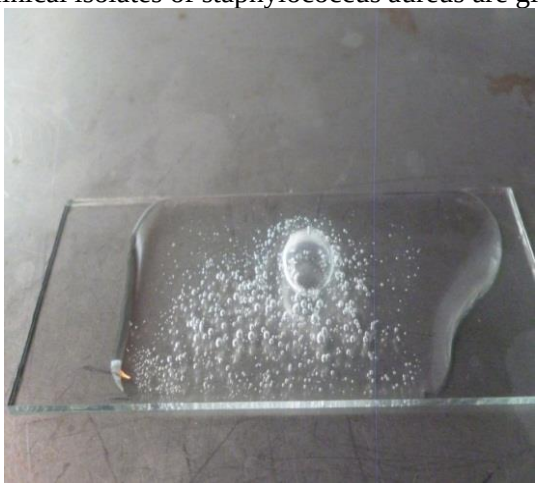

Image 1: Positive results of catalase test

Results indicated that the DNase test was positive in all isolates (Image 2).

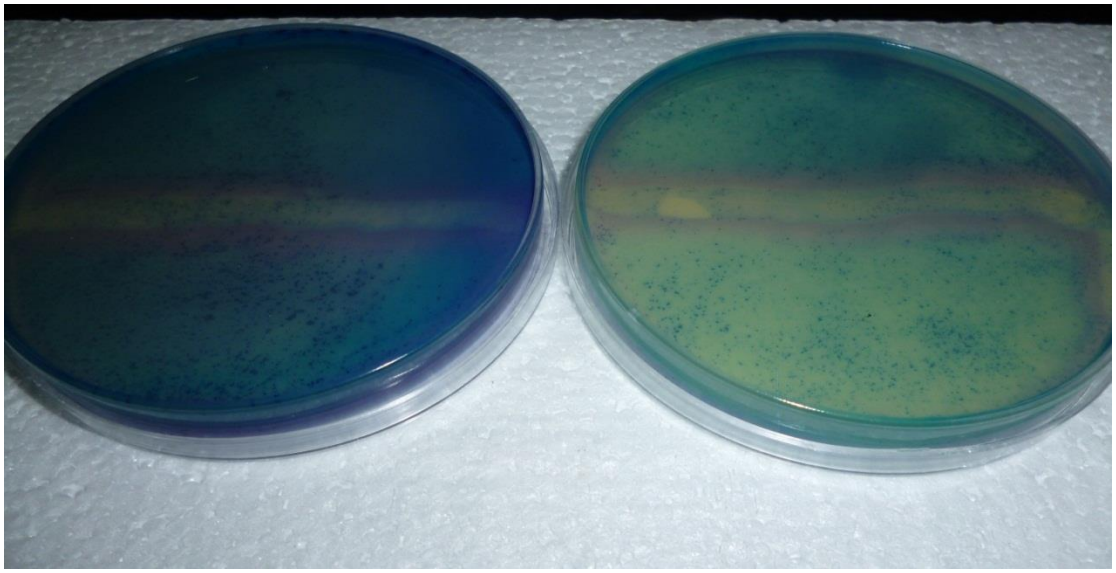

Image 2: Creation of a transparent halo around the bacterium indicating the positive DNase of it

Gram Staining Results

For diagnosis, gram staining was performed on all samples, with staphylococcus aureus isolates seen in grape purple-colored clusters under the microscope (Image 3).

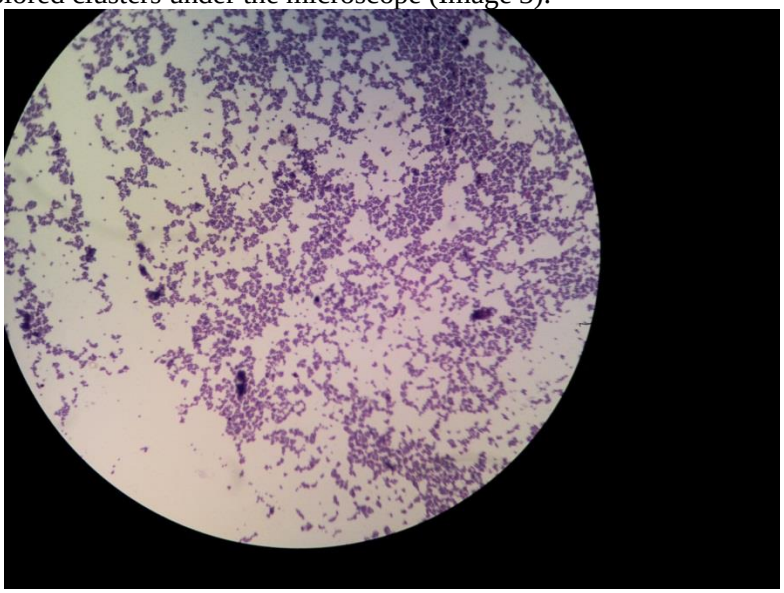

Image 3: This picture indicates staphylococcus aureus stained by gram staining method under the optical microscope

DNA Extraction Results

Image 4 shows the electrophoresis of the genomic DNA extracted from the clinical isolates of Staphylococcus aures.

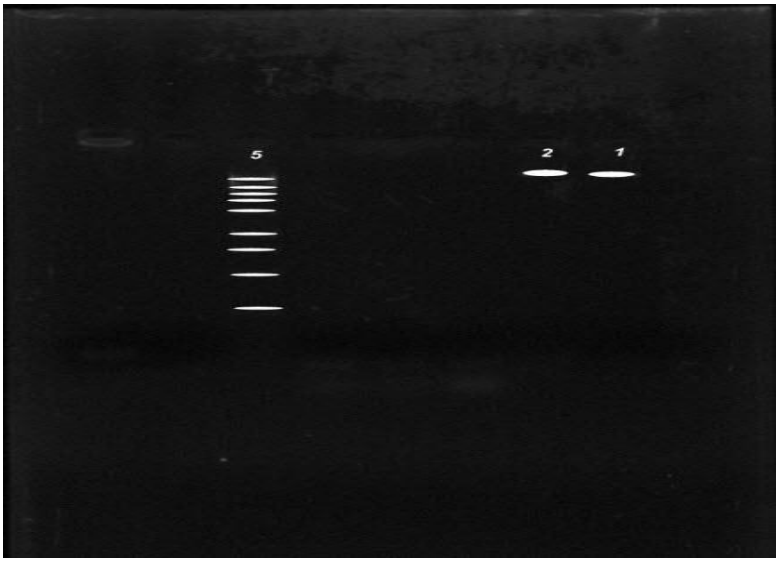

Image 4: The electrophoresis of the extracted genomic DNA product on the agar gel(columns 1 and 2: extracted DNA bands, and Column 5: Marker)

Molecular Identification Results

All Staphylococcus aureus samples were molecularly identified by using a 23SrRna primer. After the agarose gel electrophoresis, bands with weights of almost 1250 bp were achieved (Image 5).

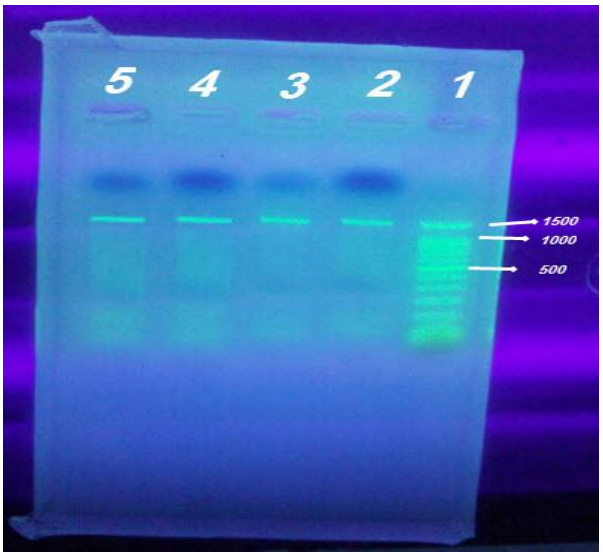

Image 5: The electrophoresis of the PCR product of 23S rRNA gene of staphylococcus aureus bacterium (Column 1: Marker, and columns 2-5: PCR product of clinical isolates of staphylococcus aureus)

PCR Results of Coa Gene

The amplification of the variable region of the coagulase gene of 30 clinical isolates of staphylococcus aureus in the PCR reaction yielded two categories of products with approximate weights of 680 bp in 19 isolates (63%) and 750 bp in 11 isolates (37%) (Images 6 and 7), with the frequency of each of the PCR products shown in Diagram 2.

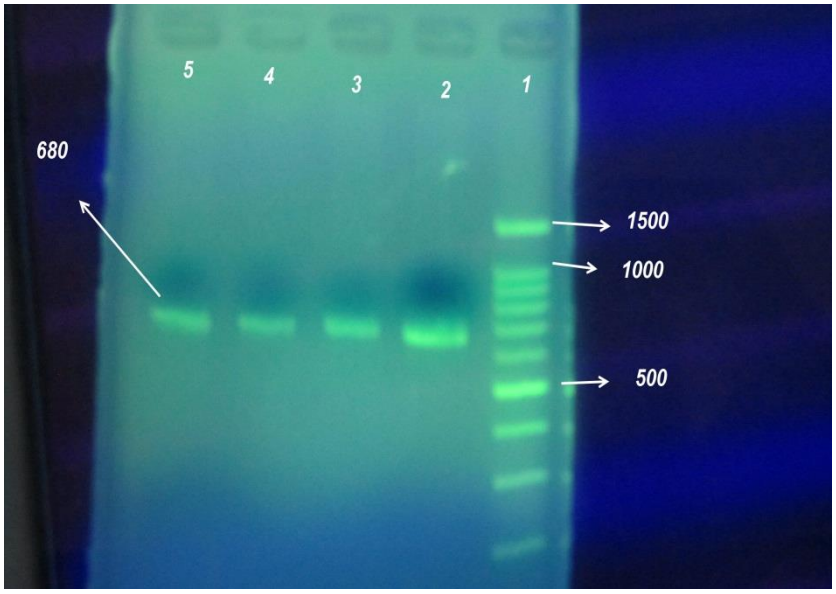

Image 6: The electrophoresis of the PCR product of the variable region of COA gene (Column 1: Marker, and Columns 2-5: a product with 680 bp)

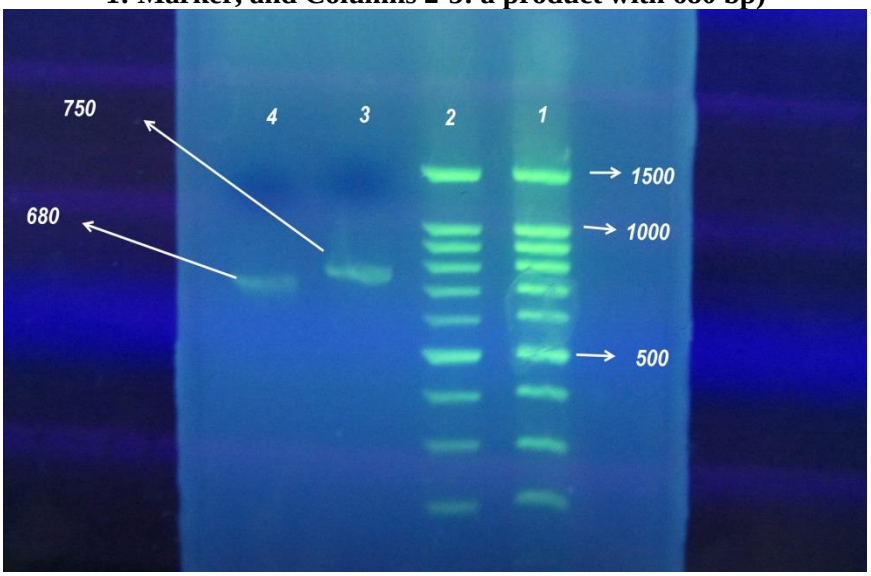

Image 7: The electrophoresis of the PCR product of the variable region of the Coa gene: (Columns 1 and 2: Marker; Column 3: the 750 bp band, and Column 4: the 680 bp band)

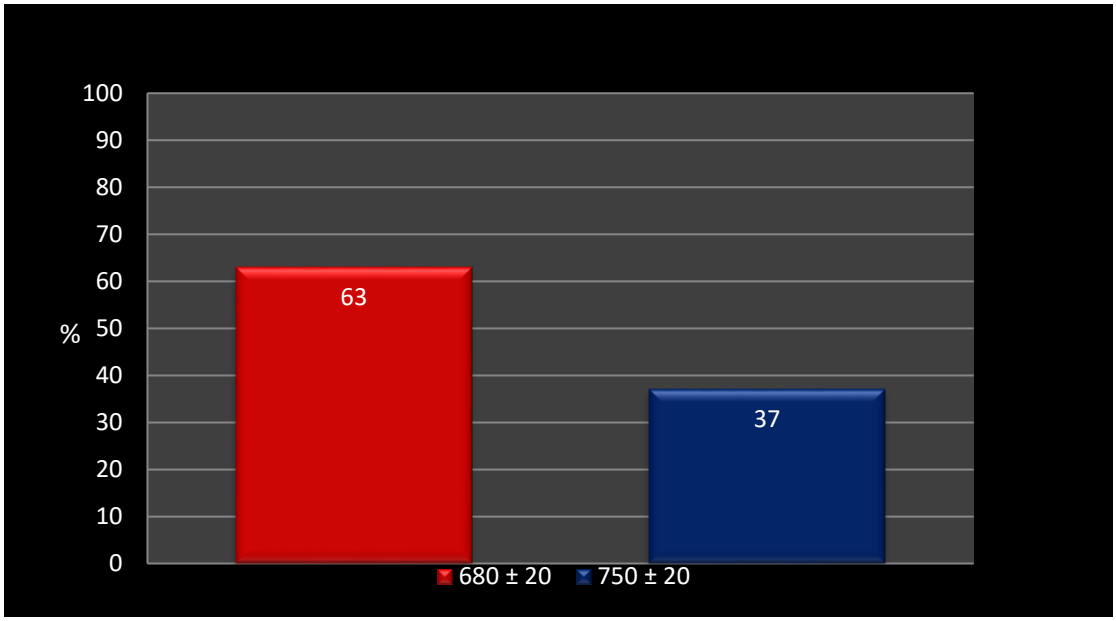

Diagram 2: This figure indicates the frequency percentage of each of the PCR products

PCR-RFLP Reaction Results

The following four different patterns were noted from the digestion of the PCR products of the variable region of the Coa gene of 30 staphylococcus aureus isolates using the Alu1 enzyme.

1. The 400+280 pattern in 13 isolates (45%)
2. The 470+280 pattern in 4 isolates (12%)
3. The 340+340 pattern in 6 isolates (19%), and
4. The no-digestion 750 patterns in 7 isolates (24%)

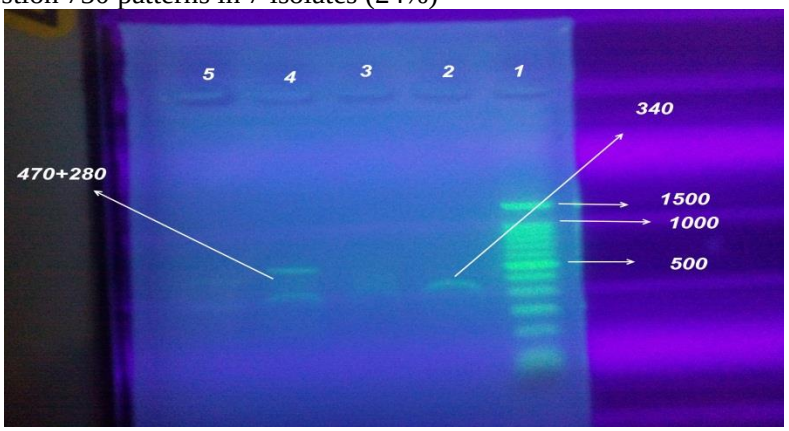

Image 8: The electrophoresis of the 470+280 and 340+340 RFLP pattern (Column 1: 100 bp marker; Column 2: 340 bp band, and Column 4: 470+280 bp band)

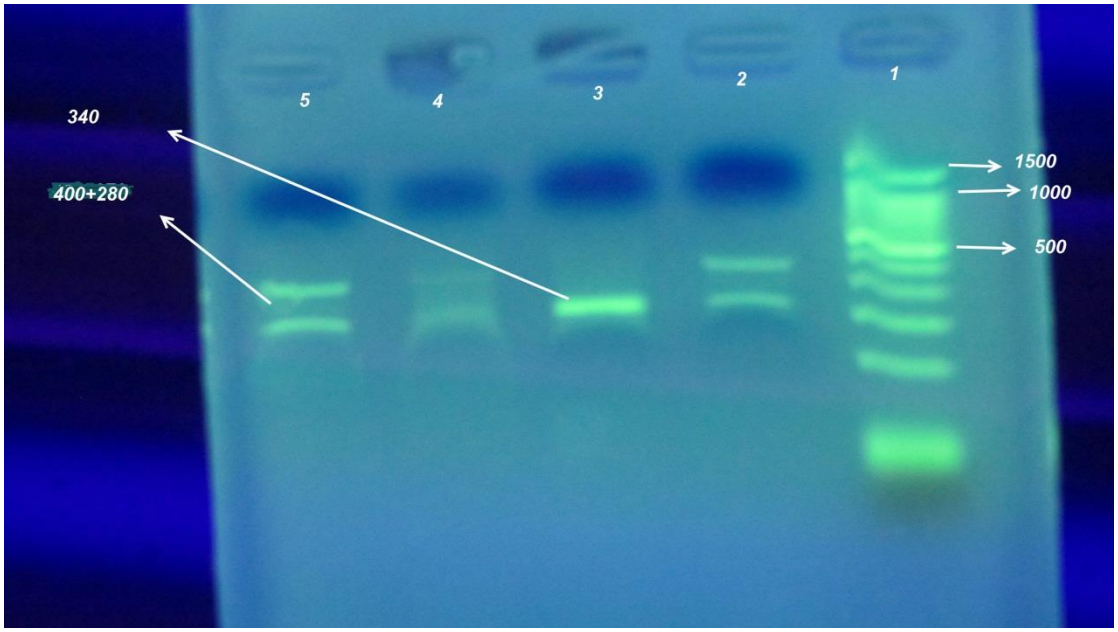

Image 9: The 400+280 and 340+340 RFLP pattern (Column 1: Marker; Columns 4, 2, and 5: 400+280 bp band, and Column 3: 340 bp band)

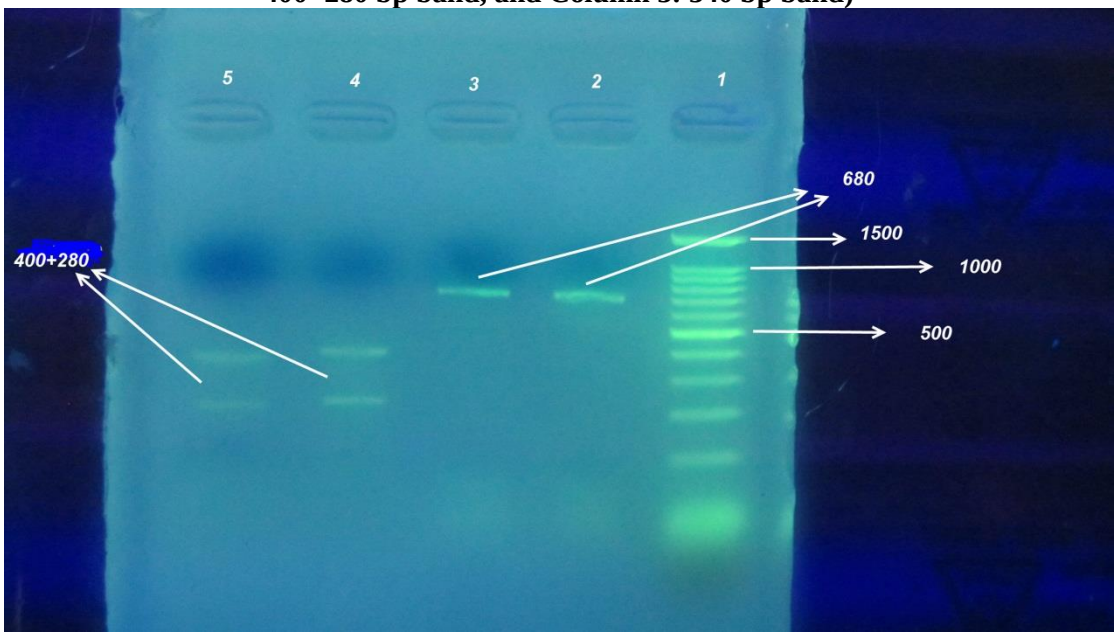

Image 10: The 400+280 RFLP pattern from the enzymatic digestion of the 680 bp PCR product (Column 1: Marker; Columns: 2 and 3: the 680 bp PCR product bands, and Columns 4 and 5: 400+280 bands of the enzymatic digestion product)

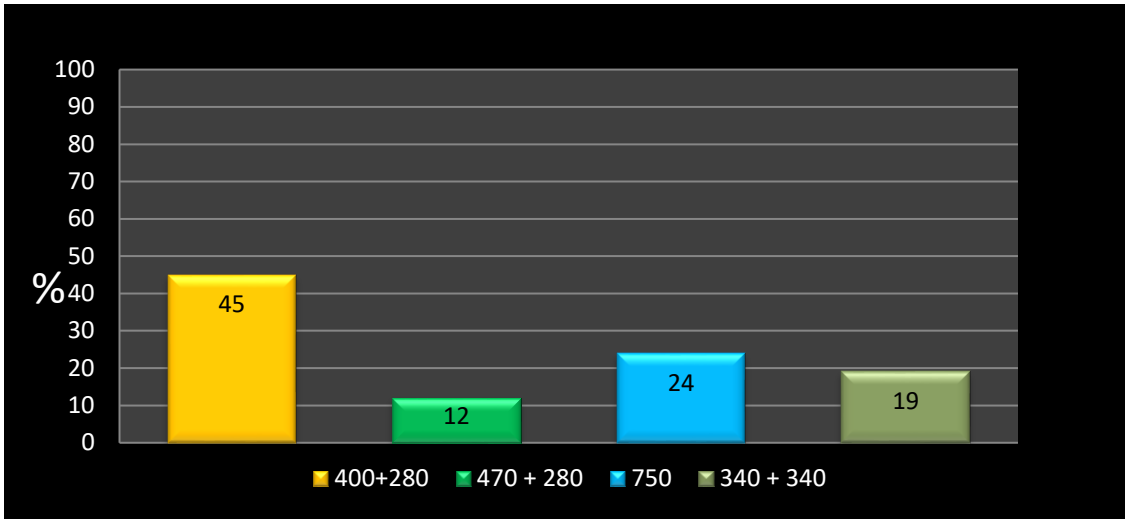

Diagram 3: Frequency percentage of each of the PCR-RFLP patterns in the studied isolates

4.11. The results of the relationship between PCR and RFLP patterns with the lesion site are respectively given in Diagrams 4 and 5. Here, the 680 bp PCR pattern (19 isolates) was respectively noted in 10 urine (34%), 3 ulcers (13%), 2 blood (7%), 2 joint liquid (7%), and 1 abscess (3%) samples. The 750 bp PCR pattern (11 isolates) was respectively noted in 7 urine (24%) samples and one sample in ulcer, blood, abdominal mass liquid, and tracheal secretions each (3%). As for the RFLP patterns, the 400+280 pattern was respectively observed in 7 urine (24%), 3 ulcers (10%), 2 abdominal liquids (8%), and 1 blood (3%) sample. The 470+280 bp RFLP pattern was noted in one sample in blood, abdominal mass liquid, urine, and tracheal secretions each, while the 750 bp RFLP pattern was noted in 6 urine (21%) samples and one ulcer (3%) sample. Meanwhile, the 340+340bp RFLP pattern was noted in 3 urine (10%) samples, and one sample in abscess, ulcer, and blood each (3%). It is noteworthy that the above percentages were calculated based on the studied samples (30 samples).

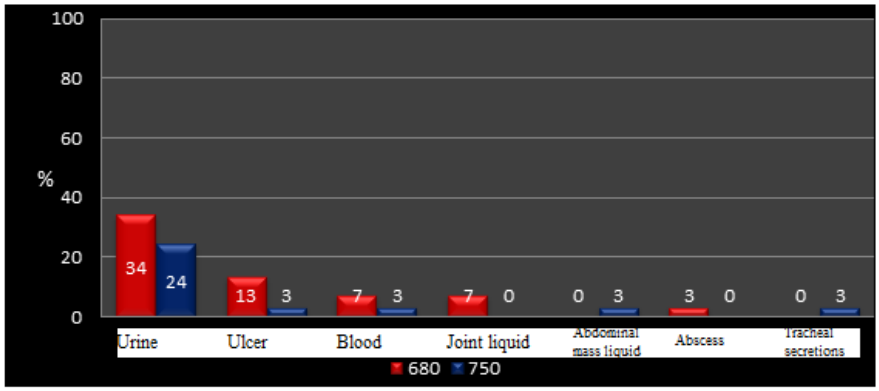

Diagram 4: This diagram indicates the percentage of each of the PCR patterns in different lesions

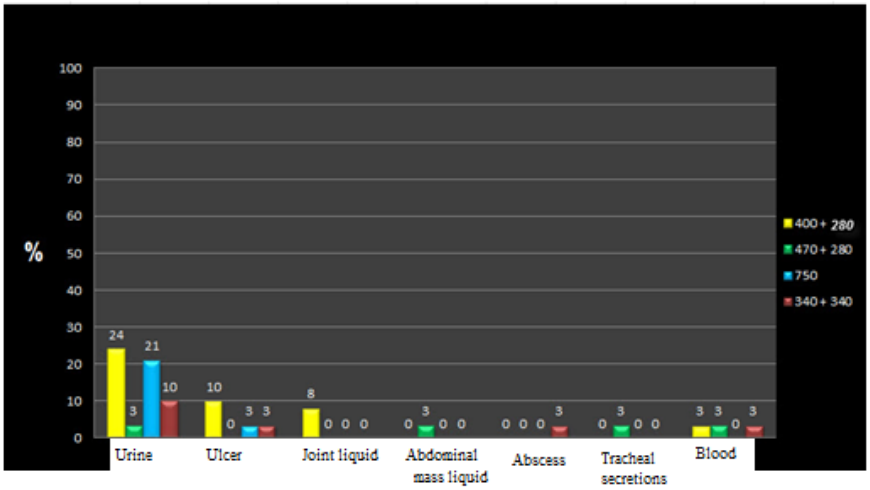

Diagram 5: This diagram indicates the percentage of each of the RFLP patterns in different lesions

Antibiogram Test Results

The antibiogram test was carried out on all staphylococcus isolates, and the resistance percentage of each of the different isolates to the antibiotics used is as follows:

Clindamycin (23%); Cefazolin (23%); Gentamicin (10%), Cloxacillin (53%), Ampicillin (73%), Nitrofurantoin (27%), Vancomycin (7%), Co-trimoxazole (13%), Ciprofloxacin (17%), Erythromycin (47%), Cefalexin (13%), Methicillin (75%), Amoxicillin (60%), Amoxiclav (27%), Cephaletin (47%), and Tetracyclin (27).

The resistance percentage of different isolates to each of the antibiotics is illustrated in Diagram 6. Also, Diagrams 7 and 8 illustrate the resistance percentage of each of the PCR and RFLP patterns to Methicillin. In the 680 bp PCR pattern, noted in 19 isolates, 12 samples (63%) were found to be resistant to Methicillin, and in the 750 bp pattern, noted in 11 isolates, 9 samples (825) were resistant to Methicillin.

As for RFLP patterns, in the 400+280 bp pattern, noted in 13 isolates, 7 samples (54%) were resistant to Methicillin. In the 470+280 bp pattern, noted in 4 isolates, all four isolates (100%) were resistant to Methicillin. In the 750 bp pattern, noted in 4 isolates (71%), five samples were resistant to Methicillin, and in the end, in the 340 bp pattern, noted in 6 isolates, 5 isolates (83%) were resistant to Methicillin.

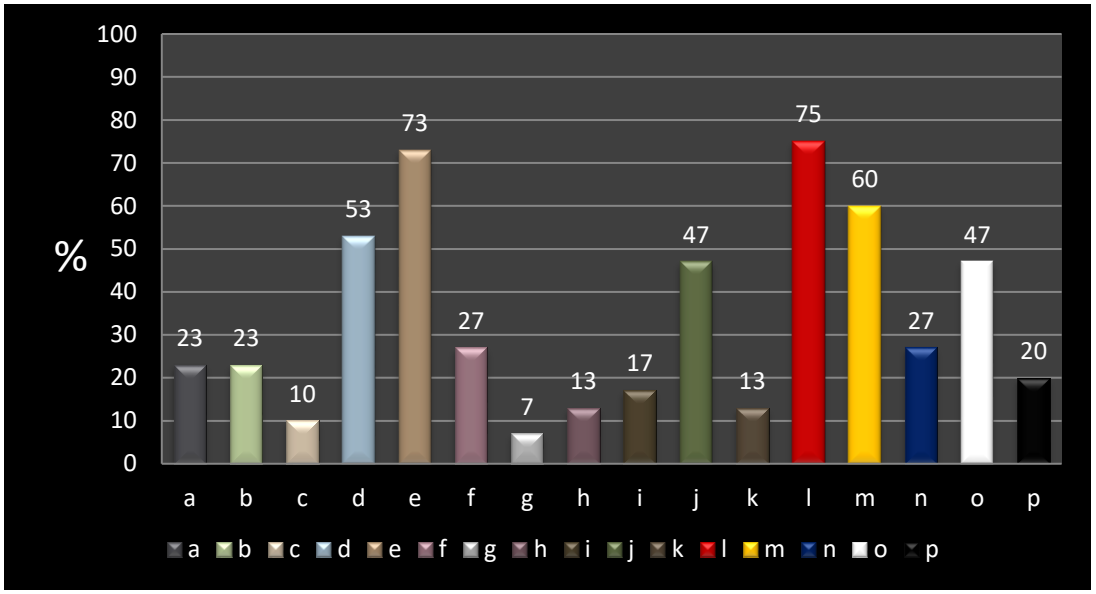

Diagram 6: This picture indicates the percentage of the resistance of each of the staphylococcus aureus isolates to different antibiotics

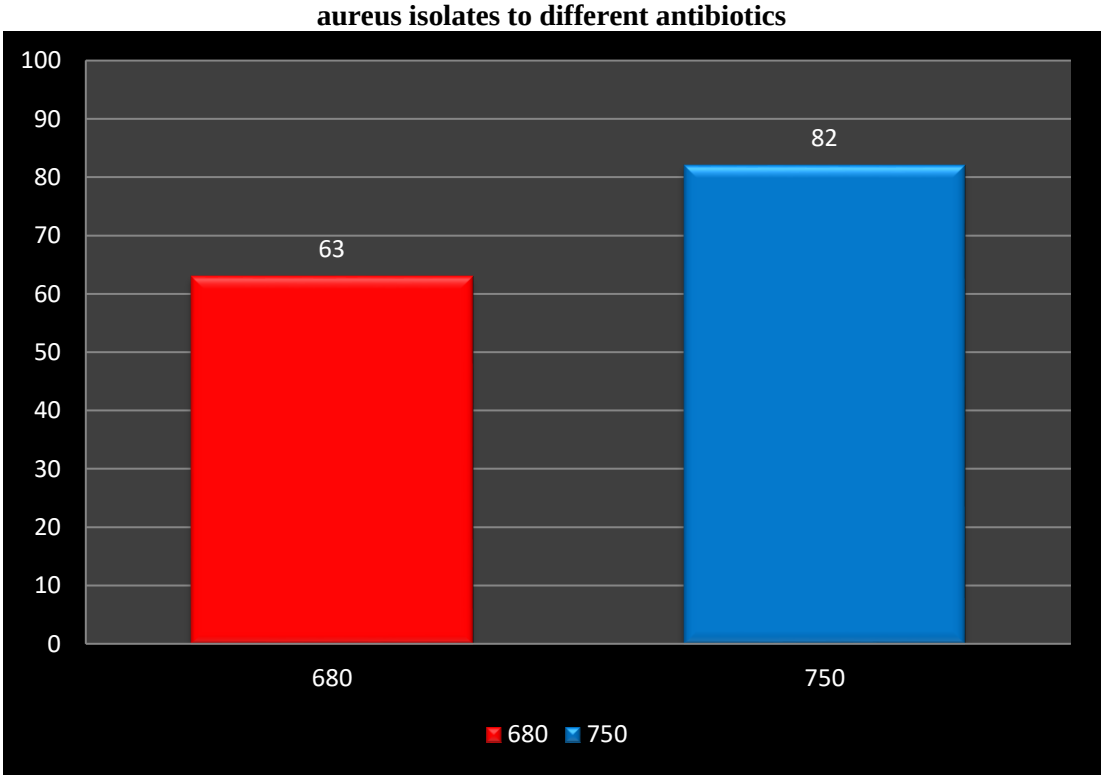

Diagram 7: It indicates the percentage of the resistance of each of the PCR patterns to Methicillin

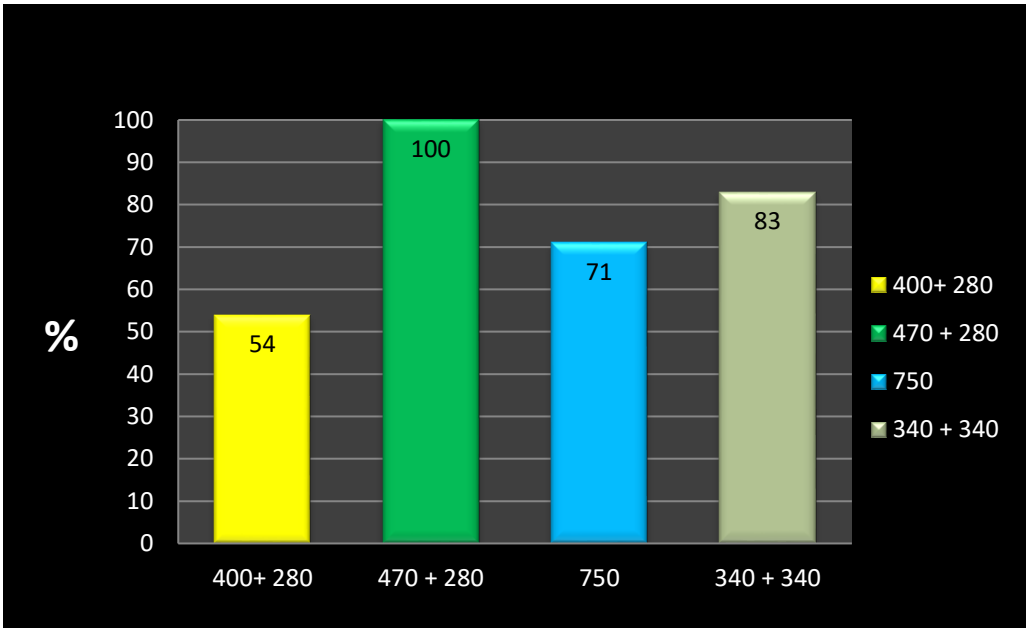

Diagram 8: This indicates the percentage of the resistance of each of the RFLP patterns to Methicillin

Discussion

This study aimed to investigate the genetic patterns of the coagulase enzyme of staphylococcus aureus isolated from clinical samples and also to investigate the relationship between the genetic patterns of the coagulase enzyme and the type of clinical infections caused by this bacterium. After Escherichia coli (E. coli), staphylococcus aureus is the second agent causing hospital-acquired infections. Since resistance to anti-microbial drugs is increasing and staphylococcus aureus shows more drug resistance than other bacteria, defense mechanisms of the immune system as anti-microbial strategies receive much attention. On the other hand, staphylococcus aureus resists to or escapes from the body's defense mechanisms by different methods. Staphylococcus aureus is one of the most important pathogenic bacteria that cause different diseases in humans. Thus, an analysis of the factors involved in the virulence of this bacterium is necessary.

The production of coagulase enzyme by staphylococcus aureus has drawn the attention of clinical microbiologists and is considered a major index in identifying this bacterium. The coagulase enzyme in staphylococcus aureus is an extracellular enzyme that has the same functionality as fibrinogen being converted to fibrin and is catalyzed by thrombin. Coagulase-thrombin not only coagulates fibrinogen but also helps proteolytic and sterolytic activities. Coagulase creates a barrier of fibrin around the lesion site to protect staphylococcus against phagocytosis by using white blood cells (8).

The Coa gene, which encodes coagulase protein, is highly polymorphic due to variable sequences in the ´3 coding region and can be used to distinguish the isolates of this bacterium. The variable region of the Coa gene is composed of repetitive sequences of 81 bp, as the number of repetitions of these sequences varies in various strains of staphylococcus aureus. In many countries, the typing of the staphylococcus aureus genotype has been a part of monitoring programs and served as a major tool to study the origins of strains, determine clonal relevance, and the epidemiology of outbreaks (9).

Because of the presence of polymorphs in the coagulase gene, which is detected by the PCR-RFLP technique, this technique can be used to study the genotype of staphylococcus aureus bacterium to detect the source and origin of these polymorphs for epidemiological reasons. The differences from these polymorphs can be due to the different levels of pathogens or differences in the reservoirs of this bacterium (17).

As stated, coagulase production is the most important phenotypic feature to identify staphylococcus

aureus. Different genotypes of the coagulase gene have been noted in different studies. These dominant genotypes may be due to their resistance to ambient conditions and also to the host's immune response.

PCR-RFLP is an effective method to control hospital-acquired infections caused by Staphylococcus aureus. This method helps to create a distinct genetic set to perform epidemiological studies. The type of prevalence of various PCR and RFLP patterns in different lesions suggests that this genetic subset can be used to match the observed patterns and the site of lesions and infections across body parts. However, the presence of several virulent genes in this bacterium warrants further research about the consistency between the pathogenicity of this bacterium in various parts of the body and the genetic patterns observed (18). Heterogenous numbers of repeat units in the Coa gene are recognized as target potentials for molecular typing in various MRSA strains (Methicillin-resistant Staphylococcus aureus) (19).

The antibiogram method and the relationship between this test and the PCR and RFLP patterns of the Coa gene is a good method for molecular typing. The problem with this method, however, is that resistance-related genes may be extrachromosomal. Therefore, for typing in this method, only the intrachromosomal resistance genes (mec A gene in MRSA) has this potential (20).

Methicillin-resistant Staphylococcus aureus (MRSA) strains are thought of serious hospital-acquired infection threats that cause treatment problems, also. Because of the severe pathogenicity of this bacterium and high treatment costs, it is necessary to take control and prevention measures. The outbreak of MRSA strains was reported in European hospitals immediately one year after the introduction of Methicillin in 1961. Currently, these strains have spread across the world. The frequencies of the MRSR strains in Asian countries, such as China, Korea, and Taiwan exceed 70% while amounting to 50%, 20%, and 50% in North America, Europe, and Iran, respectively.

The present study investigated 30 staphylococcus aureus isolates for polymorphisms in the Coa gene, and examined the presence or absence of a significant relationship between the Coa genotype and the type of clinical infection; also, the relationship between observed genetic patterns and Coa gene with the resistance and sensitivity of strains to Methicillin was studied.

In this study, after the studied samples were collected, diagnostic tests such as the coagulase test, catalase, DNase, and gram staining were carried out on each of the samples. For the complementary diagnosis, the molecular identification of the studied isolates was performed by using the 23SrRNA primer of staphylococcus aureus, with all the isolates demonstrating almost 1250 bp bands on the agarose gel. Using a specialized primer of the variable region of the Coa gene in the PCR reaction, two categories of PCR products of 680 and 750 bp lengths were observed that indicated the presence of polymorphisms in the studied isolates. Here, the frequencies of the 680 bp and 750 bp patterns were 63% and 37%, respectively, suggesting the high genotypic prevalence of the former pattern in the staphylococcus aureus isolates in the city of Rasht, Iran.

It should be pointed out that both genetic patterns fall under dominant patterns, as stated by literature in Iran and across the world (of course with a difference of ±20 bp). Concerning observed patterns as cited by Iranian studies, one can refer to findings by Dastmalchi et al. (2019) who reported the 680 and 780 bp PCR patterns (21), and Momtaz et al. (2019) who reported the 730 bp patterns (16). As well, Hookey et al (2015), Janwithayanuchit et al. (2016), Aslantas et al. (2016), Moon et al. (2016), and Sanjiv et al. (2018) observed a 660 bp pattern (22), 654 and 735 bp patterns (23), 730 bp patterns (24), 670 bp patterns (25), and 680 bp patterns (26), respectively. However, studies done on Coa gene polymorphisms have usually yielded 4 or more 4 genetic patterns resulting from the PCR reaction. These polymorphs may be due to the high number of samples in the studies across the world. Following the PCR reaction, enzymatic digestion (RFLP) was performed by using the restrictive Alu1 enzyme on the PCR product of the Coa gene of the studied isolates. As a result of the enzymatic digestion reaction, four different genetic patterns were noted, including 1. The 400+280 bp pattern with a frequency of 45%; 2. The 340 bp pattern with a frequency of 19%; 3. The 470+280 bp pattern with a frequency of 12%, and 4. The non-digestion 750 bp pattern with a frequency of 24%.

According to the four observed patterns resulting from the RFLP reaction, the highest prevalence pertained to the 400+280 pattern, while the lowest prevalence pertained to the 470+280 pattern, with

patterns almost similar to these two genotypes noted in Momtaz et al.'s study (2012), who observed the 490+240 pattern, and Aslantas et al.'s study (2006), who observed the 490+240 pattern (14, 24). The single-band 340 bp pattern (resulting from the enzymatic digestion of the PCR 680bp product) indicates two overlapped 340 bp bands, an example of which was observed in Sanjiv et al.'s study (2008) involving a single-band 300 bp resulting from the enzymatic digestion of the 600 bp PCR pattern (26). In the end, the non-digestion 750 bp pattern, which is probably due to mutation in the cutting region of the Coa gene by the restrictive Alu1 enzyme, was observed in studies by Yaser et al. (2012), and Guler et al. (2015) (20, 21).

Concerning the relationship between PCR and RFLP patterns and the lesion site, the distribution of the mentioned patterns was observed at different lesion sites, suggesting the lack of a significant relationship between the lesion site and the intended patterns. This finding is consistent with those of Talebi et al. (2012). In Talebi et al.'s study, around 70% of skin and urinary tract infections pertained to a special genetic pattern that demonstrated a greater prevalence of a genetic pattern at special lesion sites (29).

Also, Tiwari et al. (2018) studied the relationship between the genetic patterns obtained with the type of the bacterium (resistant or sensitive to Methicillin), the lesion site, and different hospital sections where the studied isolates had come from, concluding that due to the type of the pattern distribution in different sections, the PCR-RFLP method was an appropriate method for molecular typing (30).

Consistent with studies by Talebi et al. and Tiwari et al.'s studies, it is concluded that a genetic subset in staphylococcus aureus isolates has been specifically well adapted to cause infections across the entire body. In the meantime, the type of genetic adaptation between various types of bacteria can be significantly different, as various factors may be involved in this type of adaptation. These factors include the presence of adhesin genes that may have a role in the bonding of a bacterium to a specific region. Such structures as teichoic acid and protein A create sterile and incomplete reactions with immunoglobulins in a specific region to create the resistance and adhesion of these microorganisms in that region. The presence of sialoproteins near bones helps create a strong capacity to establish MRSA, whose epidemic prevalence is reflected through skin-to-skin contact. Concerning the prevalence of urinary infections, this may be caused by the intracellular adhesion gene and also the hematogenous spread from other regions to the urinary tract. Also, the presence of amino acid arginine in some of the strains (which may cause the bacterium to survive at low skin PH) could increase the affinity and resistance of the bacterium in the skin region.

Despite some specialized studies providing epidemiological insight among various isolates of staphylococcus aureus, it is requited to conduct large-scale studies on wider sample volumes of clinical samples and genetic analyses. Concerning the relationship between PCR and RFLP patterns and the sensitivity and resistance of isolates to Methicillin, 82% of the isolates of the 750 bp PCR patterns and 63% of the isolates of the 680 bp PCR patterns were found to resist Methicillin; meanwhile, in the RFLP patterns, the isolates of the 470+280 pattern resulting from the enzymatic digestion of the 750 bp PCR patterns showed 100% resistance to Methicillin. In the RFLP pattern, the isolates of the 400+280 patterns resulting from the enzymatic digestion of the 680 bp PCR pattern demonstrated 54% resistance to Methicillin.

Similar studies were done by Hookey et al. (2019), after performing the enzymatic digestion of the Coa gene on various bacteria, 10 different patterns were created, four of which were observed in MRSA isolates (22). Janwithayanuchit et al. (2016) and Tiwari et al. (2018)
also observed the relative prevalence of special PCR-RFLP patterns in MRSA isolates.

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