Evaluation of the Conventional Test Methods for Diagnosis of Methicillin-Resistant *Staphylococcus aureus* (MRSA)

by

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CERTIFICATE

This is to certify that the dissertation entitled

"Evaluation of the Conventional Test Methods for Diagnosis of Methicillin-resistant *Staphylococcus aureus* (MRSA) in HUSM"

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Abbreviation

MRSA : Methicillin-resistant *Staphylococcus aureus*

MSSA : Methicillin-susceptible *Staphylococcus aureus*

MSO : Mannitol salt agar-oxacillin

MSC : Mannitol salt agar-cefoxitin

ORSAB : Oxacillin Resistance Screening Agar Base

CHROMagar :Chromogenic agar medium

MRSA-screen : MRSA-screen latex agglutination test

PCR : Polymerase Chain Reaction

PPV : Positive predictive value

NPV : Negative predictive value

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a multiple drug-resistant bacterium, which responsible for nosocomial and community-acquired infection. This study compared the mannitol salt agar-oxacillin (MSO), mannitol salt agar-cefoxitin (MSC), oxacillin resistance screening agar base (ORSAB), chromogenic agar medium (CHROMagar), and MRSA-screen latex agglutination test to *mecA* PCR method as the "gold standard" assay in order to determine the most simple, reliable and cost effective test method for diagnostic purpose. 58 MRSA strains were isolated form 144 samples collected from HUSM. The MSO, MSC, ORSAB, CHROMagar, and MRSA-screen gave sensitivity with 94%, 95%, 97%, 95% and 93% respectively, specificity with 98%, 87%, 97%, 95% and 99% respectively, positive predictive value (PPV) with 98%, 83%, 95%, 93% and 98% respectively, negative predictive value (NPV) with 95%, 96%, 98% 97% and 96% respectively, and cost with RM1.24, RM 1.38, RM 1.42, RM 8.50 and RM 12.50 respectively for one sample. All the test methods were agreed with PCR. In conclusion, ORSAB have the highest diagnostic value, followed by MSO, MRSA-screen, CHROMagar and lastly MSC. The MSO was the cheapest test method, followed by MSC, ORSAB, CHROMagar and then MRSA-screen. ORSAB can be the most suitable diagnostic test method with high diagnostic test value and low cost.

Abstrak

"Methicillin-resistant Staphylococcus aureus" merupakan sejenis bacteria yang rintang terhadap pelbagai antibiotik, menyebabkan jangkitan nosocomial dan jangkitan "community-acquired". Projek penyelidikan ini membuat perbandingan antara ujian "mannitol salt agar-oxacillin"(MSO), "mannitol salt agar-cefoxitin"(MSC), "Oxacillin resistance screening agar base"(ORSAB), "chromogenic agar medium"(CHROMagar), dan "MRSA-screen latex agglutination test"(MRSA-screen) dengan ujian PCR sebagai kaedah "gold standard". 58 strain MRSA telah diperolehi daripada 144 specimen yang didapati dari HUSM. Setelah analisis statistik, didapati bahawa MSO, MSC, ORSAB, CHROMagar dan MRSA-screen mempunyai sensitiviti 94%, 95%, 97%, 95% dan 93%, spesifisiti 98%, 87%, 97%, 95% dan 99%. Manakala, nilai anggaran positif pula adalah 98%, 83%, 95%, 93% dan 98%, nilai anggaran negatif 95%, 96%, 98%, 97% dan 96%. Harga untuk setiap ujian adalah RM 1.24, RM 1.38, RM 1.42, RM8.50, dan RM 12.50. Sebagai kesimpulan, ORSAB mempunyai nilai diagnostik yang paling tinggi antara kelima-lima kaedah ujian, diikuti dengan MSO, MRSA-screen, CHROMagar, dan MSC. Kaedah ujian yang paling murah adalah MSO, diikuti dengan MSC, ORSAB, CHROMagar dan MRSA-screen. ORSAB boleh menjadi kaedah ujian diagnostik yang paling sesuai disebabkan oleh nilai diagnsotik yang tinggi dan harganya yang murah.

1 Introduction

1.1 *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA)

Staphylococcus aureus is an aerobic or facultative anaerobic, coagulase-positive organism. It appears as gram positive clusters on Gram stain. Taxonomically, the genus *Staphylococcus* is in the Bacterial family *Staphylococcaceae*, which includes three lesser known genera, *Gamella*, *Macrococcus* and *Salinicoccus*, most of them living as harmless commensalisms on the skin and mucosa of mammals and birds (Kenneth, 2005). *Staphylococci*, however, also belong to the most frequently isolated microorganisms in medical microbiology, and *Staphylococcus aureus* is one of the species with the greatest clinical impact. It is an important human pathogen, which is responsible for nosocomial and community acquired infections (Karsten *et al.*, 2007; Sule *et al.*, 2007).

Methicillin-resistant *Staphylococcus aureus* is a multiple antimicrobial drug-resistant bacterium (Jayaratne and Ruthrford, 1999). Beside methicillin and most other beta-lactam antibiotics, MRSA strains are also often resistant to other antibiotics such as aminoglycosides, tetracyclines and quinolones (Daniel *et al.*, 2007; Fusun *et al.*, 2007).

1.2 Epidermiology

The first report of a penicillin- resistant strain of *S. aureus* was published in 1945, revealing its association with penicillinase enzyme produced by the bacteria (Spink and Ferris, 1945). The development of methicillin antibiotics, synthetic penicilinase resistant penicillin, followed within 2 years, *S. aureus* developed resistance to methicillin shortly there after (Stewart and Holt, 1962; Bareett *et al.*, 1968). Since the first report of methicillin-resistant *S. aureus* (MRSA) in the UK and Europe in the1960, and in the US in 1968, the incidence of infections caused by MRSA has continues to increase worldwide (Karsten *et al.*, 2007). It was recognized as an important nosocomial infection in the US in the late 1960's. It became endemic in some health care setting. The prevalence of MRSA infections varies from 5% to more than 50% depending on the characteristics and size of the hospitals. In Malaysia, the prevalence of MRSA has increased from 23% in 1991 to 33% in the year 2000. The prevalence of MRSA in Hospital Universiti Sains Malaysia (HUSM) was 40.4% in year 2003 (Sarimah, 2005).

1.3 Pathogenesis

Staphylococcus aureus causes a variety of suppurative (pus-forming) infections and toxinoses in humans. It causes superficial skin lesions such as boils, styes and furunculosis. It also causes more serious infections such as pneumonia, mastitis,

phlebitis, meningitis, and urinary tract infections; and deep-seated infections, such as osteomyelitis and endocarditis. It is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices. *S. aureus* causes food poisoning by releasing enterotoxins into food, and toxic shock syndrome by release of superantigens into the blood stream (Kenneth, 2005).

Staphylococcus aureus expresses many potential virulence factors: surface proteins that promote colonization of host tissues, invasins that promote bacterial spread in tissues (lekocidin, kinases, hyaluronidase), surface factors that inhibit phagocytic engulfment (capsule, protein A), biochemical properties that enhance their survival in phagocytes (carotenoids, catalase production), immunological disguises (Protein A, coagulase, clotting factor), and membrane-damaging toxins that lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin), exotoxins that damage host tissues or otherwise provoke symptoms of disease (SEA-G, TSST, ET) and inherent and acquired resistance to antimicrobial agents (Kenneth, 2005).

The mechanisms of methicillin resistance in the heterogenous and homogenous population of MRSA are diverse (Lyon and Skunay, 1987; Berger, 1995; Kayser, 1996; Araj *et al.*, 1999; Chamber, 1997). Primarily, its strong resistance is a result of its penicillin-binding protein 2a, which has a low affinity for beta-lactam antibiotics (Berger, 1994). Penicillin-binding protein 2a is encoded by the *mecA* gene which is in

the chromosome of methicillin resistant strains of many staphylococcal species (Lyon and Skunay, 1987; Archer and Niemeyer, 1994; Berger, 1995; Kayser, 1996; Chamber, 1997). Other genes such as *femA*, *femB* and *mecR1* can also contribute to MRSA resistance (Lyon and Skunay, 1987; Berger, 1995; Kayser, 1996; Chamber, 1997).

1.4 The Importance and Challenge for Detection of MRSA

Detection of MRSA in clinical sample is very important in diagnostic microbiology because bacteremia caused by MRSA increases the length of hospital stay, is responsible for rising health care cost, and has a high morbidity and mortality rate when compared with bacteremia caused by methicillin-susceptible *S. aureus* (MSSA). Therefore, a rapid and accurate detection of MRSA in clinical sample is essential to control the spread of MRSA in the hospital and plays an important role in early therapeutic decisions (Sule *et al.*, 2007).

A wide range of methods has evolved for the detection of MRSA in the clinical laboratory (Lee *et al.*, 2004). However, the detection of methicillin resistance in clinical microbiology laboratories is stills a considerable challenge to microbiologists because of the nature of the resistance. The expression of methicillin resistance is affected by different factors including incubation temperature, inoculum size, incubation time, salt concentration and a host of other factors. For these reasons, a number of conventional test methods have been used to detect accurately methicillin

resistance of both *S. aureus* and coagulase-negative staphylococci, but none has been able to detect it in all resistant strains. DNA methods that detect the genes encoding methicillin resistance (*mecA*) or its product, penicillin-binding protein 2a, are reliable and specific, however, they are technically demanding and expensive, which restricts their use in research or reference laboratories. Therefore, there is a need to have an evaluation of conventional test methods in order to identify the simplest, cost effective and reliable test that detects methicillin resistance rapidly (Udo *et al.*, 2000).

1.5 Conventional Test Methods

Several conventional methods, included the automated rapid method are used to isolate and identify MRSA. The methods are mannitol salt agar-oxacillin, mannitol salt agar-cefoxitin, oxacillin resistance screening agar base (ORSAB), chromogenic agar medium(CHROMagar), and MRSA-screen latex agglutination test (Sakoulas *et al.*, 2001; Jochen, 2002; Perry *et al.*, 2004; Lee *et al.*, 2004; Smyth and Kahlmeter , 2005; Louie *et al.*, 2006).

Mannitol salt agar supplemented with oxacillin (4mg/l) and cefoxitin (4mg/l) were developed as a selective medium for MRSA. Mannitol salt agar-oxacillin is widely used method but has shown limited sensitivity and specificity in some studies (John *et al.*, 2004). On other hand, cefoxitin has been reported as a better agent for prediction of methicillin-resistant in *S. aureus* (Smyth and Kahlmeter, 2005). Thus,

both methods were included in this study for evaluation. A modified version of mannitol salt agar, oxacillin resistance screening agar base (ORSAB), is more selective due to the presence of lithium chloride and polymyxin and contains aniline blue as a pH indicator (Perry *et al.*, 2004). CHROMagar is a chromogenic media incorporating chromogenic enzymatic substrate and a variety of antimicrobial agents. It has become available for detection of *S. aureus*, including methicillin-resistant strains (Louie *et al.*, 2006). It offered a high degree of sensitivity and specificity compared to conventional methods (Perry *et al.*, 2004). Lastly, the MRSA-Screen is a rapid and sensitive slide latex agglutination test for the detection of PBP2a present in MRSA. For the test, latex particles sensitized with a monoclonal antibody raised against the PBP2a protein agglutinate visibly when mixed with a cell extract of methicillin-resistant Staphylococcal strains (Udo *et al.*, 2002).

All these methods were evaluated and compared to PCR test results for *mecA* as the "gold standard" assay (Sakoulas *et al.*, 2001). Therefore, the objectives of this study were:

1. To isolate the MRSA from the specimen obtain from HUSM
2. To identify MRSA by using different conventional test methods and PCR
3. To evaluate the sensitivities, specificities, positive predicted value, and negative predicted value of different conventional test for the diagnosis of MRSA
4. To determine the cost effectiveness of each conventional test methods
5. To determine the most suitable test method for detection of MRSA

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2 Review of literature

For humans, *Staphylococcus aureus* are important causes of food poisoning, pneumonia, wound infection, and nosocomial bacteremia. Staphylococcal isolates are frequently resistant to penicillinase-resistant penicillins. Organisms exhibiting this type of resistance are referred to as methicillin (oxacillin)-resistant staphylococci (MRS). These bacteria are also frequently resistant to most of the commonly used antimicrobial agents, including the aminoglycosides, macrolides, chloramphenicol, tetracylcline, and fluoroquinolones. In addition, MRS strains should be considered to be resistant to all cephalosporins, cephems, and other beta-lactams, such as ampicillin-sulbactam, amoxicillin-clavulanic acid, ticarcilin-clavulanic acid, piperacillin-tazobactam, and thecarbapenems, regardless of the *invitro* test results obtained with those agents (Lee *et al.*, 2004).

According to Sarimah (2005), MRSA, one of the major concerns of nosocomial infections, is particularly alarming because due to its association with multiple resistance to other antibiotics. The current requirement for prolonged parenteral antibiotic therapy, particularly intravenous vancomycin for MRSA bacteremia, remains a major constant in reducing the length of acute hospitalization and costs of patient care. The high treatment costs indicate that the economic burden associated with MRSA is substantial and these costs are likely to increase if the rate of MRSA

continues to rise further.

Accurate and rapid identification of MRSA in clinical specimens is essential for timely decisions on isolation procedures and effective antimicrobial chemotherapy. Early detection of MRSA permits timely implementation of preventive infection control strategies and reduces costs. Standard procedures in clinical microbiology lead to difficulties in attempting to identify MRSA within time frames that allow routine grouping of newly admitted patients. Conventional processing of screening samples takes 2 or 3 days. PCR for *mecA* and *femB* provides reliable and unequivocal results for MRSA identification within 18 hours (Jonas *et al.*, 2002).

The tube coagulase test is a valid means of identifying *S. aureus*, provided that only a firm clot that does not move when the tube is tipped is consider as a positive reaction (Sperber and Tatini, 1975).

According to Kobayashi *et al.*, (1998), MRSA is defined by the production of a specific penicillin-binding protein (PBP), PBP-2a that has a reduced binding affinity for beta-lactam compound. PBP-2a functions as a transpeptidase in cell wall synthesis in MRSA at high concentrations of beta-lactam antibiotics that inhibit the growth of methicillin susceptible strains with normal PBPs. This additional PBP is encoded by the structural gene *mecA* on the chromosome, which has also been detected in methicillin-resistant strains of other staphylococcal species. The *mecA* gene is a

component of a large DNA fragment designated to *mec* DNA, which is located at the specific site of the *S. aureus* chromosome and has been suggested to be transmitted from other bacterial species. The acquisition of *mecA* DNA is considered to be the first genetic requisite for methicillin resistance of Staphylococci.

The gene encoding the altered PBPs is primarily the *mecA* gene. Other genes such as *femA*, *femB* and *mecR1* can also contribute to MRSA resistance. However, production of the PBP2a, an induced 76kDa PBP that determines methicillin resistance, was found to be the most commonly encountered mechanism (Araj *et al.*, 1999).

Several conventional methods have been used to detect the MRSA isolates including the 1mg oxacillin disk diffusion test, agar plate screen, the microbroth dilution and the E-tests. To enhance detection of methicillin resistant *Staphylococcus* isolates, most of these methods include media supplemented with sodium chloride. The reliability of these conventional methods is reported to be between 80 to 95%; results can only be read 24h after pure colonies of the organism have been obtained (Araj *et al.*, 1999).

Low-level MRSA colonization may be missed due to the overgrowth of normal flora. However, by using selective media, it is possible to increase the rate of recovery of MRSA isolates. Mannitol salt agar (MSA) supplemented with oxacillin as well as

oxacillin resistance screening agar base (ORSAB) rendered more selective by the presence of lithium chloride and polymycin, and containing aniline blue as a pH indicator are widely used. However, these media have shown limited sensitivity and specificity in several studies. The lack of sensitivity is due in part to the delay in the expression of oxacillin resistance, which explains why these media perform well only after 48h of incubation (Nsira *et al.*, 2006)

Smyth and Kahlmeter (2005) showed that the cefoxitin is a better agent for prediction of methicillin resistance in *S. aureus*, and disk susceptibility testing with cefoxitin now replaces disk susceptibility testing with methicillin and oxacillin in an increasing number of centers. Their results demonstrate that cefoxitin is far superior to oxacillin when it is used in this particular mannitol salt medium. By the use of 48h incubation and 10^2-CFU/ml inoculum, the sensitivity and specificity of the cefoxitin MSA plate method were 100% (compared to 90.7% with oxacillin) and 100% (compared to 96.0% for oxacillin), respectively, when the comparison was made for the strains used in both studies. Mannitol-salt agar with cefoxitin appears to be superior for MRSA screening, probably because cephamycins are better inducers of PBP2a (Smyth and Kahlmeter, 2005).

Several factors contribute to the successful isolation of MRSA on selective media. These include the ability of the medium to support the growth of MRSA, the sensitivity of the indicator system, and the effective inhibition of the competing

bacterial flora. The results for pure control strains at small inocula suggest that cefoxitin may be a superior selective agent to oxacillin with respect to the growth of MRSA. The author also reported that a concentration of 4mg of cefoxitin/liter allowed the growth of all MRSA strains and the inhibition of all MSSA strains (Lee *et al.*, 2004).

Since methicillin resistance can be found both in *S. aureus* and coagulase-negative Staphylococci (CoNS), rapid identification of *S. aureus* is required in addition to the detection of methicillin resistance. For this purpose, the use of media containing chromogenic enzyme substrates has been proposed. Studies have reported adaption of CHROMagar *S. aureus* for the specific isolation of MRSA by the inclusion of methicillin or oxacillin. These media were tested with strains of MRSA from collection and were found to be specific, but again more sensitive after 48h compared with 24h of incubation (Nsira *et al.*, 2006). Genetic confirmation of positive findings based on detection of the *mecA* gene has also been reported (Lee *et al.*, 2004).

MRSA-Screen is a rapid slide latex agglutination assay produced by Denka Seiken Co. Ltd. (Tokyo, Japan). It detects the PBP2a of methicillin-resistant staphylococci where the results showed that MRSA-Screen detected PBP2a accurately in all MRSA isolates tested. It correctly identified the MRSA an isolate classified as intermediate resistant by conventional disk diffusion method.

Furthermore, it correctly classified all MSSA isolates as methicillin susceptible with no false positive results. Results of the MRSA-Screen for MRSA isolates agreed with those obtained with *mecA* PCR and the conventional methods, making it a very reliable method for detecting methicillin resistance in *S. aureus* (Udo *et al.*, 2000). The MRSA-Screen latex agglutination test for PBP2a was easy to perform, gave results rapidly (15 to 20 min), was amenable to the processing of large numbers of samples, and approached the accuracy of PCR for *mecA* with respect to sensitivity (100%) and specificity (99.1%). Induction of PBP2a by beta-lactam (cefoxitin) seems to increase the sensitivity of the MRSA-Screen latex agglutination test, especially with coagulase-negative staphylococci (Sakalous *et al.*, 2001).

3 Materials and Methods

3.1 Sample Size Determination

The sample size of this study was calculated by using the following formula:

$$\text{Sample size} = \frac{Z^2(p)(1-p)}{C^2}$$
$$= \frac{1.96^2\,(0.4)(1-0.4)}{0.08^2}$$
$$= 144$$

Where,

Z = Z value (e.g. 1.96 for 95% confidence level)

p = percentage picking a choice, expressed as decimal

c = confidence interval, expressed as decimal

Thus, the total of sample that needed to be collected was 144.

3.2 Sample Collection.

3.2.1 Materials

Contents	Amount
Peptone water	10ml
Sterile swab	1pc

Table 3.1 List of materials for sample collection

3.2.2 Methods

There are mainly two sources of sample included in this study. 40 MRSA samples were collected from Medical Microbiology and Parasitology Laboratory's stock in HUSM. These MRSA isolates were recovered from colonies on Mullen Hinton Agar for susceptibility testing which diagnosed as MRSA positive. A total of 104 clinical samples were collected from different wads in HUSM. The samples were obtained from the nasal swab of patients, relative of patient and staff nurse in the ward. Then, the swabs media were kept in the bottle that contains peptone water as preservative and transported to the research lab for further processing immediately. Isolates were characterized as MRSA by several test method as described below. ATCC 43300 standard strains were used as positive control strains for *S. aureus*. ATCC 33591 standard strains were used as positive control strain for MRSA.