بسم الله الرحمن الرحيم

Sudan University of Science and Technology
College of Graduate Studies

Detection of mecA gene among Methicillin Resistant *Staphylococcus aureus* (MRSA) Isolated from raw cow milk at Ghebaish Locality Western Kordofan State –Sudan

الكشف عن جين ميك (أ) في المكورات الذهبية المعزولة من ألبان الأبقار بمحلية غبيش ولاية غرب كردفان ـ السودان

A Thesis Submitted as a requirement for the degree of PhD in Molecular biology

By:
Gasm Alseed Alnazeer Gasm Alseed
(B.Sc. Faculty of Science)
University of Juba (2010)
(M.Sc in Microbiology)
Sudan University of Science and Technology (2014)

Supervisor:

Muna Osman Elhaj
Associate professor, Central Veterinary Research Laboratory, Soba

Co Supervisor:

GalalEldin Alazhari Mohammed
Professor, College of Veterinary Medicine (SUST)

April – 2019

بسم الله الرحمن الرحيم

قال تعالى: (**وَإِنَّ لَكُمْ فِي الأَنْعَامِ لَعِبْرَةً نُّسْقِيكُم مِّمَّا فِي بُطُونِهِ مِن بَيْنِ فَرْثٍ وَدَمٍ لَّبَناً خَالِصاً سَآئِغاً لِلشَّارِبِينَ**).

سورة النحل (الآية 66)

Dedication

This work is dedicated to:

My father, mother, brothers and sisters, my wife and my daughters who gave me continuous support and encouragement to continue my studies.

Acknowledgement

First and foremost, my heartfelt thanks to Almighty Allah for giving me the strength, patience and willpower to complete this challenging task.

I would like to express my sincere appreciations and thanks to my supervisors Prof. Muna Osman Elhaj and Prof. Galal Eldin Alazhari Mohammed who encouraged me to carry out this research and provided me with advice and suggestions towards completion of this study successfully.

Special thanks are connived to Prof. Hisham Nouraldaiem Dr.Abdurahman Adam, Dr.Rawda Sherbek, Dr.Howaida Dako, Dr.Yassir Taha, Ustz. Altaj, Ustz. Ahmed Sughier, Ustz. Alsadig muhana, Ustz. Manal, Ustz. Ali Ismail, Ustz. Abdurahman Abdalhammed, Ustz. Mohammed Jamma, My wife. Marwa Salah Eldin and Ustz. Hawa Burma who provided valuable support.

Best gratitude is extended to the staff of the College of Veterinary Medicine for all the help I got from them.

Abstract

Staphylococcus aureus is one of the important pathogens involved in food related diseases and common community associated infections. This organism proliferates in food and causes food-borne illnesses. Milk serves as an ideal medium for growth of many microorganisms including *Staphylococcus aureus*.

This study was conducted to isolate *Staphylococcus aureus* from raw cow milk and studying its antibiotics susceptibility and was to detect the specific mecA gene in the *Staphylococcus aureus* isolates.

Two hundred raw cow milk samples were collected from four areas throughout Ghebaish locality under aseptic precautions and processed under standard bacteriological techniques. The samples were investigated for the presence of *Staphylococci*. The Baird Parker Agar and Mannitol salt Agar were used as selective media for isolation. The presumptive isolates were identified on the basis of their morphological, cultural and biochemical characteristics. The sensitivity pattern of *S. aureus* with different antimicrobial agents was evaluated by disk diffusion method. Forty five (22%) *Staphylococci* isolates were obtained and confirmed by biochemical tests, 33 (73%) isolates were *Staphylococcus aureus*, 12 (26%) isolates were *Staphylococcus epidermities*.

The results of sensitivity test revealed high resistance of antibiotics used. The two organisms were showed resistant to Streptomycin (0%), Vancomycin (78% - 100%), methicillin (18% - 25%) and penicillin (100%).

Bacterial DNA was extracted from each isolate using boiling method. PCR was used to detect mecA gene. The results indicated 23 isolates were positive to mecA gene (69%).The mecA gene sequence of *S. aureus* showed high similarities with those recorded in gene bank.

It is concluded that, *S. aureus* appeared to be a major frequent bacterial contaminant of raw cow milk reflecting potential public health threat, the result of antibiotics sensitivity testing showed variable response, multi-drug resistance, Bacterial DNA extracted from *Staphylococcus aureus* reflected appearance of mecA gene which was responsible of resistance to methicillin. and it is recommended that the full dose treatment need be observed and ensured as well as raising awareness of sanitary behavior.

الـــخـــلاصـــة

تعتبر الأمراض ذات الصلة ببكتريا المكورات العنقودية من أكثر الأمراض إنتشاراً في بقاع السودان المختلفة ومن أكثرها شيوعاً وتأثيراً على الصحة العامة تلك الأمراض والأعراض ذات الصلة ببكتريا المكورات العنقودية الذهبية.

وللتعرف على مدى إنتشار هذه الأمراض ومدى تجاوبها أو مقاومتها لبعض المضادات الحيوية تم إختيار محلية غبيش بولاية غرب كردفان لدراسة الوضع حيث جمعت مئتا عينة حليب من الأبقار وتم إخضاعها للفحص المختبري لدراسة وجود البكتريا العنقودية في هذه العينات و إجراء الإختبارات الكيموحيوية. لقد أظهرت خمسة وأربعون عينة وجود البكتريا العنقودية . تم التعرف على نوعين من البكتريا العنقودية حيث كانت ثلاثة وثلاثون عزلة من المكورات العنقودية الذهبية واثنتي عشرة عزلة من المكورات العنقودية البشروية.

تم إختبار حساسية العزلات لمجموعة من المضادات الحيوية لمعرفة نمط إستجابة العنقوديات للمضادات الحيوية , أظهرت النتائج إختلاف مقاومة البكتريا للمضادات الحيوية بإختلاف المضاد الحيوي ونوع البكتريا حيت كانت نسب المقاومة لأجناس البكتريا العنقودية الذهبية والعنقودية البشروية للمضادات الحيوية كما يلي:

(0%) للإستربتوميسين , للفانكوميسين (78% , 100%) , للميثيثيلين (18% , 25%) و (100%) من المعزولات كانت مقاومة للبنسلين.

تم إستخلاص الحمض النووي الديوكسي رايبوسي بتقنية الغليان من معزولات بكتريا المكورات العنقودية الذهبية وأستخدمت نظرية التفاعل التسلسلي المتعدد للتعرف على وجود الجين (ميك أ) وقد أظهرت النتائج وجود هذا الجين بنسبة 23 (69%) . تم إجراء دراسة التسلسل النووي لهذا الجين وبينت النتيجة التشابه العالي للتسلسل الذي تمت دراسته مع ذاك الموجود في بنك الجينات.

خلصت الدراسة والبحث إلى أن أجناس بكتريا المكورات شائعة في البيئة المحيطة بالحيوانات وقادرة على إحداث الأمراض المعدية مع إكتسابها مقاومة للمضادات الحيوية .. أوصت الدراسة بضرورة إستخدام المضادات الحيوية وفقاً للجرعة الكاملة والمقررة مع ضرورة رفع الوعي بأهمية إتباع المعاييرالصحية للحد من إنتشار الأمراض.

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CHAPTER ONE
INTRODUCTION

1.1 General background

The interaction between microorganisms, plant and animals are natural and constant. The ecological role of microorganisms and their importance in all the geochemical cycles in nature is documented. Since human food supply consists of plant and animal or products derived from them, it's understandable that the food supply can contain microorganisms. In most cases microorganisms use human food as a source of nutrients for their own growth, this is of course can result in a deterioration of the food by the increasing in normal activity of microorganisms and its consequences (Frazier, 1992).

During most of the 20th century *Staphylococcus aureus* has been recognized as a major life-threatening pathogen. Although the clinical use of penicillin (1940s) initially led to a dramatic reduction in mortality from *S. aureus* infections, penicillin resistant strains soon emerged (Cookson *et al*., 2003). Concern was not only confined to the increase mortality rate among patients, but also to the spread of skin infections amongst health-care workers. Furthermore, the ability of *S. aureus* to acquire multiple antibiotic resistances further reduced the effectiveness of chemotherapy for the treatment of staphylococcal infections. Subsequently, the development of new antibiotics, such as methicillin in 1960, led to the expectation that infections caused by this bacterium would be treatable. However, *S. aureus* rapidly developed resistance to methicillin and the first methicillin-resistant strains of *S. aureus* (MRSA) were reported in the UK in 1961. Since then, the frequency of isolation of MRSA strains has increased significantly every year worldwide (Jevons, 1961; Grundmann *et al*., 2006).

It is worth noting that MRSA is no more virulent than other *S. aureus* strains, but its infections are significantly more difficult to treat due to their resistance to front-line antibiotics (Rozgonyi *et al*., 2007). The glycopeptides have emerged as the most effective anti-MRSA agents, although the emergence of MRSA strains with reduced sensitivity to vancomycin has led to increasing concerns about the use of this antibiotic as the last resort for the treatment of MRSA infections (Hiramatsu *et al*., 1997).

1.2 Problem justification and hypothesis

This study is intended to examine the spread of methicillin resistance of the bacterium *S. aureus* isolated from raw cow milk at Ghebaish Locality.

1.3 Objectives

1.3.1 The overall objectives:

(i) To detect the presence of methicillin resistant Staphylococci.

1.3.2 Specific objectives:

(i) To isolate and characterize *Staphylococcus aureus* from raw milk samples.

(ii) To study the antibiotic resistance and sensitivity pattern of the isolates.

(iii) To detect the specific mecA gene in the isolates those is responsible for the methicillin resistance and do sequencing of this gene.

CHAPTER TWO
LITERATURE REVIEW

2.1 Antimicrobial agents

The study of antimicrobial agents embraces not only antibacterial compound but also antiviral, antifungal, antiprotozoal and even antihelminthic agents. Antibiotics are naturally occurring microbial products and/or synthetic compounds such as sulphanomides quiolones nitrofuras and imidazoles. They should strictly be referred to as chemotherapeutic agents. However, same antibiotics can be manufactured synthetically while others are the products of chemical manipulation of naturally accruing compounds.

Antibiotics are defined as low molecular weight metabolite which kill or inhibit the growth of susceptible bacteria (Queen, *et al* .2002).

2.1.1 Establishment of bacteria

A bacterium organism protects itself from enemies in various ways. It may produce metabolic waste products, which change the conditions in the medium, such as pH, osmotic pressure and surface tension making the environment unfavorable to the growth of less tolerant organisms. It may elaborate specific toxic substances, which interfere with the metabolism of other organisms to an extent that they are either be killed or prevented from multiplying. These specific toxic substances are called antibiotics (Queen, *et al* .2002).

Heritage, *et al* (1996) defined antibiotics as a substance that was produced by microorganisms that is in very low concentration inhibits or kills the growth of other microorganisms.

2.1.2History and discovery of antibiotics

Pasteur and Joubert (1877) noted that a culture of Anthrax bacilli was killed if it was contaminated by common air-borne organisms. They realized that such phenomenon might well have therapeutic possibilities (Thomas, 1993).

On the agar plate culture of *Staphylococcus aureus*, Fleming (1929) obtained a mold contaminated that culture and produced a green pigment which prevented bacterial growth some distance around it. He cultivated the mold in a liquid medium and found that a filtrate of the culture had the power, even when greatly diluted to prevent growth of a number of Gram-positive pathogenic bacteria. Since the mold proved to be species of penicillium he named the antibiotic penicillin; subsequently shown to be more effective against a number of infections than the sulpha drugs. The antibiotic was found to be so nontoxic that amounts beyond the effective curative dose could be safely administrated (Salle, 1971).

In 1940, Chain, *et al* at Oxford University succeeded in obtaining penicillin preparations of high antibacterial activity. These preparations were highly effective in controlling experimental infections in animals. The remarkable clinical potentialities of penicillin were quickly demonstrated (Thomas, 1993).

Dubos (1939) isolated from the soil spore-producing bacillus that was capable of destroying living Gram-positive cocci, the organism was found to be *Bacillus brevis* a large Gram-positive rod similar to *Bacillus subtilis.* He named the antibiotic gramicidin (Salle, 1971).

Most antibiotics are produced by Streptomyces. A few are produced by *Bacillus* spp., actinomycetes and fungi. Several antibiotics are semi-synthetic

in origin e.g. cloxacillin and ampicillin are prepared from naturally produced 6-aminopenicillanic acid (Thomas, 1993).

Thousands of antibiotics have been isolated and studied. Some are useful clinically; others are not satisfactory for clinical application but more useful for other purposes (Salle, 1971).

The field of antibiotics offers unlimited possibilities in medicine. Powerful antibiotics, such as penicillin, have proven to be of tremendous importance that an ever-increasing search is going on in the hope that agents superior to those now in use might be isolated (Salle, 1971).

2.1.3 Classification and mechanism of action

Since the antibiotics were first discovered in the 1920s, much knowledge has been acquired on their mode of action and the significance of this action on their relative merits in the therapy of man and animals.

Antibacterial agents can be divided into four groups based on this effect of synthesis of nucleic acid, protein, the formation of cell wall and permeability of cell membrane.

2.1.3.1 Inhibitors of bacterial cell wall synthesis

Since most bacteria possess a rigid cell wall that is lacking in mammalian cells. This structure is a prime target for agents that exhibit selective toxicity and ability to inhibit or destroy the bacterial species without harming the host. However, the bacterial cell wall can also prevent access of agents that would otherwise be effective. Thus, the complex outer envelope of gram- negative bacteria is impermeable to large hydrophilic molecules which, may be prevented from reaching and other wise susceptible target.

Inhibitors of bacterial cell wall synthesis act on the formation of peptidoglycan layer. Bacteria that lack peptidoglycan such as *mycoplasmas* are resistant to this agent (Wax, 2008).

2.1.3.1.1 β-lactam agents

2.1.3.1.1.1 Penicillins

Benzyl penicillin exhibits unrivalled activity against *Staphylococci*, *neisseriae*, *spirochetes*, and certain other organisms. However, resistance, normally due to the production of β-lactamase has undermined its activity against staphylococci and to lesser extent gonococci. Bacteria that exhibit reduced susceptibility to penicillin by anon-enzymic mechanism are also encountered (John, 2002).

Among the most important penicillins are:

- Phenoxg methyl penicillin which can be given orally.
- Procaine penicillin – along acting of benzyl penicillin.
- Flucl0xacillin- an anti staphylococcal compound.
- Ampicillin and amoxicillin –which is active against some enterobacteria.
- Ticarcillin, azlocillin and piperacillin which are active against *pseudomonas aeruginosa* (John, 2002).

2.1.3.1.1.2 Cephalosporins

Cephalosporins are close relatives of the penicillins but β- lactam ring is fused to a six- member dihydrothiazine ring rather than the five member thiazolidine ring of penicillin-cephalosporin carry an a cetoxymethyl group on the extra carbon. This can be removed by hepatic enzymes to yield a less active derivative but is doubtful when this has any therapeutic significance. Other cephalosporins (cephamandol, cefoperozone) possess amethyltetrazole

subsistent. Use of compounds with this feature has been associated with hypoprothrombinaemia and bleeding in some patients.

Cephalosporins are generally stable to staphylococcal penicillinase but they lack activity against entercocci. They exhibit a broader spectrum that most Penicillin is less prone to cause hyper sensitivity reaction, among the most important are:

- Cefaloxin and cefaclor which can be given orally.
- Cefuroxin and cefaxitin.
- Cafolaxime and ceftriaxone combine B-lactam stability with intrinsic activity.
- Ceftazidime and cefpirome. (John, 2002).

2.1.3.1.1.3 Other β-lactam agents

Various agents with diverse properties share the structural feature of the β-lactam ring with penicillin and cephalosporins.

- Mona bactams – eg aztreonam.
- Carbapenemens – eg imipenem and meropenem.
- Oxa- cephems- eg latamoxef.
- The clavam, clavulanic acid,
- The sulphones, sulbactam and tazobactam.

2.1.3.1.1 Other inhibitors of bacterial cell- wall synthesis

- Fostomysin is a naturally occurring antibiotic with a simple phoshponic acid structure. It exhibits a fairly broad spectrum, notably against gram-negative bacilli and is mainly used for the treatment of urinary tract infection.
- Bacitracin is active against gram- positive bacteria but highly toxic for systemic use it's found in many topical preparations.

- Cycloserine is an analogue of D- alanine used only as a second line agent in infections with multi resistant strains of *Mycobacterium tuberculosis.*
- Isoniazid and some other compound used in tuberculosis probably act by interfering with formation of the mycolic acid of the *mycobacterium* cell wall (John, 2002).

2.1.3.2 Inhibitors of bacterial protein synthesis

Antibiotic classes that act by inhibiting protein synthesis include aminoglycosides e.g. gentamycin, tobramycin, kanamycin, streptomycin, tetracycline and chloramphenicol macrolides, e.g. erythromycin, azithromycin, and lincosamides e.g. clindamycin. Most are true antibiotics produced by Stryptomyces species or other soil organisms (John, 2002).

2.1.3.2 .1 Tetracyclines

These are broad-spectrum agents with important activity against *Chlamydia, rickettsia, mycoplasmas* and surprisingly, Malaria parasites, as well as most conventional gram- positive and gram negative bacteria. They prevent binding of amino- acyl transfer RNA to the Ribosome and inhibit but do not kill susceptible bacteria. Resistance has limited the value of tetracyclines against many gram positive and gram- negative bacteria, but not against *rickettsia*.

2.1.3.2 .2 Chloramphenicol

This compound and the related thiamphenicol also possess a very broad antibacterial spectrum, they act by blocking the growth of the peptide chain. Use of it has been limited to typhoid fever, meningitis and a few other clinical indications because of the occurrence of a rare but fatal side effect a plastic anaemia (John, 2002).

2.1.3.2 .3 Aminoglycolside (e.g. Streptomycin)

It is the first antibiotic to be discovered by random screening of soil organisms. It is predominantly active against enterobacteria and *M. tuberculosis*. It has no useful activity against streptococci and intracellular bacteria. They inhibit formation of the ribosomal initiation complex and cause misreading of messenger RNA. Resistant may arise from ribosomal changes or alterations in drug uptake (Jaggi, 2003).

2.1.3.2 .4 Macrolides

They are antibiotics in which a large macrocyclic lactone ring is substituted with some unusual sugars. They act by interfering with the translocation of RNA on the bacterial ribosome. They are mainly used as anti-staphylococcal and anti-streptococcal agents. Though they have wider applications, they have no useful activity against enteric gram- negative bacilli. The original macrolide erythromycin is unstable in gastric acid and is usually administrated orally as the stearate salt or as an esterified poor – drug (Kayser, 2005).

2.1.3.3 Inhibitor of nucleic synthesis

A number of important antibacterial agent act directly or indirectly on DNA or RNA synthesis.

2.1.3.3.1 Sulphonamides and diaminopyrimidines

These agents affect DNA synthesis because of their role in folic acid metabolism. Sulphonamides are analogues of para-aminobenzoic acid. They prevent the condensation of this compound with dihydro pteridine during formation of folic acid. Sulphonamides and diaminopyrimidines thus act at sequential stage of the same metabolic pathway and interact synergically. But in bacterial infection trimethoprim is generally sufficiently effective and less

toxic, when used alone. Sulphonamides are broad-spectrum antibacterial agents but resistance is common and the group also suffers from problems of toxicity (John, 2002).

2.1.3.3.2 Quinolones

This drugs act on the g subunit of DNA gyrase. Their properties allow them to be roughly categorized into three groups such as Nalidixic acid and its early congener's narrow- spectrum agent active only against gram- negative bacteria, Later quinolones such as ciprofloxacin and afloxacin which are 6-Fluoro derivatives, display much enhanced activity and a boarder spectrum, although activity against some-positive cocci, notably *Streptococcus pneumonia* is unreliable (John, 2002).

2.1.3.3.3 Nitroimidazoles

These Azoles derivatives feature prominently among antifungal antiprotozoal and antihelmenthic agents. Those that exhibit antibacterial activity are 5- nitroimidazoles. At low redox values they are reduced to a short –lived intermediate that causes strand breakage in DNA. They are active only against anaerobic bacteria and anaerobic protozoa. Among the most important Nitroimidazoles are: Nitro furans which used exclusively in urinary tract infection, Novobiocin which acts on the B subunit of DNA gyrase and Rifampycins which act by inhibiting transcription of RNA from DNA (John, 2002).

2.2 Antimicrobial Resistance

Most of the antimicrobial resistance, which is now making it difficult to treat some infectious diseases, is due to the extensive use and misuse of antimicrobial drugs which have favored the emergence and survival of resistance strains of microorganism.

Drugs resistant strains are common among *Staphylococci*, *Gonococci*, *Enterococci*, gram negative bacteria *salmonella, shigella, kelebsilla*, and *pseudomonas* and *Mycobacterium tuberculosis*.

Bacteria become resistant to antimicrobial agents by a number of mechanisms.

The commonest being:

- Production of enzymes which inactivate or modify antibiotic.
- Occurrence of changes in the bacterial cell membrane which prevent uptake of antimicrobial drug.
- Modification of the target so that it no longer interacts with the antimicrobial drug.
- Development of metabolic pathways by bacteria which enable the site of antimicrobial action to be by passed.

The fundamental cause of antibiotic resistance is a strong selective pressure favoring resistant bacteria in the presence of the drugs. Resistant bacteria survive an otherwise lethal onslaught of an antibiotic, which places a premium on developing resistance. Since bacteria reproduce so quickly, a single resistant bacterium can become millions of resistant bacteria in hours. (Patrick, 2007).

To acquire these new properties bacteria must undergo a genetic change. Such a genetic change may occur by mutation or by the acquisition of new genetic material. New genetic material is acquired by the transfer of resistance genes from one bacterium to another. Some plasmids encode for resistance several antibiotic can be transferred between bacterial species e.g. from *Escherichia coli* to *Shigella dysenteriae* (Cheesbrough, 2000).

2.3 Antibiotic sensitivity tests

The aim of bacterial culture is to diagnose the pathogenic bacteria which cause the infection. Nevertheless, diagnosis is not enough to cure the infection. For that the appropriate antibiotics should be given. To find out which antibiotic kills a particular pathogenic bacterium, an antibiotic sensitivity test which determines the sensitivity or resistance pattern of bacteria to the various antibiotics should be performed (Goldman, 2008).

The minimal inhibitory concentration (MIC) is the lowest concentration of drug that inhibits the growth of the bacteria isolated from the patient. In this test though, it isn't known if the inhibited bacteria have been killed or just stopped growing (Barazandeh, 2008).

2.3 .1 Definition

An antibiotic sensitivity test is an in vitro test to determine the sensitivity or resistance of a particular bacterium to the group of antibiotics that we have tested. In other words it finds out which antibiotic can kill the tested bacteria and cure the infection (Goldman, 2008).

2.3 .2 Aim of antibiotic sensitivity test

The aim for antibiotic sensitivity test is to prescribe antibiotic which can kill the bacteria and cure the infection. The appropriate antibiotic should produce at the site of infection a concentration high enough to kill or inhibit the growth of the pathogen without having a significant toxic effect. The different strains of a few pathogen species have constant sensitivity characters like *Streptococcus pyogenes* but strains of most pathogenic character have to be tested each time (Bhatia, 2004).

2.3 .3 Type of sensitivity test

Antibiotic sensitivity test are of two types which are diffusion test and dilution test.

2.3 .3.1 Diffusion test

It is the most widely used test for routine sensitivity testing of isolates from patients. In the diffusion test drug is allowed to diffuse through a solid medium so that a gradient is established the concentration being highest near the site of application of the drug and decreasing with distance.

The tested bacterium is seeded on the medium and its sensitivity to the drug is determined by inhibition of its growth. The drug is incorporated into discs and applied on the surface of the medium two type of discs diffusion test are used which are Stokes method and Kirby –Bauer's method (Jaggi, 2003).

2.3 .3.2 Dilution test

In this method, serial dilutions of antibiotic solutions are made and inoculated with overnight broth culture of the test strain. The minimum inhibitory concentration of the antibiotic that is the minimum concentration of the antibiotic which can kill the microorganisms is calculated. The test can be carried out in an agar or both. Dilution test is not used routinely for determination of antibiotic sensitivity test or resistance but only for standardization of procedures or for regulation of therapeutic dose accurately (Arrabty, 2008).

2.4 The Emergence of Resistance

It is irrefutable that antibiotic use promotes resistance development. However, some difficulty arises when trying to quantify the specific contribution of antibiotic use to resistance as its now evident that social economic and genetic factors also, impact the establishment, maintenance

spread of resistance. It is not only the amount of antibiotic used which selects for resistance, but also the duration and dosage used. For instance, low- doses over long periods were more selective for the carriage of penicillin resistance. As more drugs are used in a particular environment, there are few susceptible bacteria remaining to compete with the resistance organisms.

The misuse of antibiotics is reflected by the rise of bacterial resistance to more than one antibiotic. Multi-drug resistance is found in virtually all commensal and disease causing bacteria. Moreover the misuse of antibiotics especially those with broad- spectrum activity; demonstrate the emergence of new pathogens such as multi-drug resistant Acinetobactor species. (Chuanchuen, 2001).

2.5 Reversal and Stability of Resistance

It is well known that removal of an antibiotic from general consumption will not necessarily result in a decline of resistant strains and the return of susceptible strains. Epidemiological data show a high level of resistance among the commensal bacteria in healthy individuals and in the environment. These observations could be explained by the accumulation of antibiotic in the environment or by accumulation of food products (Chuanchuen, 2001). A main lesson from the last six decades of antibiotic use is that, bacterial resistance is evolutionary. Better understanding of the problem can be achieved by well designed surveillance programs and epidemiological studies which define the molecule and genetic mechanisms behind antibiotic resistance. This knowledge can be used to develop new drugs and effective alternative clinical approaches to treat bacterial disease. More important instituting strategy is to encourage and maintain a return of susceptible strains to our environment (Chuanchuen, 2001). Some bacteria produce their own antibiotic to protect themselves against other micro organisms. Of course, a bacterium will be resistant to its

own antibiotic. However, sometimes the DNA that gives that bacterium resistance to its own antibiotic can be transferred to a bacterium of another species. Genetic transfer may be induced by the bacteria involved that is the source and the destination bacteria. One model suggests that when DNA resistance plasmid released by one bacterium is accepted by a different species. . The recipient may be stimulated to release its own plasmid. The process is known as retro- transfer (Choemaker, 2001).

Resistant genes occur not only in bacteria that cause a disease, but also in commensal bacteria (those living within the same environment: soil, water and digestive tract, benefiting from each other). Eating meat or milk from animals that have been exposed to antibiotics brings the antibiotic and or resistant bacteria in contact with bacteria in the digestive tract. The interaction between bacteria can then allow for antibiotic resistance to the bacteria in your intestines (Layer, 2006).

2.6 *Staphylococcus aureus*

Pasteur and Koch were the first to discover and culture *Staphylococcus* but without carrying out any detailed studies. Ogston in 1881 and Rosenbach in 1884 performed the first detailed studies on Staphylococcus. Ogston gave Staphylococcus its genus name when he saw these bacteria formed grape-like clusters in human pus. Thereafter, Rosenbach isolated *Staphylococcus aureus* in pure culture, adding its species name aureus (=gold). Rosenbach also reported that *S. aureus* caused some wound infections whereas *S. epidermities* lived on the skin as a colonizer (Cookson *et al*., 2003).

2.6 .1 General description

Staphylococcus. aureus is a member of the *Staphylococcaceae* family (Firmicutes), Gram-positive facultative anaerobes that exhibit a coccal morphology, and are non-motile and non-sporulating. Its cells are 0.5-1.5μm in diameter and forms grape-like clusters. It is catalase and coagulase positive and some strains produce capsules. On blood agar, colonies of S. aureus appear golden (caused by staphyloxanthin, a membrane-bound carotenoid) surrounded by haemolytic zones (Wieland *et al*., 1994). In addition, *S. aureus* strains secrete various extracellular virulence factors such as coagulase and enterotoxins. Despite the fact that *S. aureus* is a normal component of the microbiota of the nasal passages, skin and mucous membranes of humans and animals, it is the cause of several important diseases (Brown *et al*., 2005; Humphreys, 2002; Bergey *et al*., 1994; Marshall and Wilmoth, 1981).

2.6 .2 Cell envelope

The cell walls of Gram-positive bacteria differ from those of Gram-negative bacteria. The *S. aureus* cell wall is a multilayered structure (20-40 nm), comprising a copolymer of peptidoglycan and teichoic acid. Peptidoglycan represents ~50% of cell wall by weight and is a polymer consisting of repeating units of sugars of 1, 4 β-linked N-acetylglucosamine and N-acetylmuramic acid. The glycan chains are cross-linked by tetrapeptide chains (L-alanine, D-glutamine, L-lysine, and D-alanine) bound to N-acetylmuramic acid and a pentaglycine bridge that links tetrapeptide chains on adjacent glycan strands. This cross-linking is catalysed by the transpeptidase (TPases) activities of penicillin banding proteins (PBPs) (Stapleton and Taylor, 2002; Lowy, 1998). Another major cell wall component is teichoic acid, an anionic polymer that consists of repeating alditol phosphate groups covalently linked to the muramic acid residue of peptidoglycan.

In some *S. aureus* strains the cell wall is coated with an extracellular polysaccharide capsule. More than 90% of clinical strains of *S. aureus* can produce polysaccharide capsules that are usually thin (<0.05μm) and consist of aminouronic acid sugars and fucosamine (Wright and Novick, 2003; Seaman *et al.*, 2004). Serotyping has identified 11 serotypes, with serotypes 5 and 8 being responsible for about 75% of human infections. Most MRSA strains are serotype 5 (Lowy, 1998).

2.6 .3 *Staphylococcus aureus* genome

The genome of *S. aureus* has been extensively studied and currently 18 staphylococcal genomes have been completely sequenced (nine are MRSA (four of which are vancomycin-intermediate resistant *S. aureus* (VISA)) and nine are MSSA) and a further 28 genomes are currently being sequenced (some of them in the finishing stage). This has provided an extraordinary glimpse into this so-called "super genome" and has led to a significant increase in our knowledge of the structure and functioning of *S. aureus*.

The staphylococcal genome is a closed circular molecule of double-stranded (ds) DNA of between 2.7 – 3.0 Mbp in length, encoding between 2509 to 2892 open reading frames (ORFs). It is composed of two domains called the core genome and the accessory genome. The core genome is inherited from the ancestors and is highly conserved in all staphylococcal species (Shittu *et al.*, 2007). The core comprises approximately 75% of *S. aureus* chromosome and it is highly conserved between strains (Lindsay and Holden, 2004). It contains all the housekeeping genes that are required for essential cell functions, such as DNA replication, proteins synthesis and core metabolism etc. The genome includes a wide variety of genes encode functions that contribute to virulence, such as toxins, exoenzymes and capsule biosynthetic cluster. The battery of virulence genes is highly strain variable (Shittu *et al.*, 2007).

The second domain, comprising of ~25% of the staphylococcal genome, is the accessory genome. The accessory genome mostly consists of mobile genetic elements (MGEs) that encode variety of non-essential components required for growth and survival. These elements include pathogenicity islands, bacteriophages, chromosomal cassettes, genomic islands, plasmids and

transposons. Many of these elements encode virulence and antibiotic-resistance determinants that are transferred horizontally between strains of clinical importance (Lindsay and Holden, 2004; Shittu *et al.*, 2007). There is preliminary evidence to indicate that some of MGEs move among isolates at a high frequency whereas others move if only rarely. Although, the transfer mechanisms are not fully understood, valuable information has been obtained about how *S. aureus* causes infection from the characterization and identification of MGEs. Several studies suggest that certain virulence and antibiotic-resistance determinants are associated with particular strains and types of infection (Lindsay and Holden, 2004).

2.6 .4 Pathogenesis and virulence factors

Although one-third of mankind is asymptotically colonized by *S. aureus*, most are asymptomatic. However, under the right conditions this versatile organism is able to cause a wide range of infections (Archer, 1998). The nature of those infections depends on several factors, such as the pathogenic characteristics of the strain, host susceptibility and the route of entry into the host. In addition, *S. aureus* infections vary in seriousness and outcome from minor skin infections such as superficial lesions (furuncles, boils) and wound infections, to life-threatening infections such as septicaemia, ostemyelitis, acute endocarditis and necrotising pneumonia (Lowy, 1998; Keane, 1992).

The ability of *S. aureus* to colonize the host and the capacity of this bacterium to exchange and obtain genetic information reflect its success as a versatile pathogen. This contributes to the fact that S. *aureus* strains can express a variety of virulence factors that play key roles in their spread and proliferation in its human and animal hosts. It is noteworthy that a virulence

factor can be multifunctional in pathogenesis and multiple virulence factors may play the same function (Decker, 2008; Gordon and Lowy, 2008)

The virulence factors of *S. aureus* can be divided mainly into three clusters: cell surface-associated factors including cell surface-bound proteins (the MSCRAMMs [microbial surface components recognizing adhesive matrix molecules]) and other surface-components (polysaccharide capsule & cell wall peptidoglycan); extracellular enzymes including coagulase and staphylokinase, and toxins such as haemolysins, leukocidins and toxic shock syndrome toxin (TSST) (Wright and Novick, 2003**;** Novick, 2006).

Most *S. aureus* strains produce a capsular polysaccharide that contributes in virulence of *S. aureus*. The capsule plays vital role in the adhesion of bacterial cells to each other and to host tissues and medical equipment. In addition, the capsule inhibits phagocytosis and restricts the ability of antibiotics to reach the bacterial cell surface. Moreover, other cell wall components (*e.g.* peptidoglycan and lipoteichoic acid) have a role in *S. aureus* pathogenicity: peptidoglycan, for example, has endotoxin activity that stimulates macrophages to release cytokines (Lowy, 1998). Lipoteichoic acid (LTA) is thought to play an important role in septic shock and other detrimental host responses (Ferry *et al.*, 2005; Fournier and Philpott, 2005; Novick, 2006).

Staphylococcal surface proteins contribute to the spread and virulence of *S. aureus*. These proteins, which include protein A, fibronectin binding proteins, fibrinogen binding proteins and collagen binding proteins are known MSCRAMMs. These proteins perform a wide spectrum of functions and many recent studies have shown that these surface proteins play essential roles in the ability of these bacteria to colonize host tissues (Wright and Novick, 2003; Lowy.1998) by promoting adhesion to the surfaces of host cells and tissues.

MSCRAMMs are covalently attached to the cell wall by sortase enzymes that recognize and cleave the Leu-Pro-X-Thr-Gly (LPXTG) motif (Schneewind *et al*., 1995; Mazmanian *et al.*, 1999). MSCRAMMs can also help the organism to evade the innate immune system and increase iron uptake (Foster, 2005). It appears that MSCRAMMs play a key role in the colonization of prosthetic-devices and endovascular infections by, for example, assisting in the formation of biofilms. Moreover, MSCRAMM protein A is a good example of immune evasion factor that binds to the Fc portion of immunoglobulin and subsequently prevents opsonisation (Gordon and Lowy, 2008).

During infection, *S. aureus* secretes a wide variety of extracellular enzymes and toxins that contribute either directly or indirectly to pathogenesis. Most, if not all *S. aureus* strains produce haemolysins, coagulases, nucleases, protease, lipases, hyaluronidase and collegenases (van Belkum, 2007). Coagulase is produced exclusively, among the staphylococci, by almost all strains of *S. aureus* (with the exception of a few strains of *S. intermedius*). Coagulase reacts with blood-prothrombin to form a staphylothrombin complex that can convert fibrinogene to fibrin. Although, coagulase covers the bacterium with fibrin to reduce its susceptibility to host defences, its contribution to pathogenesis is not clear (Lowy, 1998; Wright and Novick, 2003). In contrast, staphylokinase disassembles fibrin by its interaction with plasminogen to form plasmin (a serine protease) that, by virtue of its ability to digest fibrin clots, allows staphylococci to spread into deep host tissue (Bokarewa *et al.*, 2006). Other enzymes secreted by *S. aureus* include proteases such as serine protease V8 and lipases that breakdown the bactericidal fatty acids produced by infected cells. Many *S. aureus* strains produce hyaluronidase that can degrade the hyaluronic acid, a component of the extracellular matrix of host tissues, and enables the bacterium to spread

through host tissues. Hyaluronidase is commonly referred as the spreading factor (Hynes and Walton, 2000; Wright and Novick, 2003). The DNA and RNA of host cells are degraded by nucleases. These enzymes almost produced by all staphylococcal strains and cleave the phosphodiester bonds of both single and double stranded DNA and RNA (Wright and Novick, 2003). Finally, *S. aureus* strains can produce β-lactamases that are responsible for the resistance of β-lactam antibiotics (Lowy, 1998). *S. aureus* produces many cytotoxins that are grouped according to their mode of action (Lowy, 1998). Haemolysins (alpha, beta, gamma and delta) are porin-like toxins that lyse a variety of host cells such as red blood cells (erythrocytes) and platelets. Another important pore-forming toxin is Panton-Valentin leukocidin (PVL) that structurally resembles alpha toxin. This cytotoxin mostly forms its pores in the outer membrane of mitochondria and kills neutrophils and macrophages. Fortunately, 5% of *S. aureus* strains produces PVL and its production is associated with cutaneous infections and more recently with necrotizing pneumonia (Wright and Novick, 2003, van Belkum, 2007; Decker, 2008). *S. aureus* also secretes enterotoxins (A-E, G-I), toxic shock syndrome toxin-1 (TSST-1) and exfoliative toxin (A, B). These toxins are responsible for diseases such as food poisoning, toxic shock syndrome and exfoliative dermatitis (scalded skin syndrome) (Wright and Novick, 2003). Enterotoxin and TSST represent pyrogenic toxin superantigens (PTSgs) and both play a role in derailing and over-stimulating components of the immune response (van Belkum, 2007). Staphylococcal virulence factors are precisely regulated by a quorum-sensing system based on the accessory gene regulator (*agr*). In order to initiate colonization of tissue sites, MSCRAMMs are expressed during exponential growth phase whereas secreted enzymes and toxins are produced

later during stationary phase so as to aid the spread of the organism (Gordon and Lowy, 2008).

2.6 .5 Antibiotic resistance

Since the late 1940s, antibiotics resistance associated with hospital-acquired infections has emerged as a major problem worldwide. In this respect it is important to distinguish between bacterial strains acquired in hospitals from those acquired in the community, since the former are more likely to exhibit a greater range of antibiotic resistances than the latter (Zhang *et al.*, 2006). Penicillin resistant strains of *S. aureus* were first detected in the 1940s, shortly after this antibiotic was introduced into clinical practice (Chambers and Deleo, 2009). This resistance was due to the production of β-lactamases that inactivate the antibiotic by hydrolyzing the β-lactam ring. The main mechanism of penicillin resistance is via the *blaZ*-encodes β-lactamases (Rohrer *et al.*, 2003).

Currently, 90% of clinical *S. aureus* isolates are penicillin-resistant and resistance is particularly prevalent in hospitals. Consequently, new generations of penicillins have been developed. The introduction of methicillin in the 1960s was followed rapidly by the emergence of methicillin-resistant isolates. Methicillin-resistance is not mediated by β-lactamases but by the production of an altered penicillin binding protein (PBP2a) that has a low affinity for β-lactam antibiotics (Johnson, 1998). The 1960s also saw the development of non-β-lactam antibiotics such as chloramphenicol, erythromycin, streptomycin and tetracycline. Although initially effective against *S. aureus,* nevertheless resistance against them developed rapidly. By 1976, resistance to kanamycin and gentamicin was reported and, by the early of 1980s, multiresistant *S.*

aureus strains were reported, responsible for nosocomial outbreaks in many countries (Thomas and Archer, 1989).

Vancomycin and teicoplanin are glycopeptide antibiotics that have been the first choice for the treatment of serious nosocomial MRSA infections for the last fifteen years. Since the emergence of vancomycin-resistant enterococci, fully vancomycin resistant strains of *S. aureus* have been expected. The first vancomycin-intermediate resistant *S. aureus* (VISA) was reported in Japan in 1996. This isolate, designated Mu50, had an MIC for vancomycin of 8μg/ml (Hiramatsu *et al*., 1997). Thereafter, clinical cases from which VISA were isolated were reported in 1997 in USA, then France and later in the UK. All these reported strains were vancomycin-intermediate *S. aureus* (VISA) and were called glycopeptide-intermediate *S. aureus* (GISA) (Cui and Hiramatsu, 2003). The National Committee for Clinical Laboratory Standard (NCCLS) suggested the definition for vancomycin-resistant terms to avoid any confusion. *S. aureus* strains that have an minimum inhibitory concentration (MIC) of 4 μg/ml or below of vancomycin are defined as vancomycin-sensitive *S. aureus* (VSSA), those that have an MIC between 8-16 μg/ml as vancomycin-intermediate *S. aureus* (VISA), and those that have an MIC of 32 μg/ml or more as vancomycin-resistant *S. aureus* (VRSA) (Srinivasan *et al.*, 2002). Although, VISA strains have been isolated, they are rare in most countries. However, 2002 witnessed the reporting of the first fully vancomycin-resistant *S. aureus* (VRSA) isolate from a renal dialysis patient in Michigan, USA (Bartley, 2002). This strain had an MIC of >128 μg/ml and carried the vancomycin-resistance gene, *vanA* (Chang *et al.*, 2003; Aires de Sousa and Lencaster, 2004). Vancomycin acts on cell-wall peptidoglycan by binding to the carboxyl-terminal D-alanyl-D-alanine residues of peptidoglycan precursor, preventing PBPs from accessing to their natural substrate. Vancomycin has to

penetrate about 20 layers of peptidoglycan to reach the cytoplasmic membrane where the transglycosylation and transpeptidation reactions of PBPs take place. In the VISA strains Mu50 and Mu3, vancomycin-resistance is associated with a thickening of the cell wall caused by excessive activation of peptidoglycan synthesis. The vancomycin molecules are trapped by high levels of free D-alanyl-D-alanine residues due to the much-reduced levels of cross-linking (Aires de Sousa and Lencaster, 2004).

In contrast, full vancomycin-resistance in *S. aureus* (VRSA) is encoded by three determinants, namely VanA, VanB and VanD, normally associated with vancomycin-resistant enterococci (VRE). Resistance is brought about by the replacement of the native D-alanyl-D-alanine (D-Ala-D-Ala) residue of the cross-linking wall peptide with a D-alanyl-D-lactate (D-Ala-D-Lac) residue, which has a very low affinity to glycopeptides. It is noteworthy that the VanA induces high level of resistance and also confers resistance to teicoplanin. The *vanA* gene cluster is carried on a large resistance plasmid (Berger-Bachi, 2002).

2.6 .6 Methicillin-resistant *Staphylococcus aureus* (MRSA)

2.6 .6.1 Emerging methicillin resistance

Methicillin-resistant *S. aureus* were first discovered in the UK in 1961 by Jevons (1961). In this study, three MRSA strains were reported among 5,440 isolates and MRSA infections were initially confined to hospital patients. The first nosocomial epidemic of MRSA was reported in 1963, when an MRSA strain was isolated from an infant who was treated unsuccessfully with penicillin. The same strain was isolated from 37 children in eight wards and from one nurse. Medical centers in several European countries (*e.g.* Denmark, France and Switzerland) described outbreaks of MRSA nosocomial infections

in the 1960s. The first US MRSA outbreak did not occur until 1971 (Chambers, 1988; Keane, 1992). More recently, a number of MRSA infections have been shown to be community- rather than hospital-acquired (Chambers, 1988).

In the UK, the number of MRSA infections remained limited for several years. At the beginning of the 1971, MRSA represented 10% of all *S. aureus* isolates at the general hospital in Birmingham. Interestingly by the mid-1970s, the number of reported MRSA cases declined to virtually zero, a decrease was thought to be due to the use of a combination of aminoglycoside antibiotics and better infection control procedures (Griffiths *et al.*, 2004; Johnson *et al.*, 2005; Grundmann *et al.*, 2006). However, in the early 1980s, concerns about MRSA were heightened with the emergence of gentamicin-resistant MRSA in the UK, Ireland and the USA.

In 1982, an epidemic strain of multi-resistant MRSA was reported in Australia, and the same strain was discovered in London in connection with a hospital outbreak. The staphylococcal reference laboratory of the UK Public Health Laboratory Service established a numerical prefix for epidemic MRSA strains and based on this system, 16 epidemic strains were identified in England and Wales up to 1995. Nevertheless, only three epidemic strains, UK EMRSA-3, UK EMRSA-15 and UK EMRSA-16, were still being recorded in the 1990s and EMRSA-15 and EMRSA-16 were behaviorally more dynamic. In the meantime, six epidemic MRSA strains have been recorded in some central Europe countries (Grundmann *et al.*, 2006). EMRSA15 and EMRSA16 strains have spread broadly and are associated with severe infections (Livermore, 2000). According to the Centers for Disease Control (CDC) and the National Nosocomial Infection Surveillance System (NNISS), the proportion of MRSA in US hospitals increased dramatically from 2.4% in 1975

to 29% in 1991 (Graffunder and Venezia, 2002). In the UK MRSA bacteraemias remained at 3% until 1992 and then rose considerably to reach 43% by 2002. Since then, the rate of isolation of MRSA strains has increased significantly every year worldwide (Grundmann *et al.*, 2006).

2.6 .6.2 Mechanism of Methicillin-resistance

Methicillin-resistance in staphylococci is due to the acquisition of a large mobile DNA element (20 to 100kb in size), the so-called "Staphylococcal cassette chromosome *mec"* (SCC*mec*) (Rohrer *et al.*, 2003). Currently, eight SCC*mec* types (I to VIII) have been described in details.

β-lactam antibiotics destroy bacteria by inhibiting bacterial cell wall synthesis. The antibiotic, an analogue of D-alanyl-D-alanine, covalently attaches to penicillin-binding proteins (PBPs). These membrane-anchored proteins catalyse one or more of three reactions (transpeptidase, endopeptidase and carboxypeptidase) involved in cell wall synthesis. MRSA produces a modified PBP2a that can complete cell wall synthesis when the transpeptidation activities of the native PBPs are inactivated by the antibiotic (Chambers and Hackbarth, 1992). Resistance is due to the fact that PBP2a has low affinity to β-lactams antibiotics. The expression of the *mecA* gene is controlled by the products of the *mecRI* and *mecI* genes. MecRI synthesis is induced upon exposure to β-lactam antibiotics. As a result, the MecI repressor is inactivated and PBP2a produced (Chambers and Hackbarth, 1992).

2.7 Epidemiology of MRSA in the community

Although MRSA is mainly associated with nosocomial infections, it is becoming an increasing problem in the community. In the 1993, community-acquired MRSA (CA-MRSA) was first reported in the Western Australia and by end of 1999; four deaths were reported among children due to CA-MRSA infections in USA (Aires de Sousa and de Lencastre, 2004). CA-MRSA strains differ from nosocomial strains in a number of respects. Firstly, CA-MRSA strains are sensitive to non-β-lactams antibiotics, for example clindamycin, trimethoprim/sulfamethoxazole and doxycycline. Secondly, these strains have genotypes that are distinct from those of nosocomial strains. Finally, they have different methicillin-resistance cassettes and often encode PVL (Grundmann *et al.*, 2006; Boyle-Vavra and Daum, 2007).

There is no standard definition for CA-MRSA and approximately eight classification systems have been applied to categorize community-acquired infections (Aires de Sousa and de Lencastre, 2004). The CDC defines CA-MRSA as an infection with MRSA that lacks the risk factors of HA-MRSA including: the isolation of MRSA more than 48 hours after admission, hospitalization history, recent surgery, previous isolation of MRSA and presence of an indwelling catheter or a percutaneous device at the time of culture (Fridkin *et al.*, 2005; Brasel and Weigelt, 2008). Most common CA-MRSA infections are bacteraemias, skin and soft tissue infections, septic arthritis, toxic shock syndrome, necrotizing fasciitis and necrotizing pneumonia (Grundmann *et al.*, 2006). CA-MRSA is also associated with the production of PVL that is encoded by phage-mediated *lukS-PV* and *lukF-PV* genes. The production of this toxin is associated with severe skin and soft tissue infections (Boyle-Vavra and Daum, 2007). The CA-MRSA infections have been

documented among homeless people, homosexuals, military recruits, competitive athletes, residents of community-based health-care institutions and children in day-care centers (Grundmann *et al.*, 2006). There are several risk factors attributed to the acquisition of CA-MRSA such overcrowding, high rates of skin infections, frequent use of broad-spectrum antibiotics and close contact with a person with these risk factors. It is assumed that health-care institutions are the most likely source for CA-MRSA strains since some similarities have been found between sporadic nosocomial-MRSA and CA-MRSA infections (Aires de Sousa and de Lencastre, 2004).

2.8 Treatment and control of MRSA

MRSA strains are resistant to most β-lactam antibiotics and several other antimicrobial agents including aminoglycosides, clindamycin, chloramphenicol fluoroquinolones and macrolides (Schmitz and Jones, 1997). The glycopeptides have emerged as the most effective agents against MRSA. However, the emergence of MRSA strains with low sensitivity to vancomycin (VISA), first reported in 1996 and thereafter VRSA in 2002, has led to an increasing concern about the use of vancomycin as the first choice for the treatment of MRSA infections (Cui and Hiramatsu, 2003).

The treatment of MRSA infections depends on the site of infection. In some cases, such as infected devices and abscess, the remove of these devices and draining of abscess are more important than antimicrobial therapy (Cunha, 2005). Recently, new guidelines for the prophylaxis and treatment of MRSA infections in the UK have been published. These guidelines recommend the use of glycopepides or linezolid as a first choice to treat MRSA pneumonia and severe skin and soft tissue infections where the risk of bacteraemia is high (Gemmell *et al.*, 2006). Linezolid is recommended for treatment of pneumonia

since has showed an excellent penetration into lungs (Conte *et al.*, 2002). The same choice of glycopepides or linezolid is recommended but with longer treatment for uncomplicated bacteraemia. In bone and joint infections glycopepides can be used particularly with multiresistant MRSA and/or in combination with rifampicin or fusidic acid. Glycopeptide prophylaxis is recommended for patients who require surgery and have a history with MRSA colonization (Gemmell *et al.*, 2006). These recommendations take in their account not only the efficacy of the antibiotics but also their toxicity, selection of resistant bacteria and cost (Ben-David and Rubinstein, 2003)

In the UK, the control of MRSA has been given a high priority among health-care professionals (Haddadin *et al.*, 2002). Although there is, as yet, no consensus approaches for the control the MRSA infections, three strategies have been proposed for this purpose. The "*Scutari strategy"* is based on the application of basic cleanliness and protective procedures. This strategy is also applicable to nursing homes and small hospitals. The "*Search and Destroy*" strategy is applicable to hospitals that do not have a major problem with MRSA but have recently experienced epidemic outbreaks. This strategy is based on isolation of all infected and colonized patients and associated attempts to eradicate MRSA from the environment. The "*SALT strategy"* (*Staphylococcus aureus* Limitation Technique) is only applicable for non-containable infections, and when resources are limited. This method is appropriate for epidemic situations in which the incidence of infection is low (Spicer, 1984; Frank, 2003)

The majority of studies have reported that the screening of infected and colonized patients, the early detection of MRSA, improved hand hygiene and the prudent use of antibiotics, are effective control methods for the MRSA infections (Frank, 2003; Hardy *et al.*, 2004; Grundmann *et al.*, 2006; Wang and

Barrett, 2007). Furthermore, the most important step in controlling MRSA outbreaks is the typing of strains to distinguish between epidemic and sporadic strains (Frank, 2003).

2.9 Surface proteins of *Staphylococcus aureus*

Bacterial surface protein plays numerous functions, including adhesion to and invasion of host cells and tissues, evasion of immune responses and biofilm formation. Thus, cell wall-anchored proteins are essential virulence factors for the survival of *S. aureus* in the commensal state and during invasive infections, and targeting them with vaccines could combat *S. aureus* infections, the most important one is protein A (Deleo *et al*, 2010).

Protein A is a 42 kDa surface protein originally found in the cell wall of the bacteria *Staphylococcus aureus*. It is encoded by the spa gene and its regulation is controlled by DNA topology, cellular osmolality, and a two-component system called ArlS-ArlR. It has found use in biochemical research because of its ability to bind immunoglobulins. It is composed of five homologous disrupts opsonization and phagocytosis considered as virulence factors. As a pathogen, *Staphylococcus aureus* utilizes protein A, along with a host of other proteins and surface factors, to aid its survival and virulence, protein A helps inhibit phagocytic engulfment and acts as an immunological disguise (Muthukrishnan*, et al* ,2011).

2.10 Staphylococcal food poisoning

Staphylococcal food poisoning is one of the most common types of food borne disease worldwide. It has been identified as the causative agent in numerous outbreaks of food poisoning, but is believed to be under reported due to the selflimiting nature of the illness and the fact that most people recover within 1-2 days of becoming ill (Adams and Moss, 2008)

The onset of symptoms in Staphylococcal food poisoning can be very rapid, generally around three hours after ingestion of the food but may be as early as one hour or as late as six hours, depending on individual susceptibility to the toxin, the amount of contaminated food eaten, the amount of toxin in the food, and the general health of the individual. The most common symptoms are nausea, vomiting, retching, abdominal cramping, and prostration. Some individuals may not demonstrate all of the symptoms associated with the illness. In more severe cases, headache, muscle cramping, and transient changes in blood pressure and pulse rate may occur. Blood may be observed in stools and vomits. Recovery generally takes two days. However, it is not unusual for complete recovery to take three days or longer in severe cases (Bremer, 2004).

A number of factors contribute to the virulence of *S. aureus*, including deoxyribonuclease (DNase), Catalase, lipases and hemolysins. However, the most notable virulence factors are the enterotoxins. Staphylococcal food poisoning results from consumption of one or more preformed enterotoxins resulting in symptoms of intoxication (Mary *et al.,* 2004). Milk products, as well as other products with a high protein content, are a good substrate for growth of coagulase positive staphylococcus. Milk products were involved in 26 % of the outbreaks due to a staphylococcal food-borne intoxication *S. aureus* can gain access to milk either by direct excretion from udders with

clinical and subclinical staphylococcal mastitis or by environmental contamination during the handling and processing of raw milk (Eurobian Commition, 2003). Other coagulase positive *Staphylococci* as *S. hyicus* was isolated from minced meat, *S. intermedius* was isolated from sausage and *S. delphini* was also isolates from sausage by (Samia, 1997).

2.10.1 Associated foods

Foods that are frequently implicated in Staphylococcal food poisoning include meat and meat products, poultry and egg products, and salads such as egg, tuna, chicken, potato, and macaroni. Also implicated are bakery products such as cream-filled pastries, cream pies, chocolate eclairs, sandwich fillings, and milk and dairy products. Foods that require considerable handling during preparation and that are kept at slightly elevated temperatures after preparation are frequently involved in Staphylococcal food poisoning (Bremer, 2004).

2.10.2 Sources of contamination

Food handlers are usually the main source of food contamination. However, the surfaces of equipment can also be a source of *S. aureus* contamination (Adams and Moss, 2008).

2.10.3 Control

Due to the high incidence of *S. aureus* carriage by humans, prevention of staphylococcal food poisoning relies on good hygienic practices to reduce the incidence of contamination of food by food handlers. This is followed by the implementation of control procedures, such as cooking or chilling, to prevent growth and toxin production by any contaminating staphylococci. In seafood processing plants, low temperature storage and the presence of competitive spoilage organisms generally ensure that *S. aureus* does not pose a health risk (Adams and Moss, 2008).

Personal hygiene of food handlers is of paramount importance. People with wound infections or inflammations such as acne or boils need to take particular care. Likewise, touching the nose when processing food, sneezing or coughing over food should be avoided. Bactericidal soaps and creams can be useful in reducing the carriage of *S. aureus* on hands. Antibiotic or antiseptic cream may be of use to treat workers who have high levels of *S. aureus* in their nose. In some instances, limiting the contact that chronic carriers have with food may be the best option. Using utensils and disposable gloves is certainly advantageous. Good temperature control can prevent the growth of staphylococci in prepared foods. Foods should be kept at 60°C or above or 7.2°C or below (Bremer, 2004).

2.11 Mastitis

Mastitis is an inflammation of the mammary glands of dairy cows that can be caused by physical or chemical agents, with the majority of cases caused by bacterial infection. Mastitis is the most common and expensive disease affecting the dairy industry worldwide (Harmon, 1994; Quinn *et al.*, 1994; Moussaoui *et al.*, 2004 ; Park *et al*., 2007).

More than 130 microorganisms are related to bovine mastitis, with mastitis-causing bacteria broadly classified as contagious or environmental pathogens (Watts, 1988; Quinn *et al.*, 1994 ; Park *et al.,* 2007). *S. aureus* is a major cause of bovine mastitis. Since gene expression of many bacteria is known to be regulated by the environment, milk may play an important role in the regulation of the early steps in the pathogenesis of bovine mastitis by *S. aureus*. To get insight into the response of *S. aureus* to the milk environment, a Tn*917-lacZ* mutant library was generated and screened for genes specifically expressed during growth in milk (Aart, 2000).

2.12 Microbial Contamination from the Exterior of the Udder

The exterior of the cows' udder and teats can contribute to microorganisms that are naturally associated with the skin of the animal as well as microorganisms that are derived from the environment in which the cow is housed and milked (Bramley, 1982; Bramley and McKinnon, 1990; Hogan *et al*., 1989; Zehner *et al*., 1986). Organisms associated with bedding materials that contaminate the surface of teats and udders include streptococci, staphylococci, Gram positive spore-formers, coliforms and other Gram-negative bacteria. Both thermoduric and psychrotrophic strains of bacteria are commonly found on teat surfaces (Bramley and McKinnon, 1990). Several studies have investigated pre-milking udder hygiene techniques in relation to the bacteria count of milk (Bramley and McKinnon, 1990; Galton *et al.,* 1984; Pankey, 1989).

2.13 Coagulase-Negative Staphylococci (CNS)

CNS is an element in the normal flora of human skin and mucosa. They are classic opportunists that only cause infections given a certain host disposition.

2.13.1 *S. epidermidis*

This is the pathogen most frequently encountered in CNS infections (70–80% of cases). CNS cause mainly foreign body infections. Examples of the foreign bodies involved are intravasal catheters, continuous ambulant peritoneal dialysis (CAPD) catheters, endoprostheses, metal plates and screws in osteosynthesis, cardiac pacemakers, artificial heart valves, and shunt valves. These infections frequently develop when foreign bodies in the macroorganism are covered by matrix proteins (e.g., fibrinogen, fibronectin) to which the staphylococci can bind using specific cell wall proteins. They then proliferate on the surface and produce a polymeric substance—the basis of the developing

biofilm. The staphylococci within the biofilm are protected from antibiotics and the immune system to a great extent. Such biofilms can become infection foci from which the CNS enter the bloodstream and cause sepsislike illnesses. Removal of the foreign body is often necessary (Harley, 2005).

2.13.2 *S. Saprophyticus*

S. Saprophyticus is responsible for 10–20% of acute urinary tract infections, in particular dysuria in young women, and for a small proportion of cases of nonspecific urethritis in sexually active men. Antibiotic treatment of CNS infections is often problematic due to the multiple resistances often encountered in these staphylococci, especially *S. hemolyticus* (Kayser, 2005).

2.14 Isolation and Identification

The most successful and widely used selective plating medium for *Staph. aureus* is the one devised by Baird- Parker in the early 1960s. It combines the virtues of a high degree of selectivity, a characteristic diagnostic reaction, and the ability to recover stressed cells. Differentiating of *Staphylococcus* species can be difficult sometimes (Goldman, 2008).

2.14.1 Colony Morphology

Colonies of *Staphylococcus* on sheep blood agar present themselves as smooth, yellow, white or off-white colonies somewhere in the area of 1 to 2 mm in diameter. Colonies may exhibit β-hemolysis and may show varying degrees of growth. Sometimes, the β-hemolysis is not evident after 24 hours of incubation and requires further incubation (Hedin, 2005).

2.14.2 Quick Tests

Perhaps the most common quick test employed to help identifying colonies of *Staphylococcus* is the catalase test. This simple test can differentiate off-white or grey colonies of *Staphylococcus* from *Streptococcus* and is an invaluable tool. The modified oxidase test is another quick test that

can differentiate Staphylococcus from Micrococcus, as is the lysostaphin test (Remel). Differentiation among the Staphylococci can be achieved by the coagulase test, which tests for both bound and free coagulase. Alternatively, there are a multitude of commercial latex agglutination tests available nowadays. Slidex Staph from bioMerieux, Bacti Staph from Remel, Staphytect from Oxoid, Staphylase from Oxoid, Staph Latex Slide Test from Arlington Scientific, Staphtex from Hardy, and Set-RPLA Latex Staph from Denka Seiken are all latex agglutination tests for the identification of *Staphylococcus aureus*. In addition, there are a few kits that utilize passive hemagglutination for the identification of *Staphylococcus aureus*, such as Staphyslide from bioMerieux, HemaStaph from Remel, and StaphyloSlide from Becton-Dickinson (Forbes, 2002).

2.14.3 Conventional Methods

Staphylococcus aureus can further be identified by its ability to produce DNase and ferment mannitol. Both DNase agar and Mannitol Salt agar are readily available. When needed to speciate coagulase-negative staphylococci (CNS), there are a number of tests that can be employed. Carbohydrate utilization such as sucrose, xylose, trehalose, fructose, maltose, mannose, and lactose, as well as such tests as urease, nitrate reduction, and phosphatase, will all aid in the identification of CNS. Staphylococcus saphrophyticus is a frequent cause of urinary tract infections and can be identified by its resistance to novobiocin. Bacitracins, as well as acid production from glucose, are tests that can be employed to differentiate *Staphylococcus* from *Micrococcus* (Goldman, 2008).

2.14.4 Identification Strips

There are a number of manufacturers that have developed identification strips containing many of the aforementioned biochemicals; API ID 32 Staph from bioMerieux and API Staph also from bioMerieux utilize 10 and 19 tests on their strips, respectively. These tests are reliable in identifying most strains of CNS (coagulase-negative staphylococci) (Layer, 2006).

Automated Methods; the Vitek GPI card from bioMerieux, the MicroScan Rapid Pos Combo Panel and Pos ID 2 Panel from Dade/MicroScan, and the Phoenix Automated Microbiology System Panel from Becton Dickinson are a few of the more common automated identification panels for staphylococcus as well as other Gram-positive organisms (Salomon, 1999).

2.15 MecA (gene)

The mecA gene is a gene found in bacterial cells which allows a bacterium to be resistant to antibiotics such as methicillin, penicillin and other penicillin-like antibiotics (Ubukata *et al*, 1989).

The most commonly known carrier of the mecA gene is the bacterium known as Methicillin-resistant *Staphylococcus aureus* (MRSA). In *Staphylococcus* species, mecA is spread on the SCCmec genetic element (Deurenberg and Stobberingh, 2009).

Resistant strains are responsible for many infections originating in hospitals, the mecA gene does not allow the ringlike structure of penicillin-like antibiotics to bind to the enzymes that help form the cell wall of the bacterium (transpeptidases), and hence the bacteria is able to replicate as normal. mecA is located on the staphylococcal chromosome cassette mec (Wielders *et al*, 2002).

The gene encodes the protein PBP2A (penicillin binding protein 2A). PBP2A has a low affinity for beta-lactam antibiotics such as methicillin and penicillin. This enables transpeptidase activity in the presence of beta-lactams, preventing them from inhibiting cell wall synthesis (Fogarty *et al*, 2015).

Also Extended-spectrum β-lactamases (ESBLs) genes have been identified in *Staphyllococcus spp* all over the world, yet there is paucity of reports on these genes in despite the phenotypic evidence of resistance to betalactam drugs, The presence of these genes underscores the potential health risk of antibiotics resistance. These genes hydrolyze Oxymino-Cephalosporines and mono bactams but not Cephamycins and sometimes they can be inhibited by Clavulanic acid and they include PER, DHMA, VEB, GES, OXA_2, ACCM, CITM, FOXM, ECBM, MOX, OXA10 SHV, TEM, and CTX. Also some other

genes such as Van gene for vancomycin resistance and Coa gene which encodes coagulase enzyme secretion were reported (Wilson *et al.*, 2010).

2.16 Polymerase Chain Reaction amplification assay

Successful treatment of methicillin resistant *S.aureus* begins with the detection and confirmation that the strain in question actually possesses the mecA gene, responsible for the resistance. The use of polymerase chain reaction (PCR) is typically used to detect the presence of the mecA gene, alternative methods can be used as that can be as specific as PCR (Ubukata *et al.*, 1992).

Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. It is an easy, cheap, and reliable way to repeatedly replicate a focused segment of DNA, a concept which is applicable to numerous fields in modern biology and related sciences **(**Bartlett and Stirling 2003)**.**

The vast majority of PCR methods rely on thermal cycling, which involves exposing the reactants to cycles of repeated heating and cooling, permitting different temperature-dependent reactions specifically, DNA melting and enzyme-driven DNA replicationto quickly proceed many times in sequence. Primers (short DNA fragments) containing sequences complementary to the target region, along with a DNA polymerase, after which the method is named, enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the original DNA template is exponentially amplified. The simplicity of the basic principle underlying

PCR means it can be extensively modified to perform a wide array of genetic manipulations. PCR is not generally considered to be a recombinant DNA method, as it does not involve cutting and pasting DNA, only amplification of existing sequences (Cheng *et al*, 1994).

2.17 Sequencing of mecA gene

Mec A gene sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four nitrogenous bases including adenine, guanine, cytosine, and thyminein a strand of DNA. DNA sequencing may be used to determine the sequence of individual genes, larger genetic regions (i.e. clusters of genes or operons), full chromosomes or entire genomes, of any organism (Olsvik *et al*, 1993).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Area

Ghebaish Locality lies in West Sudan in the western south part of West Kordofan State between Longitudes 27^0 35" and 28^0 30" East and Latitudes 11^0 30" and 12^0 30" North and about 149 kilometers far from El-Nuhud city. The study area is dominated by the poor savannah rainfall covering the area of 15.950 square kilometers with population estimated by 250.619 inhabitants according to Population Census(2010), the most of them are specialized in sheep breeding, grow food and cash crops including sesame, millet, groundnuts and watermelon. Gum Arabic trees are a part of the ecosystem covering a considerable belt from the study site.

3.2 Collection of samples

A total of 200 raw cow's milk samples were collected in sterile clean containers. This was done by applying gentle pressure with fingers on the udders for the presence of swelling, hardness, redness, heat, and pain. Moreover, the physical characteristic of the milk from each quarter was checked for any alterations. Before milk samples were collected, each quarter was washed with tap water and dried.. The teats were swabbed one after the other with cotton soaked in 70% ethanol. 10 ml of milk was then collected aseptically from the udders into sterile universal bottles after discarding the first three milking streams. The samples were immediately transported on ice to Microbiology Research Laboratory of the College of Veterinary Medicine, West Kordofan University, where standard bacteriological assays followed. The study was conducted between July 2017 and December 2018.

3.3 Media for isolation and identification

3.3.1 Peptone water (Oxoid, CM9)

The medium prepared according to manufacturer.

3.3.2 Mannitol Salt Agar (Oxoid, CM85)

The medium prepared according to manufacturer.

3.3.3 Baird-Parker Agar (Oxoid, CM275)

The medium prepared according to manufacturer.

3.3.4 Blood Agar Base (Oxoid, CM55)

The medium prepared according to manufacturer.

3.3.5 Glucose phosphate medium MR -VP medium (Oxoid, CM43)

The medium prepared according to manufacturer.

3.3.6 Christensen's Urea medium (Oxoid, CM0053)

The medium prepared according to manufacturer.

3.3.7 Nutrient Broth (Oxoid, CM1)

The medium prepared according to manufacturer.

3.3.8 Nutrient Agar (Oxoid, CM3)

The medium prepared according to manufacturer.

3.3.9 DNAse agar (Oxoid, CM321)

The medium prepared according to manufacturer.

3.3.10 Mueller Hinton Agar (Oxoid, CM337)

The medium prepared according to manufacturer.

3.4 Methods of isolation

3.4.1 Nourishment

One milliliter of each sample is inoculated into 9 ml of sterile peptone water and incubated overnight, that is according to the procedure described by (Abera *et al*., 2010)..

3.4.2 Isolation of Staphylococci

A loop full of peptone water broth is streaked on blood agar base enriched with sheep blood, mannitol salt agar and Baird-Parker using the quadrant streaking method for each quarter.

Colonial morphology, pigmentation and haemolysis of isolates were observed in all media used. In general, on Baird-Parker` medium, all coagulase-positive staphylococci showed small to moderate size colonies. *S. aureus* showed two diagnostic characteristics. Firstly, a clear zone appears around the colonies (due to proteolysis). Secondly, opaque zones appear within the clear zones (caused by lipase).

On blood agar coagulase-positive staphylococci grows forming convex, circular golden-yellow and opaque colony after 18-24 hours incubation at 37°C (FDA, 2008).

3.4.3 Identification of Staphylococci

Isolated staphylococci in this study were identified using the primary and secondary tests as described in Barrow and Felltham (2003).

3.4.3.1 Gram stain

A part of a separate colony from pure culture picked by sterile loop then diluted in a drop of distilled water in a clean grease free slide and spread on about one cubic centimeter area and let to dry in air then fix by passing onto the flame. The film is flooded with crystal violet solution for one minute then washed by tap water and drain the excess water. Add iodine solution for one

minute, wash with tap water then add decolorizer and wash quickly with tap water stain with safranin for one minute then wash with tap water and , let the stained smear to dry and examined under the oil immersion lens (Tang and Charles, 2006).

3.4.3.2 Vogues proskauer test

Dissolve Phosphate buffered peptone water powder medium in distilled water, adjust the pH to 7.5 then distribute it into suitable containers and sterilize with the autoclave at 121˚C for 15 minutes. When it is cooled, add glucose sugar then mix well and dispense into 2 ml amounts in small tubes then sterilize by autoclaving at 115˚C for 10 minutes. Inoculated with 2 ml of *Staphylococcus spp*, and then incubated at 37^{o}C for 5days (Cheesbrough, 2000).

After completion of incubation time add 0.6 ml of 5% α-naphthol solution and 0.2ml 40% KOH aqueous solution to inoculated Phosphate Buffered Peptone Water medium tube , shake well and slope (to increase the area of the air-liquid interface), then examine after one hour. A positive reaction seen by strong red color (Barrow and Felltham,2003).

3.4.3.3 Coagulase Test

To test the ability of isolates to produce coagulase enzyme that convert fibrinogen to fibrin, measure 0.5 ml of plasma in to sterile test tube then inculcate with test organism then incubate at 37^{o}C for 1 day, Coagulation of plasma indicates positive result (Brooks, *et al* 2004).

3.4.3.4 Urease test

This test is based on production of urease enzyme and break down of urea into CO_2 and NH_3. This test performed by streaking of the examined organisms onto slant surface of Christensen's medium and then incubated at

37°C for 24 hours. The test is positive when the color changes to pink (Collins, *et al* 2004).

3.4.3.5 Oxidase test

A portion of the tested organism smeared on the oxidase paper disk that impregnated with tetra methyl para-phenylenediamine dihydro chloride. Production of a purple color immediately indicates positive test. (Cheesbrough 2000).

3.4.3.6 Sugars fermentation

The tested organism is inoculated into peptone water containing the BTB indicator and the desired sugar such as Lactose, Sucrose and Mannitol and incubated at 37°C for 24 hours. Production of yellow color indicates positive result (Barrow and Felltham, 2003).

3.4.3.7 Deoxyribonuclease (DNAse) test

DNAse test was used to differentiate *Staph. aureus* which produce the deoxyribonuclease (DNAse) enzyme from other staphylococci. The test was performed by culturing the organism on DNAse agar which incubated over night at 37º C. DNAse production was tested by flooding the plate with 1M hydrochloride acid solution. The acid precipitates unhydrolyzed DNA. DNAse producing colonies were surrounded by clear areas indicating DNA hydrolysis. (Barrow and Felltham, 2003).

3.4.3.8 Identification Strips

There are a number of manufacturers that have developed identification strips containing many of the aforementioned biochemical; API ID 32 Staph from bioMerieux and API Staph also from bioMerieux utilize 10 and 19 tests on their strips, respectively. These tests are reliable in identifying most strains of CNS (coagulase-negative staphylococci) (Layer, 2006).

3.4.3.9 Antibiotic susceptibility testing

Antibiotic resistance pattern of all isolates are determined by disks diffusion method as described by (Barrow and Fellthamֺ, 2003).

Sensitivity of Staphylococcus isolates to 4 antibiotics discs was determined using Kirby-Bauer disc diffusion assay, the following antibiotics were used: Sterptomycin, Vancomycin, Methicillin and Penicillin.

Pure culture colonies (3-5) of isolated bacteria were suspended in a test tube containing Nutrient broth and incubated at 37°C for 24 hours. Two ml of each culture were spreaded over Muller-Hinton agar, the plate was left to dry for 15 minutes and the excessive fluid was aspirated. The commercially prepared antibiotic disks were placed on the agar surface using sterile forceps and pressed gently to ensure full contact with the surface of the culture medium. The plates were then incubated at 37°C for 24 hours. The area showing no visible growth was taken as the zone of growth inhibition and as measured in millimeters from the underside of the plate.

3.5 Molecular methods

3.5.1 Preparation of reagents

3.5.1.1 Primers

Primers for PCR were used to amplify specific product as in table (1).

Table.1. Primer used in characterizing Mec A gene.

Name	Sequence (5' -------- 3')	Product size	Annealing	References
Mec A -R	5'- AGT TCT GCA GTA CCG GAT TTG C -3'	533 bp	55 °C	(Geha, *et al*, 1994)
Mec A- F	5'-AAA ATC GAT GGT AAA GGT TGG C -3'			

3.5.1.2 Preparation of 10x TBE buffer

Amount of 108g Tris base was weighted and added to 55g of boric acid and 9.3g EDTA then dissolved into one liter of distilled water.

3.5.1.3 Preparation of 1x TBE buffer

10ml of 10x TBE buffer was added to 90ml of distilled water and heated until completely dissolved.

3.5.1.4 Preparation of Agarose gel

One gram of agarose was dissolved into 100 ml 1x TBE then 5µl of Ethidium Bromide was added before pouring the liquid agarose gel into the gel casting tray which was equipped with suitable comb and its open sides were closed and left to solidify, the comb was gently removed and the closure from the opened sides were removed.

3.5.1.5 Preparation of Ethidium Bromide

Five milligrams of Ethidium Bromide powder dissolved into 500µl distilled water and kept into a brown bottle.

3.5.1.6 Preparation of loading dye

Three ml of glycerol were added to seven ml of distilled water and 25g of bromophenol blue was dissolved into 100ml distilled water, the mixture was used as loading dye.

3.5.2 DNA extraction

According to the methods described by Dewanand *et al*. (2007) and Brakstad *et al*. (2009).The genomic DNA is extracted by boiling method. In brief for the extraction of genomic DNA, a single colony of *S. aureus* was taken in 100 µL of distilled water, mixed well and boil for 10 min. After boiling the tubes were placed immediately on ice for cooling followed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant containing DNA is collected which is further used as template DNA.

3.5.3 Detection of DNA

Five micro liters of DNA extract were mixed with 0.5µl of loading dye by using automatic pipette (0.5-10µl) then transferred to the wells of the gel. This conducted into electrophoresis tank after1% of agarose gel was prepared. Then the gel was poured after fixed the spacer and comb, let it to solidify, few buffers were added to facilitate the removal of the comb. Gel electrophoresis system and tank was filled with electrophoresis buffer, separation of DNA was carried out at a constant voltages of 75 volts for 30 minutes. After running, the gel was photographed by using UV light.

3.5.4 Measurement of DNA Concentration

The concentration of extracted DNA was read using the spectrophotometer, (Bioeppendorf), Spectrophotometric analysis is based on the principles that nucleic acids absorb ultraviolet light in a specific pattern. In the case of DNA and RNA, a sample that is exposed to ultraviolet light at a wavelength of 260 nanometres (nm) will absorb that ultraviolet light.

3.5.5 Polymerase Chain Reaction "PCR" techniques

3.5.5.1 Preparation of master mix

The primers were prepared. The desired master mix consists of buffer, MgCL2, dNTBs, and Tag polymerase was prepared for 25 reactions, and this minimizes reagent loss and enables accurate pipetting. As 10µl of dNTBs(0.4µl), 6.25µl of Tag polymerase(0.25µl), 37.5µl of MgCL2 (1.5µl),62.5µl buffer (2.5µl), 333,75µl of distilled water (13.35µl), 12.5µl of primers forward(0.5µl), 12.5µl of primers reverse(0.5µl). The total volume was 21µl, then completed up to 25µl by 4µl of sample (template). Master Mix without template DNA was used as negative control. All these reactions performed into PCR tubes (0.2ml capacity).

3.5.5.2 PCR amplification

For identification of mecA gene Two different primers pairs are used F (5'-AAA ATC GAT GGT AAA GGT TGG C -3') and R (5'- AGT TCT GCA GTA CCG GAT TTG C -3'), the amplification protocol is performed with thermocycler (Mastercycler personal®- Eppendorf® Germany) under conditions of an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, anneling temperature of primers is 55°C for 45 seconds and extension at 72°C for 1 min. The amplified PCR products are resolved by electrophoresis in 1.8% agarose gel at 100 V for 30 min, stain with

ethidium bromide and finally visualize and under UV trans-illuminator (UVsolo TS® Imaging System, Biometra®, Germany) (Geha, *et al*, 1994) .

3.5.5.3 Visualization of PCR products

The gel casting tray was put into the electrophoresis tank flooded with 1xTBE buffer just to cover the gel surface, 5µl of PCR products from each samples was mixed with 0.5µl of loading dye and then a gel of electrophoresis was runed. 5µl of DNA ladder (Marker) was mixed with 0.5µl loading dye and were added to the well in each run. The gel electrophoresis apparatus was connected to power supply (Prime, 125v, 500µA, UK). The electrophoresis was carried at 75 V for 30 minutes, after electrophoresis period, the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized by UV Trans illuminator (Uvitee- UK).

The gene was identified by sequencing of plasmid using chain terminator (Sanger) method. An approximately~533 bp region of the mecA gene was sequenced with the set of primers by (RIKEN BRC –Egypt).

In brief, in each reaction there is a population of partially synthesized radioactive DNA molecules, each having a common 5'-end, but each varying in length to a base-specific 3' end. After a suitable incubation period, the DNA in each mixture is denatured, electrophoresed side by side, and the radioactive bands of single-stranded DNA detected by autoradiography. The sequence can then be read off directly from the autoradiograph, (Mwangi *et al*., 2007).

CHAPTER FOUR
RESULTS

4.1 Laboratory analysis

4.1.1 Isolation of Staphyllococci

Out of 200 cow milk samples were collected from four areas throughout Ghebaish locality. 45(23%) samples were found positive to *Staphyllococcus sp*, growth on special media like Mannitol Salt Agar, Baird-Parker Agar and Blood Agar Base was observed (fig.1) and (fig.2).

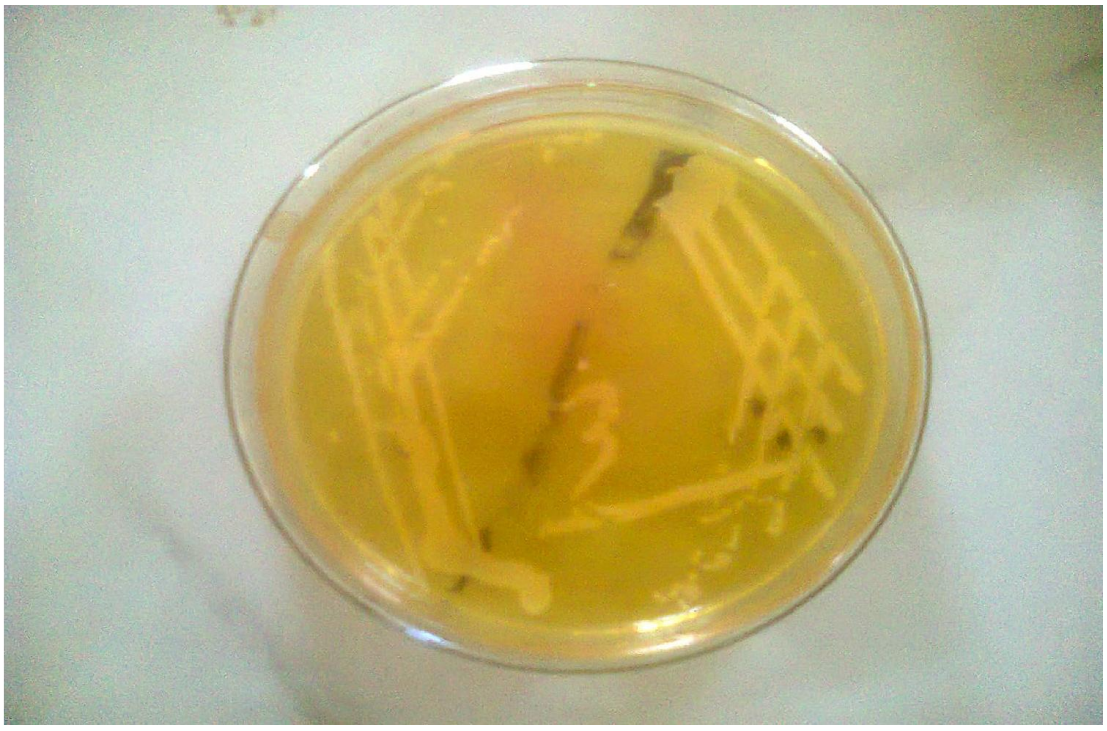

Figure.1.Growth of *Staphyllococcus aureus* on mannitol salt agar

The test organism is salt resistant and mannitol has been fermented and the phenol red pH indicator in the medium has changed colors as a result of the acid from sugar breakdown.

Figure.2. Growth of *Staphyllococcus aureus* on blood agar

Hemolysis and breakdown of red blood cells due to the production of hemolysins enzymes. 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.

4.1.2 Identification of Staphyllococci

Conventional methods for identification of *Staphyllococcus sp* isolated using gram's stain, morphology, catalase test, oxidase test, coagulase test, Vp test, urease and DNase tests revealed two Staphyllococcus species namely *Staphyllococcus aureus* 33(73%) and *Staphyllococcus epidermities* 12(26%).Percentages of different Staphyllococci species isolated were shown in (tab.2), (fig.3),(fig .4) and (fig.5) .

Table.2. Biochemical properties of *Staphylococcus spp* isolated

Staphyllococcus sp	***S. aureus***	***S .epidermities***
Tests	**33 isolates**	**12 isolates**
Gram stain	+	+
Shape	Cocci	Cocci
Catalase	+	+
Oxidase	-	-
Blood heamolysis	β	γ
Coagulase	+	-
Urease	+	+
Vogues proskauer	+	+
DNase	+	+

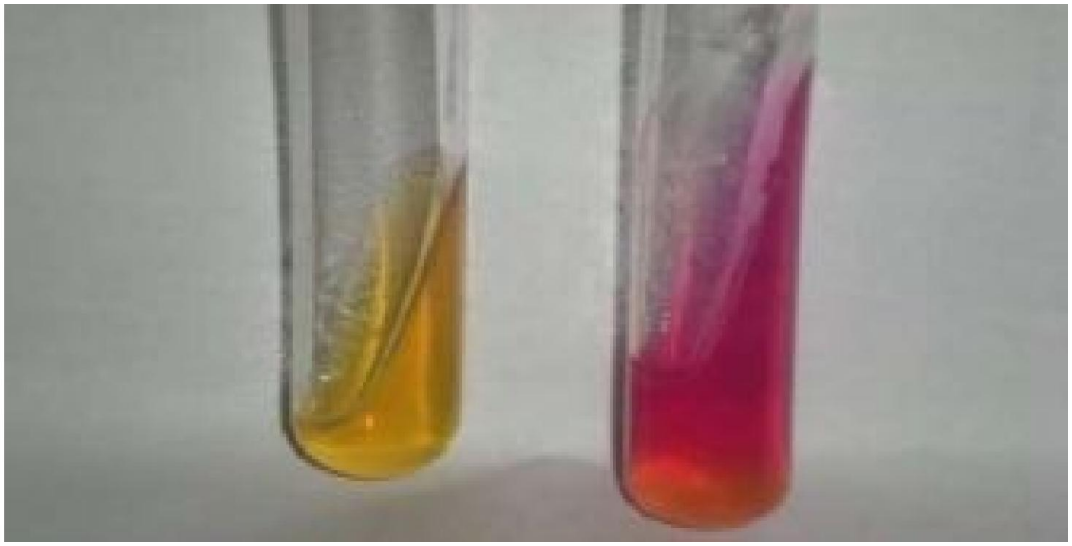

Figure.3. Reaction of *staphylococcus spp* in urease test

From left to right:

Tube 1 = Standard control without inoculation.

Tube 2 = positive result is pink.

Figure.4. Show reaction of *Staphylococcus spp* in Vogues proskauer test

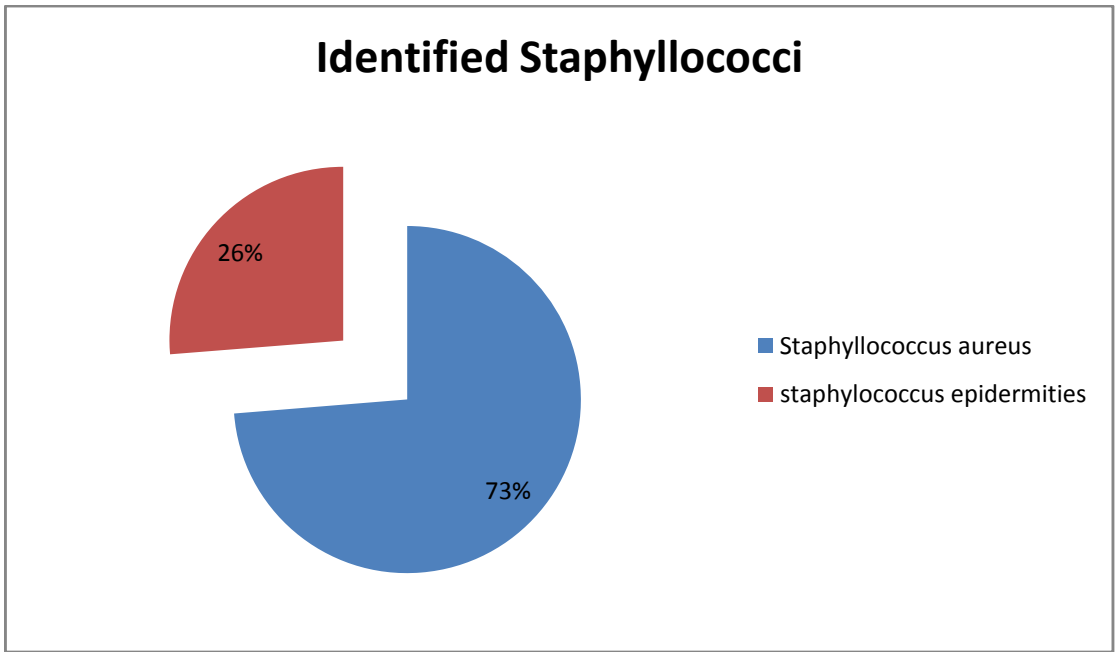

Figure.5. Percentage of characterized Staphylococcus species

4.1.3 Confirmation

By using API-Staph Strips the results revealed that *Staphyllococcus aureus* 33(73%) and *Staphyllococcus epidermities* 12(26%) were identified (fig.2).All results on API- Staph Strips showed negative reactions to ONPG, ARA, and RAF, while they showed positive reactions to VP, URE, ARG, SUC, LAC, ALK and MAL, however different reactions were obtained from TRE and MAN (tab.3) and (fig.6).

Table.3. Analytical Profile Index (API) for *Staphylococcus spp* isolates

Staphyllococcus sp	***S. aureus***	***S .epidermities***
Tests	**33 isolates**	**12 isolates**
VP	+	+
Alkaline phosphatase	+	+
ONPG	-	-
Urease	+	+
Arginine	+	+
Mannitol	+	-
Sucrose	+	+
Lactose	+	+
Arabinose	-	-
Raffinose	-	-
Trehalose	+	-
Maltose	+	+

Figure.6. Reaction of biochemical tests of *Staphyllococcus spp* on API-Staph strips.

4.1.4 Distribution of Staphyllococci isolates

This study revealed that *S. aureus* showed higher prevalent 33(73%) out of the total isolates distributed as 18 (81%) 10(66%) and 5 (62%) isolated from Eastern sector, Almansoura sector and Western sector respectively. Also 4 (18%), 3(37%) and 5(33%) *S. epidermities* was isolated from Eastern sector, Western sector respectively and Almansoura sector respectively (tab.4) and (fig.7).

Table.4. *Staphyllococcus spp* isolated from different sectors in Ghebaish Locality.

Area	No of samples	No of positive	*S.aureus*	*S.epidermities*
Eastern Sector	70	22	18 (81%)	4(18%)
Western Sector	58	8	5(62%)	3(37%)
Almansoura Sector	47	15	10 (66%)	5(33%)
Aljaflah Sector	25	0	0	0

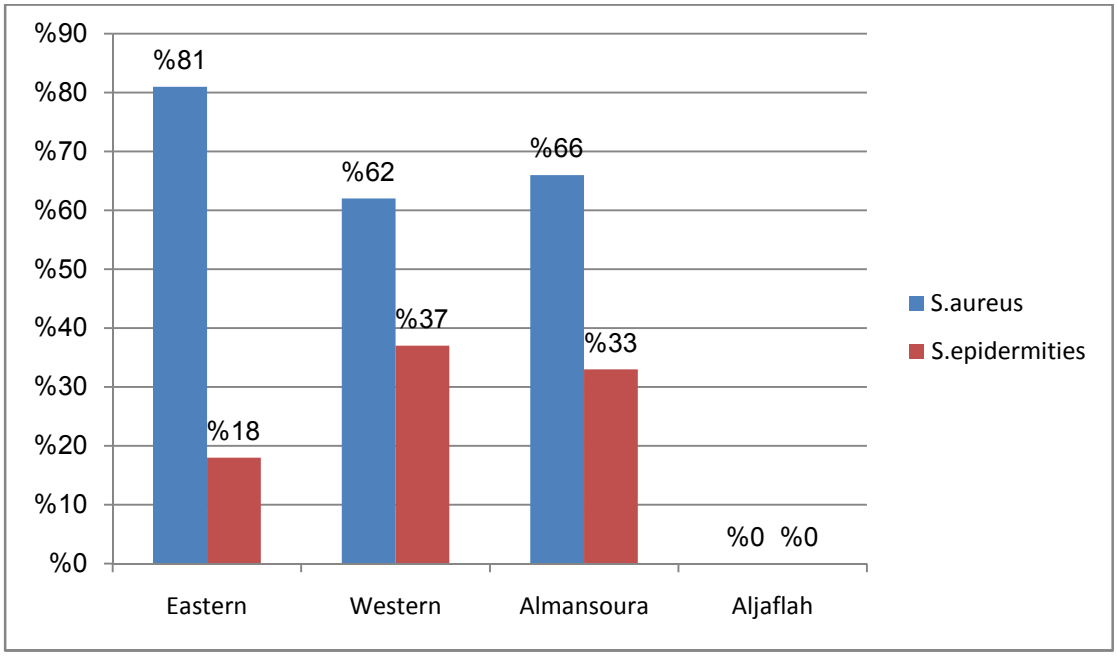

Figure.7. Distribution of *Staphyllocous .spp* in terms of percentage

4.2 Results of antimicrobial susceptibility tests

Overall resistance of *Staphyllococcus sp* isolates to different antibiotics is shown in (tab.5), (fig.8) and (fig.9).

Individual resistance of each *Staphylococcus sp* isolates to four antibiotics among the sectors are shown in (tab.6), (tab.7), (tab.8), (fig.10), (fig.11) and (fig.12).

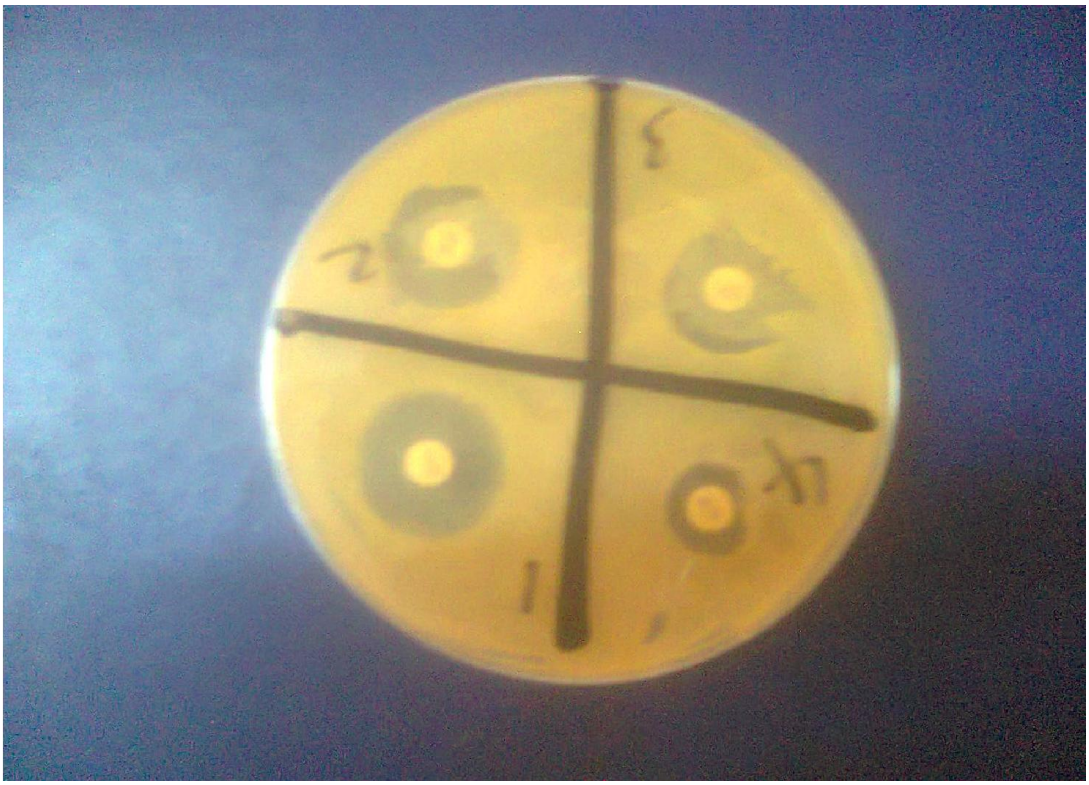

Figure.8. Multidrug resistance of *Staphyllococcus sp* (Sterptomycin, Vancomycin, Methicillin and Penicillin)

Table.5. Resistance of *Staphylococcus* isolates to four antibiotics in terms of percentage.

Antibiotic	Sterptomycin	Vancomycin	Methicillin	Penicillin
Total *S.aurus* (R)	0 (0%)	26 (78%)	6 (18%)	33 (100%)
Total *S.aurus* (S)	31(93%)	7 (21%)	3 (9%)	0 (0%)
Total *S.aurus* (I)	2 (6%)	0 (0%)	24 (72%)	0 (0%)
Total *S.epidermities* (R)	0 (0%)	12(100%)	3 (25%)	12 (100%)
Total *S.epidermities* (S)	12 (100%)	0 (0%)	0 (0%)	0 (0%)
Total *S.epidermities* (I)	0 (0%)	0 (0%)	9 (75%)	0 (0%)

Where**: R** = Resistance**, S** = Sensitive**, I** = Intermediate Resistance

Total = Total number of isolates interacts with antibiotic.

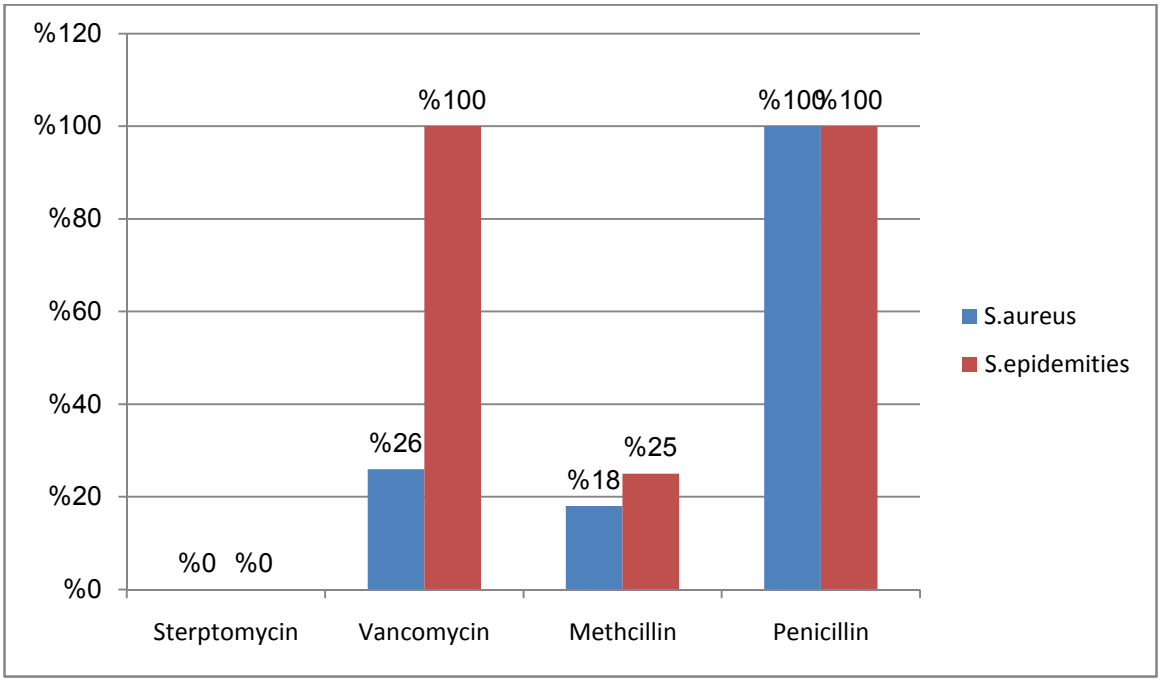

Figure.9. *Staphylococcus* species resistance rates to different antibiotics

Table.6. Sensitivity of the isolates to different antibiotics (Eastern sector)

No	Isolate	Streptomycin	Vancomycin	Methicillin	Penicillin
1	*S. aureus*	15 (S)	10(R)	11(I)	8(R)
2	*S. aureus*	17(S)	19(S)	10(I)	10(R)
3	*S. aureus*	16(S)	16(S)	11(I)	7(R)
4	*S. aureus*	20(S)	10(R)	10(I)	7(R)
5	*S. aureus*	20(S)	11(R)	10(I)	7(R)
6	*S. aureus*	20(S)	8(R)	7(R)	7(R)
7	*S. aureus*	17(S)	10(R)	12(I)	11(R)
8	*S. aureus*	20(S)	11(R)	10(I)	7(R)
9	*S. aureus*	18(S)	11(R)	7(R)	7(R)
10	*S. aureus*	21(S)	8(R)	9(R)	7(R)
11	*S. epidermities*	20(S)	14(R)	13(I)	12(R)
12	*S. aureus*	15(S)	10(R)	11(I)	8(R)
13	*S. epidermities*	20(S)	11(R)	10(I)	7(R)
14	*S. aureus*	15(S)	10(R)	10(I)	8(R)
15	*S. aureus*	21(S)	11(R)	10(I)	7(R)
16	*S. aureus*	20(S)	11(R)	10(I)	7(R)
17	*S. aureus*	19(S)	10(R)	10(I)	8(R)
18	*S. aureus*	20(S)	11(R)	10(I)	7(R)
19	*S. aureus*	23(S)	20(S)	13(I)	20(R)
20	*S. epidermities*	21(S)	11(R)	10(I)	8(R)
21	*S. aureus*	20(S)	11(R)	10(I)	7(R)
22	*S. epidermities*	17(S)	12(R)	9(R)	11(R)

Where: **R**= Resistance, **I** = Intermediate susceptibility, **S** =Sensitive.

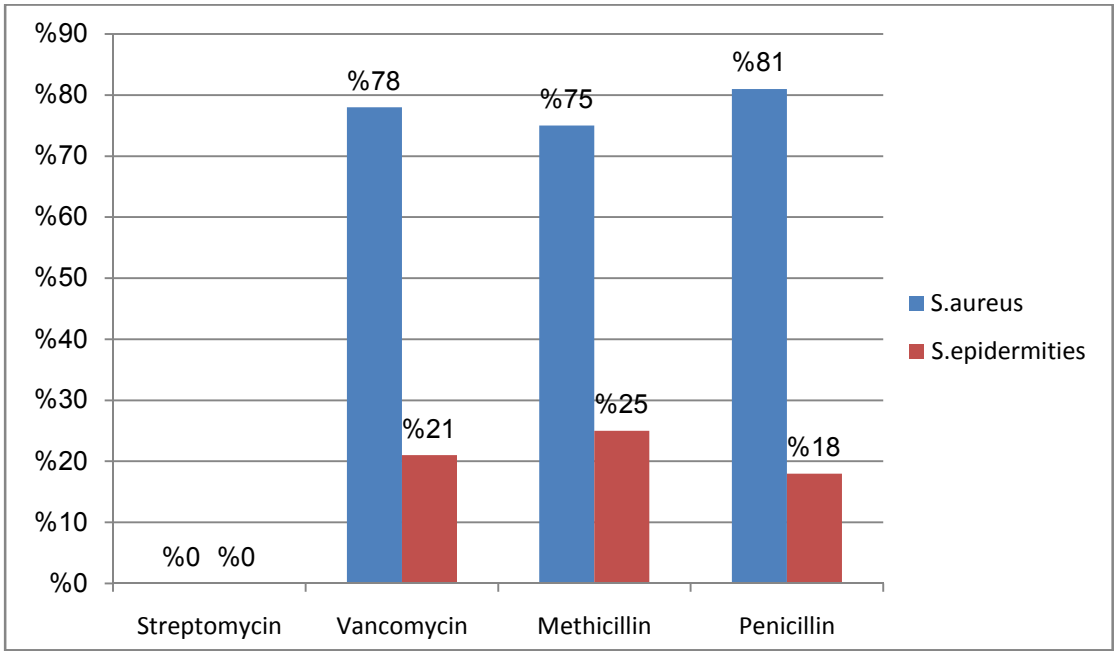

Figure.10. Levels of *Staphyllococcus sp* resistance to different antibiotics (Eastern sector)

Table.7. Sensitivity of the isolates to different antibiotics (Western sector)

No	Isolate	Streptomycin	Vancomycin	Methicillin	Penicillin
1	*S. epidermities*	16 (S)	7(R)	9(R)	7(R)
2	*S. aureus*	21(S)	19(S)	18(S)	8(R)
3	*S. aureus*	17(S)	18(S)	19(S)	13(R)
4	*S. aureus*	20(S)	12(R)	10(I)	7(R)
5	*S. epidermities*	20(S)	9(R)	9(R)	8(R)
6	*S. aureus*	22(S)	10(R)	10(I)	7(R)
7	*S. epidermities*	15(S)	11(R)	10(I)	7(R)
8	*S. aureus*	13(I)	9(R)	14(S)	12(R)

Where: **R**= Resistance, **I** = Intermediate susceptibility, **S** =Sensitive.

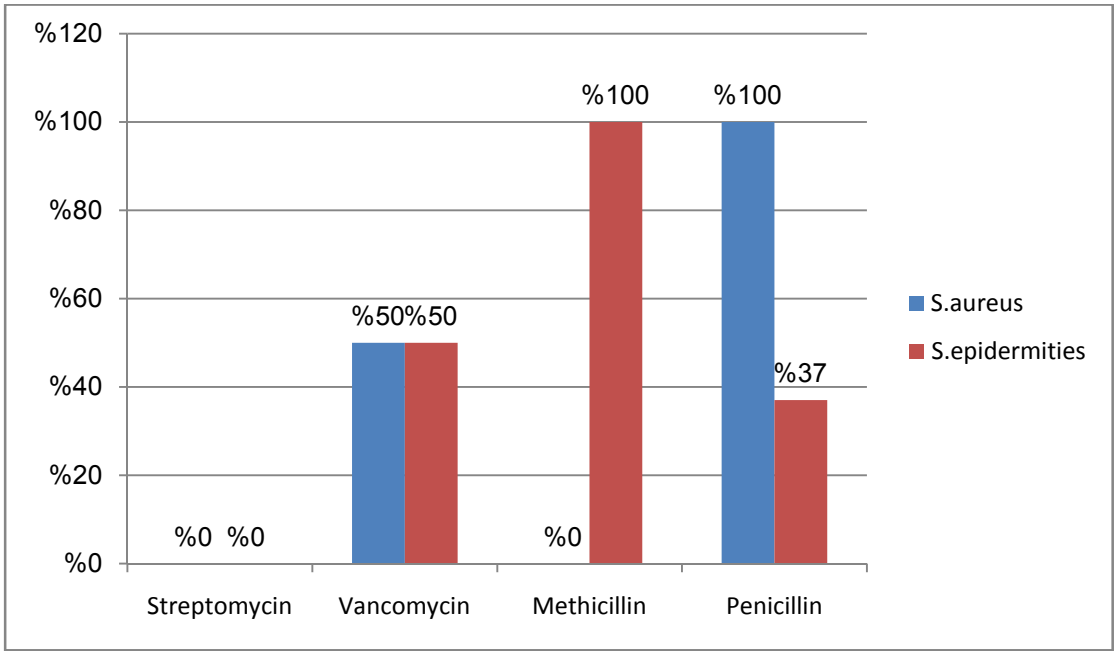

Figure.11. Levels of *Staphyllococcus sp* resistance to different antibiotics (Western sector)

Table.8. Sensitivity of the isolates to different antibiotics (Almansoura sector)

No	Isolate	Streptomycin	Vancomycin	Methicillin	Penicillin
1	*S. epidermities*	20(S)	11(R)	10(I)	7(R)
2	*S. aureus*	21(S)	11(R)	10(I)	7(R)
3	*S. epidermities*	20(S)	10(R)	10(I)	7(R)
4	*S. epidermities*	20(S)	11(R)	10(I)	7(R)
5	*S. aureus*	15(S)	10(R)	11(I)	8(R)
6	*S. aureus*	19(S)	13(R)	12(I)	7(R)
7	*S. epidermities*	21(S)	12(R)	10(I)	7(R)
8	*S. epidermities*	15(S)	11(R)	10(I)	8(R)
9	*S. aureus*	15(S)	11(R)	9(R)	7(R)
10	*S. aureus*	23(S)	17(S)	10(I)	7(R)
11	*S. aureus*	22(S)	12(R)	11(I)	7(R)
12	*S. aureus*	21(S)	11(R)	10(I)	7(R)
13	*S. aureus*	16(S)	11(R)	10(I)	8(R)
14	*S. aureus*	19(S)	15(R)	9(R)	8(R)
15	*S. aureus*	14(S)	19(S)	7(R)	9(R)

Where: **R**= Resistance, **I** = Intermediate susceptibility, **S** =Sensitive.

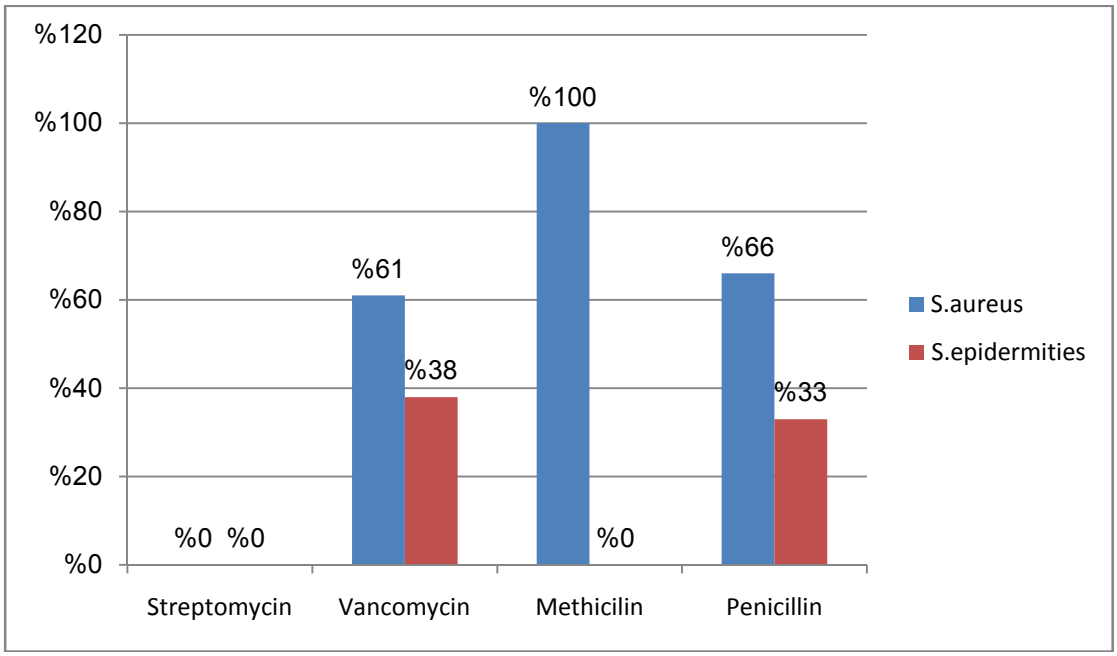

Figure.12. Levels of *Staphyllococcus sp* resistance to different antibiotics (Almansoura sector)

4.3 DNA extraction

Detection of extracted DNA bands from antimicrobial resistant *S. aureus* was done using gel electrophoresis (fig.13)

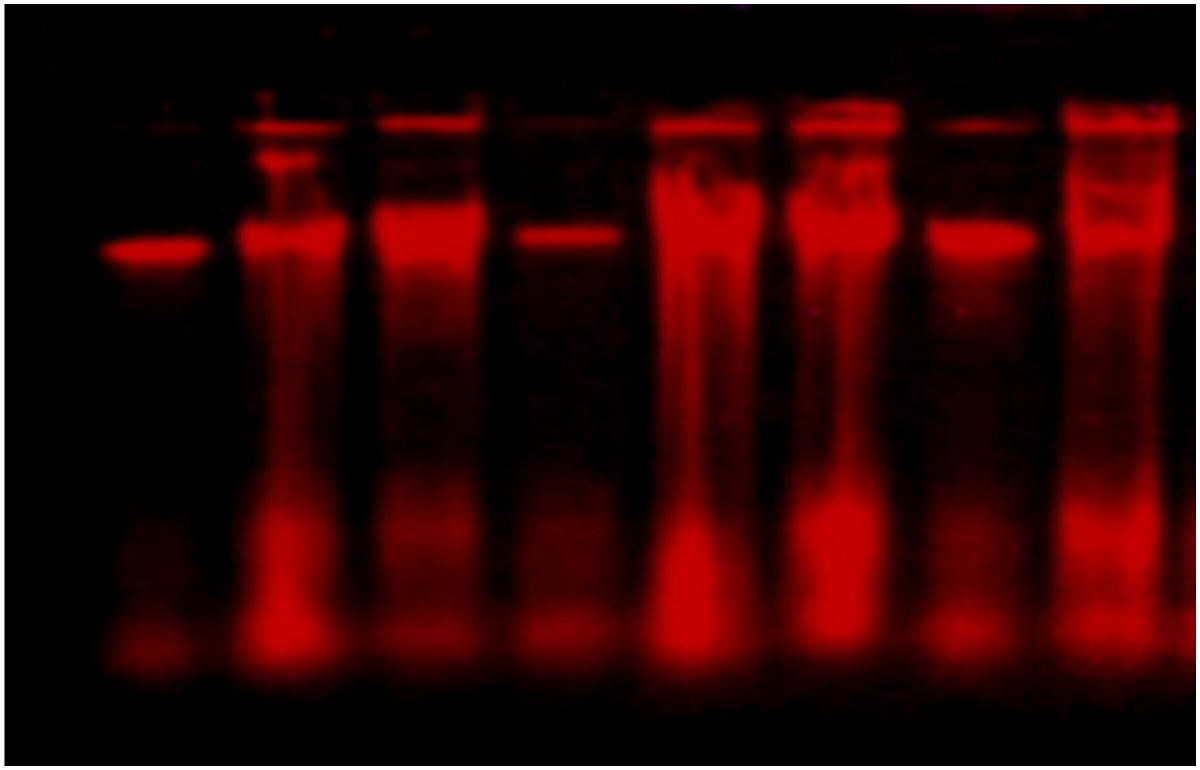

Figure.13. DNA detection with gel electrophoresis

4.4 Determination of Mec A gene

Amplification of thirty three DNA extracts from *Staphyllococcus aureus* and isolates with Mec A specific primers and Marker (M) are shown in figures (14-18).

PCR results were showed that 23(69%) isolates were positive to mecA gene (fig.19).

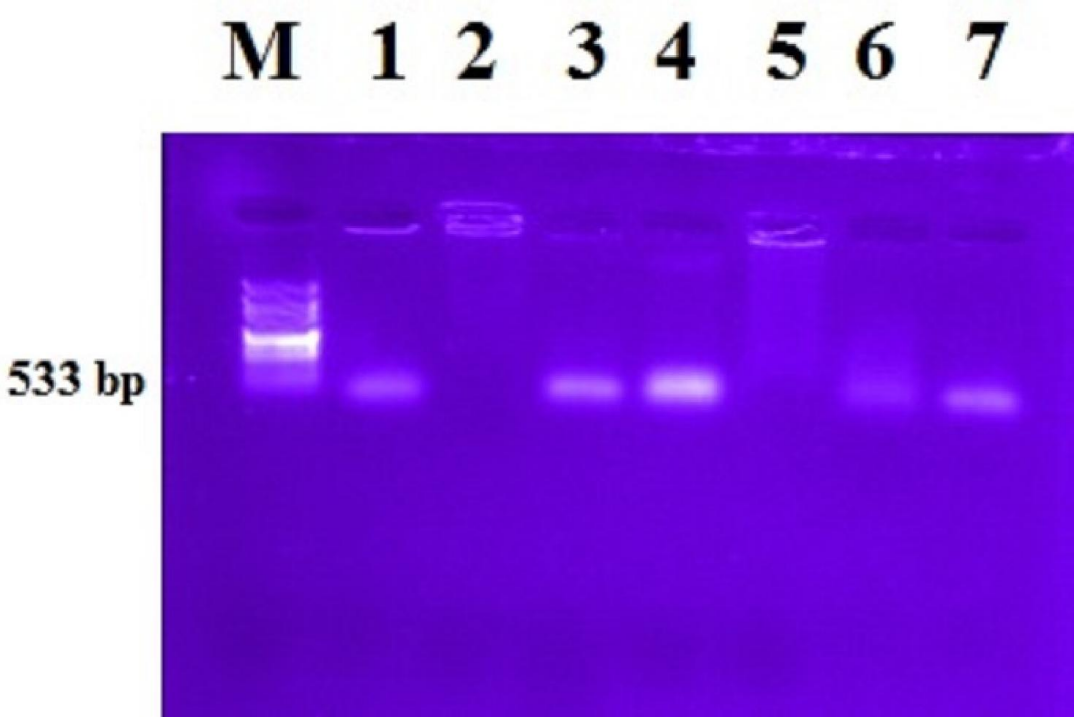

Figure.14. PCR amplification of the mec A gene for Methicillin resistance

Agarose gel (1%) used for separation of PCR products. Amplification of seven DNA extracts of *Staphyllococcus aureus* isolates with mecA specific primers and Marker (M) with different bands, lanes 1,3,4,6 and 7were positive to the mecA genes lanes 2and 5 were negative.

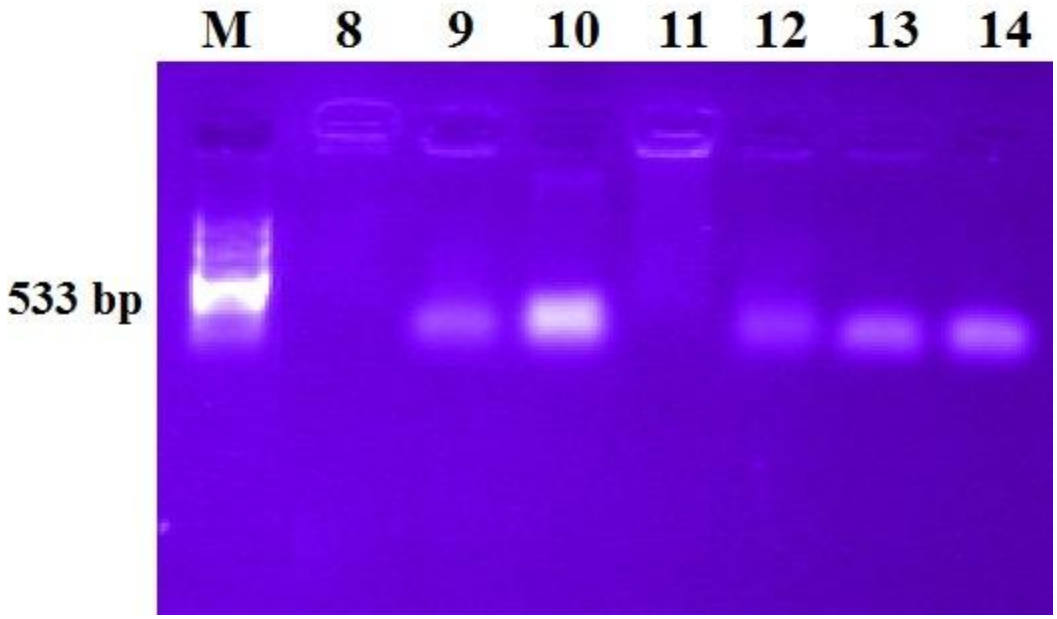

Figure.15. PCR amplification of the mec A gene for Methicillin

Agarose gel (1%) used for separation of PCR products. Amplification of seven DNA extracts of *Staphyllococcus aureus* isolates with mecA specific primers and Marker (M) with different bands, lanes 9,10,12,13 and 14were positive to the mecA genes lanes 8 and 11 were negative.

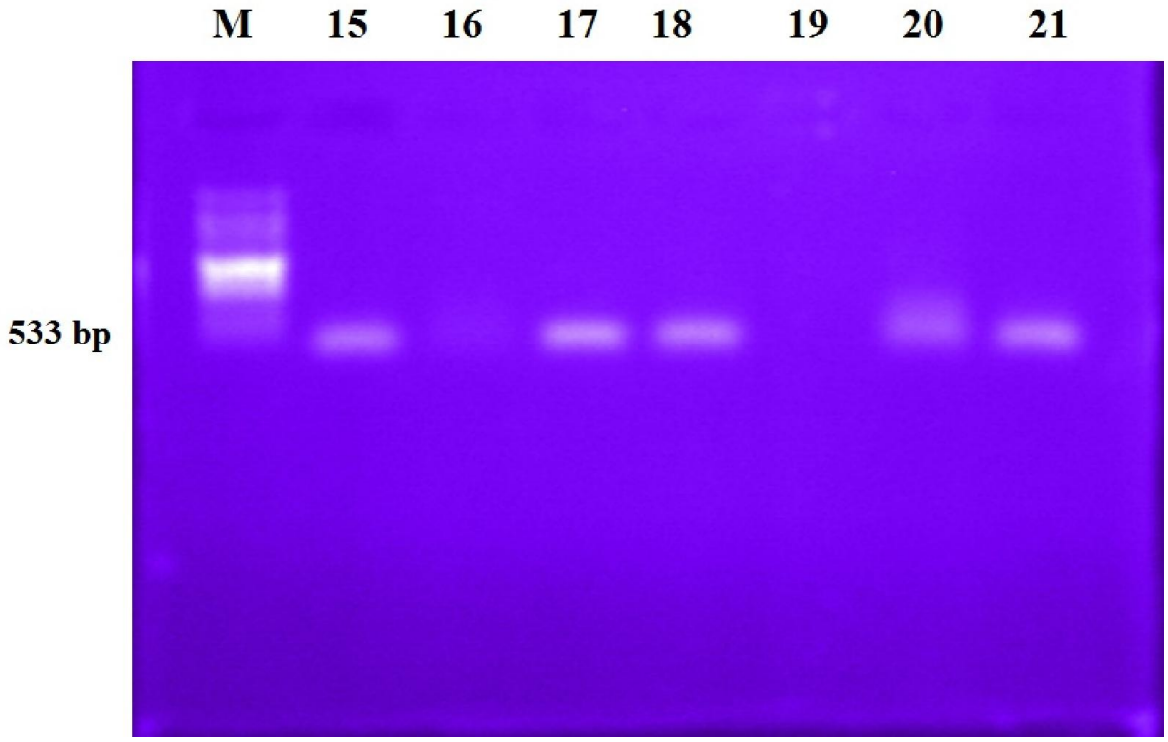

Figure.16. PCR amplification of the mec A gene for Methicillin

Agarose gel (1%) used for separation of PCR products. Amplification of seven DNA extracts of *Staphyllococcus aurus* isolates with mecA specific primers and Marker (M) with different bands, lanes 15,17,18,20 and 21were positive to the mecA genes lanes 16 and 19 were negative.

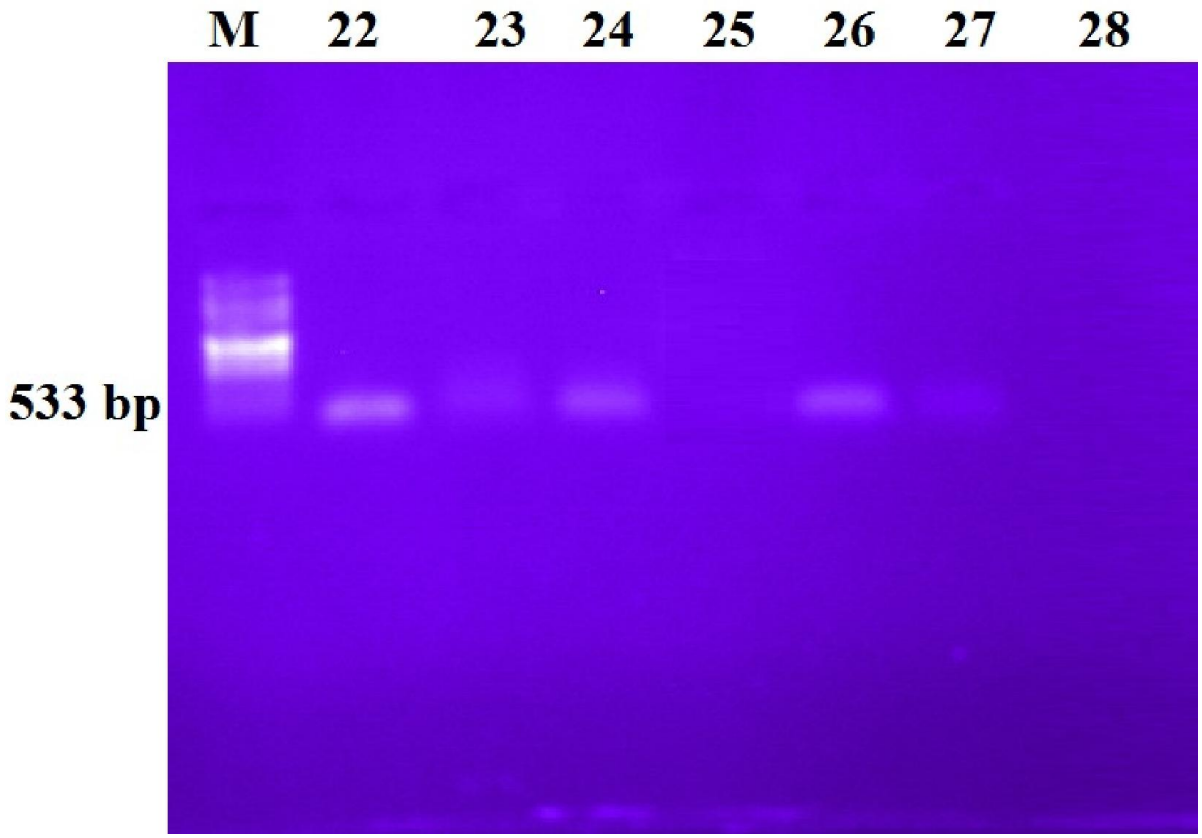

Figure.17. PCR amplification of the mec A gene for Methicillin

Agarose gel (1%) used for separation of PCR products. Amplification of seven DNA extracts of *Staphyllococcus aurus* isolates with mecA specific primers and Marker (M) with different bands, lanes 22, 24,26 and 27were positive to the mecA genes lanes 23, 25 and 28 were negative.

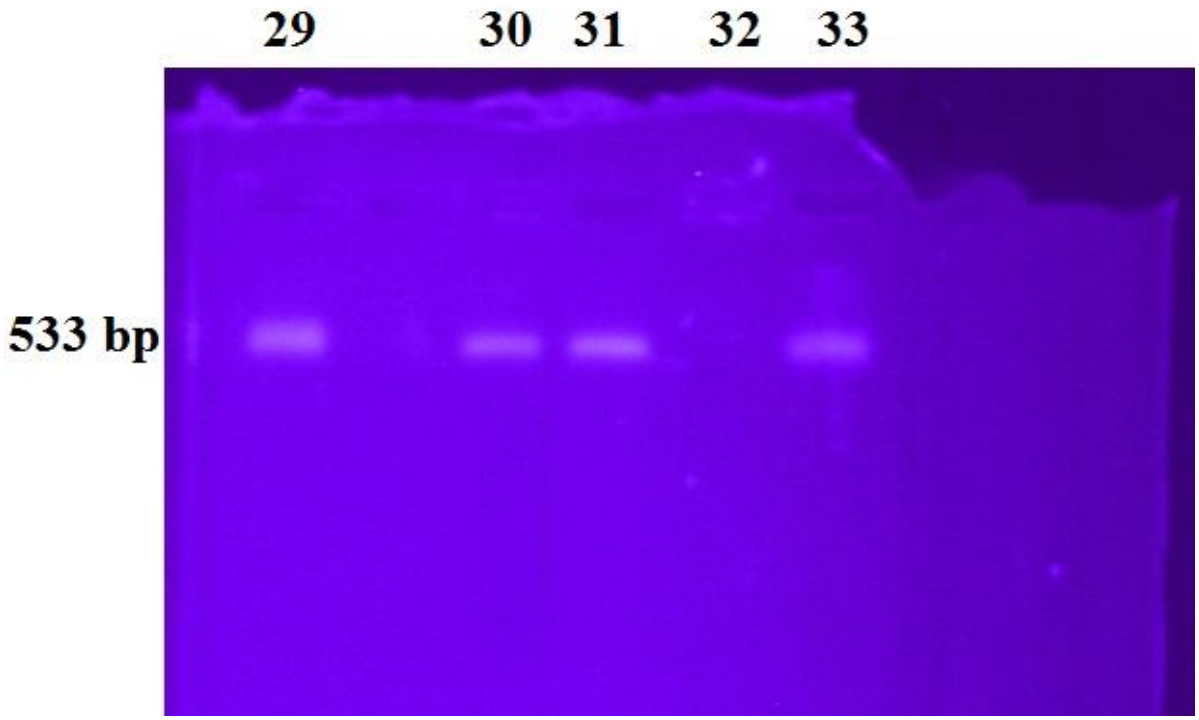

Figure.18. PCR amplification of the mec A gene for Methicillin

Agarose gel (1%) used for separation of PCR products. Amplification of five DNA extracts of *Staphyllococcus aurus* isolates with mecA specific primers and Marker (M) with different bands, lanes 29,30,31 and 33were positive to the mecA genes lane 32 was negative.

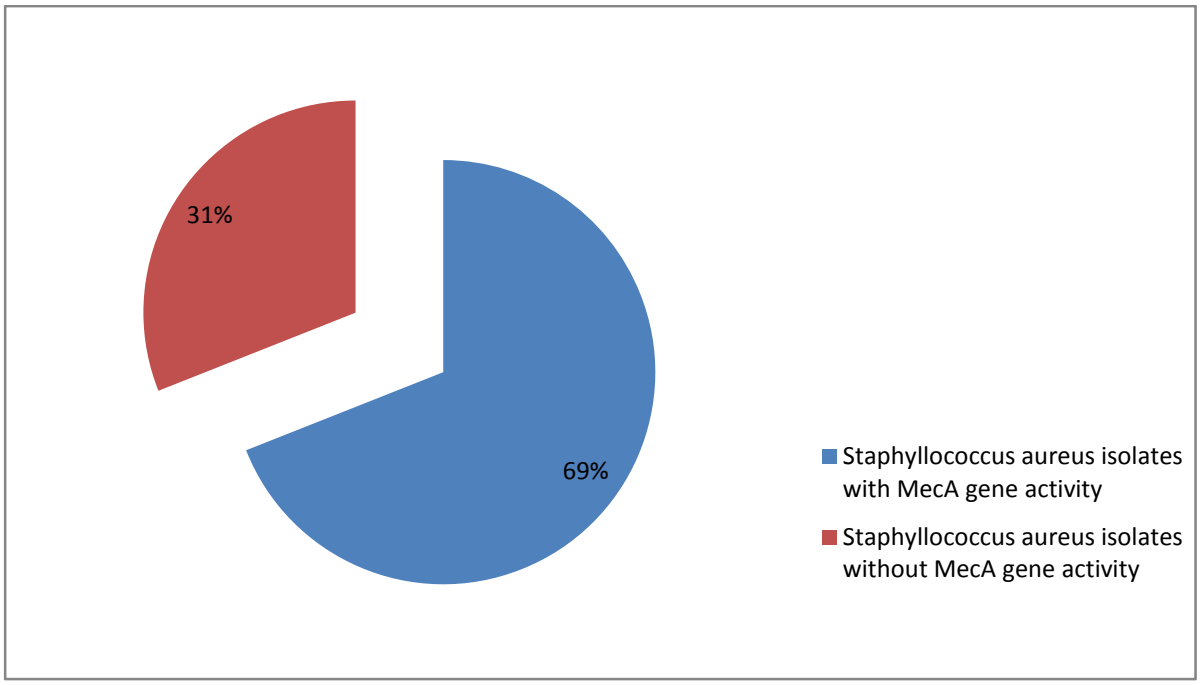

Figure.19. Genotypic resistance pattern of *Staphyllococcus aureus* isolates

4.5 Sequencing of Mec A gene

The sequence had “perfect” match (similarity, 99%) with sequences of their corresponding gene from gene bank as determined by using BLAST (version 2.7),(fig.20).

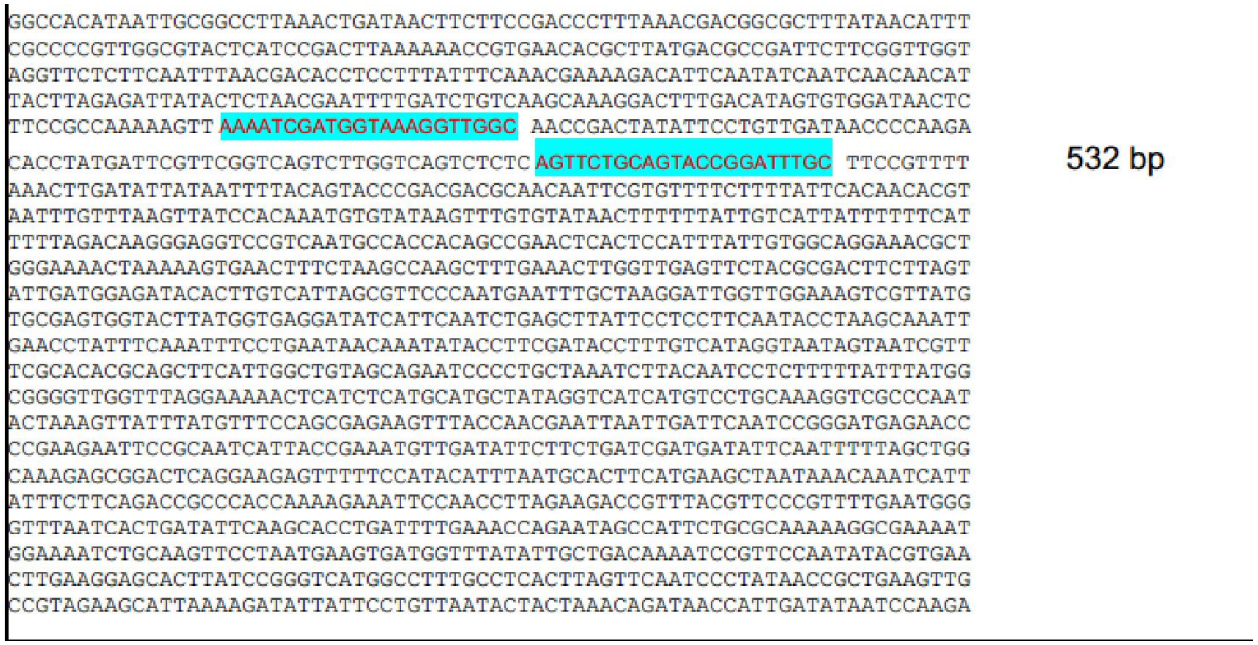

Figure.20. Nitrogen bases sequencing of the *mecA* gene in *Staphylococcus aureus* isolates

CHAPTER FIVE
DISCUSSION

5.1 Discussion

Although Staphylococci are normal flora of skin and mucous membranes of man and animal body, some of them have been associated with many health problems. They might cause disease by their presence in the animal body or by contaminating the food. Staphylococcal infections cause significant morbidity and mortality in both the community and hospital setting (Cosgrove *et al*., 2003).

Milk is normally sterile in the udder of the cow and buffalo provided they do not suffer from mastitis (udder infection). If they have mastitis, a large number of generally Gram positive bacteria such as *Streptococcus* and *Staphylococcus* spp. may be present in milk when it leaves the udder (Holm and Jespersen, 2003).

The results obtained from this research indicate the occurrence rate of subclinical mastitis among the 200 cows was 22.5 % (45 cows). This report is lower than the report of Sharma (2014) with a prevalence of 30.9% in subclinical mastitis and that of Ameh *et al*. (1999) with a prevalence of 31% from settled herds in Zaria and 57.7% in cows in Maiduguri by Bamayi and Aniesona (2013). The presence of *S. aureus* also shows deficient sanitary conditions of the cattle herd given that *S.aureus* is predominant as the cause of most cases of mastitis.

Many coagulase-negative staphylococci were isolated in this study. Recently, the coagulase negative staphylococci have been studied extensively because of their pathogenicity and involvement in some kinds of

human and animal diseases (Buttery *et al*., 1997; Kloos and Bannerman 1994; Mahoudeau *et al*., 1997; Pfaller and Herwaldt, 1988).

In this study all isolates showed resistance to penicillin this can be explained by the fact that penicillin was the most frequently used antibiotic in Sudan in many applications. Another study in Bangladesh conducted by Begum *et al*. (2007) revealed that *S. aureus* was 82.86% resistant to Penicillin, however in our study, we noticed that the antibiotic was 100% resistant by *S. aureus* and *S.epidermities*, indicating increasing resistance of the organism against Penicillin. Similar types of resistance pattern also reported by Islam *et al*. (2007).

All isolates of *S. epidermities* were resist vancomycin while only 26 (78%) isolates of *S.aureus* resist it, which is higher than (13%) recorded by Ahmed (2004).

The resistance of *S. aureus* to methicillin was found to be 18%. Usually methicillin is not widely used in livestock treatment, for that reason may be; some isolates were found to be at intermediate resistance to methicillin. Also (25%) from *S. epidermities* isolates were resist methicillin.

The isolates were fully sensitive to streptomycin in contrast to (57%) resistance obtained by Anueyiagu and Isiyaku (2015). The antibiotic sensitivity pattern of the isolates were significantly interesting and alarming for livestock and public health sector in ghebaish locality; but the attempts were unable to identify any streptomycin resistant *S. aureus* in the study.

Methicillin resistant *Staphylococcus aureus* (MRSA) is a major cause of hospital acquired (HA-MRSA) infections and the most significant multi resistant pathogens worldwide (Van *et al*, 2011). In this study, we used phenotypic (disk diffusion test) and genotypic (PCR method for mecA gene) methods for detection of MRSA. Rapid and accurate detection of methicillin resistance in *S. aureus* is important for the control of spread of MRSA strains and use of appropriate antimicrobial therapy.

For the strains included in this study, PCR assay was used for detection of methicillin resistance in 33 isolates of *S. aureus*, 23 (69%) mecA positive and 10(30%) mecA negative. this result disagreed with that reported by Tenover *et al* ,(1994) which was (8.7%) and less than (93.6%) reported by (Al-Khulaif *et al* 2009), in Saudi Arabia ,microbiological susceptibility testing and PCR results did not showed concordant results. Detection of mecA gene was considered the gold standard for MRSA confirmation (Chambers, 1997). The mecA gene, which is responsible for this resistance, is often associated in-vitro with resistance to all ß-lactam antibiotics. MRSA strains are frequently resistant to other classes of antibiotics and results compared with conventional methods of MRSA detection. Previous studies have reported discrepancies, noting that some strains lacking mecA displayed phenotypic resistance to methicillin while others containing mecA showed phenotypic susceptibility (Voss *et al*., 1994).

The prevalence of MRSA (69%) identified in this study was higher than that identified in Kwazulu-Natal province and in other major cities in South Africa such as Johannesburg (33%) and Cape Town (43%) (Shittu and Lin, 2006).

The results of this study showed that 6 (18%) of *S. aureus* isolates were recognized as MRSA by disc diffusion test method phenotypically, whenever 23 (69%) of isolates were mecA-positive in PCR genotypically, some of them were methicillin sensitive in disk diffusion test. This could be attributed to not consistently expression of mecA gene, this results were higher compared to that recorded by Safa and Ali,(2018) among 50 *S. aureus* isolates and mecA gene prevalence was 21 (42%) .

The rise of methicillin resistance may be due to antibiotic-resistant genes spread in the community, hospitals and healthy staff (Lederer *et al,* 2007). But the common thread among all of these studies, illustrate the variety of mecA gene in the risk of occurrence of resistant staphyllococcal infections. Thus, health plans and control infection measures should be taken to prevent this problem. Vancomycin has been the drug of choice for MRSA infections, but vancomycin-resistant *S. aureus* (VRSA) also emerged as a new challenge in infection management (Aligholi *et al,* 2008). However, in this study 26(78%) of *S. aureus* isolates was found to be resistant.

This work thus provides an initial database for genes responsible for methicillin resistance in *Staphyllococcus* strains isolated from raw cow milk from Ghebaish locality (west Kurdufan). The findings in this work expose the possible health risk in terms of transfer of drug resistance from these food animal to man. Methicillin as well as other Beta lactams is still the drug of choice in treating some life threatening infections in developing countries (Naas *et al*., 2005). It is important to monitor the emergence of resistant bacteria from animal foods, such animals may be important source of these resistant bacteria which can be spread from their products directly to man, it can jeopardize success of effective treatment thus constituting a potential grave public health hazard.

5.2 Conclusions

The study could be concluded in the followings:

- The study showed that *Staphylococcus* spp. in general, and *S. aureus* in particular, are common in raw cow milk in the study area and may impose a public health hazard (73%).
- Different Staphylococcus sp shows various responses to antimicrobial agents.
- Significant increase in the prevalence of methicillin-resistance in the *S. aureus* strains caused by the indiscriminate and excessive use of antibiotics during the last decade.
- PCR method is a useful method for detection of mecA genes which leads to rapid detection and identification of MRSA.
- Bacterial DNA extracted from *S. aureus* isolates reflected appearance of mecA gene which was responsible of methicillin-resistance.
- Also it is concluded that there is a widespread mecA gene activity in and around the Cows, causing antibiotic resistance of Staphyllococci and other species of bacteria.
- There is a real need for the control of indiscriminate antibiotic use which encourages antibiotic resistance, and thus exacerbating an existing global problem of antibiotic resistance.

5.3 Recommendations

It is recommended that:

1. Staphylococcal infections need to be prevented through proper boiling of milk and pasteurization.
2. Raising awareness of proper and correct sanitary behavior is to be observed.
3. Watching and preventing of contamination and pollution that may affect food and water resources.
4. Increase of hygienic measures among worker in the field of animal production, food and water resources.
5. Drug sensitivity test must be applied during diagnosis to determine the most effective drug.
6. When antimicrobial drugs are administered they should be given in full therapeutic doses for adequate period.
7. Antibiotics should not be provided without prescriptions.
8. Antibiotics with low effectiveness must be excluded.
9. Isolation and quarantine of sick animals from healthy animals.
10. Molecular detection and identification of mecA gene and other resistance plasmids should be put in mind for further research.
11. Further extensive work should be carried out in West Kurdufan state and its localities to survey the prevalence of antibacterial drug resistance genes of bacteria.

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APPENDIX

Figure .21. Ghebaish locality management

Figure .22.West Kurdufan University – College of Veterinary Medicine

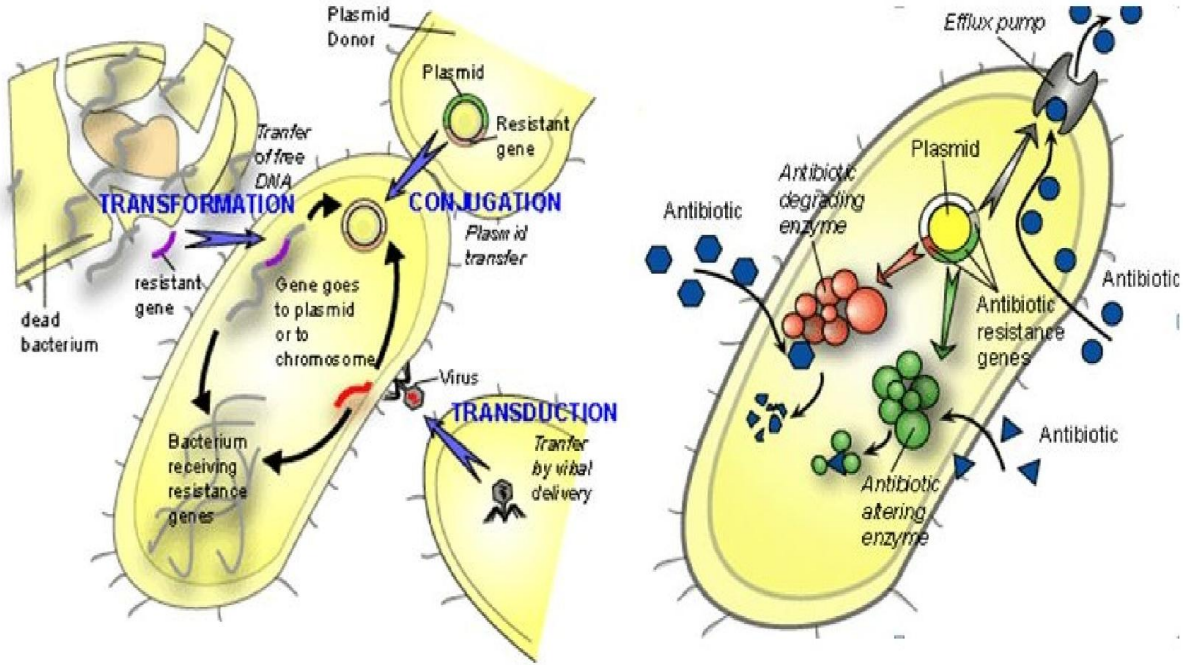

Figure .23. Mechanisms of Antibiotics resistance (genetic transfer)

REAGENTS

(1) Oxidase reagent:

1%-naphthol in 95% ethanol.

Tetramethyl-p-phenylenediamine dihydrochloride was prepared freshly each time by adding a loop full of it to 3 ml of D.W.

(2) Bromothymol blue:

Bromothymol sulfonephthalein 1%, BTB indicator in acidic medium (yellow), neutral (green), and alkaline (blue) according to pH concentration. Dissolve 0.10g in 8.0 cm^3 N/50 NaOH and dilute with water to 250 cm^3.

(3) V.P reagent:

Composed of 5%α-naphthol solution and 0.2ml 40%KOH aqueous solution.

(4) Stains (Gram stain):

a. Crystal violet

Crystal violet dye 10g, absolute methanol 500ml, dissolves the dye gently and stored at room temperature in screw caped bottle.

b. Safranines

Safranine dye 10g dissolved in 1000 ml – D.W.

c. Lugol's iodine

The 5% solution of lugol's iodine consists of 5% (wt/v) iodine (I2) and 10% (wt/v) potassium iodide (KI) mixed in distilled water. It is used as a mordant.

d. Ethanol

Used as a decolorized with absolute concentrations.

CULTURE MEDIA

A- Peptone water (Oxoid, CM9)

The medium contains (per liter) 10g of Peptone, 5g of Sodium chloride and final pH adjusted to 7.2±0.2 at 25°C.

Prepared by dissolving 10 grams of media in 1 liter of distilled water, heated to boiling to dissolve completely. Sterilization was done by autoclaving at 121°C, 15Ibs for15 minutes. The medium was mixed well and poured into sterile containers under aseptic conditions.

B- Mannitol Salt Agar (Oxoid, CM85)

The medium contains (per liter) 10g of Peptone, 1g of Lab-Lemco' powder, Mannitol 10.0g, Sodium chloride 75.0g, Phenol red 0.025g, Agar 15.0 and final pH adjusted to 7.2±0.2 at 25°C.

Prepared by dissolving 111 grams of media in 1 liter of distilled water and heated to boiling to dissolve completely. Sterilization was done by autoclaving at 121°C, 15Ibs for15 minutes. The medium was mixed well and poured into sterile petri dishes under aseptic conditions.

C- Baird-Parker Agar (Oxoid, CM275)

The medium contains (per liter) 10g of Tryptone, 5g of `Lab-Lemco' powder, Yeast extract 1.0g, Sodium pyruvate 10.0g, Glycine 12.0g, Lithium chloride 5.0g, Agar 20.0g and final pH adjusted to 6.8±0.2 at 25°C.

Prepared by dissolving 63 grams of media in 1 liter of distilled water then heated to boiling to dissolve completely. Sterilization was done by autoclaving at 121°C, 15Ibs for15 minutes. After cooling, 50ml of Egg Yolk-Tellurite Emulsion was added and mixed well before pouring.

D- Blood Agar Base (Oxoid, CM55)

The medium contains (per liter) `Lab-Lemco' powder 10.0g, Peptone Neutralised 10.0g, Sodium chloride 5.0g Agar 15.0, and final pH adjusted to 7.3±0.2 at 25°C.

Prepared by dissolving 40 grams of media in 1 liter of distilled water and heated to boiling to dissolve completely. Sterilization was done by autoclaving at 121°C, 15Ibs for15 minutes. For blood agar, cool the Base to 50° C, 7% of Defibrinated Horse Blood was added. Mixed with gentle rotation and poured into sterile petri dishes.

E- Glucose phosphate medium MR -VP medium (Oxoid, CM43)

The medium contains (per liter) 7g of peptone, 5g of Glucose,5g of K_2HPO_4 and final pH adjusted to 6.9 at 25°C.

Prepared by dissolving 5 grams of media in 1 liter of distilled water, heated to boiling to dissolve completely. Sterilization by autoclaving at 121°C for 15 minutes. Mixed well and poured into sterile containers under aseptic conditions.

F- Christensen's Urea medium (Oxoid, CM0053)

The medium contains (per liter)1.0g of Peptone,1.0 g of Glucose,5.0 g of Sodium chloride,1.2 g of Disodium phosphate,0.8g of Potassium dihydrogen phosphate,0.012 g of Phenol red, 15.0 g of Agar, pH 6.8 ± 0.2 at 25°C.

Prepared by dissolving 2.4 grams of media in 1 liter distilled water, heated to boiling to dissolve completely, sterilized by autoclaving at 121°C for 15 minutes. The medium cooled to 50°C and aseptically 5ml of sterile 40% urea solution was added. Mixed well, distribute 10ml amounts into sterile containers and allowed to set in slope position.

G- Nutrient Broth (Oxoid, CM1)

The medium contains (per liter) meat 5.0 g of peptone, 3.0 g of meat extract and final pH adjusted to 7.0± 0.2. It was prepared by dissolving 28.0 grams of powder (Oxoid) in 1 liter of distilled water, heated to boiling to dissolve completely. Sterilized by autoclaving at 121°C, for 15 minutes. Mixed well and poured into sterile containers under aseptic conditions.

H- Nutrient Agar (Oxoid, CM3)

The medium contains (per liter) Lab-Lemco' powder 1.0g, Yeast extract 2.0g, Peptone 5.0, Sodium chloride 5.0, Agar 15.0 and final pH at 25°C adjusted to 7.4±0.2.

The medium was prepared by suspending 28 grams in 1 liter water, heated to boiling to dissolve completely, sterilized by autoclaving at 15Ibs pressure at 121°C for 15 minutes.

I- DNAse agar (Oxoid, CM321)

Forty-two grams of DNAse agar (Oxoid) were suspended in one litre of distilled water and boiled to dissolve completely. The media was sterilized by autoclaving at 121ºC for 15 minutes then poured into sterile plates in 15 ml amounts.

J- Mueller Hinton Agar (Oxoid, CM337)

The medium contains (per liter) 300.00g of Beef- infusion, 17.50g of Cosein acid hydrolysate, 1.50g of Starch, 17.00g of Agar and pH adjusted to 7.4±0.2.

The medium was prepared by suspending 38.0 grams in 100 ml distilled water, and then heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 121°C for 15 minutes the medium was mixed well before pouring.