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Detection of *Staphylococcus aureus*-Specific Gene and Simultaneous Confirmation of Methicillin Resistant *Staphylococcus aureus* (MRSA) by Polymerase Chain Reaction

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Abstract

Background: The differentiation of Methicillin resistant *Staphylococcus aureus* (MRSA) strains from other strains of *S. aureus* has important implications for the treatment and management of patients with *S. aureus* infections. Detection of the *mecA* gene by PCR has been described as a rapid method for the identification of MRSA. *Objectives:* A molecular assay for the simultaneous detection of a *Staphylococcus aureus*-specific gene and the *mecA* gene, responsible for the resistance to methicillin in staphylococci, was evaluated. *Methods:* In a clinical study, 100 isolates of *Staphylococcus aureus* were investigated. Polymerase chain reaction (PCR) was used (with a Techne TC-5000 instrument-Bibby Scientific Ltd.) to amplify both the *S. aureus* specific sequence gene and *mec*A gene of 100 isolates with the amplicon size of 107 and 532bp. To accelerate the procedure of identification in clinical microbiology laboratories, simple and rapid method for DNA extraction directly from a single colony was employed. The assay included a rapid DNA extraction protocol conducted in 15 minutes and PCR conducted. The performance and robustness of the assay was evaluated with a control strain of methicillin susceptible *Staphylococcus aureus*-ATCC 25923 (MSSA). The specificity of the new molecular assay was tested with a bacterial strain of methicillin susceptible *Staphylococcus aureus*-ATCC 25923 (MSSA). *Results:* All clinical the isolates gave positive results for the *S. aureus*-specific genomic target, and only five isolates (5.0%) were positive for the *mecA* gene. *Conclusion:* The new rapid DNA extraction protocol was found to be quick, robust, and labor saving and it proved to be suitable for a routine molecular diagnostic laboratory. On the basis of this finding; establishment of molecular diagnostic laboratory in secondary and tertiary health units is urgently required.

1. Introduction

Staphylococcus aureus has been known to be a major pathogen causing a wide spectrum of clinical manifestations, such as wound infections, pneumonia, septicemia, and endocarditis, with beta-lactam antibiotics being the drugs of choice for therapy. Since the introduction of methicillin into clinical use in 1961, the occurrence of methicillin-resistant *S.*

aureus (MRSA) has steadily increased and nosocomial infections caused by such isolates have become a serious problem worldwide (Benner and Kayser, 1968; Lowy, 1998).

The differentiation of MRSA strains from other strains of *S. aureus* has important implications for the treatment and management of patients with *S. aureus* infections, and glycopeptides are the drugs of choice for infections caused by MRSA strains. Furthermore, evidence of MRSA requires

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extensive hygienic precautions to limit the spread of such strains (Wenzel *et al.*, 1998; Chaix *et al.*, 1999).

In the clinical laboratory, *S. aureus* is identified by growth characteristics and by the subsequent detection of catalase and coagulase activities. Conventional susceptibility testing of *S. aureus* reliably detects resistance to methicillin or oxacillin if agar dilution tests, disk diffusion tests, or agar screening methods are used according to the standards of the National Committee of Clinical Laboratory Standards (NCCLS) (NCCLS, 1993; NCCLS, 1999). Standard susceptibility tests, however, are time-consuming. Because the phenotypic expression of methicillin resistance in vitro is heterogeneous and sometimes difficult to induce, false-negative results may be observed (Ubukata *et al.*, 1989; Ribeiro *et al.*, 1999). Furthermore, *S. aureus* strains may show a false-negative or non-interpretable result when commercially available kits for coagulase testing (Ruane *et al.*, 1986; Weeres-Pothoff *et al.*, 1987; Fournier *et al.*, 1989; Unal *et al.*, 1994; Wilkerson *et al.*, 1997) are used.

The main mechanism of methicillin resistance is induced by the presence of an additional low-affinity penicillin-binding protein, PBP2a (encoded by the *mec*A gene), or, in rare cases, induced by the hyper production of β-lactamase (Waxman and Strominger, 1983; Hartman and Tomasz (1984; Chambers, 1993). Detection of the *mec*A gene by PCR has been described as a rapid method for the identification of MRSA (Tokue *et al.*, 1991; Brakstad *et al.*, 1992; Tokue *et al.*, 1992; Unal *et al.*, 1994; Barski *et al.*, 1996; York *et al.*, 1996; Kearns *et al.*, 1999; Martineau *et al.*, 1998; Kohner *et al.*, 1999).

In the present study, a molecular assay for the rapid identification of MRSA was established and evaluated. The new assay targeted both the *S. aureus* gene and the *mecA* gene within a single PCR and was based on automated DNA isolation and real-time PCR.

2. Materials and Methods

2.1. The Study of Areas

A total of 8 health institutions located in six states in Northwestern Nigeria, were enrolled in the study. The health institutions were two teaching hospitals [Aminu Kano Teaching Hospital (AKTH) and Ahmadu Bello University Teaching Hospital (ABUTH)], three Federal Medical Centres [Federal Medical Centres Birnin Kudu (FMCB), Federal Medical Centre Gusau (FMCG) and Federal Medical Centre Katsina (FMCK) located in Jigawa, Zamfara and Katsina state in Northwestern Nigeria respectively], two Specialist Hospitals [Murtala Muhammad Specialist Hospital, Kano (MMSH) and Specialist Hospital Sokoto (SHS) located in Kano and Sokoto states of Northwestern Nigeria respectively] and the Infectious Diseases Hospital (IDH) located in the city of Kano, Kano State.

2.2. Specimen Collection

A total of 100 consecutive non – duplicated *Staphylococcus aureus* isolates were obtained from clinical samples in 8 health institutions across Northwestern Nigeria. The isolates were collected from the eight hospitals during two years period from February 2008 to April 2010. Routine clinical microbiology specimens were selected during the period. The quality control and rejection criteria of specimen were followed (Isenberg, 1998). *Staphylococcus aureus* ATCC 25923 was used as a reference control organism. Specimens were processed within 2 hours of collection by the standard microbiology technique. The sheep blood agar and mannitol salt agar were used for inoculation and the agar plates were then incubated at 35^0C for 18-24 hours in aerobic atmosphere (NCCLS, 2003).

2.3. Study Design

A molecular assay for the detection of MRSA based on rapid DNA extraction method was established. The new assay was based on rapid DNA extraction and PCR with a Techne TC-5000 instrument (Bibby Scientific Ltd).In the first step, 100 clinical isolates of *Staphylococcus aureus* were tested. *S. aureus* strains were identified by their characteristic growth morphologies, Gram stain characteristics, reaction to catalase, and coagulase production were detected. After identification, the strains were stored at −80°C [Micro bank (Diagnostic pro-lab)]. For molecular testing, clinical specimens were thawed and recultured on blood agar overnight at 37°C. Portions of individual bacterial colonies were suspended in 25µl of sterile distilled water. In the second step, the specificity of the new molecular assay was determined with a suspension of the *S. aureus* strain of methicillin susceptible *Staphylococcus aureus*-ATCC 2592 (American Type Culture Collection, Manassas, Va.). In the third step, the molecular assay of the 100 Staphylococcal isolates was determined.

2.4. DNA Extraction

After overnight culture on brain heart infusion (Difco Laboratories) agar plates, one colony of each sample was resuspended in 25 µl of sterile distilled water and the suspension was then placed in a 100°C heat block for 15 min. From this suspension, a 5-µl volume was directly used as a template for PCR amplification (Pérez-Roth *et al.,* 2001).

2.5. Primers

The oligonucleotide primers used in this study have been

previously described (Martineau *et al*., 1998; Meshref *et al.,* 2011) and were obtained from a commercial source (Inqaba Biotechnical Industries (Pty) Ltd., South Africa). The 3-end region of the *S. aureus* specific gene was amplified using A 30 nucleotide forward primer 5'- AATCTTTGTCGGTACACG ATA TTCTTCACG -3' and A30 nucleotide reverse primer, 5'-CGTAAT GAG ATT TCAGTA GAT AATACAACA-3' (which hybridize to 5-34 and (112-83), respectively, (Martineau *et al*., 1998). While

The 3-end region of the *mecA* gene was amplified using A 22nucleotide forward primer 5'- AAA ATC GAT GGT AAA GGTTGG C - 3' and A22 nucleotide reverse primer, 5'-AGTTCTGCAGTACCG GAT TTG C-3' (which hybridize to sites 1282-1301 and 1814-1793) (Robert Koch institute, 2003) (Table 1). *Staphylococcus aureus* specific gene and *mecA* gene have the amplicon size of 107 and 532 bp using primers described by (Meshref *et al.,* 2011).

Table 1. Oligonucleotide Primers used in the PCR assay

Oligonucleotide	Sequence	Target
Position	Nucleotide	gene
A30fwd5-34	AATCTTTGTCGGTACACGGATATTCTTCACG	Sa
A30 rev112-83	CGTAATGAGATTTCAGTAGATAATACAACA	Sa
A22fwd1282-1301	AAAATCGATGGTAAAGGTTGGC	mecA
A22 rev1814-1793	AGTTCTGCAGTACCGGATTTGC	mecA

2.6. PCR Amplification

PCR assays were all directly performed from the bacterial suspension obtained after the rapid DNA extraction method described above. An aliquot of 5 µl of this suspension was added to 95 µl of PCR mixture consisting of 1× reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mMTris-HCl (pH 8.8)], a 0.5mM concentration of each of the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP) (Inqaba Biotechnical Industries (Pty) Ltd., South Africa), 1.0µM of each primer, and *mecA* primer, and 1.25 U of The Dream Taq™ Green PCR Master Mix (2x) (Fermentas Life Sciences, supplied by Inqaba Biotechnical Industries (Pty) Ltd., South Africa) is a ready-to-use solution containing Dream Taq™ DNA polymerase, optimized Dream Taq™ Green buffer and 4mMMgCl₂. For each sample, one reaction was performed with the pair of primers to identify *S. aureus* specific sequence gene and with the *mecA* pairs of primers to detect both resistance gene (*mec*A). In order to reduce the formation of nonspecific extension products, a hot-start PCR protocol was used; the tubes were placed in the thermal cycler when the denaturing temperature was reached. All PCR assays were carried out with a negative control containing all of the reagents without DNA template. DNA amplification was carried out in a Techne PCR system TC-5000 thermo cycler (Bibby Scientific Ltd.) with the following thermal cycling profile: an initial denaturation step at 94°C for 5 min was followed by1 cycle of amplification this was followed by denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s ending with a final extension step at 72°C for 5 min. After PCR amplification,5 µl was removed and subjected to agarose gel electrophoresis(1.5% agarose, 1× Tris-borate-EDTA, 100 V, 40 min) to estimate the sizes of the amplification products by comparison with a 100-bp O' GeneRuler™ 100bp molecular size standard DNA Ladder, ready-to-use designed by

Ferment as Life sciences (supplied by Inqaba Biotechnical Industries (Pty) Ltd., South Africa) The gel was stained with ethidium bromide, and the amplicons were visualized using a UV light box. This protocol, including the rapid DNA extraction method from a single colony and electrophoretic analysis of the amplified products on an agarose gel, was completed within 4 hours.

3. Results

When a suspension of an MRSA strain with a turbidity equivalent to a McFarland standard of 0.5 was repeatedly tested by a molecular assay based on the rapid DNA extraction protocol and rPCR on an instrument, the crossing points of both targets were always found to be within one cycle. All the isolates (n=100) expressed *S. aureus* specific sequence gene in their PCR products which confirmed the assumption that all the strains were *Staphylococcus aureus***.** Fig. 1 shows a representative agarose gel electrophoresis of PCR products. Only 5 isolates (5.0%) were confirmed as MRSA based on the detection of *mec*A gene. No contamination was observed at any time during the study. The size of the amplicon was expected to be 532 base pairs (bp). An amplicon of 532bp was seen in only 5 of the 100 isolates (which were found to be MRSA isolates using the by phenotypic method) tested. PCR amplification of *mec*A gene demonstrating amplicon of 532bp products are given in Fig. 2.

The 100 *Staphylococcus aureus* of isolates were derived from wounds (31 samples), the urine samples (26 samples), the high vaginal swabs (12 samples), the sputum samples (11 samples), the ear swabs (9 samples), the blood cultures (8 samples), the urethral swabs (2 samples), and the semen samples (1 sample). Among the MRSA strains, three MRSA isolate was detected in wound samples. The remaining 2 MRSA isolates were detected in urine samples. The overall

distribution of *Staphylococcus aureus* used in the PCR assay according to the study area is given in Table 2.
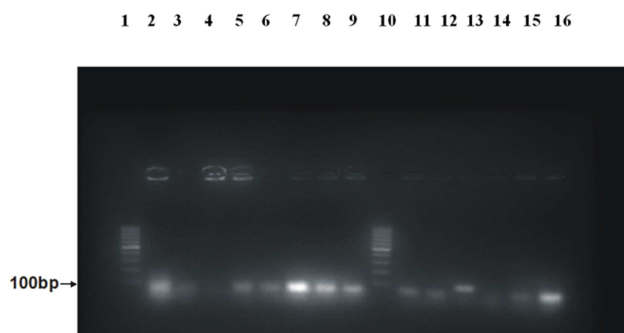
Figure 1. Agarose gel electrophoresis patterns showing PCR amplification products from *S. aureus* isolates. A 100 molecular weight size marker (O' GeneRuler™ 100bp ladder) was applied at the lane 1 and 10 of the gel to identify the isolated genes. A negative control (methicillin susceptible *S. aureus*, ATCC 25923) was applied at the lane 13. PCR products of the test samples were applied on lane 2,3,4,5,6,7,8,9,11,12,14,15 and 16 which showed clear bands confirmed that all the isolates were *Staphylococcus aureus*.
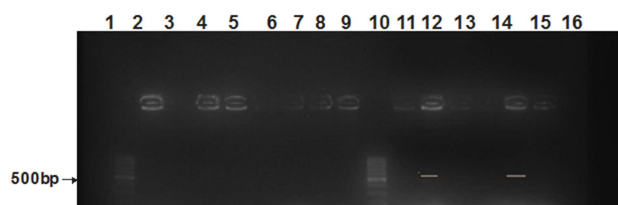
Figure 2. Representative agarose gel electrophoresis of PCR products. Lanes 12, 14 are positive for *mec*A as indicated by 532bp PCR product (Methicillin Resistant *S. aureus* isolates), Lane 2-9, 11, 13, 15, are negative for *mec*A (Methicillin Susceptible *S. aureus* isolates), Lane 16: negative control (methicillin susceptible *S. aureus*, ATCC 25923); Lane 1, 10: molecular weight size marker.

Table 2. Distribution of *Staphylococcus aureus* isolates according to the type of specimen.

Specimen type	*Staphylococcus aureus* isolates		
	No. of *mec*A$^+$ (%)	No. of *mec*A$^-$(%)	Total (%)
Wound swabs	3 (9.7)	28 (90.3)	31 (31.0)
Ear swabs	0 (0.0)	9 (100)	9 (9.0)
Blood culture	0 (0.0)	8 (100)	8 (8.0)
Urine	2 (7.7)	24 (92.3)	26 (26.0)
High vaginal swabs	0 (0.0)	12 (100)	12 (12.0)
Sputum	0 (0.0)	11 (100)	11 (11.0)
Semen	0 (0.0)	1 (100)	1 (100)
Urethral swabs	0 (0.0)	2 (100)	2 (100)
Total	**5 (5.0)**	**95 (95.0)**	**100 (100)**

Mean = 0.285714, SE = 0.285714, SD = 0.755929, CL (95.0%) =0.699118

4. Discussion

Since the introduction of semi synthetic penicillins, such as methicillin and oxacillin, for the therapy of infections caused by *S. aureus*, the occurrence of *S. aureus* strains resistant to methicillin has steadily increased and MRSA strains have become major nosocomial pathogens (Panlilio *et al*., 1992;

Voss *et al*., 1994). Infections with MRSA strains require treatment with glycopeptide antibiotics, which can be nephro- and ototoxic. Additionally, a diagnosis of MRSA infection has important implications for the management of patients since an extensive set of hygienic precautions must be taken to limit the spread of MRSA (Boyce, 2001).

Therefore, a rapid and reliable diagnosis of infection by MRSA is of major importance. Although *S. aureus* is relatively easy to cultivate, conventional identification methods may yield false-positive or false-negative results (Wallet *et al.,* 1996; Frebourg *et al*., 1998). Standard susceptibility tests are time-consuming. The correct identification of *S. aureus* and the detection of the *mec*A gene based on molecular methods have evolved as the method of choice for definitive identification. Earlier studies, however, involved more or less complicated manual DNA extraction protocols, followed by single or multiplex PCR with detection of amplification products on agarose gels (Barski *et al*., 1996; Brakstad *et al*., 1992; Tokue *et al*., 1991; Tokue *et al*., 1992; Unal *et al*., 1994; York *et al*., 1996; Martineau *et al*., 1998; Kearns *et al*., 1999; Kohner *et al*., 1999). Those assays, however, were time-consuming, prone to contamination, and not suitable for routine diagnostic laboratories because of the lack of a hybridization technique.

Recently, a real-time PCR technique for the detection of MRSA with two separate PCRs based on a manual DNA extraction protocol was described (Tal *et al*., 2001). In that study, however, no internal control was employed. (Reischl *et al*., 2000) described a manual DNA extraction protocol followed by multiplex real-time PCR for the simultaneous detection of the *mec*A gene and an *S. aureus*-specific gene which served as an internal control. In comparison to the conditions used in that study, primer compositions and concentrations had to be changed in our study to balance PCR efficiencies for both of the targets by optimization of primer concentrations and product lengths. Because variations in lot-to-lot primer concentrations may exist, it is advisable to adjust the concentrations for each primer lot prior to its first use in routine diagnostics.

In spite of the growing consensus for the use of molecular methods, they are not yet available in the majority of routine diagnostic laboratories because of their elevated technical requirements. In the present study, a new molecular assay that targets both an *S. aureus*-specific gene and the *mec*A gene within a single PCR was established and evaluated. This assay includes a rapid DNA extraction protocol and PCR on a Techne TC-5000 instrument.

5. Conclusion

The new molecular assay was found to be rapid and robust.

Because it is a largely automated assay, less hands-on work is needed and it can be incorporated into the workflow of a routine diagnostic laboratory. On the basis of this finding; establishment of molecular diagnostic laboratory in secondary and tertiary health units is urgently required.

Acknowledgments

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