A research report, submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in the branch of Clinical Microbiology

Johannesburg, 2009
Detection of Bacteraemic Methicillin-Resistant *Staphylococcus aureus*

**DECLARATION**

I, Nelesh Premapragasan Govender, declare that this research report is my own work. It is being submitted, in partial fulfilment of the requirements for the degree of Master of Medicine in the branch of Clinical Microbiology, in the University of the Witwatersrand, Johannesburg. It has not been submitted before, for any degree or examination, at this or any other University.

___________________________

Nelesh Premapragasan Govender                                                Date: 21 July 2009
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**PUBLICATIONS AND/OR PRESENTATIONS ARISING FROM THE RESEARCH REPORT**

ABSTRACT

Introduction: The clinical laboratory is required to rapidly identify *Staphylococcus aureus* as a cause of bacteraemia, and in particular, to detect methicillin resistance amongst bacteraemic isolates, to facilitate prompt initiation of appropriate therapy which may directly impact on patient survival, and to allow for implementation of appropriate infection control measures. Hence, the laboratory needs to choose tests to detect methicillin-resistant *S. aureus* (MRSA) bacteraemia which are rapid, accurate, simple, cost-effective and appropriate for the setting.

Primary study objective: To determine the accuracy of four phenotypic susceptibility tests to directly detect MRSA from blood culture specimens (BC) compared with detection of the *mecA* gene by the polymerase chain reaction (PCR) from *S. aureus* cultured from the same BC.

Materials and Methods: BCs were selected from patients with incident, *S. aureus* bacteraemic episodes at two hospitals, during January and February 2006. *S. aureus* was identified by standard phenotypic tests, including the presence of a deoxyribonuclease (DNAse). Direct susceptibility tests (DST) were performed (oxacillin (1µg) and cefoxitin (30µg) disk diffusion (DD), oxacillin Etest® (AB bioMérieux) and CHROMagar®-MRSA (CHROMagar® Microbiology)), and repeated on stored cultures. Detection of *nuc* and *mecA* genes by PCR confirmed *S. aureus* and methicillin resistance respectively. The sensitivity and specificity of the DST were calculated with reference to the *mecA* PCR result, to fulfil the primary study objective.
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**Results:** During the two-month study period, 9,400 BC were submitted to the clinical laboratories at the 2 hospitals; *S. aureus* was isolated from 156 specimens. Of these, 89 BC from 89 incident cases were included in the study, and 65 were subjected to all tests, including PCR. Of the 65 *nuc*-positive *S. aureus* isolates from 65 BC, all were positive with the direct DNAse test, and 25 (38%) were meca positive. Compared to PCR, sensitivity and specificity for the direct oxacillin DD, cefoxitin DD, oxacillin Etest® and CHROMagar®-MRSA was 100% and 90%, 98% and 100%, 100% and 100%, and 96% and 42% respectively.

**Discussion:** In this study, we found that, compared to PCR for the *nuc* and meca genes, the combination of a direct DNAse test and oxacillin Etest®, facilitated accurate detection of MRSA bacteraemia. The direct oxacillin Etest® result did not appear to be influenced by a non-standardised inoculum, in contrast to the other direct tests, and provided an oxacillin minimum inhibitory concentration. The direct cefoxitin DD test produced more accurate results than the direct oxacillin DD test, was easier to read and distinguished MRSA from MSSA with zone diameters clustering into more clearly defined susceptibility categories. Although the chromogenic agar performed well when used to identify methicillin resistance amongst cultured *S. aureus* isolates, it was apparent that this test, read at 24 hours, could not be used reliably as a DST. Since the Etest® is more costly than the DD test; its use should be reserved for BC from patients in “high-risk” hospital areas, e.g. intensive care units. The direct cefoxitin DD could be used for all BC positive for GPCC, and could be used without a direct identification test because of its lower cost; it is further recommended that the direct cefoxitin DD test replace the direct oxacillin test.
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1. INTRODUCTION

1.1. Background

*Staphylococcus aureus*, which forms part of the normal, human commensal flora, causes serious, invasive, hospital- and community-associated infections, including bacteraemia, of which an increasing proportion is caused by methicillin-resistant *S. aureus* (MRSA) (1-3). The clinical laboratory is required to rapidly identify *S. aureus* as a cause of bacteraemia, and in particular, to detect methicillin resistance amongst bacteraemic isolates, to facilitate prompt initiation of appropriate therapy which may directly impact on patient survival (4), and to allow for implementation of appropriate infection control measures. In order to fulfil this responsibility, the laboratory needs to choose tests to detect MRSA bacteraemia which are rapid, accurate, simple, cost-effective and appropriate for the setting. In this background section, the pathogenesis of *S. aureus* is briefly reviewed to provide an understanding of how this wily organism is able to make the transition from coloniser to deadly, invading pathogen. In addition, the emergence and mechanisms of methicillin resistance, the global and local epidemiology of *S. aureus* and MRSA bacteraemia, and the adverse impact of MRSA bacteraemia are discussed. Finally, the strategies used by clinical laboratories to detect MRSA bacteraemia are summarised to provide context for this study.

1.1.1. Pathogenesis of Staphylococcal Bacteraemia

Colonisation provides a reservoir from which *S. aureus* may be transmitted person-to-person, or cause invasive disease (5). The anterior nares, which provide a major ecological niche for *S. aureus*, are colonised persistently in one-fifth of healthy people and intermittently in 30% of people (1). It has been suggested that colonisation may also induce protective immunity, specific to the colonising strain, and that this immunity may contribute to improved patient
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outcome if the same strain subsequently invades into the bloodstream (5). *S. aureus* has more than 100 well-characterised virulence factors which allow the organism to invade (1;6). These virulence factors, which include both structural and secreted products, facilitate attachment of *S. aureus* to host tissues, persistence of the organism outside of colonising sites, evasion or destruction of host defences, and development of sepsis. Staphylococcal surface proteins, collectively known as “Microbial Surface Components Recognising Adhesive Matrix Molecules” or MSCRAMMs, facilitate attachment of the organism to host tissue or foreign material. Outside of colonising sites, *S. aureus* is able to persist within biofilms (7), and in the context of in-vitro experiments, the organism is able to persist within host cells (8). These niches may also serve as a launch pad for bacteraemic infection. Key virulence determinants which assist the organism to evade the host immune response, at various stages of infection, include its anti-phagocytic capsule, surface-associated protein A which binds to the Fc portion of immunoglobulin and inhibits opsonisation, and secreted proteins which block neutrophil recruitment to the infection site (9). *S. aureus* also produces a range of enzymes, e.g. proteases, lipases, which facilitate penetration of tissue and subsequent, disseminated infection, and superantigens which may induce a sepsis-like syndrome by initiating a massive outpouring of cytokines (10). Expression of genes, which encode these virulence factors, is finely orchestrated by the organism, depending on the stage of infection, e.g. MSCRAMMs are expressed during the logarithmic (growth) phase, facilitating the establishment of colonisation, while secreted proteins, like enzymes, are produced during the stationary phase, facilitating spread of infection (1). The nature of infection also seems to influence expression of virulence genes, e.g. increased surface-associated protein A production is only associated with invasive disease, in the context of community-associated MRSA (CA-MRSA) infection (11). Host and environmental factors also play an important role in the pathogenesis of bacteraemia; patients with the following, well-described risk factors have a substantially
increased chance of developing staphylococcal bacteraemia: haemodialysis, peritoneal dialysis, HIV infection, intravenous-drug use, diabetes mellitus and alcohol abuse (12-15).

1.1.2. **Methicillin Resistance in \textit{S. aureus}**

MRSA was first isolated one year after methicillin was introduced into clinical practice in 1960 (16), reflecting what had happened twenty years earlier, with the rapid emergence of penicillin resistance amongst \textit{S. aureus} isolates (17;18). Methicillin was the first ß-lactam antibiotic designed to be resistant to hydrolysis by staphylococcal penicillinase, but was subsequently replaced by more potent and less toxic agents like cloxacillin, which could also be administered orally. Although methicillin is no longer used in clinical practice or to determine resistance in the laboratory (for reasons mentioned in paragraph 1.1.3.3 below), the term “methicillin resistance” is still used to denote resistance of staphylococci to all penicillinase-stable ß-lactams in current clinical use, and MRSA is a widely used acronym (19). High-level methicillin resistance in \textit{S. aureus} is mediated by acquisition of the \textit{meca} gene, which resides on a mobile genetic element called the staphylococcal cassette chromosome (SCC) \textit{mec}, along with its regulator genes, \textit{mecI} and \textit{mecR} (20;21). Methicillin-susceptible staphylococci have no allelic equivalent of the \textit{meca} gene, although clinical strains of methicillin-susceptible \textit{S. aureus} (MSSA) with this gene have been documented (22). The \textit{meca} gene codes for an alternative penicillin-binding protein (PBP), known as PBP2a or PBP2', which has reduced affinity for ß-lactam antibiotics (21). The interaction between a native PBP and a ß-lactam antibiotic starts with the formation of a non-covalent Michaelis complex, followed by formation of a more stable, covalent, acylated PBP-ß-lactam intermediate complex, with subsequent deacylation and dissociation of the intermediate complex, and generation of free, inactive PBP and hydrolysed ß-lactam. PBP2a has an altered interaction with ß-lactam antibiotics, with a reduced rate-constant for acylation, which results
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in resistance to β-lactam antibiotics (23). PBP2a retains its ability to cross-link peptidoglycan moieties, although less efficiently than native PBP (23), which may explain why expression of the *meca* gene is strongly regulated and is only “switched on” in the presence of β-lactam antibiotics. Heterogenous expression of the *meca* gene, within a staphylococcal population, is influenced by other genes which are also present in methicillin-susceptible strains, and confers “borderline” or “low-level” phenotypic resistance (24). Less commonly, “low-level” methicillin resistance has been described in strains which hyper-produce β-lactamase (20), or have altered native PBP (25). There have been no reports of therapeutic failure amongst patients, with infections due to penicillinase-hyper-producing strains, who are treated with penicillinase-stable β-lactams, so it appears that this laboratory phenomenon is of limited clinical importance. The clinical relevance of infrequently-isolated, clinical strains with altered native PBP is unknown.

1.1.3. Global and Local Epidemiology of *S. aureus* Bacteraemia

1.1.3.1. Hospital-associated Bacteraemia

While *S. aureus* has long been associated with hospital-associated infections, MRSA became a frequently-isolated, nosocomial pathogen in the mid-1980s, more than two decades after it first emerged (26). There is considerable geographic variation in the incidence of hospital-associated *S. aureus* bacteraemia across the world (26). Between 1995 and 2002, *S. aureus* was the second commonest cause of nosocomial bloodstream infection (BSI), reported by a hospital-based surveillance programme in the United States, accounting for 20% of all BSI cases, with a reported incidence rate of 10.3 BSI per 10,000 admissions, and a crude mortality rate exceeding 25% (3). In addition, amongst all *S. aureus* isolates, the proportion of MRSA increased from 22% in 1995 to 57% in 2002. In the United States, approximately 74,000 people were hospitalised for treatment of *S. aureus* bacteraemia from 1999 through 2000, with
more than 40% of the isolates identified as MRSA (27). A significant increase in the incidence of hospital-associated *S. aureus* bacteraemia at two large hospitals in the United Kingdom, between 1997 and 2003, was clearly associated with an increase in MRSA infection (28). In contrast, some countries in Western Europe have reported a consistently low prevalence of hospital-associated MRSA (HA-MRSA) infection (29). There are few incidence data for hospital-associated *S. aureus* bacteraemia in South Africa. The Antibiotic Study Group of South Africa (ASG), comprising investigators from public-sector laboratories affiliated to academic hospitals, reported that 1,889 unique, bacteraemic *S. aureus* episodes were diagnosed at 7 hospitals during 1983, accounting for 15% of all reported episodes (30). In 1999, the same group reported data from 8 hospitals; of 15,155 bacteraemic isolates, 1,692 (11%) were *S. aureus* (31). In 2007, the National Antimicrobial Surveillance Forum (NASF), including investigators at public-sector and private-sector laboratories, reported that almost one-fifth (2,040/10,467) of reported bacteraemic isolates at public-sector, academic hospital-affiliated laboratories were *S. aureus* (32). It is important to note that only data for selected pathogens, causing bacteraemia, were reported by the ASG and NASF; this limits any conclusions drawn about the rank order of *S. aureus* as a cause of bacteraemia amongst hospitalised South African patients. The proportion of bacteraemic *S. aureus* isolates, reported to ASG and NASF from two academic hospitals in Johannesburg, which was methicillin-resistant, remained fairly constant between 1983 and 2003: 32% (1983), 33% (1999); 30% (2001), 31% (2002), 35% (2003) (32;33). In 2007, an increased proportion (49%) of bacteraemic isolates was reported to be MRSA; this increase may reflect a nosocomial outbreak or may represent a reporting artefact (32).
1.1.3.2. Community-associated Bacteraemia

In the 1990s, MRSA emerged as an epidemic-prone infection amongst patients with no classic risk factors or direct hospital contact in developed countries like the United States (26). Although most patients with CA-MRSA infections present with skin and soft tissue involvement, other distinct syndromes, including bacteraemia, have been described (34). Unexpectedly, CA-MRSA has emerged more recently amongst hospitalised patients with classic risk factors for HA-MRSA infection (34). Very little epidemiological work has been performed to look at the role of CA-MRSA in South Africa. However, Oosthuysen and colleagues recently characterised 314 MRSA isolates, obtained from South African clinical laboratories (35). Of 302 isolates where SCC \textit{mec} type was established, 37% were SCC \textit{mec} type IV, which is classically associated with CA-MRSA. The genes associated with Panton-Valentine leukocidin (PVL), a putative virulence factor in CA-MRSA, were detected in 2 isolates. The proportion of bacteraemic \textit{S. aureus} episodes, in hospitalised South African patients, caused by CA-MRSA strains, has not been documented.

1.1.4. Adverse Impact of MRSA Bacteraemia

Although there is no evidence at present that MRSA is more virulent than MSSA (1), patients with MRSA infection are still likely to have a worse overall outcome, compared to patients with MSSA infection (36;37). Several factors may adversely impact on patient outcomes: (a) initiation of appropriate antimicrobial therapy may be delayed with risk of treatment failure (4;38); (b) vancomycin, which remains the mainstay of therapy for MRSA bacteraemia, is associated with slower bacterial clearance and poorer response rates, compared to anti-\textit{staphylococcal} beta-lactams (39); (c) the patient with MRSA bacteraemia may be subject to additional investigations and procedures; and (d) may be hospitalised for a longer period of time, with attendant complications (40). The hospital is also adversely affected by frequent
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development of MRSA bacteraemia amongst patients, e.g. cost of infection control procedures to minimise horizontal transmission, including use of isolation and laboratory screening, use of broad-spectrum empiric antimicrobial therapy, and longer length of hospitalisation (40). In addition, MRSA may spread in the community, with the need for re-admission to hospital (26). Overall, MRSA infections have an adverse impact on the patient, the hospital and the community.

1.1.5. **Detection of MRSA Bacteraemia by the Clinical Laboratory**

1.1.5.1. **Direct Susceptibility Testing**

MRSA bacteraemia is diagnosed by most clinical laboratories when positive blood culture specimens, detected by automated blood culture systems, are microscopically examined for the presence of Gram-positive cocci in clusters (GPCC), and inoculated onto solid agar media to allow growth, followed by conventional identification and susceptibility test procedures. Direct susceptibility testing (DST), which is performed directly on blood culture specimens, offers the advantage of a greatly reduced turn-around time, and has been applied widely in the field of microbiology for many years, particularly to rapidly-growing pathogens, like *S. aureus*, observed in normally-sterile body fluids (41;42). When applied to positive blood culture specimens where GPCC are observed, DST facilitates rapid initiation of appropriate antimicrobial therapy for potentially life-threatening MRSA BSI, and implementation of infection control measures to limit nosocomial transmission of this drug-resistant pathogen. DST also has the advantage of testing a more representative bacterial population, obtained directly from the clinical specimen, which may be important for detection of “borderline” resistance, where only a small proportion of the bacterial cell population expresses resistance (43).
1.1.5.2. Genotypic Methods

The reference standard for detection of high-level methicillin resistance in *S. aureus* is detection of the *mecA* gene by molecular techniques, e.g. the polymerase chain reaction (PCR) (44). The inherent limitation of this method is that it will not detect “low-level” resistance mediated by β-lactamase hyper-production, or by alteration of native PBP. These rare *mecA*-negative phenotypes may be detected by phenotypic tests which determine that the oxacillin minimum inhibitory concentration (MIC) ≥4µg/mL (19). This method may also falsely report methicillin resistance in rare circumstances, where *S. aureus* strains which contain the *mecA* gene, do not express the gene, due to repression by the product of the *mecI* gene (22).

1.1.5.3. Phenotypic Methods

Although tests which detect the *mecA* gene or the novel PBP2a most accurately predict methicillin resistance, most clinical laboratories still depend on conventional phenotypic tests to identify MRSA, even from blood culture specimens. Phenotypic detection of methicillin resistance is influenced by the *S. aureus* strain which is tested, by varying antimicrobial susceptibility test (AST) conditions, like test agent, medium, inoculum, and incubation, and by the manner in which the tests are read (44). Despite not providing an MIC and despite its lower sensitivity and specificity for predicting resistance (45), disk diffusion is the most widely-used method for AST for reasons of convenience and cost. Resistance can be most reliably detected using either methicillin or oxacillin. However, methicillin, which was originally used to determine resistance to penicillinase-stable β-lactams, is no longer manufactured. Oxacillin is more stable than methicillin in storage, and is less resistant to hydrolysis by staphylococcal β-lactamases than methicillin, which facilitates its ability to detect low-level resistance. Oxacillin and more recently, cefoxitin, which provides
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comparable (46-48), but easier-to-read results, are currently recommended by the Clinical and Laboratory Standards Institute (CLSI) for standard disk diffusion testing for *S. aureus* (19). Tests performed on Mueller-Hinton and Columbia agar produce equally accurate results which surpass those produced by other agar formulations (44). Increased salt (NaCl) content of the agar medium has increased sensitivity of the disk diffusion test for MRSA detection, but has simultaneously reduced specificity (45). For disk diffusion testing, CLSI recommends Mueller-Hinton agar without added NaCl, although NaCl-containing Mueller-Hinton broth is recommended for broth microdilution testing (19). In contrast, the British Society for Antimicrobial Chemotherapy (BSAC) recommends use of 2% NaCl-containing Mueller-Hinton or Columbia agar for both disk diffusion and broth microdilution tests (24). Although resistant strains are more reliably detected at lower incubation temperatures, CLSI recommends that a standard incubation temperature (35°C +/- 2°C) is used for the disk diffusion test (19). Both CLSI and BSAC recommend that plates are incubated for 24 hours before reading to allow for detection of resistant sub-populations which may grow more slowly (19;24). Although a large inoculum also increases the chances of detection of heterogenous resistance, this practice may lead to over-reporting of resistance. The disk diffusion test to detect MRSA has been modified as a DST, and has produced results which correlated well with standard test results; however, careful inoculum standardisation was required to ensure this correlation (49). The Etest® (AB bioMérieux, Solna, Sweden), a commercial gradient-diffusion test, is as simple to set up as a disk diffusion test, but also provides an MIC. The oxacillin Etest® requires modified Mueller-Hinton agar (with 2% NaCl), and a 24-hour incubation period, at 35°C (50). Hong and co-workers showed that the Etest®, performed directly from blood culture specimens positive for GPCC (and which subsequently cultured *S. aureus*), was able to accurately determine MICs for 5 antimicrobial drugs, 24 hours earlier than conventional testing and with no requirement for inoculum
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standardisation; however, oxacillin MICs were not determined in this study (43). Commercia
Commercially-available chromogenic agar, which selects for growth of \textit{S. aureus} and is able
to differentiate MSSA and MRSA, usually by incorporation of a cephapenic antibiotic, is a
convenient, increasingly used detection tool (51), and has been reported to have a sensitivity
of 97.6\% and specificity of 99.9\%, at a 24-hour reading, for detection of MRSA directly from
blood cultures (52). The accuracy of other phenotypic test methods like agar and broth
dilution, agar and broth breakpoint and agar screening methods, as well as commercial
methods are reviewed elsewhere (44), but will not be discussed further in this report.

1.2. Statement of Problem

The clinical laboratory is required to provide reliable and rapid information to clinicians,
infection control practitioners and hospital epidemiologists to ensure that MRSA, a common
cause of bacteraemia amongst hospitalised patients, is detected early and managed
appropriately. This information facilitates initiation of appropriate and prompt antimicrobial
therapy to improve patient survival (38), and appropriate infection control measures to
minimise the chances of nosocomial spread between patients.

1.3. Justification for Study

Approximately one-third of bacteraemic \textit{S. aureus} isolates was determined to be methicillin
resistant at two academic hospital-affiliated laboratories in Johannesburg in recent years (32).
The availability of continuously-monitored, automated blood culture instruments at these
clinical laboratories has already substantially decreased the time required to detect positive
blood cultures. In addition, the oxacillin disk diffusion test, and more recently, the oxacillin
Etest® and cefoxitin disk diffusion test have been performed directly from positive blood
culture specimens, to reduce time to detection of MRSA. However, this routine laboratory
practice was not formally evaluated on-site, against standardised methods (19). This study also provided an opportunity to evaluate the accuracy of direct inoculation of a commercially-available chromogenic agar formulation to rapidly detect MRSA bacteraemia.

1.4. Study Hypothesis

We hypothesised that the four, selected, direct susceptibility tests (oxacillin and cefoxitin disk diffusion, oxacillin Etest® and chromogenic agar) would differ in their ability to accurately detect bacteraemic MRSA from case patients, compared with meca gene detection by PCR, and with conventional susceptibility tests.

1.5. Study Objectives

The primary study objective was to determine the accuracy of the four abovementioned, phenotypic susceptibility tests to directly detect MRSA from blood culture specimens, compared with detection of the meca gene by PCR from S. aureus cultured from the same blood culture specimens. Secondary objectives were: (a) to compare the DST results with those obtained by performing the susceptibility tests on cultured S. aureus isolates, and (b) to evaluate the performance of two methods (plasma-mannitol-NaCl broth and DNAse agar) to directly identify S. aureus from blood culture specimens positive for GPCC, against detection of the nuc gene from cultured isolates.
2. MATERIALS AND METHODS

2.1. Study Design

This prospective, descriptive, cross-sectional study, which was conducted at two public-sector laboratories linked to tertiary academic hospitals in Johannesburg, was designed to determine the accuracy of four phenotypic susceptibility tests, to rapidly and directly detect MRSA from blood culture specimens, compared with PCR detection of the meca gene from S. aureus isolates. The study was conducted from 1 January 2006 through 28 February 2006. The study included patients who were hospitalised at either Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) or Chris Hani Baragwanath Hospital (CHBH), and who had an incident, laboratory-confirmed S. aureus bacteraemic episode during the study period.

2.2. Study Population

The study population included both adult and paediatric patients, who were hospitalised at either CMJAH or CHBH, and who had an incident bacteraemic episode from 1 January 2006 through 28 February 2006. Both university-affiliated, tertiary-care hospitals are located in an urban setting, with an on-site clinical microbiology laboratory.

2.3. Sampling

Consecutive patients, diagnosed with incident, laboratory-confirmed episodes of S. aureus bacteraemia at CMJAH during the study period, were included in the study. At CHBH, a convenience sample of patients, diagnosed with bacteraemic episodes during the study period, was included. Case patients were identified by the investigator when a blood culture from that patient grew S. aureus, as identified by the laboratory. If an isolate, grown from a blood culture from case patient, was subsequently determined by the study investigator to not be S.
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*aureus*, the case patient was excluded from the study. Case patients with polymicrobial bacteraemia were also excluded from the study.

2.4. Definition of an Incident Episode of *S. aureus* Bacteraemia

An incident episode of *S. aureus* bacteraemia was defined as occurring in a case patient if a blood culture specimen submitted to the laboratory yielded growth of *S. aureus*, confirmed by routine phenotypic tests. An incident episode was considered to span four weeks, after the date of first isolation of *S. aureus* from a blood culture specimen from a case patient. This period was arbitrarily defined for the purposes of this study, because it was recognised that the duration of an incident episode could only be accurately determined by careful review of patient clinical records, which was not done. Four weeks was chosen because it was assumed that a *S. aureus* bacteraemic infection, unless complicated, could be eradicated within this time frame with appropriate treatment. Subsequent blood culture specimens, submitted during the course of the episode and which grew *S. aureus*, were not included in the study. However, if a subsequent blood culture specimen, from the same patient, yielded growth of *S. aureus* after four weeks, this was considered to be a new episode and the blood culture specimen was included in the study.

2.5. Laboratory Methods

2.5.1. Collection of Blood from Case Patients

Blood culture specimens were obtained from patients, admitted to CMJAH or CHBH, following clinical assessment by the attending clinician for evidence of bacteraemia or sepsis. Standard collection techniques were used. The volume of blood inoculated into each blood culture container, and the number of blood culture specimens submitted per episode, was left to the discretion of the clinician or phlebotomist, although recommendations were provided
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by the laboratory. Blood was inoculated into blood culture containers (BacT/ALERT®, Organon Teknika Corporation, Durham, North Carolina, U.S.A.) at the bedside, and submitted immediately to the on-site clinical laboratory.

2.5.2. **Identification and Storage of Cultures**

Upon receipt at the laboratory, blood culture specimens were incubated at 35°C in the automated, continuously-monitored BacT/ALERT® system. A Gram stain was performed directly on broth from positive blood culture specimens. If Gram-positive cocci were observed, arranged in clusters, broth was inoculated onto 5% horse blood and MacConkey agar plates (Diagnostic Media Products (DMP), National Health Laboratory Service (NHLS), Sandringham). After overnight incubation at 35°C, *S. aureus* was identified by growth of typical, cream-coloured to gold, smooth, convex colonies on 5% horse blood agar (24). The catalase test, which is consistently positive for all staphylococci, was performed on MacConkey agar. In addition, broth was directly inoculated onto deoxyribonucleic acid (DNA)-containing agar plates and into specially-prepared tubes containing plasma-mannitol-NaCl broth (DMP, NHLS, Sandringham). The plasma-mannitol-NaCl tubes were incubated in a 37°C water bath and read at 2 hours and 24 hours. Growth in the presence of NaCl, fermentation of mannitol with an indicator colour change, and presence of a visible clot indicated the presence of *S. aureus*. *S. aureus* can be distinguished from coagulase-negative staphylococci, by the presence of a deoxyribonuclease (DNAse). The activity of the staphylococcal DNAse was visualised by precipitation of DNA in the agar: after adding hydrochloric acid to the surface of the agar plate which had been incubated overnight at 35°C, precipitated DNA appeared opaque and a zone of clearing around growth of an organism indicated digested DNA and hence, *S. aureus*. Selected cultured isolates were identified by use of a latex agglutination (Staphaurex®, Remel, Lenexa, U.S.A.) or biochemical kit (API 20
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Staph®, bioMérieux, Marcy-l’Etoile, France). A sterile wire loop was used to inoculate five to six, pure colonies of *S. aureus* into semi-solid agar bottles (DMP, NHLS, Sandringham); these were refrigerated between 2°C and 8°C for up to six months. When required for further work, stored cultures were inoculated onto 5% horse blood agar, incubated overnight at 35°C, and assessed for purity. If contamination was suspected, identification procedures were performed, as above, to confirm the presence of *S. aureus*.

2.5.3. **Direct Susceptibility Tests**

2.5.3.1. **Oxacillin (1µg) and Cefoxitin (30µg) Disk Diffusion**

No attempt was made to standardise the inoculum used for DST. Using a sterile, cotton-tipped swab, a few drops of blood culture broth were evenly spread on the surface of a Mueller-Hinton agar plate (DMP, NHLS, Sandringham). Two disks, one containing 1µg oxacillin and the other containing 30µg cefoxitin, were applied, spaced apart, to the surface of the inoculated agar plate, which was then incubated for 24 hours, in an aerobic atmosphere, at 35°C. Zone diameters were read after incubation, using callipers, to the nearest whole millimetre. Cefoxitin and oxacillin zone diameters were read using reflected and transmitted light respectively. Zone diameters were categorised as susceptible, intermediate or resistant based on breakpoints, defined by CLSI (19).

2.5.3.2. **Oxacillin Etest®**

Using a sterile, cotton-tipped swab, a few drops of blood culture broth were evenly spread on the surface of a Mueller-Hinton + 2% NaCl agar plate (DMP, NHLS, Sandringham). An Etest® strip (AB bioMérieux, Solna, Sweden), containing oxacillin, was applied to the surface of the inoculated agar plate, which was then incubated for 24 hours, in an aerobic atmosphere, at 35°C. The MIC was read visually, after incubation, at the point where the
elliptical zone of inhibition intersected with the Etest® strip (50). The MIC was categorised as susceptible, intermediate or resistant based on breakpoints, defined by CLSI (19).

2.5.3.3 Chromogenic agar

A few drops of blood culture broth were inoculated directly onto the CHROMagar®-MRSA agar plate (CHROMagar® Microbiology, Paris, France), which was incubated overnight, in an aerobic atmosphere, at 35°C. The growth of rose-pink (or mauve) colonies, on the chromogenic agar, indicated the presence of MRSA, whereas growth of MSSA was inhibited.

2.5.4 Indirect Susceptibility Tests

Five to six, single *S. aureus* colonies were touched with a sterile wire loop and inoculated into sterile saline (0.45% NaCl) (DMP, NHLS, Sandringham), to make a suspension equivalent to a 0.5 McFarland standard. Apart from preparation of a standardised inoculum, all indirect tests were performed as detailed above (paragraph 2.6.3).

2.5.5 Detection of *nuc* and *mecA* genes by PCR

The presence of the nuclease (*nuc*) and *mecA* genes, detected by PCR performed on stored cultures, confirmed the identification of *S. aureus* and resistance to methicillin respectively. A rapid lysis procedure was used to extract the DNA from the isolates (53). PCR was performed in a 25µl volume containing 0.5µM of the *mecA* (54) and 0.3 µM *nuc* (55) primers, 2µl DNA and 1X reaction Master Mix (Fermentas Life Sciences, Hanover, MD, U.S.A.). The thermocycling conditions were: 94°C for 1 minute, 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes, followed by a final extension phase of 72°C for 10 minutes. Amplicons were separated by agarose-gel electrophoresis in 2% agarose gels made with 1X Tris-acetate-EDTA (TAE) containing ethidium bromide. The gels were visualised using a UV-transilluminator. Control organisms included *S. aureus* RSA 3/45 (MRSA) and
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HPV107 (MRSA), as well as a negative control (no organism). Cultures which were negative for the *nuc* gene and which did not demonstrate typical phenotypic characteristics of *S. aureus* were discarded, and all data, for the corresponding case patient, were excluded from the final analysis.

2.5.6. **Quality Control**

Appropriate positive and negative controls were used for all phenotypic and genotypic procedures. The oxacillin and cefoxitin disk diffusion tests were subjected to quality control (QC) procedures recommended by CLSI, with testing of QC strain, *Staphylococcus aureus* ATCC® 25923, performed in parallel (19). The oxacillin Etest® was also subjected to QC procedures recommended by AB bioMérieux (50). The study was performed in a clinical laboratory, which was accredited by the South African National Accreditation System (SANAS), and which regularly participated in external proficiency testing schemes.

2.6. **Data Analysis, including Statistical Methods**

Data, obtained through a laboratory record review and from tests performed for the purposes of this study, were entered into a password-restricted Microsoft Access database. Data were exported to Microsoft Excel for analysis. The sensitivity and specificity of the DST were calculated with reference to the *mecA* PCR result, to fulfil the primary study objective. For negative predictive value (NPV) and positive predictive value (PPV) calculations, the prevalence of methicillin resistance, amongst bacteraemic *S. aureus* isolates at CMJAH and CHBH, was assumed to be 33% based on recent data from these sites (32). In addition, categorisation of an isolate as susceptible, intermediate or resistant to methicillin, by direct and indirect susceptibility testing, was compared, with reference to the *mecA* PCR assay result. A very major error occurred, if the patient was determined, by a phenotypic
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susceptibility test, to have a bacteraemic episode caused by MSSA, when the PCR assay
detected the presence of the *mecA* gene (falsely susceptible). Conversely, a major error
occurred, if the patient was determined, by a phenotypic test, to have a bacteraemic episode
caused by MRSA, and the PCR assay did not detect the presence of the *mecA* gene (falsely
resistant). A minor error occurred, if a phenotypic test classified an isolate as intermediately
resistant, and the PCR assay did not detect the presence of the *mecA* gene. To fulfil a
secondary objective, the correlation between results, obtained through direct and indirect
susceptibility testing, was determined. Complete correlation occurred if: (a) zone diameter
results, obtained through direct and indirect testing, were placed in the same susceptibility
category, and (b) MIC results, obtained through the direct and indirect Etest®, were placed in
the same susceptibility category.

2.7. Ethics Approval

An application for ethics approval, submitted to the Human Research Ethics Committee
(Medical), University of the Witwatersrand, Johannesburg, was considered, and approved
unconditionally on 4 April 2005 (Protocol number M05-03-13; Reference R14/49 Govender).

2.8. Funding

Funds to perform laboratory tests, specific to the study, were provided by the Department of
Clinical Microbiology and Infectious Diseases, School of Pathology, University of the
Witwatersrand. The CHROMagar®-MRSA plates and Staphaurex® kits were supplied for the
study by local distributors of the products.
3. RESULTS

3.1. Study Population and Sampling

From 1 January 2006 through 28 February 2006, 5,022 blood culture specimens were submitted to the clinical laboratory at CMJAH (Table 3.1). Of these, GPCC were observed in 527 (10.5%) blood culture specimens. Of the GPCC-positive specimens, 64 (12%) yielded isolates identified by CMJAH laboratory as S. aureus. Forty-eight consecutive patients, with incident episodes of S. aureus bacteraemia, were included in the study. Five blood culture specimens were determined to be duplicates, i.e. occurring within the same incident episode. Eleven blood culture specimens, belonging to patients with incident bacteraemic episodes, were missed. During the study period, 8,548 blood culture specimens were submitted to the CHBH laboratory (Table 3.1). The proportion of blood culture specimens with observed GPCC was not available. Of all submitted specimens, 92 (1%) grew isolates identified by CHBH laboratory as S. aureus. Forty-one patients, with an incident S. aureus bacteraemic episode, were selected from this site. No isolates from a second bacteraemic episode were included at either CMJAH or CHBH, and no blood culture specimens had to be excluded due to polymicrobial infection.

3.2. Laboratory Identification and Storage of S. aureus Isolates

Of 89 isolates, obtained from 89 patients at CMJAH and CHBH with incident bacteraemic episodes, two (2%) were excluded due to misidentification by the clinical laboratory. In these 2 cases, stored cultures were determined not to possess the typical phenotypic characteristics of S. aureus and lacked the nuc gene (Table 3.1); one isolate was identified as Staphylococcus haemolyticus and the other as Staphylococcus capitis subsp. urealyticus. Of the remaining 87 isolates which were confirmed to be S. aureus, 22 (25%) did not survive storage or were
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contaminated after storage. Hence, indirect susceptibility testing and *nuc* and *mecA* PCR assays could not be performed. Data from the corresponding case patients were excluded from further analysis. In total, 65 bacteraemic isolates were confirmed as *S. aureus*, subjected to all identification, susceptibility and molecular tests and were included in the final analysis (Table 3.1). All 65 *nuc*-positive isolates were positive with the direct DNAse test, which was read at 16 to 24 hours post-incubation. The direct plasma-mannitol-NaCl test was performed for 44 of 65 *nuc*-positive isolates (68%): 16 (36%) were positive at the 2-hour reading and a further 22 (50%) at the 24-hour reading.
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**Table 3.1**: Selection of *S. aureus* isolates from patients with incident bacteraemic episodes at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and Chris Hani Baragwanath Hospital (CHBH) for this study.

<table>
<thead>
<tr>
<th></th>
<th>CMJAH</th>
<th>CHBH</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of consecutive blood culture specimens processed by the laboratory during the 2-month study period</td>
<td>5,022</td>
<td>8,548</td>
<td>9,400</td>
</tr>
<tr>
<td>Number of blood culture specimens with observed Gram-positive cocci in clusters (GPCC)*</td>
<td>527</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Number of blood culture specimens which yielded growth of <em>S. aureus</em></td>
<td>64</td>
<td>92</td>
<td>156</td>
</tr>
<tr>
<td>Number of <em>S. aureus</em> bacteraemic isolates which were not included# in the study</td>
<td>16</td>
<td>51</td>
<td>67</td>
</tr>
<tr>
<td>Number of <em>S. aureus</em> isolates, from patients with incident bacteraemic episodes, which were stored for further processing</td>
<td>48</td>
<td>41</td>
<td>89</td>
</tr>
<tr>
<td>Number of stored cultures which were subsequently confirmed to not be <em>S. aureus</em></td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Number of stored cultures which were contaminated or non-viable</td>
<td>11</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>Number of incident bacteraemic isolates which were subjected to PCR$^5$ to detect the <em>nuc</em> and <em>mecA</em> genes, and included in the final data analysis</td>
<td>37</td>
<td>28</td>
<td>65</td>
</tr>
</tbody>
</table>

*Number of GPCC-positive blood culture specimens: Data were not available at CHBH; #Exclusion of *S. aureus* bacteraemic isolates: At CMJAH, 5 *S. aureus* duplicate isolates were not included in the study, according to the incident bacteraemic episode case definition, as were 11 isolates, which were missed by the study investigator. At CHBH, 51 isolates were missed; $^5$PCR: Polymerase chain reaction.
3.3. Prevalence of Confirmed Methicillin Resistance Amongst Tested Isolates

Of 65 nuc-positive S. aureus isolates which were subjected to mecA PCR, 25 (38%) were confirmed as MRSA (mecA positive); the remainder lacked the mecA gene and were considered to be methicillin-susceptible (Figure 3.1 and 3.2).

![Agarose gel showing polymerase chain reaction (PCR)-amplified, 279bp product of the nuc gene from 3 bacteraemic isolates, with phenotypic characteristics of S. aureus.](image)

Lane 1: 50bp Fermentas Marker; Lane 2: negative control; Lane 3: positive control (HPV107); Lane 4: positive control (RSA 3/45); Lane 5: nuc positive (confirmed S. aureus); Lane 6: nuc positive (confirmed S. aureus); Lane 7: nuc positive (confirmed S. aureus).
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**Figure 3.2**: Agarose gel showing polymerase chain reaction (PCR)-amplified, 162bp product of the *mecA* gene from 3 bacteraemic *nuc*-positive *S. aureus* isolates.

Lane 1: 50bp Fermentas Marker; Lane 2: negative control; Lane 3: positive control (HPV107); Lane 4: positive control (RSA 3/45); Lane 5: *mecA* negative (methicillin susceptible); Lane 6: *mecA* negative (methicillin susceptible); Lane 7: *mecA* positive (methicillin resistant).

### 3.4. Accuracy of Direct Susceptibility Tests

One major error and 3 minor errors occurred with the direct oxacillin disk diffusion test (Table 3.2). Compared to PCR results, sensitivity and specificity for this direct test was 100% and 90% respectively (Table 3.3). For the direct cefoxitin disk diffusion test, one major error occurred (Table 3.4); hence, sensitivity was 98% and specificity was 100%. The direct oxacillin Etest® had a sensitivity and specificity of 100%, with no categorisation errors (Table 3.5). For the direct chromogenic agar method, sensitivity and specificity was 96% and 42% respectively, with 1 very major error and 21 major errors, when read at 24 hours (Table 3.6).
Table 3.2: Comparison of susceptibility categories, determined by the direct oxacillin (1µg) disk diffusion test, with presence of the mecA gene, detected by the polymerase chain reaction, for 65 bacteraemic S. aureus isolates.

<table>
<thead>
<tr>
<th>Category</th>
<th>Susceptible*</th>
<th>Intermediate*</th>
<th>Resistant*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>mecA gene positive</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>mecA gene negative</td>
<td>36</td>
<td>3</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>0</td>
<td>29</td>
<td>65</td>
</tr>
</tbody>
</table>

*Clinical and Laboratory Standards Institute interpretive breakpoints for zone diameters (ZD), determined by the oxacillin (1µg) disk diffusion test - Susceptible: ZD ≤10mm; Intermediate: ZD =11-12mm; Resistant: ZD ≥13mm; Boldface type indicates isolates which were incorrectly categorised by the direct oxacillin disk diffusion test.

Table 3.3: The sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) for four direct susceptibility tests performed on 65 bacteraemic S. aureus isolates.

<table>
<thead>
<tr>
<th>Direct Susceptibility Test</th>
<th>Sensitivity* (%)</th>
<th>Specificity* (%)</th>
<th>NPV* (%)</th>
<th>PPV* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin (1µg) disk diffusion</td>
<td>100</td>
<td>90</td>
<td>100</td>
<td>83</td>
</tr>
<tr>
<td>Cefoxitin (30µg) disk diffusion</td>
<td>98</td>
<td>100</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>Oxacillin Etest®</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Chromogenic agar</td>
<td>96</td>
<td>42</td>
<td>96</td>
<td>45</td>
</tr>
</tbody>
</table>

*The reference standard method was detection of the mecA gene by the polymerase chain reaction, and for NPV and PPV calculations, it was assumed that the overall prevalence of methicillin resistance, amongst isolates from incident bacteraemic S. aureus episodes at CMJAH and CHBH, was 33%.
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**Table 3.4:** Comparison of susceptibility categories, determined by the direct cefoxitin (30µg) disk diffusion test, with presence of the *mecA* gene, detected by the polymerase chain reaction, for 65 bacteraemic *S. aureus* isolates.

<table>
<thead>
<tr>
<th>Number of <em>S. aureus</em> isolates</th>
<th>Category</th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible*</td>
<td>Resistant*</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>mecA</em> gene positive</td>
<td>0</td>
<td>25</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td><em>mecA</em> gene negative</td>
<td>39</td>
<td>1</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>26</td>
<td></td>
<td>65</td>
</tr>
</tbody>
</table>

*Clinical and Laboratory Standards Institute interpretive breakpoints for zone diameters (ZD), determined by the cefoxitin (30µg) disk diffusion test - Susceptible: ZD ≤21mm; Resistant: ZD ≥22mm; Boldface type indicates isolates which were incorrectly categorised by the direct cefoxitin disk diffusion test.

**Table 3.5:** Comparison of oxacillin minimum inhibitory concentrations (MIC), determined by the direct oxacillin Etest®, with presence of the *mecA* gene, detected by the polymerase chain reaction, for 65 bacteraemic *S. aureus* isolates.

<table>
<thead>
<tr>
<th>Number of <em>S. aureus</em> isolates</th>
<th>Oxacillin MIC* (µg/mL)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>256</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.125</td>
<td>0.19</td>
<td>0.25</td>
<td>0.38</td>
<td>0.5</td>
<td>0.75</td>
</tr>
<tr>
<td><em>mecA</em> gene positive</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>mecA</em> gene negative</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>15</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>15</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

*Interpretive breakpoints for oxacillin MIC (Clinical and Laboratory Standards Institute) - Susceptible: MIC ≤2µg/mL; Resistant: MIC ≥4µg/mL; Thick vertical line indicates separation of susceptibility categories.
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**Table 3.6:** Comparison of direct chromogenic agar (CHROMagar®-MRSA) test, with presence of the mecA gene, detected by the polymerase chain reaction, for 60\(^\#\) bacteraemic *S. aureus* isolates.

<table>
<thead>
<tr>
<th>Category</th>
<th>Susceptible*</th>
<th>Resistant*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>mecA gene positive</td>
<td>1</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>mecA gene negative</td>
<td>15</td>
<td>21</td>
<td>36</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>16</td>
<td>44</td>
<td>60</td>
</tr>
</tbody>
</table>

\(^\#\)Direct chromogenic agar testing was not performed for 5 isolates; \(^\ast\)Methicillin-resistant *S. aureus* colonies were rose-pink on chromogenic agar whereas growth of methicillin-susceptible *S. aureus* was inhibited; Boldface type indicates isolates which were incorrectly categorised by the direct chromogenic agar test.

### 3.5. Accuracy of Indirect Susceptibility Test Results

Compared to mecA PCR results, sensitivity, specificity, NPV and PPV was 100%, for all indirect tests, with no categorisation errors recorded.

### 3.6. Correlation Between Direct and Indirect Susceptibility Test Results

Although all methicillin-resistant isolates, as determined by the indirect oxacillin disk diffusion test, were correctly categorised by the direct test, the direct test falsely over-read resistance, placing three susceptible strains in the intermediate category (zone diameter = 11mm or 12mm), and one in the resistant category (zone diameter = 9mm). In contrast, the zone diameter was 6mm, with both direct and indirect oxacillin disk diffusion tests, for all 25 mecA-positive MRSA isolates. The direct cefoxitin disk diffusion test correlated perfectly with the indirect test when categorising resistant isolates (zone diameter = 6mm for all 25 mecA-positive MRSA isolates); however, the direct test also falsely over-read resistance, placing one susceptible isolate (zone diameter = 20mm) in the resistant category. Complete
correlation was demonstrated between direct and indirect oxacillin Etest® results.

Interestingly, the same MIC was obtained for 32 of 65 isolates (49%) with both the direct and indirect Etest® (Table 3.7). The direct Etest® produced higher MIC results for 28 isolates (43%), and although this MIC was ≥2-fold higher than the indirect Etest® MIC for 13 of 28 isolates, it remained well within the susceptible category. The direct chromogenic agar method performed poorly, compared to the indirect method, falsely categorising 22 isolates.

**Table 3.7:** Comparison of direct and indirect oxacillin Etest® minimum inhibitory concentrations (MIC) for 65 bacteraemic *S. aureus* isolates.

<table>
<thead>
<tr>
<th>Susceptibility Test</th>
<th>Direct Etest®</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Same</td>
<td>Higher MIC</td>
<td>Lower MIC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MIC &lt;2-fold</td>
<td>≥2-fold</td>
<td>&lt;2-fold</td>
<td>≥2-fold</td>
</tr>
<tr>
<td>Indirect Etest®</td>
<td>32</td>
<td>15</td>
<td>13</td>
<td>5</td>
</tr>
</tbody>
</table>
4. DISCUSSION

4.1. Summary of Study Findings

*S. aureus* is a common cause of hospital- and community-associated bacteraemia. This fact, coupled with the high prevalence of methicillin resistance amongst bacteraemic isolates, means that the clinical laboratory needs to be able to rapidly and accurately detect MRSA bacteraemia to facilitate initiation of appropriate antimicrobial therapy and infection control interventions. In this study, we found that, compared to PCR for the *nuc* and *meca* genes, the combination of a DNAse test and an oxacillin Etest®, performed directly on 65 blood culture specimens where GPCC were observed, facilitated accurate detection of MRSA bacteraemia, 24 hours after the blood culture specimen was flagged positive. The direct oxacillin Etest® result did not appear to be influenced by a non-standardised inoculum, in contrast to the other direct tests. The direct cefoxitin disk diffusion test produced more accurate results than the direct oxacillin disk diffusion test, was easier to read and distinguished MRSA from MSSA with zone diameters clustering into more clearly defined susceptibility categories. Although the chromogenic agar performed well when used to identify methicillin resistance amongst cultured *S. aureus* isolates, it was apparent that this test, read at 24 hours, could not be used reliably as a DST.

4.2. Direct Susceptibility Testing to Detect MRSA Bacteraemia

DST facilitates rapid identification of MRSA bacteraemia and allows a more representative staphylococcal population to be tested. Although more recent studies have focused on the development of molecular tools, e.g. real-time PCR (56,57), to directly identify MRSA (within hours) from blood culture specimens positive for GPCC, expertise and specialised equipment are usually required to include these tests in a clinical laboratory. Modification of
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conventional phenotypic methods to directly detect MRSA bacteraemia has the advantages of low costs, simplicity and ease of inclusion into a diagnostic laboratory testing algorithm (58). Two major issues need to be addressed when a conventional method is modified for DST: (a) selection of appropriate specimens for DST, and (b) inoculum preparation (59). Specimens from normally-sterile body sites, e.g. blood culture specimens, are most suited to DST because a single organism is usually cultured, and reading of susceptibility results is easier (41). When attempting to rapidly identify MRSA bacteraemia, blood culture specimens positive for GPCC are often selected for DST to improve specificity. However, because coagulase-negative staphylococci (CNS) are frequently isolated from blood culture specimens as contaminants or occasional pathogens, tests that rapidly differentiate *S. aureus* from CNS can assist to select appropriate specimens for DST. Previous studies have indicated that the 2-hour tube coagulase test, applied directly to blood culture specimens, is cheaper and more reliable than immunologic tests (60;61), and that direct Gram stain characteristics may be able to differentiate *S. aureus* from CNS (62). In our study, we compared two readily-available, in-house preparations to rapidly identify *S. aureus* from blood culture specimens which were positive for GPCC: plasma-mannitol-NaCl broth and DNAse agar. Although the plasma-mannitol-NaCl broth had a lower sensitivity at a 24-hour reading than the DNAse agar, the broth had the advantage of an additional 2-hour reading which could assist with selection of specimens for DST. Unfortunately, the low reported 2-hour sensitivity precludes routine use of this in-house test to screen GPCC-positive blood culture specimens for DST. Previous investigators have made attempts to standardise the inoculum for DST from blood culture specimens, including varying the inoculum volume (63), dilution of the inoculum with saline to diminish the inhibitory effects of blood itself and components within the blood culture medium (58), and centrifugation of the blood culture specimen and suspension of the resulting bacterial pellet in saline to make a 0.5 McFarland standard (64). In our study, we tested a non-
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standardised inoculum, partly to simplify the testing algorithm, and partly to determine which DST methods were inoculum-independent. Although the lawn of bacterial growth obtained for the direct disk diffusion test and Etest® varied in confluence from specimen-to-specimen in our study, from our results it is apparent that the Etest® method was least affected by inoculum effects, with perfect correlation with *mecA* PCR results, and with the same MIC recorded by the direct and indirect Etest® for almost half of the tested isolates. This correlates with the findings of previous investigators who used a non-standardised inoculum, and reported that Etest® readings are largely inoculum-independent (43). Those investigators determined an optimal inoculation amount, for easy reading of the Etest®, based on the BACTEC® NR-660 instrument (Becton Dickinson Diagnostic Instrument Systems, Sparks, Maryland, U.S.A.) used in the study, and suggested that laboratories tailor the inoculum for DST to the in-use instrument (43). The continuously-monitored BacT/ALERT® system, used in this study, may require a larger inoculum for DST, because the system detects positive blood cultures earlier than older instruments and the broth may contain a lower organism concentration. Overall, the direct cefoxitin disk diffusion test was more accurate than the oxacillin disk diffusion test. As has been previously reported, the cefoxitin disk diffusion test is also simpler to read, and less influenced by test conditions (24), and may facilitate detection of heterogenous resistance (48). The sensitivity and specificity for the direct cefoxitin disk diffusion test, which we report here, are in keeping with findings from other studies (49). The CLSI recommends that isolates, which are categorised as intermediately resistant with the oxacillin disk diffusion test, be re-tested using another more accurate method (like the cefoxitin disk diffusion test) and that the results of the repeat test be reported (19); this potentially delays results for up to 24 hours. In our study, we recorded minor categorisation errors for 3 isolates with the direct oxacillin disk diffusion test, which could have been averted by using the cefoxitin test upfront. Although a major error was recorded for 1 isolate
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with both direct oxacillin and cefoxitin disk diffusion tests, these false-resistant isolates had zone diameters greater than 6mm, in contrast to the 25 *mecA*-positive isolates which all grew up to the edge of the antimicrobial-containing disk. It is, therefore, possible for such categorisation errors to be detected by an astute laboratory worker. The chromogenic agar formulation, which was evaluated in this study, performed poorly as a DST, with 21 false-positive results when read at 24 hours. This reported specificity is much lower than the specificity of 99.9%, reported by Pape, Wadlin & Nachamkin (52); in that study, 2 false-positive results occurred with blood culture specimens which yielded CNS. All our false-positive results occurred with MSSA isolates, which had oxacillin MIC <1µg/mL, and inexperience with reading the chromogenic agar directly from blood culture specimens may have contributed to over-reporting MRSA. It is possible that reading the direct chromogenic agar test after 48 hours could have improved our reported sensitivity to 100% (52), but this delayed reading would have negated the reason for performing a rapid DST, and other workers have suggested that prolonged incubation could lead to more false-positive results (65). Newer formulations of chromogenic agar have been developed recently, and have been reported to detect MRSA from blood culture specimens with more accuracy (100% sensitivity and specificity, compared to conventional cefoxitin disk diffusion testing) (66).

### 4.3. Study Strengths and Limitations

We selected tests, for evaluation in this study, which were simple, easily-available at relatively low cost, and which could be incorporated into a clinical microbiology laboratory with no need for expertise or specialised equipment. All phenotypic tests were evaluated against a reference standard (PCR), which enabled accurate calculations of sensitivity, specificity, NPV and PPV. Although the 65 *S. aureus* isolates, which were selected to assess the accuracy of the four DST to detect MRSA, were cultured from patients with incident
bacteraemic episodes, we made no attempt to link patients epidemiologically, and
unrecognised nosocomial outbreaks caused by clonal strains may have occurred during this
time. Given that S. aureus strain types may influence phenotypic detection of MRSA (44), it
is possible that the sensitivity and specificity of the tests, reported in this study, may not be
applicable to other settings. Although we excluded blood culture specimens with more than
one organism from this study, we made no attempt to standardise the inoculum for DST; this
could have adversely affected the accuracy of our results. We also made no attempt to
evaluate the accuracy of different inoculation volumes and dilutions. We did not perform
direct identification tests on GPCC-positive blood culture specimens from which CNS were
subsequently cultured; therefore, we could not determine the specificity for these tests.

4.4. Suggestions for Future Work
This study focused on the modification of currently used, phenotypic methods to rapidly
detect MRSA bacteraemia in a diagnostic laboratory. Further work needs to be done to
identify a more rapid, cost-effective, practical, and sensitive method to distinguish S. aureus
from CNS in GPCC-positive blood culture specimens for use in local laboratories; this will
facilitate selection of appropriate specimens to perform rapid DST. Since commercial,
automated systems are increasingly used in clinical laboratories to confirm the identity and
provide AST results for organisms cultured from blood culture specimens, recent studies have
evaluated the option of direct inoculation of commercial identification and AST systems, e.g.
VITEK® 2 (bioMérieux, Marcy-l’Etoile, France) (59). Such studies should be replicated in
local laboratories to evaluate in-use automated systems. In addition, it would be useful to
evaluate a simple molecular assay which can identify MRSA directly from positive blood
cultures, and which could be incorporated into the workflow of a clinical laboratory, with
minimal adjustment (67). The focus of this study was laboratory-based, with no evaluation of
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de the impact that rapid DST had on case patient management and outcome. A future study, evaluating a rapid DST for MRSA bacteraemia, could include prospective follow-up of case patient to evaluate if the DST result was associated with improved patient outcomes, although such an analysis could be confounded by organism as well as patient factors, e.g. underlying co-morbid illness, complicated MRSA infection, *etc.*

**4.5. Conclusions and Recommendations**

In summary, we found that the direct oxacillin Etest® most reliably predicted methicillin resistance amongst tested bacteraemic *S. aureus* isolates, compared to *mecA* PCR, and provided an oxacillin MIC. However, since the Etest® is more costly than disk diffusion testing, it would make sense to use this test in conjunction with a direct identification test, which is as reliable as the direct DNAse test but more rapid, or reserve its use for blood culture specimens from patients in “high-risk” hospital areas, e.g. intensive care units. The direct cefoxitin disk diffusion test was almost as accurate as an Etest®, much less costly and easier to read. This test could be used for all blood culture specimens positive for GPCC, and could be used without a direct identification test because of its lower cost; it is further recommended that the direct cefoxitin disk diffusion test replace the direct oxacillin test. Given the equivalent accuracy of the indirect disk diffusion, Etest® and chromogenic agar test, we suggest that the most convenient and least costly method (i.e. cefoxitin disk diffusion) also be used for identification of methicillin resistance amongst cultured *S. aureus* isolates.
5. References


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