Genotypic characterization of prevalent methicillin resistant Staphylococcus aureus *in Pakistani community hospitals**

Caracterización genotípica de *Staphylococcus aureus* resistente a la meticilina prevalente en hospitales de Pakistán

Caracterização de Staphylococcus aureus *resistente à meticilina prevalecente em hospitais da comunidade no Paquistão*

Zahid Majeed[1], Yasrab Arafat[2], Zainab Ajab[1], Salman Akbar Malik[2], Waseem Shehzad Abbasi[2], Huma Ajab[1]

[1] M. Phil
[2] PhD

* Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

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Summary

Protein A gene was used as a genetic marker for the characterization of Pakistani methicillin resistant Staphylococcus aureus *(MRSA) isolates. Out of a total of 130* Staphylococcus aureus *isolates, 90 were identified as MRSA and of these 90 MRSA, 81 MRSA isolates were characterized by* spa *typing. All of these isolates were collected from five National Community Hospitals. Two different sets of primers were used to amplify the X-region of Protein A gene in MRSA strains. One set of primers i.e,* spa*-F/*spa*-R identified three types of different repeats viz., 7 repeats (*spa *2), 8 repeats (*spa *3) and 10 repeats (*spa *4) and another set of primers i.e.* spa*-1113F/*spa*-1514R identified 4 types of different repeats viz., 6 repeats (*spa *1), 15 repeats (*spa *6), and 17 repeats (*spa *7) and 19 repeats (*spa *8). Repeat 11 (*spa *5) was identified with both sets of primers. Sporadic MRSA types carrying 6, 7, 10, 17 and 19 repeats were less prevalent, while the epidemic MRSA with 8, 11 and 15 repeats were more prevalent and considered to be involved in transmission among the patients within different hospitals. Research work concludes that* spa *technique is efficient enough to differentiate* spa *strains carrying variations in general and those slowly transforming from sporadic to epidemic outbreak in particular.*

Keywords: genotyping** * Staphylococcus aureus * **Protein A** * spa **typing** * **Polymorphism

Resumen

El gen de la proteína A se usó como marcador genético para la caracterización de aislados de *Staphylococcus aureus* resistentes a la meticilina (SAMR). De un total de 130 aislados de *Staphylococcus aureus*, 90 fueron identificados como SAMR y 81 de éstos se pudieron caracterizar por tipificación spa. Todos estos aislados fueron obtenidos de cinco Hospitales Nacionales de la Comunidad. Se utilizaron dos juegos diferentes de cebadores para amplificar la región-X del gen de la proteína A en las cepas de SAMR. Un conjunto de cebadores, *spa*-F/*spa*-R ha identificado tres tipos de repeticiones diferentes, a saber, 7 repeticiones (*spa* 2), 8 repeticiones (*spa* 3) y 10 repeticiones (*spa* 4) y otro conjunto de cebadores, *spa*-1113F/*spa*-1514R ha identificado 4 tipos de repeticiones diferentes, a saber, 6 repeticiones (*spa* 1), 15 repeticiones (*spa* 6), y 17 repeticiones (*spa* 7) y 19 repeticiones (*spa* 8). Se identificó la repetición 11 (*spa 5*) con ambos conjuntos de cebadores. Los tipos de SAMR esporádicos que portaban las repeticiones 6, 7, 10, 17 y 19 fueron poco prevalentes mientras que los SAMR epidémicos con 8, 11, y 15 repeticiones fueron más prevalentes y se los consideró involucrados en la transmisión entre los pacientes dentro de los diferentes hospitales. Este trabajo concluye que la técnica spa es lo suficientemente eficiente como para diferenciar las cepas epidémicas, esporádicas y aquéllas que se transforman lentamente de esporádicas a epidémicas.

Palabras clave: genotipo * *Staphylococcus aureus* * proteína A * tipificación *SPA* * polimorfismo

Resumo

O gene da proteína A foi usado como marcador genético para a caracterização de isolados de Staphylococcus aureus *resistentes à meticilina (SAMR). De um total de 130 isolados de* Staphylococcus aureus, *90 foram identificados como SAMR e 81 destes puderam se caracterizar por tipificação* spa*. Todos estes isolados foram obtidos de cinco Hospitais Nacionais da Comunidade. Utilizaram-se dois jogos diferentes de cevadores para amplificar a região-X do gene da proteína A nas cepas de SAMRUn conjunto de cevadores,* spa*-F/*spa*-R tem identificado três tipos de repetições diferentes, a saber, 7 repetições (*spa *2), 8 repetições (*spa *3) e 10 repetições (*spa *4) e outro conjunto de cevadores,* spa*-1113F/*spa*-1514R tem identificado 4 tipos de repetições diferentes, a saber, 6 repetições (*spa *1), 15 repetições (*spa *6), e 17 repetições (*spa *7) e 19 repetições (*spa *8). Foi identificada a repetição 11 (*spa *5) com ambos os conjuntos de cevadores. Os tipos de SAMR esporádicos que tinham as repetições 6, 7, 10, 17 e 19 foram pouco prevalecentes enquanto que os SAMR epidêmicos com 8, 11, e 15 repetições foram mais prevalecentes e são considerados envolvidos na transmissão entre os pacientes dentro dos diferentes hospitais. Este trabalho conclui que a técnica* spa *é o suficientemente eficiente como para diferenciar as cepas epidêmicas, esporádicas e aquelas que se transformam lentamente de esporádicas em epidêmicas.*

Palavras chave: genótipo * Staphylococcus aureus * proteína A * tipificação SPA * polimorfismo

Introduction

Staphylococcus aureus is a gram-positive coccus bacterium which is a major human pathogen. Over the years this species has developed a resistance to methicillin which poses a significant risk to patients and contributes to a substantial financial burden on healthcare resources (1), particularly in underdeveloped countries including Pakistan. Large number of clinical infections like folliculitis, furunculosis, cellulitis, mastitis, impetigo, osteomyelitis, acute endocarditis, meningitis are linked to *S. aureus* (2). The pathogenesis of *S. aureus* has been attributed to its ability to produce a diverse range of proteins. Staphylococcal surface protein known as protein A, sizes 40-60 kD, is encoded by *spa* gene and it is present both in methicilin-susceptible and methicillin-resistant strains. This protein protects the bacteria from host defense mechanism (3).

The *spa* gene is composed of approximately 2,150 bp and harbors a number of functionally distinct regions (Figure 1) which includes, Fc-binding region of immunoglobulin G (IgG), X-region and C-terminus (4), The IgG Fc-binding region is composed of five 160 bp repeats. The X-region contains a varying number of 24 bp repeats and is located immediately upstream of the region encoding the C-terminal cell wall attachment sequence (5) (6).

Typing is an important pre-requisite for control measures for the clinically important micro-organisms. There are many genotyping techniques standardized to identify the geographically isolated and distinct *S. aureus* strains. These are Pulse Field Gel Electrophoresis (PFGE), Multilocus Enzyme Electrophoresis (MLEE) and Multilocus Sequence Typing (MLST), coagulase A gene (*coa*) typing and microarray technique. Compared with these typing techniques, *spa* typing is single locus marker for rapid

identification of *S. aureus* strains in general. X-region of the Protein A is a single locus molecular marker used in *spa* typing. *spa* typing is advantageous as its data can easily be compared globally through online Ridom StaphType software developed (7).

Very rare published literature is available within Pakistan on the local MRSA strains. Earlier works (8) (9) (10) (11) (12) (13) are incomplete and does not provide any molecular epidemiology of clinically important MRSA. The non-availability of the pre-typed strains, their storage and access are the major impediments that must be streamlined.

The application of several new molecular typing techniques gave many new in depth understanding of the epidemiology and population structure of *S. aureus* in geographically isolated regions of the world. In clinical set-up, routine drug therapy requires, low cost, exact and rapid identification of bacterial pathogenic strains. We performed genotypic characterization of prevalent methicillin resistant *Staphylococcus aureus* (MRSA) in Pakistani community hospitals and presented *spa* types analysis.

Materials and Methods

MRSA SAMPLING

Out of the total 130 *S. aureus* isolates, 90 were identified as MRSA and from these 90 MRSA we were able to characterize 81 MRSA isolates by *spa* typing. *S. aureus* samples were collected during 2007-2009 from five different community hospitals. Details on number of *S. aurues* isolates available from each hospital are given in Table I. The control NCTC strain 11561 was received from Army Medical College (AMC), Rawalpindi (13). These strains were re-confirmed through recommended biochemical and antibiotic sensitivity tests. *S. aureus* resistance to Methicillin was determined through disc diffusion methods (14). The resistant and susceptible MRSA were recognized on the basis of standard criteria (14).

The detailed view of number of identified MRSA isolates with their origin of infection is given in the Table III.

GRAM STAINING AND BIOCHEMICAL TESTS

Gram staining and biochemical tests were performed according to the National Standard Method developed by Health Protection Agency, UK (http://www.hpa-standardmethods.org.uk/).

DNA EXTRACTION

Method (15) was adopted with some modification. Brain Heart Infusion (BHI) broth (Oxoid, England), containing bacterial culture, incubated overnight for 18-24 hours, was taken into 1.5 mL eppendorf tube. The bacterial culture was centrifuged at 14,000 rpm for 10-minutes at 4 ºC. The supernatant was discarded and pellet was re-unsuspended into 467 µL of Tris-EDTA buffer by repeated pipetting. Sodium Dodecyl Sulphate (SDS), 30 µL of 10% and proteinase K (MB, Biochemicals Inc.), 3 µL of 20 µg/mL, was added. This solution was mixed gently and incubated for 1-hour at 37 °C. An equal volume of Phenol and Chloroform (1:1) was added and mixed very gently to avoid shearing of the DNA by inverting the tube until the two phases were mixed completely and spun at 12,000 rpm for 10- minutes. Then upper aqueous phase was transferred to a new eppendorf tube. Again, in aqueous phase added

Table I. *MRSA isolates collected from different hospitals.*

Sources	*Total received* S. aureus *isolates (n=130)*	*MRSA Isolates (n=90)*	*Clinical Samples Type*
Army Medical College (AMC), Rawalpindi*	50	38	Pus, Urine, Sputum, Blood Ear, Tissue Fluid
Pakistan Institute of Medical Science (PIMS), Islamabad*	35	25	Ear, Tissue, Catheter tips, Blood, Spinal Cord
Khyber Hospital, Peshawar			Pus and Blood
Kahuta Research Laboratories (KRL), Hospital, Islamabad.	25	16	Urine
Federal Government Services Hospital, (FGSH), Islamabad	10	1	
	10	1	Uncharacterized

* Higher rate of clinical samples were available from AMC and PIMS due to their comprehensive MRSA surveillance program.

an equal volume of Phenol and Chloroform (1:1), mixed well and centrifuged at 14,000 rpm for 10 minute at 4 °C. Again upper aqueous phase was transferred to a new tube and added 30 µL of 3M sodium acetate. DNA was precipitated with 99% cold ethanol followed by 3-4 minutes centrifugation at 10,000 rpm. DNA pellet was suspended in 50 µL Tris-EDTA buffer (pH 8) and stored at -20 ºC.

DNA QUANTIFICATION

A DNA concentration (ng/µL) was determined by measuring absorbance of the sample at 260 nm wavelength. Each DNA sample was diluted 300 times and quantified through UV spectrophotomer (Series 8453, Agilant, USA). The absorbance wavelength range was used between 200nm to 340nm. Apply the following formula to calculate the final quantity of the DNA in the sample.

DNA (ng/µL) = [OD_{260} x Dilution Factor × 50 ×Ratio (260/280)]/180

CHARACTERIZATION OF THE PROTEIN A

In MRSA, the X-region of the *spa* gene was amplified with two sets *spa* specific primers first, *spa* 1113F 5'-TAAAGACGATCCTTCGGTGAGC-3', *spa* 1514R 5'-CAGCAGTAGTGCCGTTTG CTT-3', second *spa* F 5'- AGCACCAAAAGAGGAAGACAA-3', *spa* R 5'-GTTTAACGACATGTACTCCGT-3' (16) (17). In 20 µL aliquot of single PCR reaction added 2 µL PCR buffer of 10X stock, 2 µL $MgCl_2$ of 25mM stock, 4 µL dNTPs of 2mM stock, 0.5U of Taq polymerase (5U/µl) (MBI, Fermentas, USA), 2 µM Primers each of 0.5 mM stock, 2 µL of template DNA (Use 10 times dilution of the concentrated DNA) and 6.8 µL of ddH_2O. PCR amplification of X-region in *spa* gene was carried out with Tpersonal Thermocycler (Biometra, Germany). The protocol included an initial denaturation at 94 °C for 5-minute, followed by 35 cycles of denaturation at 94 °C for 30s, annealing at 55 °C for 30s, and extension at 72 °C for 1-minnute, and final extension at 72 °C for 10-minute. Used the strain NCTC-11561 as *S. aureus* positive control while strain EMRSA-15 as *spa* positive control in the PCR. Master mixture without DNA template was used as a negative control.

AGAROSE GEL ELECTROPHORESIS

PCR products were run for electrophoresis on 2% agarose in the presence of Ethidium Bromide (EtBr) in 1X TBE (Tris-borate-EDTA) buffer for 80-minute and 120 Volt. DNA ladder, 50 bp (Fermentas, USA) and 100 bp (Pomega), were included in each run. The DNA bands were visualized on UV trans-illuminator and photographed. Band patterns were visually evaluated, analyzed as sized by Quantity One software (version 4.2, Bio-Rad, Germany). MRSA strains which showed a distinct band between 200 bp to 600 bp was considered as a distinct *spa* type. The number of repeats determined as described in succeeding section.

VARIABLE NUMBER OF TANDEM REPEATS CALCULATION

The amplicons analyzed on the gel, were used to calculate the number of variable tandem repeats (VNTRs) in the X region of spa gene. Amplicon of 263 bp contains 9 repeats in the X-region of *spa* gene in *S. aureus* (18). This is used as a reference amplicon for the calculation of repeats in this study. In all samples the number of repeats were calculated according to the criteria given below:

- Size of single repeats in the X-region = 24 bp
- Size of nine repeats in the X-region = 24 bp x 9 repeats = 216 bp
- Size of nine repeats in the X-region (18) = 263 bp
- Extra region 263 - 216 = 47 bp
- Amplicon size in this study = X
- Amplicon size used for the calculation of the repeats = X-47= Y
- No. of repeats determined in sampled MRSA strains = Y/24 = R repeats

Figure 1 shows the extra region (47bp) and repeats region used for the calculation of variable number of tandem repeats in amplified X-region of protein A gene.

spa INCLUSION AND EXCLUSION CRITERIA

Inclusion and exclusion criteria for the *spa* typing were also established. MRSA pure cultures, in which specific *spa* identified were subsequently subcultured at

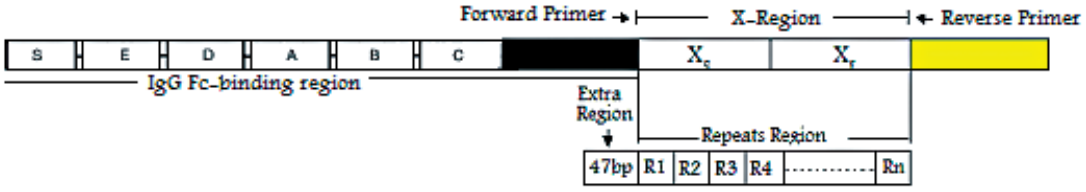

Figure 1. *Protein A gene map (23) (25) reproduced with some modifications.*

least thrice to reconfirm the specific *spa* in term of number of repeats following the steps mentioned earlier in the materials and methods. If *spa* resolved on the gel then it confirmed the number of repeats. It resulted in *spa* inclusion otherwise excluded.

CRITERIA OF EPIDEMIC (EMRSA) AND SPORADIC (SMRSA) METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS DIFFERENTIATION

In our study, in addition to the earlier proposed cut-off value of 7 repeats (23), we characterized MRSA epidemic and sporadic on the following criteria.

If specific *spa* type is epidemic it should be recovered from more than one hospital from large number of clinical samples. Recovery from more than one hospital is very valid first point to say that *spa* type is epidemic.

If *spa* type spread is only limited within a hospital and recovered from a less number of patients samples it is called sporadic.

If the sporadic strain is well spread in only one hospital but very litter in any other hospital it can be thought that it is in transformation from sporadic to epidemic spread.

STATISTICAL ANALYSIS

Chi-square test

Applying Chi-square test at significance level of 95% assessed difference in the *spa* types (n=81). The following formula was used and the values were calculated manually.

$\chi^2 = (o-e)^2/e$ where o = Observed values e = Expected value

Hierarchical clustering

Numeric repeats data were analyzed using the Hierarchical Clustering UPGMA (Unweighted Pair Group Method with Arithmetic Mean) using the version 15 of Statistical Package for Social Sciences (SPSS). The clusters were analyzed at a suitable similarity distance, representing the *spa* types. Amplicon size and their respective repeats were used in the hierarchical clustering to define the different types of the MRSA groups.

Results

TYPABILITY

Two different sets of primers were used for typing of the MRSA strains. It was found that Primer "*spa*-1113F/*spa*-1514R" typed 74 MRSA isolates (91.35%) while Primer "*spa*-F and *spa*-R" typed 61 MRSA isolates (75.30%) (see Table II). There was a highly significant difference (Chi-square test, p <0.005) between the number of typed and non-typed MRSA. In this study, *spa* gene showed high variability as eight different MRSA *spa* types were obtained on the basis of their X-region polymorphism. We found eight different PCR products which ranged from 200 bp to 500 bp. PCR product of Primer "*spa*-F/*spa*-R" showed three distinct types of amplicons of sizes 225 bp, 250 bp, 275 bp. PCR products of Primer "*spa*-1113F/*spa*-1514R" showed four distinct types of amplicons of sizes 200 bp, 400 bp, 450 bp and 500 bp. PCR product of 300 bp was shown by both primers. We found that primer selection might change the criteria for the strain characterization.

In this study we observed that Primer *spa*-F/*spa*-R could be used where interest was to identify MRSA types with amplicon of 200, 225 bp, 250 bp and 275 bp. While Primer "*spa*-1113F/*spa*-1514R" was more suitable epidemiological marker where interest was to find MRSA types with amplicon sizes of 400 bp, 450 bp and 500 bp. It is evident that the MRSA types with 300 bp can be typed by either primer "*spa*-R/*spa*-F" or "*spa*-1113F/*spa*-1514R" (Figure 2 and 3).

ALLELIC POLYMORPHISM

In a particular MRSA type, numbers of repeats were assessed for the X-region. The repeats calculated for eight different PCR products corresponded to eight types of allelic polymorphism in the *spa* gene. The allele with 6 repeats, named type *spa* 1 (n = 2, 2.46%) and the allele with 7 repeats named the *spa* 2 (n = 1, 1.23%). Type *spa* 1 was associated with clinical samples of urine and pus while the type *spa* 2 was linked only with the clinical samples of bone fracture. The more important *spa* types were *spa* 3, *spa* 5 and *spa* 6 as these types were recovered from multiple samples (Table III). The allele with 8 repeats, named type *spa* 3 (n = 25, 30.86%), was associat-

Table II. *Comparison of typability and non-typability of MRSA by spa specific primers.*

Primers	*Typeable MRSA*	*Non-Typeable MRSA*	*p value*
spa-F/*spa*-R	61 (75.30%)	20 (24.69%)	p < 0.005
spa-1113F/*spa*-1514R	74 (91.35%)	7 (8.64%)	p < 0.005

*Less number of isolates makes the prevalence biased. Therefore their prevalence have not been calculated.

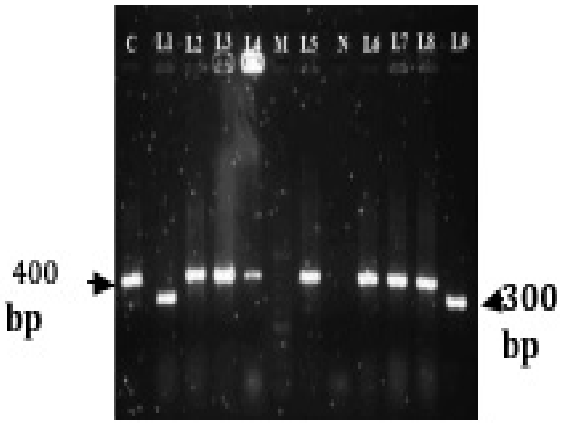

Figure 2. *Agarose gel electrophoresis (3%) with two spa types found with Primer "spa-1113F/spa-1514R" (M = 50 bp). Primer "spa-1113F/spa-1514R" showed amplicons of 300 bp and 400 bp. MRSA which are shown in lane number 1 and 9 were sized to 300 bp. MRSA in the lane number 2 to 8 were sized to 400 bp. Lane C has positive control of EMRSA-15 which sized to 400 bp. Lane N has negative control.*

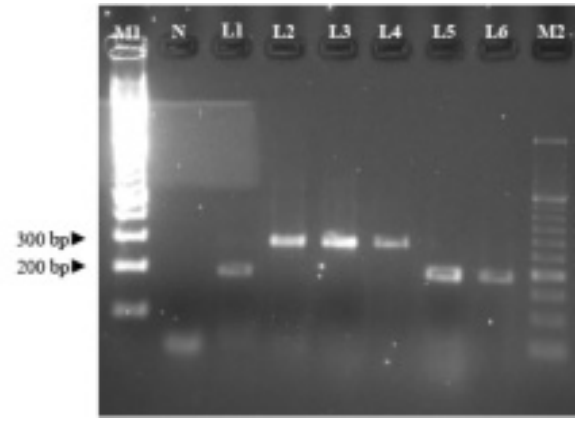

Figure 3. *Agarose gel electrophoresis (3%) with two spa types found with Primer "spa-R/spa-F" (M1=100 bp, M2= 50 bp). Primer "spa-R/spa-F" showed the MRSA types with 200 bp and 300 bp. MRSA in lane number 1, 5 and 6 were sized to 200 bp. MRSA strains in lane 2, 3 and 4 were sized to 300 bp.*

ed with clinical samples of non-directed bronchoalveolar lavage (NBL), catheter tip (CT), pus, tissue fluid, urine and sputum. The allele with 11 repeats, named type *spa* 5 (n=36, 44.44%) was linked with clinical samples of urine, central nervous system (CNS), ear and pus. The allele with 15 repeats, named type *spa* 6, is the only type which has been recovered from maximum number of clinical samples like pus, CNS, bone fracture, spinal cord, NBL, tissue fluid, sputum and urine**.** The allele with 17 repeats, named type *spa* 7 (n=2, 2.46%) was associated only with clinical samples of pus. The allele with 19 repeats, named type *spa* 8 (n=4, 4.93%) was found in clinical samples of pus, sputum and catheter tip.

EMRSA AND SMRSA

There was a highly significant difference (chi square test $p<0.005$) in the number of *spa* types; *spa* 3, *spa* 5 and *spa* 6 (Table III). These *spa* types were also more prevalent. There was non-significant difference (chi square test $p > 0.05$) in the number of *spa* types; *spa* 1, *spa* 2, *spa* 4, *spa* 7 and *spa* 8. It was found that *spa* types; *spa* 1, *spa* 2, *spa* 4, *spa* 7 and *spa* 8 were found to be less prevalent (Figure 4). The highest prevalence was observed for type *spa* 6 (71.60%) while the least prevalence was observed for type *spa* 2 (1.23%). According to our set epidemiological criteria, it was found that *spa* types; *spa* 3 (8 repeats), *spa* 5 (11 repeats) and *spa* 6 (15 repeats) were spread minimum 3 to maximum 5 hospitals (AMC, PIMS, Khyber, KRL, FGSH) and number of isolates for each *spa* types were also high. These *spa* types also possessed more than seven repeats. Hence their number of repeats along with spatial and temporal isolates dispersal among different hospitals showed these *spa* types as EMRSA. In SMRSA *spa* types; *spa* 1 (6 repeats), *spa* 2 (7 repeats) were spread within a single hospital (AMC) of their origin and these *spa* types also have less than ≤7 repeats in X-region and number of isolates bearing these repeats were very less. *spa* 4 (10 repeats) from PIMS, *spa* 7 (17 repeats) from AMC and *spa* 8 (19 repeats) from AMC and PIMS hospitals did not follow the rule of ≤ 7 however their spatio-temporal spread was limited to minimum 1 to maximum 2 hospitals with less number of isolates. Further, they were infrequent in occurrence (Table III). These later three *spa* types were exceptional to the rule set to discriminate SMRSA and EMRSA.

Clinical samples of pus involved variety of *spa* types compared to other samples. There are seven *spa* types of MRSA that were specifically associated with the clinical samples of pus except *spa* 2. *spa* 1, 2, 4, 7 and 8 carrying the 6, 7, 10, 17 and 19 repeats respectively were observed in very low numbers. *spa* types 3, 5 and 6 with 8, 11 and 15 repeats respectively, were more prevalent in the different clinical samples.

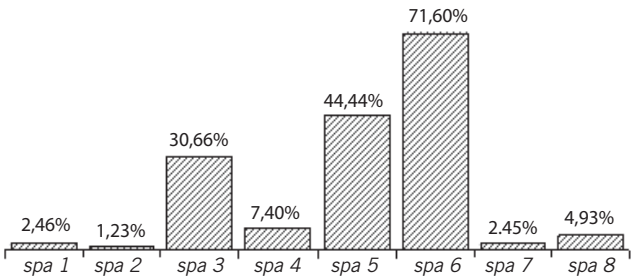

Figure 4. *Prevalence (%) of the different spa types of MRSA*

Table III. *Different spa types of MRSA identified from different clinical samples.*

Hospital	*spa types*	*PCR Product Size (bp)*	*Repeats*	*Clinical Samples*
Khyber	*spa* 1	200	6	Pus, Urine
AMC	*spa* 2	225	7	Bone fracture
AMC, PIMS, Khyber, KRL, FGSH	*spa* 3*	250	8	Catheter tip , NBL, Pus, Sputum,
PIMS	*spa* 4	275	10	Tissue fluid, Urine
AMC, PIMS, Khyber	*spa* 5*	300	11	Blood Pus, Spinal cord, Swab
AMC, PIMS, Khyber, KRL	*spa* 6*	400	15	Tissue fluid
	spa 7			CNS, Ear, Pus, Urine
AMC	*spa* 8	450	17	CNS, Bone fracture, NBL ,Pus,
AMC, PIMS		500	19	Spinal cord Sputum, Tissue fluid, Urine
				Pus
				Catheter tip, Pus, Sputum,

* Chi-square test for EMRSA, spa types is highly significant ($p < 0.005$).

INTER AND INTRA HOSPITAL PREVALENCE

The Primer "*spa*-F/*spa*-R" showed that type *spa* 3 and *spa* 5, were found with percentage prevalence of 62.50% and 37.50% respectively in AMC, Rawalpindi, hospital. These two strains were also found in Khyber hospital, Peshawar but percentage prevalence was significantly differed from AMC, hospital, Rawalpindi. In later, type *spa* 3 was less prevalent (26.66%) and *spa* 5 was highly prevalent (73.33%). *spa* 2 and *spa* 4 were only detected from PIMS hospital. Type *spa* 2 was only identified from one sample with percentage prevalence of 5.00% (Table IV).

Typed MRSA with Primer "*spa*-1113F/*spa*-1514R" showed that type *spa* 6 was present in all hospitals except FGSH but most prevalent type in both AMC and PIMS hospitals. Type *spa* 5 was observed in a few samples. This type was little more prevalent in PIMS hospital (8.0%) compared to AMC (3.22%) hospital. Type *spa* 6 was the most prevalent type detected from AMC, PIMS and Khyber hospitals with prevalence of 80.64%, 88.00% and 68.75% respectively (Table V). Type *spa* 1 was only available from the Khyber hospital, Peshawar. Types *spa* 2, *spa* 3, *spa* 4 can be identified with primer "*spa*-F/*spa*-R" while types *spa* 1, *spa* 6, *spa* 7 and *spa* 8 can be identified with primer "*spa*-1113F/*spa*-1514R". Type *spa* 5 can be identified by either of two primers (Table IV and V).

Primer "*spa*-F/*spa*-R" grouped 61 typed MRSA isolates into four clusters (Figure 5) using Hierarchical Clustering at the similarity value of 14%. Similarly, "*spa*-1113F/*spa*-1514R" grouped 74 typed MRSA into five distinct clusters represented by *spa* types using Hierarchical Clustering at similarity distance of 10% (Figure 6).

Table IV. *spa types of MRSA (n = 61) typed with primer "spa-F/spa-R"*

MRSA (n)	*PCR Product Sizes (bp)*	*No. of repeats*	*spa types (n)*	*Prevalence of spa types*
AMC (24)	250	8	*spa* 3 (15)	62.50
	300	11	*spa* 5 (9)	37.50
	225	7	*spa* 2 (1)	5.00
PIMS (20)	250	8	*spa* 3 (4)	20.00
	275	10	*spa* 4 (6)	30.00
	300	11	*spa* 5 (9)	45.00
Khyber (15)	250	8	*spa* 3 (4)	26.66
	300	11	*spa* 5 (11)	73.33
KRL (1)*	250	8	*spa* 3 (1)	-
FGSH (1)*	250	8	*spa* 3 (1)	-

*Less number of isolates makes the prevalence biased. Therefore their prevalence have not been calculated.

Table V. *spa types of MRSA (n = 74) typed with primer "spa-1113F/spa- 1514R".*

MRSA (n)	*PCR Product Sizes (bp)*	*No. of repeats*	*spa types (n)*	*Prevalence of spa types*
AMC (n=31)	300	11	*spa* 5 (1)	3.22
	400	15	*spa* 6 (25)	80.64
	450	17	*spa* 7 (2)	6.44
	500	19	*spa* 8 (3)	9.67
PIMS (n=25)	300	11	*spa* 5 (2)	8.00
	400	15	*spa* 6 (22)	88.00
	500	19	*spa* 8 (1)	4.00
Khyber (n=16)	200	6	*spa* 1 (2)	6.25
	300	11	*spa* 5 (3)	12.25
	400	15	*spa* 6 (11)	68.75
KRL (n=1)*	400	15	*spa* 6 (1)	-
FGSH (n=1)*	300	11	*spa* 5 (1)	-

*Less number of isolates makes the prevalence biased. Therefore their prevalence have not been calculated.

Discussion and Conclusion

TYPABILITY

Primer fidelity to the X-region sequence may vary to any length, as one strain not detectable by one primer is detectable by other different primer. This problem needs to be reduced by standardizing the particular primer for the particular strains. However, X-region response may be variable which is beyond our control compared to primer selection and designing. This aspect is being ignored for defining the strain characterization in many typing techniques which needs the attention of the scientific community.

Percentage typability showed that primer "*spa*-1113F/*spa*-1514R" tended to be more specific and possessed high fidelity for X-region of *spa* gene compared to the other primer "*spa*-F/*spa*-R". We found that probability of more number of MRSA types could be achieved when used former primer. Primer "*spa*-1113F/*spa*-1514R" typability was high 91.35% (n=74) compared to *spa*-F/*spa*-R" which was 75.30% (n=61). This *spa* typability results are considerably close the study where *spa* typed 29 (80.55%) out of 36 MRSA strains (21). We observed non-typability percentages of 8.64% and 24.69% for the primer "*spa*-1113F/*spa*-1514R" and primer "*spa*-F/*spa*-R" respectively. It is conceived that in non-typable isolates, the X-region of the Protein A might be absent (20). Mutation of Protein A gene could also hide the *spa* typing (22).

Single primer set has frequently been used to study the *spa* typing (21) (23) (24) which make the *spa* type's comparability and discrimination through PCR ambiguous and subjective. As observed in our studies different primer amplified different PCR products of different sizes for the same "X-region". However, it might be advantageous to predict more reliably different *spa* types. Further, it is also an important consideration that sometimes a single primer may not be compatible due to conformational changes in the specific region of the DNA where actually the primer is to bind. These factors could affect the typability results.

In order to compare our work with the other published work, we calculated *spa* types based on amplicon size and use as a reference 263 bp, which consist of 9 repeats as suggested by (18). We found PCR products which were ranged from 200 bp to 500 bp. PCR amplification of X-region of protein A gene yielded amplicon of sizes ranging from 90±10 to 350±10 bp in *S. aureus* isolates (25). In the *spa* typing (21), observed single amplicon of 200 to 600 bp. PCR products that ranged from 250 bp to 637 bp in length for the various numbers of repeats in the X-region of *spa* gene (24). In our study, the difference in the amplicon size was related with difference in the repeats which may be due of deletion, point mutations, duplication or insertion of the repeats in the X-region (16) (26).

ALLELIC POLYMORPHISM

Spa typing showed eight types of allelic polymorphism in the X-region of the *spa* gene. In a study (25) 12 *spa* types have been classified on the basis of the am-

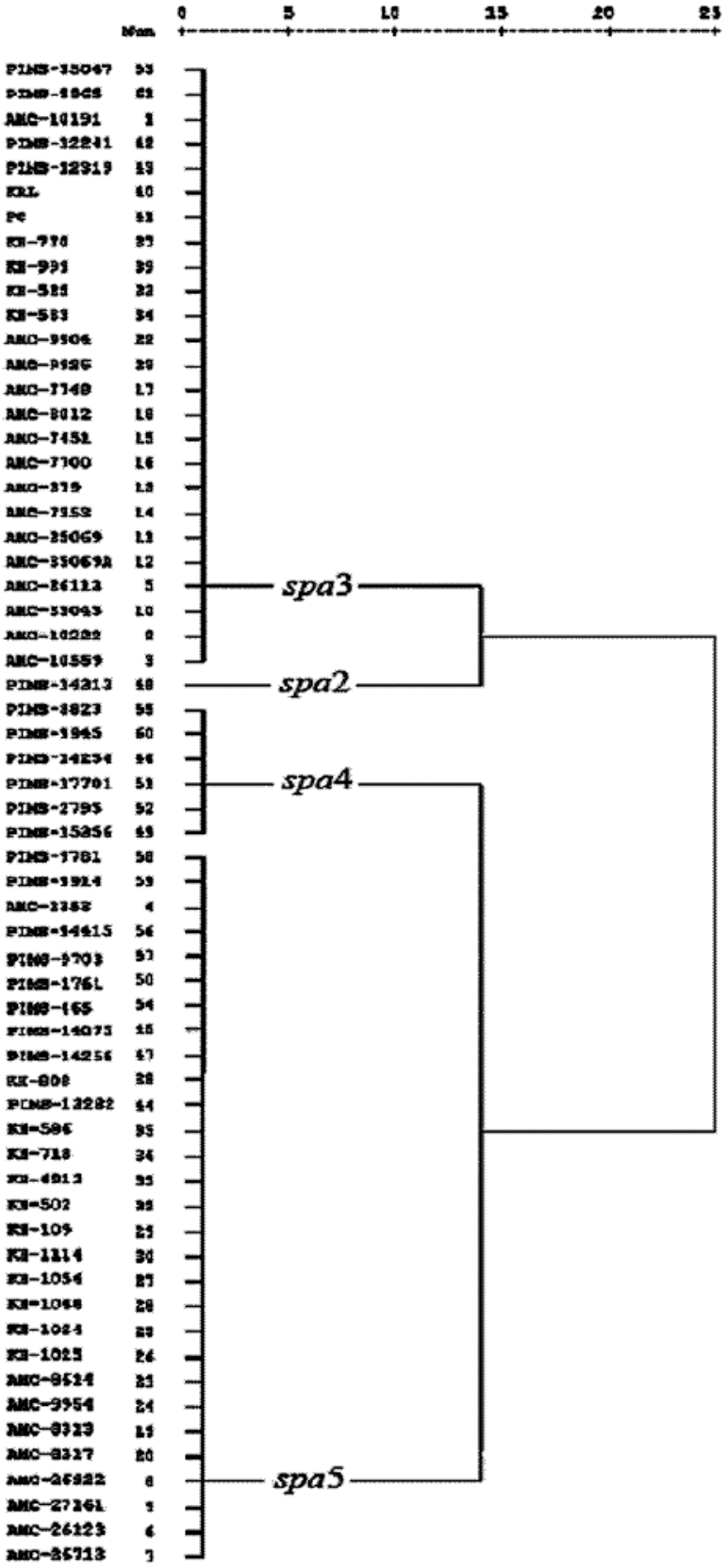

Figure 5. *Dendrogram shows the spa types based on the number of repeats in the X-region of spa gene with primer spa-F/spa-R.*

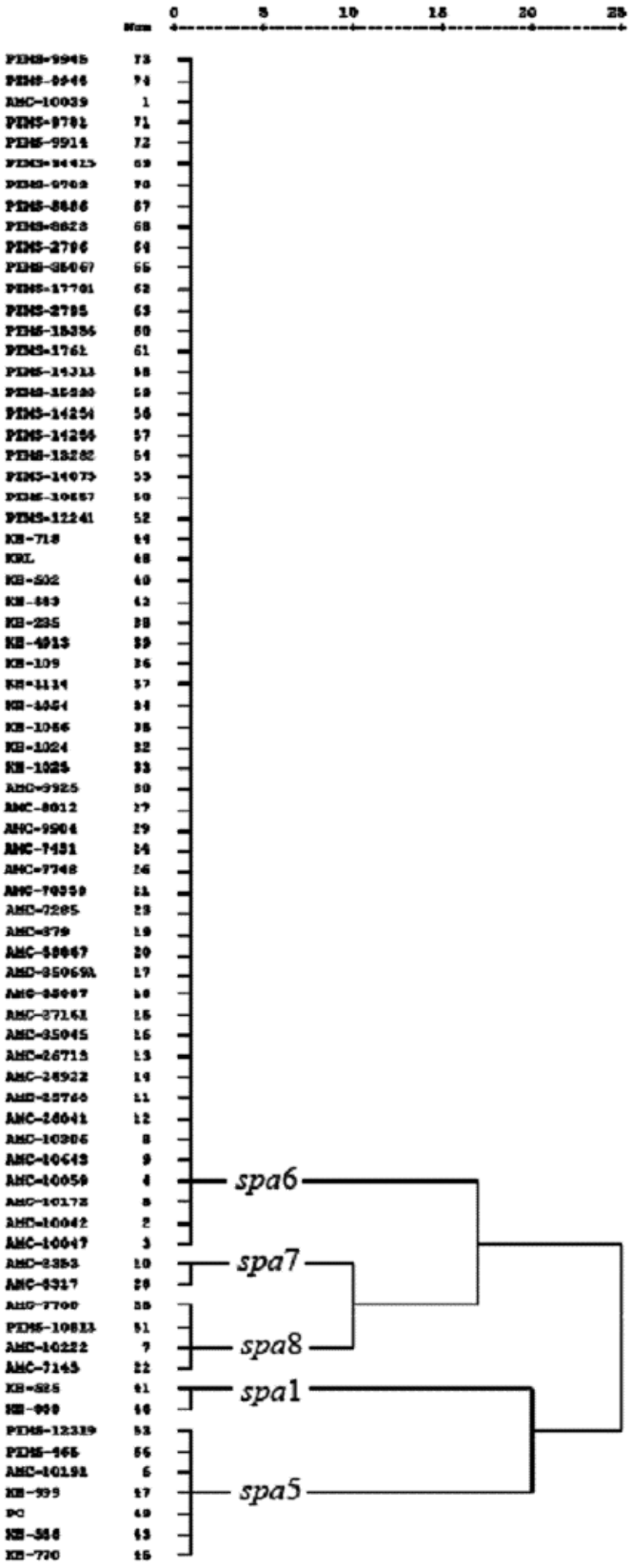

Figure 6. *Dendrogram shows the spa types based on the number of repeats in the X-region of spa gene with primer "spa-1113F/spa-1514R".*

plicon size and described 2 to 13 numbers of repeats. In our study, the types carrying the 6, 7, 8, and 11 repeats are 100 percent in agreement with earlier studies. However types with 15, 17 and 19 repeats are unique. Various studies described various repeats for different types of MRSA strains. A study reported 4 to 13 repeats in 142 isolates (27). MRSA strains, in which X-region composed of 4, 5, 7, 8, 9, 10, and 11 repeats (24). In their studies the most common *spa* type, had 10 repeats corresponds to 556 bp. They also observed types with 2, 7, 17 and 21 repeats in some samples. In our study, number of repeats calculated for different *spa* types through analysis of the PCR products is in close agreement with these studies.

In another study (18) characterized the *spa* types based on the amplicon size and categorized into four *spa* types. Their study showed that type *spa* 1 (11 repeats, n = 92.72%), was the most common and it was involved in most of the epidemic outbreaks. Types *spa* 2 (10 repeats; 20.16%) were linked with rare cases of outbreak in hospitals. Types *spa* 3 (9 repeats, 14.11%) and *spa* 4 (5 repeats, 2.2%) was observed only in sporadic cases. In our study, the most common type was *spa* 6 (15 repeats, 71.60%), which was highly prevalent. The highly prevalent *spa* types; *spa* 3, *spa* 5 and *spa* 6 significantly (chi square test $p < 0.005$) vary from other less prevalent *spa* types, *spa* 1, *spa* 2, *spa* 4, *spa* 7 and *spa* 8. In this study, seven *spa* types were identified from clinical samples of pus. The identification of high prevalence of the *spa* types associated with the pus may be due to maximum samples of the pus available from hospitals. Other reason may be the preference of the clinical practitioners to take pus samples for MRSA screening.

EMRSA AND SMRSA ANALYSIS

Spa typing emerged as a single locus marker for typing the MRSA. The types *spa* 3 (8 repeats), *spa* 5 (11 repeats) and *spa* 6 (15 repeats) were and *spa* 1 (6 repeats), *spa* 2 (7 repeats) and *spa* 3 (8 repeats), *spa* 4 (11 repeats), *spa* 7 (17 repeats) and *spa* 8 (19 repeats) were SMRSA. A rule has been suggested (23) for discrimination between EMRSA and SMRSA on the basis of protein A gene polymorphism. They found that presence of seven or fewer repeats in the X-region tended to be sporadic while more than seven repeats was indicative of epidemic character of the MRSA strain. Pakistani EMRSA isolates fulfilled the criteria defined in the materials and methods section and was in agreement with the earlier studies (19) (23).. All EMRSA have repeats more than seven but few SMRSA do not completely follow the criteria of ≤7 repeats. These SMRSA; *spa* 4 (10 repeats), *spa* 7 (17 repeats) and *spa* 8 (15 repeats) are exceptional to the rule. It seems these types are inherently SMRSA and somehow due to duplication or insertion in X-region they have acquired larger number of repeats. MRSA isolates with these *spa* types show less epidemiological prevalence among the clinical samples which indicates acquisition of longer X region may not necessarily correlated positively with spread of infection. Longer X region allows a more-favorable exposition of the Fc-binding regions at the cell surface, facilitating infection and important for epidemic spread (23) may not be corroborated for transient SMRSA which have longer X-region but have restricted spread. Evidence of Longer X region is advantageous to SMRSA in getting transformed into the Epidemic MRSA. But this aspect of SMRSA yet need more research. These considerations need attention and long term *spa* surveillance. Difference in our and earlier studies (23) may be due to the fact that there is no exact criteria for classifying MRSA into EMRSA and SMRSA precisely. Here it is important consideration that Epidemic and Sporadic character may not only depend on the number of repeats but also depend upon source of inter/intra hospital transmission which may be due to paramedical staff and patients traveling among hospitals. The reason to restrict an epidemic spread of SMRSA is linked with stringent hygienic conditions. Multiple loci on the chromosome may contribute to the epidemic character and the X-region of the *spa* gene may be associated one of these loci. In an earlier study it was found that all the epidemic outbreaks corresponded to strains with *spa* types carrying 10 and 11 repeats while *spa* isolates with either 5 or 9 repeats were only detected in sporadic cases (19). More number of repeats may provide an advantage/natural selection to the strains and help in the epidemic spread of the strains A longer X region, is important for epidemic character of a strain (23).

PREVALENCE OF THE CHARACTERIZED TYPES IN PAKISTANI HOSPITALS

Inter and intra hospital prevalence of different *spa* types showed that both *spa* 3 and *spa* 5 were prevalent strains in AMC Hospital, Rawalpindi and Khyber hospital, Peshawar. *spa* 5 was more prevalent in Khyber vs. AMC (73.33 % vs. 62.50%) hospital. However, *spa* 3 was more prevalent in AMC vs. Khyber hospital (62.50% vs. 26.66%). It showed that *spa* 3 spread was high in AMC hospital while *spa* 5 spread was high in Khyber hospital. *Spa* 2 and *spa* 4 were only detected from PIMS hospital which showed that these types have newly emerged in response to different antibiotics treatment to the patients. PIMS is a major hospital, which received patients from all the four districts of the country. In these *spa* types (*spa* 2 and *spa* 4), chances of occurrence due to new admissions of outdoor patients could not be ignored.

Type *spa* 5 was observed in a few samples but was more prevalent in PIMS (8.0%) as compared to AMC (3.22%) hospital. Type *spa* 6 carried 15 repeats in its X-

region and was the most prevalent types detected from AMC, PIMS and Khyber Hospital with significant level of prevalence. This *spa* type was isolated from most of different clinical samples. It could be considered that *spa* 6 has more diversity which leads to more spread in the hospitals that are distantly located (Khyber hospital Peshawar vs. other hospitals). Type *spa* 1 was only available from Khyber hospital, Peshawar that showed it was rare or might be a newly emerging type due to some unknown reasons, which needs further studies. We concluded that current study has provided the basic reliable information on epidemiologica prevalence of various MRSA *spa* types involed in community hospitals in the country. Albeit, s*pa* typing can provide supplementary knowledge for the hospital epidemiologist, but this tecahnques is efficient enough to differentiate the new *spa* strains carrying variations in general and those slowly transforming from sporadic to epidemic outbreak in particular.

CORRESPONDENCE

Zahid Majeed C/o Prof. Dr. Salman Akbar Malik, Chairman
Department of Biochemistry
Faculty of Biological Sciences
Quaid-i-Azam University, ISLAMABAD, Pakistan.
P.O Box No. 44000 Tel.: +92 51 90613012, +92 51 2601164
E-mail: zahidfdb@gmail.com

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