Comparison of identification and antimicrobial resistance pattern of Staphylococcus aureus isolated from Amassoma, Bayelsa state, Nigeria.

Funmilola Abidemi Ayeni[1], Tombari Gbarabon[2], Camilla Andersen[3], Niels Nørskov-Lauritsen[3]

1. University of Ibadan, Faculty of Pharmacy, Department of Pharmaceutical Microbiology. Ibadan, Nigeria
2. Niger Delta University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Amassoma, Nigeria
3. Aarhus University Hospital, Department of Clinical Microbiology, Aarhus, Denmark

Abstract

Background: Staphylococcus aureus is often responsible for fatal infections and recent upsurge of resistant strains has resulted in therapeutic failure. The identification of this microorganism is a major challenge to medical microbiologists in developing countries.

Methods: One hundred and eighty five isolates which had been previously isolated from the nares of 185 healthy college students' volunteers in Amassoma, Bayelsa State, South Nigeria were identified by MALDI TOF mass spectrometry, and PCR amplification of the spa gene. The identified isolates were compared with presumptive identities obtained by growth on MSA, tube coagulation and slide agglutination tests. Antimicrobial susceptibility testing of S. aureus isolates was performed by Kirby Bauer technique while MRSA was screened for by growth on chromIDTM MRSA plate and confirmed by PCR-amplification of mecA/mecC genes.

Results: From the 185 staphylococci that grew with yellow colonies on MSA, 24 were positive in the slide coagulase test, while 17 were positive in the tube coagulase test; MALDI TOF mass spectrometry and PCR amplification of the spa gene showed excellent concordance with the tube test, as all tube coagulase-positive strains were identified as S. aureus, while tube coagulase-test negative isolates in all cases were designated as other staphylococcal species by MALDI-TOF mass spectrometry and were spa PCR test negative. All S. aureus isolates were susceptible to clindamycin, vancomycin, fusidic acid, rifampicin and linezolid, while observed resistance to penicillin and trimethoprim were high. Only one MRSA strain was detected

Conclusion: The study confirms that the tube coagulase test is an accurate diagnostic method for identification of S. aureus, while growths on MSA and slide agglutination tests are inaccurate. We found a low prevalence of MRSA and a high rate of trimethroprim-resistance in the studied population.

Keywords: Antibiotics, coagulase, identification, S. aureus.

Introduction

Staphylococcus aureus is an important pathogen causing a wide range of infections e.g abscesses, furuncles, osteomyelitis, infective endocarditis and bacteremia. It is a major cause of severe nosocomial and community-acquired infections[1]. Its carriage in the anterior nares, considered to be the ecologic niche, plays a key role in the epidemiology of this pathogen[2].

Corresponding author:
Funmilola Abidemi Ayeni,
University of Ibadan,
Department of Pharmaceutical Microbiology
Email: funmiyeni@yahoo.co.uk

S. aureus can acquire resistance to many anti-microbial agents. Of particular concern is the synthesis of penicillin-binding protein (PBP) 2A that confers resistance to all β-lactam antimicrobial agents. Such strains are designated methicillin-resistant S. aureus (MRSA). PBP 2A is encoded by the mecA gene cassette, that can be detected by PCR and other DNA-based methods. Recently, a novel mecA homologue mecCLGA251 has been described[3]. The novel mecA gene also confers resistance to β-lactam antibiotics but escapes detection by molecular diagnostic tests currently used for identification of MRSA[3]. Genetic techniques have been used to study the evolution of the MRSA clones that have emerged since the early 1960s, and to study their worldwide dissemination. The early MRSA clones were hospital-associated (HA-MRSA). However, there

is now an evolution of community-associated MRSA (CA-MRSA) clones. CA-MRSA mostly causes skin or soft-tissue infections as well as deep-seated infections such as necrotizing pneumonia.

The culture characteristics of S. aureus on various selective media have served as the basis for its presumptive identification[4]. In an attempt to achieve presumptive isolation of pathogenic staphylococci from clinical samples in a single step, Mannitol Salt Agar (MSA) was developed in 1945[5,6]. Several modifications of MSA were subsequently described[7,8]. The key to the presumptive identification of S. aureus on MSA is the selective growth in the presence of 5 - 6.5% NaCl and yellow pigmented colonies resulting from fermentation of mannitol[9].

The molecular identification of S. aureus strains can be established by detecting the presence of staphylococci protein A, (spa). The spa typing method is based on sequencing of the polymorphic X region of the protein A gene present in all strains of Staphylococcus aureus[10] Also, a rapid and accurate method for identification of microorganisms is the matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry.[11]

In some developing countries, S. aureus is usually identified by growth and production of yellow colonies on MSA and the presumptive identification may be confirmed by a rapid agglutination kit or tube coagulase test. However, it has been observed that some of the results are often misleading because organisms that were identified as S. aureus with these methods may not be S. aureus but other staphylococci. There is therefore the need to compare commonly used methods of identification in Nigeria with other identification methods to determine the degree of accuracy of these commonly used methods. The aims of this study therefore are to compare the diagnostic accuracy of growth on MSA, tube coagulase, latex agglutination slide test with presence of spa gene and MALDI TOF for identification of S. aureus and to determine the resistance pattern of isolated S. aureus strains to selected antibiotics with screening for MRSA in the isolated S. aureus strains and determination of its susceptibility pattern.

Methodology
Bacterial strains
One hundred and eighty five non duplicated strains which were previously isolated from the nares of 185 healthy college students volunteers aged between 16 and 35 years of Niger Delta University, Ammasoma, Bayelsa State, (a state in South-South Nigeria) between January and August, 2011 had been presumptively identified as S. aureus through growth and production of yellow colonies on MSA. MSA is regarded as a presumptive media in the identification of S. aureus and differentiation of coagulase-positive staphylococci from coagulase-negative staphylococci. For the present study, all the strains were repeatedly streaked on MSA (Oxoid, UK) and incubated for 48 hours at 37[0]C. All strains that grew on MSA with characteristic yellow colonies were used in further tests.

Identification of strains
Coagulase tests
The strains that produced bound coagulase (BC) were detected in all strains that grew on MSA with yellow colonies by a rapid agglutination kit for identification of S. aureus: (Slidex® Staph PLUS. bioMérieux, France) according to the manufacturer's instructions. Slidex Staph Plus is based on a triple detection system: blue latex particles sensitized with human fibrogen and monoclonal antibodies simultaneously detect clumping factor, Protein A and group-specific antigen bound to the S. aureus-specific peripheral structure. All strains that grew on MSA with yellow colonies were also inoculated into a tube containing horse plasma (SSI, Denmark) and incubated at 37[0]C for 4 hours. The tube coagulase test detects free cogulase produced by the staphylococci by coagulating plasma.

Detection of Spa gene.
The polymorphic X region of the staphylococcal protein A gene present in almost all S. aureus described by Harmsen et al,[10] was amplified for all isolated strains in a total volume of 50 µl by adding 1µl of a 1:500 dilution of genomic DNA, 1 µl of 25Mm $MgCl_2$, 1 µl of forward primer, 1 µl of reverse primer, 21 µl of water and 25 µl of AmpliTaq Gold® 360 Mastermix into a 0.2-ml PCR tube. Primers used were spa-1113F (5'-TAAA-GACGATCCTTCGGTGAGC-3') and spa-1415R (5'-CAGCAGTAGTGCCGTTTGCTT-3'). A negative control (plain mastermix) and a positive control (from our laboratory's S. aureus collection) were included. Tubes were capped and placed in a Veriti Thermal Cycler (Applied Biosystems). Thermal cycling conditions included an initial 10 min at 95°C followed by 32 cycles

of 30s at 94°C, 30 s at 55°C, and 60 s at 72°C; and a final extension at 72°C for 10 min. PCR products were resolved by agarose (1 %) gel electrophoresis previously stained with GelRed (Biotium Inc, Hayward, CA, USA) and run at approximately 40 mAmp for 45 min. The molecular marker used was a pUC mix Marker 8. The size of the PCR products was 400-600 bp for the spa gene. This test is the first gold standard to compare the accuracy of tube and slide coagulase test and growth on MSA.

MALDI mass spectrometry.
Identification of all isolated strains was carried out by MALDI mass spectrometry. A thin smear of 18 h old culture was deposited on a MALDI plate. The samples were overlaid with 1 µl of matrix solution (saturated solution of cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid). The matrix sample was crystallized by air drying at room temperature. Measurements were performed with a Microflex mass spectrometer (Bruker Daltonik, Bremen, Germany) using FlexControl software (version 3.0). Spectra were recorded in the positive linear mode (laser frequency, 20 Hz; ion source 1voltage, 20 kV; ion source 2 voltage, 18.4 kV; lens voltage, 9.1 kV; mass range, 2,000 to 20,000 Da). For each spectrum 240 shots in 40-shot steps from different positions of the target spot were collected and analyzed.

Results of the pattern-matching process were expressed as proposed by the manufacturer with scores ranging from 0 to 3. For each isolate, the highest score of a match against a spectrum in the database was used for identification. Scores below 1.7 were considered not to have generated a reliable identification; a score of 1.7 was considered identification to genus, and a score of 2.0 was used for species identification. This test is the second gold standard to compare the accuracy of tube and slide coagulase test and growth on MSA

Antibiotic susceptibility testing
Antibiotic susceptibility testing of all S. aureus strains was performed by Kirby Bauer technique according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (www.eucast.org) using standard antibiotic discs (Oxoid, UK namely: fusidic acid, linezolid, ampicillin, norfloxacin, rifampicin, penicillin, clindamycin, erythromycin, cefoxitin, tobramycin, trimethoprim and vancomycin were placed firmly on an agar plates by a disc dispenser. The plates were incubated at 37⁰C for 24 hrs and examined for clear zones of inhibition around the discs. The diameter of inhibition was measured and compared with standard zones to determine resistance

Detection of MRSA
Growth on MRSA plates
All S. aureus strains that were resistant to cefoxitin were subcultured on a chromogenic medium, chromIDTM MRSA plate (bioMérieux, France). After incubation, strains that grew on the plates with green colouration were selected for further studies.

SLIDEX MRSA detection
All S. aureus strains that were resistant to cefoxitin were tested for the presence of Penicillin Binding Protein-2 (PBP-2). This was performed with Slidex MRSA Detection Kit (bioMérieux, France) according to the manufacturer's instructions. This is a slide agglutination assay that detects PBP2a in MRSA by use of latex sensitised with a monoclonal antibody directed against PBP2a

Detection of MecA and coagulase gene
PCR amplification of mecA/(mecC(LGA251)) gene. PCR assay was performed for all suspected MRSA strains to amplify a region of mecA including the novel homologue mecC (mecALGA251) according to García-Álvarez et al (3) . Primers were as follows: Fw, 5´ TCACCAGGTTCAACY]CAAAA 3´; and Rv, 5´ CCT-GAATCW]GCTAATAATATTTC 3´.
PCR reaction contained 50 µl reaction volume of AmpliTaq Gold® 360 Mastermix (2x stock including PCR buffer, TAQ-polymerase, MgCl2 and dNTPs) with 0.5 µl of each primer (stock for 100 pmol/µl (LGA251) and 25 pmol/µl (Coa)) and 10 µl template for each. The mixture was prepared as freezer-mix (ready-to-use just as add 10 µl template to 40 µl mix freshly thawed).
A negative control, with no target DNA, was included in the PCR and run in the Veriti Thermal Cycler (Applied Biosystems) and a positive control the amplification programme consisted of an initial denaturation step at 95°C for 10 min; 40 cycles of denaturing at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min; and a final extension at 72°C for 5 min. PCR products were resolved by agarose (1 %) gel electrophoresis previously stained with GelRed (Biotium Inc, Hayward, CA, USA) and run at approximately 40 mAmp for 45 min. The molecular marker used was a pUC mix Marker 8. The size of the PCR products were 356 bp for the mecA/mecC gene.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of antibiotics were determined in the MRSA and selected methicillin susceptible S. aureus (MSSA) for comparison using Gram-positive (GP) AST P-580 antibiotics sensitivity cards (bioMérieux, France) by Vitek-2 apparatus (bioMérieux) according to the manufacturer's instructions. The system determined the susceptible and resistant strains according to the software program.

Results

The nasal carriage rate of S. aureus strains in the studied bacterial population is 9%. All 185 strains grew as yellow colonies on MSA and all the strains were identified by MALDI TOF as staphylococci. 168 were coagulase negative staphylococci (CNS) while 17 strains from the total strains were identified as S. aureus.

The presence or absence of spa gene in the 185 strains were detected by PCR amplification of the gene. There was amplification only in 17 strains and the strains were identified as S. aureus by MALDI TOF (Table I). All the 185 strains were tested for ability to coagulate plasma using agglutination kit and tube coagulase test. All the strains identified by MALDI TOF as S. aureus were also tube-coagulase positive (Table I). Twenty four isolates were positive in agglutination kit test. Isolates positive in agglutination kit but negative in tube coagulase test were identified as S. xylosus. . Three strains had positive result in tube coagulase test, spa-PCR and identified as S. aureus by MALDI TOF but had negative result in slide agglutination test.

Table 1: Comparison of Accuracies of Identification Methods for Isolated *S. aureus* Strains

Gold Standards	Other Tests		
MALDI and *spa*	Tube Coagulase	Slide agglutination	MSA
17 *S. aureus*	17 *S. aureus*	14 *S. aureus*	185 P. *S. aureus*
168 CNS	168 CNS	171CNS	-
	Sensitivity 1	0.82	0.1
	Specificity 1	0.95	-

Note=P is presumptive identified S.aureus which is only correct for 17 out of 185 isolates

The studied S. aureus strains were generally susceptible to the tested antibiotics except penicillin and trimethoprim. All the tested strains were resistant to penicillin except 3 strains. 53% of the isolates were also completely resistant to trimethoprim with no zone of inhibition. All the tested isolates were susceptible to clindamycin, vancomycin, fusidic acid, rifampicin and linezolid. 7% of the tested strains were resistant to erythromycin and cefoxitin while 24% of the tested strains were resistant to norfloxacin. (Table II).

Table II: Percentage Resistance of Isolated *S. aureus* strains to antibiotics

Antibiotics	No of Resistant Strains	% Resistance
Ampicillin	3	18
Norfloxacin	4	24
Penicillin	13	77
Erythromycin	1	6
Cefoxitin	1	6
Tobramycin	2	12
Trimethoprim	9	53

Only one MRSA strain was detected in the studied population and the MRSA carriage in the studied population is 0.5%. The MRSA strain (S. aureus FA91) grew on chromIDTM MRSA plate, produced PBP-2 by Slidex MRSA Detection Kit and was confirmed as MRSA by detection of mecA/C by PCR. Comparison of the susceptibility pattern of the isolated MRSA with 3 MSSA revealed that the isolated MRSA was relatively susceptible to tested antibiotics with resistance observed only to beta lactams and trimethoprim. (Table III).

Table III: Comparison of Resistance Pattern of Isolated MRSA and Selected MSSA by Determination of MIC.

Antibiotics	Minimum Inhibitory Concentration of MRSA and MSSA			
	MRSA FA91	MSSA FA28	MSSA FA213	MSSA FA293
Cefoxitin screen	+	-	-	-
Penicillin	R > 0.5	R > 0.5	S	R > 0.5
Oxacillin	R 1	S	S	S
TMP/SMX	R 160	R > 320	S	R > 320
Gentamycin	S	R 16	S	S
Tobramycin	S	R 16	S	S
Levofloxacin	S	R 8	S	S
Moxifloxacin	S	R 2	S	S
Tetracycline	S	R 16	S	S

Note- MRSA: Methicillin Resistant *Staphylococcus aureus*
MSSA: Methicillin Susceptible *Staphylococcus aureus*
R- Resistant
S:-Susceptible

Discussion

Microbiologists in developing countries rely on biochemical tests and growth on selective media to identify pathogens. Often, these identifications are inaccurate resulting in inappropriate prescription and use of antibiotics for misidentified pathogens[12]. From the studied 185 organisms that grew with yellow colonies on MSA in this study, 168 are CNS while 17 were identified as S. aureus by MALDI TOF. Furthermore, the accuracy of identification of S. aureus strains by coagulase tests was tested with amplification of spa gene and MALDI TOF. The spa gene is specific to S. aureus and expression of this gene provides useful strain identification [10,13]. Identification using whole cell protein profiles by MALDI-TOF–MS has been found useful and accurate in identifying most bacterial strains including S. aureus strains[11].

The tube coagulase test (horse plasma) is 100% accurate in identifying S. aureus strains, however slide agglutination method is not only positive for S. aureus but also for S. xylosus strains and three S. aureus strains were negative in slide agglutination test. Therefore, the slide agglutination test and growth on MSA is inaccurate for sole identification of S. aureus while the tube coagulase test is totally accurate for identification of S. aureus strains.

The S. aureus strains isolated from this study were generally susceptible to the tested antibiotics except trimethoprim. This observed susceptibility may be because the strains were isolated from healthy carriers in the community. The high rate of resistance to trimethroprim by S. aureus strains found in this study is very disturbing. The combination of trimethoprim and sulfamethozole (co-trimoxazole) is widely used for various infections in Nigeria because of its low cost compared with other antibiotics. It is also commonly used as prophylaxis for HIV infected patients who have a high occurrence in Nigeria. This may account for a possible higher selective pressure on the agent therefore, accounting for increased rate of resistance. Huovinen et al.[14] observed a clear trend in the resistance to TMP-SMX with strains isolated in the developing world being more often resistant than the strains isolated in the developed countries. The clinical significance of this finding is that resistance to co-trimoxazole has been associated with concurrent resistance to other antibiotics resulting in multi-drug resistant pathogens.[15]

There are conflicting reports about the prevalence of MRSA strains in Nigeria. Ghebremedhin et al.[1] reported a prevalence rate of 20.23% of MRSA and 47% of CA-MRSA in Nigeria, Taiwo et al.[16] reported a prevalence rate of 29% while 1.4% prevalence rate was reported by Adesida et al.[17]. Only one MRSA was detected in this study. This shows a low prevalence rate of MRSA in the studied population.

The MRSA isolated in this study is resistant to trimethoprim and beta lactams while generally susceptible to other tested antibiotics. Interestingly, an MSSA strain was highly resistant to tested antibiotics while the studied MRSA was very susceptible to tested antibiotics. The MRSA has the susceptibility pattern of CA-MRSA according to Fridkin et al.[18] who reported that CA-MRSA are generally susceptible to most antimicrobial agents with the exception of beta-lactams and erythromycins. Lack of multidrug resistance in an MRSA may be an indication that it is CA-MRSA.

This investigation confirms that coagulation of horse plasma in tube coagulase test is reliable and accurate identification method for identifying S. aureus strains and that slide agglutination test is not totally accurate. It also confirms that growth with mannitol fermentation (yellow) colonies on MSA cannot differentiate between S. aureus and CNS colonies on MSA. This observation was confirmed by Adebayo et al.,[9] who observed growth of CNS on MSA. It also showed low prevalence of MRSA in the studied population. The high rate of resistance to trimethroprim by S. aureus strains found in this study calls for control in the use of the antibiotics in Nigeria

Conflict of interest disclosure
There is no conflict of interest in the manuscript

References
1. Ghebremedhin B, Olugbosi MO, Raji AM, Layer F, Bakare RA, König B, König, W (2009) Emergence of a Community-Associated Methicillin-Resistant Staphylococcus aureus Strain with a Unique Resistance Profile in Southwest Nigeria. *J Clin Microbiol* 47(9): 2975–2980
2. von Eiff C, Becker K, Machka K, Stammer H, Peters G, for the Study Group (2001) Nasal carriage as a source of Staphylococcus aureus bacteremia. *N Engl J Med* 344: 11–16
3. García-Álvarez L, Holden MT, Lindsay H, Webb CR, Brown DF, Curran MD, Walpole E, Brooks K, Pickard DJ, Teale C, Parkhill J, Bentley SD, Edwards GF, Girvan EK, Kearns AM, Pichon B, Hill RL, Larsen AR, Skov RL, Peacock SJ, Maskell DJ, Holmes MA (2011) Meticillin-resistant Staphylococcus aureus with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. *Lancet Infect Dis* 11(8): 595-603
4. Stoakes L, Reyes R, Daniel J, Lennox G, John MA, Lannigan R, Hussain Z (2006) Prospective comparison of a new chromogenic medium, MRSASelect, to CHROMagar MRSA and mannitol-salt medium supplemented with oxacillin or cefoxitin for detection of methicillinresistant Staphylococcus aureus. *J Clin Microbiol* 44: 637– 639
5. Chapman GH (1945) The significance of sodium chloride in studies of staphylococci. *J Bacteriol* 50: 201– 203
6. Blair EB, Emerson JS, Tull AH (1967) A new medium, salt mannitol plasma agar, for the isolation of Staphylococcus aureus. *Am J Clin Pathol* 47:30– 39
7. Safdar N, Narans L, Gordon B, Maki DG (2003) Comparison of culture screening methods for detection of nasal carriage of methicillin-resistant Staphylococcus aureus: a prospective study comparing 32 methods. *J Clin Microbiol* 2003; 41:3163– PubMed ;3166
8. Smyth RW, Kahlmeter G (2005) Mannitol salt agar–cefoxitin combination as a screening medium for methicillin-resistant Staphylococcus aureus. *J Clin Microbiol* 43:3797–3799
9. Adebayo Shittu, Johnson Lin, Donald Morrison and Deboye Kolawole (2006) Identification and molecular characterization of mannitol salt positive, coagulase-negative staphylococci from nasal samples of medical personnel and students. *J Med Microb* 55: 317–324
10. Harmsen D, Claus H, Witte W, Rothgänger J, Claus H, Turnwald D, Vogel U (2003) Typing of methicillin-resistant Staphylococcus aureus in a university hospital setting by using novel software for spa repeat determination and database management. *J Clin Microbiol* 41(12):5442-5448
11. Madhava Charyulu E., Arumugam Gnanamani and Mandal A.B. (2012) Identification and Discrimination of Methicillin Resistant Staphylococcus aureus Strains Isolated from Burn Wound Sites Using PCR and Authentication with MALDI-TOF–MS. *J Microbiol* 52(3) :337–345
12. Ayeni FA, Adeniyi BA, Ogunbanwo ST, Tabasco R, Paarup T, Peláez C, Requena T (2009) Inhibition of uropathogens by lactic acid bacteria isolated from dairy foods and cow's intestine in western Nigeria. *Arch Microbiol* 191 (8): 639-648

13. Walker J, Borrow R, Edwards-Jones IV, Oppenheim BA, Fox AJ (1998) Epidemiological characterization of methicillin-resistant staphylococcus aureus isolated in the North West of England by protein A (spa) and co-agulase (coa) gene polymorphisms. *Epidemiol Infect* 121: 507-514

14. Huovinen P, Sundstrom L, Swedberg, G, Skold O (1995) Trimethoprim and sulfonamide resistance. Antimicrob Agents Chermother 39: 279-289

15. Zhanel GG, Karlowsky JA, Harding GKM, Carrie A, Mazzulli T, Low DE. The Canadian Urinary Isolates Study Group, and Daryl J,Hoban DJ (2000) A Canadian National Surveillance Study of Urinary Tract Isolates from Outpatients; Comparison of the Activities of Tri-methoprim-Sulfamethoxazole, Ampicillin, Mecillinam, Nitrofurantoin and Ciprofloxacin. Antimicrob. Agents Chermother. 44: 1089-1092

16. Taiwo SS, Bamidele M, Omonigbehin EA, Akinsinde KA, Smith SI, Onile BA, Olowe AO (2005) Molecular epidemiology of methicillinresistant Staphylococcus aureus in Ilorin, Nigeria. *West Afr J Med* 24:100–106

17. Adesida SA, Boelens H, Babajide B, Kehinde A, Snijders S, Van Leeuwen W, Coker A, Verbrugh H, van Belkum, A (2005) Major epidemic clones of Staphylo-coccus aureus in Nigeria. *Microb Drug Resist* 11:115–121

18. Fridkin SK, Hageman JC, Morrison M, Sanza LT, Como-Sabetti K, Jernigan JA (2005) Methicillin-resist-ant Staphylococcus aureus disease in three communi-ties. *N Engl J Med* 352: 1436-1444