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Incidence of Methicillin and Multidrug Resistant *Staphylococcus aureus* (MRSA) in Hunters in Maiduguri Metropolis, Borno State, Nigeria

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Abstract: *Examination of a total of 174 swab samples from the nostril and skin of hunters used for detection of S. aureus revealed 48(27.5%) positive isolates. Microscopic examination of gram stained colonies showed gram positive cocci arranged in irregular grape-like clusters, some appearing in singles while others in pairs, short chain or tetrads. Results of catalase and coagulase positive isolates were respectively recorded as 60(82.2%) and 48 (68.8%). However 48(68.8%) identified as catalase-coagulase positive were confirmed as S. aureus by the outcomes of incubation on Mannitol salt agar medium with yellowish colonial appearance. The values of S. aureus isolated from the nostril and skin of hunters is18 (20.9%) and 30 (34.1%). Chi square analysis (Fisher's exact test) shows that the distribution of MRSA from skin and nasal cavity of hunters was significantly different (P≤ 0.05).*
The phenotypic characteristics of organisms were detected using microscopic technique, colony morphology, catalase-coagulase tests and the use of Mannitol salt agar, Oxacillin Resistance Screening Agar Base (ORSAB) and Antibiotic Susceptibility Testing methods. Genotypically, DNA extraction and the presence of nuc and mecA gene were detected by PCR. Examination of 174 swab samples from nasal and skin regions of hunters revealed a total of 48 (27.5%) of S. aureus, out of which 18 (10.3%) were MRSA. Molecular analysis revealed that 7 nuc gene bands specific for S. aureus from 20 presumptive MRSA assayed were all mecA PCR negative. The isolates were sensitive to Gentamycin and Ciprofloxacin but were highly resistant to Cefoxitin and Oxacillin. Conclusively MRSA was found to exist in hunters with high isolation rate on the skin.
Key word: *Methicillin, Multidrug resistant, Staphylococcus aureus, Hunters*

I. Introduction

Staphylococcus aureus are ubiquitous species of the genus Staphylococcus that are major resident or transient colonizer of the skin and the mucosa of humans and primates, these organisms are gram positive, nonmotile,catalase positive and coagulase positive in nature (Cheesbrough, 2002; Talaro and Talaro, 2002; Pantosti, 2012).

Resistance to Methicillin that indicates resistance to all beta-lactam agents – was first reported in 1961, which marked the appearance of Methicillin-resistant S.aureus (MRSA) (Pantosti et al., 2007). MRSA is becoming a public health concern because companion animals often are in close physical contact (touching, petting and licking) with their owners, exposing them to infection with pathogenic bacteria (Guardabassi et al., 2004). Dogs are usually colonized by MRSA strains from humans (Loeffler and Lloyd, 2010; Lin et al., 2011).

From the foregoing, it therefore follows that hunting dogs and their owners could both be colonized by MRSA. Colonization of Methicillin-resistant Staphylococcus aureus (MRSA) in dogs and their owners have been extensively studied in Europe and most parts of the world. However, in Nigeria there is very little documented information on (MRSA) colonization of dogs and human.

Staphylococcus aureus produces an extracellular thermo-stable nuclease, encoded by *nuc* gene. The *nuc* gene is one of the most distinguishing characteristic features that is used to successfully differentiate S. aureus from other Staphylococcus spp. This suggests that *nuc* gene is a specific marker gene and PCR is a useful method for identifying this gene in S. aureus (Zhang et al., 2004).

Methicillin resistance is due to the acquisition of the *mecA* gene, that encodes a new protein designated Penicillin Binding Protein, belonging to a family of enzymes necessary in building the bacterial cell wall. Penicillin Binding Protein (PBP2a) has a very low affinity for β-lactam antibiotics and confers resistance to Methicillin and the other beta-lactams (Pantosti et al., 2007). The *mecA* gene is located on a mobile genetic element, named staphylococcal cassette chromosome mec (SCCmec) inserted in the S. aureus chromosome upstream orf X (Katayama et al., 2000). The aim of this study is to determine the presence of Methicillin and multidrug resistant *Staphylococcus aureus* and the incidence of *mecA* gene *Staphylococcus aureus* in hunters in Maiduguri.

II. Materials And Methods

Study Area

Maiduguri is the capital and the largest urban Centre of Borno State, North Eastern Nigeria. The State lies between latitude 11^0 32' North and 11^0 40' North and latitude 13^0 20' East and 13^0 25'East between the Sudan savannah and Sahel savannah vegetation zones, characterized by short rainy season of 3-4 months (June-September) followed by a prolonged dry season of more than 8 months duration(BMLS, 2007).

Sample Collection

A total of 174 swab samples were collected from the four major hunting rendezvous in Maiduguri Metropolis. Eighty six (86) and Eighty eight (88) swab samples from the nostril and skin region were collected respectively from hunters and analyzed. Cotton swab sticks were used to collect all the samples according to laboratory standard (Cheesbrough, 2010). Each sample was labeled with an identification number and date of collection. The samples were kept on ice pack and transported to the research laboratory, Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Maiduguri.

Isolation and Identification

An enriched solid medium of 7% Blood agar (Sigma® Switzerland) was prepared according to the manufactures instruction and the swabs were inoculated onto it. A sterile wire loop was used to streak the inoculums in order to get discrete colonies. The inoculated plates were incubated aerobically at 37°C for 24hours and observed for yellowish white colonies with smooth slightly raised surfaces. Some positive colonies have complete zones of hemolysis while others were non-hemolytic.

Colony Morphology

Gram staining of the collected samples was performed to identify Staphylococci by their Gram reaction. Samples that were cocci arranged in grape like clusters were subjected to biochemical tests (Catalase and Coagulase tests). The positive isolates were streaked onto Mannitol salt agar (MSA, Oxoid) which is a selective medium for Staphylococcus aureus and the plates were incubated aerobically at 37°C for 24hours.The appearance of yellowish colonies on MSA was presumptively considered as *Staphylococcus aureus*.

Oxacillin Resistance Screening Agar Base (ORSAB test)

Oxacillin Resistance Screening Agar Base is a medium for the screening of Methicillin resistant Staphylococcusaureus (MRSA), the medium is nutritious, selective and contains peptones for growth. It has a high salt and lithium chloride concentration to suppress non-staphylococcal growth; with Mannitol and aniline blue, for the detection of Mannitol fermentation. The antibiotics contained in ORSAB Selective Supplement are Oxacillin at 2 mg/liter to inhibit Methicillin sensitive Staphylococcus aureus (MSSA) and Polymyxin B for the suppression of other bacteria that are able to grow at such a high salt concentration. eg. Proteus spp. typical colonies of MRSA are intense blue in color on a colorless background enabling the organism to be more easily identified in mixed culture than the pale yellow colonies seen on Mannitol Salt Agar.

Antibiotic Susceptibility Testing

The Antibiotic Susceptibility Testing (ATS) of MRSA isolates was determined according to the method of Bauer-Kirby (Bauer et al., 1966) by using commercially prepared disc (Oxoid, UK) with known concentration of antibiotics. Freshly sub-cultured MRSA and well isolated colonies from ORSAB plates were emulsified in 3-4 m of sterile normal saline. The turbidity of the suspension was adjusted to the turbidity of standard equivalent to 0.5 McFarland (CLSI, 2011). Muller Hinton Agar Medium was prepared and a sterile cotton swab stick was dipped into the suspension. Excess fluid was removed by pressing and rotating the swab against the side of the tube above the suspension. The dried surface of the Mueller-Hinton Agar was inoculated by streaking the swab evenly over the surface of the medium in three directions, rotating the plate approximately 60° to ensure even distribution (Cheesbrough, 2010). Five antimicrobial discs were dispensed into each inoculated plates and incubated at 35°C for 24hrs. Zone of inhibition were measured in millimeters (mm) using verniercaliper. The sizes of the zones of inhibition were interpreted according to CLSI (2011) criteria. The following ten antibiotics were tested; Cefoxitin (FOX) 30ug, Clindamycin (DA) 2ug, Sulphadiazine and Trimetoprine (SXT) 25ug, Ciproflaxacin (CIP) 5ug, Erythromycin (E) 15ug, Cephazoline (KZ) 30ug, Chloramphenicol (C) 30ug, Gentamycin (CN) 10ug, Tetracyclin (TE) 30ug, and Oxacillin (OX) 1gm (Oxoid,UK). For the interpretation of susceptibility towards Oxacillin disc, growth within the zone of inhibition was considered indicative of Methicillin resistance. According to the classification criteria given by CLSI (2011), a diameter of inhibition zones of ≤ 10, 11-12, and ≥ 13 by 1ug of Oxacillin is categorized as resistant (R), intermediate (I) or susceptible (S) to Oxacillin accordingly. For Cefoxitin disc, a diameter of inhibition zones of ≤ 24 and ≥ 25mm correspond to the class of Staphylococci considered as resistance or susceptible for Oxacillin,

accordingly. There is no intermediate category of classification for staphylococci using the Cefoxitin disc diffusion test (CLSI, 2011).

Genotypic Characterization

Eighty (80) phenotypically detected MRSA from hunting dogs within which twenty (20) samples were randomly selected for the genotypic analysis using PCR for the detection of *S. aureus* specific gene (*nuc* gene) and the *mecA* gene encoding the resistance as described by Perez-Roth et al.(2001).

DNA Extraction

A loop full MRSA isolates was scooped into 1.5ml tube that contained 400µl of lysis buffer and 4µl proteinase k and was vortexed (Fisher brand, Allied Fisher Scientific, USA) for 2 minutes to get a homogenous mixture. This was followed by incubation at 55°C for 3 hours in the thermo-cycler (Stratagene, USA). Four hundred microliters (400µl) of PC (Phenol/Chloroform) was added to the tube and mixed gently for 1minute. It was then spun in a micro centrifuge for 10 minute at maximum speed (10,000 rpm). The supernatant was transferred to another tube and 400µl of PC (Phenol/Chloroform) was added, vortexed and then it was spun for 10 minutes at 10,000rpm. The supernatant was transferred to a new tube, 300µl of chloroform was added and vortexed, spun for 1 minute at 10,000 rpm, and the supernatant was also transferred to another tube. Eight hundred and twenty five microliters (825µl) of 100% ethanol and 25µl of sodium acetate was added and incubated in the freezer overnight.

The following day, the samples were centrifuged using high speed refrigerated centrifuge (Harvey Instruments Inc. USA) for 20 minutes and the supernatant was discarded. One (1ml) of 70% ethanol was added to the samples, mixed and centrifuged for 20 minutes at 13500 rpm, the supernatant was discarded and dry spun for 1 minute and the residual ethanol were removed. The DNA pellets were allowed to dry at room temperature. The pellets were resuspended in 20µl of water. After the DNA extraction, 10µl of loading dye was mixed with 5µl of the DNA pellets and pipetted into the wells on the gel, finally electrophoresis was carried out to determine the presence of DNA.

PCR Primers Dilution

Primers that corresponded to nuc gene specific for S. aureus and mecA gene were obtained from Integrated DNA Technologies, USA. The primers were resuspended in sterile distilled water and the diluted primers were stored at -20^{0}C

Detection of *nuc* and *mecA* Gene by Polymerase Chain Reaction (PCR)

The PCR amplification was done according to Perez – Roth et al. (2001) using the following primers that will detect the *nuc* gene in S. aureus and *mecA* gene with the amplicon size of 276 bp and 533 bp. The *nuc* primers were 5'- GCG ATT GAT GGT GAT ACG GTT-3' and 5'- AGC CAA GCC TTG ACG AAC TAA AGC- 3', while the *mecA* primers were 5'- AAA ATC GAT GGT AAA GGT TGG C-3' and 5'- AGT TCT GCA GTA CCG GAT TTG C- 3'. The PCR amplification mixture consisted of 10µl of PCR premix, 2µl DNA template,2µl primers and 16µl of distilled water. A total of 40 cycles were used to amplify 533bp of *mecA* gene and 276bp of *nuc* gene specific for S. aureus. Deoxyribonucleic acid (DNA) pre-denaturation occur at 94^{0}C for 5min, DNA denaturation at 30sec to 1min primers annealing at 55^{0}C for 30sec. approximately 5^{0}C below primers temperature, extension of the two strands at 72^{0}C for 60 sec and a final extension at 72^{0}C for 4 minute.

Ten microliter (10µl) each of the PCR products for *mecA* and *nuc* gene was analyzed separately on 2% agarose gel (Biogene, UK). Electrophoresis was performed in TBE buffer at 180 volts for 1 hour and the gel was subsequently stained with 3µl of ethidium bromide (Sigma, UK).Deoxyribonucleic acid (DNA) bands were visualized using UV-light with camera (Gel Doc 2000, Bio-Rad) and photographed.

Data Analysis

Fisher's exact test (Graph pad® software Inc) was used to determine the probability and significance of MRSA detection from the skin, perineal region and nasal cavity of hunting dogs. The values were considered significant ($P < 0.05$).

III. Results

Examination of a total of 174 swab samples from the nostril and skin of hunters of S. aureus revealed 48 (27.5%) positive isolates. Microscopic examination of gram stained colonies showed gram positive cocci arranged in irregular grape-like clusters, some appearing in singles, while others in pairs, short chain or tetrads. Results of catalase and coagulase positive isolates (plate 2 and 3) is 60 (82.2) and 48 (68.8%) respectively. Table 4.1 shows results of Gram staining, Biochemical test, ORSAB screening of Staphylococcal isolates in Hunters in Maiduguri Metropolis, Borno State. The values of *S. aureus* isolated from the nostril and skin of

hunters were 18 (20.9%) and 30 (34.1%). The values of MRSA isolated from the nostril and skin of hunters were 3 (3.5%) and15 (17.0%) respectively (Table 4.2). The result of antimicrobial susceptibility test indicated that the isolates were highly resistant to Cefoxitin (99%), Oxacillin (88.7%), Tetracycline (73.4%), Cephazoline (70.0%) while they were highly susceptible to Gentamycin (98%), Ciprofloxacin (90.8%), Chloramphenicol (87.6%), Sulphamethozole trimethoprim (87.8%), Erythromycin (81.6%) and Clindamycin (79.6%) as presented in Table 4.3.Multi drug Resistance (MDR) Profile of MRSA isolated from hunting dogs and their owners in Maiduguri Metropolis is shown in Table 4.4. Twenty (20) Presumptive MRSA isolates assayed 7 bands showed evidence expression of *nuc* gene specific for S. aureus with a molecular weight of 276bp which is presented in fig 7. This confirms the assumption based on phenotypic detection that some of the strains were *S. aureus*. The result of PCR based on targeted *mecA* gene revealed that none of the isolates possessed *mecA* gene as represented in fig 8.

Table 4.1: Results of Gram staining, Biochemical test, ORSAB screening test of Staphylococcal isolates in hunters in Maiduguri Metropolis, Borno state.

Test	Result of positive Isolates (%)	Result of Negative Isolates (%)	Total
Gram reaction	73 (41.9)[a]	101 (58.0)[b]	174
Catalase test	60 (82.2)[c]	13(17.0)[d]	73
Coagulase test	48 (68.8)	25(34.2)	73
ORSAB Screening	18 (37.5)[k]	30 (62.5)[y]	48

KEY: Figures in brackets are percentage occurrence of Staphylococcal isolate species. Values denoted by different superscripts for a given parameter are significantly different (P < 0.05)

Table 4.2: Distribution of S. aureus and MRSA in percentage isolated from hunters Maiduguri Metropolis

Source	Site	S. aureus (-ve)	S. aureus (+ve)	MRSA (+ve)	No. of samples
Dog	Nasal	68	18 (20.9)	3(3.5)	86
	Skin	58	30 (34.1)	15(17.0)	88
Total		126	48 (27.5)	18 (10.3)	174

Table 4.3: Antibiotic Susceptibility Pattern of MRSA isolated from hunting dogs in Maiduguri Metropolis, Borno state.

	Susceptibility pattern		
Antibiotics	Resistant No of isolates (%)	Intermediate No of isolates (%)	Susceptible No of isolates (%)
Cefoxitin	97 (99.0)	0 (0.0)	1 (1.0)
Cephazolin	69 (70.0)	6 (6.1)	23 (23.5)
Chloramphenicol	8 (8.2)	4 (4.1)	86 (87.8)
Ciprofloxacin	3 (3.1)	6 (6.1)	89 (90.8)
Clindamycin	15 (15.3)	5 (5.1)	78 (79.6)
Erythromycin	14 (14.3)	4 (4.1)	80 (81.6)
Gentamycin	1(1.0)	1 (1.0)	96 (98.9)
Oxacilin	87 (88.8)	1 (1.0)	10 (10.2)
Tetracycline	72 (73.4)	4 (4.1)	22 (22.4)
Sulpha/Trimethoprim	7 (7.1)	5 (5.1)	86 (87.8)

Table 4.4: Multi drug Resistance (MDR) Profile of MRSA isolated from hunting dogs in Maiduguri Metropolis

Number of drugs resisted	Percentages (%)
0	0.00
1	2.04
2	6.12
3	34.7
4	35.7
≥5	21.4

IV. Discussion

Higher MRSA colonization among hunters (10.3%) was recorded in the current study probably due to the close association between the dogs and their owners. This is however higher than the findings of Lu et al. (2005) who reported the occurrence of 7.6% in Taiwan, but is lower than the values recorded by Loeffler and Lloyd (2010) where the rate of 27% of MRSA detection was reported in U.K.

Several epidemiological studies and case reports have posited that MRSA transmission from human to companion animals and vice versa is possible (van Duijkeren et al.,2004; Hanselman et al.,2008). People with direct contact to animals such as pet owners, farmers, veterinarians, stockmen and staff of slaughter houses showed a higher prevalence of colonization compared to unexposed people (Khanna et al., 2008). Similarly, in 2002, Saxana et al. reported a higher colonization rate in healthcare workers compared to non-healthcare workers as a result of exposure to the pathogens. The outcome of the present study therefore concurred with these assertions.

Antimicrobial susceptibility pattern of the MRSA isolates have also been investigated indicating high level of resistance to Cefoxitin and Oxacillin. This therefore implies that Cefoxitin based assays are particularly important for low level Oxacillin resistant MRSA detection (Witte et al., 2007). Phenotypic antimicrobial susceptibility test needs particular care when using Oxacillin as a test substrate, because of heterogeneous in vitro expression of Methicillin-resistance (hetero-resistance) in nearly all of currently disseminated MRSA clonal lineages. Heteroresistance can be either detected using high inocula as recommended by the clinical laboratory standard institutes (CLSI), or by use of Cefoxitin disks since this antibiotic is less affected by heterogeous expression (CLSI, 2011).

The MRSA isolates were highly susceptible against Ciproflaxacin and Gentamycin. Ciproflaxacin, a member of the fluoroquinolones which are newer drugs with mode of action on DNA inhibition are relatively expensive and less available for abuses (Onanuga et al., 2005). In addition Gentamycin an aminoglycoside also showed high activity against MRSA which may be as a result of complexity of the aminoglycoside and the route of administration (Onanuga et al., 2005).

It was concluded that 11 out of 18 isolates of MRSA were multi-drug resistant in the current study. This was supported by the work of Abeer et al. (2007) who reported that 14 out of 51 MRSA isolates were multi-drug resistant. Methicillin-resistant coagulase negative Staphylococci from healthy dogs in Nsukka, Nigeria isolated revealed that 13 out of 109 isolates of MRCoNS were multi-drug resistant (MDR) (Chah et al., 2014).

The PCR analysis did not reveal any *mecA* positive samples and this might indicate the presence of *mecA* PCR negative MRSA isolates in Maiduguri. A previous study by Garcia-Alvarez et al. (2011) had reported a novel allele of the *mecA* gene encoding an alternative penicillin binding protein that mediates Methicillin resistance among bovine S. aureus isolates, and humans in UK, Denmark and Germany, that were Methicillin-resistant but mecA PCR negative.

These novel alleles of the *mecA* gene ($mecA_{LGA251}$) have 70% nucleotide identity to the archetypal mecA gene. Moreover, the findings highlight the possibility that additional mecA allele are in circulation in the environment and therefore could be acquired by S. aureus and lead to the emergence of new MRSA strain. However, antimicrobial susceptibility testing and other routine culture will identify S. aureus isolates encoding $mecA_{LGA251}$ as Methicillin resistant (Garcia-Alvarez et al., 2011). Furthermore, Kriegeskorte et al. (2012) also found MRSA isolates with novel genetic homolog among human in a study of human MRSA isolates in Germany.

The results of the PCR analysis in the current study which revealed *nuc* genes at 276 bp tallied with the findings recorded by Merlino et al. (2002), Saiful et al. (2006), Szczepanic et al (2007), who used modified PCR analysis (multiplex PCR) for the detection of *mecA* and nuc genes in MDR (multi-drug resistance) and NMDR (non-multi-drug resistance) MRSA.

V. Conclusion

Conclusively Methicillin-resistant *Staphylococcus aureus* was phenotypically detected with significant ($P < 0.05$) isolation rate in the hunters. Microbiological and PCR results confirm the presence of MRSA in hunters in Maiduguri Metropolis, Borno State, Nigeria. Higher percentages (30.0 %) of MRSA were detected from the skin than nasal cavity in hunters (18.0%).

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Plate 1: Catalase test, showing the appearance of bubbles (right) which indicates the presence of enzyme catalase while catalase negative bacteria give no reaction (left)

Plate 2: Coagulasetest, showing clumping of bacteria with plasma (left) indicates the presence of enzyme coagulase while coagulase negative gives no reaction (right)

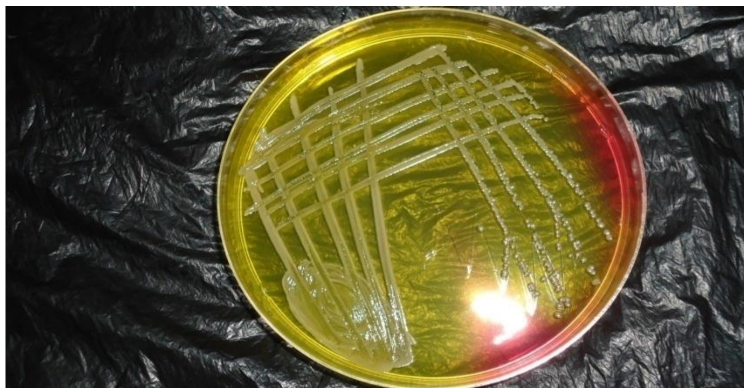

Plate 3: Yellowish Colonial appearance of S. aureus on Mannitol Salt Agar

Plate 4: An intense blue coloration of MRSA on ORSAB Media

Plate 5: Antibiotic susceptibility pattern of MRSA isolates showing various zones of inhibition

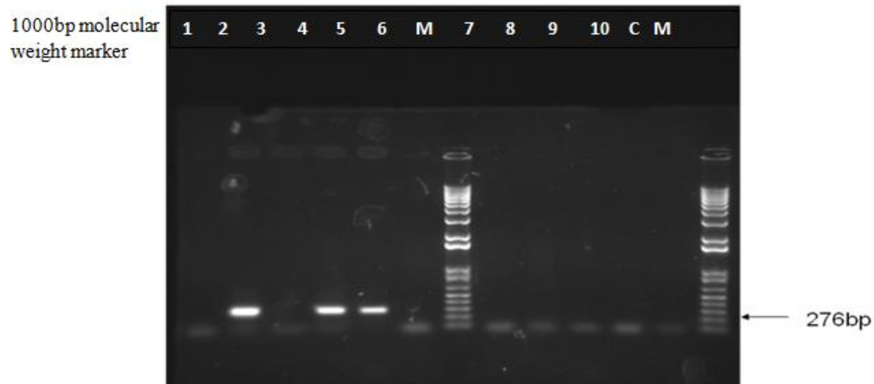

Plate 7: Agarose gel electrophoresis of PCR of MRSA isolate.
Lanes 2, 4 5 are positive for *nuc* gene as indicated by 276bp
Lane 1, 3, 6, 7, 8, 9 and 10 are negative samples
Lane M is the molecular weight maker
Lane C is the negative control

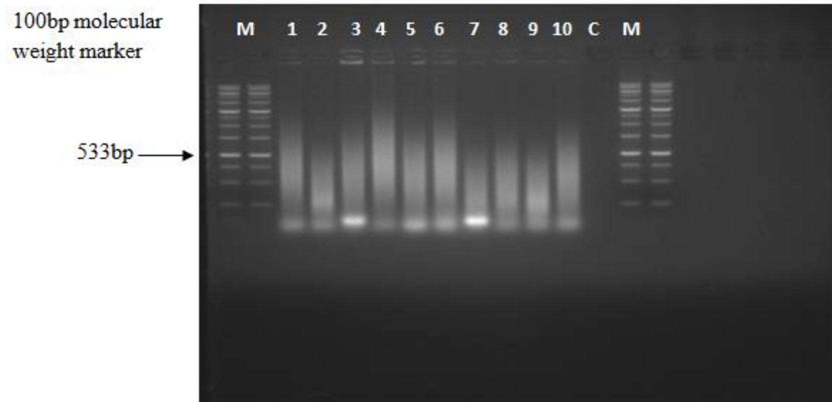

Plate 8: Agarose gel electrophoresis pattern of PCR of MRSA isolate
Lane M: molecular weight marker
Lane 1 - 10: negative for *mecA*
Lane C: negative control