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Detection of genetic mutations by insertion sequence IS256 in *Staphylococcus aureus* under the stress of Vancomycin and Ciprofloxacin

A Thesis

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

((يَا أَيُّهَا الَّذِينَ ءَامَنُوا إِن تَنصُرُوا اللَّهَ يَنصُرْكُمْ

وَيُثَبِّتْ أَقْدَامَكُمْ)) (٧)

سُورَةُ مُحَمَّدٍ

آيَةُ رَقْم ٧

Certification

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Dedication

Who has been a constant source of support and encouragement during the challenges of graduate study and life.

My husband....

Whose shared a dream with me, their encouragement and prayers have guided me, without whom I would not have come this far.

To my parents....

Who their love flows in my veins. Whose they always I find them when I need them.

To my brothers and sisters....

Rasha

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Summary

A total of 109 clinical specimens were collected from burns, nose, urine, joint fluid, vagina, blood, sputum and tip drainage and soft tissues from surgical operations for patients attends Baghdad teaching hospital and the division of laboratories/ private nursing hospital in Baghdad Governorate for the period from July/2019 to September/2019. From these samples a total of 109 bacterial isolates were obtained after culturing on McConkey agar and Blood agar plates. After screening these bacterial isolates, it was found 47 of them were Gram positive and 62 Gram negative bacteria. Twenty-two of G⁺ve bacteria were identified as *Staphylococcus aureus* according to their ability to cause β -heamolysis, monnitrol fermentation, they were positive for catalase, lisithinase, urease, while they were negative for oxidase. Identification of these isolates was confirmed by using Viteck-2.

Susceptibility of *S.aureus* isolates to different antibiotics was determined. Results showed these isolates have different resistant profile for different antibiotics, where all the isolates (100%) were resistant to penicillin and methicillin, 77% were resistant to ampicillin, 53% were resistant to erythromycin, 45% to azithromycin, trimethoprim and chloramphenicol, 36% were resistant to tetracycline, 23% to ciprofloxacin, doxycillin and lincomycin, 18% to refampin and 4% for vancomycin.

Genomic DNA was extracted from all *S.aureus* isolates with high purity and concentration, then insertion sequence 256 (IS256) was detected by using specific primers. Results showed that IS256 was detected in 18 isolates, and the prevalence of this transposable element in bacterial isolates from clinical samples was 100% in UTI isolates, nose, wounds, tip drainage and soft tissues isolates, 66% in respiratory isolates, 33% in joint

fluid isolates, and this element was not founded in vagina and blood isolates.

S.aureus S1 was selected from the other bacterial isolates because it was multidrug resistant and the most antibiotic resistant isolate to study the stress of vancomycin and/or ciprofloxacin on transposition frequency by IS256 and inactivate antibiotic resistance genes by pretreatment of this isolate with the subinhibitory concentration of vancomycin and / or ciprofloxacin for six hours intervals, then aliquots were taken each hour and spread on Brain- Heart infusion agar containing the antibiotic that the bacterial isolate was resist and calculation the transposition frequency and survival fraction (%). Results showed that the pretreatment with vancomycin was more effective than ciprofloxacin and both vancomycin and ciprofloxacin according to higher transposition frequency and inactivating antibiotic resistance genes, and decrease the survival fraction after this pretreatment.

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List of Abbreviations

Abbreviate	Meaning
a _w	water activity
BHI	Brain Heart Infusion
bp	Base Pair
CLSI	Clinical Laboratory Standards Institute
FnBPs	Fibronectin-binding proteins
IR	inverted repeat
IS	insertion sequence
Kb	Kilo base
MRSA	methicillin resistant <i>S. aureus</i>
MBC	Minimal Bactericidal Concentration
MDR-SA	multidrug-resistant <i>S.aureus</i>
MGEs	mobile genetic elements

MIC	Minimum Inhibitory Concentration
MSA	Mannitol Salte Agar
PBS	Phosphate Buffer Saline
PEC	Paired-End Complex
PVL	Panton-Valentine leukocidin
SEC	Single-End Complex
SF	Survival Fraction
TEs	Transposable elements
TF	Transposition Frequency
Tn	transposition
TNFR-1	tumor necrosis factor 1
TSB	Tryptone soya broth
TSST-1	Toxic shock syndrome toxin
μ	Mutation rate
VRSA	vancomycin resistant <i>S.aureus</i>
vWF	von Willebrand factor

1. Introduction

Staphylococcus aureus is the most dangerous of the many common staphylococcal bacteria. This gram positive, sphere shaped (coccal) bacteria often causes skin infections but can cause pneumonia, endocarditis, osteomyelitis, bacteremia (Zycinska *et al.*, 2008).

Increasing trend of antimicrobial resistance in bacteria that causes infectious diseases is a global problem, although resistance significantly varies between geographical regions. The common bacterial pathogens can be resistant to all known antimicrobial agents (Prestinaci *et al.*, 2015). Infections caused by resistant *S. aureus* can be difficult to treat, resulting in a greater risk of death. Especially Orthopaedic infections, such as *S. aureus*. Most clinical cases of orthopaedic surgeries have shown that patients infected with antibiotic resistant bacteria, such as methicillin resistant *S. aureus* (MRSA), are associated with increased morbidity and mortality (Walsh, 2016).

The date of multidrug-resistant *S.aureus* (MDR-SA) have been isolated from both adults and children around the world (Thapaliya *et al.*, 2018), they are resistant to, methicillin, extended-spectrum beta-lactamase-producing bacteria, streptomycin, gentamycin, tetracycline and vancomycin (Schaumburg *et al.*, 2016). The emergence of antibiotic resistance among bacterial pathogens is a major problem in treatment of infectious disease in both community and in healthcare settings throughout the world (Kleinert *et al.*, 2017).

Many virulent factors of *S. aureus* increase its susceptibility of antibiotics. The virulent factors is including adherence to host proteins do promotes attachment to blood clots and traumatized tissue (Thomer, 2016), and *S. aureus* is avoidance of host defenses by many defense mechanism such as capsule, teichoic acids, Protein A, fibronectin and

collagen binding protein (Tankeshwar, 2019). *S. aureus* is known of its capability to produce extra cellular toxins include haemolysis, leukcidin, enterotoxin, exfoliative and TSST-1 (Bukowski *et al.*, 2010).

Staphylococcus aureus has enzymes to help opsonization and phagocytosis to infection varies tissues include coagulase, staphylokinase, hyaluron -idase and deoxyribonuclease (Berends *et al.*, 2010).

Methicillin resistant *S.aureus* (MRSA), vancomycin –resistant (VRSA) and multidrug-resistant have the ability to acquire mobile genetic elements and transposition of IS256 has been investigated in *S.aureus*, where to play an important role in biofilm formation and antibiotic resistance (Gregorio *et al.*, 2016).

IS256 has been detected in the genome of several clinical isolates of *S. aureus* in multiple copies, alone or flanking both ends of the amino -glycoside resistance transposon Tn4001. Tn4001 is composed of 1.9 Kb central region flanked 1.3 Kb (1324 bp) (Moffatt *et al.*, 2011) inverted repeats, the IRs flanking the resistance determinant of Tn4001 have been designated IS256. Transposon can transposes by a copy and paste mechanism (Vandecraen *et a l.*, 2017).

Gene inactivation is Possible affects of IS transposition in the function and expression of a target gene. Many cases have been described illustrating the modulation of resistance, virulence and metabolic activities by IS-mediated gene inactivation (Vandecraen *et al.*, 2017).

According to what mentioned above, this study was aimed to detection of genetic mutations by insertion sequence IS256 in *Staphylococcus aureus* under the stress of Vancomycin and Ciprofloxacin antibiotics. This was achieved according to the following steps:

1. Collection of specimens from clinical sources (boil, wounds, nasal cavity, urine, vagina, blood, operation tip drainage, soft tissue, joint fluid and sputum).
2. Identification of *S. aureus* according to their morphological characteristic and biochemical tests.
3. Investigating the antibiotic resistance of bacterial isolates against different antibiotics from different groups, and detection of the minimum inhibitory concentration MIC and the minimum bactericidal concentration MBC for vancomycin and ciprofloxacin antibiotics.
4. Detection of IS256 in bacterial isolates.
5. Choosing bacterial isolate which contained IS256 and multi drug resistant and high minimum inhibitory concentration to Vancomycin and Ciprofloxacin.
6. Investigating the genetic mutation and survival fraction IS256 in *S. aureus* under the influence of stress Vancomycin and Ciprofloxacin antibiotics on penicillin, Ampicillin, Azethromycin, Trimethoprim, Erythromycin, Rifampin, Gentamycin and Lincomycin.

2. Literature Review

2.1 Genus *Staphylococcus*

Staphylococci (staph) are Gram-positive spherical bacteria that occur in microscopic clusters resembling grapes. Bacteriological culture of the nose and skin of normal humans invariably yields staphylococci. In 1884, Rosenbach described the two pigmented colony types of staphylococci and proposed the appropriate nomenclature: *Staphylococcus aureus* (golden) and *Staphylococcus albus* (white). The latter species are now named *Staphylococcus epidermidis*. Although more than 30 species of *Staphylococcus* are described in Bergey's Manual (2015), only *Staphylococcus aureus* and *Staphylococcus epidermidis* are significant in their interactions with humans. *S. aureus* colonizes mainly the nasal passages, but it may be found regularly in most other anatomical locales, including the skin, oral cavity and gastrointestinal tract. *S. epidermidis* is an inhabitant of the skin. Although the cooking process kills them, the enterotoxins are heat-resistant and can survive boiling for several minutes. *Staphylococci* can grow in foods with relatively low water activity (Ijabadeniyi, 2017).

There are five organisms to consider as potential human pathogens in this genus: *S. aureus*, *S. epidermidis*, *S. saprophiticus*, *S. haemolyticus*, and *S. hominis* but the first three are the most common isolates. the coagulase-negative staphylococci (*S. epidermidis* and *S. saprophiticus*) are much less frequently found as pathogens but are occasionally associated with endocarditis, prosthetic joint infections, and wound infections, just to name a few (Reynolds, 2019).

Reynolds (2019) mentioned that Types of species differ in their susceptibility to growth in this case based on high sodium chloride (MSA and SM1 10 are both selective media for the isolation of Staphylococci-

7.5% NaCl). A selective medium has an inhibitory agent which favors the growth of certain bacteria by inhibiting others. MSA contains an additional indicator for monitoring mannitol fermentation, which makes it a differential media also. The bacteria which can grow in the presence of high NaCl, some are halophilic (requiring a certain concentration of salt to grow) while other is haloduric (do not use the salt, but can tolerate it). *Staphylococcus* is not halophilic, but rather haloduric, in that it can live in or endure high NaCl concentrations. The high salt content in SM1 10 and MSA inhibits other common skin microorganisms.

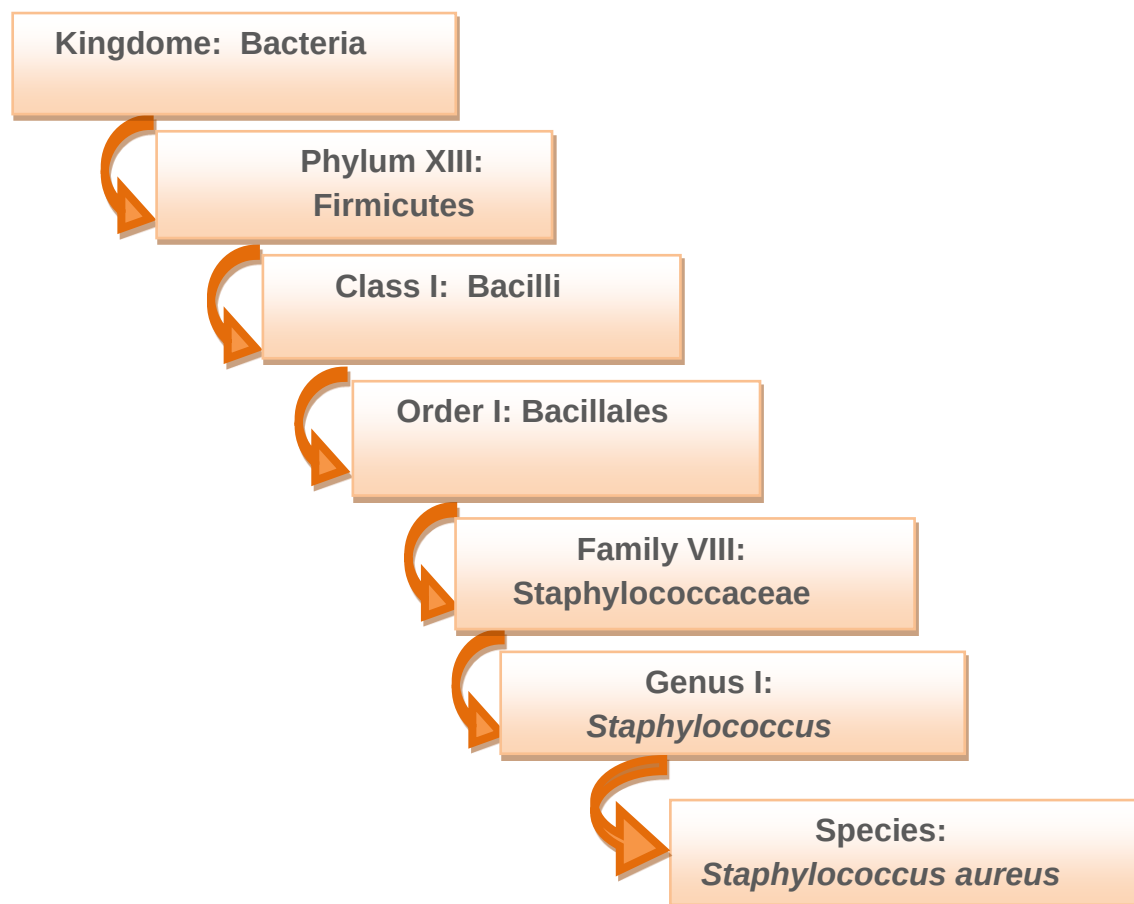
Not only salt resistant, *Staphylococcus* is always facultatively anaerobic. When stained, it will be seen in small clusters (staphylo = cluster). *Staphylococcus* is usually either beta hemolytic or not hemolytic at all (called gamma hemolysis). Pathogenic Staphylococci can produce a variety of virulence factors, including toxins, coagulase, leucocidins, and hydrolytic enzymes that can damage host tissues.

Hemolysis is the breakdown of red blood cells. Hemolysins are enzymes produced by some bacteria and are released into the medium around the bacterial colony. It can be a complete breakdown of the cells, with the release of hemoglobin and a clearing of the red from the surrounding medium around the colony, or the hemolysis can be a partial breakdown, resulting in a greenish or green-yellow zone around the colony (Naushad *et al.*, 2019).

2.2 *Staphylococcus aureus*

Staphylococcus aureus, literally "Golden Cluster Seed" and also known as golden staph, is the most common cause of staph infections. It is a spherical bacterium, frequently living on the skin or in the nose of a person. Approximately 20–30% of the general populations are "staph

carriers" (Miller and Geffen, 2020). *S. aureus* is a Gram-positive coccus, which appears as grape like clusters when viewed through a microscope and has large, round, golden yellow colonies, often with β -hemolysis, when grown on blood agar plates, *S. aureus* is a facultative anaerobe and opportunistic pathogen (Taylor, 2019). According to Bergey's Manual of Systematic bacteriology, *S. aureus* was classified as follows:



The characteristics used to identify *S. aureus* include Gram stain morphology, cell morphology, production of catalase, coagulase production, pigment production, susceptibility to lysostaphin and lysozyme, and anaerobic production of acid from glucose (Habib *et al.*, 2015). There are several other commercially available methods that allow

strains to be biochemically characterized. *S. aureus* is unique in its ability to clot plasma (coagulase).

2.3 Growth conditions of *S. aureus*

The nutritional requirements of *S. aureus* are complex and vary from strain to strain (Krismer *et al.*, 2014). In general, *S. aureus* grows between 7 and 47°C, with an optimum of 30-37°C. Enterotoxins are produced between 10 and 46°C, with an optimum of 35-45°C. Enterotoxin production is substantially reduced at 20-25°C. It is generally accepted that enterotoxin production is unlikely to occur at temperatures below 10°C (Medved'ová *et al.*, 2017). Enterotoxins can be narrower than the range over which it will grow. It is possible for *S. aureus* to grow without producing enterotoxin. Optimum enterotoxin production occurs at pH 6-7 and is influenced by atmospheric conditions, carbon and nitrogen source and salt level. Reduced levels of water activity (a_w), may also inhibit toxin synthesis more than growth. Optimum growth and toxin production occur at a_w levels >0.99 . Toxin production has been reported at as low as $a_w = 0.86$. *S. aureus* can grow in the presence or absence of oxygen, but grows best under aerobic conditions (Claes *et al.*, 2014).

2.4 Pathology

Staphylococcus aureus may occur commensally on human skin (particularly the scalp, armpits, and vagina), it also occurs in the nose and throat (in about 25% of the population) and less commonly in the colon and in urine. The occurrence of *S. aureus* does not always indicate infection and therefore does not always require treatment (Zycinska *et al.*, 2008). *S. aureus* is normally found colonizing many parts of human's body such as nose, mouth, mammary gland, hair and upper respiratory tract. *S. aureus*

usually colonizes these parts of the body without causing harm. Although it is considered as a normal flora in some parts of human's body, *S. aureus* causes a wide range of infections in the skin, wound and deep tissues to life-threatening conditions such as: endocarditis, pneumonia, septic arthritis and septicemia. These bacteria also most commonly cause nosocomial infections. Furthermore, *S. aureus* can also cause joint and bone infections, food poisoning, scalded skin syndrome and toxic shock syndrome, through the production of many toxins (Tong *et al.*, 2015; Akindele *et al.*, 2010). Figure (2-1) shows the site of infection and disease caused by *S. aureus* both according to its location in body.

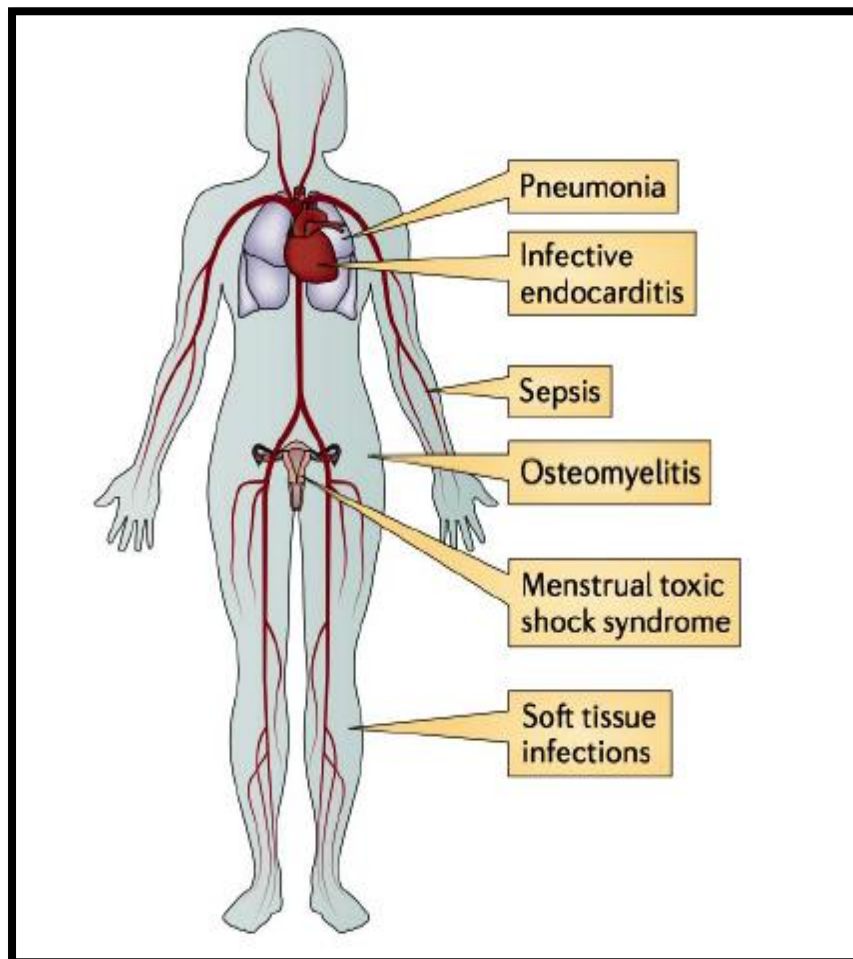


Figure (2-1): Sites of infection and diseases caused by *Staphylococcus aureus* (Chung, 2018).

2.5 Antimicrobial agents

The development of resistance to many antibiotics by *S. aureus* has involved acquisition of determinants by horizontal gene transfer of mobile genetic elements (Jensen and Lyon, 2009). These determinants may have evolved in antibiotic producers to protect them from potentially inhibitory molecules, or in their competitors. Analysis of the soil resistome shows that the express of bacterial resistance to antibiotics are widespread (Nesme and Simonet, 2015).

Resistance also can by two ways:

- Emerge by mutations that alter the drug binding sites on molecular targets such as penicillin and vancomycin.
- Increasing expression of endogenous efflux pumps.

The development of resistance by mutation can in principle be reduced by using combinations of inhibitors that target different sites or for two or more mutations. Penicillin-resistant strains of *S. aureus* emerged shortly after the introduction of the antibiotic in the early 1940s (Peacock and Paterson, 2015; Walsh, 2016). *S. aureus* β -lactamase responsible for resistance to penicillin (Lowy, 2003). The β -lactamase structural gene *blaZ* gene is carried by the transposon Tn552 (Jensen and Lyon, 2009). The transposon is either located on a large plasmid, the prototype of which is pI524, or is integrated into the bacterial chromosome. The altered PG structure might also account for the inability of the global accessory gene regulator (Agr) to be induced in some MRSA strains (Rudkin *et al.*, 2012). Increase the resistance to methicillin was indicated in various hospitals in the world. In many MRSA strains, the genes have been truncated by insertion sequences which should result in constitutive expression of genes that the β -lactamase regulators (Walsh, 2016).

Resistance of *S. aureus* to streptomycin, gentamycin and tetracycline has been increased after a few years of using them emergence of high-level resistance encoded by mobile genetic elements (Jensen and Lyon, 2009). While the tetracycline efflux pumps or the ribosomal protection proteins can be altered by mutations to generate resistance determinants are typically encoded on chromosomally located conjugative transposons such as Tn916 and Tn1545 (Jensen and Lyon, 2009).

Fifth generation of semisynthetic cephalosporins, ceftaroline fosamil and ceftobiprole, exhibited significant inhibitory activity against the Pencillin Binding Protein 2a of MRSA. Only to treat *S. aureus* infections, resistance to the drug with mutations that cause amino acid substitutions both close to the active site and at the allosteric site of PBP2a (Long *et al.*, 2014; Lahiri and Alm, 2016; Schaumburg *et al.*, 2016).

Since 1996, vancomycin was considered as the drug of choice against *S. aureus*, but the emergence of reduced susceptibility of bacteria to this antibiotic increases the possibility that some strains will become fully resistant to this antibiotic. The *Van* genes are located on mobile genetic elements (Courvalin, 2006; Zeng *et al.*, 2016).

Plasmid – mediated antibiotic resistance in *S. aureus* has been reported. The most troublesome source of resistance is the transfer of genes from resistant organisms to those that are sensitive by the mechanisms: conjugation, transduction and transformation (Howden *et al.*, 2010; Gardete and Tomasz, 2014; Howden, Peleg and Stinear, 2014; Zeng *et al.*, 2016). The resistance gene that code for enzymes that inactivate the antimicrobial are often found on conjugative plasmids called resistance plasmids or R plasmids. Because of the ease with which antibiotics resistance on R plasmids can be transferred through conjugation (Foster,

2017). Stages of evolution of antimicrobial resistance to *S. aureus* were illustrated in figure (2-2).

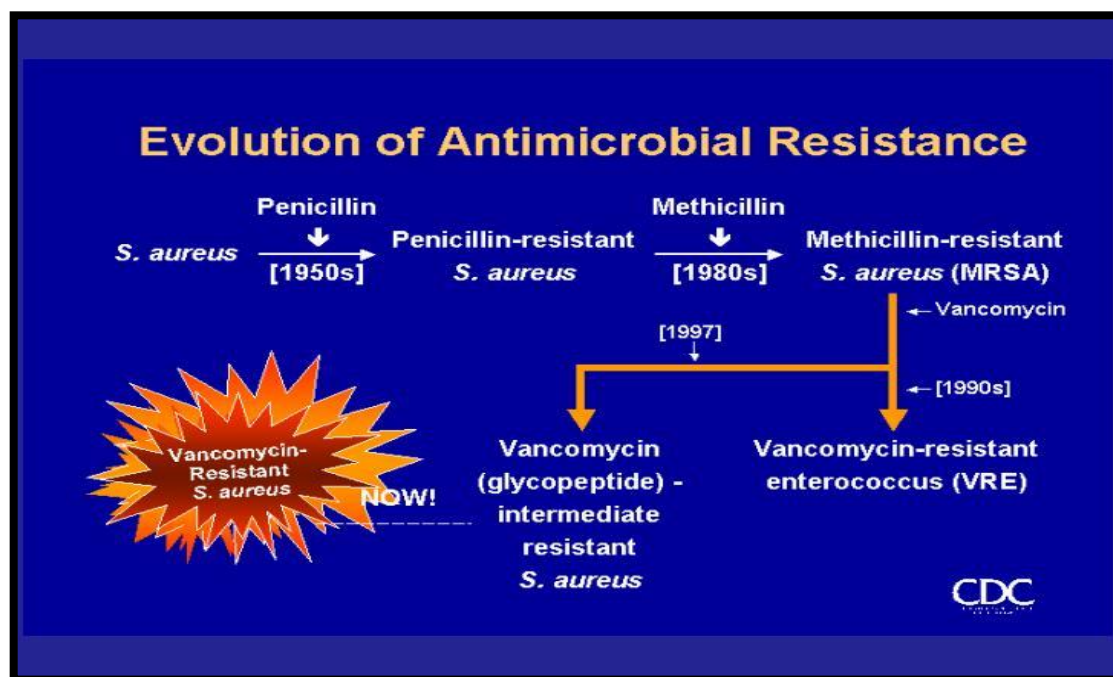


Figure (2-2) Evolution of antimicrobial resistance to *S. aureus* (Lacovino, 2011).

2.6 *Staphylococcus aureus* virulence factors

Staphylococcus aureus expresses a variety of extracellular proteins and polysaccharides, some of which are correlated with virulence. Virulence results from the combined effect of many factors expressed during infection as show in figure (2-3). Antibodies will neutralize staphylococcal toxins and enzymes; however, vaccines are not available (Liu, 2009).

2.6.1 Adherence to host proteins

Staphylococcus aureus cells express on their surfaces proteins that promote attachment to host proteins such as laminin and fibronectin that form part of the extracellular matrix. Fibronectin is present on epithelial and endothelial surfaces as well as being a component of blood clots. In

addition, most strains of *S. aureus* express a fibrinogen/fibrin binding protein (the clumping factor) which promotes attachment to blood clots and traumatized tissue (Tankeshwar, 2019).

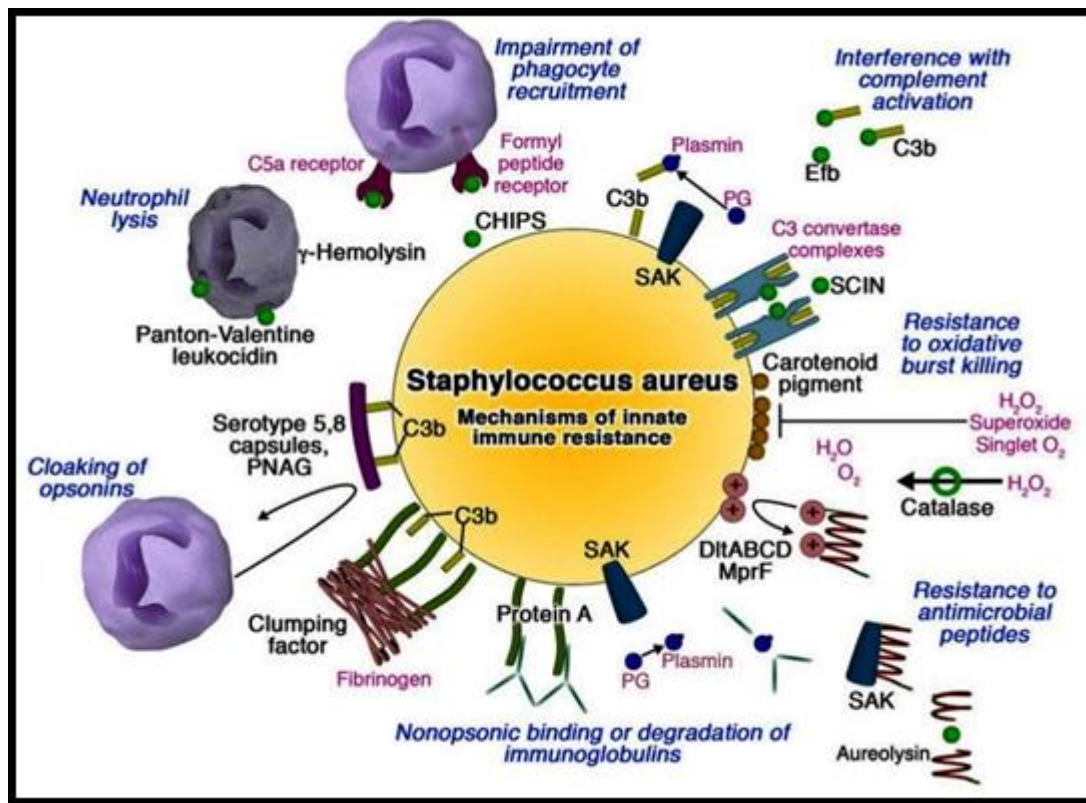


Figure (2-3): Virulence determinants of *Staphylococcus aureus* (Tankeshwar, 2019).

S. aureus can adhere to the surface of cultured human endothelial cells and become internalized by a phagocytosis-like process and because infections associated with indwelling medical devices ranging from simple intravenous catheters to prosthetic joints and replacement heart valves (Tankeshwar, 2019).

2.6.2 Avoidance of Host Defenses

S. aureus expresses a number of factors that have the potential to interfere with host defense mechanisms such as a capsular polysaccharide,

teichoic acids, protein A, Fibronectin- binding proteins and Collagen - binding protein.

2.6.3 Extacellular Toxins

2.6.3.1 Heamolysis

S. aureus is known of its capability to produce three decomposition types (alpha, beta and gamma) of haemolysis, which have different antigenic and chemical effects on the red blood cells. Type beta dissolves whole blood in a narrow range, while type alpha causes a wider range partial hemolysis, while gamma does not induce hemolysis (Tankeshwar, 2019).

2.6.3.2 Leukocidin

Leukocidin is a protein damages polymorphonuclear leucocytes and necrosis, (Holzinger, 2012).

2.6.3.3 Enterotoxins

Heat stable toxin is responsible for *Staphylococcal* food poisoning. Once formed enterotoxin is not destroyed even if food is heated sufficiently to kill all viable *Staphylococci* (Argudin *et al.*, 2010).

2.6.3.4 Exfoliative (epidermolytic toxin)

Two forms of epidermolytic toxins (ETA and ETB) of *S. aureus* split human skin at a site in the upper epidermis. Epidermolytic toxins are responsible for *Staphylococcal* scalded skin syndrome in which the outer layer of epidermis gets separated from the underlying tissue (Bukowski *et al.*, 2010).

2.6.3.5 Toxic shock syndrome toxin (TSST-1)

Toxic shock syndrome toxin (TSST-1) is a Staphylococcal superantigen that play major roles inducing cytokine release from macrophage and T- lymphocytes, it is also capable of enhancing the toxic effects of endogenous endotoxin, produce leakage of endothelial cells, penetrate mucosal barrier and *Staphylococcal* superantigen is responsible for almost all menstrual toxic shock syndrome cases (Bukowski *et al.*, 2010).

2.6.3.6 Enzymes

Enzymes responsible for pathogenesis such as coagulase is causing the plasma to clot by conversion from fibrinogen to fibrin, (Tankeshwar, 2019). So Staphylokinase is Fibrinolytic enzyme, which breaks fibrin clot and allow the spread of infection to contiguous tissues (Argudin *et al.*, 2010). Hydrolyses hyaluronic acid present in the intercellular ground facilitating the spread of the organism to adjacent areas (Bukowski *et al.*, 2010).and Deoxyribonuclease often degrades DNA. Other enzymes responsible for pathogenesis are lipase degrades lipids, Phospholipases degrades phospholipase and Proteases causes proteolysis (Berends *et al.*, 2010).

2.7 Transposable genetic elements

Transposable elements (TEs) were first identified more than 50 years ago by geneticist Barbara McClintock of Cold Spring Harbor Laboratory in New York.

Transposable elements (TEs), also known as "jumping genes". There are three types of transposable elements described in prokaryotes (Haaber *et al.*, 2017).

- Insertion Sequences (IS elements)
- Composite transposons
 - Simple
 - compose
- Tn3 elements (Integron)

2.7.1 Composite transposons

Transposon elements are a stem from two IS elements that insert near one other. The regions (sequences) between the two elements can be "mobilized" by the joint action of the two IS elements. This is of significance in that many Tn elements possess genes that confer resistance to antibiotics between the two IS elements show in figure (2-4) (Malachowa, 2010).

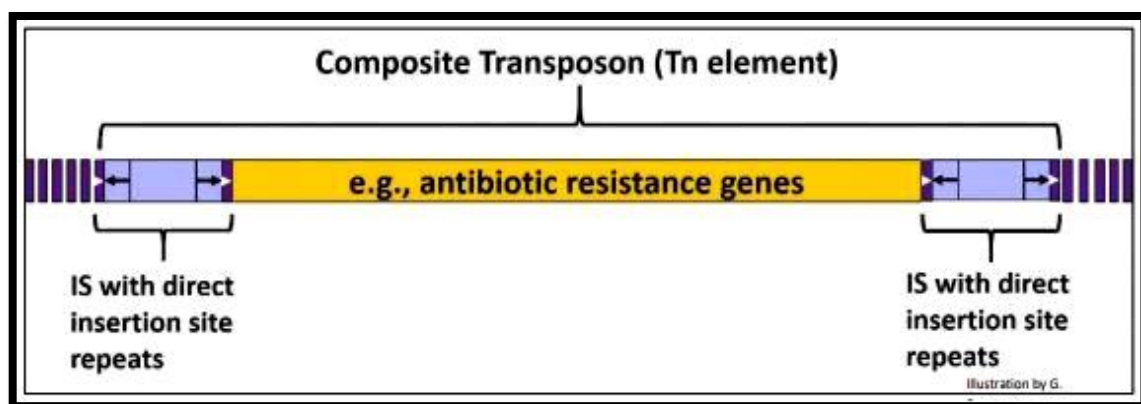


Figure (2-4): Composite transposon show in this schematic blue region IS with direct insertion site, yellow region indicates antibiotic resistance genes (Malachowa, 2010).

Tn transposition is regulated by a "repressor" that appears to exist to keep the elements somewhat quiescent. *Tn3* elements are simply large transposable elements that are not generated by flanking IS elements (as

in Tn elements). They are generally ~5,000 bp, have ~386 bp inverted repeats at both ends, and carry antibiotic resistant (Bourque *et al.*, 2018). Transposable elements are able to move from one location to another in the genome. Tns have been identified in all organisms, prokaryotic and eukaryotic, and can occupy a high proportion of a species' genome. For example, transposable elements comprise approximately 10% of several fish species, 12 %, transposable elements have accumulated over time and continue to shape genomes through their mobilization (Hood *et al.*, 2001). The mobilization of Tn is termed transposition or retrotransposition, depending on the nature of the intermediate used for mobilization. There are several ways in which the activity of Tn can positively and negatively impact a genome; for example, Tn mobilization can promote gene inactivation, modulate gene expression or induce illegitimate recombination. Thus, Tns have played a significant role in genome evolution. However, from a strictly theoretical point of view, Tn can be considered as selfish DNA or junk DNA, and the existence of these elements in a genome represents the fight between selfish DNA (to be perpetuated) and the host (Tn is able to produce various genetic alterations upon insertion as a consequence of the transposition process (insertions, excisions, duplications or translocations in the site of integration). For example, DNA transposons can inactivate or alter the expression of genes by insertion within introns, exons or regulatory regions (Clegg *et al.*, 2003). In addition, Tn can participate in the reorganization of a genome by the mobilization of non-transposon DNA or by acting as recombination substrates. This recombination would occur by homology between two sequences of a transposon located in the same or different chromosomes, which could be the origin for several types of chromosome alterations. Indeed, Tn can participate in the loss of genomic DNA by internal deletions

or other mechanisms (Symer *et al.*, 2002) to curtail their spread and its consequences.

A type of TE, RNA transposons (Class I), function via reverse transcription of an RNA intermediate (replicative mechanism) and can be further subdivided in two main groups depending on the presence of Long Terminal Repeats (LTR) flanking the retroelement main body.

DNA transposons (Class II) generally move by a cut-and-paste mechanism in which the transposon is excised from one location and reintegrated elsewhere. Most DNA transposons move through a non-replicative mechanism, although there are exceptions. DNA transposons consist of a transposase gene that is flanked by two Terminal Inverted Repeats (TIRs). The transposase recognizes these TIRs to perform the excision of the transposon DNA body, which is inserted into a new genomic location. Upon insertion, target site DNA is duplicated, resulting in Target Site Duplications (TSDs), which represent a unique hallmark, for each DNA transposon (Lopez and Perez, 2010).

Within both classes of Tns (Class I and II), they do not encode proteins required for their mobilization), but they are presumably dependent on autonomous transposons for mobility. As an example, Miniature Inverted-repeat Transposable Elements (MITEs) are short (80-500 bp) DNA transposon-like elements present in bacteria (Yang *et al.*, 2006). Although they have TIRs and are flanked by TSDs, lack transposase coding potential and are thus presumably dependent on autonomous DNA transposons for their mobilization (Lopez and Perez, 2010).

2.7.1.1 Transposition Mechanism

The mobilization of Tc1/mariner elements is a non-replicative transposition process that operates by a cut-and-paste mechanism and consists of the following steps:

- a. Two transposase molecules recognize the TIRs and bind to them via their HTH motifs, forming the Single-End Complex (SEC).
- b. Both transposases cleave the 5'-ends of the TIRs by hydrolysis of the phosphodiester bond to liberate the non-transferred strands (5'-P extremes), which do not participate further in the transposition process.
- c. The two-transposase molecules interact and bring together the transposon ends to form the PAIRED-END COMPLEX (PEC) generating a transposase dimer show in figure (2-5). At this point, the phosphodiester bond undergoes a hydrolysis in the 3'-ends to produce the transferred strands (3'-OH extremes).
- d. The PEC binds to target DNA forming the TARGET CAPTURE COMPLEX, at which insertion takes place as in figure (2-5). The target in Tc1/MARINER elements is any TA dinucleotide. Therefore, the transposase selects a random TA where the transposon insertion will be carried out. The 5'-end in the target DNA undergoes a nucleophilic attack from the transposon transfer strand 3'-OH. The host, generating canonical TSDs flanking the new transposon insertion (Lopez and Perez, 2010), fills the gaps in the transposon 5'-ends.

2.7.2 Insertion sequence

Insertion sequences (IS) are among the simplest mobile genetic elements (MGEs) and widespread in all field of life. They can occur in a wide range of copy numbers in a particular genome and can move within a genome (between different DNA molecules or within an individual DNA molecule) or horizontally between genomes as part of other mobile genetic elements MGE vectors such as phages and plasmids. More than 4500 IS

belonging to 29 families have been identified to date (Siguier *et al.* 2006, 2014).

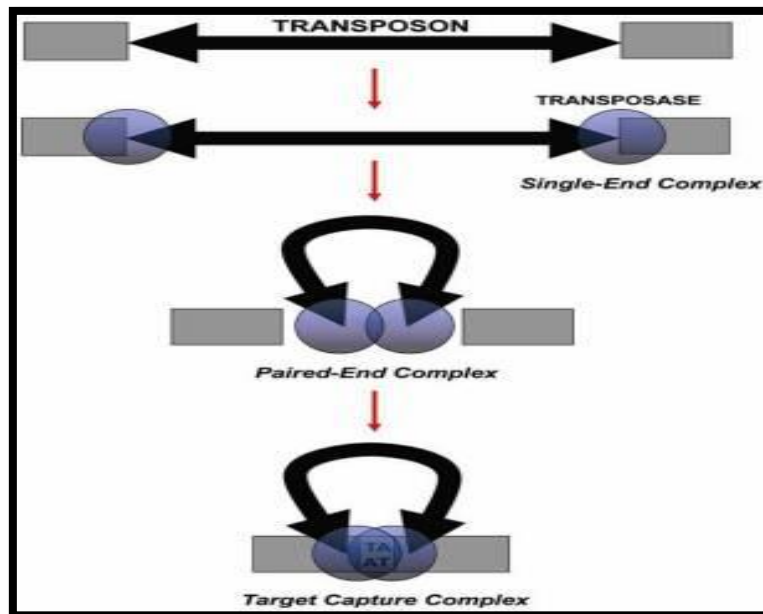


Figure (2-5): Transposition steps. Representation of the transposition mechanism performed by the transposase proposed for Tc1/*mariner* elements. The process begins with the binding of two transposase monomers to the TIRs, forming the *Single-End Complex*. Then, the transposon ends are brought together by both transposase monomers that form a dimer, generating the *Paired-End Complex*, and transposon excision takes places. Finally, the transposase dimer recognises a TA dinucleotide, joins it, and forms the *Target Capture Complex* to carry out the insertion (Lopez and Perez, 2010).

These small and compact elements (typically less than 3 kb), are capable of independent transposition, and generally flanked by short terminal inverted repeats (IR) and carry one or two open reading frames encoding gene products essential for their mobility (Siguier *et al.*, 2014). DNA cleavage (at the transposon tips) and strand transfer (of the transposon DNA into the target) necessary for transposition are catalyzed by a transposon-specific enzyme, the transposase. The

inverted repeats (IR) are necessary for transposase-binding, donor DNA cleavage and strand transfer. Often, short directly repeated sequences of the target DNA, from 2 to 14 bp long and whose length is specific for a given element, are generated at the point of insertion (Mahillon and Chandler, 1998). IS varying in the degree of sequence specificity in choice of their DNA target-site. Depending on the element, this can vary from highly sequence-specific to near random insertion (Craig, 1997). For some elements, the potential DNA structure of the target site is also important (Hu and Derbyshire, 1998). Perhaps the most common effect of IS transposition is gene inactivation shown in figure (2-6) is Possible effects of IS transposition in the function and expression of a target gene as mentioned by Vandecraen *et al.* (2017). Many cases have been described illustrating the modulation of resistance, virulence and metabolic activities by IS-mediated gene inactivation. A variety of cases where IS elements affect antibiotic/ xenobiotic resistance by directly inactivating uptake, ISs are widespread and can occur in very high numbers in prokaryotic genomes (Aziz *et al.*, 2010).

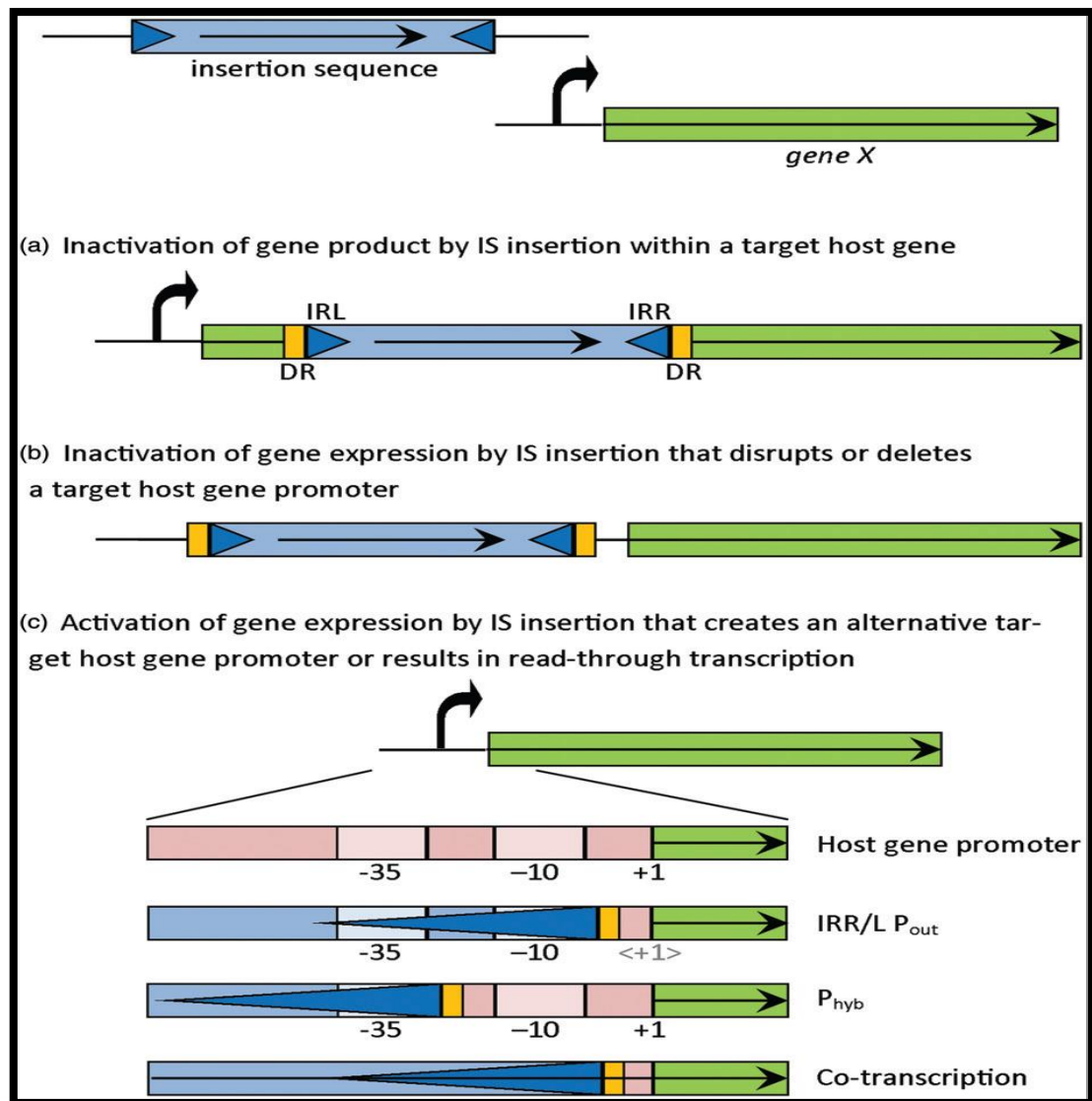


Figure (2-6): Possible effects of IS transposition in the function and expression of a target gene. The IS is shown as a blue rectangle delimited by short terminal inverted repeats (IR; blue triangles) and flanked by directed repeats (DR; yellow rectangles) and the target gene as a green rectangle. mRNA transcripts are represented by straight arrows, the target gene promoter sequence is represented by a curved arrow and detailed by -35 and -10 components, and transcriptional start site (+1). For IS elements carrying a complete outward-directed promoter, the target gene transcription start is altered and denoted as <+1> (Vandecraen, 2017).

2.8 IS256 Family

A total of 33 members of the IS256 family, including 2 isoforms, have been identified from a wide range of organisms. They are 1,298 bp (ISRm3) to 1,486 bp (IS6120) long. IS256 was originally isolated as a component of the compound transposon Tn4001 (Gregorio *et al.*, 2016) show figure (2-7). Members carry related terminal IRs of between 24 and 41 bp, and most generate 8 bp direct target repeats but some 9 bp duplications. It has been suggested that translational frameshifting may occur to generate a single transframe T_{pase}. This subgroup also includes most cases of 9-bp target repeats. The putative MurA gene product of the autonomous mutator element of *Zea mays*, MuDR exhibits some weak similarities to the T_{pases} of this group.

MuDR generates 9-bp target repeats resistance to the aminoglycosides gentamicin (*Gmr*), tobramycin (*Tmr*) and kanamycin (*Kmr*) in strains of *S. aureus* isolated mediated by the transposon Tn4001.

The 1.35 kb inverted repeat of this transposon exhibits many of the characteristics of an insertion sequence and has consequently been designated IS256. Tandem duplication of IS256 contiguous with Tn4001 results in an increase in the level of *GmrTmrKmr*, thereby implying that the element possesses strong promoter sequences. Both contiguous and independent insertions of IS256 into the *Staphylococcal* chromosome have been observed, the latter suggesting that the element may play a role in molecular rearrangements of the genome (Vandecraen *et al.*, 2017).

In *S. aureus* and *S. sciuri*, IS256 transposition into an upstream region of, respectively, the *llm* and *mecA* gene resulted in a hybrid promoter that enhanced their transcription and elevated methicillin resistance (Couto *et al.*, 2003). In *Burkholderia cepacia* isolates, the ability to utilize lactose and the herbicide 2, 4, 5-trichlorophenoxyacetic

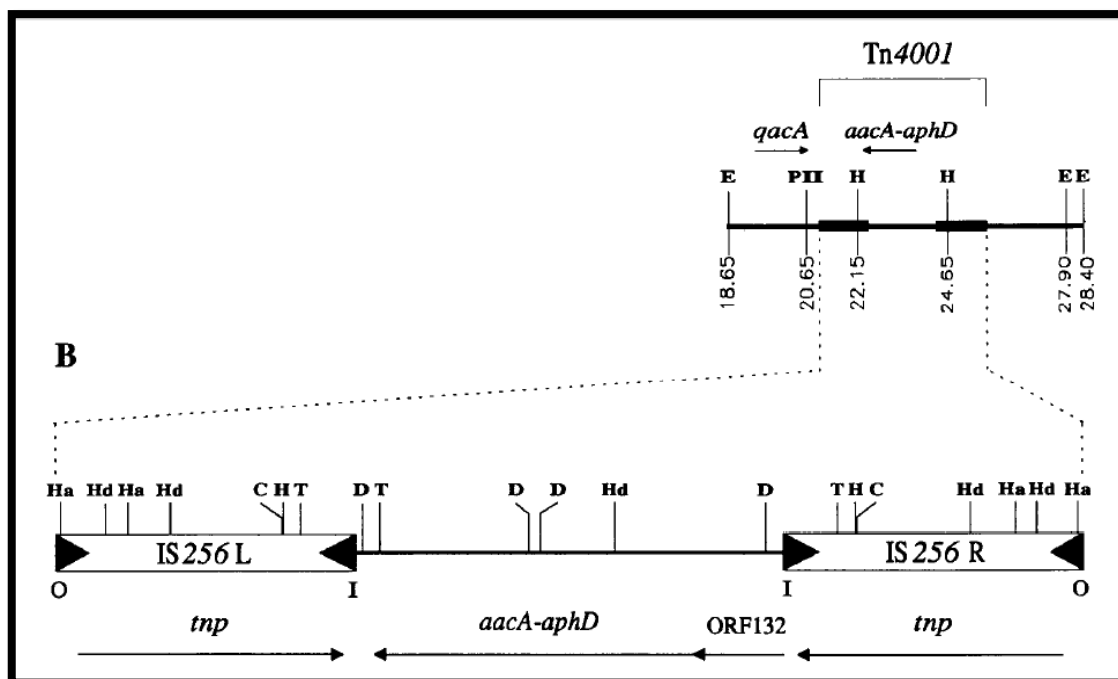


Figure (2-7): Genetic maps of Tn4001. (A) which mediates resistance to acriflavine, ethidium bromide and quaternary ammonium compounds; *aacA-aphD* on the transposon Tn4001, are indicated by arrows. IRS of IS256 on Tn4001 is represented by thick lines. Coordinates (in kb) of the restriction sites used in generating clones for sequencing Tn4001 are shown below the map. (B) Expanded map of Tn4001. IS256 left (L) and right (R) IR sequences are represented by boxes. Within each IS256, O and I indicate outside and inside ends of IS256 in Tn4001, respectively, and the bold arrowheads represent the 26-bp imperfect terminal IRS. The resistance gene *aacA-aphD*, which specifies GmTmKmR via a 56.9-kDa bifunctional AAC (6')-APH (2'') aminoglycoside modifying enzyme, ORF132 of unknown function, and putative *tnp* genes are shown with thin arrows indicating their extents and directions of transcription. On both maps restriction sites are indicated by Bg (*BglII*), C and I, D (*DruI*), E (*EcoRI*) (Marry *et al.*, 1989).

acid as a sole carbon source was promoted by IS406 and IS1490, respectively. IS406 promoted expression of the otherwise silent *lac* operon by formation of a hybrid promoter comprising a -35 promoter box at the right IR of IS406 and a duplicated -10 box from the native *lac* promoter (Wood *et al.*, 1991).

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IS406 promoted expression of the otherwise silent lac operon by formation of a hybrid promoter comprising a -35 promoter box at the right IR of IS406 and a duplicated -10 box from the native lac promoter (Wood *et al.*, 1991). Whereas insertion of IS1490, 110 bp upstream of tftA, coding for 2, 4, 5-trichlorophenoxyacetic acid oxygenase, created a fusion promoter which is responsible for high level constitutive transcription (Hubner and Hendrickson, 1997).

Determinants have been described. Examples, which are detailed in Table (2-1), include increased carbapenem resistance because of IS transposition into the porincoding oprD gene of numerous *Pseudomonas aeruginosa* isolates (Al Bayssari *et al.*, 2014) and *Pseudomonas putida* (Neyestanaki *et al.*, 2015), the porin-coding carO gene of *Acinetobacter baumannii* (Mussi *et al.*, 2005), the porin-coding ompE36 gene of *Enterobacter aerogenes* (Chen *et al.*, 2008b), and the porin-coding ompK36 gene of *Klebsiella pneumoniae* (Lee *et al.*, 2007).

Furthermore, IS-mediated inactivation of ompK36 also resulted in increased cefoxitin resistance. In contrast, inactivating efflux can result in hypersensitivity, e.g. to solvents due to insertional inactivation of acrB, part of the AcrAB-TolC efflux pump, by IS30 in *Escherichia coli* (Kobayashi *et al.*, 2001).

In addition to IS influence on transport, antibiotic/xenobiotic processing or target sites can also be affected by IS transposition (Table (2-1)). For instance, IS-mediated inactivation (ISAb11, IS701 family) of lipid A biosynthesis genes in *Acinetobacter baumannii* results in high level

colistin resistance because of complete loss of lipopolysaccharide production, the initial binding target of colistin (Moffatt *et al.*, 2011). Insertional inactivation of the thymidylate synthase-encoding *thyA* gene in *Deinococcus radiodurans*, the 16S rRNA methyltransferase- encoding *rsmG* gene in *Thermus thermophilus* HB8 and the *lytH* gene involved in the biogenesis of the bacterial cell and the membrane protein-coding *tcaA* gene in *S. aureus* results in increased trimethoprim (Mennecier *et al.*, 2006), streptomycin (Gregory and Dahlberg, 2008), methicillin (Fujimura and Murakami, 2008) and glycopeptide resistance, respectively. Next to a direct effect, IS elements can also affect regulatory pathways, thereby by modulating expression of determinants leading to resistance (Table 1-1). IS-mediated derepression of efflux pumps has been observed in *P. aeruginosa*, by IS21 (IS21 family) insertion into the *mexR* repressor resulting in increased transcription of the *mexAB-oprM* efflux pump and increased β -lactam resistance in *P. putida*, by IS12 insertion into the *srpS* repressor resulting in increased expression of the *srpAC* efflux pump and constitutive tolerance to toxic organic solvents (Sun and Dennis, 2009), and in *Cupriavidus metallidurans* AE126, by inactivation of *cnrY* or *cnrX*, coding, respectively, for a membrane-bound anti-sigma factor (*CnrY*) and a sensor protein (*CnrX*), resulting in release of the extracytoplasmic function family (ECF) sigma factor *CnrH* that directs transcription of the *cnrCBAT* efflux pump and increased (non-specific) Zn_2 efflux (Vandecraen *et al.*, 2016). Other examples are the insertional inactivation of the regulatory *mgrB* gene in *K. pneumoniae* and *K.*

Table (2-1): IS-mediated gene inactivation affecting virulence, resistance, and metabolism

Phenotype affected	Gene	IS element	IS family	Organism	Reference
Antibiotic/xenobiotic resistance					
β-lactam ^R	<i>ampD</i>	IS1669	IS5	<i>P. aeruginosa</i>	Bagge et al. (2002)
	<i>mexR</i>	IS21	IS2	<i>P. aeruginosa</i>	Boutoille et al. (2004)
Carbapenem ^R	<i>carO</i>	ISAb125	IS30	<i>A. baumannii</i>	Mussi et al. (2005)
		ISAb825	IS982	<i>A. baumannii</i>	Mussi et al. (2005)
	<i>ompE36</i>	IS903-like	IS903	<i>E. aerogenes</i>	Chen et al. (2008b)
	<i>ompK36</i>	IS5	IS5	<i>K. pneumoniae</i>	Lee et al. (2007)
	<i>oprD</i>	ISPa133	IS13	<i>P. aeruginosa</i>	Ruiz-Martinez et al. (2011)
		ISPa1328	IS256	<i>P. aeruginosa</i>	Al-Bayssari et al. (2015) and Wolter et al. (2004)
		ISPa46	IS256	<i>P. aeruginosa</i>	Diene et al. (2013)
		ISPPu22	IS3	<i>P. putida</i>	Kalantar-Neyestanaki et al. (2015)
		ISPa1635	IS4	<i>P. aeruginosa</i>	Wolter et al. (2004)
		ISPa45	IS4	<i>P. aeruginosa</i>	Rojo-Bezares et al. (2014)
		ISPa26	IS5	<i>P. aeruginosa</i>	Evans and Segal (2007)
		ISPa8	IS5	<i>P. aeruginosa</i>	Fowler and Hanson (2014)
		ISPre2-like	IS5	<i>P. aeruginosa</i>	Al Bayssari et al. (2014)
Cefoxitin ^R	<i>ompK36</i>	IS26	IS6	<i>K. pneumoniae</i>	Hernandez-Alles et al. (1999)
		IS903	IS5	<i>K. pneumoniae</i>	Hernandez-Alles et al. (1999)
		IS1	IS1	<i>K. pneumoniae</i>	Hernandez-Alles et al. (1999)
		IS5	IS5	<i>K. pneumoniae</i>	Hernandez-Alles et al. (1999)
Colistin ^R	<i>lpxA/lpxC</i>	ISAb11	IS701	<i>A. baumannii</i>	Moffatt et al. (2011)
	<i>mgrB</i>	IS1F-like	IS1	<i>K. pneumoniae</i>	Cannatelli et al. (2014)
		ISKpn14	IS1	<i>K. pneumoniae</i>	Cannatelli et al. (2014), Poirrel et al. (2015)
		IS5-like	IS5	<i>K. pneumoniae</i>	Cannatelli et al. (2014) and Poirrel et al. (2015)
		ISKpn13	IS5	<i>K. pneumoniae</i>	Poirrel et al. (2015)
		ISKpn26-like	IS5	<i>K. oxytoca</i>	Jayol et al. (2015)
Methicillin ^R	<i>lytH</i>	IS1182	IS1182	<i>S. aureus</i>	Fujimura and Murakami (2008)
Silver ^R	<i>ompR</i>	IS3	IS3	<i>E. coli</i>	Graves et al. (2015)
Solvents ^S	<i>acrB</i>	IS30	IS30	<i>E. coli</i>	Kobayashi et al. (2001)
Streptomycin ^R	<i>rsmG</i>	IS7th7	IS5	<i>T. thermophilus</i>	Gregory and Dahlberg (2008)
Trimethoprim ^R	<i>thyA</i>	ISDra2	IS200/IS605	<i>D. radiodurans</i>	Mennecier et al. (2006)
Toluene ^R	<i>srpS</i>	ISS12	IS21	<i>P. putida</i>	Sun and Dennis (2009) and Wery et al. (2001)
Zinc ^R	<i>cnrYX</i>	IS1086	IS30	<i>C. metallidurans</i>	Vandecraen et al. (2016)
		IS1087B	IS3	<i>C. metallidurans</i>	Collard et al. (1993), Tibazarwa et al. (2000), Vandecraen et al. (2016)
		IS1088	IS30	<i>C. metallidurans</i>	Vandecraen et al. (2016)
		IS1090	IS256	<i>C. metallidurans</i>	Vandecraen et al. (2016)
		ISRme3	IS3	<i>C. metallidurans</i>	Vandecraen et al. (2016)
		ISRme5	IS481	<i>C. metallidurans</i>	Vandecraen et al. (2016)
		ISRme15	IS3	<i>C. metallidurans</i>	Vandecraen et al. (2016)
Virulence					
Biofilm formation	<i>icaC</i>	IS256	IS256	<i>S. aureus</i>	Kiem et al. (2004)
	<i>icaC</i>	IS256	IS256	<i>S. epidermidis</i>	Ziebuhr et al. (1999)
	<i>rsbU</i>	IS256	IS256	<i>S. epidermidis</i>	Conlon et al. (2004)
	<i>sarA</i>	IS256	IS256	<i>S. epidermidis</i>	Conlon et al. (2004)
Capsule biosynthesis	<i>siaA</i>	IS1301	IS5	<i>N. meningitidis</i>	Hammerschmidt et al. (1996)
Capsule O-acetylation	<i>oatWY</i>	IS1301	IS5	<i>N. meningitidis</i>	Claus et al. (2003)
Cell adhesion	<i>rickA</i>	ISRpe1	IS481	<i>R. peacockii</i>	Simser et al. (2005)
Cytotoxin expression	<i>rot</i>	IS256	IS256	<i>S. aureus</i>	Benson et al. (2014)
Eps biosynthesis	<i>gumM</i>	ISXo2	IS1595	<i>X. oryzae</i>	Rajeshwari and Sonti (2000)
		ISXo1	IS5	<i>X. oryzae</i>	Rajeshwari and Sonti (2000)
Gelatinase (biofilm)	<i>fsrC</i>	IS256	IS256	<i>E. faecalis</i>	Perez et al. (2015)
Pneumolysin	<i>ply</i>	IS1515	IS5	<i>S. pneumoniae</i>	Garnier et al. (2007)
Porin (antigen)	<i>porA</i>	IS1301	IS5	<i>N. meningitidis</i>	Newcombe et al. (1998)
Protease	<i>prtP</i>	IS195	IS982	<i>P. gingivalis</i>	Lewis and Macrina (1998)
Metabolism					
Cellulose production	<i>cel1</i>	IS1031	IS5	<i>A. xylinum</i>	Coucheron (1991)
Fe ²⁺ oxidation	<i>resB</i>	ISTfe1	ISL3	<i>T. ferrooxidans</i>	Cabrejos et al. (1999)
Glucose utilization	<i>rpoS</i>	IS1	IS1	<i>E. coli</i>	Gaffe et al. (2011)
Indole production	<i>tna operon</i>	IS1	IS1	<i>Shigella</i>	Rezwan et al. (2004)
Lactocin 5 production	<i>las operon</i>	IS1163	IS3	<i>L. sakei</i>	Skaugen and Nes (1994)
		IS1520	IS3	<i>L. sakei</i>	Skaugen and Nes (2000)
Lactose utilization	<i>lac operon</i>	ISL4	IS110	<i>L. delbrueckii</i>	Lapierre et al. (2002)
Lipid A biosynthesis	<i>lpxA/lpxC</i>	ISAb11	IS701	<i>A. baumannii</i>	Moffatt et al. (2011)
Naphthalene dissimilation	<i>nahH</i>	ISPst9	ISL3	<i>P. stutzeri</i>	Christie-Oleza et al. (2008)
Poly-γ-glutamate production	<i>comP</i>	IS48su1	IS4	<i>B. subtilis</i>	Nagai et al. (2000)
Protocatechuate catabolism	<i>pcaH pobR</i>	IS1236	IS3	<i>A. calcoaceticus</i>	Gerischer et al. (1996)
o-, m-, p-xylene catabolism	<i>xyl/tou</i>	ISPst2	ISL3	<i>P. stutzeri</i>	Bolognese et al. (1999)

oxytoca resulting in colistin resistance (Poirel *et al.*, 2015), of the *ompR* regulator in *E. coli* resulting in decreased porin expression and increased silver resistance (Randall *et al.*, 2015) and of the amidase-coding *ampD* gene resulting in high-level β -lactam resistance (Bagge *et al.*, 2002).

1.9 IS expansion, elimination and genome streamlining

Perhaps, one of the most striking concerns IS expansion. ISs can undergo massive expansion and loss accompanied by gene inactivation and decay, genome rearrangement and genome reduction. Clearly, host lifestyle strongly influences these IS-mediated effects on genome structure, presumably by determining the level of genetic isolation of the microbial population. Factors affecting this include: whether the bacteria are ectosymbionts, primary endosymbionts having long evolutionary histories with their hosts, or secondary endosymbionts with more recent associations; whether they are transmitted in a strictly vertical manner or pass through a step of horizontal transfer via reinfection or passage through a second host vector (Moya *et al.*, 2008).

IS expansion has been commonly observed in bacteria with recently adopted fastidious, host-restricted lifestyles. Those which may have more ancient host-restricted lifestyles *Wiggles worthia* in the Tsetse fly; *Buchnera aphidicola* in the aphid; *Blochmannia floridanus* in the ant (tend to possess small streamlined genomes with few pseudogenes or MGEs) (Moya *et al.*, 2008), supporting Information, Table (2-1).

One view is that IS expansion is an early step in this genome reduction process as shown in figure (2-8).

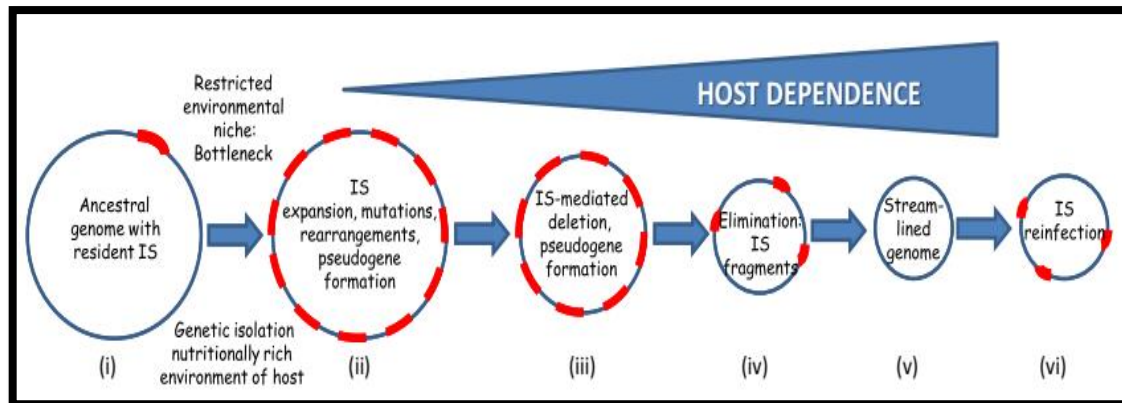


Figure (2-8): IS expansion, elimination and genome ‘streamlining’. The figure shows schematically from left to right events leading to the evolution of host-dependence in bacteria. (i) The parental (ancestral) chromosome including a low number of resident IS (red arcs). Note that the entire genome might also include transmissible plasmids carrying their own IS load, which can in principle undergo transposition into the chromosome. (ii) IS expansion occurs as a result of isolation and the formation of population bottlenecks within a host organism. This is accompanied by mutation promoted by insertion of new IS copies and by their related transposition activities of deletion and rearrangement. These genome rearrangements can also occur by homologous recombination between identical IS copies. (iii) With time IS will have a tendency to undergo deletion with adjacent DNA sequences in the absence of direct selection. This leads to a reduction in genome size. (iv) Eventually, extensive deletion will lead to the generation of nonautonomous IS fragments and their elimination. (vi) This gives rise to stream lined, IS ‘free’ genomes which may become ‘reinfected’ by IS on rare contact with other, IS-carrying strains or infection by IS-carrying bacteriophage (v). (Plague *et al.*, 2008 .)

This results from a decrease in strength and efficacy of purifying selection due to the shift from free to intracellular lifestyles (Moran and Plague, 2004). It is reinforced by a phenomenon known as Muller’s ratchet, which leads to the irreversible accumulation of mutations in a confined intracellular environment (Silva *et al.*, 2003).

In the nutritionally rich environment of the host, many genes of freeliving bacteria are inessential. Enhanced genetic drift would allow fixation of slightly deleterious mutations in the population, facilitated by the occurrence of successive population bottlenecks. The more genetically

isolated the bacterial population, the more acute would be the effect. Indeed, many examples of this can be found among intracellular endosymbionts. This initial stage of transition from free-living to host-dependence would therefore result in the accumulation of pseudogenes, which will eventually be eliminated by so-called deletional bias (Mira *et al.*, 2001). Clearly, the activities of MGEs and of ISs in particular, make them important instruments in these processes. IS expansion would contribute to pseudogenisation by IS-mediated intrachromosomal recombination and genome reduction (Mira *et al.*, 2001) by their capacities to generate deletions (Mahillon and Chandler, 1998). Such deletions would also eventually lead to complete or partial elimination of the ISs themselves. These processes are shown schematically in Figure (2-8).

3. Materials and Methods

3.1 Materials

3.1.1 Apparatus and Equipment

Apparatus and equipment used in this study with their company and origin are listed in table (3-1).

Table (3-1): Apparatus and equipment used in this study

Apparatus and Equipment	Company /origin
Autoclave	Hirayama /Japan
Balance	Sartorius /Germany
Compound light microscope	Meiji /Japan
Cooled Centrifuge	Eppendrof /Germany
Oven	Memmert /Germany
Incubator	
Laminar air flow cabinet	
Water bath	
pH-meter	Radiometer /Denmark
Vortex	Scientific Industries /USA
Refrigerator	Eshtar /Iraq
Vitek 2 compcat system	Biomerieux /France
Optical density measured	DensiCHEK /France
Magnetic stirrer with hot plate	Labtech/Korea
Nanodrop Spectrophotometer	Thermo scientific /USA
Gel documentation system	Bio-Rad /USA
Gel electrophoresis unit	Techne / USA
Millipore filter unit	Millipore Core /Germany

Apparatus and Equipment	Company /origin
Microwave oven	LG / China
Mini centrifuge	A and B lab/UK
Master cycler gradient PCR	TECHNE/England
Shaker incubator	Gyrmax /USA
Screw capped test tubes	BBL /USA
Sterile syringes	Hagg /UAE
Inoculating loop	Fisher scientific /UK
Tips	Sterellin Ltd. /UK
Sterilized cotton swabs	
Micropipette	Dragon/China
Water distiller	GFL/ Germany
PCR premix tubes	Promega /USA

3.1.2 Chemicals and Biological Materials

Chemicals and Biological Materials used in this study with their company and origin are listed in table (3-2).

Table (3-2): Chemicals and biological materials used in this study with their company and origin

Chemicals and Biological Materials	company /origin
Absolute ethanol	Fluka /Switzerland
Gram stain set including crystal violet, iodine and safranin.	Institute of sera and vaccines /Iraq
Human blood, Human serum	Blood bank in private nurse hospital / Iraq
Normal saline	Qatar Pharma/ Qatar

Isopropyl alcohol	Buchs SG / Germany
Ethyl alcohol % 70	API /raq
Urea, Tetramethyl-p-phenylenediamine	CDH /India
Vancomycin, ciproflaxacin, PenicillinG, Ampicillin, Methicillin, Azthromycin, Trimethoprim/Sulfamethoxazole, Erythromycin antibiotic powders	Direvo Industrial Biotechnology /Germany
Tris- EDTE buffer	Sigma / USA
Agarose	Stratagene/ USA
Phosphate Buffer Saline	Chemical paint/ Germany
TBE Buffer 10X	Promega / USA
TE buffer	
Lysozyme	Bioneer/ Korea
Agar agar	BDH / UK
Glycerol	
Standard MacFarland 's solution	Himedia /India

3.1.3. Ready – to – use Media

Ready – to – use Media listed in table (3-3) were prepared according to manufacturer's instructions. These media were sterilized by autoclaving.

Table (3-3): Culture media used in this study

Medium	Company/Origin
Tryptone soya broth	Salucea / Netherland
Blood agar medium	
Brain heart infusion Agar (BHIA)	
Nutrient broth	LabM / UK
Mannitol salt agar	Himedia / India
Muller-Hinton agar	Biomark / India

Urea agar base	Oxoid / England
Muller-Hinton broth	
MacConkey agar	

3.1.4. Antibiotic discs

Antibiotic discs mentioned in table (3-4) were used to determine the antibiotic susceptibility of bacterial isolates (Bioanalyse /Turkey). Results of inhibition zone of *S. aureus* were compared with NCCLS (2013) as shown in table (3-4).

Table (3-4): Antibiotic discs used for determining the antibiotic susceptibility of *S. aureus* isolates against according to NCCLS (2013).

Antibiotic	Symbol	Concentration (µg/disk)	Inhibition zone (mm)		
			R	I	S
Penicillin G	P	10	19	20-27	28
Ampicillin	AM	10	13	14-16	17
Methicillin	ME	10	12	13-14	15
Azithromycin	AZM	15	13	14-17	18
Tirmethoprim/ sulfamethoxazole	SXT	25	19	16-18	15
Ciproflaxacin	CIP	5	15	16-20	21
Chloramphenicol	C	30	12	13-17	18
Tetracycline	TE	30	14	15-18	19
Erythromycin	E	15	13	14-22	23
Doxycyline	D	30	12	13-15	16
Rifampin	RA	5	16	17-19	20
Gentamicin	GM	10	12	13-14	15
Vancomycin	VA	30	9	10-11	12
Lincomycin	L	2	9	10-14	15

R: Resistant I: Intermediate S: Sensitive

3.1.5 Kits

1. Wizard genomic DNA purification kit (Promega/USA) was used for extraction of *S.aureus* genomic DNA. This kit consists of Cell lysis solution, Nuclei lysis solution, protein precipitation solution, DNA rehydration solution and RNase solution.
2. Go Taq master mix kit (Promega/USA), consists of Mg-free Buffers, $MgCl_2$, dNTPs and Taq DNA polymerase.

3.1.6 Dyes

All dyes used in this study were prepared by the manufacturer company (Promega /USA)

- Ethidium bromide was used for visualizing DNA in agarose gels.
- Blue/Orange Loading Dye was used for loading DNA samples into wells of agarose gel and tracking migration during electrophoresis.

3.1.7 Buffer and solutions

3.1.7.1 Phosphate buffered saline

This buffer solution was prepared by dissolving 9.86 mg of phosphate buffered saline in 100ml sterilized deionized water then complete the volume to 1000 ml sterilized and deionized D.W.

3.1.7.2 TBE Buffer solution (1X)

This buffer solution was prepared by adding 50ml of TBE buffer (10X) to 450 ml D.W, mixed gently and stored at 4°C.

3.1.7.3 Lysozyme solution (0.6 mg/ml)

Lysozyme solution was freshly prepared by dissolving 6 mg of lyophilized lysozyme in 10 ml of sterilized deionized D.W until clear solution was formed, used immediately.

3.1.7.4 Urea stock solution

A urea stock solution was prepared according to Chakraborty *et al.* (2012) by dissolving two g of urea powder in 100 ml of D.W, and sterilled by filtration using 0.22µm Millipore filter unit.

3.1.8 Reagents

- **Catalase reagent**

Catalase reagent was prepared to be consisting of 3% hydrogen peroxide (H₂O₂).

- **Oxidase reagent**

Oxidase reagent was freshly prepared by dissolving 1g of N,N,N,N-tetramethyl-P-phenylene-diamine dihydrochloride in 100 ml of distilled water .

3.1.9 Sterilization methods

- **Autoclaving**

Media and solutions were sterilized by autoclaving at 121°C for 15 minute, 15 (psi) pound per sequire inch.

- **Dry heat sterilization**

Glassware were sterilized in an oven at 180°C for Three hours.

- **Membrane sterilization**

Crysrol, Urea and antibiotic solution were sterilized by filtration using 0.22µm Millipore filter unit.

3.1.10 Preparation of media

3.1.10.1 Ready to use media

All ready to use media mentioned in table (3-3) were prepared as recommended by the manufacturing companies and sterilized by autoclaving.

3.1.10.2 Laboratory – prepared media

•Blood agar medium

Blood agar medium was prepared according to Buxon (2005) by adding 40 g of blood agar base to one liter of distilled water and sterilized by autoclaving. After cooling to 45°C, 5% of blood was added to the media, mixed well, and poured in to sterile Petri dishes. This medium is suitable for the primary isolation and cultivation of *S. aureus* and for the detection of haemolysis type.

•Urea base medium

Urea base agar was prepared according to Chakraborty *et al.*(2011) by dissolving 2.4 g of urea base in 95ml of distilled water and sterilized by autoclaving. After cooling to 45°C, 5ml of urea was added to the media, mixed well and poured into sterile vials. This medium was used for detection of urease production.

•Egg yolk agar medium (10%)

Egg yolk agar medium was prepared according to Aryal (2019) by adding 10ml of egg yolk emulsion to nutrient agar after autoclaving then cooled to 45°C, mixed well, and poured into sterile Petri dishes. This medium was used to detect Lecithinase produced by *S. aureus*.

3.1.10.3 Antibiotic solutions

Antibiotic solutions were prepared according to (Louis, 2019) as follow:

• Vancomycin solution (10 mg/ml)

Vancomycin stock solution was prepared in a concentration of 10 mg/ml by dissolving 1g of this antibiotic in 100 ml of D.W, then double serial dilutions were prepared (1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 µg/ml) and sterilized by filtration.

- **Ciprofloxacin solution (10 mg/ml)**

Ciprofloxacin stock solution was prepared in a concentration of 10 mg/ml by dissolving 1g of this antibiotic in 100 ml of D.W, then double serial dilutions were prepared (1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 µg/ml) and sterilized by filtration, (CLSI, 2013).

3.1.11 Oligonucleotide Primers:

Primer pair used for detection of IS256 in bacterial isolates of *S.aureus* is listed in table (3-5).

Table (3-5): Primers used for detection of IS256 in bacterial isolates (Kozitskay *et al.*, 2003)

Primer	Sequence (5' → 3')	T _m (°C)	Product size (bp)
F	GTAGAATTTCGGATAAAGTCCGTATAATTGTGT	56.6	1324
R	TGTTCTAGCTATAACAATGTTTTTACCATTTTC	56.6	1324

3.2 Methods

3.2.1 Specimen collection

3.2. 1.1 Isolation of *S. aureus*

Clinical specimens were obtained from the division of Microbiology/ National center for educational Laboratories in the Medical City and the division of Microbiology Laboratories/ Private Nursing Hospital in Baghdad governorate, Iraq. These samples were collected from boil, wounds, nasal, urine, vagina, blood, and sputum, by using cotton swabs and transferred by cooling box to the laboratory. Tip drainage and joint tissue specimens were received in laboratory, which they were taken

from surgery operations. After that, cultured in brain heart infusion broth at 37 °C for 24 hour then all specimens were cultured on blood agar, MacConkey agar and nutrient agar already prepared for this purpose, and incubated at 37°C for 24 hours.

3.2.2.2 Identification of *S. aureus*

Isolates of *S. aureus* were identified according to their cultural, morphological and biochemical tests as follow:

3.2.3 Cultural and morphological characteristics

Bacterial isolates were identified according to their morphological characteristics, as staining ability in addition to colonial characterization including, size, shape, edge, color and transparency (Shujat *et al.*, 2020).

3.2.3.1 Microscopic examination

Bacterial isolates were subjected to Gram staining to detect their response to stain and to represent their shapes and arrangement (Enci *et al.*, 2020).

3.2.3.2 Biochemical tests

- **Coagulase test (Aryal, 2020)**

Human plasma diluted with distilled water (1:6)(v:v) was inoculated with single colony of each bacterial isolate, and incubated at 37°C. Clot formati -on within 4 hours indicates a positive result.

3.2.3. 3 Growth on mannitol salt agar (Aryal, 2019).

This test was achieved by inoculating mannitol agar medium with each bacterial isolate, and incubated at 37°C for 24 hours. Color change of medium to yellowish indicates a positive result.

3.2.3.4 Catalase test (Ankur, 2019)

This test was achieved by placing clump of bacterial cells from pure culture of each bacterial isolate on to microscopical slide, then two drops of 3% hydrogen peroxide solution were added on bacterial cells. Presence of gaseous bubbles within few seconds indicates a positive result.

3.2. 3.5 Oxidase test (Khan *et al.*, 2019)

This test was performed by moistening filter paper with few drops of freshly prepared oxidase reagent (mentioned in item 3.1.8), then a clump of bacterial cells was picked up from pure colony of each bacterial isolate by using sterile wooden stick applicator and smeared on the moistened filter paper. Development of violet to purple color within few seconds indicates a positive result.

3.2.3.6 Blood haemolysis test (Karthikayalu *et al.*, 2010)

This test was achieved by inoculating human blood agar medium prepared in item (3.1.10.2) with single colony of each bacterial isolate, and then plates were incubated at 37°C for 24 hours. Presence of halo of hydrolysis around colonies indicates a positive result ,while type alpha causes a wider range partial hemolysis, gamma does not induce hemolysis.

3.2.3.7 Urease test (Pylori, 2016)

This test was achieved by inoculating slants of urea base agar medium prepared in item (3.1.10.2) with single colony of each bacterial isolate, and incubated at 37°C for 24 hours. Presence of a pink color indicates a positive result.

3.2.3.8 Lecithinase test (Aryal, 2019)

This test was achieved by inoculating egg yolk agar medium prepared in item (3.1.10.2) with single colony of each bacterial isolate, then plates were incubated at 37°C for 24 hours. Presence of white opaque zone of precipitation that spreads beyond the edge of the colony indicates a positive result.

3.2.4 Automated of biochemical test (VITEK 2 system)

VITEK-2 system (Biomereux, 2010) was used in this study for the diagnosis of *S.aureus* isolates, which involved many steps as follows:

- **Preparation of bacterial suspension:**

A sterile swab was used to transfer a sufficient number of *S. aureus* colonies of a pure culture and separately suspended in 3 ml of sterile saline in clear plastic test tubes. The turbidity was then adjusted up to 0.5-0.63 O.D.

- **Inoculation of identification card:**

Identification card was inoculated with *S.aureus* isolate suspension using an integrated vacuum apparatus. A test tube containing the Staphylococci suspension was placed into a special rack (cassette) and the identification card was placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. The cassette can accommodate up to 10 tests or to 15 tests. The filled cassette was placed either manually or transported automatically into a vacuum chamber station. After the vacuum was applied and air was re-introduced into the station, the Staphylococci suspension was forced through the transfer tube into micro-channels that fill all the test wells.

- **Card sealing and incubation:**

Inoculated card was passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator. The carousel incubator can accommodate up to 30 or up to 60 cards, all card types

are incubated on-line at $35.5 \pm 1.0^\circ \text{C}$. each card is removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings, and then returned to the incubator until the next reading time. Data were collected at 15 minute intervals during the incubation period.

3.2.5 Maintenance of bacterial strains

Bacterial isolates were maintained according to Maniatis *et al.*, (1982) as follow:

1. Short term storage

Bacterial isolates were maintained for few weeks on nutrient agar plates. The plates were tightly wrapped in parafilm and stored in refrigerator at 4°C .

2. Medium term storage

Bacterial isolates were maintained in nutrient agar for few months. Such culture was prepared in small screw-capped tubes containing 5-8 ml of nutrient agar and stored in refrigerator at 4°C .

3. Long term storage

Bacterial isolates were maintained for few years on media containing 15% glycerol at low temperature without significant loss of viability, this was done by adding 1.5 ml of glycerol to 10 ml of an exponential growth culture of each bacterial isolate in sterile screw capped tubes with final volume of 10ml and stored at -20°C .

3.2.6 Antibiotic susceptibility test

Susceptibility of bacterial isolates to different antibiotics was achieved according to (National Committee for Clinical Laboratory Standards, 2013) as follow:

- a. Single colony of each bacterial isolate was selected and transferred into about 5ml sterile normal saline. The turbidity of bacterial suspension was compared with the turbidity of McFarland standard to obtain an approximate number equal to 0.5 O.D.
- b. Sterile cotton swab was dipped into the standardized inoculums, streaking was done on the entire agar surface of the plate with the swab three times by turning the plate at 60 degrees between each streaking, then inoculum was allowed to dry for 5-15 minutes.
- c. Antibiotic discs were applied by using aseptic conditions; the discs were deposited with centers at least 24mm apart.
- d. The plates were incubated immediately at 37°C and examined after 18- 24 hours or later if necessary. Zones of inhibition were measured and the diameters of the zones were recorded to the nearest millimeter (National Committee for Clinical Laboratory Standards, 2013).

3.2.7 Determination of minimum inhibitory concentration and minimum bactericidal concentration of Vancomycin and Ciprofloxacin against *S. aureus* (CLSI, 2016)

The minimum inhibitory concentration (MIC) is the lowest concentration that inhibits the visible growth of bacteria. This test was achieved by preparing serial dilutions of antibiotics between (0.5-1024µg/ml) of Vancomycin and Ciprofloxacin. The experiment included the 3 control tubes: a tube containing sterile broth (Sterility control), a tube containing broth and bacterial isolate (Growth control) and a tube containing antibiotic and sterile broth. After 18-24 hours incubation at 37 C°, the tubes were examined for growth. The MIC of Vancomycin and Ciprofloxacin were taken as the lowest concentration that showed no growth. Additionally, the minimal bactericidal concentration (MBC) values were determined by

subculturing the content of each tube without any growth on Mueller Hinton agar medium with looking for any bacterial growth.

3.2.8 Molecular study

3.2.8.1 Extraction of Genomic DNA

Genomic DNA was extracted from *S.aureus* bacterial isolates according to the protocol of Wizard Genomic DNA purification kit, (Promega, USA) as in the following steps:

- An overnight culture of each bacterial isolate was pelleted at 15000 rpm for two minutes, then supernatant was discarded.
- Pellet was suspended in 480 µl of 50 mM EDTA, then 120 µl of the lytic enzyme was added to cell suspension in a total volume of 120µl, and gently mixed by pipetting for weakening the cell wall, then 120 µl of lysozyme solution was added and mixed by pipetting.
- Microcenterifuge tubes were incubated at 37°C for 30 minutes in water bath, then tubes were centrifuged at 15000 rpm for two minutes.
- After centrifugation, supernatant was discarded and the pellet was resuspended in 600µl of Nuclei Lysis Solution, and mixed by pipetting.
- Microcenterifuge tubes were incubated at 80°C for 5 minutes for complete cell lysis, then cooled to room temperature.
- For RNA lysis, 3µl of RNase Solution was added to each tube cell, and the contents were mixed by inverting, and Microcenterifuge tubes were incubated at 37°C for 30 minutes.
- After cooling to room temperature, aliquot of 200µl of Protein Precipitation Solution was added to cell lysate in each microcenterifuge

tube, mixed by vortexing for 20 seconds and incubated for 5 minutes on ice.

- Tubes were centrifuged at 15000 rpm for 3 minutes, then DNA solution was transferred to a new microcentrifuge tube containing 600µl of room temperature isopropanol, and gently mixed by inversion until the thread-like strands of DNA form a visible mass.
- Microcentrifuge tubes were centrifuged at 15000 rpm for two minutes, then supernatant was drained on filter paper.
- Aliquot of 600µl of 70% ethanol was added, mixed by inverting for washing DNA pellet.
- Tubes were centrifuge at 15000 rpm for 2 minutes, then alcohol layer was carefully aspirated, then it was cleaned on filter paper and the pellet was allow to air dry for 10 minutes.
- The pellet was rehydrated in 100µl of DNA rehydration for one hour at 65°C.

3.2.8.2 Preparation of Primers solution

Primers were supplied in a lyophilized form and reconstituted by dilution with TB buffer to get a final concentration of pmol/µl amplification as follow:

- a. Primer tubes were centrifuged at 8000 rpm for 30 seconds to get all the lyophilised and to the bottom of the tube.
- b. Primers were dissolved in TB solution (For Primer forward 677 µl, and 906 µl for reverse primer) to get a final concentration of 100 µM , then tubes were mixed by vortexing, and left to stand for 2 minutes.
- c. Tubes were centrifuged at 8000 rpm for 30 seconds then 100 µl of the primer solution was added to 900 µl of TB buffer to get 10 %, working stock solution and stored at -20°C.

3.2.8. 3 Preparation of agarose gel

- **Agarose Gel Electrophoresis**

After PCR amplification, agarose gel electrophoresis was used to detect genomic DNA and the amplified products.

3.2.8.4 Preparation of Agarose Gel (1% and 0.8%)

- a. A volume of 100ml of 1X TBE buffer solution was placed in 250ml conical flask, then 1.0g and 0.8g of agarose gel were added to TBE buffer separately to get final concentrations of 1% and 0.8%, respectively.
- b. Contents of each flask were heated to boiling in a microwave oven until the gel particles were completely dissolved.
- c. Agarose gel solutions were cooled to 55-60, aliquots of 2 μ l of ethidium promide stain was added to each flask, then contents were mixed thoroughly.

- **Casting of the Horizontal Agarose Gel**

Agarose gel solution was poured into the gel tray and left to stand at room temperature for 30 minutes until complete solidification, then the previous fixed comb was carefully removed and the tray was placed in the tank filled with 1X TBE buffer until it reached 2-3mm over the surface of the gel.

- **Gel Electrophoresis (Lee *et al.*, 2012)**

Aliquot of 5 μ l of pure DNA solutions were mixed carefully with 2 μ l of 6x loading dye, then DNA samples were loaded into the wells and the electrophoresis was carried out for 1-2 hours at 75volts/cm². DNA bands were visualized under a UV-light transilluminator.

3.2.8.5 Quantitation of DNA concentration (Carlos, 2001)

The DNA concentration ($\mu\text{g/ml}$) was measured by using nanodrop spectrophotometer. A drop (two μl) of DNA solution was placed in a hole and the gate was closed.

Pure DNA was obtained when the ABS 260/280 ratio nm was equal to (1.8 – 2.0).

3.2.8.6 Amplification of IS256

A PCR solution was prepared according to the instructions of the manufacture company as indicator in table (3-6).

Table (3-6) PCR master mixture used for amplification IS256

Component	Volume (μl)
Go Taq G ₂ Green Master mix	12.5
Forward primer 10 μM	2
Reverse primer 10 μM	2
DNA Template	5
Nuclease- Free water	3.5
Total	25

PCR tubes were mixed by vortexing for 5 seconds, then the amplification was taken place by using thermocycler.

3.2.8.7 Optimization of DNA amplification

Optimum conditions for amplification of Insertion sequence 256 was described in table (3-7).

Table (3-7): PCR program for amplification of IS256.

Step	Temperature (°C)	Time (min:sec)	No. of cycles
Initial	95	02:00	1
Denaturation	95	00:30	35
Annealing	56.6	00:30	
Extension	72	01:00	
Final extension	72	05:00	1

3.2.8.8 Agarose gel electrophoresis solutions

A. Tri -EDTA (TE)

TE buffer (10X) was prepared according to the supplier company instructions. Tris HCl and EDTA are the components of TE buffer. pH was adjusted to 8.3 and the volume was completed to 1000 ml distilled water and stored at 25°C until use. For preparing 1X TE working solution, 100 ml of TE (10 X) was added to 900 ml of D.W.

B. Tris-borate EDTA Buffer (TBE)

This buffer solution was prepared to be consisting of the following components:

Component	Quantity
Tris-Base	108 g
Boric acid	55 g
EDTA (0.5 M)	40 ml

All components were dissolved in 900 ml of distilled water, pH was adjusted to 8.0, then volume was completed to 1000 ml with distilled water.

3.2.9 Determination of IS256 insertion and excision Frequency (Nagel, *et al.*, 2011)

3.2.9.1 Determination of transposition frequency

Transposition frequency of IS256 under different antibiotic stresses was achieved according to Nagel *et al.* (2011) as in the following procedure:

- Aliquot of 100µl of bacterial isolate was grown in 10 ml TSB in presence of vancomycin (8µg / ml) and ciprofloxacin (4µg / ml) and incubated at 37 °C for 6 h with shaking.
- Aliquots of the 100µl were withdrawn at each hour interval, and spread on plates of BHI agar containing subinhibitory concentration of antibiotics and incubated at 37 °C to 24 h.
- The frequency of transposition was determined as the number of Antibiotics - resistant colonies.
- Transposition frequency was determined as the median of:

$$\text{Transposition frequency} = r / Nt$$

r: number of mutants in culture.

Nt: number of viable cells in the culture

3.2.9.2 Determination percentage survival fraction

- a. A single bacterial colony was picked and diluted in 3 ml of phosphate buffer saline (PBS), and set with a McFarland 0.5 tube to get 1.5×10^8 CFU/ml of the bacterial suspension (Wahl and Zhu, 2015).
- b. To investigate whether ciprofloxacin or vancomycin elevate the mutation rate of S1.

- a) Ciprofloxacin was added at a concentration of 4 µg /ml for bacterial isolate.
- b) Vancomycin was added at a concentration of 8µg /ml for bacterial isolate.
- c. After 6 h of incubation at 37°C withdrawn 100µl cultures plated on BHI containing antibiotic incubation overnight at 37°C.
- d. The mutation rate (μ) of bacterial isolate was mentioned by Wahl and Zhu, (2015) as in the following formula:

$$\mu = m/Nt$$

m: number of mutational events per culture.

Nt: total number of viable cells in culture.

- e. Percentage Survival fraction was determined as in the following formula:

$$SF_x = s / N_x \times 100$$

SF= Survival fraction

s= Number of survival cells after Treatment with mutagenesis

N_x= Total count at the same hour without adding an antibiotic

X= Time (h).

3.2.10 Statistical Analysis:

The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Least significant difference–LSD test (Analysis of Variation-ANOVA) was used to significant compare between means in this study.

4. Results and Discussion

4.1 Clinical specimens

A total of 109 clinical specimens were collected from different sources including, wounds, nasal, urine, operation Joint fluid, in addition to operation tip drainage, sputum, Vagina and blood from patients attends to Baghdad Medical City in Baghdad Governorate, Iraq. Specimens were collected during the period from June 18th to September 9th 2019. Collected description of clinical samples used for isolation *S.aureus* was indicated in table (4-1).

Table (4-1): Number and percentage of clinical specimens used for isolation *S.aureus*.

Specimens	No. of specimens	(%)
Blood	31	28.4
Operation Soft tissue	17	15.5
Urinary tract infection	11	10.3
Joint fluid	11	10.3
wound	10	9.1
sputum	10	9.1
Vagina	8	7.3
Nasal cavity	8	7.3
Operation Drainage tip	3	2.7
Total	109	100

4.2 Isolation of bacterial isolates

Swabs from each clinical specimens were cultured on BHI at 37° C for 24 hours under aerobic condition. After growing, a total of 109 isolates were obtained and characterized first according to growth in MacConkey

agar (selective media for gram negative bacteria) and no growth was appeared by using this medium. Results indicated in table (4-2) and figure (4-1A) showed that 47 isolates (43.1%) out of the total isolates were Gram positive (G^{+ve}) bacteria, while the other 62 isolates (56.9%) were Gram negative (G^{-ve}) bacteria. G^{+ve} isolates were further characterized according to type of blood haemolysis on blood agar, and it was found that 30 isolates (63.8%) were β - haemolysis producers as they produce a clear zone around their growth, while the other 17 isolates (36.2 %) were non β -haemolysis as shown in figure (4-1B).

Table (4-2): Prevalence of bacterial isolates in Clinical specimen characterized according to growth in MacConkey medium.

Source of specimen	Gram positive No. (%)	Gram negative No. (%)
Blood	8(17.1)	23(37.0)
Soft tissue	12(25.5)	5(8.0)
Urinary tract infection	2(4.3)	9(14.5)
Joint fluid	7(14.8)	4(6.5)
wound	4(8.5)	6(9.7)
sputum	3(6.4)	7(11.3)
Vagina	4(8.5)	4(6.5)
Nasal	4(8.5)	4(6.5)
Drainage	3(6.4)	0(0)
Total	47(100)	62(100)

4.2.1 Identification of bacterial isolate

Bacterial isolates suspected to be *Staphylococcus* spp were grown on mannitol salt agar under aerobic conditions, and identified according to their cultural, microscopial, examined gram and biochemical test.

Results showed that all *Staphylococcal* isolates were able to grow on mannitol salt agar medium due to their ability to ferment mannitol (alcoholic sugar) to carboxylic acid that decrease pH of the medium and from yellow color around their growth as indicated in figure (4-2).

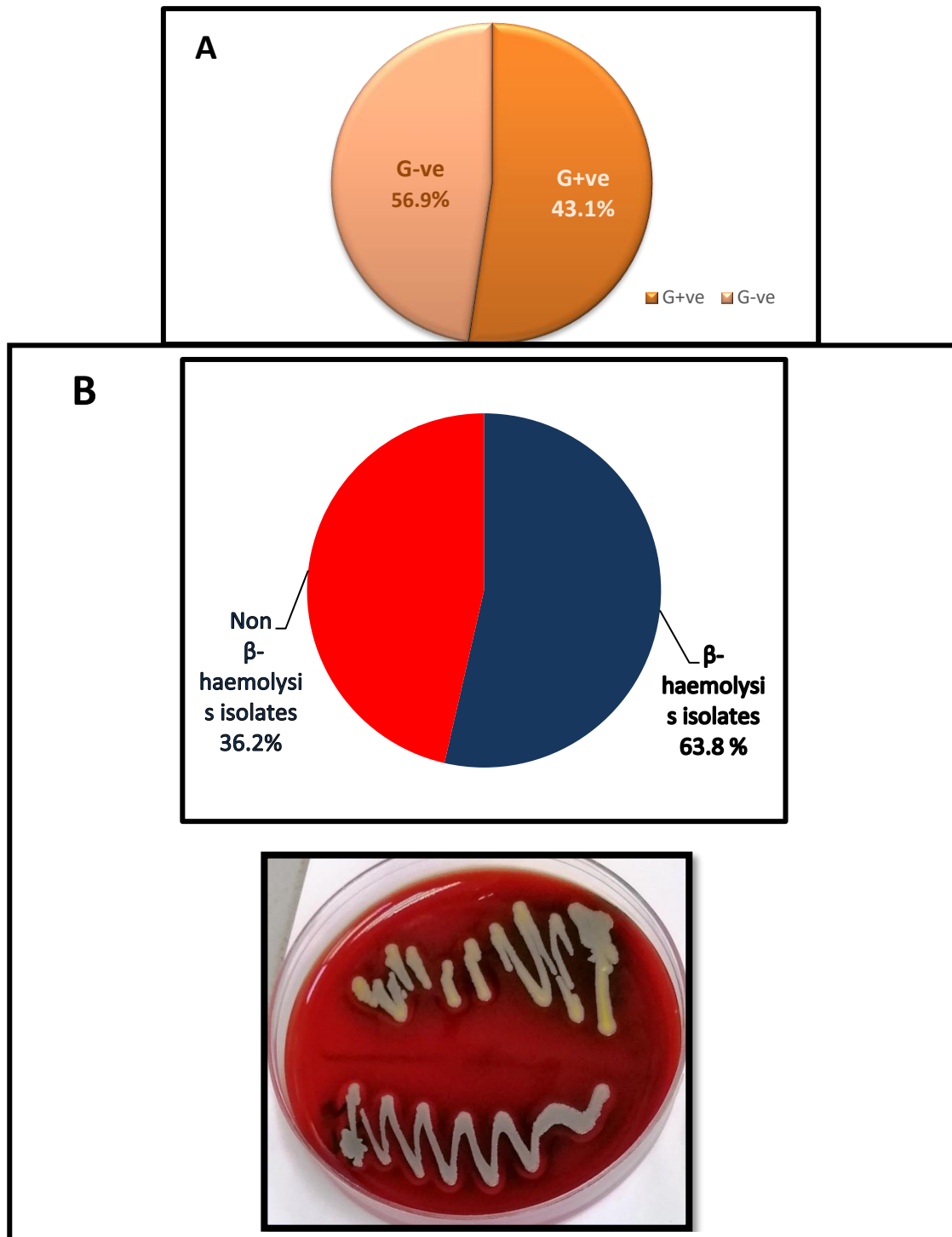


Figure (4-1): Characterization of bacterial isolates according to non growth in MacConkey agar (A), and β - haemolysis (B).

On the other hand it was found that 22 isolate (73.3%) out of the total isolates of *Staphylococcus* (30 isolate) were coagulase positive that distinguish between *S. aureus* and other species of *Staphylococcus* (coagulase negative) as mentioned by Mohan *et al.* (2011). Results also showed that these isolates were cocci and arranged in grape shape, Gram's + ve cocci, appears as grape like clusters under light microscope. Colonies of these isolates are smooth, convex, opaque, shiny yellow and circle on BHI.



Figure (4-2): Growth of *Staphylococcus* spp on mannitol salt agar medium producing yellow color.

Bacterial isolates suspected to be *Staphylococcus aureus* were further identified according to their biochemical reactions. Results indicated in table (4-3) showed that these isolates were negative for oxidase (cytochrome oxidase) and can use oxygen as a terminal electron acceptor in aerobic respiration. Results also showed that these isolates are catalase positive and able to reduce hydrogen peroxide to water and free oxygen as mentioned by Brooks *et al.* (2007). This test was usually used to differentiate between *Staphylococcus* spp and other Gram's positive

Table (4-3): Biochemical test for identification of *S. aureus*

Tsolate symbol	Test						
	Oxi	Cata	Hem	Coag	Min	Urea	Lici
S1	-	+	β	+	+	+	+
S2	-	+	β	+	+	+	+
S3	-	+	β	+	+	+	+
S4	-	+	β	+	+	+	+
S5	-	+	β	+	+	+	+
S6	-	+	β	+	+	+	+
S7	-	+	β	+	+	+	+
S8	-	+	β	+	+	+	+
S9	-	+	β	+	+	+	+
S10	-	+	β	+	+	+	+
S11	-	+	β	+	+	+	+
S12	-	+	β	+	+	+	+
S13	-	+	β	+	+	+	+
S14	-	+	β	+	+	+	+
S14	-	+	β	+	+	+	+
S15	-	+	β	+	+	+	+
S16	-	+	β	+	+	+	+
S17	-	+	β	+	+	+	+
S18	-	+	β	+	+	+	+
S19	-	+	β	+	+	+	+
S20	-	+	β	+	+	+	+
S21	-	+	β	+	+	+	+
S22	-	+	β	+	+	+	+

(+): positive, (-): Negative ;Oxi: oxidase; Cata:catalase;Hem: heamolysis; coag:coagulase; Min:mannitol; Ure:urease; Lici: lecithinase.

bacteria (*Streptococcus* spp and *Micrococcus* spp). Results also showed that *S. aureus* isolates are positive for urease, the enzyme catalyzed the conversion of urea to ammonia and carbon dioxide. They were positive for lecithinase, the enzyme degrades lecithin present in egg yolk and from

opaque precipitate around their colonies. On the other hand it was found these isolates were positive for citrate and able to utilize citrate as a sole source of carbon for their metabolism.

4.2.3 Identification of bacterial isolates by compact system (Vitek-2)

To confirm the identification of *S.aureus*, Vitek-2 was used as the best tool for identifying all types of bacteria cause different diseases in a short period with high accuracy. To confirm the identification of bacterial isolates of *S.aureus*, biochemical characteristics of these isolates were also examined by using Vitek-2. Results depend on six groups of tests. (Spanu *et al.*, 2003). Vitek-2 system was used according to the manufacturer's instructions; ID-Gram Positive Cocci cards were used for identification. Result of identification indicated in appendix (1) showed that these isolate were positive for acid production from lactose , N-acetyl-glucosamine, D-terhalose , D- mannitol and D- maltose , while they were negative for acid production from amygdaline , arbutine, D- galactose , D- glucose, D-melibiose ,D-raffinose ,D-sorbitol , D-xylose , glycerol , L-arabinose , and salicin. They were also positive bacitracin resistance, urease and growth in 6.5% NaCl. According to these results 22 isolates were confirmed as *S.aureus*.

Results also showed that the 22 isolate were *S.aureus* as primarily identified, while the other eight isolates are identified as *Staphylococcus lentus*, *Staphylococcus xylosus*, *Staphylococcus gallinarum* and *Staphylococcus haemolyticus*.

The prevalence of *S.aureus* isolates was distributed among different clinical cases and environmental sources and is mostly in nasal infections and other clinical sources as indicated in table (4-4).

Table (4-4): Prevalence of *S.aureus* isolates among in clinical specimens

Source of specimen	Isolate symbol	No. (%)
Operation soft tissue	S5,S6,S14	3(13.6)
Urinary tract infections	S2,S15	2(9.1)
Joint fluid	S18,S20,S22	3(13.6)
Wound	S1,S3,S10,S16	4(18.2)
Sputum	S7,S9,S17	3(13.6)
Vagina	S21	1(4.6)
Nasal	S4,S8,S11,S19	4(18.2)
Operation drainage	S12,S13	2(9.1)
Total	22(100)	

4.3 Antibiotics susceptibility of *S.aureus*

Treatment of *S.aureus* has become difficult owing to the global rise in the prevalence of antibiotic resistance, particularly against first-line antibiotics such as Penicillin and methicillin (Foster, 2017).

Illustrated in figure (4-3) showed the antibiotic susceptibility to different antibiotics by using disc diffusion method Muller – Hinton agar and incubation at 37°C for 18-24 hours.

On the other hand, results indicated in appendix (2) showed that the multidrug resistance was spread among *S.aureus* isolates as these isolates gave different resistance. Results also showed that the isolate *S.aureus* S1 was resistant to 11 antibiotics (78.5%), while the isolate S2 was resistant to ten antibiotics (71%), then the isolates S5 and S8 were resistant to nine antibiotics (64%), S10 and S15 were resistant to eight antibiotics (57%), S11 was resistant to seven antibiotics (50%), S4, S7, S14 and S21 were resistant to six antibiotics (43%), S18 was resistant to five antibiotics (36%), S12, S16, S17 and S20 were resistant to four antibiotics (28%), and finally S6, S13, S19, and S22 were resistant to three antibiotics (21%).

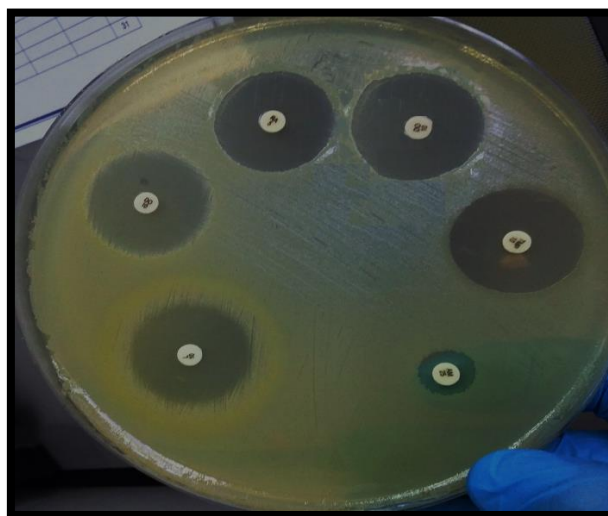


Figure (4-3): Antibiotic susceptibility of *S.aureus* on Muller- Hinton agar and incubation in 37° C to 24 hour.

Furthermore results indicated in Table (4-5) showed that 82% of *S.aureus* isolates were multidrug resistant as the resist antibiotics belong to three groups or more. Infections caused by multidrug resistant microorganisms are correlated with high mortality compared to those caused by susceptible bacteria and carry important economic burdens (O'Neill, 2016). Resistance and multi-resistance pattern to different antibiotics has been reported in several studies, mostly with one or more of the antibiotics penicillin G, methicillin and ampicillin.

These results agree with Timothy (2017) who found that *S.aureus* have the resistance to β – lactame antibiotics. In which there is perform *S. aureus* β -lactamase an enzyme responsible for the resistance to penicillin and he found that the β -lactamase structural gene (*blaZ*) is carried by the transposon Tn552 elements. Transposon is either located on a large plasmid (the prototype of which is pI524), or integrated into the bacterial chromosome (Sanchis *et al.*, 2018).

table (4-5): Pattern of antibiotic susceptibility of *S.aureus* isolates against different antibiotics.

Isolate No.	Antibiotic														MD R
	P	AP	ME	AZ	TM	CIP	C	TE T	E	DO	RA	GN	V	L	
S1	R	R	R	R	R	R	R	S	R	S	R	R	S	R	+
S2	R	R	R	S	R	R	R	R	R	R	S	R	S	S	+
S3	R	S	R	S	R	R	S	R	S	R	S	S	S	S	+
S4	R	R	R	R	S	S	R	S	R	S	S	S	S	S	+
S5	R	R	R	R	S	S	S	R	R	R	S	R	S	R	+
S6	R	S	R	S	S	S	R	S	S	S	S	S	S	S	-
S7	R	R	R	R	S	S	R	S	R	S	S	S	S	S	+
S8	R	R	R	R	R	S	S	S	R	S	R	S	R	R	+
S9	R	R	R	S	R	R	R	R	R	R	S	S	S	S	+
S10	R	R	R	R	S	S	R	R	R	R	S	S	S	S	+
S11	R	R	R	R	R	R	R	S	S	S	S	S	S	S	+
S12	R	R	R	S	R	S	S	S	S	S	S	S	S	S	+
S13	R	S	R	S	S	S	S	S	S	S	S	S	S	R	-
S14	R	R	R	S	R	S	S	R	S	S	S	S	S	R	+
S15	R	R	R	R	R	S	S	R	R	S	S	R	S	S	+
S16	R	S	R	S	S	S	R	R	S	S	S	S	S	S	+
S17	R	S	R	R	S	S	S	S	R	S	S	S	S	S	+
S18	R	R	R	S	S	S	R	S	S	S	R	S	S	S	+
S19	R	R	R	S	S	S	S	S	S	S	S	S	S	S	-
S20	R	R	R	S	S	S	S	S	R	S	S	S	S	S	+
S21	R	R	R	R	R	S	S	S	R	S	S	S	S	S	+
S22	R	R	R	S	S	S	S	S	S	S	S	S	S	S	-
Total (%)															18 (82)

R:Resistance; S:Sensitive; P:penicillinG;AP:Ampicillin; ME:Methicillin; AZ: Azethromycin; TM:Trimethoprim;CIP: Ciprofloxac -in; C:Chloramphenicol; TET:Tetracycline; E:Erythromycin; Do:Doxycycline ; RA: Rifampin ; GN: gentamycin; V:vancomycin; L: Lincomycin; MRD:Multidrug resistant; (+): positive; (-): negative.

Results indicated in figure (4-4) showed that the resistance of bacterial isolates to different antibiotics was distributed among them as follows: 100% to Penicillin and Methicillin, 77% to Ampicillin, 54% to Erythromycin, 45% to Azithromycin, Tirmethoprim and Chloramphenicol, 36% to tetracycline, 23% to Ciprofloxacin, Lincomycin and Doxycycline, 18% to Gentamicin, 13% to Rifampin, 4% to vancomycin.

In other study carried out by Kareem (2015), who performed on 74 MRSA isolates collected from hospitalized patients in Baghdad, found different pattern of antibiotic susceptibility toward Oxacillin, Tetracycline, Vancomycin, Ciprofloxacin and Gentamicin were 100%, 31.14%, 16.4%, 13.11% and 13.11% respectively. Recently, in a prospective, descriptive, and investigative study performed in Diyala government, it was found that methicillin resistant *S.aureus* clinical isolates exhibit different antibiotic resistance rate to tetracycline (70.9%), vancomycin (58.2%), chloramphenicol (34.5%), and Ciprofloxacin (32.7%) as mentioned by Iman (2020). This study was also in agreement with Ashraf and Nahla (2018) who mentioned that 80% of MRSA was resistance to tetracycline (Ashraf and Nahla, 2018).

In contrast, in 2011, in study conducted in India and focused on the antibiotic susceptibility of *S.aureus* isolated from pus of the post operative pus patients admitted to burn and wound section of Medical college and Hospital, it was found that 20% of the isolates were resistant to kanamycin and 46% were resistant to oxacillin and there is an increase in antibiotic resistance rates so that the development and spread of bacterial strains that are resistant to antibacterial drugs emerged as a global problem (Subhankari *et al.*, 2011). In other study carried out by 2019 Salmanov in Ukrain, information is available on the occurrence and antimicrobial susceptibility of *S. aureus* isolated from patients with surgical site

infection by the ENT departments of Kyiv hospitals, it was found that a high level (98,4%) of the isolates were resistant to trimethoprim, 97.6% , to mupirocin, 95.9%, to vancomycin and fosfomicin, 90.6% to moxifloxacin, 89.1% to gentamycin, while 85.5% of the isolates were resistant to rifampicin 85.5%, cefoxitin 84.6%, erythromycin 82.6% , tetracycline 76.3%, and clindamycin 75.4% was observed to be some lower. Resistance to oxacyllin *S.aureus* (MRSA) came up to 21.1 % (Salmanov *et al.*, 2019).

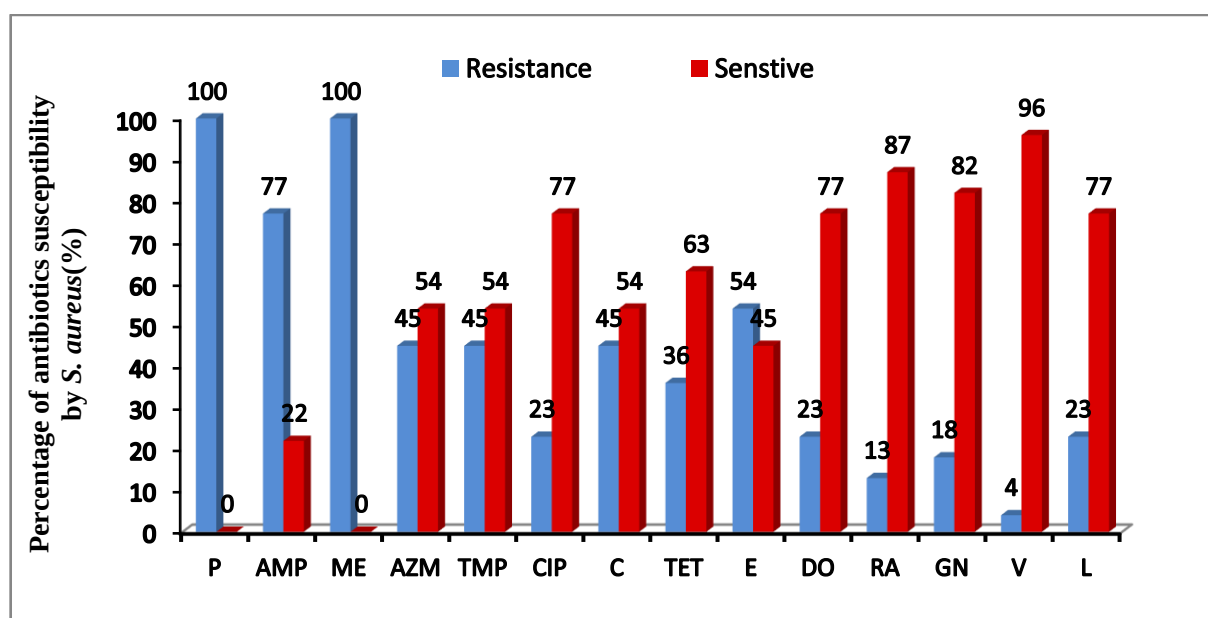


Figure (4-4): Antibigram of *S.aureus* isolates and the susceptibility percentage against different antibiotics. P :penicillinG; AMP:Ampicillin; ME: Methicillin; AZM: Azethromycin; TMP/SMZ: Trimethoprim; CIP: Ciprofloxacin; C:Chloramphenicol; TET: Tetracycline; E:Erythromycin; Do: Doxycycline ; RA: Rifampin; GN: gentamycin; V: vancomycin; L: Lincomycin.

The development of antibiotics resistance by *S. aureus* has involved acquisition of determinants by horizontal gene transfer of mobile genetic elements (Jensen and Lyon, 2009). These determinants may have evolved in antibiotic producers to protect them from potentially inhibitory molecules, or in their competitors. Analysis of the soil resistome shows that bacteria that express resistance to antibiotics are widespread (Nesme and

Simonet, 2015). Insertion sequences have been instrumental in the development of resistance, since they represent the active component of transposons and, thus, mobilize resistance genes for horizontal gene transfer or modulate the expression of resistance conferring genes (Schreiber *et al.*, 2013). The activity of transposition of IS256, which is one of the factors contributing to a genetic flexibility that, in turn, may accelerate the development of resistance in *S.aureus* (Schreiber *et al.*, 2013).

4.4 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against *S. aureus*

Determination of MIC and MBC was done by using Muller Hinton agar plate in two fold dilution method. MIC values for each isolate were determined by choosing the lowest concentrations (in µg/ml) of on anti-biotic that inhibits the growth of a given isolate. Additionally, the minimal bactericidal concentration (MBC) was examined by determining the lowest concentration (in µg/ml) of an antibiotic required by subculturing the content of each tube to present growth.

4.4.1 Vancomycin

Vancomycin was selected as the antibiotic to detect the MIC and MBC for *S. aureus* depended the break point from CLSI (2016). Vancomycin is a glycopeptides antibiotic that is widely used to treat serious infections caused by MRSA strains in hospital patients. It binds to the dipeptide D-Ala, 4-D-Ala5 of lipid II and prevents transglycosylation and transpeptidation catalysed by PBP2 and PBP2a and antagonises peptidoglycan remodeling (Foster, 2017). According to results indicated in table (4-5) and figure (4-3), it was found that they must majority of *S.aureus* isolates (95%) were sensitive to Vancomycin. On the other, it was found that the MIC and MBC of Vancomycin to *S. aureus* isolates were

ranged between 4 µg/ml (for isolate S5) and 256 µg/ml (for isolate S4) as indicated in table (4-6), and it was variable for different isolates in developing mechanisms of Vancomycin treatment.

Table (4-6): The MIC and MBC value of Vancomycin and Ciprofloxacin against *S.aureus* isolates.

Isolate No.	Vancomycin (µg/ml)		Ciprofloxacin (µg/ml)	
	MIC	MBC	MIC	MBC
S1	16	16	256	256
S2	16	16	512	512
S3	128	128	64	64
S4	256	256	256	256
S5	4	4	128	128
S6	8	8	256	256
S7	32	32	256	256
S8	16	16	32	32
S9	8	8	256	256
S10	16	16	256	256
S11	64	64	512	512
S12	16	16	32	32
S13	16	16	16	16
S14	16	16	16	16
S15	16	16	64	64
S16	64	64	256	256
S17	8	8	16	16
S18	16	16	128	128
S19	8	8	4	4
S20	16	16	16	16
S21	32	32	8	8
S22	64	64	64	64

Abbreviations: MIC: Minimum Inhibitory Concentration; MBC: Minimum Bacterial Concentration S, *S.aureus* isolate no.

The data confirmed previous results from different study conducted on patients in Bagdad by Esaa (2012), who recorded that MIC of *S.aureus* isolates was ranged between (16-512) µg/ml. Dezfulian *et al.* (2012) also found during his study on the isolates of *S.aureus* from various patients in Iran that the MIC values 512 µg/ml who described HLR-VRSA. While Schreiber (2013) mentioned that MIC to *S.aureus* was ranged between 4-8 µg/ml in a study achieved in Germany, and the same result was recorded in a study conducted in Bufflo ,USA by mentioned that McEvoy *et al.* (2013). Other study achieved in Leipzig, Germany by Kleinert *el.* (2017) mentioned that the value of MIC to *S. aureus* was 6 µg/ml.

A common form of treatment failure with vancomycin is due to strains that emerge during prolonged treatment by acquiring multiple mutations in chromosomal genes that affect cell wall biosynthesis and homeostasis (Foster, 2017).

4.4.2 Ciprofloxacin

Ciprofloxacin was selected as the antibiotic to detect the MIC and MBC for *S. aureus* depended the break point from CLSI (2016). Ciprofloxacin is fluoroquinolones antibiotic that was inhabiting two enzymes (DNA Topoisomerases and DNA gyrase) involved in bacterial DNA synthesis that were essential for bacterial DNA replication, thereby enabling these agents to be specific and bactericidal .

Resistance to fluoroquinolones in clinical isolates of *S. aureus* involves both mutational changes to the topoisomerases that reduce drug binding efficiency and elevated expression of endogenous efflux pumps (Hooper 2002; Hooper and Jacoby 2015).

According to results indicated in table (4-5) and figure (4-3), it was found that *S.aureus* isolates (77%) were sensitive to Ciprofloxacin. On the

other, it was found that the MIC and MBC of Ciprofloxacin to *S.aureus* isolates were ranged between 4 µg/ml (for isolate S19) and 512 µg/ml (for isolate S2) as indicated in table (4-6), and it was variable for different isolates in developing mechanisms of Ciprofloxacin treatment. These result was agree with study from a tertiary teaching hospital at Kuala Lumpur, Malaysia by Liu *et al.* (2013), who recorded that MIC values of MRSA strains were ranged between 1 and 256 µg/ml. Tavakoli *et al.* (2019) also found that the MIC values was ranged between 64 and 512 µg/ml of ciprofloxacin alone against *S. aureus* isolated from different patients. While Wamba *et al.* (2018) mentioned that the value of MIC to *S.aureus* was between (4-32) µg/ml and value of MBC between Two folds to three folds of MIC.

So according Marangon *et al* (2013) in University of Miami School of Medicine, Miami, Florida 2013 in specimens for culture were obtained by scraping with a blade or spatula. found the value of MIC to Ciprofloxacin was ranged between (0.5-4) µg/ml.

In *Staphylococci*, to develop resistance that exceeds the MIC breakpoint, changes in both enzymes are required two mutational changes involves both mutational changes to the topoisomerases that reduce drug binding efficiency and elevated expression of endogenous efflux pumps (Foster, 2017).

4.5 Molecular study

4.5.1 Isolation of genomic DNA

In order to detect IS256 transposable element, genomic DNA was extracted from each bacterial isolate of *S.aureus* by using Wizard Genomic DNA purification kit (Promega/USA). Results indicated in appendix (2) showed that DNA concentration was ranged between 4-195 ng/ml, and purity between 1.8-2. The quality and integrity of DNA

solutions were checked by electrophoresis on 1% agarose gel. Results of electrophoresis illustrated in figure (4-5) showed that there is a sharp DNA bands mentioning a high DNA quality for amplification of IS256 by using PCR technique.

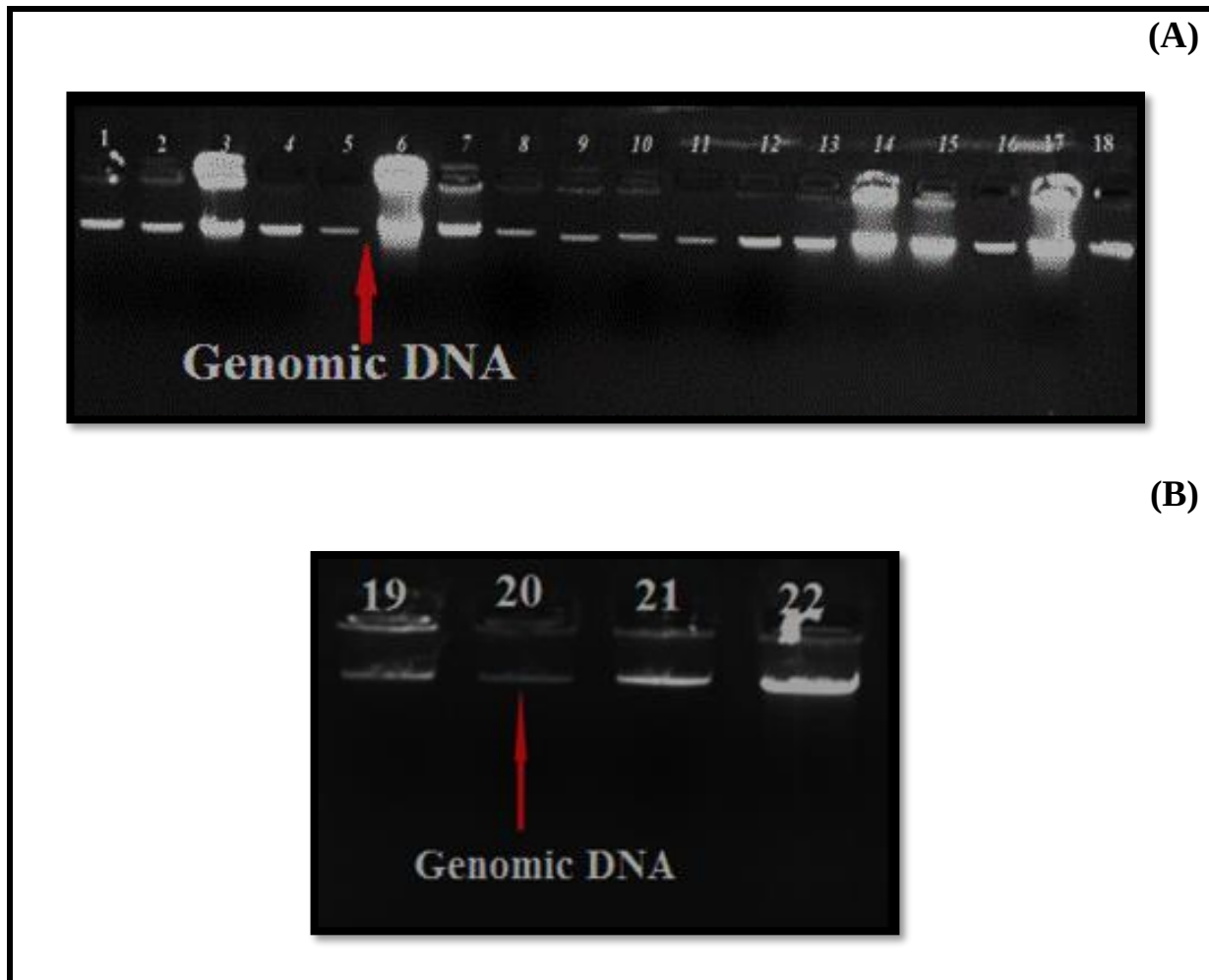


Figure (4-5): Genomic DNA of *S.aureus* isolates after electrophoresis on agarose gel (1%) for 1 hour, at 70 volt., lanes (1-18) *S.aureus* isolates number (A) and lanes (19-22) *S.aureus* isolates number (B).

4.5.2 Detection of Insertion sequence 256 element in *S. aureus* isolates

IS256 occurs in multiple free copies in the genome of *S. aureus*, and it has been identified as a typical marker of hospital acquired multi-resistant causing opportunistic infections in immunocompromised patients (Hennig and Ziebuhr, 2010). Results illustrated in figure (4-6) represents

that IS256 was detected in the genome DNA of 18 isolates (82%) of *S. aureus*, where an amplified product of 1324 bp was detected after electrophoresis on agarose gel electrophoresis.

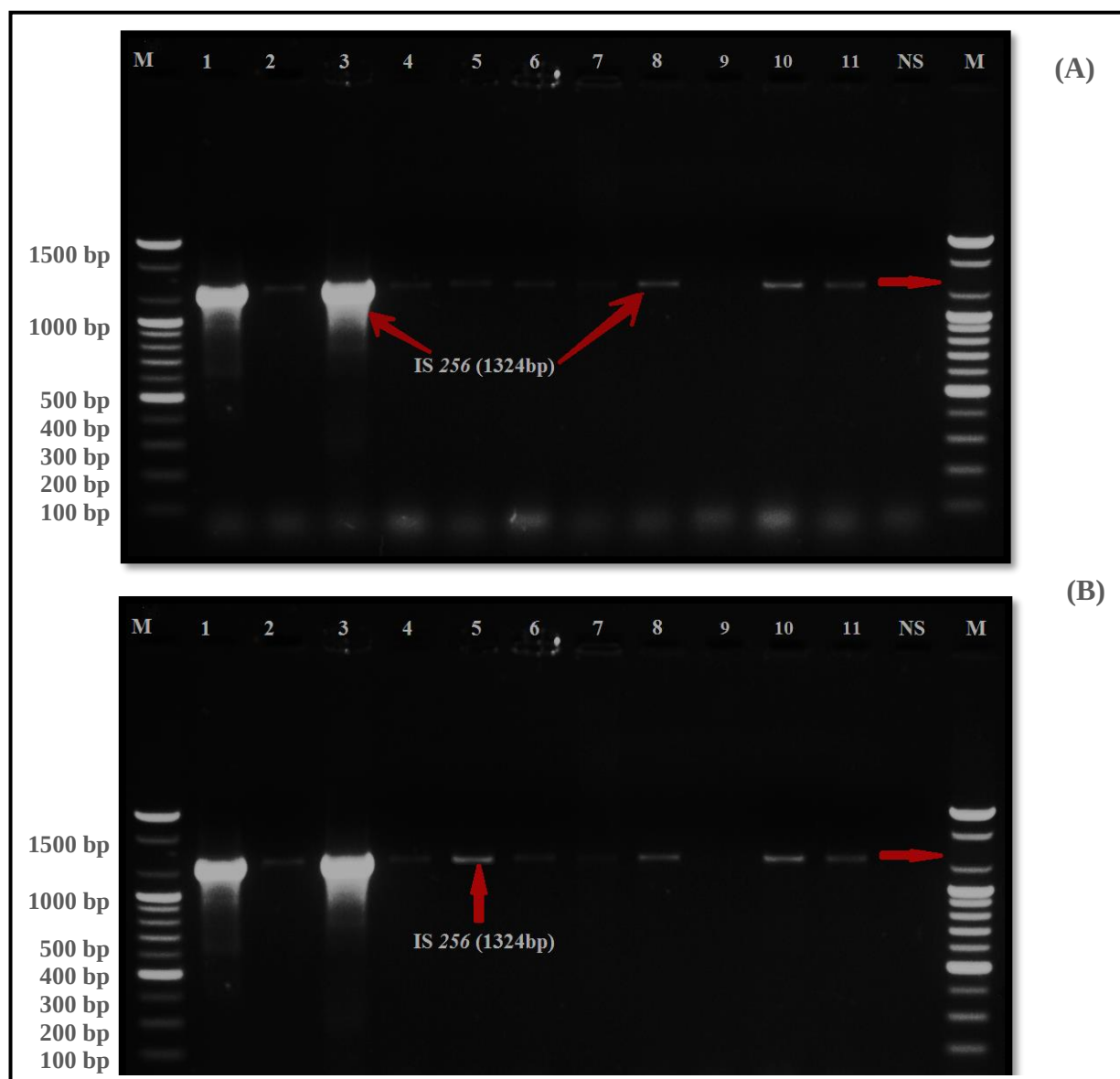


Figure (4-6): IS256 element detection after amplification of genomic DNA of *S.aureus* isolates by using specific primers and electrophoresis on agarose gel (1%) for one hour.

Lane (M): Ladder marker.

Lanes (1-11): bacterial isolates S1-S11 (A).

Lanes (12-22): bacterial isolates S12-S22 (B).

Lane (NS): Negative sample.

This result was in accordance with Kleinert (2017) who found that the IS256 with the same length can transposes by a "copy and paste" mechanism thereby leading to an accumulation of copies in the genome, since every transposition will increase the copy number by one.

The integration of a single IS element into a gene or its promoter may result in inactivation or over expression of the effected gene. The distribution of IS256 among *S.aureus* isolates from different clinical sources was illustrated in figure (4-7). The prevalence of IS256 in these isolates was 2 isolates (100%) from urinary tract infections, 2 isolates (100%) from operation drainage tip, 4 isolates (100%) from nasal cavity, 4 isolates (100%) from wound, 3 isolates (100%) from operation soft tissue, 2 isolates (66.66%) from sputum, one isolates (33.33) from joint fluid isolates, while IS256 was not detected in vagina isolate (0%).

From these results, it was found that IS256 reresents a specific element that is more likely to occur in isolates from nasal swab, urinary tract infection, wound, operation drainage and operation soft tissue than in isolates from sputum, fluid, and vagina respectively.

The presence of IS256 might play a role in the flexibility of the genome of multi-resistant *S.aureus* and could represent an advantage in the rapid adaptation of the bacterium to changing environmental conditions, and the underlying genetic mechanisms (Kozitskya *et al.*, 2004). IS256 element is present in multiple copies in the *Staphylococcal* genome, either flanking the Tn 4001 or independent of it. Inter IS256 PCR analysis is proposed as an efficient molecular typing assay for epidemiological studies of MRSA (Deplano *et al.*, 1997).

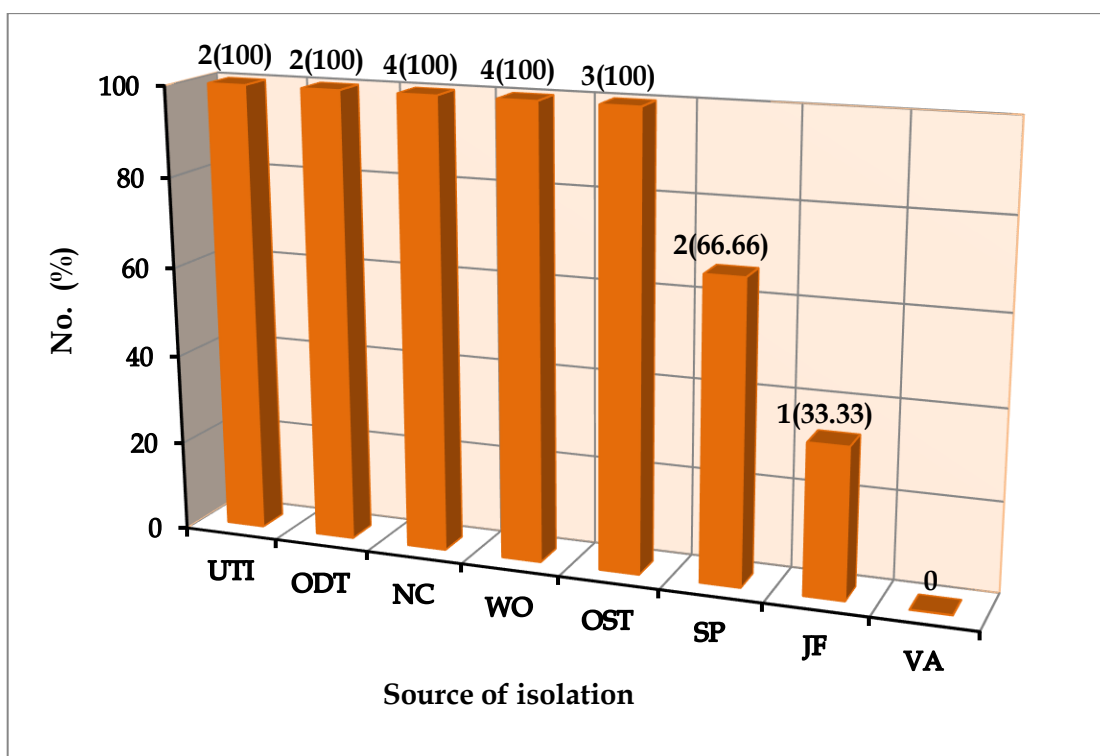


Figure (4-7): Distribution of IS256 among *S.aureus* isolates from different clinical samples. UTI: urinary tract infection; ODT: operation drainage tip; NC: nasal cavity; WO: wound; OST: operation soft tissue; SP: sputum; JF: joint fluid; VA: vagina.

In a study carried out by Schreiber *et al.* (2013), they observed that *S.aureus* strains contain multiple copies of IS256 as a component of the composite transposon Tn4001; it had played a pivotal role in development of aminoglycoside resistance.

Nagel *et al.* (2010) was also found that multiple copies of IS256 are also present in *S. aureus* strains that display intermediate resistance to vancomycin, the MRSA *S. aureus* are resistant to macrolides, rifampicin, quinolones, tetracycline, clindamycin, aminoglycosides, and chloramphenicol. Hennig and Ziebuhr (2010) showed that IS256 is an insertion sequence wide spread in the genomes of multi-resistant *Enterococci* and *staphylococci*. So the element, which is 1324 bp in size, consists of a single open reading frame encoding a transposase protein flanked by non-coding regions harboring imperfect inverted repeats (IRs).

IS256 occurs in multiple free copies in its host genomes, but is also known to form the ends of composite transposon Tn4001 conferring aminoglycoside resistance. Multiple genomic IS256 copies may serve as crossover points for homologous recombination events and thereby play an important role in genome flexibility, adaptation, and evolution of genomic (Tasi *et al.*, 2016).

According to the results of antibiotic susceptibility of *S.aureus* isolates and both of MIC and MBC, *S.aureus* S1 isolate was selected to study the influence of Vancomycin and Ciprofloxacin stress on mutations by IS256, as this isolate was the most resistant isolate to different antibiotics kept it as a multi-drug resistance by IS256, as this isolate was the most resistant isolate to different antibiotics kept it as multidrug resistance and harbouring IS256 transposable element in its genomic DNA (Sally *et al.*, 2018).

4.6 Effect of Vancomycin and Ciprofloxacin in induction of genetic mutations by IS256.

In order to study the antibiotics stress of vancomycin and/or ciprofloxacin in induction of genetic mutations by IS256, *S.aureus* S1 was treated with subinhibitory concentration of these antibiotics and incubated for six hours at 37°C, then mutation rate (the probability of a mutation event per cell and per generation), transposition frequency, and percentage survival fractions were determined.

Results illustrated in figure (4-8) formerly showed that there is a morphological changes in bacterial growth on BHI after 24 hrs of incubation with these antibiotics at 37 °C represented by color change of bacterial colonies from yellow to white color, reduction in the size of colonies, and in haemolysis type from β - haemolysis to α - haemolysis.

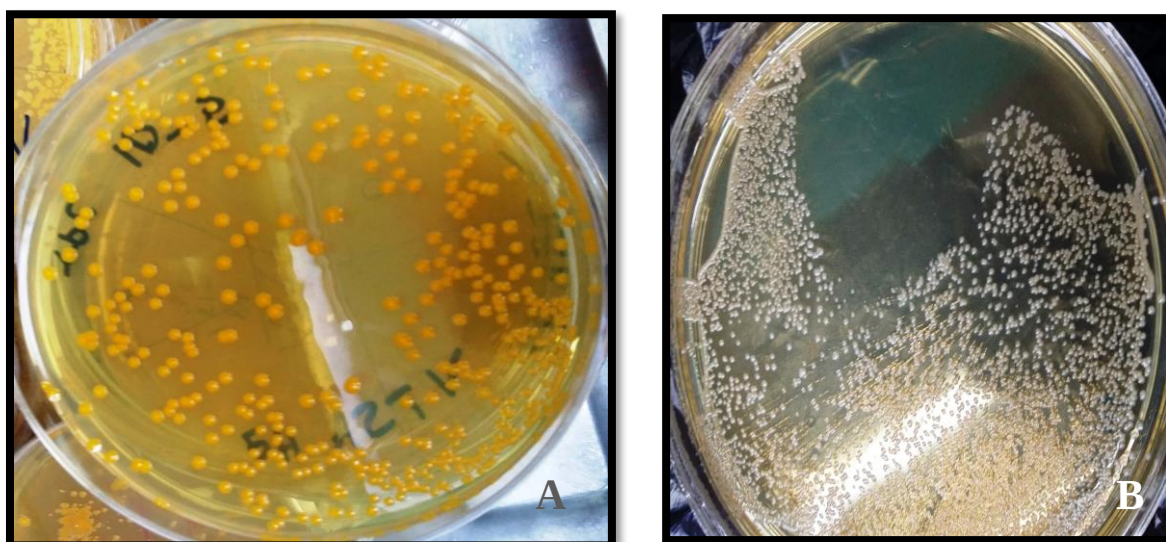


Figure (4-8): Growth of *S.aureus* S1 on BHI agar for 24 hrs at 37°C.(A): Growth without antibiotics represented by yellow and large size colonies; (B): Growth under the stress of vancomycin and/ or ciprofloxacin represented by white and small colonies.

These results agreed with Schaaf *et al.*(2002), and Schreiber *et al.* (2013) who mentioned that the growth characteristics of *S.aureus* were changed by increasing number of small white colonies with or without reduced β - haemolysis observed after treatment with aminoglycosides induced mutations due to inactivation of hem *hly* gene.

4.6.1 Effect of transposition on Erythromycin resistance of *S. aureus* S1

Results illustrated in figure (4-10) showed that the number of transposition events after pre-incubation in the presence of subinhibitory concentrations of vancomycin and/ or ciprofloxacin. These results represents that the transposition frequency after pretreatment with vancomycin is most effective to induce genetic mutations by transposon IS256 and inactivate erythromycin gene compared with pretreatment with ciprofloxacin or ciprofloxacin and vancomycin as shown in figure (4-10b), as the survival fraction was significantly decreased ($P \leq 0.01$) during the six

hours intervals of incubation with subinhibitory concentration of vancomycin from 30% to 2% and the transposition frequency was increased from 1.3 per 10^8 to 1.8 per 10^8 cells. According to these results, vancomycin enhanced the transposition activity, whereas the effect of ciprofloxacin alone and both vancomycin and ciprofloxacin was noticed but significantly ($P \leq 0.01$) lower. Furthermore, the maximum number of colonies carrying transposon insertions per 10^8 cells of culture was reached after incubation for six hours in presence of subinhibitory concentration of vancomycin. On the other hand, results illustrated in figure (4-10a) showed that the transposition frequency after pretreatment with subinhibitory concentration of ciprofloxacin was most effective after two and six hours of incubation with the antibiotic (1.8 per 10^8 and 1.7 per 10^8 cells) respectively compared with the pretreatment with both vancomycin and ciprofloxacin ($P \leq 0.01$), where the transposon frequency was 1.5 per 10^8 after treatment for the same periods as shown in figure (4-10c). Furthermore, treatment with subinhibitory concentrations of both antibiotics resulted in activation of transposition frequency of the insertion element IS256 in *S.aureus* S1. Transposition was dependent on presence of a functional transposase enzyme and the activation of transposition as mentioned by Nagel *et al.* (2011). Results of statistical analysis indicated in table(4-7) showed that the transposition frequency of IS256 in erythromycin gene after pretreatment with ciprofloxacin was significantly higher ($P > 0.05$) than pretreatment with vancomycin and/or ciprofloxacin during the first five hours of incubation, while the survival fraction was significantly different ($P \leq 0.01$) among the pretreatment of these antibiotics during the six hours of incubation as indicated in table (4-8).

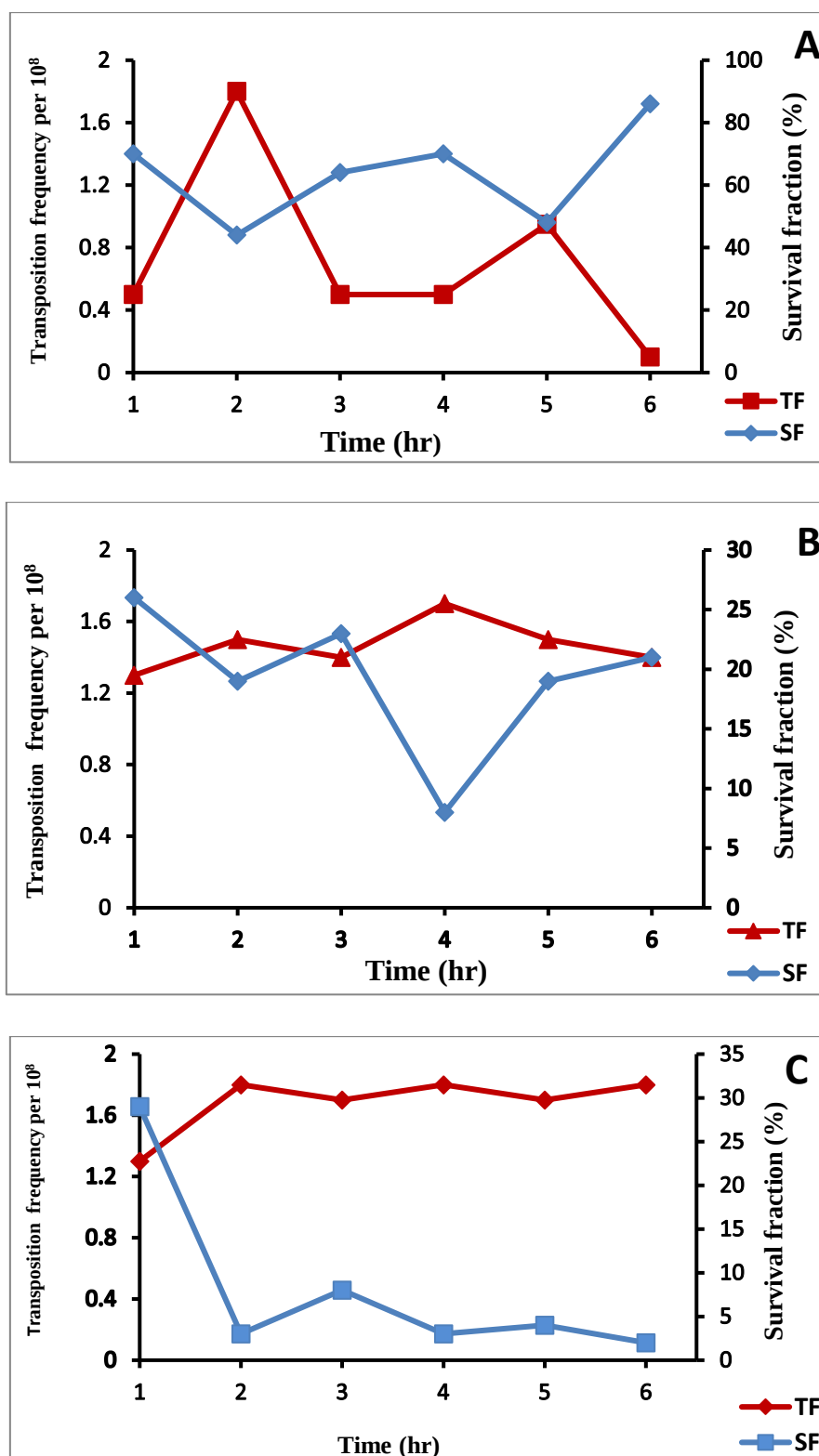


Figure (4-9): Transposition frequency of IS256 in erythromycin gene of *S.aureus* S1 and survival fraction after pretreatment with vancomycin and/or ciprofloxacin for six hours intervals.(A): pretreatment with Ciprofloxacin; (B): pretreatment with Vancomycin; (C): pretreatment with vancomycin and ciprofloxacin.

Table (4-7): Statistical analysis for transposition frequency of IS256 in erythromycin gene after pretreatment of *S.aureus* S1 with ciprofloxacin and /or vancomycin for six hour intervals.

Time (hr)	Transposition Frequency per 10 ⁸ cells			LSD
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	0.5 ^b	1.3 ^a	1.3 ^a	0.602 [*]
2	1.8 ^a	1.8 ^a	1.5 ^a	0.433 NS
3	0.5 ^b	1.7 ^a	1.4 ^a	0.619 [*]
4	0.5 ^b	1.8 ^a	1.7 ^a	0.532 [*]
5	0.95 ^b	1.7 ^a	1.5 ^a	0.495 [*]
6	0.1 ^b	1.8 ^a	1.4 ^{ab}	0.439 [*]

Different letters in rows represents a significant difference, *(P≤0.05),

NS: Non significance.

Appendix (4-8): Statistical analysis for survival fractions of *S.aureus* S1 after pretreatment with ciprofloxacin and/or vancomycin for six hours intervals.

Time (hr)	Survival fraction (%)			LSD value
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	70 ^a	29 ^b	26 ^b	7.64 ^{**}
2	44 ^a	3 ^c	19 ^b	6.88 ^{**}
3	64 ^a	8 ^c	23 ^b	6.71 ^{**}
4	70 ^a	3 ^b	8 ^b	7.09 ^{**}
5	48 ^a	4 ^c	19 ^b	7.52 ^{**}
6	86 ^a	2 ^c	21 ^b	7.49 ^{**}

Different letters in same row represents a significant difference, **(P≤0.01).

4.6. 2 Effect of transposition by IS256 on Ampicillin resistance of *S. aureus* S1

Effect of vancomycin and ciprofloxacin on the resistance of *S. aureus* S1 was also studied. Results illustrated in figure (4-11) showed that the transposition of IS256 in genomic DNA of *S. aureus* S1 was induced under the stress of vancomycin and / or ciprofloxacin, and it was found the transposition frequency was most effective after pretreatment with both vancomycin and ciprofloxacin to induce genetic mutations in *S. aureus* S1 genomic DNA and inactivate ampicillin gene confer to the bacterial resistance to ampicillin. Results illustrated in figure (4-11b,c) showed that the transposition frequency was increased from 1.55 per 10^8 cells in the first hour of incubation under the stress of vancomycin and both vancomycin and ciprofloxacin to 1.8 per 10^8 cells in the five hours of incubation, while the transposition frequency per 10^8 was increased gradually from 0.9 per 10^8 cells in the first hour of incubation to 1.8 per 10^8 cells in the last hour of incubation as shown in figure(4-11a).

On the other hand, results illustrated in figure (4-11) showed that the percentage of survival fraction was significantly decreased ($P \leq 0.01$) from 10% after one hour of pretreatment with vancomycin and both vancomycin and ciprofloxacin to 0.01 % after six hours of in incubation with these antibiotics as mentioned in figures (4-11b,c) compared with the decrease in survival fraction after pretreatment with vancomycin alone for the same times intervals as shown in figure (4-11a).

Furthermore results indicated in table (4-9) showed that the transposition frequency of IS256 was increased significantly ($P \geq 0.05$) after pretreatment with vancomycin and both vancomycin and ciprofloxacin

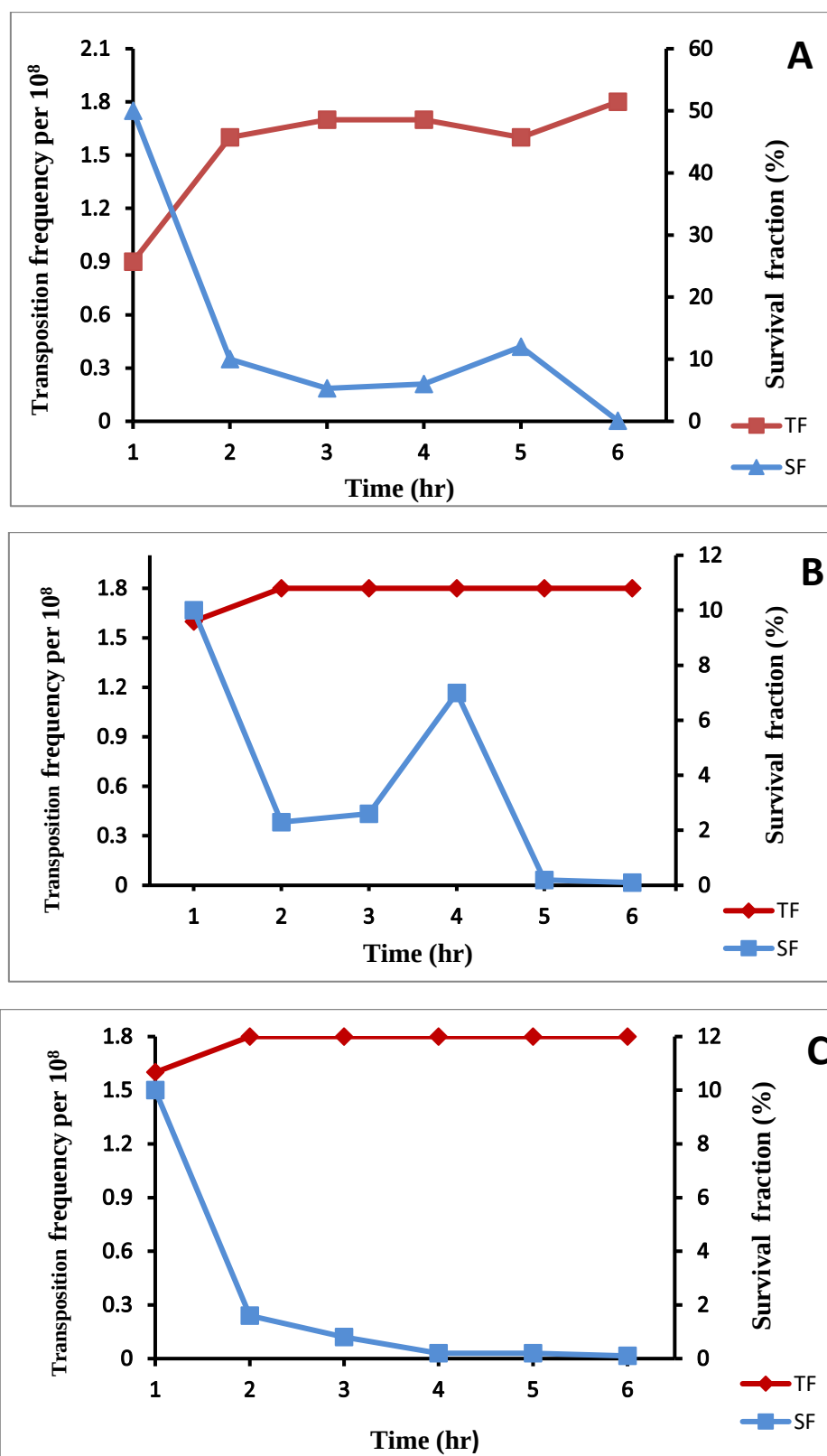


Figure (4-10): Transposition frequency of IS256 in Ampicillin gene of *S. aureus* S1 and survival fraction after pretreatment with vancomycin and/or ciprofloxacin for six hours intervals. (A): pretreatment with Ciprofloxacin; (B): pretreatment with Vancomycin; (C): pretreatment with vancomycin and ciprofloxacin.

Table (4-9): Statistical analysis for transposition frequency of IS256 in ampicillin gene after pretreatment of *S.aureus* S1 with ciprofloxacin and /or vancomycin for six hour intervals.

Time (hr)	Transposition Frequency per 10 ⁸ cells			LSD
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	0.9 ^b	1.6 ^a	1.6 ^a	0.438*
2	1.6 ^a	1.8 ^a	1.8 ^a	0.302 NS
3	1.7 ^a	1.8 ^a	1.8 ^a	0.298 NS
4	1.7 ^a	1.8 ^a	1.8 ^a	0.298 NS
5	1.6 ^a	1.8 ^a	1.8 ^a	0.281 NS
6	1.8 ^a	1.8 ^a	1.8 ^a	0.275 NS

Different letters in rows represents a significant difference, *($P \leq 0.05$).

Table (4-10): Statistical analysis for survival fractions of *S.aureus* S1 after pretreatment with ciprofloxacin and / or vancomycin for six hours intervals.

Time(hr)	Survival fraction (%)			LSD value
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	50 ^a	10 ^b	10 ^b	6.59**
2	10 ^a	2.3 ^b	1.6 ^b	3.72**
3	5.3 ^a	2.6 ^b	0.8 ^b	2.37**
4	6 ^a	7 ^a	0.2 ^b	3.07**
5	12 ^a	0.2 ^b	0.2 ^b	3.19**
6	0.1 ^a	0.1 ^a	0.1 ^a	0.055 NS

Different letters in same rows represents a significant difference, **($P \leq 0.01$),
NS: non-significant

compared with the pretreatment with ciprofloxacin, and the survival fraction was significantly decreased ($P \leq 0.01$) after pretreatment with vancomycin and both ciprofloxacin and vancomycin in the first five hours of incubation compared with the decrease with ciprofloxacin after the same times intervals as indicated in table (4-10).

The accumulation of multiple copies of IS256 in the genome is due to its transposition process which affects flexibility and resistance to antibiotics by *S.aureus* as mentioned by Reipert *et al.* (2003). Results of this study was agree with Nagel *et al.* (2011) who mentioned that the subinhibitory concentrations of ciprofloxacin and vancomycin lead to the activation of IS256 transposition.

4.6.1.3 Effect of transposition by IS256 on azithromycin resistance of *S.aureus* S1

Effect of transposition by IS256 on azithromycin resistance of *S.aureus* S1 under the stress of vancomycin and /or ciprofloxacin was studied by incubation of the bacterial isolate with the subinhibitory concentrations of these antibiotics. Results illustrated in figure (4-12) showed the transposition frequency of IS256 in azithromycin gene and survival fractions after incubation for six hours intervals. As shown in figure (4-12b) and table (4-11), it was found that the transposition frequency of IS256 in azithromycin gene was significantly increased ($P < 0.01$) after two hours of pretreatment with the subinhibitory concentration of vancomycin from $0.4 \text{ per } 10^8$ to $2.3 \text{ per } 10^8$, then slightly increased to $2.5 \text{ per } 10^8$ in the next four hours of incubation. On the other hand, survival fractions of *S.aureus* S1 was significantly decreased ($P \leq 0.01$) after two hours of incubation with vancomycin from 19% to 2%, then slightly decreased to 1% in the next hours of incubation. Results

illustrated in figure (4-12c) showed that the synergistic effect of both vancomycin and ciprofloxacin on transposition frequency of IS256 in azithromycin gene was significantly higher ($P < 0.01$) than vancomycin alone in the first two hours of incubation as the transposition frequency in azithromycin gene was increased from 1.8 per 10^8 in the second hour, then oscillating dropped was occurred in the next four hours. Results illustrated in figure (4-12b) and table (4-12) also showed that there is a significant decrease ($P < 0.01$) in survival fractions of *S.aureus* S1 after two hours of incubation with vancomycin from 20% to 2%, then slightly decreased to 1% in the next four hours of incubation, while less effect of ciprofloxacin on transposition frequency of IS256 in azithromycin gene, and survival fractions of *S.aureus* S1 after incubation for six hours intervals as shown in figure (4-12a).

These results agree with Schreiber *et al.* (2013) who mentioned that the transposition of IS256 and accumulation of multiple copies of this insertion sequence was activated by incubation of *S.aureus* with subinhibitory concentrations of chloramphenicol, linezolid, and spectinomycin, and the transposition frequency is due to the enzymatic activity of the functional transposase enzyme.

Furthermore, statistical analysis indicated in table (4-11) and (4-12) showed the influence of vancomycin and both vancomycin and Ciprofloxacin was significantly higher ($P \leq 0.01$) than the influence of ciprofloxacin alone on transposition frequency of IS256, and inactivation of azithromycin gene, and on survivals of the bacterial isolate.

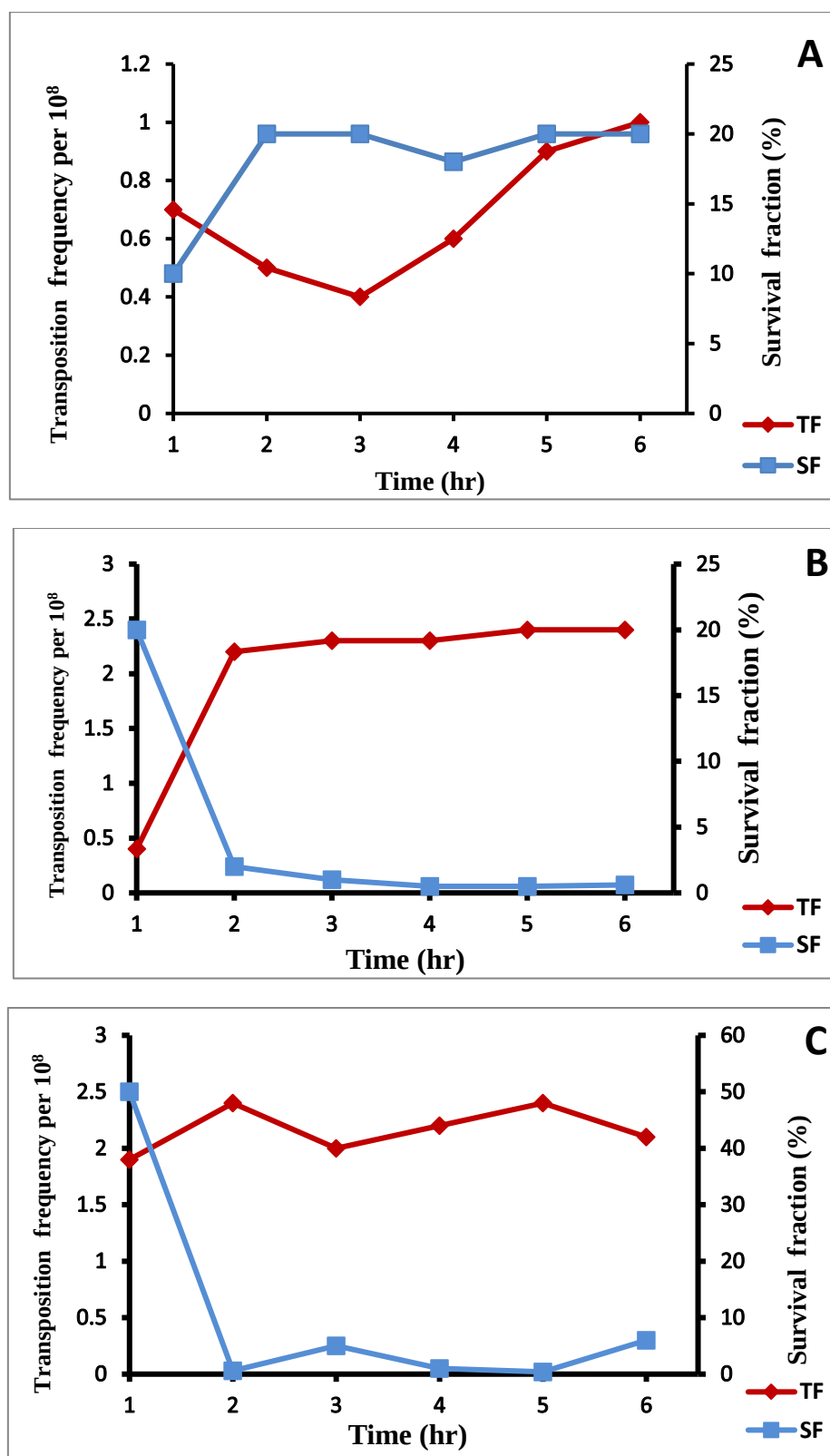


Figure (4-11): Transposition frequency of IS256 in azithromycin gene of *S.aureus* S1 and survival fraction after pretreatment with vancomycin and/or ciprofloxacin for six hours intervals.(A): pretreatment with Ciprofloxacin; (B): pretreatment with Vancomycin; (C): pretreatment with vancomycin and ciprofloxacin.

Table (4-11): Statistical analysis for transposition frequency of IS256 in azithromycin gene after pretreatment of *S.aureus* S1 with ciprofloxacin and /or vancomycin for six hour intervals.

Time (hr)	Transposition Frequency per 10 ⁸ cells			LSD
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	0.7 ^b	0.4 ^b	1.9 ^a	0.482*
2	0.5 ^b	2.2 ^a	2.4 ^a	0.407*
3	0.4 ^b	2.3 ^a	2.0 ^a	0.348*
4	1 ^b	2.3 ^a	2.2 ^a	0.392*
5	0.9 ^b	2.4 ^a	2.4 ^a	0.501*
6	1 ^b	2.4 ^a	2.1 ^a	0.452*

Different letters in same rows represents a significant difference, *($P \leq 0.05$).

Table (4-12): Statistical analysis for survival fractions of *S.aureus* S1 after pretreatment with ciprofloxacin and / or vancomycin for six hours intervals.

Time(hr)	Survival fraction (%)			LSD value
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	10 ^b	20 ^a	5 ^c	4.82**
2	20 ^a	2 ^b	0.6 ^b	5.39**
3	20 ^a	1 ^b	5 ^b	5.08**
4	10 ^a	0.5 ^b	1 ^b	4.36**
5	20 ^a	0.5 ^b	0.4 ^b	5.92**
6	20 ^a	0.6 ^c	6 ^b	5.16**

Different letters in rows represents a significant difference, **($P \leq 0.01$).

4.6.1.4 Effect of transposition by IS256 on trimethoprim resistance of *S.aureus* S1

Effect of transposition by IS256 on trimethoprim resistance of *S.aureus* S1 under the stress of vancomycin and/or ciprofloxacin was studied. Results illustrated in figure (4-13) showed the transposition frequency of IS256 and survival fraction of *S.aureus* S1 after pretreatment with the subinhibitory concentration of vancomycin and/or ciprofloxacin for six hours intervals. Results showed that the transposition frequency of IS256 was significantly increased ($P \leq 0.01$) from 2 per 10^9 cells after six hours of incubation as shown in figure (4-13b) due to the inactivation of trimethoprim gene due to transposition of IS256, and converted bacterial cells of *S.aureus* S1 from trimethoprim resistance to trimethoprim sensitive. On the same time, transposition of IS256 in the genome of *S.aureus* S1 caused significant decrease ($P \leq 0.01$) in the survival fractions from 100% at the zero time of incubation with vancomycin to 10% after one hour of incubation, then to 1% after six hours of incubation due to the insertion of IS256 into different structural genes and inactivate these genes essentials for DNA replication and enzymes responsible for bacterial metabolic pathways upregulation of error-prone polymerases/ and or by the down regulation of mismatch repair system as mentioned by Nagel *et al.* (2011).

On the other hand, results illustrated in figure (4-13c) showed the most effective transposition frequency of IS256 after pretreatment of *S.aureus* S1 with the subinhibitory concentrations of vancomycin and ciprofloxacin together, as the transposition frequency was significantly increased ($P \leq 0.01$) from 19 per 10^9 cells after one hour of incubation to 22 per 10^9 cells after six hours of incubation and inactivation of trimethoprim gene. At the same time, survival fractions of *S.aureus* S1 bacterial cells were significantly decreased ($P \leq 0.01$) from 100% at the zero time of

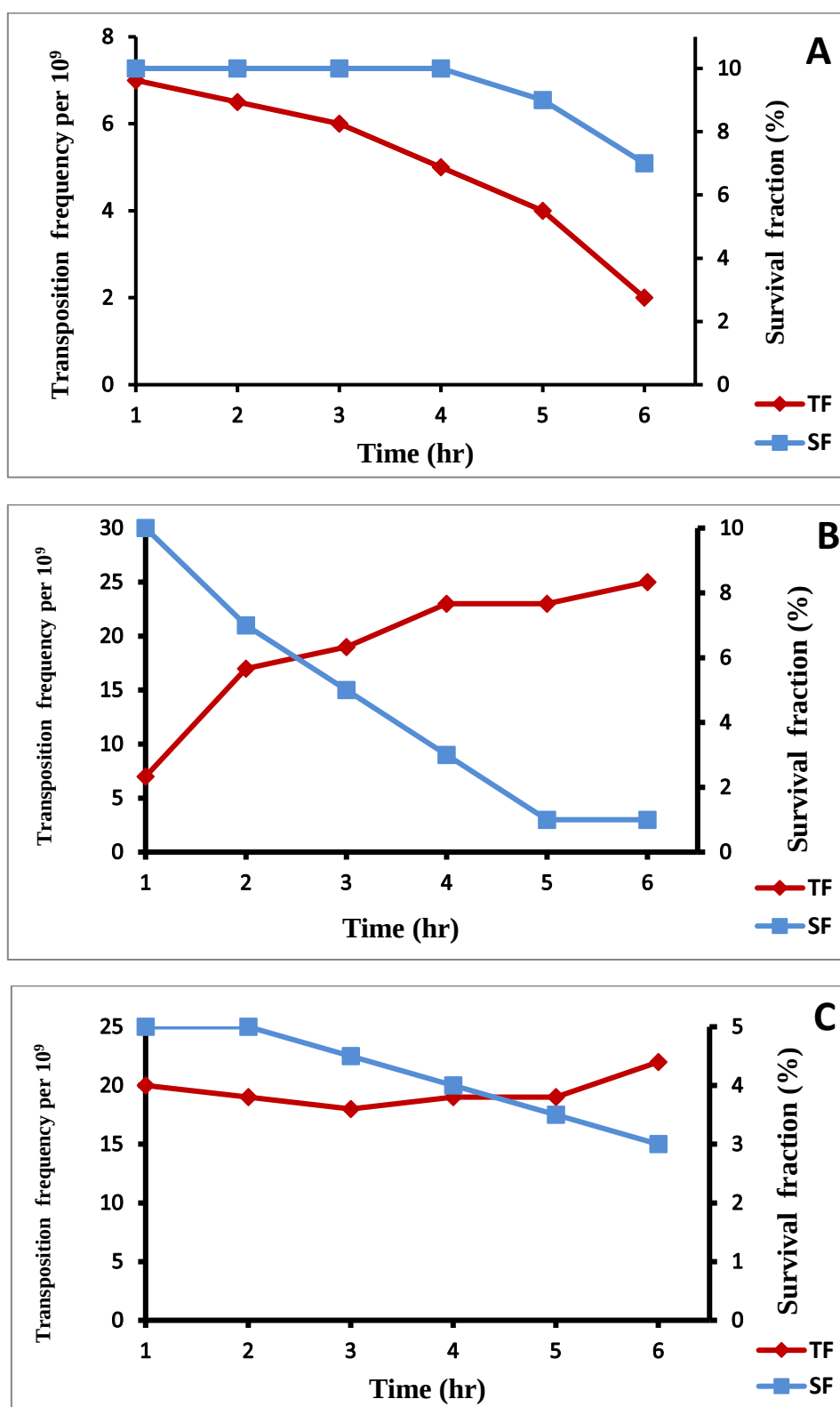


Figure (4-12): Transposition frequency of IS256 in trimethoprim gene of *S.aureus* S1 and survival fraction after pretreatment with vancomycin and/or ciprofloxacin for six hours intervals.(A): pretreatment with Ciprofloxacin; (B): pretreatment with Vancomycin; (C): pretreatment with vancomycin and ciprofloxacin.

Incubation with the subinhibitory concentration of vancomycin and ciprofloxacin to 5% after one hour of incubation, then to 3% after six hours of incubation. Results illustrated in figure (4-13 a) showed less effect of ciprofloxacin on transposition frequency of IS256 in trimethoprim gene compared with vancomycin and synergistic effect of vancomycin and ciprofloxacin or vancomycin alone.

Furthermore results indicated in table (4-13 and 4-14) showed the significant influence ($P \leq 0.01$) of both vancomycin and ciprofloxacin on the transposition frequency of IS256 and survival fractions of *S.aureus* S1 compared with the mono effect of ciprofloxacin or vancomycin.

The presence of IS256 confers a strong genomic plasticity that is useful for adaptation to antibiotic stress (Kallies *et al.*, 2017) that explain the reversion was caused by excision of IS256 in a small number of fast growing clones that quickly outcompeted the Trimethoprim-Sulfamethoxazole in transposonal frequency. As mentioned by Kleinert *et al.* (2017), *S. aureus* has acquired resistance to nearly all antibiotics used in clinical practice. The mutants were isolated after long-term exposure to trimethoprim-sulfamethoxazole in vivo. Here, they show for the first time that via insertions of IS elements, mutations may also give rise to unstable auxotrophic SCVs. Other genetic mechanisms that can confer fast phenotypic switching, like insertion or deletion of single bases or short repeats by slipped-strand mispairing, have also been discussed previously for phase variation in virulent resistance of antibiotics to *S.aureus* (Kleinert *et al.*, 2017).

Table (4-13): Statistical analysis for transposition frequency of IS256 in Trimethoprim gene after pretreatment of *S. aureus* S1 with ciprofloxacin and /or vancomycin for six-hour intervals.

Time (hr)	Transposition Frequency per 10 ⁹			LSD
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	7 ^b	7 ^b	20 ^a	1.337 **
2	6.5 ^b	17 ^a	19 ^a	2.062 **
3	6 ^b	19 ^a	18 ^a	2.471 **
4	5 ^c	23 ^a	19 ^b	3.152 **
5	4 ^c	23 ^a	19 ^b	2.983 **
6	2 ^b	25 ^a	22 ^a	3.219 **

Different letters in rows represents a significant difference ($P \leq 0.01$).

Table(4-14): Statistical analysis for survival fractions of *S.aureus* S1 after pretreatment with ciprofloxacin and / or vancomycin for six hours intervals.

Time(hr)	Survival fraction %			LSD value
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	10 ^a	10 ^a	5 ^b	1.750 **
2	10 ^a	7 ^b	5 ^c	1.064 **
3	10 ^a	5 ^b	4.5 ^b	1.502 **
4	10 ^a	3 ^b	4 ^b	1.266 **
5	9 ^a	1 ^c	3.5 ^b	1.095 **
6	7 ^a	1 ^c	3 ^b	1.267 **

Different letters in rows represents a significantly difference ($P \leq 0.01$).

4.6.1.5 Effect of transposition on Penicillin resistance of *S.aureus* S1

In order to test the effect of vancomycin and/or ciprofloxacin on the transposon frequency of IS256 and Percentage of survival fraction of *S.aureus* S1. Bacterial cells were incubated in the presence of subinhibitory

concentrations of both (vancomycin and ciprofloxacin and, combination from them in for six hours intervals, then aliquots of each culture were plated onto BHI agar plates—containing penicillin G.

Results illustrated in figure (4-14) showed the number of transposition events after preincubation in the presence of subinhibitory concentration (s) of vancomycin and/ or ciprofloxacin. These results represents that the transposition frequency after pretreatment with both vancomycin and ciprofloxacin is most effective to induce genetic mutations by transposon IS256 and inactivate penicillin gene compared with pretreatment with vancomycin alone as shown in figure (4-14c), as the survival fraction was strongly decrease during the six hours intervals of incubation with subinhibitory concentration of vancomycin and ciprofloxacin from 10% to 0.5% and the transposition frequency was increased from 20 per 10^9 to 7 per 10^9 cells.

According to these results, vancomycin enhanced the transposition activity, whereas the effect of ciprofloxacin alone was noticed but significantly ($P \leq 0.01$) lower. Furthermore, the maximum number of colonies carrying transposon insertions per 10^9 cells of culture was reached after incubation for six hours in presence of subinhibitory concentration of vancomycin. On the other hand, results illustrated in figure (4-14a) shows that the transposition frequency after pretreatment with subinhibitory concentration of ciprofloxacin was most effective after one and six hours of incubation with antibiotic (8 per 10^9 and 2 per 10^9) cells respectively compared with the pretreatment with both vancomycin and ciprofloxacin and vancomycin alone in ($P \leq 0.01$), where the transposonal frequency was 2 per 10^9 after pretreatment for the same periods as shown in figure (4-14a).

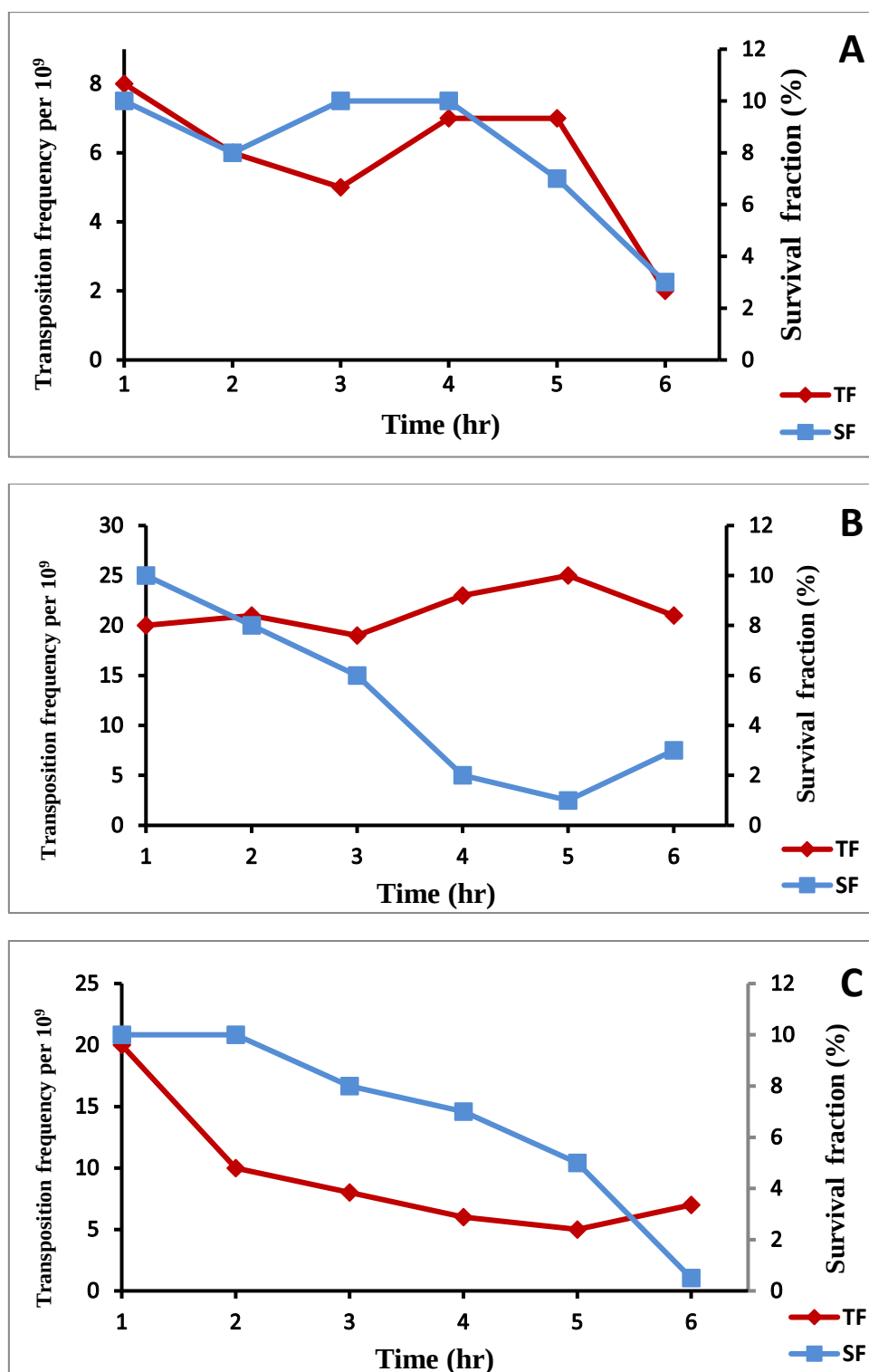


Figure (4-13): Transposition frequency of IS256 in pncillin gene of *S. aureus* S1 and survival fraction after pretreatment with vancomycin and/or ciprofloxacin for six hours intervals. (A): pretreatment with Ciprofloxacin; (B): pretreatment with Vancomycin; (C): pretreatment with vancomycin and ciprofloxacin.

Furthermore, results of statistical analysis indicated in table (4-15) showed that the transposition frequency of IS256 in penicillin gene after pretreatment with ciprofloxacin was significantly higher ($P \leq 0.01$) than pretreatment with vancomycin and/or ciprofloxacin during the first five hour of incubation, while the survival fraction was significantly different ($P \leq 0.05$) among the pretreatments of these antibiotics during the six hours of incubation as indicated in table (4-16).

In study achieved by Gregorio *et al.*(2016) conducted in Buenos Aires, Argentina on a patient suffering from bone and joint infection, before and after 40 days of vancomycin treatment, showed an increase in copies of IS256 due to "copy and paste" mechanism and increase in the number of IS256 copies, and mutants occurred at frequencies of around 10^{-7} - 10^{-8} in all strains even in the presence of subinhibitory concentrations of vancomycin, It is known that transposition of IS256 may impact in virulence impact of transposition events on virulence and antibiotic resistance in *S. aureus*.

Table (4-15): Statistical analysis for transposition frequency of IS256 in Penicillin G gene after pretreatment of *S.aureus* S1 with ciprofloxacin and /or vancomycin for six hour intervals.

Time (hr)	Transposition Frequency per 10^9			LSD
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	8 ^b	20 ^a	20 ^a	1.420 **
2	6 ^c	21 ^a	10 ^b	2.447 **
3	5 ^c	19 ^a	8 ^b	2.091 **
4	7 ^b	23 ^a	6 ^b	2.153 **
5	7 ^b	25 ^a	5 ^b	2.758 **
6	2 ^c	21 ^a	7 ^b	3.026 **

Different letters in rows represents a significant difference ($P \leq 0.05$).

Table(4-16): Statistical analysis for survival fractions of *S.aureus* S1 after pretreatment with ciprofloxacin and / or vancomycin for six hours intervals .

Time(hr)	Survival fraction %			LSD value
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	10	10	10	0.500 NS
2	8	8	10	2.288 ^{NS}
3	10 ^a	6 ^c	8 ^b	1.625 [*]
4	10 ^a	2 ^c	7 ^b	2.116 ^{**}
5	7 ^a	1 ^c	5 ^b	1.358 ^{**}
6	3 ^a	3 ^a	0.5 ^b	0.750 ^{**}

Different letters in rows represents a significant difference ($P < 0.01$).

4.6.1.6 Effect of transposition by IS256 on Lincomycin resistance of *S.aureus* S1

Effect of vancomycin and ciprofloxacin on the resistance of *S. aureus* S1 was also studied. Results illustrated in figure (4-15) showed that the transposition of IS256 in genomic DNA of *S.aureus* S1 was induced under stress of vancomycin and / or ciprofloxacin, and it was found the transposition frequency was most effective after pretreatment with vancomycin to induce genetic mutations in *S.aureus* S1 genomic DNA and inactivate Lincomycin gene confer to the bacterial resistance to Lincomycin. Results illustrated in figure (4-15b) showed that the transposition frequency was increased from 24 per 10^9 cells in the one hour to 8 per 10^9 cells after six hours of pretreatment with vancomycin. Results illustrated in figure (4-15c) results that the transposition frequency was pretreatment with both vancomycin and ciprofloxacin was 24 per 10^9 cells in the second hour of incubation, while the transposition frequency per 10^9 was increased gradually from 21 per 10^9 after six hours of incubation.

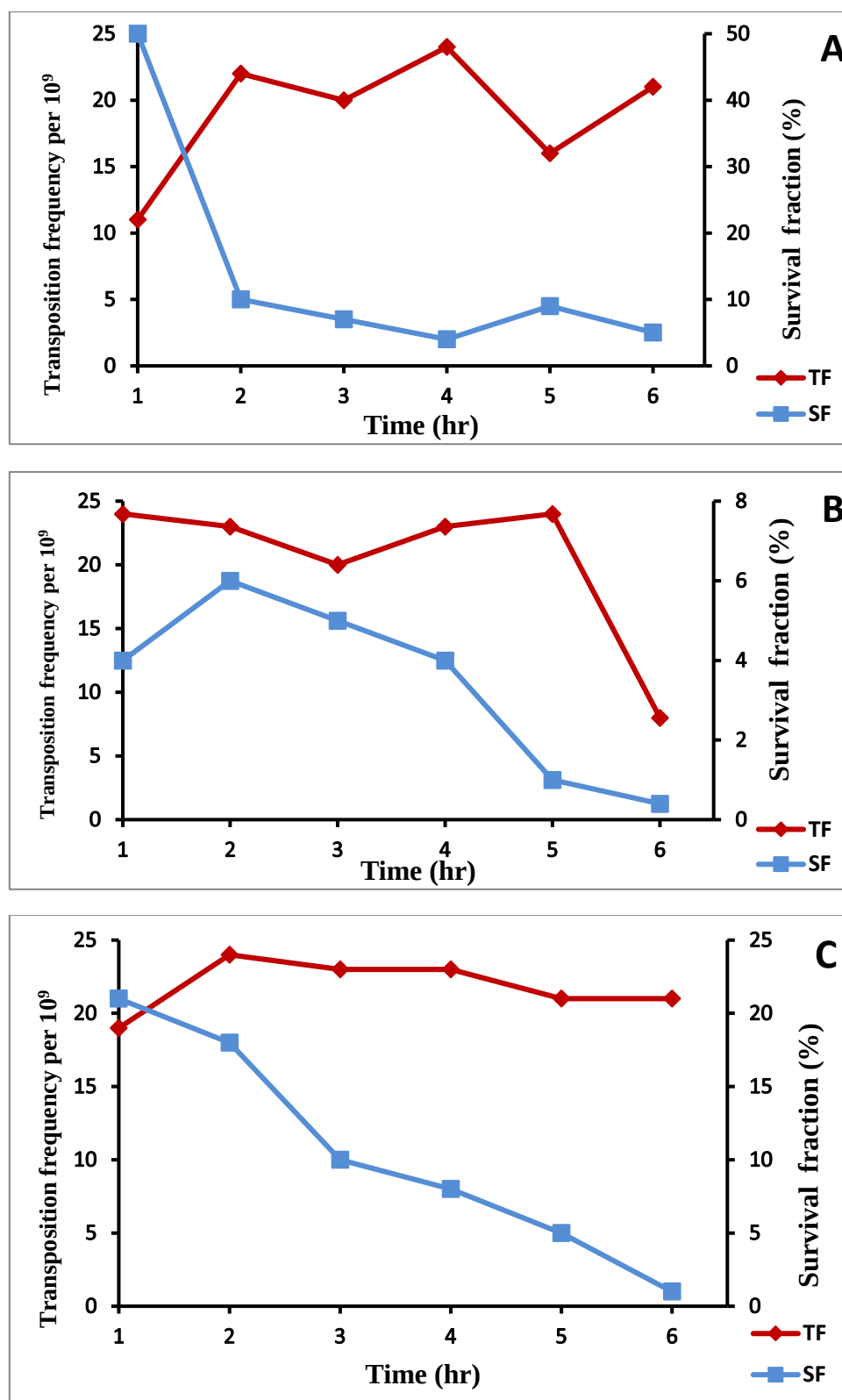


Figure (4-14): Transposition frequency of IS256 in licomycin gene of *S.aureus* S1 and survival fraction after pretreatment with vancomycin and/or ciprofloxacin for six hours intervals.(A): pretreatment with Ciprofloxacin; (B): pretreatment with Vancomycin; (C): pretreatment with vancomycin and ciprofloxacin.

On the other hand, results illustrated in figure (4-15b) showed that the survival fraction (%) was significantly decreased ($P \leq 0.01$) from 4% after one hour of treatment with vancomycin to 0.4 % after six hours of incubation with these antibiotics compared with the decrease in survival fraction after pretreatment with both vancomycin and ciprofloxacin or ciprofloxacin alone for the same times intervals as shown in figure (4-15a,c). Furthermore results indicated in table (4-17) showed that the transposition frequency of IS256 was increased significant ($P \leq 0.01$) after pretreatment with vancomycin and both ciprofloxacin compared with the pretreatment with ciprofloxacin, and the survival fraction was significantly decreased ($P \leq 0.01$) after pretreatment with vancomycin and both ciprofloxacin and vancomycin in the first six hours of incubation compared with the decrease with ciprofloxacin after the same times intervals as indicated in table(4-18). Barceló (2018) describes the disparate effects of bacteriophages on antibiotic-resistant bacteria that *S. aureus* specialized transduction. As for other species, bacterial genes spread by phages can be part of particular mobile genetic elements functional antibiotic resistance activity.

Table (4-17): Statistical analysis for transposition frequency of IS256 in Lincomycin gene after pretreatment of *S.aureus* S1 with ciprofloxacin and /or vancomycin for six hour intervals.

Time (hr)	Transposition Frequency per 10^9 cells			LSD
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	11 ^c	24 ^a	19 ^b	3.672 ^{**}
2	22 ^a	23 ^a	24 ^a	4.610 NS
3	20 ^a	20 ^a	23 ^a	3.071 NS
4	24 ^a	23 ^a	23 ^a	2.613 NS
5	16 ^b	24 ^a	21 ^a	3.026 ^{**}
6	21 ^a	8 ^b	21 ^a	2.983 ^{**}

Different letters in rows represents a significantly difference, ^{**}($P \leq 0.01$), NS: non-significant

Table (4-18): Statistical analysis for survival fractions of *S.aureus* S1 after pretreatment with ciprofloxacin and / or vancomycin for six hour intervals.

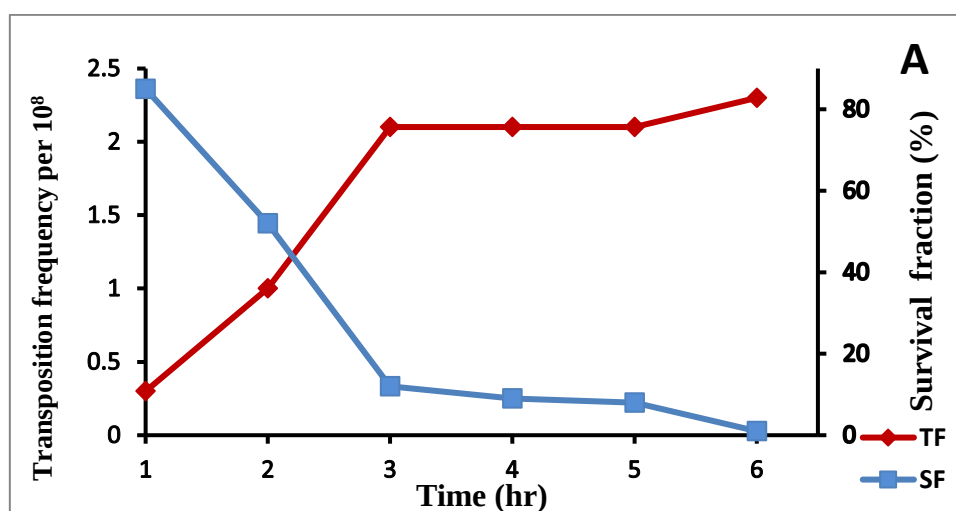
Time(hr)	Survival fraction (%)			LSD value
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	50 ^a	4 ^c	21 ^b	6.92 ^{**}
2	10 ^b	6 ^c	18 ^a	3.850 ^{**}
3	7 ^b	5 ^b	10 ^a	2.339 ^{**}
4	4 ^b	4 ^b	8 ^a	1.703 ^{**}
5	9 ^a	1 ^c	5 ^b	2.094 ^{**}
6	5 ^a	0.4 ^b	1 ^b	0.766 ^{**}

Different letters in rows represents a significantly difference, ^{**}($P \leq 0.01$).

4.6.1.7 Effect of transposition by IS256 on Rifampin resistance of *S.aureus* S1

Effect of transposition by IS256 on rifampin resistance of *S.aureus* S1 under the stress of vancomycin and /or ciprofloxacin was also studied by incubation of the bacterial isolate with the subinhibitory concentrations of these antibiotics. Results illustrated in figure (4-16) showed the transposition frequency of IS256 in rifampin gene and survival fractions after incubation for six hours intervals. As shown in figure (4-12b) and table (4-18), it was found that the transposition frequency of IS256 in rifampin gene was significantly increased ($P \leq 0.05$) after two hours of incubation with the subinhibitory concentration of vancomycin from 2.18 per 10^8 cells, then slightly increased to 1.7 per 10^8 cells in the next six hours of incubation. On the other hand, survival fractions of *S.aureus* S1 was significantly decreased ($P \leq 0.01$) after one hours of incubation with vancomycin from 70% to 20%, then slightly decreased to 0.1% in the next hours of incubation. On the other hand, results illustrated in figure (4-16c) showed that the synergistic effect of both vancomycin and ciprofloxacin on transposition frequency of IS256 in rifampin gene was significantly

higher ($P \leq 0.05$) than vancomycin alone in the first two hours of incubation as the transposition frequency in rifampin gene was increased to 2.14 per 10^8 cells in the second hour, then oscillating dropp was occurred in the next four hours. Results illustrated in figure (4-16a) and table (4-19) also showed that there is a significant decrease ($P \leq 0.01$) in survival fractions of *S.aureus* S1 after two hours of incubation with ciprofloxacin from 85% to 52%, then slightly decreased to 1% in the next four hours of incubation, while less effect of ciprofloxacin on transposition frequency of IS256 in rifampin gene, and survival fractions of *S.aureus* S1 after incubation for six hours intervals as shown in figure (4-16a). These results agree with Nagel *et al.* (2011) who studied influence of ciprofloxacin and vancomycin on mutation rate and transposition of IS256 in *S. aureus* by pretreatment of bacterial cells with subinhibitory concentrations of the antibiotics, then plated onto rifampicin-containing agar plates, and the mutation rate was determined by fluctuation analysis. The results showed the mutation rate



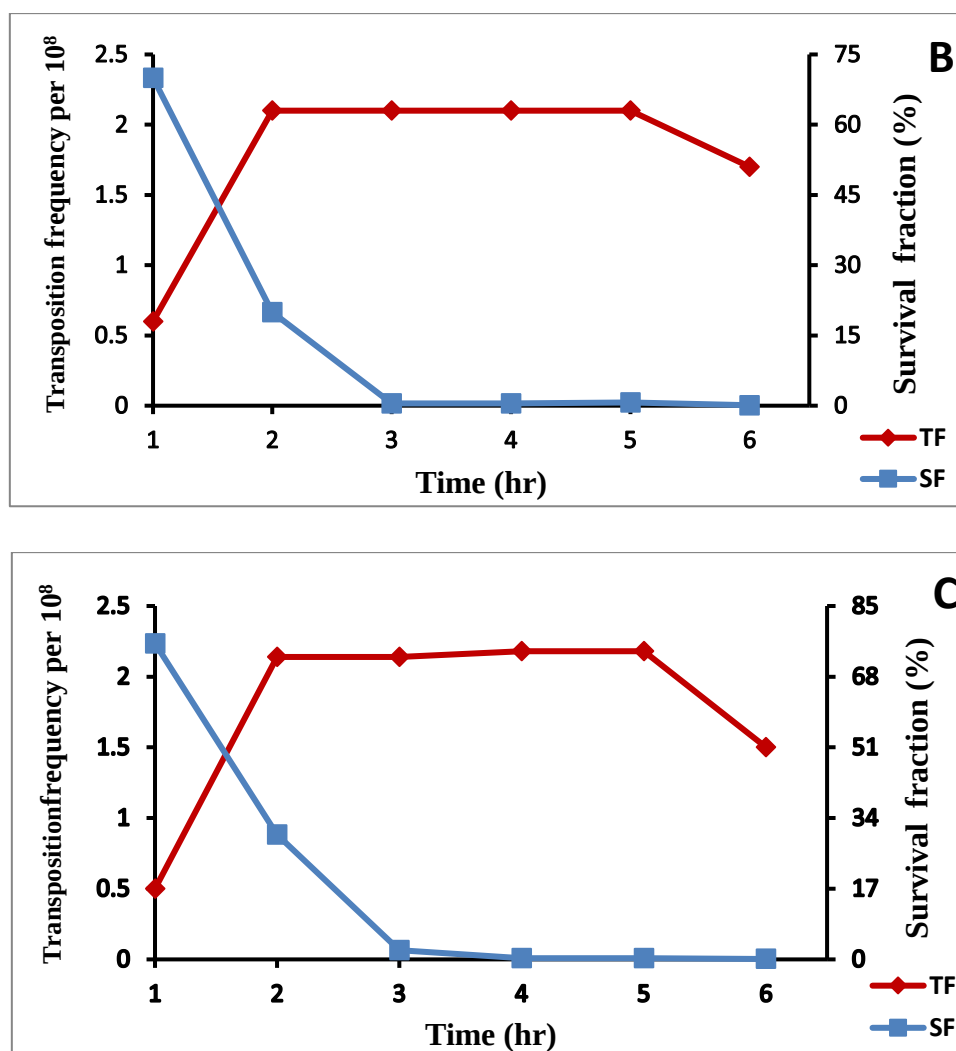


Figure (4-15): Transposition frequency of IS256 in rifampin gene of *S.aureus* S1 and survival fraction after pretreatment with vancomycin and/or ciprofloxacin for six hours intervals.(A): pretreatment with Ciprofloxacin; (B): pretreatment with Vancomycin; (C): pretreatment with vancomycin and ciprofloxacin.

was enhanced by ciprofloxacin and vancomycin, as the mean values of the mutation frequencies are 5.9×10^{-6} to 2.3×10^{-6} cells in the presence of ciprofloxacin and 4.1×10^{-6} to $0. \times 10^{-6}$ cells in the presence of vancomycin. The transposition of IS elements confer genomic plasticity and adaptability to staphylococci in the presence of antibiotics. Moreover, theoretically, both Processes might act synergistically. Thus, antibiotic stress may lead to an activation of transposition of IS256 that such a strain in turn becomes hypersensitive to antibiotic stress and, in consequence, is prone to resistance development as shown previously (Schaaff *et al.*, 2002).

Furthermore, statistical analysis indicated in table (4-19) and (4-20) showed the influence of vancomycin and both vancomycin and ciprofloxacin was significantly higher ($P \leq 0.01$) than the influence of ciprofloxacin alone on transposition frequency of IS256, and inactivation of rifampin gene, and on survivals of the bacterial isolate.

Table (4-19): Statistical analysis for transposition frequency of IS256 in Rifampin gene after pretreatment of *S.aureus* S1 with ciprofloxacin and /or vancomycin for six hour intervals.

Time (hr)	Transposition Frequency per 10^8 Cells			LSD
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	3.3 ^a	0.6 ^b	0.5 ^b	0.389*
2	1 ^b	2.18 ^a	2.14 ^a	0.503*
3	2.18 ^a	2.18 ^a	2.14 ^a	0.286 NS
4	2.18 ^a	2.18 ^a	2.18 ^a	0.500 NS
5	2.18 ^a	2.18 ^a	2.18 ^a	0.500 NS
6	2.3 ^a	1.7 ^b	1.5 ^b	0.497*

Different letters in rows represents a significantly difference, * ($P \leq 0.05$), ** ($P \leq 0.01$), NS: non-significant

Table (4-20): Statistical analysis for survival fractions of *S.aureus* S1 after pretreatment with ciprofloxacin and / or vancomycin for six hour intervals.

Time(hr)	Survival fraction (%)			LSD value
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	85 ^a	70 ^b	76 ^b	6.93**
2	52 ^a	20 ^c	30 ^b	6.46**
3	12 ^a	0.5 ^b	2.2 ^b	4.87**
4	9 ^a	0.5 ^b	0.3 ^b	4.02**
5	8 ^a	0.7 ^b	0.3 ^b	3.97**
6	1 ^a	0.1 ^b	0.1 ^b	0.582*

Different letters in rows represents a significantly difference, * ($P \leq 0.05$); ** ($P \leq 0.01$), NS: non-significant

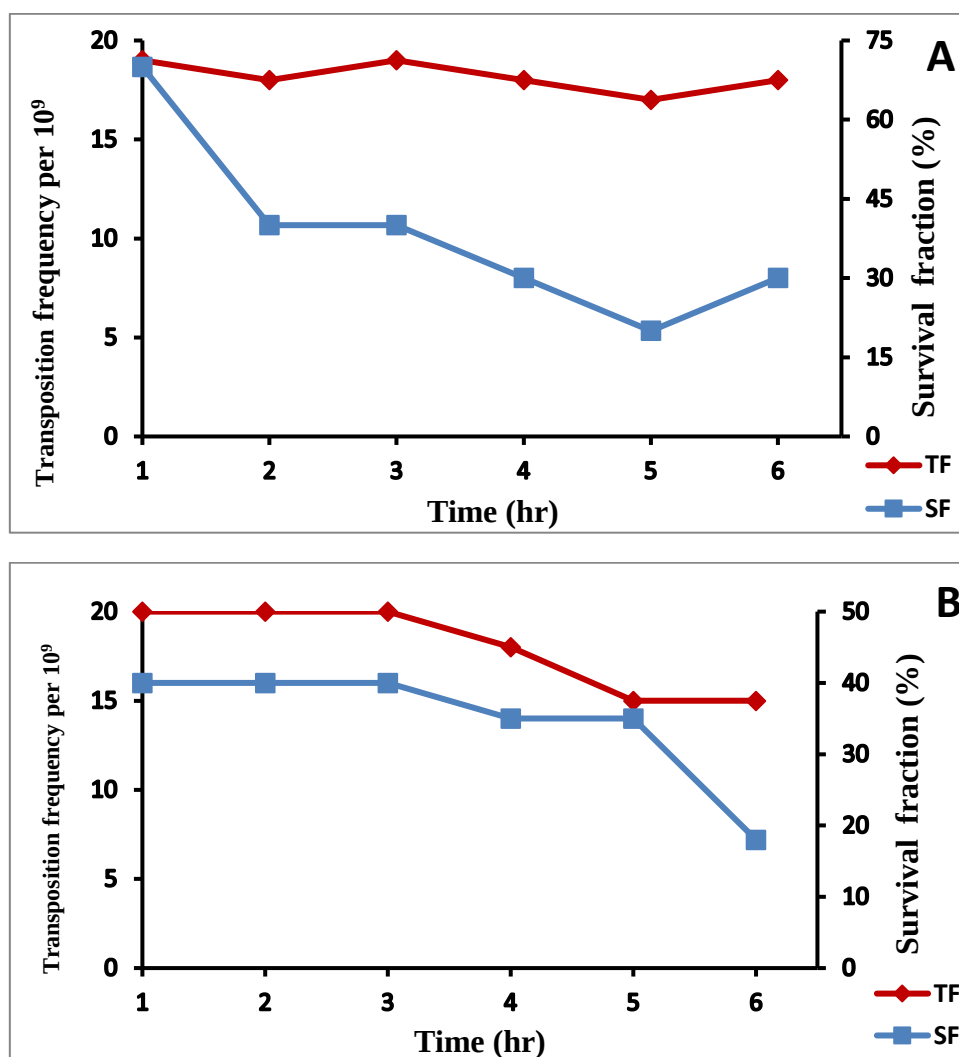
4.6.1.8 Effect of transposition by IS256 on Gentamicin resistance of *S.aureus* S1

Effect of transposition by IS256 on gentamicin resistance of *S.aureus* S1 under the stress of vancomycin and/or ciprofloxacin was studied. Results illustrated in figure (4-17) showed the transposition frequency of IS256 per 10^9 cells and survival fraction of *S.aureus* S1 after incubation with the subinhibitory concentration of both vancomycin and ciprofloxacin together for six hours intervals. It was found that transposition frequency of IS256 was significantly increased ($P \leq 0.01$) to 4 per 10^9 cells after six hours of incubation as shown in figure (4-17c) due to the inactivation of gentamicin gene by transposition of IS256, and converted bacterial cells of *S.aureus* S1 from gentamicin resistance to gentamicin sensitive. On the same time, transposition of IS256 in the genome of *S.aureus* S1 caused significant decrease ($P \leq 0.01$) in the survival fractions from 100% at the zero time of incubation with vancomycin to 50% after one hour of incubation, then 4% after six hours of incubation due to the insertion of IS256 into different structural genes and inactivate these genes essentials for DNA replication and enzymes responsible for bacterial metabolic pathways upregulation of error-prone polymerases/ and or by the down regulation of mismatch repair system as mentioned by Nagel *et al.* (2011).

On the other hand, results illustrated in figure (4-17b) showed the most effective transposition frequency of IS256 after incubation of *S.aureus* S1 with the subinhibitory concentrations of vancomycin, as the transposition frequency was significantly increased ($P \leq 0.01$) from 20 per 10^9 cells after one hour of incubation to 15 per 10^9 cells after six hours of incubation and inactivation of gentamicin gene. At the same time, survival fractions of *S.aureus* S1 bacterial cells were significantly decreased ($P \leq 0.01$) from 100% at the zero time of incubation with the subinhibitory

concentration of vancomycin and ciprofloxacin to 40% after one hour of incubation, then to 18% after six hours of incubation. Results illustrated in figure (4-17 a) showed less effect of ciprofloxacin on transposition frequency of IS256 in gentamicin gene compared with vancomycin and synergistic effect of vancomycin and ciprofloxacin or vancomycin alone.

Further more results indicated in table (4-21 and 4-22) showed the significant influence ($P \leq 0.01$) of both vancomycin and ciprofloxacin on the transposition frequency of IS256 and survival fractions of *S.aureus* S1 compared with the mono effect of ciprofloxacin or vancomycin.



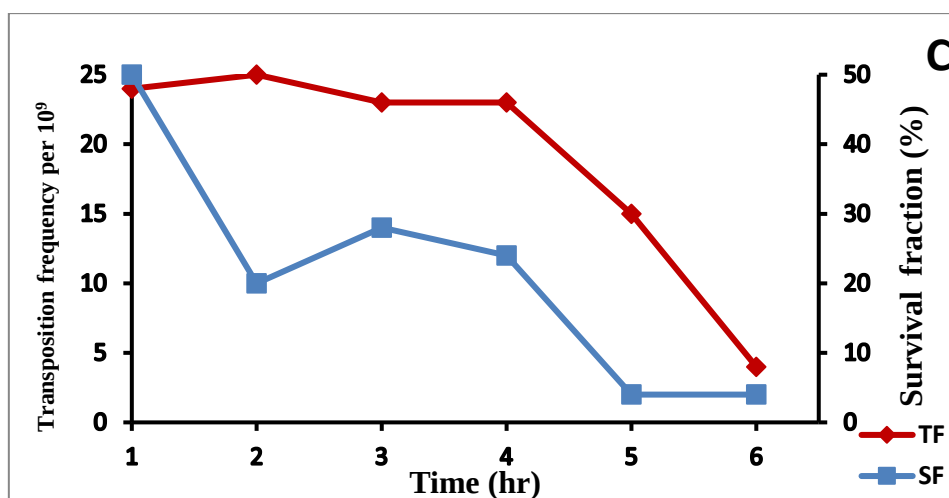


Figure (3-16): Transposition frequency of IS256 in gentamicin gene of *S.aureus* S1 and survival fraction after pretreatment with vancomycin and/or ciprofloxacin for six hours intervals.(A): pretreatment with Ciprofloxacin; (B): pretreatment with Vancomycin; (C): pretreatment with vancomycin and ciprofloxacin.

The presence of IS256 confers a strong genomic plasticity useful for adaptation to antibiotic stress, which explains the reversion was caused by excision of IS256 in a small number of fast-growing clones that quickly outcompeted the gentamicin in transposonal frequency (Kallies *et al.*, 2017). While Kleinert *et al.* (2017) mentioned that *S. aureus* has acquired resistance to nearly all antibiotics used in clinical practice. The mutants were isolated after long-term exposure to gentamicin in vivo. Here, they show for the first time that via insertions of IS elements, mutations may also give rise to unstable auxotrophic SCVs.

Table (4-21): Statistical analysis for transposition frequency of IS256 in Gentamicin gene after pretreatment of *S.aureus* S1 with ciprofloxacin and /or vancomycin for six hour intervals.

Time (hr)	Transposition Frequency per 10 ⁹ Cells			LSD
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	19 ^b	20 ^b	24 ^a	2.185*
2	18 ^b	20 ^b	25 ^a	2.563*
3	19 ^a	20 ^a	23 ^a	4.014 NS
4	18 ^b	18 ^b	23 ^a	3.402*
5	17 ^a	15 ^a	15 ^a	2.192 NS
6	18 ^a	15 ^a	4 ^b	3.411**

Different letters in rows represents a significantly difference, * (P≤0.05), ** (P≤0.01), NS: non-significant

Table (4-22): Statistical analysis for survival fractions of *S.aureus* S1 after pretreatment with ciprofloxacin and / or vancomycin for six hour intervals.

Time(hr)	Survival fraction (%)			LSD value
	Ciprofloxacin	Vancomycin	Cipro.+Vanco.	
1	70 ^a	40 ^c	50 ^b	6.024**
2	40 ^a	40 ^a	20 ^b	5.750**
3	40 ^a	40 ^a	28 ^b	5.821**
4	30 ^a	35 ^a	24 ^b	5.619**
5	20 ^b	35 ^a	4 ^c	6.840**
6	30 ^a	18 ^b	4 ^c	4.783**

Different letters in rows represents a significantly difference, *(P≤0.01), **(P≤0.01)

Conclusions and recommendations

Conclusions

1. Multidrug resistance *S. aureus* is widespread in nosocomial infections.
2. Insertion sequence IS256 was widespread in clinical isolates of *S. aureus*.
3. IS256 element affects antibiotic resistance genes in *S. aureus* isolates by direct transposition and inactivation of these genes.
4. Transposition of IS256 in genomic DNA of *S. aureus* can be induced under the stress of vancomycin and/or ciprofloxacin.
5. Transposition frequency of IS256 was highly increased after pretreatment of *S. aureus* with sub-inhibitory concentration of vancomycin compared with the pretreatment with sub-inhibitory concentration of ciprofloxacin or both of vancomycin and ciprofloxacin.
6. Survival fraction of *S. aureus* was highly decreased after pretreatment of *S. aureus* with sub-inhibitory concentration of vancomycin compared with the pretreatment with sub-inhibitory concentration of ciprofloxacin or both of vancomycin and ciprofloxacin.
7. Mutation rates due to transposition of IS256 were proven to play a significant role in reduces pathogenicity of *S. aureus*.

Recommendations

1. Detection the distribution of IS256 in other species of Staphylococcal isolates.
2. Studying the relationship between the presence of IS256 and biofilm formation and other virulence factors in *S. aureus* nosocomial pathogens.
3. Determining the association of IS256 with the aminoglycoside resistance transposon Tn4001.
4. Detection of other mobile elements in Staphylococcal isolates and other bacteria.
5. Detection of IS256 in a wide range of natural hosts including important human and animal pathogens.

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Appendix (2): Concentration of genomic DNA extracted from *S.aureus* isolates.

Isolate No.	Concentration (µg/ml)
1	37.6
2	42.2
3	50
4	32
5	35
6	31
7	43.6
8	53.2
9	42.8
10	72.7
11	87.6
12	90.5
13	107.9
14	95.51
15	146.7
16	187.5
17	13
18	7.4
19	14.9
20	9.5
21	4.5
22	40

Appendix (11): Statistical analysis for transposition frequency of IS256 in Penicillin G gene after pretreatment of *S.aureus* S1 with ciprofloxacin and/or vancomycin for six hour intervals.

المئوية للبكتريا المتبقية في كل ساعة بأخذ عينات من وسط النمو ونشرها على مستخلص القلب والدماغ المتصلب بمادة الأكار الحوي على مضادات الحياة التي تقاومها البكتريا كل على أفراد. وقد أظهرت النتائج أن تأثير كل من الفانكوميسين و السبروفلوكساسين والتأثير التآزري لكليهما كان له دور في فقدان العزلة البكتيرية *S.aureus* S1 لصفة المقاومة لجميع المضادات الحيوية التي كانت مقاومة لها وكان للفانكوميسين تأثيرا أكبر من تأثير السبروفلوكساسين والتأثير التآزري لكليهما على أساس ارتفاع تردد القفز وتعطيل جينات المقاومة لمضادات الحياة ، وانخفاض النسبة المئوية لبقاء البكتريا بعد تلك المعادلة.

الخلاصة

جمعت 109 عينة سريرية مختلفة شملت كل من الجروح والأنف والأذنين والسائل المفصلي والمهبل والدم فضلاً عن الى تصريف العمليات الجراحية و الأنسجة الرخوة في العمليات لمرضى رافدين والمراجعين لمستشفى بغداد التعليمي و مستشفى دار التمريض الخاص في محافظة بغداد للفترة من تموز/ 2019 الى أيلول/ 2019 . وقد تم الحصول على 109 عزلة بكتيرية من تلك العينات بعد التنمية على أوساط المكوني و آكار الدم، منها 47 عزلة موجبة لصبغة غرام و 62 عزلة سالبة لصبغة غرام، وقد شخصت 22 عزلة من العزلات البكتيرية الموجبة لصبغة غرام على أنها *Staphylococcus aureus* لقابليتها على تحلل الدم نوع بيتا و انزيم التجلط وتخمر المانيتول ، وموجبة لأختبار الكاتليز والسيثينييز واليوريز ، وقد تم تأكيد تشخيص العزلات باستخدام نظام الفايترك (vitek.2).

أختبرت حساسية عزلات بكتريا *S.aureus* لمضادات الحياة المختلفة ، وقد أشارت النتائج الى تباين تلك العزلات في نمط المقاومة ، حيث كانت جميع العزلات (100%) مقاومة للبنسيلين و الميثيسيلين ، فقد كانت نسبة العزلات المقاومة للأمبسيلين 77% و للأثرثرومايسين 53% ولكل من الأثرثرومايسين والترايميثوبرايم و الكلورامفينيكول 45% وللتترايسايكلين 36% ولكل من السبروفلوكساسين والدوكسيسايكلين واللكنومايسين 23% وللجنتامايسين 18% والريفامبسين 13% والفانكومايسين 4%.

أستخلص الدنا المجيني من عزلات بكتريا *S.aureus* بنقاوة وتركيز عاليين، تم بعدها التحري عن تعاقب الغرس IS256 باستخدام بادئات نكليوتيدية متخصصة ، وقد أشارت النتائج الى وجود هذا العنصر في 18 عزلة من العزلات البكتيرية، وقد كانت نسبة انتشار العنصر في البكتريا المعزولة من المجاري البولية و التجويف الأنفي والجروح و من مياه انابيب التصريف العمليات الجراحية و الانسجة الطرية للعمليات كانت 100 % والبكتريا المعزولة من الجهاز التنفسي 66.6% و نسبة انتشار العنصر من عينات سائل المفاصل 33% و لم تظهر في عينات الدم والمهبل.

أختبرت العزلة البكتيرية *S.aureus* S1 المتعددة المقاومة لمضادات الحياة لدراسة تأثير الشد بمضادي الفانكومايسين و/أو السبروفلوكساسين على تردد الانتقال والتطهير بالعنصر القافز IS256 . وتعطيل جينات المقاومة لمضادات الحياة وذلك بحضنها مع تحت التركيز المثبط الأدنى للفانكومايسين و/أو السبروفلوكساسين لمدة ستة ساعات ثم حساب تردد الانتقال والنسبة



وزارة التعليم العالي والبحث العلمي

جامعة ديالى

كلية التربية للعلوم الصرفة

التحري عن الطفرات الوراثية بالعنصر القافز IS256 في بكتريا

***Staphylococcus aureus* تحت تأثير الشد بمضادي**

الفانكوميسين و السبروفلوكساسين

رسالة مقدمة الى

مجلس كلية التربية للعلوم الصرفة/جامعة ديالى

وهي جزء من متطلبات نيل درجة الماجستير في علوم الحياة

من قبل

رشا كفاح حسن

بكالوريوس علوم حياة/كلية التربية للعلوم الصرفة ابن الهيثم/جامعة بغداد 2001

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