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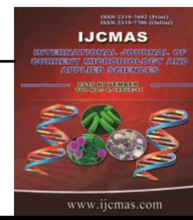

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Original Research Article

Randomly Amplified Polymorphic DNA Assay of Methicillin Resistant *Staphylococcus aureus* Isolated from Clinical Samples from Bengaluru, India

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Keywords

Staphylococcus aureus, MRSA, Plasmid, RAPD-PCR, Dendrogram

A B S T R A C T

Staphylococcus aureus is one of the most common nosocomial pathogens which cause disease in humans, when the immune system becomes compromised. Out of 323 isolated *S. aureus*, 97 strains were found to be Methicillin Resistant *S. aureus* (MRSA). Antibiotic sensitive pattern showed that isolated MRSA were 100% resistant to oxacillin, 90% resistant to ampicillin, 84.5% to penicillin, 76.2%to erythromycin, 71% to cephalexin and 40.2% to co-trimoxazole. Here, we observed ten different multi-drug resistant patterns among the isolated MRSA strains. When the 97 MRSA isolates were subjected for plasmid isolation, only 39 (40.20%) strains had plasmids. RAPD analysis of the MRSA isolates using the primers OLP6, OLP11 and OLP13 revealed 44, 42 and 43 bands respectively. A dendrogram based on UPGMA analysis using the RAPD data generated by primer OLP6, OLP11, OLP13 clustered the MRSA isolates into different groups and classes based on their genetic relation.

Introduction

Staphylococcus aureus is an opportunistic pathogen that causes a nosocomial infections ranging from self-limiting to life-threatening in both developing and developed countries (Deleo *et al.,* 2010; Kennedy *et al.,* 2009; Rehm, 2008; Franklin lowy, 2003). Due to its high clonal variability, *S. aureus* has the ability to develop a resistance towards common antibiotics by renovating their genomes through gene mutations or horizontal gene transfer (Kuroda *et al.,* 2001).

The incidence of Methicillin Resistant *S. aureus* (MRSA) varies from 25% to 50% in western and southern parts of India respectively (Patel *et al.,* l2010; Joshi *et al.,* 2013). The intrinsic resistance of *S.aureus* towards methicillin is due to expression of mecA gene, whose product is a 78-kDa protein called penicillin binding protein 2a (Shahkarami *et al.,* 2014).

Recently, it has been demonstrated that MRSA isolates often holds a variety of

resistance genes which displays resistance to most common antibiotics. Moreover, the percentage of MRSA isolates that show resistant to several classes of antibiotics is significantly higher than methicillin susceptible *S. aureus* (MSSA) (Intrakamhaenga *et al.,* 2012). Now, in India the MRSA is endemic (Joshi *et al.,* 2013). The widespread occurrence of MRSA with increasing resistance to a wide range of antibiotics other than methicillin imposes a serious threat to patients and health care setups (Diep *et al.,* 2008). Even though the occurrence of MRSA is higher in developing countries, but the data about the genetic variability are scarce (Gerardo Alvarez-Uria and Raghuprakash Reddy, 2012). Today, there are numerous conventional methods available such as bacteriophage typing, ribotyping and capsular typing to determine the genetic variability among the clinical isolates (Gaurav Kumar Singh *et al.,* 2014). But the situation warrants a fast and trustworthy genetic characterisation method for MRSA (Idil *et al.,* 2014).

Randomly Amplified Polymorphic DNA (RAPD), a simple PCR based technique, has been extensively used for epidemiological analysis. Moreover, RAPD primers can effectively scans the whole chromosomal DNA for the presence of small inverted repeats and amplifies the intervening DNA segments of variable length that can be used for identifying genetic variation and establishing strain-specific fingerprints. Also the assay can be performed with low concentration of DNA using short synthetic oligonucleotide primers in length (Idil N *et al.,* 2014). Based on their versatility and easy handling, PCR based RAPD is widely used in epidemiological study of MRSA. Moreover, it is very useful to have a precise microbial databases linking genetic marker and their clinical outcomes in order to control their spread.

The objectives of the present study are to examine the plasmid profile using gel electrophoresis and analyse the molecular diversity of the multidrug resistant MRSA isolated from various clinical samples using RAPD-PCR.

Material and Methods

Collection and identification of Methicillin Resistant *S. aureus*

A total of 671 clinical samples including urine, pus, sputum, semen, ET secretion, and swabs from vagina, umbilical, nasal, throat, ear, eye were collected and screened for *S. aureus* during 2011-2015 from different hospitals located in Bengaluru. All the samples were aseptically handles and processed. The morphotypes were done for all the collected samples using Gram`s staining method. The colonies of Gram-positive cocci in clusters were further inoculated on to the blood agar plates (aerobic with 5% carbon-di-oxide), MacConkey agar and Robertson cooked meat medium (RCM) for further sub culturing and incubated at 37°C for 24 hours. All the confirmed *S. aureus* strains were subsequently tested for methicillin resistance based on Kirby-Bauer disk diffusion method using oxacillin disc contained from Hi-Media Laboratories Pvt. Ltd. and the results were evaluated according to the Clinical and Laboratory Standards (CLSI). The isolates were considered methicillin resistant if the zone of inhibition was 10 mm or less.

Plasmid DNA extraction

Extraction of the plasmid DNA from all isolates was carried out using alkaline lysis method (Sambrook *et al.,* 1989). Briefly, a single colony is inoculated in nutrient broth and incubated at 37°C. Then the overnight grown cells are harvested and suspended in

200µl of solution A (100mM glucose; 50mM Tris hydrochloride (pH 8); 10mM EDTA) containing 10 mg of lysozyme per ml and incubated for 30 minutes at 37°C. 400µl of freshly prepared 1% sodium dodecyl sulfate in 0.2 N NaOH is added and the samples are mixed gently by inverting tubes, 300µl of a 30% potassium acetate solution (pH 4.8) is added and the samples are mixed by vortexing. After incubating the tube on ice for 5 minutes, the debris is removed by centrifugation. The supernatant is collected and extracted once with a phenol-chloroform mixture (1:1) and precipitated with an equal volume of isopropanol. The pellet that has plasmid DNA is then dissolved using TE buffer and stored at appropriate temperature.

Analysis of plasmid DNA by Gel electrophoresis

The isolated plasmids were loaded on a 1% agarose gel in Tris-Acetate-EDTA (TAE) buffer and run two hours at 100 v in 1× TAE buffer. Then the gel is stained with 0.5 µg/mL ethidium bromide and photographed using gel documentation system. The molecular weight of the plasmid was determined by running λ phage DNA as ladder digested with EcoRI/HindIII restriction enzyme.

Isolation of chromosomal DNA for RAPD-PCR

Multidrug resistant strains of MRSA were selected for chromosomal DNA isolation. Genomic DNA was extracted using in-house standardized method then estimated using standard spectrophotometer method. Briefly, colonies of pure MRSA isolates are harvested from each agar plates and suspended in 3 ml of nutrient broth and incubated overnight at 37°C. Then 1.5 ml of overnight grown culture was centrifuged and the pellet was suspended in 600 µl lysis buffer and vortexed. Then equal volume of phenol/chloroform was added and mixed gently. After centrifugation, the upper aqueous phase was transferred into a clean microfuge tube and 3 volumes of ice cold ethanol was added. Then the tube was incubated at -20 °C for 30 min and centrifuged for 15 min at 4°C. The pellet was washed with 70% ethanol and dissolved in TE buffer. The purified chromosomal DNA was stored at appropriate temperature for further experiments.

RAPD fingerprinting and analysis

RAPD-PCR assay was carried out as described by Williams *et al.* (1990). Approximately 25 ng of chromosomal DNA was used per reaction. Amplifications were performed in 25µl of buffer solution containing 3µM of oligonucleotides, 200µM of each deoxynucleoside triphosphates (Promega, Madison, USA), 3.5mM $MgCl_2$ and 2.5U of DNA Taq polymerase (Promega). The oligonucleotides OLP6 (5′-GAGGGAAGAG-3′), OLP11 (5′-ACGATGAGCC-3′) and OLP13 (5′-ACCGCCTGCT-3′), with 60–70% of G-C content and containing non-palindromic sequences were used for DNA amplification. The amplifications were performed in a PCR Express thermal cycler (Hybaid, United Kindom). The amplification consisted of a cycle of predenaturation at 94°C for 5 min, followed by 40 cycles of 1 min at 93°C, 1.30 min at 37°C and 1 min at 72°C. A final extension step of 72°C for 8 min is included in all amplifications. A negative control of the same reaction mixture with water instead of chromosomal DNA was included in each run. In addition, a positive control, containing the same reactive with chromosomal DNA as a well characterized reference strain (S. aureus strain ISP479) was also included. Each isolate was tested

under the same conditions at least twice with the selected oligonucleotides. Amplified products were separated by electrophoresis in a 1.5% agarose gel (Promega) in 0.5 X TBE buffer at a constant voltage of 4 V/cm and stained with ethidium bromide (0.5 µg/ml) and photographed. The photographed RAPD PCR banding patterns generated with each primer were analysed using similarity and distance matrix. The dendrogram were generated using Pearson coefficient (formula: d = (1 - r) *100) and Jaccard method.

Results and Discussion

The extensive use of broad-spectrum antibiotics has led to the wide spread occurrence of nosocomial infections by multi drug resistant microorganisms (Courvalin and Weber, 2005; Chikere *et al.,* 2008). There are many typing systems available, in order to obtain the information about the source and mode of transmission of the nosocomial pathogen. Among that, antibiogram draws an enough attention due to its simple method and it widely used to indicate the emergence and prevalence of the new multidrug strains. But, the problem is the susceptibility patterns of the nosocomial pathogen, for example MRSA is tend to vary very often, since the antibiotic resistance are acquired through R plasmid which can easily gained or cured. On the other hand, the sensitivity patterns are poorly discriminative, since the antibiotics misuse may favour the emergence of resistance concurrently in independent strains (Andrasevic *et al.,* 1999). So, in this present study we aimed to analyse the plasmid profile using gel electrophoresis and to examine the molecular diversity of the multidrug resistant MRSA isolated from various clinical samples using RAPD-PCR.

First, we performed the prevalence and antibiotic susceptibility study using identified *S. aureus* strains from clinical specimens such as urine, pus, sputum, semen, ET secretion, and swabs from vaginal, umbilical, nasal, throat, ear and eye. A total number of 671 clinical specimens were subjected for *S. aureus* screening. Out of which 323 (48.14 %) were identified as *S. aureus*. Then, all the confirmed *S. aureus* isolates were subjected for methicillin resistance screening using Kirby-Bauer disk diffusion method. Out of 323 identified *S. aureus*, 226 (70 %) and 97 (30%) isolates were confirmed as MSSA and MRSA respectively (Figure 1).

The drug resistance patterns of the isolated MRSA were found to be highly variable (Table 1). The antibiotics resistance patterns among the MRSA and MSSA isolates showed 100% resistant to oxacillin, 90% resistant to ampicillin, 84.5% to Penicillin-G, 76.2% erythromycin, 71% to cephalexin and 40.2% to cotrimoxazole. However, here we reported that 7.22% and 28.87% of the identified MRSA strains are resistance to linezolid, and vancomycin respectively. The other contemporary reports state that all the MRSA isolated in major southern districts of Tamilnadu are 100% susceptible to vancomycin and most of them were susceptible to linezolid (Rajaduraipandi *et al.,* 2006). Similarly, lower resistance of MRSA strains towards to linezolid was also reported by Tsiodras (2001).

Higher percentage of intermediate resistance was noted against antibiotics such as gentamycin and ciprofloxacin. Except for amoxicillin, penicillin and tetracycline, MSSA isolates exhibited intermediate resistance to all antibiotics, suggesting that more isolates can become resistant in the near future (Data not shown). Recent study on the spectrum of antimicrobial resistance among MRSA reported the ciprofloxacin

resistance was as high as 90% (Qureshi AH *et al.,* 2004). In contrast, here we reported that 59.79% of the MRSA strains resistant to ciprofloxacin and a further lower resistant rate to amikacin (15.46%). An increase of gentamicin resistance from 0% before 1996 to 80% after 1996 has been reported. However, it was reported that only 8% MRSA were resistance to gentamicin as against our present data (Pulimood TB *et al.,* 1996). Moreover, here we observed 10 different multi-drug patterns among MRSA samples collected from various clinical samples respectively (Figure 2).

Then all the isolated 97 MRSA strains were subjected for plasmid isolation using alkaline lysis method. Here out of 97 only 39 (40.20%) isolates had plasmids. The representative plasmid profile photograph is shown in figure 3. Plasmid analysis of representative MRSA isolates also demonstrates the presence of a wide range of plasmid sizes, with no consistent relationship between plasmid profiles and resistance phenotypes. Plasmid profile analysis appears to be of very low discriminatory capacity in the investigation of MRSA epidemiology because of the non-detection of plasmids in 60 % of the MRSA isolates. Based on the antibiotic resistance patterns (Table 1, Figure 2) and the plasmid profiling (Figure 3), 10 MRSA strains were selected based the strain that shows resistance to maximum number of antibiotics (more than 7 antibiotics). RAPD-PCR technique was used as a tool to assess the genetic relationship of those selected MRSA strains. The selected 10 MRSA strains are isolated from two different locations i.e., 5 strains namely S1, S2, S3, S4, and S5 from location 1 and S6, S7, S8, S9 and S10 from location 2 (Table 2).

It's well documented that RAPD-PCR is one of the most widely used method to investigate the genetic variability of any given nosocomial pathogen, moreover in RAPD the power of designated and discriminatory primers can be been easily assessed (Hojo *et al.,* 1995; van Belkum *et al.,* 1995; Neela *et al.,* 2005; Nikbakht *et al.,* 2008). The discriminatory powers of primers are evaluated based on the revealed banding profiles which provide the genotype of the isolates. So the choice of the RAPD primes is one of the most critical factors that helps in the occurrence of different banding patterns. Based on the above said criteria, in this study we have chosen three oligonucleotides that produce a high number of bands and a limited number of low intensity bands were used (Table 3). The dendrogram was constructed individually for the banding profiles of the three RAPD primers represent a novel approach for rapid screening of the multidrug resistant strains. This data and method will also provide the opportunity for monitoring the emergence and dissemination of the multidrug resistant MRSA strains between the locations.

RAPD profile of the multi-drug resistant MRSA chromosomal DNA with the primers OLP6 and OLP11 resulted in DNA fragments size ranged from 100 to 1200 bp were as with the primer OLP13 the amplified fragment size in the range between 300-2000 bp (Figure 4). The number of amplified fragments from each samples were ranged between 1 to 6 and high molecular weight bands (size between 1500-1599 and 1900-1999 bp) were amplified by primer OLP13 (Figure 4). The total number of bands amplified in 10 multi-drug resistant MRSA by the primers OLP6, OLP11 and OLP13 were 44, 42 and 43 respectively (Figure 4). Dendrograms constructed with each oligonucleotide showed that most the selected multidrug strains could be easily distinguished from each other. In addition, the strains that are

clustered together sometimes may differ depending on the nature of the oligonucleotide was used. The dendrogram with genetic variability of 10 multidrug resistance strains using Jacard coefficient method for RAPD data using primer OLP6 OLP11 and OLP13 (Figure 5,6,7).

Cluster analysis of the binary matrix distance based on RAPD data sets were transferred into dendrogram using Pearson coefficient method. A dendrogram based on UPGMA analysis using the RAPD data generated by primer OLP6 is shown in figure 5A. Ten multidrug resistant MRSA isolates were grouped into two and clustered into three classes. The large cluster comprised S1, S8, S9, S4 and S6; a second cluster included strains of the S3, S7 and S10; and the third remaining clusters corresponded to the S1 and S5. The primer OLP6 dendrogram showed that the highest percentage of similarity was between S7 and S10; S8 and S9; S4 and S6 strain, whereas the lowest percentage of similarity was between S1 and S10 strain. The similarity matrixes were obtained for each pairwise comparison of RAPD fragments is shown in figure 5B. The highest similarity value 138.925 to 84.848 recorded between 9 and S10 and lowest values of 66.265 to 127.273 between S7 and S8 samples.

Table.1 Multi-drug resistance pattern of MRSA isolated from clinical isolates

Antibiotics	Resistance (%)	Sensitive (%)	Intermediate (%)
Ampicillin	90.72	9.28	0.00
Amikacin	15.46	84.54	0.00
Ciprofloxacin	59.79	30.93	9.28
Cephalexin	71.13	28.87	0.00
Cephotaxime	23.71	76.29	0.00
Cotrimoxazole	40.21	59.79	0.00
Erythromycin	76.29	22.68	1.03
Gentamycin	31.96	57.73	10.31
Linezolid	7.22	92.78	0.00
Oxacillin	100.00	0.00	0.00
Pencillium G	84.54	15.46	0.00
Vancomycin	28.87	71.13	0.00

Table.2 Antibiotic resistant pattern of the selected ten multidrug resistant MRSA strains

Sample #	Location	Resistant pattern
S1	1	AMP, AMK, CIP,LEX,CTX,CTZ,ERY,GEN,LZD,OXA,PEN,VAN
S2	1	AMP, AMK, CIP,LEX,CTX,CTZ,ERY,GEN,OXA,PEN,VAN
S3	1	AMP,CIP,LEX,CTX,CTZ,ERY,OXA,PEN,VAN
S4	1	AMP, CIP,LEX,CTX,CTZ,ERY,GEN,LZD,OXA,PEN,VAN
S5	1	AMP, AMK, CIP,CTX,CTZ,ERY,GEN,OXA,PEN,VAN
S6	2	AMP, AMK, CIP,LEX,CTX,CTZ,ERY,LZD,OXA,PEN,VAN
S7	2	AMP, CTX,CTZ,ERY,GEN,OXA,VAN
S8	2	AMP, CIP,LEX,CTZ,ERY,GEN,OXA,PEN,VAN
S9	2	AMP, CIP,LEX,CTZ,OXA,PEN,VAN
S10	2	AMP, AMK, CTZ,ERY, OXA,PEN,VAN

Ampicillin (AMP); Amikacin (AMK); Ciprofloxacin (CIP); Cephalexin (LEX); Cefotaxime (CTX); Cotrimoxazole (CTZ); Erythromycin (ERY); Gentamicin (GEN); Linezolid (LZD); Oxacillin (OXA); Penicillin (PEN); Vancomycin (VAN)

Figure.1 Pie showing the prevalence rate of MRSA in the collected clinical samples

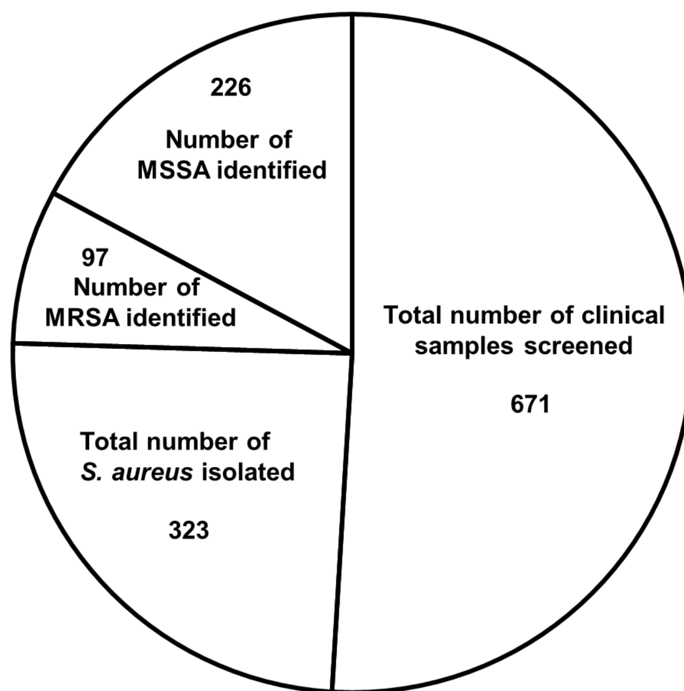


Figure.2 Frequency of multidrug resistant pattern of MRSA isolated from clinical samples. Number denote the antibiotics to which the strains were resistant

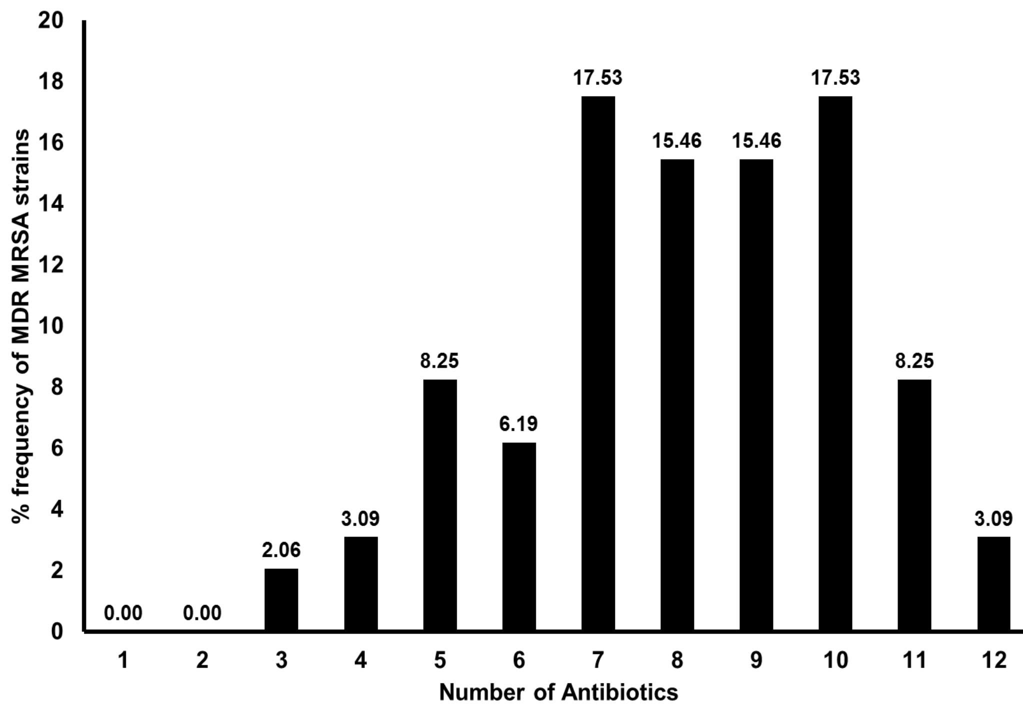


Figure.3 The representative plasmid profile photograph showing plasmids isolated from MRSA

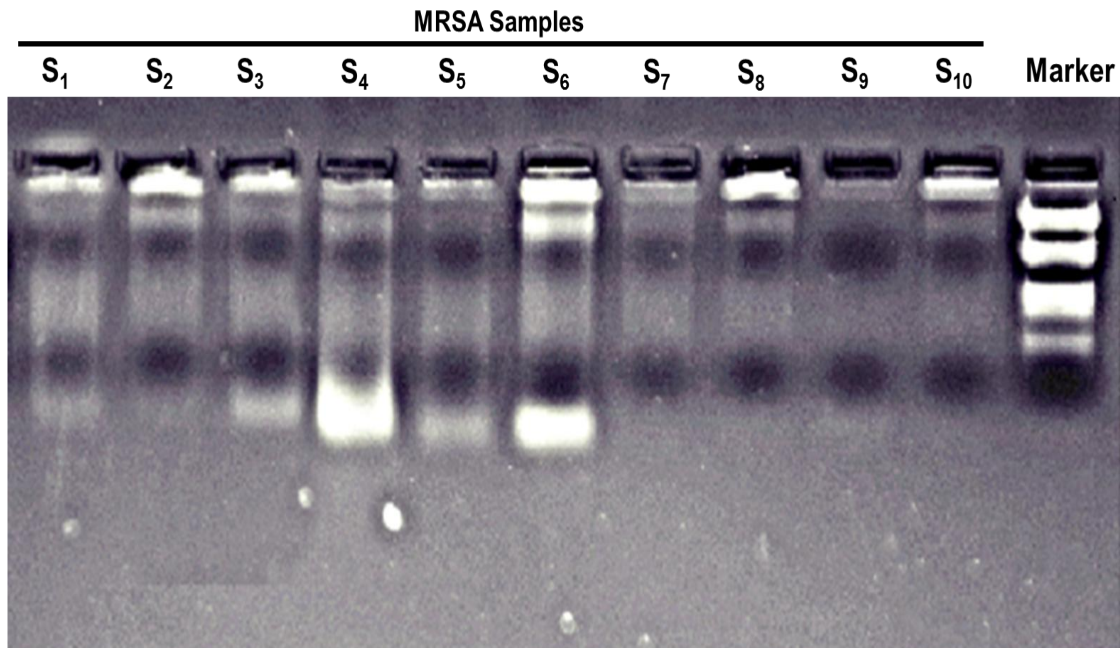
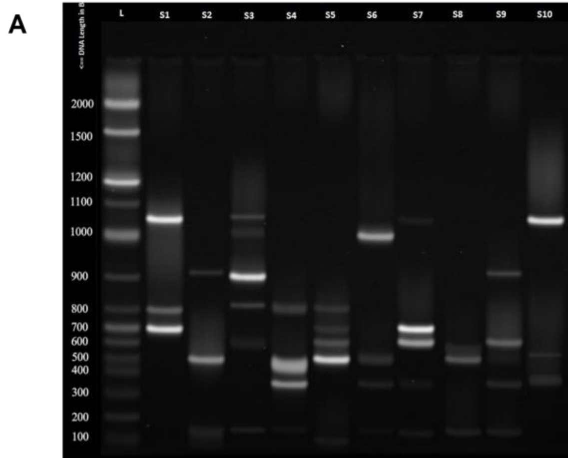


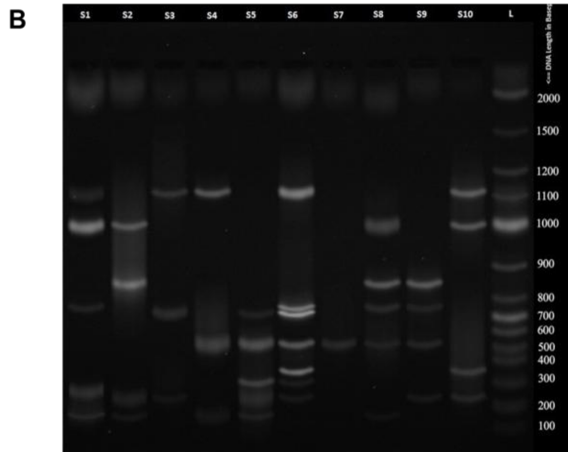
Figure.4 RAPD-PCR profiles and the banding pattern of multi drug resistant *S. aureus* selected from two locations and generated with each primer. (A) RAPD banding patterns obtained with primer OLP6, (B) RAPD banding patterns obtained with primer OLP11 and (C) RAPD banding patterns obtained with primer OLP13. M: DNA ladder. Lanes 1–5: MRSA isolates from location 1; lanes 6-10: MRSA isolates from location 2

A



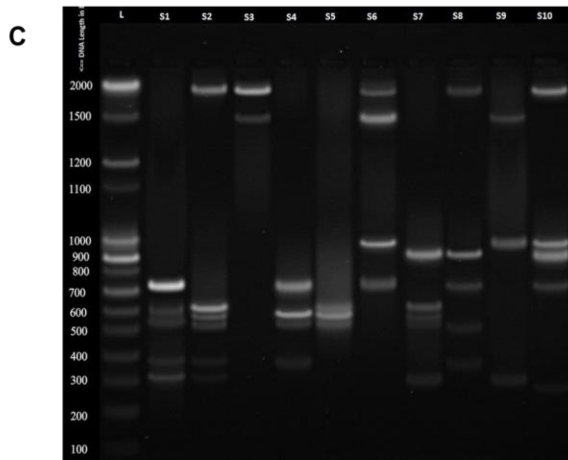
Band size (bp)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
1000-1099	+	-	++	-	-	+	+	-	-	+
900-999	-	+	+	-	-	-	-	-	+	-
800-899	+	-	+	+	+	-	-	-	-	-
700-799	+	-	-	-	+	-	+	-	-	-
600-699	-	-	+	-	+	-	+	+	+	-
500-599	-	+	-	+	+	+	-	+	+	+
400-499	-	-	-	-	-	-	-	-	-	-
300-399	-	-	-	+	-	+	+	-	+	+
200-299	-	-	-	-	-	-	-	-	-	-
100-199	-	++	+	+	+	+	+	+	+	-

B



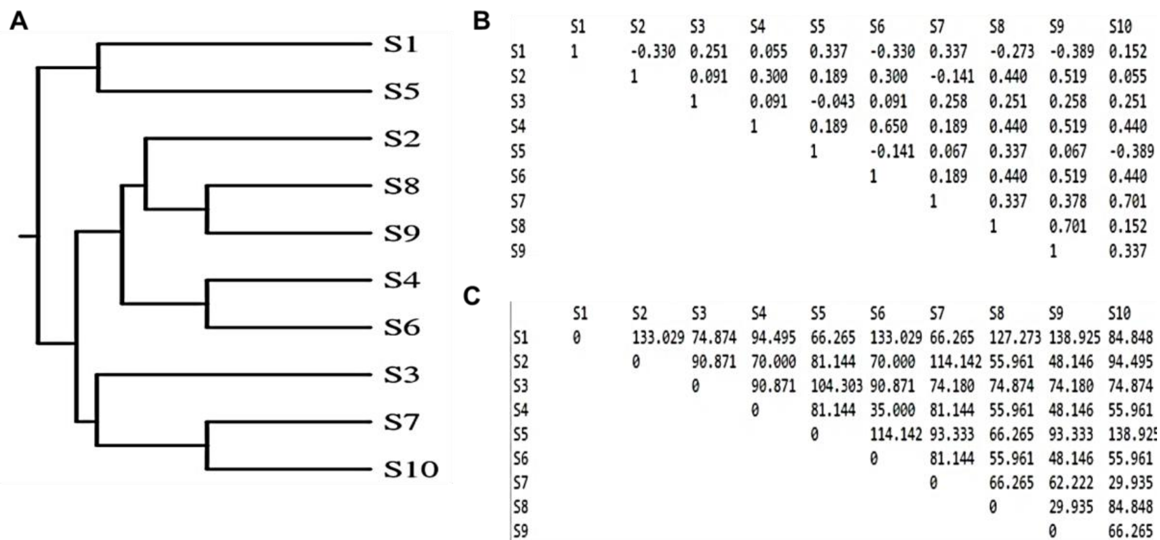
Band size (bp)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
1100-1199	+	-	+	+	-	+	-	-	-	+
1000-1099	+	+	-	-	-	-	-	+	-	+
900-999	-	-	-	-	-	-	-	-	-	-
800-899	-	+	-	-	-	-	-	+	+	-
700-799	+	-	+	-	+	++	-	+	+	-
600-699	-	-	-	-	-	-	-	-	-	-
500-599	-	-	-	+	+	+	+	+	+	-
400-499	-	-	-	-	-	+	-	-	-	+
300-399	+	-	-	-	+	+	-	-	-	-
200-299	-	+	+	-	+	+	-	-	+	+
100-199	+	+	-	+	+	-	-	+	-	-

C



Band size (bp)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
1900-1999	-	+	+	-	-	+	-	+	-	+
1500-1599	-	-	+	-	-	+	-	-	+	-
1000-1099	-	-	-	-	-	+	-	-	+	+
900-999	-	-	-	-	-	-	+	+	-	+
800-899	-	-	-	-	-	-	-	-	-	-
700-799	+	-	-	+	-	+	-	+	-	+
600-699	+	+	-	-	+	-	+	-	-	-
500-599	+	++	-	++	++	-	++	+	-	-
300-399	++	++	-	+	-	-	+	+	+	+

Figure.5 A. Genetic relationship between 10 *S. aureus* isolates, from two locations, as estimated by clustering analysis of RAPD profiles obtained with the primers OLP6. S1–S5: MRSA isolates from location 1; S6-S10: MRSA isolates from location 2. The dendrogram was generated by the unweighted pair group method with arithmetic averages (UPGMA). B. Similarity matrix showing genetic similarity of 10 MRSA strains using Pearson coefficient method for RAPD data using OLP6. C. Distance matrix showing genetic divergence of 10 MRSA strains using Pearson coefficient method for RAPD data using OLP6



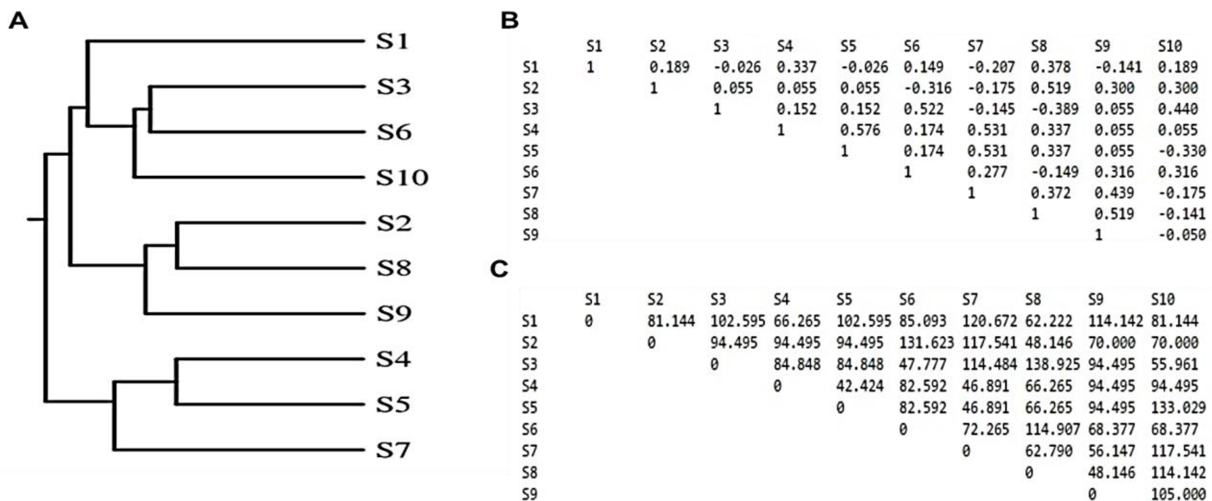
B

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1	1	-0.330	0.251	0.055	0.337	-0.330	0.337	-0.273	-0.389	0.152
S2		1	0.091	0.300	0.189	0.300	-0.141	0.440	0.519	0.055
S3			1	0.091	-0.043	0.091	0.258	0.251	0.258	0.251
S4				1	0.189	0.650	0.189	0.440	0.519	0.440
S5					1	-0.141	0.067	0.337	0.067	-0.389
S6						1	0.189	0.440	0.519	0.440
S7							1	0.337	0.378	0.701
S8								1	0.701	0.152
S9									1	0.337

C

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1	0	133.029	74.874	94.495	66.265	133.029	66.265	127.273	138.925	84.848
S2		0	90.871	70.000	81.144	70.000	114.142	55.961	48.146	94.495
S3			0	90.871	104.303	90.871	74.180	74.874	74.180	74.874
S4				0	81.144	35.000	81.144	55.961	48.146	55.961
S5					0	114.142	93.333	66.265	93.333	138.925
S6						0	81.144	55.961	48.146	55.961
S7							0	66.265	62.222	29.935
S8								0	29.935	84.848
S9									0	66.265

Figure.6 A. Genetic relationship between 10 *S. aureus* isolates, from two locations, as estimated by clustering analysis of RAPD profiles obtained with the primers OLP11. S1–S5: MRSA isolates from location 1; S6-S10: MRSA isolates from location 2. The dendrogram was generated by the unweighted pair group method with arithmetic averages (UPGMA). B. Similarity matrix showing genetic similarity of 10 MRSA strains using Pearson coefficient method for RAPD data using OLP11. C. Distance matrix showing genetic divergence of 10 MRSA strains using Pearson coefficient method for RAPD data using OLP11



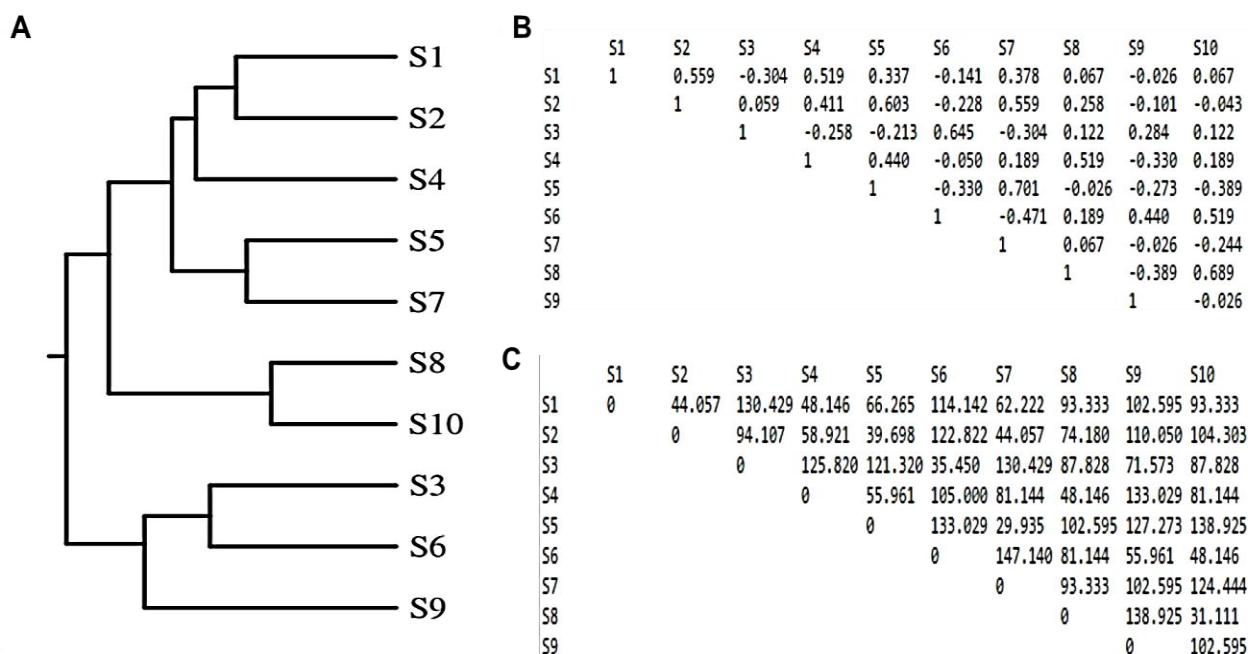
B

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1	1	0.189	-0.026	0.337	-0.026	0.149	-0.207	0.378	-0.141	0.189
S2		1	0.055	0.055	0.055	-0.316	-0.175	0.519	0.300	0.300
S3			1	0.152	0.152	0.522	-0.145	-0.389	0.055	0.440
S4				1	0.576	0.174	0.531	0.337	0.055	0.055
S5					1	0.174	0.531	0.337	0.055	-0.330
S6						1	0.277	-0.149	0.316	0.316
S7							1	0.372	0.439	-0.175
S8								1	0.519	-0.141
S9									1	-0.050

C

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1	0	81.144	102.595	66.265	102.595	85.093	120.672	62.222	114.142	81.144
S2		0	94.495	94.495	94.495	131.623	117.541	48.146	70.000	70.000
S3			0	84.848	84.848	47.777	114.484	138.925	94.495	55.961
S4				0	42.424	82.592	46.891	66.265	94.495	94.495
S5					0	82.592	46.891	66.265	94.495	133.029
S6						0	72.265	114.907	68.377	68.377
S7							0	62.790	56.147	117.541
S8								0	48.146	114.142
S9									0	105.000

Figure.6 A. Genetic relationship between 10 *S. aureus* isolates, from two locations, as estimated by clustering analysis of RAPD profiles obtained with the primers OLP13. S1–S5: MRSA isolates from location 1; S6-S10: MRSA isolates from location 2. The dendrogram was generated by the unweighted pair group method with arithmetic averages (UPGMA). B. Similarity matrix showing genetic similarity of 10 MRSA strains using Pearson coefficient method for RAPD data using OLP13. C. Distance matrix showing genetic divergence of 10 MRSA strains using Pearson coefficient method for RAPD data using OLP13.



B

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1	1	0.559	-0.304	0.519	0.337	-0.141	0.378	0.067	-0.026	0.067
S2		1	0.059	0.411	0.603	-0.228	0.559	0.258	-0.101	-0.043
S3			1	-0.258	-0.213	0.645	-0.304	0.122	0.284	0.122
S4				1	0.440	-0.050	0.189	0.519	-0.330	0.189
S5					1	-0.330	0.701	-0.026	-0.273	-0.389
S6						1	-0.471	0.189	0.440	0.519
S7							1	0.067	-0.026	-0.244
S8								1	-0.389	0.689
S9									1	-0.026

C

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1	0	44.057	130.429	48.146	66.265	114.142	62.222	93.333	102.595	93.333
S2		0	94.107	58.921	39.698	122.822	44.057	74.180	110.050	104.303
S3			0	125.820	121.320	35.450	130.429	87.828	71.573	87.828
S4				0	55.961	105.000	81.144	48.146	133.029	81.144
S5					0	133.029	29.935	102.595	127.273	138.925
S6						0	147.140	81.144	55.961	48.146
S7							0	93.333	102.595	124.444
S8								0	138.925	31.111
S9									0	102.595

A dendrogram on the RAPD data generated by primer OLP11 is shown in figure 6. The analysis revealed that 10 multidrug resistant MRSA isolates were grouped into two and clustered into two classes. Large cluster comprised of strains that include S1, S3, S6, S10, S2, S8, S9; and the smaller cluster included strains of the S4, S5 and S7. The dendogram based on primer OLP11 showed that the highest percentage of similarity was between S2 and S8; S4 and S5, whereas the lowest percentage of similarity was between S1 and S7 strain. The similarity matrixes were obtained for each pairwise comparison of RAPD fragments is shown in figure 6B. The highest similarity value 0.378 recorded between S8 and S9 and lowest values of 0.026 to 0.149 between S3 and S7 samples.

UPGMA analysis for the dendrogram made based on the RAPD data generated by primer OLP13 were performed and shown in figure 7. Analysis showed that the 10 multidrug resistant MRSA strains were grouped into two and clustered into three classes. The large cluster comprised the S1, S3, S6, and S10; a second cluster included strains of the S2, S8 and S9; and the third remaining clusters corresponded to the S4, S5 and S7. The highest percentage of similarity was observed between S8 and S10 whereas the lowest percentage of similarity was between S1 and S9 strain in the dendogram by primer OLP13. The similarity matrixes were obtained for each pairwise comparison of RAPD fragments is shown in figure 7B. The highest similarity value 0.559 to 0.519 recorded between S2 and S4 and lowest values of 0.026 to 0.067 between S9

and S10 samples. Similar observations have been reported in earlier studies also (Neela *et al.,* 2005; Nikhbakt *et al.,* 2008; Idil *et al.,* 2014). The dendrogram indicated that the strains are from two different locations within in the city and placed in the different groups based on their genetic similarities. The reason for this situation especially in developing countries might be due to the factors such as antibiotic misuse, shortfalls in infection control and moreover urban migration might increase the chance of dissemination of resistant strains in to the health care setup as well as in the community. Recent studies in Nigeria and Nepal have demonstrated that urban residents are more likely to harbour resistant bacteria than people residing in rural or provincial areas (Lamikanra and Okeke, 1997; Walson *et al.,* 2001).

In conclusion, it is important to have fast and reliable epidemiological typing method to monitor the inter or intra-spread of multi-drug resistant MRSA strains. Here, three primers were used for the analysis of multi-drug resistant MRSA using RAPD-PCR. All the three dendogram obtained here clearly demonstrates the distinct clustering of the multi-drug resistant strains are due to the intra-spread of the MRSA strains between locations. Moreover, RAPD-PCR could be used to track the routes of transmission, which could be used in controlling the spread of strains within hospital, and between the hospitals, and especially preventing the nosocomial infections caused by the multi-drug resistant MRSA.

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