PREVALENCE OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) IN LAHORE, PAKISTAN ON THE BASIS OF STAPHLOCOCCAL PROTEIN A (*SpA*) TYPING

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ABSTRACT

Staphylococcus aureus has become a common nosocomial infectious agent throughout the world, causing a wide range of infections. Epidemiological studies suggest that hospitals of all sizes are facing the problem of the resistant form of *Staphylococcus aureus* that is Methicillin Resistant *Staphylococcus aureus* (MRSA). Protein A of *Staphylococcus aureus* is a virulence factor which is encoded by *spa* that shows a variation in length among different strains of MRSA. In this study, ninety-six strains of MRSA were collected from private and public sector hospitals of Lahore. Sixty-seven (70%) strains were confirmed as MRSA by DNase test and Kirby-Bauer disk diffusion test. The remaining (30%) isolates were misdiagnosed as other *Staphylococcus* species. Subsequently, polymorphic X region of the *spa* gene was amplified using specific primers of *spa* gene by the polymerase chain reaction (PCR). Four different banding patterns, ranging in length between 300 to 500 bp, showed polymorphism of the *spa* gene. Most strains showed 98 % homology with isolate *spa* 7 *Staphylococcus aureus* IgG protein. Those sequences can serve as a rapid diagnostic tool for the identification of local MRSA during outbreaks.

Keywords: Methicillin Resistant *S. aureus* (MRSA), Staphylococcal Protein A (Spa)

INTRODUCTION

Staphylococcus aureus is the main reason of nosocomial human infections and bacteremia. These infections are due to susceptible strains of *S. aureus* which are associated with a 20-40% mortality rate (Sorge *et al*., 2013). *S. aureus* strains which show resistance towards penicillin and other β-lactam antibiotics are called methicillin resistant strains of *S. aureus* (Enright, 2008). In strains of MRSA, the resistance to methicillin evolves due to the changes in penicillin binding proteins (also denoted as PBP2α and PBP2') which reduces the cell wall sensitivity to β-lactam antibiotics (Wilke *et al*., 2005). The microbial surface attachment proteins are produced in logarithmic growth phase during cell wall synthesis. These microbial surface components recognize adhesive matrix molecules (MSCRAMMs) (Gordon and Lowy, 2008) including fibronectin binding protein, fibrinogen binding protein, collagen binding protein, and clumping factor.

There is another surface protein production during cell wall synthesis known as staphylococcal Protein A (*Spa)* that may has a role in evasion of host defense mechanisms. Although it's biological function is to bind with the Fc region of the IgG antibody (Foster, 2005). Spa is a 42- kDa protein which covalently anchored in the bacterial cell wall surface. It belongs to the MSCRAMM family, because it has the ability to attach with a larger glycoprotein name von Willebrand factor that mediates the adhesion of platelets at the site of endothelial damage. It comprises of IgG binding region having five domains i.e. E, D, A, B and C and X region with repeated regions of 24 base pairs (Bien *et al*., 2011). Frenay *et al.* (1996) have used the sequence of polymorphic region X of the *S. aureus* protein A (*spa*) gene to develop a single locus sequence typing method for MRSA. The *spa* locus comprises of a number of (mainly 24-bp) repeats and its diversity is attributed to deletions and duplications of the repeats and more seldom, to point mutation (Kahl *et al*., 2005). The discriminative power of *spa* typing lies between that of PFGE and MLST (Malachowa *et al*., 2005) and in contrast to MLST, *spa* typing can be used to investigate both the molecular evolution and hospital outbreaks of MRSA (Koreen *et al*., 2004). The main advantage of *spa* typing over MLST is its rapid diagnosis, since it involves sequencing of only a single locus. Another advantage of *spa* typing is that several laboratories can use different sequencing platforms and analyze the resulting sequence chromatograms using special software. By this means, typing is made accessible not only to reference laboratories, but also to local laboratories. Comparability and a common nomenclature with excellent quality of data are available (Aires *et al*., 2006).

Very little published reports on *spa* local MRSA strains are available from Pakistan. Earlier works 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. This study was

designed to provide the information about the *Spa* typing of local isolates of MRSA from various healthcare settings of Lahore, Pakistan.

MATERIALS AND METHODS

Different strains of Methicillin Resistant *Staphylococcus aureus* (MRSA) were collected from labs of Sheikh Zayad Hospital, Mayo Hospital, Services Hospital, Itifaq Hospital, Chugtais and Al Razi. The identification of all hospital strains were reconfirmed by the biochemical tests, including Gram's staining, Catalase test, DNase test and the growth on Mannitol salt agar (Cappuccino and Sherman, 2007) . The susceptibility pattern of antibiotic was determined by the Kirby-Bauer disc diffusion method on Muller Hinton agar following NCCLS guidelines, against different antibiotics like Oxacillin/Methicillin, Cefoxitin and Vancomycin. Bacterial DNA was isolated by CTAB method (Ausubel *et al*., 2002). Amplification of the *spa* variable repeat region was performed using primers *spa*-1113f (5'-AGACGATCCTTCGGTGAGC-3') and *spa*-1514r (5'-AGCAGTAGTGCCGTTTGCTT-3'). Extracted DNA from selected strains and master mix was prepared. The 50 µl PCR reaction mixture was prepared by adding 5 µL PCR buffer (10X), 1 µL dNTPs, 1.5 µL $MgCl_2$ (50mM), 2.5 µL of forward and reverse primer, 0.2 µL Taq polymerase, 5 µL DNA and 32.3 µL water. PCR conditions were: initial denaturation was 80^{o}C for 5 minutes, amplification of 45 cycles at 94^{o}C for 45seconds, annealing at 60^{o}C for 45 seconds, extension at 72^{o}C for 90 seconds with a final extension of 10 minutes at 72^{o}C .

RESULTS

Ninety-six different strains of *S. aureus* were obtained from three hospitals and laboratories of Lahore. After screening, sixty-seven were confirmed as MRSA and four methicillin sensitive strains (MSSA; Fig.1). Out of total 96 strains received, 67 (70%) strains from private sector and 25 (30%) strains from public sector were taken.

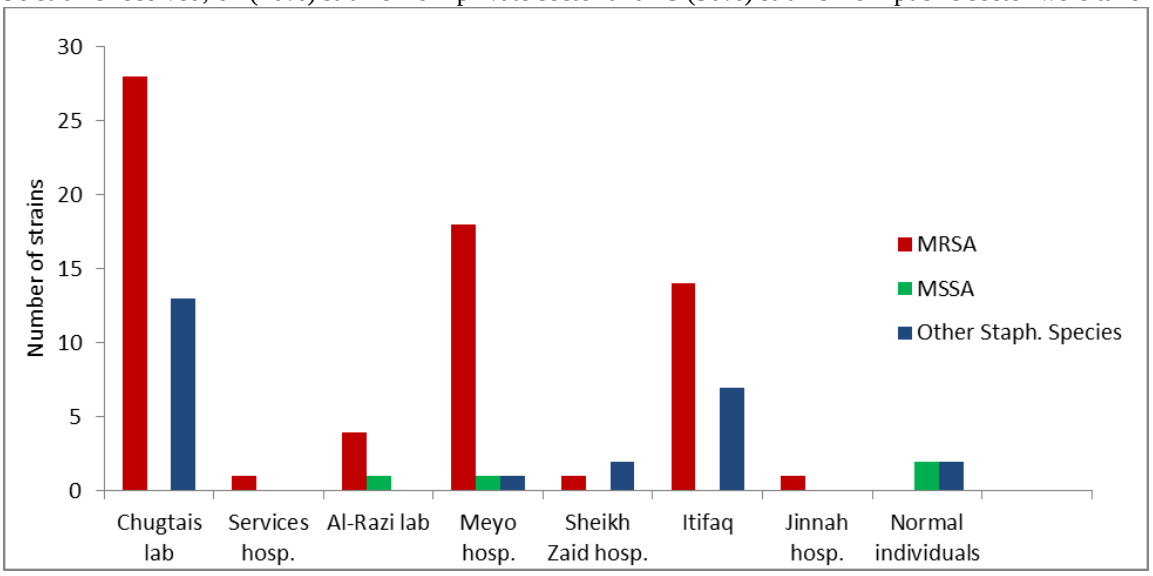

Fig. 1. Distribution of strains from various hospitals of Lahore.

AMPLIFICATION OF *Spa* GENE

The DNA was isolated by CTAB method and one set of primer was used for the typing of MRSA. 33 MRSA strains were typed. Four different PCR amplicons which ranged from 300 bp to 500 bp were found. In this study four different MRSA *spa* types were obtained on the basis of X-region polymorphism. *Spa* type composed of repeats and each type of *spa* which interpret 24 nucleotides. 33 MRSA strains were showed bands of different sizes. In 28 strains of MRSA a single band of *spa* gene was observed with the 400 bp (14 repeats) while 300 bp (10 repeats) *spa* gene band was observed in 4 samples. One of the strains of skin swab had 500 bp (18 repeats) *spa* band whereas 450 bp (16 repeats) band was also observed only in one strains from pus. The frequency of samples having 400 bp was in greater proportion than 300 bp bands (Fig. 3).

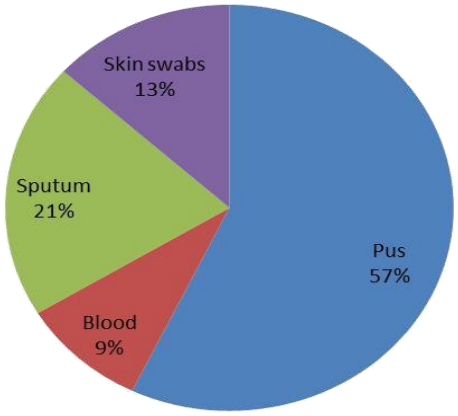

Fig. 2. Anatomical size of MRSA from clinical samples of Lahore labs.

CALCULATION OF VARIABLE TANDEM REPEATS (VNTRs)

The number of variable tandem repeats (VNTRs) in the X region of *spa* gene was calculated by the PCR amplicons on the gel. In X-region of *spa* gene of *S. aureus* 263 bp contains 9 repeats (Sabat *et al*., 2003). In all samples the number of repeats was 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 (Majeed *at al*., 2012).

ALLELIC POLYMORPHISM

In a particular MRSA type, numbers of repeats were assessed for the X-region. The repeats calculated for four different PCR products corresponded to four types of allelic polymorphism in the *spa* gene. The allele with 10 repeats, named type *spa* 1 and the allele with 14 repeats named the *spa* 2. *Spa* 3, *spa* 4 have 16 and 18 repeats respectively in their polymorphic region of X. Type *spa* 1 was associated with clinical samples of pus and blood while *spa* 3 and *spa* 4 were found only with the clinical samples of pus and skin swab respectively. The more important *spa* types were *spa 2* was recovered from multiple samples (Fig.3). Prevalent % data showed that *spa* 2 was widespread (81%) while other *spa* types are less common. Out of all clinical samples pus samples were more prone to *spa* 2 type (Fig.4, 5).

The *spa* typing (Hoefnagels *et al*., 1997), showed single amplicon of size 200 to 600 bp. In 28 strains of MRSA of our study, single band of *spa* gene was determined with the size of 400 bp (14 repeats) while 300 bp (10 repeats) *spa* gene band was observed in 4 samples. One of the strains of skin swabs had 500 bp (18 repeats) *spa* bands. 450 bp (16 repeats) was also observed only in one strain from pus. The frequency of samples having 400 bp was more than 300 bp bands. The most common *spa* gene band in MRSA was about of 400 bp. In 25 cases of 400 bp bands, 17 strains were from pus, 2 strains from blood, 3 strains from skin and 3 strains from sputum was observed. 3 cases out of 4 showed that 300 bp was from pus and remaining 1 sample was from blood. So the differences in the amplicon sizes is due to the variability in the repeats which is related to the deletion, duplication, point mutation or insertion of the nucleotide bases in the X-region (Majeed *et al*., 2012; Japoni *et al*., 2003).

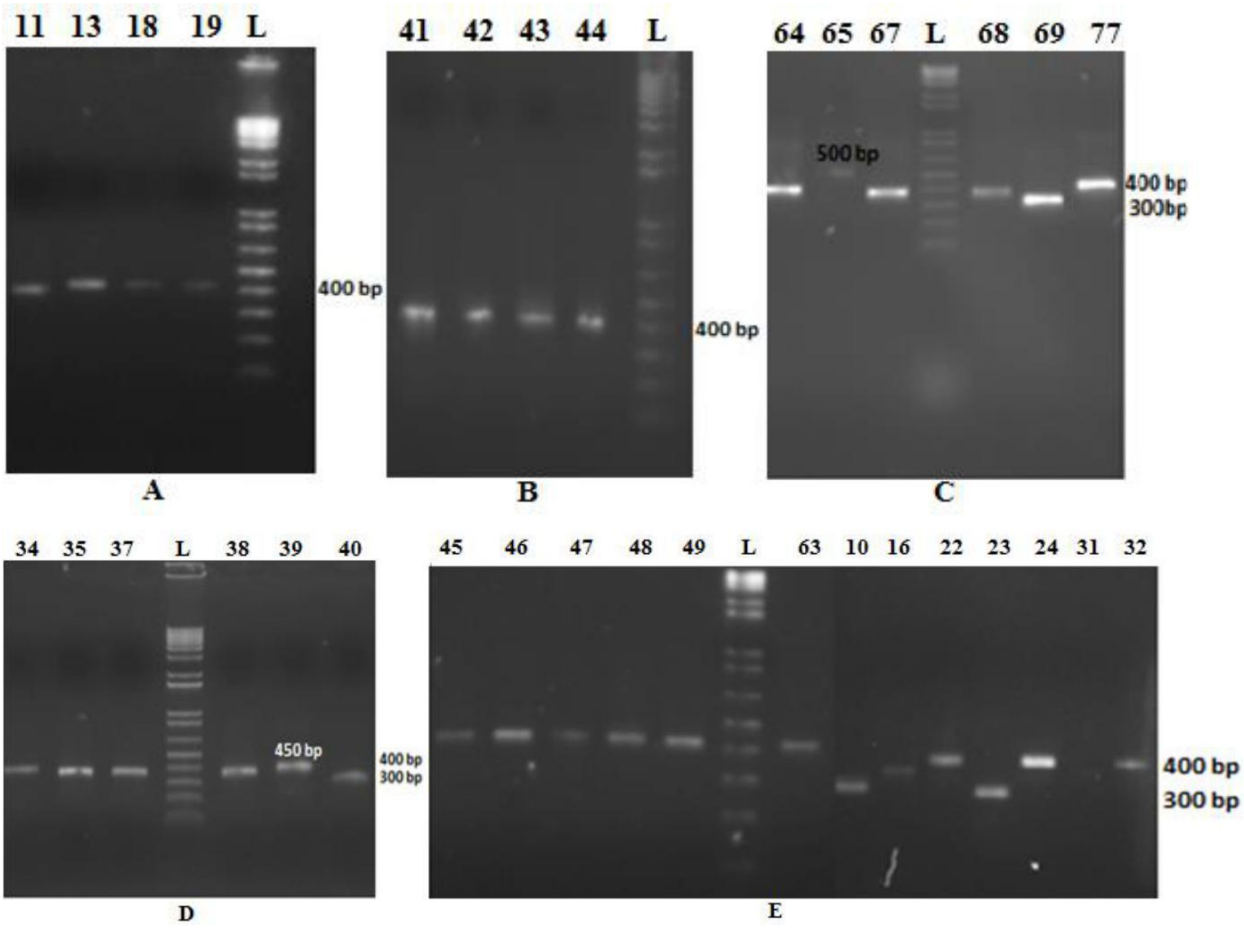

Fig. 3. Representation of *spa* gene on agarose gel electrophoresis**.** L: DNA ladder **(A)** *spa 2* type showing 400 bp repeats in sample # 11, 13, 18, 19. **(B)** *spa 2* type showing 400 bp repeats in sample number 41, 42, 43, 44. **(C)** *spa 1* showing 300bp repeats in sample # 69. *spa 2* showing 400 bp repeats in sample # 64, 67, 68, 77. *spa 4* showing 500 bp repeat in sample # 65. **(D)** *spa 1* type showing 300 bp repeats in sample# 40. *spa 2* type showing 400 bp repeats in sample number 34, 35, 37, 38. *spa 4* type showing 450 repeat in sample number 39. **(E)** *spa 1* type showing 300 repeats in sample # 10, 16, 23. *spa 2* showing 400 repeats in sample # 45, 46, 47, 48, 49, 63, 22, 24, 32. Sample # 31 showing no repeats.

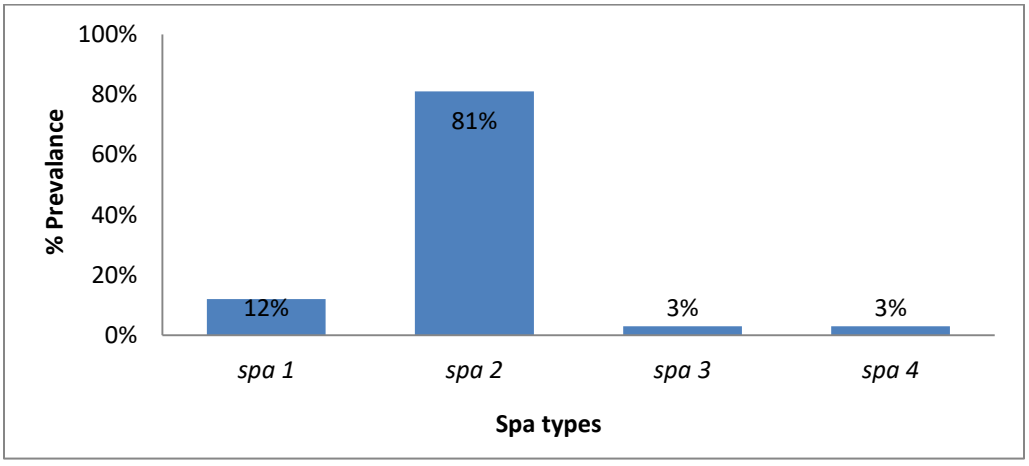

Fig. 4. Graphical Representation of the prevalence (%) of the different *spa* types of MRSA.

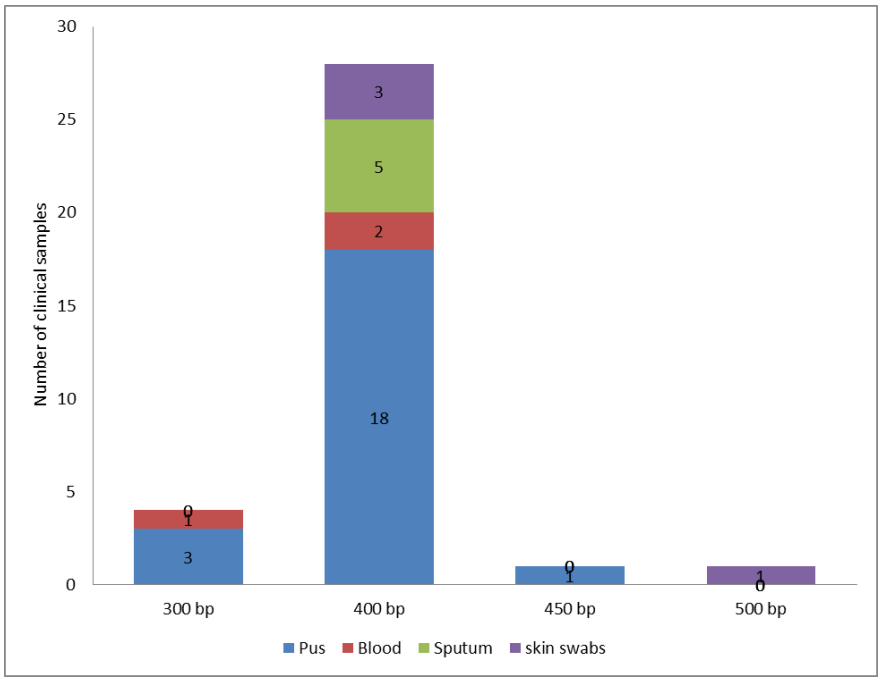

Fig. 5. Contribution of clinical samples for variable amplicons size.

In a particular MRSA type, numbers of repeats were assessed for the X-region. The repeats calculated for four different PCR products corresponded to four types of allelic polymorphism in the *spa* gene. The allele with 10 repeats, named type *spa* 1 and the allele with 14 repeats named the *spa* 2. *Spa 3* and *spa* 4 have 16 and 18 repeats respectively in their polymorphic region of X. Type *spa* 1 was associated with clinical samples of pus and blood while the type *spa 3* and *spa 4* were linked only with the clinical samples of pus and skin swabs. The more important *spa, spa 2* was recovered from multiple samples.

DISCUSSION

In Pakistan the reported prevalence of MRSA is to be 42% (Arif *et al*., 2007). The triggering factor for the emergence of antibiotic resistant bacteria in Pakistani hospitals is due to irrational use of antibiotics. So increase in the use of antibiotic prescription causes the evolution of resistant bacteria at comparatively high rate (Perveen *et al*., 2013). In a multicenter study, the varying distribution of MRSA was observed in different cities of Pakistan. MRSA was more prevalent in Lahore i.e. 61% followed by 57.5% in Karachi, 46% in Islamabad/Rawalpindi, 36% in Peshawar and only 2% in Sukkur (Hafiz *et al*., 2002). In our study 70 % strains of MRSA were found in different regions of Lahore. Highest percentages of these isolates were from pus and specimen of pus swabs (57%) while rest of them were obtained from sputum samples (21%), skin swabs (13%) and blood (9%). The occurrence of every isolate increases from 1996 to 2003 (Panlilio *et al*., 1992). In the last two decades there is readily increase in the prevalence of MRSA throughout the world (Siddique *et al*., 1999), including Pakistan (Perveen *et al*., 2013). In 1999, the analyses of various clinical samples in Sargodha had 23% MRSA (Siddique *et al*., 1999). In other studies from Mayo hospital Lahore (Khatoon *et al*., 2002)and Karachi (Perwaiz *et al*., 2007)revealed 38.5% and 69% isolates of MRSA respectively from diverse clinical samples. In our study out of 96 strains 67 (70%) were MRSA which were collected from various sectors of Lahore. The higher proportion of MRSA was attained from private sector of Lahore (Chugtais Lab, Al Razi and Itefaq Hospital). This somehow correlates with the other studies and indicate an emerging trend of MRSA.

In Pakistan this is only due to self-medication and higher availability of antibiotics (Idrees *et al*., 2009). The study has shown that the prevalence of MRSA infections has increased gradually over the years (Orrett *et al*., 2006). Although initially the MRSA infections were traditionally restricted to hospitals, but with the increasing time the community-associated cases of MRSA (CA-MRSA) were also reported (DeLeo *et al*., 2010). CA-MRSA infections occurred in patient having at least 1 of the following health care risk factors: (1) presence of an invasive device at time of admission; (2) history of MRSA infection or colonization; (3) history of surgery, hospitalization, dialysis, or residence in a long-term care facility in previous 12 month preceding culture date. Community-associated Cases of

MRSA occurred with no documentation of community-onset health care risk factor. 8987 cases of invasive MRSA were observed during the surveillance period. Most MRSA infections were health care–associated: 5250 (58.4%) were community-onset infections, 2389 (26.6%) were hospital-onset infections; 1234 (13.7%) were community-associated infections, and 114 (1.3%) could not be classified. Invasive MRSA infection affects certain populations disproportionately. It is a major public health problem primarily related to health care but no longer confined to intensive care units, acute care hospitals, or any health care institution (Kleven *et al*., 2007)

In this study the DNA of strains were isolated by CTAB method and one set of primer was used for the typing of MRSA. Single primer set has often been used to study the *spa* typing which make the *spa* type's comparability and discrimination through PCR. The factor which affects the typing results is the significance of single primer that may not be compatible due to conformational changes in the specific region of the DNA where actually the primer is to bind (Majeed *et al*., 2012).

When this study was compared with already published work, the *spa* types were calculated on the basis of amplicon size and use 263 bp as a reference which consist of 9 repeats as suggested by (Sabat *et al*., 2003). After PCR four different band sizes ranging from 300 bp to 500 bp were found in *S. aureus*. Thus four different *spa* types of MRSA were obtained on the basis of X-region polymorphism. *Spa* type composed of repeats and each type of which interpret 24 nucleotides. Majeed *et al.,* 2012 yielded amplicons of size ranging from 100 to 400 bp in *S. aureus* isolates after PCR amplification variable fragment of protein A gene (Majeed *et al*., 2012).

The epidemiological prevalence of our study (70%) compares favorably well with other studies done within the country i.e. 61% (Hafiz *et al*., 2002) and 60.40% (Perveen *et al*., 2013). Our study has several limitations concerning the extent of representation of the MRSA population in Pakistan. First, our represented samples lacked a significant number from various parts of the city. Second, despite the collection instructions, the proportion of clinical samples is not even from all centers. Maximum samples were collected from Chugtais lab, Mayo Hospital and Itefaq Hospital and very few samples were collected from other centers. The clinical samples of skin swabs and blood cultures isolates were not even present in all centers.

Our study defines molecular epidemiological features that our MRSA isolates have in common with those in other parts of the country (Majeed *et al*., 2012). Thus the introduction of new strain is changing our local epidemiology. It also represented that prevalence of MRSA strains in our region has reached up to 70%. Maximum contribution of strains was from private sector. Molecular *spa* typing of strains showed the *spa* band size of 400 bp is significantly more in Lahore region. The MRSA *spa* gene isolated from pus samples showed their average length more as compared to other clinical samples.

REFERENCES

Aires de Sousa, M., K. Boye, H. de Lencastre, A. Deplano, M.C. Enright, J. Etienne and A. Friedrich (2006). High inter laboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. *Journal of Clinical Microbiology.* 44: 619-621.

Ali, A. M., S.A. Abbasi, S. Arif and I.A. Mirza (2007). Nosocomial infections due to methicillin resistant *Staphylococcus aureus* in hospitalized patients. *Pakistan Journal of Medical Sciences,* 23: 593-596.

Ausubel F. B. R., R.E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl (2002). *Short Protocols in Molecular Biology*. John Wiley & Sons, Inc., New York, USA.

Bien, J., O. Sokolova and P. Bozko (2011). Characterization of virulence factors of *Staphylococcus aureus*: Novel function of known virulence factors that are implicated inactivation of airway epithelial proinflammatory response. *Journal of Pathogens,* 2011: 1-13.

Butt, T., R.N. Ahmad, M. Usman and A. Mahmood (2004). Methicillin resistant *Staphylococcus aureus*, Pakistan, 1996–2003. *Emerging Infectious Disease.* 10(9): 1692 – 1693.

Cappuccino, J. and N. Sherman (2007). *Microbiology: A Laboratory Manual.*7th ed. Pearson Education.

DeLeo, F. R., M. Otto, B.N. Kreiswirth and H.F. Chambers (2010). Community-associated methicillin-resistant *Staphylococcus aureus*. *Lancet.* 375(9725):1557-1568.

Enright, M. C. (2008). The population structure of *Staphylococcus aureus*. In: Lindsay, J. A. (Ed.) *Staphylococcus: molecular genetics,* Norfolk, Caister Academic Press.

Foster, T. J. (2005). Immune evasion by staphylococci. *Nature Reviews Microbiology.* 3(12): 948–958.

Frenay, H. M., A.E. Bunschoten, L. M. Schouls, W. J. van Leeuwen and C.M. Vandenbroucke-Grauls (1996). Molecular typing of methicillin-resistant *Staphylococcus aureus*on the basis of protein A gene polymorphism. *European Journal of Clinical Microbiology and Infectious Diseases.* 15:60.

Gordon, R. J. and F.D. Lowy (2008). Pathogenesis of Methicillin-Resistant *Staphylococcus aureus* Infection. *Clinical and Infectious Disease.* 46:350–359.

Hafiz, A. N., H.L. Ali, A. S. Chughtai, B. Memon, A. Ahmed and S. Hussain (2002). Methicillin resistant *Staphylococcus aureus*: a multicenter study. *Journal of Pakistan Medical Association.* 52:31.

Hoefnagels-Schuermans, A., W.E. Peetermans, M. J. Struelens, S. Van Lierde and J. Van Eldere (1997). Clonal analysis and identification of epidemic strains of methicillin-resistant *Staphylococcus aureus* by antibiotyping and determination of protein A gene and coagulase gene polymorphisms. *Journal of Clinical Microbiology.* 35: 2514-2520.

Idrees, F., K. Jabeen, M. Khan and A. Zafar (2009).Antimicrobial resistance profile of methicillin resistant Staphylococcal aureus from skin and soft tissue isolates. *Journal of Pakistan Medical Association.* 59(5): 266-269.

Japoni, A., A. Alborzi, M. Rasouli and B. Pourabbas (2003). Modified DNA Extraction for rapid PCR detection of Methicillin resistant *Staphylococci*. *Iranian Biomedical Journal.* 8(3): 161-165.

Kahl, B. C., A. Mellmann, S. Deiwick, G. Peters and D. Harmsen (2005). Variation of the Polymorphic Region X of the Protein A Gene during Persistent Airway Infection of Cystic Fibrosis Patients Reflects Two Independent Mechanisms of Genetic Change in *Staphylococcus aureus. Journal of Clinical Microbiology.* 43:502.

Khatoon, N., H. Bukhari, J.R. Riaz, and A. S. Sheikh (2002).Prevalence of Methicillin Resistant *Staphylococcus aureus* (MRSA) infection laboratory study at Mayo Hospital Lahore. *Biomedica*, 18:49-52.

Klevens, M., R. Edwards, L. Richards, C. Horan, P. Gaynes, A. Pollock and M. Cardo (2007). Estimating health care-associated infections and deaths in US hospitals, 2002. *Public health reports*. 122(2): 160.

Koreen, L., S.V. Ramaswamy E. A. Graviss S. Naidich J.M. Musser and B.N. Kreiswirth (2004). Use of a single marker to detect genetic microsec- and macrovariation. *Journal of Clinical Microbiology.* 42: 792.

Majeed, Z., Y. Arafat, Z. Ajab, S.A. Malik, W. A. Abbasi and H. Ajab (2012). Genotypic characterization of prevalent methicillin resistant *Staphylococcus aureus* in Pakistani community hospitals .*Acta Bioquim Clin Latinoam.*46 (2): 271 – 283.

Malachowa, N., A. Sabat, M. Gniadkowski, J. Krzyszton-Russjan, J. Empel, J. Miedzobrodzki, K. Kosowska-Shick, P.C. Appelbaum and Hryniewicz (2005). Comparison of multiple-locus variable-number tandem-repeat analysis with pulsed-field gel electrophoresis, spa typing, and multilocus sequence typing for clonal characterization of *Staphylococcus aureus* isolates. *Journal of Clinical Microbiology.* 43:3095.

Orrett, F. A. and M. Land (2006). Methicillin-resistant *Staphylococcus aureus* prevalence: Current susceptibility patterns in Trinidad. B. M. C. *Infectious Diseases.* 6: 6-83.

Panlilio, A. L., D.H. Culver, R. P. Gaynes, S. Banerjee, T. S. Henderson and J. S. Tolson (1992). Methicillin-resistant *Staphylococcus aureus* in US hospitals, 1975-1991. *Infection Control and Hospital Epidemiology.* 13:582-586.

Perveen, I., A. Majid, S. Knawal I. Naz, S. Sehar and S. Ahmed (2013). Prevalence and Antimicrobial Susceptibility Pattern of Methicillin-Resistant *Staphylococcus aureus* and Coagulase-Negative Staphylococci in Rawalpindi, Pakistan. *British Journal of Medicine & Medical Research.* 3(1):198-209.

Perwaiz, S., Q. Barakzi, B.J. Farooqi, N. Khursheed and N. Sabir (2007). Antimicrobial susceptibility pattern of clinical isolates of Methicillin Resistant *Staphylococcus aureus*. *Journal of Pakistan Medical Association.* 57: 2.

Sabat, A., J. Krzyszton-Russjan, W. Strzalka, R. Filipek, K. Kosowska and W. Hryniewicz (2003). New method for typing *Staphylococcus aureus* strains: multiple-locus variable-number tandem repeat analysis of polymorphism and genetic relationships of clinical isolates. *Journal of Clinical Microbiology.* 41:1801–1804.

Siddique, G. M., K.A. Karamat and A. Hannan (1999). Prevalence of Methicillin Resistant *Staphylococcus aureus*: A study at PAF Hospital Sargodha. *Pakistan Journal of Pathology*, 10:26-28.

Sorge, N. M., C.B. Federico, G. Ivan, J. G. David, V. K. B. Maren and Sabina (2013). Methicillin-resistant *Staphylococcus aureus* bacterial nitric oxide synthase affects antibiotic sensitivity and skin abscess development. *Journal of Biological Chemistry*. 288(9): 6417-6426.

Wilke, M. S., A.L. Lovering and N.C. Strynadka (2005). Beta-lactam antibiotic resistance: a current structural perspective. *Current Opinion of Microbiology.* 8: 525-533.

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