Genotypic and Phenotypic Methods for Detection of Methicillin Resistant *Staphylococcus aureus*

By

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Dedication

To my Mother, Father, Brother, Sisters, Aunt

To my small sweet family

To all people who helped me
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**Abbreviations**

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<td>MRSA</td>
<td>Methicillin Resistant <em>Staphylococcus aureus</em></td>
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<td>ORSA</td>
<td>Oxacillin Resistant <em>Staphylococcus aureus</em></td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>HA-MRSA</td>
<td>Health care associated Methicillin Resistant <em>Staphylococcus aureus</em></td>
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<td>LA-MRSA</td>
<td>Livestock associated Methicillin Resistant <em>Staphylococcus aureus</em></td>
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<td>SCCmec</td>
<td>Staphylococcal chromosomal cassette mec</td>
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<td>PBP2a</td>
<td>Penicillin binding proteins 2a</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>MH</td>
<td>Mueller Hinton</td>
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<tr>
<td>NacL</td>
<td>Sodium chloride</td>
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<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
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<td>CLSI</td>
<td>Clinical and Laboratory Standard Institute</td>
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Abstract

**Background:** Methicillin-resistant *Staphylococcus aureus* (MRSA) is a global public health problem. MRSA strains are frequently resistant to different class of antibiotics. Multi drug resistance among MRSA is a matter of concern for professional health workers worldwide. Therefore, an accurate detection of MRSA in microbiology laboratory is essential and of high value for patient management and epidemiological purpose including hospital infection control.

The present study was undertaken to compare various phenotypic methods including antimicrobial resistance tests (oxacillin disc diffusion, Cefoxitin disc diffusion and E-test Minimum Inhibition Concentration (MIC) oxacillin for detection of MRSA), comparing to the Genotypic methods by using Polymerase Chain Reaction (PCR) technique as gold standard method.

**Materials and Methods:** One hundred strains of *S. aureus* clinical isolates were used in the study. PCR for the presence or absence of the mecA gene and routine antibiotic susceptibility testing were performed including oxacillin (1µg), Cefoxitin (30µg), Amoxicillin (25µg), Erythromycin (15µg), Cefotaxime (30µg), E-test MIC oxacillin. All the isolates were tested for antibiotic susceptibility testing using kirby bauer disc diffusion method against a predefined panel of antimicrobials, and interpretation was done according to Clinical and Laboratory Standards Institute (CLSI) guidelines.

**Results:** Out of the 100 *Staphylococcus aureus* clinical isolates, 40 mecA gene positive and 60 mecA gene negative by PCR. Cefoxitin (30µg) disc diffusion test showed 97.5% sensitivity and 100% specificity while Oxacillin (1µg) disc diffusion test showed 92.5% sensitivity and 100% specificity. The resistance percentage of MRSA isolate to Amoxicillin (25µg), Erythromycin (15µg), and Cefotaxime (30µg) was 92.5%, 82.5%, 90% respectively. E-test MIC oxacillin showed 92.5% sensitivity and 100% specificity.

**Conclusion:** Results of Cefoxitin disc diffusion test is in concordance with the PCR for mecA gene. Thus, the test can be an alternative to PCR for detection of MRSA in resource constraint settings.
المستخلص

الخلفية: المكورات العنقودية الذهبية المقاومة للميثيسيلين (م.ر.أس.) هي مشكلة صحية عامة عالمية. وكثيراً ما تكون (م.ر.أس.) مقاومة للمضادات الحيوية. تعدد مقاومة دواء مضادات الميكروبات ما بين (م.ر.أس.) في مجال الصحة المهنية ومن أمثلة ذلك؛ الكشف الدقيق في مجال الاحياء الدقيقة ضروري، وذات قيمة عالية لإدارة المرضى والاوبئة بما في ذلك السيطرة على العدوى بالمستقبلات.

هذه الدراسة قامت بمقارنة معايير مختلفة واسع النطاق بما في ذلك اختبار مقاومة مضادات الميكروبات (نشر القرص اوكسيثيلين، نشر القرص سيفوكستين) واختبار لإلكترونات لثبيط اوكسيثيلين للكشف عن (م.ر.أس.) مقاومة بالأساليب الجينية باستخدام طريقة (تفاعل البلمرة المتسلسل) واستخدامه كأسلوب المعيار الذهبي.

منهجية البحث: مئات سلالة من المكورات العنقودية الذهبية المعزولة سريرياً، التي استخدمت في الدراسة، أُختبرت بواسطة تفاعل البلمرة المتسلسل لوجود أو غياب الجين ميغ أ (طريقة المعيار الذهبي) والاختبار الوراثي لحساسية المضادات الحيوية تضم اوكسيثيلين (امغ) سيفوكستين (30مغ) الاموكسيثيلين (25مغ) الايرثرومايثين (21مغ) سيفوتاكسيم (30مغ). اختبار الكترونات لثبيط اوكسيثيلين. كل العينات المعزولة تم اختبارها لحساسية المضادات الحيوية بطريقة كبري بنشر القرص المحددة مسبقاً في الجدول لمضادات المكورات وتصنيفها وفق معهد المعايير السريرية والمختبرية.

النتائج: من بين 100 من المكورات العنقودية الذهبية المعزولة سريرياً، إيجابية للجين ميغ أ و 60 منها سلبية للجين ميغ أ بواسطة تفاعل البلمرة المتسلسل. في اختبار نشر القرص السيفوكستين (30مغ) أظهرت حساسية 47.5% وتحديد 100%، بينما في اختبار نشر القرص الاوكسيثيلين (1مغ) أظهرت حساسية 92.5% وتحديد 100%.

الخلاصة: تتوافق نتائج اختبار نشر القرص سيفوكستين مع اختبار تفاعل البلمرة البوليميرات. وهكذا يمكن أن يكون الاختيار بديل لاختبار سلسلة البوليميرات في أعداد مصارع القيد.
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Chapter one

Introduction and Literature Review
1.1 Introduction

*Staphylococcus aureus* is considered as one of the most common causes of nosocomial and community-based infections, leading to serious illnesses with high rates of morbidity and mortality[1]. In recent years, increasing the number of bacterial strains that show resistance to methicillin become a serious clinical and epidemiological problem because of methicillin antibiotic is considered as the first option in the treatment of *staphylococci* infections, and because resistance to this antibiotic implies resistance to all beta-lactam antibiotics. For these reasons, accuracy and promptness in the detection of methicillin resistance is of key importance to ensure correct antibiotic treatment in infected patients as well as control of methicillin Resistant *Staphylococcus aureus* (MRSA) isolates in hospital environments, to avoid them spreading [1].

(MRSA) is a bacterium responsible for several difficulties in treating infections in humans. It is also called oxacillin-resistant *Staphylococcus aureus* (ORSA). MRSA is any strain of *Staphylococcus aureus* that has developed, through the process of natural selection, resistance to beta-lactam antibiotics, which include the penicillin (methicillin, dicloxacillin, nafcillin, oxacillin, etc.) and the cephalosporin [2]. Strains unable to resist these antibiotics are classified as methicillin-sensitive *Staphylococcus aureus*, or MSSA. The evolution of such resistance does not cause the organism to be more intrinsically virulent than strains of *S. aureus* that have no antibiotic resistance, but resistance does make MRSA infection more difficult to treat with standard types of antibiotics and thus more dangerous [2]. (MRSA) strains emerged soon after the introduction of methicillin into clinical practice. In addition to being a nosocomial pathogen, MRSA has become a community pathogen. Strains that possess *mecA* gene are either heterogeneous or homogeneous in their expression of resistance. The heterogeneous expression occasionally results in minimal inhibitory
Concentrations that appear to be borderline and consequently the isolates may be interpreted as susceptible [3].

1.2 Pathogenesis

Most MRSA infections occur in people hospitalized for more than 48 h in health centres. When it occurs in these settings, it's known as health care-associated MRSA (HA-MRSA). HA-MRSA infections typically are associated with invasive procedures or devices, such as surgeries, intravenous tubing or artificial joints. MRSA is especially troublesome in hospitals, prisons, and nursing homes, where patients with open wounds, invasive devices, and weakened immune systems are at greater risk of nosocomial infection than the general public. MRSA began as a hospital-acquired infection, but has developed limited endemic status and is now sometimes community-acquired. The terms HA-MRSA (healthcare-associated MRSA) and CA-MRSA (community-associated MRSA) reflect this distinction [2]. (CA-MRSA) infections most commonly manifest as skin and soft tissue infections, but more invasive infections, including sepsis syndrome, necrotizing pneumonia, and fasciitis, also occur [4,5]. Outbreaks of CA-MRSA infection have occurred among prisoners, sports participants, military recruits, and healthy full-term newborns [6,7]. In a population-based study in Atlanta and Baltimore, the incidence of CA-MRSA infection was highest among children <2 years old [4]. An infection was defined as healthcare-onset if the culture was obtained >48 hours after hospital admission. The proportion of S. aureus infections caused by CA-MRSA increased from 4% to 23% over 2 years; the proportion caused by healthcare-associated MRSA (HA-MRSA) decreased from 25% to 5%. Livestock-associated MRSA (la-MRSA). The scope of MRSA infection is not limited to human medicine only but also in Veterinary Medicine [8], MRSA was first considered a human infection until when it was isolated in a dairy cow with mastitis [9] and in pigs [10].
1.3 Clinical Signs

Infection with MRSA results in the same syndromes as *S. aureus*, which can cause a wide variety of suppurative infections. MRSA has been specifically isolated from various skin and wound infections including abscesses, dermatitis including severe pyoderma, exudative dermatitis in pigs, postoperative wound infections, fistulas, and intravenous catheter or surgical implant infections. The presence of suture material or orthopaedic implants seems to be linked to persistent infections in dogs and cats. MRSA has also been found in other conditions including pneumonia, rhinitis, sinusitis, otitis, bacteraemia, septic arthritis, osteomyelitis, omphalophlebitis, metritis, mastitis (including gangrenous mastitis) and urinary tract infections. Both *Bordetella bronchiseptica* and MRSA were isolated outbreak of fatal respiratory disease; the role of MRSA in the outbreak was uncertain. The post-mortem lesions of MRSA infections are those seen with any purulent bacterial infection, and vary with the organ system or tissue involved [11].

1.4 Aetiology

*Staphylococcus aureus* is a Gram positive, coagulase positive coccus in the family *Staphylococcaceae*. Methicillin-resistant *S. aureus* strains have acquired the mecA gene, which is carried on a large mobile genetic element called the staphylococcal chromosomal cassette mec (SCCmec). This gene codes for a penicillin binding protein, PBP2a, which interferes with the effects of beta lactam antibiotics (e.g. penicillins and cephalosporins) on cell walls. It confers virtually complete resistance to all beta-lactam antibiotics including the semisynthetic penicillins. Acquisition of mecA seems to have occurred independently in a number of *S. aureus* strains. Some clonal lineages of *S. aureus* have a tendency to colonize specific species,
and may be adapted to either humans or animals. Other lineages (“extended host spectrum genotypes”) are less host-specific, and can infect a wide variety of species. Some MRSA strains, called epidemic strains, are more prevalent and tend to spread within or between hospitals and countries. Other “sporadic” strains are isolated less frequently and do not usually spread widely. There are also MRSA strains that produce various exotoxins (e.g., toxic shock syndrome toxin 1, exfoliative toxins A or B, and enterotoxins) associated with specific syndromes, such as toxic shock syndrome. Community-associated MRSA strains that express a toxin called Panton Valentine leucocidin (PVL) have been linked to skin and soft tissue infections and severe necrotizing pneumonia. It is possible that PVL are associated with increased virulence in general, although this remains to be proven. Phenotypic methicillin resistance and/or the mecA gene has been reported occasionally in various animal-adapted Staphylococcus species. Some of these organisms can cause zoonotic infections or colonize people asymptotically. There are also concerns that they may transfer mecA to human-adapted staphylococci [11].

1.5 Transmission and Spreading

MRSA infections are contagious from person to person; occasionally direct contact with a MRSA-infected person is not necessary because the bacteria can also be spread by people who touch materials or surfaces contaminated with MRSA organisms. There are two major ways people become infected with MRSA. The first is physical contact with someone who is either infected or is a carrier (people who are not infected but are colonized with the bacteria on their body) of MRSA. The second way is for people to physically contact MRSA on any objects such as door handles, floors, sinks, or towels that have been touched by a MRSA-infected person or carrier. Normal skin tissue in people usually does not allow MRSA infection to develop; however, if there are cuts, abrasions, or other skin flaws such as psoriasis (a chronic
inflammatory skin disease with dry patches, redness, and scaly skin), MRSA may proliferate. Many otherwise healthy individuals, especially children and young adults, do not notice small skin imperfections or scrapes and may be lax in taking precautions about skin contacts. This is the likely reason MRSA outbreaks occur in diverse types of people such as school team players (like football players or wrestlers), dormitory residents, and armed-services personnel in constant close contact. A recent example of this spread of MRSA occurred in three NFL football players, all members of the same team, Tampa Bay. Three players got skin infections, and one had to undergo foot surgery to rid the player of recurrent MRSA infection [12].

1.6 Risk Factors

People with higher risk of MRSA infection are those with obvious skin breaks (for example, patients with surgical or traumatic wounds or hospital patients with intravenous lines, burns, or skin ulcers) and people with depressed immune systems (infants, the elderly, or HIV-infected individuals) or those with chronic diseases (diabetes or cancer). People with pneumonia (lung infection) due to MRSA can transmit MRSA by airborne droplets. Health-care workers as a group are repeatedly exposed to MRSA-positive patients and can have a high rate of infection if precautions are not taken. Consequently, health-care workers and patient visitors should use disposable masks, gowns, and gloves when they enter the MRSA-infected patient's room [13]. As long as people, including carriers, have MRSA organisms in wounds or droplets that are shed into the environment, they are contagious. Carriers must be very careful about personal hygiene (especially coughs, itching or scratching skin, and sneezing) as they may be contagious indefinitely.

*S. aureus* are reported to remain viable for 46 hours on glass, 17 hours in sunlight, and less than 7 days on floors under laboratory conditions. Environmental contamination with MRSA has been
reported in some veterinary practices, even at times when MRSA patients were not detected. Both animal-associated and human-associated MRSA strains have been found in meat. MRSA can also occur in raw milk and cheese. S. aureus is not ordinarily invasive when eaten, except under rare and unusual circumstances, and these organisms are mainly of concern in contributing to carriage or infection by direct contact [13].

1.7 Prevention and Control

1.7.1 Preventing HA-MRSA

In the hospital, people who are infected or colonized with MRSA often are placed in isolation as a precaution to prevent the spread of MRSA. Visitors and health care workers caring for people in isolation may be required to wear protective garments and must follow strict hand hygiene procedures. Contaminated surfaces and laundry items should be properly disinfected.

1.7.2 Preventing CA-MRSA

- **Washing hands.** Careful hand-washing remains your best defence against germs. Scrub hands briskly for at least 15 seconds, then dry them with a disposable towel and use another towel to turn off the faucet. Carry a small bottle of hand sanitizer containing at least 62 percent alcohol for times when you don't have access to soap and water.

- **Keep wounds covered.** Keep cuts and abrasions clean and covered with sterile, dry bandages until they heal. The pus from infected sores may contain MRSA, and keeping wounds covered will help keep the bacteria from spreading.

- **Keep personal items personal.** Avoid sharing personal items such as towels, sheets, razors, clothing and athletic equipment. MRSA spreads on contaminated objects as well as through direct contact.
- **Shower after athletic games or practices.** Shower immediately after each game or practice. Use soap and water. Don't share towels.

- **Sanitize linens.** If you have a cut or sore, wash towels and bed linens in a washing machine set to the hottest water setting (with added bleach, if possible) and dry them in a hot dryer. Wash gym and athletic clothes after each wearing [14].

### 1.8 MecA gene

MecA is responsible for resistance to methicillin and other β-lactam antibiotics [15]. After acquisition of mecA, the gene must be integrated and localized in the *S. aureus* chromosome [16]. mecA encodes penicillin-binding protein 2a (PBP2a), which differs from other penicillin-binding proteins as its active site does not bind methicillin or other β-lactam antibiotics. As such, PBP2a can continue to catalyse the transpeptidation reaction required for peptidoglycan cross-linking, enabling cell wall synthesis in the presence of antibiotics. As a consequence of the inability of PBP2a to interact with β-lactam moieties, acquisition of mecA confers resistance to all β-lactam antibiotics in addition to methicillin [16,17]. MecA is under the control of two regulatory genes, mecI and mecR1. MecI is usually bound to the mecA promoter and functions as a repressor [18,19]. In the presence of a β-lactam antibiotic, MecR1 initiates a signal transduction cascade that leads to transcriptional activation of mecA. This is achieved by MecR1-mediated cleavage of MecI, which alleviates MecI repression mecA is further controlled by two co-repressors, BlaI and BlaR1. blaI and blaR1 are homologous to mecI and mecR1, respectively, and normally function as regulators of blaZ, which is responsible for penicillin resistance. The DNA sequences bound by MecI and BlaI are identical: therefore, BlaI can also bind the mecA operator to repress transcription of mecA [20].
1.9 Diagnostic of MRSA

There are several methods for detecting methicillin resistance including classical methods for determining MICs (disc diffusion, E-test, or broth dilution), screening techniques with solid culture medium containing oxacillin, and methods that detect the mecA gene or its protein product (PBP2a protein) [22,23]. Detection of the mecA gene is considered as the reference method for determining resistance to methicillin [24]. However, many laboratories throughout the world do not have the capacity or the experienced staff required to develop molecular techniques for detecting MRSA isolates and it is therefore essential that other, more useful, screening methods are incorporated into routine clinical practice. The main objective of this study was to evaluate some of these methods in relation to the detection of mecA and to compare and contrast their suitability as routine methods for detecting MRSA isolates in clinical microbiology laboratories.
The recent development of Cefoxitin disc diffusion tests is likely to alter the recommendations for these methods as studies all suggest that tests with Cefoxitin are more reliable than those with oxacillin [25,26]. It is suggested that no special medium or incubation temperature is required with Cefoxitin [25,27], although some effect of temperature has been reported [26] there may be medium effects and the effects of inoculum have not been reported.

In assessing the performance of susceptibility testing methods the MIC determined by a dilution method has traditionally been the reference method; but methicillin MICs are affected by test conditions and some reports of erroneous results in studies of MRSA detection methods may actually be due to failure to detect resistance with the reference MIC tests. MIC methods have now been replaced as the reference method by molecular methods, which detect the mecA gene. Disc diffusion methods remain the most widely used in routine clinical laboratories, although some commercial systems for detection of methicillin resistance are available and automated methods are increasingly used. With phenotypic tests, in vitro conditions, such as test agent, temperature and NaCl concentration, have long been known to affect the expression of resistance [28, 29] and have been reviewed previously [30].

1.9.1 Oxacillin disk diffusion test

Accurate detection of oxacillin /methicillin resistance can be difficult due to the presence of two subpopulations (one susceptible and the other resistant) that may coexist within a culture of staphylococci [31]. All cells in culture may carry the genetic information for resistance, but only a small number may express the resistance in vitro. Oxacillin 1µg on Muller Hinton agar plate, incubate 35°C for 24 h then Zone diameter will measured Any growth after 24 h was considered oxacillin resistant [32,33].
1.9.2 Cefoxitin disc diffusion test and other Antibiotic

Cefoxitin 30µg has recently been investigated as an alternative agent for detection of resistance by disc diffusion and all studies indicate that tests are more reliable than those with oxacillin [25,26]. It has been suggested that no special test conditions are needed when testing Cefoxitin but the range of conditions tested has been very limited and results with one set of conditions should not be extrapolated to others without investigation to ensure that resistant and susceptible populations are adequately discriminated. And Erythromycin 15µg, Cefotaxime 30µg, and Amoxicillin 25µg on Muller Hinton agar plate, incubate 37°C for 24 h then Zone diameter will measured. The zone size was interpreted according to the National Committee for Clinical Laboratory Standards (NCCLS).

1.9.3 E-test Methods

The E-test method (AB Biodisk, Solna, Sweden) gives an MIC result and is affected by test conditions in a similar way to other MIC and diffusion methods. The test (bioMe´rieux, Marcy l’Etoile, France) conditions recommended by the manufacturer are based on providing results comparable with NCCLS methods and include MH, an inoculum density equivalent to 0.5–1.0 McFarland standards, application of inoculum with a strips and incubation at 35°C for 24 h. The E-test has an advantage over other MIC methods in that it is as easy to set up as a disc diffusion test.

1.9.4 Molecular methods (PCR)

The fact that high-level resistance to penicillinase-resistant penicillins is generally related to the presence of the mecA gene means that a radioactive label, enabling the safer utilization of the test system in a diagnostic laboratory, but even when used in either a dot blot or colony.
blot format, DNA probing involves a number of time consuming manipulations resulting in delayed reporting. These early probe studies also highlighted occasional discrepancies, in that borderline-resistant *S. aureus* isolates with a methicillin MIC of 8 µg/L were sometimes probe-negative, but produced large amounts of b-lactamase, which accounted for the elevated methicillin MIC [34]. More recently, PCR-based methods have been used routinely by reference laboratories as their standard method for detecting the mecA gene [35]. Occasional susceptible strains carrying a non-functional or non-expressed mecA, will also be detected, but the presence of mecA is generally considered to indicate a potential for resistance and is used as a marker to identify MRSA. Borderline resistance, which is not mediated by mecA, will not be detected and although they are uncommon such discrepancies have been highlighted in several studies [36,37] discrepant results can also arise because of a locally present strain which may lack the target sequence. It is important therefore that thorough local evaluation is made in different geographical regions. This comment also applies to molecular screening methods. Generally speaking, MRSA PCR assays that use a single amplification step are both robust and simple to perform. However, simple assays of this type are vulnerable to the presence of inhibitors, which will lead to a false-negative result, and the addition of a second set of primers to amplify a gene which is always present within *staphylococci* has been a very common control method. Primers directed against the nuc, coa and gyrA genes have been used for this purpose [38,39,40]. An alternative internal control involves the amplification of *S. aureus*-specific 16S rRNA [41]. This basic principle of the development of assays was applied to a new sensitive and specific molecular assay which has recently been described for the direct detection of MRSA [42]. Commercial kits are available that successfully identify the mecA gene in organisms previously identified as *S. aureus* [43] but these generally work only with purified cultures or enrichment screening broths [44,45].
1.2 Literature Review:

Methicillin-resistant *Staphylococcus aureus* (MRSA) were first reported in 1961 [46] and have since become a major nosocomial pathogen worldwide [47,48]. Guidelines for the control of MRSA infections in the UK have been previously published by a joint Working Party of the British Society for Antimicrobial Chemotherapy (BSAC), and the Hospital Infection Society (HIS) in 1986 [49], 1990 [50] and together with the Infection Control Nurses Association (ICNA) in 1998 [51]. Virtually all MRSA produce an additional penicillin-binding protein, PBP2a or PBP2 [52,53] which confers resistance to all currently available β-lactam agents. PBP2a is encoded by the mecA gene [54]. Additional genes, which are also found in susceptible isolates, can affect the expression of methicillin resistance in *S. aureus*, resulting in heterogeneity of resistance and making detection of resistance difficult [55,56].

In (2004) found that Cefoxitin disks were better than oxacillin disks in detecting methicillin resistant *S. aureus* [26]. In (2009) among 50 *S. aureus* strains found 28 MRSA by routine oxacillin disk diffusion test. They also showed that 30 isolates were MRSA in oxacillin agar screening and 32 isolates were resistant with Cefoxitin disk diffusion test and in these 32 isolates mecA gene was detected by PCR method. Their study showed that sensitivity and specificity for Cefoxitin disk diffusion test was 100% but other tests have less sensitivity and specificity. Some of the reports have shown different sensitivity and specificity for these three phenotypic tests for detection of MRSA [57]. In (2007) reported that the sensitivity and specificity of the Cefoxitin and oxacillin disk diffusion test were 84.6, 84.6, 87.5 and 79.2%; respectively. Also they found that the oxacillin agar screening was 92.3% sensitive and 45.8% specific [58]. In (2008) the sensitivity and specificity of the Cefoxitin and oxacillin disk diffusion test were 94.44, 100, 95.83 and 58.33%; respectively [59]. In (2010) showed the Cefoxitin and oxacillin disk diffusion
test and oxacillin agar screening was 100% specific but only the Cefoxitin and oxacillin disk diffusion test had 100% sensitivity. Also they found the oxacillin agar screening had the lowest sensitivity 82.2% [60].

Molecular typing techniques have been used with increasing frequency in studies of the epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) and also for a better understanding of the evolutionary relationships among MRSA clones [61,62,63,64]. One of the conclusions emerging from these studies was that a complete characterization of MRSA lineages requires not only identification of the genetic background of the bacteria but also identification of the structural types of the large and heterologous mec element, which carries methicillin resistance determinant mecA [64,65,66]. Studies by Ito et al. have elucidated the complete structure of three major mec elements, also referred to as the staphylococcal chromosomal cassette (SCCmec). Type I (34 kb) was identified in the first MRSA strain isolated in 1961 in the United Kingdom (strain NCTC10442), type II (52 kb) was identified in an MRSA strain isolated in 1982 in Japan (strain N315), and type III (66 kb) was identified in an MRSA strain isolated in 1985 in New Zealand (strain 82/2082) [67,68].

More recently, a smaller fourth mec element, SCCmec type IV (20 to 24 kb), was independently identified among representatives of the Pediatric clone [64] and in two community-acquired MRSA strains. In a recent report we described the characterization of the structural types of the mec elements carried by 28 MRSA isolates belonging to five widespread epidemic clones, which were all characterized by multilocus sequence typing (MLST) as single-locus or double-locus variants of two completely different genetic backgrounds [64]. The full structure of each mec element carried by these MRSA isolates was determined by Southern blot analysis using three restriction enzymes and several key probes specific for each SCCmec type, by PCR detection of
several loci covering the entire *mec* element structures, and by DNA sequencing, based on the information described in references [67,68]. However, these methods are laborious and time-consuming. In this paper we describe a multiplex PCR strategy which was designed to detect the structural variations observed in the *mec* element in our previous study. Such a method should provide a useful tool for the rapid tentative identification of the structural type of the *mec* elements in MRSA isolates.

In the recent years, MIC methods have been replaced by molecular methods which detect mecA gene become a gold standard for determining MRSA. However, there use is largely restricted to reference laboratories [69,70].
1.3 Rationale

Methicillin-resistant *Staphylococcus aureus* (MRSA) was emerged soon after once methicillin introduced in regular clinical practice and has become a one of leading cause of nosocomial infections as well as community pathogen [71]. Most of the times accurate detection of methicillin resistance *S.aureus* becomes difficult due to the presence of either heterogenous or homogenous in their gene expression of resistant [72]. Also heteroresistance grow more slowly than homogenous and that is why CLSI recommended incubating isolates tested against these antibiotics, for full 24 hours [31]. The gold standard for identifying MRSA is to detect the mecA gene, or its product, PBP 2a, by latex agglutination [73]. However performing this test is costly and may not be possible to be done at all the labs mainly in developing countries.

In recent years there are multiple published report suggest the use of Cefoxitin as surrogate marker for the detection of MecA gene mediated Methicillin-resistant *Staphylococcus aureus*. CLSI guidelines recommended Cefoxitin to be used to identify MRSA. According to CLSI Recommendation a 30 µg of Cefoxitin disk is used and a zone of less than 19 mm or equal is considered as resistant strain [31].

Also search through previous studies have found one study linked to the existence of PCR and phenotypic methods for detection of methicillin resistant *Staphylococcus aureus* outside Sudan and another studies are evaluating of a disk diffusion method with Cefoxitin (30 µg) for detection of methicillin-resistant *Staphylococcus aureus* and other study Identification of methicillin-resistant strains of *Staphylococci* by polymerase chain reaction. To best of our Knowledge no studies were conducted in Sudan for comparison of Genotypic and Cefoxitin disc diffusion test as phenotypic marker for MRSA.
The aim of our study was to evaluate the efficacy of Cefoxitin disc diffusion test and Oxacillin disc diffusion test as phenotypic methods for MRSA and compare with molecular detection of mecA gene by PCR.

1.4 Objectives

1.4.1 General objective:

The main goal of this study to compare of different phenotypic methods with Genotypic detection of MecA gene for disclosing of Methicillin-Resistant Staphylococcus aureus (MRSA).

1.4.2 Specific objective:

1. Comparison of Cefoxitin disc diffusion test, E-test method, and PCR for mecA gene for detection of MRSA.

2. To evaluate the efficacy of Cefoxitin disc diffusion test as phenotypic marker for MRSA and compare it with molecular detection of Mec A gene by PCR.

3. To evaluate the efficacy of Cefoxitin disc diffusion test as phenotypic marker for MRSA and compare it with E-test.
Chapter Two

Materials and Methods
2. Materials and Methods

2.1 Test organism

Standard bacterial control strains of *Staphylococcus aureus* was tested for their susceptibility to reference antibiotic were used and included Positive control *S. aureus* and negative control *S. aureus*.

2.2 Clinical bacterial isolate

The methicillin resistance *Staphylococcus aureus* was isolate from different clinical sample

2.3 Study design and study period

This is analytical descriptive a cross sectional laboratory based study was carried out during the period from March to October 2015.

2.4 Sample Size

One Hundred clinical isolates of *S. aureus* were collected From Soba University Hospital in Khartoum State. The sample size required for the study was calculated based on an estimated population prevalence of MRSA of 10%. A smaller sample size of 100 would give a confidence level of 90%.
2.5 Methods

2.5.1 Microscopical examination

All isolates were subjected to microscopical examination to study their stain properties (using Gram’s stain technique) to determine the shapes and arrangements of their cells.

In principle the bacterial film is fixed and stained with a Crystal violet, in conjunction with iodine solution and subsequently treated with an organic solvent such as Acetone alcohol. Bacteria which retain the dye are designated Gram positive. Decolorized organisms are rendered visible by the application of a counter stain of suitable color e.g. safranin red. Shapes and arrangements of the cells were also considered (74).

2.5.2 Biochemical tests

The biochemical activities of the purified isolates were then studied for identification and confirmation of these organisms. The biochemical tests that were carried out include the followings

2.5.2.1 Catalase test:

**Required:** hydrogen peroxide (3% H₂O₂) and wooden stick.

**Principle:** Catalase acts as a catalyst in the breakdown of hydrogen peroxides (H₂O₂) to water and oxygen. An organism is tested by bringing it into contact with hydrogen peroxides bubbles of oxygen are released if the organism is catalase producer.

**Methods:** Pour 2-3 ml of the hydrogen peroxides solution into test tube by using sterile wooden stick remove a good growth of the test organism and immerse it in the hydrogen peroxides solution and look for immediate bubbling.
**Results:** The positive test indicated by of active bubbling. [74].

### 2.5.2.2 Coagulase test:

**Required:** Undiluted human plasma, slide and physiological saline.

**Principle:** Coagulase lead to plasma clotting by converting fibrinogen to fibrin.

Two type of coagulase are produced by most strains of staphylococcus aureus:

A. Free coagulase detected by tube method

B. Bound coagulase (clumping factor) which converts fibrinogen directly to fibrin without requiring a coagulase reacting factors (CRF). It can be detected by the clumping of bacterial cells in the rapid slide test.

**Method:** Place a drop of physiological saline in each end of slide, emulsify a colony of the test organism in each of the drops to make two thick suspension and add a drop of plasma to one of the suspension and mix gently by rotating. Look for clumping of the organism within 10 seconds.

**Result:** Positive reaction was indicated by clumping within 10 seconds [74].

### 2.5.2.3 Deoxyribonuclease (DNase) test:

**Required:** DNase agar plate, 1ml of hydrochloric acid (1%HCL)

**Principle:** Deoxyribonuclease hydrolyze deoxyribonucleic acid (DNA)

**Method:** The test organism was cultured on a medium which contain DNA. After overnight incubation, the colonies are tested for DNase production by flooding the plate with a weak hydrochloric acid solution. The acid precipitates unhydrolyzed DNA, DNase producing colonies
are therefore surrounded by clear areas indicating DNA hydrolysis.

**Result:** Positive reaction was indicated by clearing around colonies [75].

### 2.5.3 Agar disk diffusion method

The disk diffusion method was adopted with some minor modifications to assess the antibacterial activity of the prepared extracts.

#### 2.5.3.1 Inoculums preparation:

Four to five of isolated colonies 16 to 24 hours of age were suspended in 0.85% saline solution to achieve a turbid suspension. Used of standard inoculum size is an important as culture purity and was accomplished by comparison of the turbidity of the organism suspension with a 0.5 Mcfarland turbidity standards. The standard provides an optical density comparable to the density of a bacterial suspension of $1.5 \times 10^8$ colony forming units (CFU) / ml [75].

#### 2.5.3.2 Inoculation of test plates:

A sterile cotton swab was dipped into the bacterial suspension standardized to match the turbidity of the 0.5 Mcfarland standard and pressed firmly on the inside of the tube to remove excess liquid. The dried surface of Muller Hinton agar plates were inoculated by streaking the entire surface and then repeated this twice, rotating the plate 60° each time. This will result in an even distribution of the inoculums. The inoculated plates were then allowed to dry within 15 min. Once the agar plate is completely dry, the disks were applied manually using sterile forceps. Each disc was lightly pressed down to ensure its contact with the agar. The discs were previously impregnated with 20 µl of extract at different concentration. Within 30 minutes of applying the discs, invert the plate and incubate it aerobically at 35°C for 18 h. After incubation the diameters
of the resultant growth inhibition zone were measured averaged and the values were tabulated [75.76]

2.5.4 Disc diffusion

Antibiotic susceptibility testing for all of *S. aureus* was performed according to the Clinical and Laboratory Standard Institute guidelines (CLSI). For phenotypic detection of MRSA, susceptibility to oxacillin (1µg), Cefoxitin (30µg), Erythromycin (15µg), Amoxicillin (25µg) and cefotaxime (30µg) were determined by Kirby-Bauer disk diffusion method. A bacterial suspension equivalent of 0.5 McFarland was inoculated onto a Muller-Hinton’s (MH) agar. Plates were incubated at 37°C for 24 h. Results were interpreted according to CLSI guidelines Reference strains included *S. aureus*.

**Medium:** Muller-Hinton (MH) agar media prepare according to the manufacturer’s instructions. After autoclaving, mix the media well and pour plates to a depth of 3-4 mm .Dry the plates and store them in refrigerator.

**Control strain:** Methicillin sensitive *S. aureus* (MSSA) ATCC 25923 and methicillin resistant *S. aureus* (MRSA) ATCC 43300 were used as negative and positive

**Incubation:** In oxacillin disc diffusion incubate at 35°C for 24 h aerobically .and in Cefoxitin disc diffusion and other antibiotics incubate at 37°C for 24 h aerobically .

**Reading:** Measure the zone to the nearest millimeter.

**Interpretation:** *S.aureus:* Oxacillin (1µg) $S \geq 12$ mm $R \leq 11$ mm, Cefoxitin (30µg) $S \geq 20$ mm $R \leq 19$ mm, Amoxicillin (25µg) $S \geq 20$ mm $R \leq 19$ mm, Erythromycin (15µg) $S \geq 23$ mm $R \leq 13$ mm, Cefotaxime (30µg) $S \geq 20$ mm $R \leq 19$ mm
2.5.4.1 Cefoxitin Disc diffusion test

All the isolates was tested for the Cefoxitin disc diffusion as CLSI protocol [31]. A 0.5 Mac Farland suspension of isolates was pleated on MHA and a 30 µg potency disc of Cefoxitin was placed plates. Zone diameter was measured after 18-20 hours of incubation. An inhibition of zone diameter of equal or less than 19 mm was considered as resistant and equal or more than 20 mm was considered as susceptible [31].

2.5.4.2 Oxacillin disc diffusion test

Mueller-Hinton agar (MHA) plates and 1 µg/ml of oxacillin was prepared. Plates was inoculated with 10 µL of 0.5 Mc Farland suspension of the isolate by streaking in one quadrant and was incubated at 35°C for 24 h. Plates was observed carefully in transmitted light for any growth. Any growth after 24 h was considered oxacillin resistant [32,33].

2.5.5 MIC by E-test Oxacillin

Reagents and equipment

- Agar plates (3.5-4.5 mm deep)
- E-test strips
- Sterile saline or appropriate broth (2-5ml)
- Sterile cotton tipped swabs
- Forceps
- Incubator with correct atmosphere at appropriate temperature
**Inoculation**

1. Subculture the organism to be tested onto a Muller Hinton agar plate and incubate for 18-24 hours to obtain a pure growth.

2. Remove the E-test package / storage tube from the -20°C freezer and allow the strips to reach room temperature for approximately 30 minutes. Replace the E-tests and container in the freezer as soon as you have finished using them. Do not use E-test past their expiry date.

3. Using a straight wire or loop, touch at least six individual colonies from the pure culture.

4. Emulsify the colonies in sterile saline or broth to give an equivalent turbidity to the appropriate McFarland Standard.

5. Within 15 minutes after adjusting the turbidity of the inoculum, immerse a sterile cotton swab into the emulsion. Press the swab against the inner side of the tube, above the fluid level, to remove excess fluid.

6. Use the appropriate plate and incubation conditions.

7. Inoculate the entire agar surface of the plate, by spreading the plate 3 times, rotating the plate 60° between the streaks and then swabbing the rim of the agar surface.

8. Place E-test of the appropriate antibiotics for the species in question (Appendix 1 of SOP MIC-001) on the plate manually using forceps.
• Do not touch the antimicrobial impregnated side of the E-test strip with your fingers.

• One or two E-test can be applied per 90 mm agar plate.

• E-tests need to be applied evenly on the dry agar surface with the MIC scale facing upward; press gently on the strip after application. Small bubbles will not affect the results.

• E-test should be applied no later than 15 minutes after the plates have been inoculated. Similarly, once the E-test are applied, they should be put in the incubator within a 15 minutes interval to prevent pre-diffusion of the antimicrobial at room temperature.

• After the incubation is complete, remove the plates from the incubator and measure the MIC, where the edge of the inhibition ellipse interests the side of the strip.

• If there is growth all along the strip and no visible ellipse, record the MIC as greater than (>) the highest value on the scale.

• If the inhibition ellipse is below the strip, then report the MIC as less than (<) the lowest value on the scale.

• For bactericidal drugs (e.g. beta-lactams), read the MIC at the point of complete inhibition of all growth including hazes, micro-colonies, and isolated colonies.

• Tilt the plate and use a magnifying glass if necessary.
• An E-test value which falls between two-fold MIC values (i.e. 0.06, 0.12, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256µg/ml etc.) on the strip should be rounded-up to the next two-fold value before categorization.

• Compare the rounded-up MIC with that for the species and antibiotic combination in SOP MIC-001. Record the MIC and the category taken from the table. If the organism and MIC is not included in the table then refer to the CLSI documents for further information. Results can usually be put into one of the categories below:

• **Susceptible (S)** includes isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used.

• **Intermediate (I)** includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels, and for which response rates may be lower than for susceptible isolates. The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated (e.g. quinolones and β-lactams in urine) or when a higher than normal dosage of a drug can be used (e.g. β-lactams). The “intermediate” category also includes a “buffer zone” which should prevent small, uncontrolled technical factors from causing major discrepancies in interpretation, especially for drugs with narrow pharmacotoxicity margins.

• **Resistant (R)** includes isolates that are not inhibited by the usually achievable
concentrations of the agent with normal dosage schedules and/or that demonstrate MICs or zone diameters that fall in the range where specific microbial resistance mechanisms are likely (e.g. β-lactamases) and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.

- **Nonsusceptible (NS)** is used for isolates for which only a susceptible interpretative criterion has been designated because of the absence or rare occurrence of resistant strains. Isolates that have MICs above the value indicated for the susceptible breakpoint should be reported as nonsusceptible. This does not necessarily mean that the isolate has a resistance mechanism: it is possible that isolates with MICs above the susceptible breakpoint that lack resistance mechanisms may be encountered within the wild-type distribution after the susceptible only breakpoint is set.

### 2.5.6 Detection of the MecA gene of *S. aureus*

This study was carried to determine the possibility of using bacterial culture for the detection of *S. aureus* using polymerase chain reaction (PCR). The set of primers for detection of MecA gene were used in this study to amplify the MecA gene with product size about 400 bp in length.

**Table 1: Primers for amplification of mecA gene**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Product size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>GCACTCGAATTAGGCAGTAAGA</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>GAGTTCTGCAGTACC GGATTT</td>
<td>400</td>
</tr>
</tbody>
</table>
2.5.6.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction was used for the amplification of specific DNA. It was first described by Mullis [77] and consists of a cyclic progression of denaturing the DNA, annealing of specific primers to the DNA and synthesis of the complementary strand by polymerase, which leads in every cycle to a duplication of DNA.

2.5.6.2 DNA extraction:

Add 2 to 3 colonies of bacteria to 200µl of phosphate buffer saline (PBS) which was pelleted at 10000 RPM for 3 min in centrifuge, and the supernatant was removed. The cell pellet was suspended in 200 µl of chelex, then the mixer put in hot plate at 100°C for 10 min ,the tube was mixed by vortex for 10 seconds during incubation period. Then cool to room temperature. finally, centrifuge for 1 min at 10000 RPM. Following centrifugation, the extracted supernatant was transferred to a sterile 1.5 ml Eppendorf tube. Genomic DNA was stored at -20°C.

2.5.6.3 Polymerase Chain Reaction (PCR) technique

Polymerase chain reaction (PCR) was carried out for the amplification of a target mecA gene by using genomic DNA template. PCR was performed in a volume of 20 µl via using ready master mix. 1µl forward primer and 1µl reverse primer were used. volume was completed to 20 µl per reaction mixed with distilled water. The master mix of all sample were mixed by vortexing in a sterile 0.2 ml PCR tube. The cycling program was initiated with a denaturation first step of ten minutes at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 seconds, Annealing at 52 °C for 30 seconds, extension at 72 °C for 1 minute, followed by a final extension at 72 °C for or 10 minute showed in ( table 2). The amplified products were checked on 1 % agarose gel,
stained with ethidium bromide, and visualized under UV light in order to determine the presence of specific band of the MecA gene.

Table 2: Standard PCR program used for MecA gene

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>52</td>
<td>30 sec</td>
<td>40</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>10 min</td>
<td>1</td>
</tr>
</tbody>
</table>

2.5.7 Data analysis:

The statistical analysis of the result was performed using the statistical package for social science (SPSS) windows version 16. Chi-square test was used for testing difference significance.
Chapter Three

Results
Results

From March to October 2015, totally, 100 strains of *Staphylococcus aureus* were isolated from different clinical specimens. A total of 100 clinical isolated of *S. aureus* were evaluated with PCR. The mecA PCR assay including the internal control allowed us to classify 40 (40%) of the isolates as *S. aureus* mecA-positive and 60 (60%) as *S. aureus* mecA-negative. The results of the some tested isolates with the positive and negative controls have been shown In (Figure 2)

![Image: Results of mecA gene PCR (400 bp product)](image_url)

**Figure 2: Results of mecA gene PCR (400 bp product)**

Lane 1: 100 bp molecular weight marker
Lane 2, 3, 4, 7, 8, 9, 10, 11 and 12: MRSA isolates
Lane 5, 6 and 13: MSSA isolates

Antimicrobial susceptibility pattern of *S. aureu* by disc diffusion method and MIC Oxacillin by E-test were conducted. High resistance was noted for erythromycin (82.5%), Amoxicillin (92.5%), Cefotaxime (90%), Cefoxitin (97.5%), and Oxacillin (92.5%) show in (table 3).
Table 3: Antimicrobial susceptibility pattern of methicillin resistant strains of *S. aureus* (MRSA) [n=100, 40 were MRSA]

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Susceptible</th>
<th>Resistant</th>
<th>Resistance %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin</td>
<td>3</td>
<td>37</td>
<td>92.5%</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>1</td>
<td>39</td>
<td>97.5%</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>4</td>
<td>36</td>
<td>90%</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>3</td>
<td>37</td>
<td>92.5%</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>7</td>
<td>33</td>
<td>82.5%</td>
</tr>
</tbody>
</table>

The results of the E-test by using supplemented MH agar is shown in (table 4). A high level of resistance (>32 µg/ml) was noted for oxacillin in 19 (19%) isolates, moderate level resistance (16-4 µg/ml) in 18 (18%) and low level resistance (<4 µg/ml) in 53 (53%) by E-test for oxacillin. The sensitivity (92.5%) and specificity (100%).

Table 4: Distribution of E-test oxacillin MIC values among *S. aureus* isolates (n=100)

<table>
<thead>
<tr>
<th>MIC value of E-test oxacillin (µg/ml)</th>
<th>Number</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;32</td>
<td>19</td>
<td>19%</td>
</tr>
<tr>
<td>16</td>
<td>7</td>
<td>7%</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>4%</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>7%</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>16%</td>
</tr>
<tr>
<td>0.5</td>
<td>11</td>
<td>11%</td>
</tr>
<tr>
<td>0.25</td>
<td>30</td>
<td>30%</td>
</tr>
<tr>
<td>0.125</td>
<td>6</td>
<td>6%</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100%</td>
</tr>
</tbody>
</table>
**Figure 3: MIC E-test oxacillin susceptibility 1.5µg/ml**

**Disk diffusion:** Results for Oxacillin 1µg disk incubation for 24 at 35 °C, in meca-positive isolates gave inhibition zone diameters of <11 mm, for meca-negative isolates, zone diameters for the 1µg disk >12 mm. The sensitivity (92.5%) and specificity (100%) shown in (table 5).

Results for Cefoxitin 30µg disk incubated for 20 to 24 h at 37 °C, in meca-positive isolates gave inhibition zone diameters of <19 mm, for meca-negative isolates, zone diameters for the 30µg disk >20 mm. The sensitivity (97.5%) and specificity (100%).
Table 5: Comparison of phenotypic method with genotypic method of detection of MRSA

<table>
<thead>
<tr>
<th>Test methods</th>
<th>Number of Detected as MRSA</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin 1µg</td>
<td>37</td>
<td>92.5</td>
<td>100</td>
</tr>
<tr>
<td>Cefoxitin 30µg</td>
<td>39</td>
<td>97.5</td>
<td>100</td>
</tr>
<tr>
<td>Amoxicillin 25 µg</td>
<td>37</td>
<td>92.5</td>
<td>98.4</td>
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<tr>
<td>Erythromycin 15µg</td>
<td>33</td>
<td>82.5</td>
<td>95.7</td>
</tr>
<tr>
<td>Cefotaxime 30µg</td>
<td>36</td>
<td>90</td>
<td>97</td>
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<tr>
<td>E-test oxacillin</td>
<td>37</td>
<td>92.5</td>
<td>100</td>
</tr>
<tr>
<td>PCR for mecA gene</td>
<td>40</td>
<td>100</td>
<td>100</td>
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</tbody>
</table>

Figure 4: Results of antibiotic disc diffusion test

OX: Resistance to Oxacillin 1 µg
FOX: Resistance to Cefoxitin 30 µg
AX: Resistance to Amoxicillin 25 µg
E: Sensitive to Erythromycin 15 µg
CTC: Resistance to Cefotaxime 30 µg
Chapter Four

Discussion
Discussion

The accurate and early determination of methicillin resistance is of key importance in the prognosis of infections caused by *S. aureus*. Although multiple methods of detection of this resistance have been developed, they are often too slow or not sufficiently sensitive or specific to ensure appropriate treatment of the MRSA-infected patients. The sensitivity and specificity values of the methods used also vary depending on the person carrying them out and the techniques used. Some of the reports have shown different sensitivity and specificity for these three phenotypic tests for detection of MRSA. Baddour *et al.* (2007) reported that the sensitivity and specificity of the Cefoxitin and oxacillin disk diffusion test were 84.6%, 84.6%, 87.5% and 79.2% respectively [58]. In another study by Jain *et al.*, (2008) the sensitivity and specificity of the Cefoxitin and oxacillin disk diffusion test were 94.44%, 100%, 95.83% and 58.33%; respectively [59]. Matos *et al.*, (2010) showed the Cefoxitin and oxacillin disk diffusion test and oxacillin agar screening was 100% specific but only the Cefoxitin and oxacillin disk diffusion test had 100% sensitivity [60]. The reported sensitivity and specificity of the oxacillin disk diffusion test for *S. aureus* is 90.4–98% and 83–99%, respectively [78, 79]. In general, in the most conducted studies, Cefoxitin disk diffusion test has shown the highest specificity compared to oxacillin disk diffusion and agar screening.

Detection of *mecA* gene or its product, penicillin binding proteins (PBP2a), is considered the gold standard for MRSA confirmation [24]. In a laboratory where it is not possible to carry out molecular method as a routine, Cefoxitin disk diffusion test is a good surrogate marker for detecting methicillin resistance. It is far superior to most of the currently recommended phenotypic method like oxacillin disc diffusion and oxacillin screen agar method. No special medium or incubation temperature is required for Cefoxitin as is required for oxacillin and
results are easy to read in both transmitted and reflected light. It is now an acceptable method for detection of MRSA by many reference groups including CLSI [80]. The present study revealed 40% methicillin resistance among *Staphylococcus aureus* isolates. Numerous studies have informed that the results of the Cefoxitin disc diffusion test correlates better with the presence of mecA compared with those of the oxacillin disc diffusion test and Oxacillin Screen agar test [26,57,81]. Similar finding were observed in present study.

In this study Cefoxitin disc diffusion was found to be 98.4% sensitive and 100% specific while the sensitivity of oxacillin disc diffusion was 92.5% and specificity 100%. On the other hand Cefoxitin is a better inducer of the expression of the mecA gene that’s why it better detect heterogeneous MRSA populations that variably express the mecA [26]. CLSI has also recently substituted the oxacillin disc with Cefoxitin disc for detection of MRSA [69]. In present study E-test MIC determination for oxacillin was found with sensitivity of (92.5%) and specificity of (100%).

Antibiotic susceptibility testing has been found to be a good epidemiological marker for MRSA phenotyping. In present study, among MRSA isolates high degree of resistance was encountered for Amoxicillin 25µg (92.5%), Erythromycin 15µg (82.5%), Cefotaxime 30µg (90%). This is similar to the finding of studies carried out by Sasirekha et al and Udo et al which also found high level of resistance to erythromycin [82,83]. MRSA become resistant to beta-lactam antibiotics due to mutation in PBP gene [54]. but resistance to other antibiotics is not well elucidated as yet.

In the recent years, MIC methods have been replaced by molecular methods which detect mecA gene which become a gold standard for determining MRSA. However, there use is largely restricted to reference laboratories [69, 70]. In current study, MIC strip test showed the
sensitivity and specificity about 91.6% and 100%, respectively. In the study of Rahbar et al., sensitivity and specificity were both 100% [84]. Three isolates in our study showed discordant results for PCR. This can be probably explained by the fact that not all S. aureus isolates express their mecA gene [85]. In the study of Farahani et al., the sensitivity and Specificity of the oxacillin disc diffusion method was 100% and 73.6%, respectively [86]. In previous study that performed by Pillai et al., the sensitivity and specificity were reported 93.5% and 83.5%, respectively [87].

Correct identification of MRSA using conventional methods is complex, and some strains are difficult to classify, a strain can appear susceptible by one method and borderline or resistant by another [88, 89]. For these reasons, several molecular methods have been developed to detect the mecA gene in MRSA clinical isolates [41,90]. Polymerase chain reaction (PCR) for amplification of the mecA gene is presently considered as the gold standard for detecting MRSA [91]. In spite of growing consensus in the literature for this method, it is not yet available in all clinical laboratories due to financial and technical constraints, therefore phenotypic methods, although dependent on many environmental and conditional factors still remains a method of choice in resource constraint laboratories [80].
Conclusion

Although the number of isolates are small, Present study revealed that the Cefoxitin disc diffusion method in routine susceptibility testing at 37 °C for 20 to 24 hours had a high sensitivity and specificity compare to other routinely used methods for detection of MRSA as compared to mecA gene detection by PCR. Hence, it can be used as an alternative to the technically demanding PCR.

Nevertheless, none of the techniques compared showed 100% sensitivity and specificity, although if the mecA gene detection method (PCR) is not accessible or available, it is advisable to combine two methods, one with high sensitivity and the other with high specificity.

Recommendation

In the absence of availability of molecular biology techniques, the Cefoxitin disc was the best predictor of methicillin resistance in S. aureus among the techniques tested.

Continuous monitoring and updating of data on antimicrobial susceptibility profile to reduce the spread of resistant strain.
References


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Appendix 1

Materials:

Equipment

Different equipment were used in this study such as Sensitive balance, Autoclave, Light Microscope with oil immersion lenses, Incubator, Hot air oven, Refrigerator, Wire loops with handles, Straight loops with handles, Cork borer (0.5 cm in diameter), Bunsen burner, Rack and Syringes.

Glass ware

Different Glass ware used in this study included Petri dishes, Flasks with different size, measuring cylinder, Beakers, Sterile containers (bijou bottle) and Test tubes.

Disposable materials

Disposable syringes, wooden application and sterile cotton Swabs were used during conduction of this study.
Appendix 2

Reagents:

Acetone-alcohol decolorizer

Content:

To make 1 liter:

Acetone ........................................... 500 ml
Ethanol or methanol, absolute ............. 475 ml
Distilled water ......................... 25 ml

Procedure:

1. Mix the distilled water with the absolute ethanol (ethyl alcohol) or methanol (methyl alcohol).
   Transfer the solution to a screw-cap bottle of 1 liter capacity.
2. Measure the acetone, and add immediately to the alcohol solution. Mix well.
3. Label the bottle, and mark it Highly Flammable. Store in a safe place at room temperature.
   The reagent is stable indefinitely.

Crystal violet Gram stain

Content:

To make 1 liter:

Crystal violet ......................... 20 g
Ammonium oxalate .................... 9 g
Ethanol or methanol, absolute ........ 95 ml
Distilled water ...................... To 1 liter

Procedure:
1. Weigh the crystal violet on a piece of clean paper. Transfer to a brown bottle premarked to hold 1 liter.

2. Add the absolute ethanol or methanol and mix until the dye is completely dissolved.

3. Weigh the ammonium oxalate and dissolve in about 200 ml of distilled water. Add to the stain. Make up to the 1 liter mark with distilled water, and mix well.

4. Label the bottle, and store it at room temperature. The stain is stable for several months.

5. Filter a small amount of the stain into a dropper bottle or other stain dispensing container.

**Kovac’s reagent**

**Content:**

Amyl or isoamyl alcohol ................. 150 ml

P-Dimethyl-aminobenzaldehyde .......... 10 g

Concentrated hydrochloric acid ........... 50 ml

**Procedure:**

Dissolve the aldehyde in the alcohol and slowly add the acid. Prepare in small quantities and store in the refrigerator. Shake gently before use.

**Lugol’s iodine solution**

**Content:**

To make 1 liter:

Potassium iodide ....................... 20 g

Iodine ................................. 10 g

Distilled water ........................ To 1 liter

**Procedure:**

1. Weigh the potassium iodide, and transfer to a brown bottle premarked to hold 1 liter.
2. Add about a quarter of the volume of water, and mix until the potassium iodide is completely dissolved.

3. Weigh the iodine, and add to the potassium iodide solution. Mix until the iodine is dissolved.

4. Make up to the 1 litre mark with distilled water, and mix well. Label the bottle, and mark it Toxic. Store it in a dark place at room temperature. Renew the solution if its colour fades.

**Physiological saline, 8.5 g/l (0.85% w/v)**

**Content:**

To make 1 liter:

Sodium chloride ......................... 8.5 g

Distilled water ......................... To 1 liter

**Procedure:**

1. Weigh the sodium chloride, and transfer it to a leak-proof bottle remarked to hold 1 liter.

2. Add distilled water to the 1 liter mark, and mix until the salt is fully dissolved.

3. Label the bottle, and store it at room temperature. The reagent is stable for several months. Discard if it becomes contaminated.

**Turbidity standard equivalent to McFarland 0.5**

**Content:**

Concentrated sulphuric acid .............. 1 ml

Dihydrate Barium chloride............... 0.5g

Distilled water ......................... 150 ml

**Procedure:**

1. Prepare a 1% v/v solution of sulphuric acid by adding 1 ml of concentrated sulphuric acid to 99 ml of water. Mix well.
2. Prepare a 1% w/v solution of barium chloride by dissolving 0.5 g of dihydrate barium chloride (BaCl₂.2H₂O) in 50 ml of distilled water.

3. Add 0.6 ml of the barium chloride solution to 99.4 ml of the sulphuric acid solution, and mix.

4. Transfer a small volume of the turbid solution to a capped tube or screw-cap bottle of the same type as used for preparing the test and control inocula.

When stored in a well-sealed container in the dark at room temperature (20–28 C), the standard can be kept for up to 6 months.

**Culture media**

**Mueller Hinton agar**

**Typical formula in g/L:**

- Beef infusion from ......................... 300g
- Casein acid hydrolysate .................. 17.5g
- Starch ....................................... 1.5g
- Agar ......................................... 17g

**Direction:**

Suspend 38g of powder in 1 Liter of distilled water heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 121 C⁰ for 15 minutes. Mix well before pouring.