

Full Length Research Paper

Molecular characterization of *staphylococcus aureus* isolated from wounds

Wurochekke A.U, Obanibi W.O and Sarkiyayi S

Department of Biochemistry, Modibbo Adama University of Technology Yola, PMB 2076, Adamawa State. Nigeria.

Accepted 21 October, 2013

This study was carried out to determine the molecular characterization of *staphylococcus aureus* isolated from wound sources in ABU teaching hospital. 10 samples were collected from the hospital. They were sub cultured, the morphological test, the microbiological test, the biochemical test and the molecular biology tests were carried out. The molecular techniques that require the isolation of genomic DNA, polymerase chain reaction (PCR) for the amplification of a gene sequence were carried out. The PCR-machine used was 312-techgene, 9 samples were amplified during the reaction, which means 9 samples were positive. The *s. aureus* strain associated with the methicillin resistance gene (*femA*gene) was amplified at 647bp. The specific gene for *femA* gene was amplified at 647bp. With the advantage of it being assayed for within 5 hours using molecular techniques compared to the conversational method of testing which take up to 4 days. *S. aureus* was present on healthy humans of all ages, sex and is a multidrug resistance microorganism which can result to complicated infection, if not diagnosed and treated earlier. This molecular technique should be employed in the hospital for early diagnosis and appropriate treatment to prevent complicated infections.

Key words: Methicillin resistance gene ,wounds, isolation, Genomic DNA, microorganism.

INTRODUCTION

Staphylococcus aureus is a gram-positive, non-motile spherical bacteria occurring in grape like clusters. Some species are saprophytes others are parasites. They are commonly present on the skin, mucous membrane, pus and wounds. Apart from causing boils and internal abscesses more serious, infections caused by the bacteria include pneumonia, bacteraemia, as well as boils, urinary tract, and wound infection (Liu et al., 2005).

The common species of *Staphylococcus* include *S. aureus* and *Staphylococcus epidermis*, which does not normally cause infection. However, either of these bacteria can cause serious infections under the right conditions (Cook et al., 2007). Following heart surgery, *S. epidermis* may cause endocarditis. It may also turn an existing abnormality in the urinary tract into cystitis (Iwase et al., 2010).

S. aureus are present in places where their food is found, like in blood stream where they can withstand

streeful conditions. This bacterium had been isolated on the human skin, in the air, wounds and pus (Cimolai, 2011). Wound can be an injury or damage to the underlying tissue that offer access to microorganism to the cells to cause infection. Through this, *S. aureus* had gain access in to human body (Encarta Dictionary, 2009).

Disease associated strains often promote infections by producing potent toxins and expressing cell surface proteins that bind and inactivate antibodies. The emergence of antibiotic-resistance forms of pathogens *S. aureus* is a worldwide problem in clinical medicine. A study conducted by Ogston, (2004) revealed that infections with a strain called methicillin-resistant *S. aureus* (MRSA) are spreading and pose a major health problem in wounds. The strain, popularly dubbed a "superbug", is resistant to the antibiotic drug methicillin, as well as to penicillin and other commonly used antibiotics. MRSA can invade tissues and organs, causing blood and bone infections, pneumonia, and inflammation of the lining of the heart (Chang, 2007). Study estimated that there were 94,360 invasive MRSA infections (Hiramatsu et al., 2005).

Conventional methods of diagnosis identification of MRSA are time consuming and delay in treating this

*Corresponding author. E-mail: sarkiyayi_shehu@yahoo.com

type of infection can have fatal consequences. New rapid techniques for identification and characterization of MRSA have been developed. Consequently, quantitative PCR technique is increasingly being employed in clinical laboratories for rapid detection and identification of MRSA strains. This work was designed to isolate and characterize the genomic DNA of *S. aureus* from wounds of patients attending Ahmadu Bello University Teaching Hospital Zaria Nigeria.

MATERIALS AND METHODS

Staphylococcus aureus.

Equipment

Sterile Eppendorf Pasteur pipette, Eppendorf Centrifuges, Thermocool micro wave oven, Incubator, microscope, tech UV-ipro, Omega Autoclave, Techgene PCR Machine

Reagents

They were obtained from the Fermentas Company, and they were all of analytical grade.

Sample collection

The samples were collected from the laboratory in Ahmadu Bello University Teaching Hospital by sub-culturing to a prepared mannitol agar salt. A total of 10 specimens were collected from wound infections for bacterial culture.

Genomic DNA Isolation

- Bacterial broth of about 2×10^9 was harvested by pipetting 2ml into sterile micro-centrifuge tube and then centrifuged for 10minutes at 700rpm and the supernatant was discarded.
- The pellet was suspended in 180 μ l of digestion solution. About 20 μ l of proteinase K solution was added and mixed thoroughly; it was then vortexed to obtain a uniform suspension.
- The samples were incubated at 56 $^{\circ}$ C and vortex until the cell was completely lysed.
- RNase solution of 20 μ l was added and mixed by vortexing and it was incubated for 10minutes at room temperature.
- Lysis solution of 200 μ l was added to each sample, mixed thoroughly by vortexing for 15s until homogeneous mixture was obtained.

- Micro-Pipette was used to add 400 μ l of 50% ethanol and it was mixed by vortexing.
- The prepared lysate was transferred to a geneJET™ Genomic DNA purification column, it was inserted into a collection tube. The column was centrifuged at 8000rpm. The flow-through solution in the collection tube was discarded. Then the GeneJET™ genomic DNA purification column was placed into a new 2ml collection tube.
- Wash buffer I of 500 μ l (with ethanol added) was centrifuged for 1minute at 1000rpm. The flow-through was discarded and the purification column was placed back into the collection tube.
- Wash buffer II of 500 μ l (with ethanol added) was added to the GeneJET™ genomic DNA purification column. It was Centrifuged for 3minutes at 14000rpm, the collection tube containing the flow-through solution was discarded and the GeneJET™ genomic DNA purification column transferred into a sterile 1.5ml micro-centrifuge tube.
- Elution buffer of 200 μ l was added into the GeneJET™ Genomic DNA purification column membranes to elute genomic DNA. It was incubated for 2minutes at room temperature and centrifuged for 1minute at 10000rpm. The purification column was discarded, then the purified DNA was used immediately or stored at -20 $^{\circ}$ C (Fermentas Company, 2011).

Agarose gel electrophoresis preparation

- TAE Buffer of 1 strength 100ml was measured into a beaker.
- Agarose gel of 1g was weighed and gently transferred into the beaker containing x1 TAE Buffer.
- It was carefully taken into a microwave oven to prevent the Agarose from getting to the wall of the beaker.
- After 2 minutes, it was removed from oven, and allowed to cool to 60 $^{\circ}$ C. 10 μ l of Ethidium bromide was added, using micro-pipette. Ethidium bromide which was responsible for absorbing DNA best at 254nm and re-emits at 590nm in red-orange spectrum which makes DNA visible and fluorescence.
- The electrophoresis tray was blocked with the edge of the electrophoretic tank.
- Then the dissolved Agarose gel which contains 10 μ l of Ethidium bromide was gently poured into the tray to prevent bubbles and when there were bubbles, it was popped out with micro-pipette tips, the comb was fixed.
- After 30minutes, it solidified and the comb was removed straight up to prevent the gel from breaking, then the wells were formed.
- 300ml of x1 TAE Buffer was poured into the Agarose gel electrophoretic tank after setting the tray, 10 μ l of DNA ladder was picked with micro-pipette and

and loaded into the first well.

- Then, 13µl of DNA sample was mixed with 5µl of loading dye (loading dye contains glycerol which makes DNA to be heavy and sink into the well). using micro-pipette on a parafilm paper or in a PCR tube
- The DNA and the loading dye was mixed and pick with micro-pipette, it was placed inside the well and slowly ejected to make sure that the mixture was put inside the well.
- After completely loading all the DNA samples into the well, the gel electrophoretic tank was covered and it was switched on for 30minutes with voltage of 100v and 140mA.
- Then, the gel was carefully placed on the transilluminator and bands of DNA was visualized, the picture was capture in the documentation unit, using UV ipro the image was visualized on computer screen and was captured, recorded, and interpreted. (Centre for Biotechnology Research and Training, 2011).

Polymerase Chain Reaction (PCR) Kit for the testing of *Staphylococcus aureus*

Protocol as it was recommended by the Manufacturer of the Kits

- (i) The dream taq PCR master mix (2x) was gently vortex and briefly centrifuged.
- (ii) The primer was reconstituted by adding nuclease-free water as specified into primer I and primer II respectively; it was spun and placed on ice.
- (iii) The PCR tube was placed on ice and 50µl of mixture components were added into each centrifuge tube as following.
- (iv) Each sample was gently vortex and spin down. The PCR programme was performed using the recommended thermal cycling conditions was optimized as outlined by the PCR kit manufacturer. The PCR machine was then started and stopped at the end of the programme (Fermentas Company, 2011). The gel electrophoresis was carried out; amplicons was visualized on the transilluminator due to the presence of Ethidium bromide in the gel. It was then documented in the documentation unit using UV ipro to capture image as obtained on the computer screen (CBRT, 2011).

Microscopic Method (Morphological Test)

Morphology is the Biological structure of an organism, it was visualized under Microscope and it was observed to be in cluster stings form.

The Microbiological Test (Gram Staining)

Gram staining, colony grown overnight was picked with a

Sterile wire loop, it was used to prepare smear. The smear was allowed to dry and the slide was gently passed sterile wire loop, it was used to prepare smear. The smear was allowed to dry and the slide was gently passed through a flame to heat fixed it.

The heat fixed smear was then covered with crystal violet for 1 minute, the stain was washed away after 1 minute with distilled water, and it was then covered with lugol iodine for 30 seconds after which it was washed with distilled water. The color of the smear was washed away with acetone (alcohol), then the slide was placed at slanted position, the acetone was washed away immediately and safaril was added for 2 minutes, which indicated that it was gram positive bacteria.

Preparation of Mannitol and Culture of *Staphylococcus aureus*

Mannitol salt Agar was prepared by weighing 11.1g of Mannitol Salt Agar into beaker, it was dissolved and made up to 100ml using distilled water, an aliquot was taken into small bottle (culturing bottle) and was autoclaved and cooled. The swab stick was used to inoculate the media from the source, it was done close to flame to prevent contamination. There was production of yellow coloured colonies which is as a result of fermentation and drop in the pH of media, which was prove for growth of *S. aureus*.

Enzyme based Tests

Catalase Tests

This was to detect the test organism producing the catalase enzyme, the enzyme break down Hydrogen peroxide into Oxygen and Water. This test is used in distinguishing *S. aureus* from other staphylococcus species; two colonies of the test organism were picked with applicator stick. The colonies were dipped into a Hydrogen peroxide on the slide. There was escape of gas bubbles indicating a *S. aureus* positive.

Coagulase Tests

Slide Test

A slide coagulase test was run with a negative control to rule out agglutination. Two drops of saline were put into the slide labeled with sample number, Test sample (T) and control (C). The two saline drops were emulsified with the test organism using a wire loop. A drop of plasma (plasma anticoagulated with EDTA was added) and placed on the inoculated saline drop corresponding to test, and mixed well, then the slide was rocked gently for about 10 seconds. Macroscopic clumping was observed in the plasma within 10 seconds indicates positive, with no clumping in the saline drop indicates negative for control.

Components	Volume
Dream Taq PCR Master Mix 2x	25µl
Forward primer	0.5µl
Reverse primer	0.5µl
Template DNA	7µl
Water, nuclease free	17µl
Total	50µl

Step	Temperature	Time	Number of Cycle
Initial denaturation	94 ⁰ C	35 cycles	5minutes
Denaturation	94 ⁰ C		30seconds
Annealing	55 ⁰ C		30seconds
Extension	72 ⁰ C		1minute/kb
Final Extension	72 ⁰ C		10minutes

Analysis of result for Genomic DNA on Agar Gel Electrophoresis.

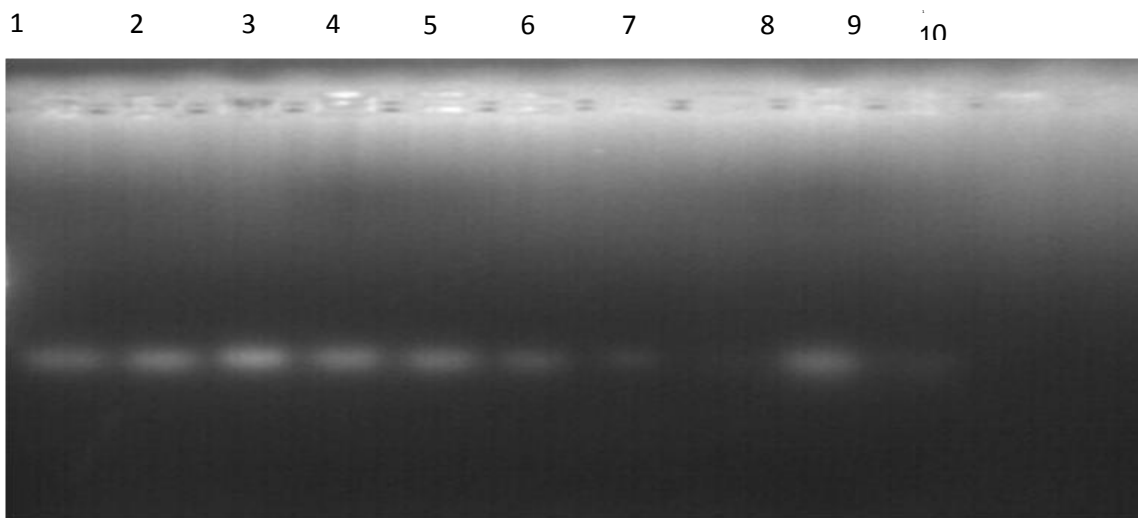


Figure 2. The Bands of Genomic DNA.

RESULTS

The result revealed that all samples were gram positive, catalase positive and coagulase positive. It was then grown in mannitol salt agar for 16 hour which was a selective media for *S. aureus*, then the next day the density had increased which means that there was growth.

The isolates were suspected as *S. aureus* morphologically on a culture, Species identification was confirmed by polymerase chain reaction (PCR) amplification as shown in Figure 2.

Microscope Method (Morphological Test)

It was positive to morphological test, visualized under

microscope to be observed in cluster stings form.

The Microbiological Test (Gram Staining)

They were positive to Gram staining.

Preparation of Mannitol and Culture of *Staphylococcus aureus*

There was production of yellow coloured colonies which was as a result of fermentation and drop in the pH of media, which was proved to be growth of *S. aureus*.



Figure 1. Catalase reaction with colonies of *Staphylococcus aureus*.

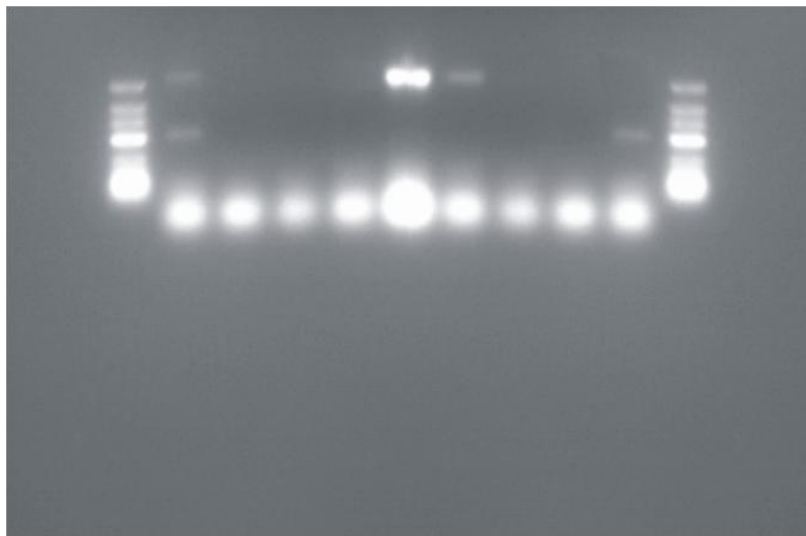


Figure 3. PCR Amplification of *femA* Gene.

Enzyme based Tests

Catalase Tests

The colonies were dipped into a Hydrogen peroxide on the slide. There was escape of gas bubbles indicating a *S. aureus* positive as shown in [Figure 1](#).

Coagulase Tests

Slide Test

Macroscopic clumping was observed in the plasma within 10 seconds indicates positive, with no clumping in the saline drop indicates negative for control.

Genomic DNA isolated from *S. aureus* Lane 1-10 are bands on the Agarose Gel Electrophoresis Lane 1-7 was visualized distinct 8 was not appearing at all and 9 distinct 10 appeared very faint (slightly) with the indication that DNA is present in the isolates.

Analysis of PCR Result as obtained by Gel Electrophoresis

Out of 9 isolates, Amplicons were visualized in 4 of the isolates. Molecular weights were calculated by comparing Hind III digested λ -phage.

DNA marker and were analyzed by comparing the molecular weight of the Amplicons with the molecular weight of the ladder.

The polymerase chain reaction (PCR) product of *femA* gene in *S. aureus*, the M is the Molecular weight ladder which were calculated with hind III digested λ -phage, 4 of the specimens were positive to the gene *femA* (Lane 1, 5, 6, 9), while 5 were negative (Lane 2, 3, 4, 7, 8). In lane 1 and 9, the light amplicons move faster than the heavier once. In lane 1, 5, 6 contain the gene for *femA* which were Amplified at 647 and 1000bp when using 100bp from Fermentas Company. The base pair of *femA* gene was Amplified at between 600 to 984bp using 100bp (Fujita, et al., 2009).

DISCUSSION

The emergence of methicillin resistance in *S. aureus* is of great concern, as MRSA strains are often multidrug resistant. Infections with MRSA are known to be associated with considerable morbidity and mortality. Standard culture methods for the identification of *S. aureus* and the determination of drug resistance susceptibility are time-consuming, usually requiring 2 to 4 days. This delay may lead to the abuse of antimicrobial agents. For these reasons, it has become important to develop rapid diagnostic tests for the detection of MRSA. In order to achieve this, PCR techniques was developed to assay for identification of antibiotic resistance genes in *S. aureus* clinical isolates, ten samples were tested for the presence of genes associated with methicillin resistance to antibiotic (*femA* gene) using the polymerase chain reaction(PCR) gene-specific primers. This assay was based on the highly conserved regions within all methicillin-resistant strains of staphylococci. The *femA* gene region was selected because it encodes a factor which is essential for methicillin resistance and is present in all *S. aureus* isolates unless if mutated. The specificity of MRSA molecular identification is based on the presence of the *mecA* gene which may be present in all Staphylococci. However, *femA* is specific gene to *S. aureus* and it does not cross react with any other bacterial species. Its presence signals existence of *S. aureus* and this was achieved within 5 hours instead of four days.

Polymerase Chain Reaction (PCR) primer had been developed to assay for Identification of antibiotic resistance in *S. aureus* had work with 35 samples, the Amplicons were visualized at 647bp for *femA* gene which produce factor that encodes methicillin resistance. The primers used were AACAGCTAAAGAGTTTGGTGCC/CATCACGATCAGCAAAAGCT to amplify the *femA* gene. The result was 100% concordance obtained in 5hours instead of four days (Flonta et al., 2009).

CONCLUSION

There was the presence of *S. aureus* among human of all age, sex attending Health Clinical ABUTH, Shika, Zaria having wound infections. The *S. aureus* prevalence was 44.4%. *S. aureus* are 57% responsible for surgical wound infections. Test for presence of *S. aureus* should be done quickly using molecular techniques so as to identify the

organism earlier to avoid multidrug resistance or mutated. In our present work, this molecular technique was achieved within 5 hours compared to the conversational method that take up to four days.

RECOMMENDATION

- (i) There should be efforts to educate the populace on the mode of infection of bacteria as well risk factor associated with the bacteria.
- (ii) Awareness programmes should be promoted on the importance of early test screening by molecular techniques because of its sensitivity and specificity to detecting of species and stains of *Staphylococcus* which will help in the reduction of mortality among patients with wound infections.
- (iii) Further studies using molecular techniques in the identification *S. aureus* should be encouraged in the institution of higher learning and hospitals.

REFERENCES

- Centre for Biotechnology Research and Training, (2011). ABU Zaria laboratory manual p.24.
- Chang S, Sievert DM, Hageman JC, Boulton ML, Tenover FC, Downes FP, Shah S, Rudrik JT, Pupp GR, Brown WJ, Cardo D, Fridkin SK (2007). "Infection with vancomycin-resistant *Staphylococcus aureus* containing the *vanA* resistance gene". *N. Engl. J. Med.* 348, (14): pp.1342–1347.
- Cimolai (2011) MRSA and the environment: implications for comprehensive control measures. *European journal of clinical microbiology & infectious diseases: official publication of the European Society of Clinical Microbiology* 27, (7) pp. 481-493
- Cook H, Furuya E, Larson E, Vasquez G, Lowy F (2007). "Heterosexual transmission of community-associated methicillin-resistant *Staphylococcus aureus*". *Clin. Infect Dis.* 44, (3): pp.410–413.
- Encyclopedia, Medical Dictionary, Microsoft Student Encarta (2009): Paalexander 13.webs.com, <http://vm.cfsan.fda.gov/~mow/c>.
- Fermentas Company (2011): www.fermentas.com.
- Flonta D, Gervaix A, Heininguer U, Berger C, Aebi C, Vaudaux B, Kind C, Gnehm HP, Hitzler M, Renzi G, Schrenzel J, François P (2009).Molecular epidemiology of the nasal colonization by methicillin-susceptible *Staphylococcus aureus* in Swiss children. *Clinical microbiology & Infection.*16: 1414-1420.
- Fujita S, Senda Y, Iwagami T, Hashimoto T (2009): Rapid identification of staphylococcal strains from positive-testing blood culture bottles by internal. transcribed spacer PCR followed by microchip gel electrophoresis. *J. Clin. Microbiol.* 43: pp.1149–1157.
- Hiramatsu K, Whitt Dixie D, Salyers, Abigail A (2005)."14". *Bacterial Pathogenesis: A Molecular Appr-*

oach (2nd ed.). USA: ASM Press. ISBN 1-55581-171-X.
Iwase, Tadayuki, Uehara, Yoshio; Shinji, Hitomi, Tajima, Akiko, Seo, Hiromi, Takada, Koji, Agata, Toshihiko, Mizunoe, Yoshimitsu (20 May 2010). "Staphylococcus epidermidis Esp inhibits *S. aureus* biofilm formation and nasal colonization". *Nature* 465 (7296): pp.346–349.

Liu GY, Essex A, Buchanan JT, Datta V, Hoffman HM, Bastian JF, Fierer J, Nizet V (2005). "Staphylococcus aureus golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity". *J. Exp. Med.* 202 (2): pp.209-215.
Ogston A (2004). "On Abscesses". *Classics in Infectious Diseases*. *Rev. Infect Dis.* 6 (1): pp.122–128.