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Typing of methicillin resistant and sensitive *Staphylococcus aureus* isolated from Tamilnadu, India using DNA fingerprints by pulsed-field gel electrophoresis

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ABSTRACT MRSA infection is alarming particularly in hospital set ups/community. We typed 43 isolates of *Staphylococcus aureus* (MRSA and MSSA) based on genomic DNA restriction fragment length polymorphisms (RFLPs). The genomic DNA of the test isolates was digested with *Sma*I enzyme, fractionated by PFGE and the patterns were assessed by dendrogram for percentage similarity. The *Sma*I restricted genomic DNA of 19 MRSA and 24 MSSA identified 27 different PFGE patterns, in which 11 and 16 were from MRSA and MSSA, respectively. Prevalence predominance was observed in few types/subtypes of MSSA (type B and subtype I-1) and MRSA (sub type A-2) and high percentage of similarity was noticed among the subtypes of PFGE types such as P and I of MSSA. During the epidemiological studies, to understand the dissemination of endemic/epidemic MRSA and MSSA, PFGE-based typing of pathogens may be used as a reliable and effective typing method.

KEY WORDS

Staphylococcus aureus
methicillin-resistant
MRSA
MSSA
PFGE
molecular epidemiology

Acta Biol Szeged 58(2):171-177 (2014)

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a global health concern and poses a continuous threat to medical care since the first strain was isolated in the United Kingdom in 1961 (Chambers 2001; Yamamoto et al. 2012; Nelson and Gallagher 2012; Uhlemann et al. 2013; Udo et al. 2014). MRSA is becoming majorly responsible for a wide spectrum of nosocomial and community associated infections worldwide (Mehendiratta and Bhalla 2012; Stryjewski and Corey 2014) and cause endemic and epidemic infections in many parts of the world including India (Pulimood et al. 1996; Stryjewski and Corey 2014).

The treatment and eradication of MRSA is becoming difficult and they may further increase the number of infections, the costs and the length of hospital stays (Vidhani et al. 2001; Patel 2007; Elixhauser and Steiner 2007). Also, MRSA strains are commonly resistant to multiple antimicrobials, in particular to all beta-lactams (except for the newer anti-MRSA compounds), which narrows the treatment possibilities and changes the spectrum of antibiotics used for therapy (Qureshi et al. 2004; Rajadurai et al. 2006; Bartoloni et al. 2014; Ben Nejma et al. 2014). Now, health care facilities of all

types experience such MRSA with significant and consistent increase in prevalence and with very obvious clinical impact. This increased prevalence of MRSA could be due to increased transmission of various strains among people or emergence of new strains (Uemura et al. 2004). Therefore, the knowledge of endemic MRSA strains in each place will be valuable in the surveillance and epidemiological investigation of MRSA clones/outbreaks for which epidemiological typing would be indispensable (Fujino et al. 2004; Bartoloni et al. 2014).

Based on PFGE typing, which is one of the most widely used molecular approaches of epidemiological analyses, the clonal nature of MRSA can be identified in order to understand the sources and routes of MRSA transmission (Hosoda et al. 2002; Goering et al. 2008). This will help the implementation of appropriate infection control measures and the effective empiric treatment protocols during the clinical management of MRSA infections, besides assisting our understanding of the diverse evolutionary trajectories of MRSA lineages globally (Bartoloni et al. 2014). As epidemiologically related isolates share the same genetic features, DNA-based typing techniques can be applied for an accurate epidemiological evaluation of MRSA. In this study we describe the application of DNA fingerprints generated by PFGE

Accepted Dec 21, 2014

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Table. Test MRSA and MSSA isolates subjected to PFGE.

S. No.	Collection centers	Type of clinical specimen	Total No. of MRSA (n=19)	Isolate number for PFGE	MRSA PFGE pattern	Type of clinical specimen	Total No. of MSSA (n=24)	Isolate number for PFGE	MSSA PFGE pattern
1	Coimbatore	Blood	1	13	F2	Pus	4	23,26,33,35	B
		Pus	1	15	S	Pus	4	1,31,41,42	I-1
		Pus	1	16	T	Pus	1	8	Q
2	Palayam	Pus	1	20	U	Throat	2	24,30	C
						Pus	1	4	P-1
						Pus	1	5	P-2
3	Salem	Pus	5	9,11,19,21,32	A-2	Urine	1	25	D
						Syn. fluid	1	28	G
4	Trichy	Urine	2	7,22	A-1	Pus	1	3	I-2
							1	6	I-3
							1	18	I-4
							1	14	I-5
5	Coonoor	Syn. fluid	2	10,29	H	Pus	1	12	R
6	Erode	Sputum	2	17,43	O	Sputum	2	37	M-1
		Blood	2	2,27	F-1	Pus	1	40	N
7	Karur	Pus	1	34	K	Sputum	1	38,39	M-2
		Pus	1	36	L				

Syn. fluid = Synovial fluid

to determine and compare the PFGE patterns of endemic MRSA and MSSA strains isolated from different centers of Tamilnadu, India.

Materials and Methods

Identification of *S. aureus*

The clinical samples collected from Microbiological Laboratory at Coimbatore, and from six of its sub-centers in Tamilnadu for a period of 24 months were aseptically handled and processed (Table 1). The morphotypes (cellular types) were done for all the samples based on the Gram staining method. The samples were also inoculated into respective preliminary screening media according to the standard laboratory procedures (Bannerman 1999) and incubated at 37 °C for 24 h. The microscopically identified isolates were further confirmed based on biochemical test results. All isolates of *S. aureus* were tested for the production of free coagulase enzyme using tube coagulase test. The results were confirmed using the standard coagulase-producing *S. aureus* strain ATCC-25923.

Screening for MRSA

All the confirmed *S. aureus* isolates were tested for methicillin resistance using 1 µg oxacillin disc on Mueller Hinton agar incubated at 35 °C and their methicillin resistance/sensitivity was documented. Furthermore, the antibiotic susceptibility pattern was studied by disc diffusion test following the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2010). The antibiotics used were Penicillin-G (10 unit); Ampicillin (10 µg); Cephalexin (30 µg); Cephotaxime (30

µg); Erythromycin (15 µg); Gentamycin (10 µg); Amikacin (30 µg); Netillin (30 µg); Ciprofloxacin (5 µg); Ofloxacin (5 µg); Norfloxacin (10 µg); Co-trimoxazole (25 µg); Vancomycin (30 µg) and Linezolid (30 µg).

Pulsed-Field Gel Electrophoresis (PFGE)

A total of forty-three isolates of *S. aureus* (19 MRSA and 24 MSSA) were selected based on antimicrobial susceptibility patterns and subjected to PFGE. Three well characterized MRSA clones, EMRSA-15 (Clonal Complex [CC22]), EMRSA-16 [CC30] and SMRSA-105 [CC5] were also included in the study for comparative evaluation. NCTC 8325 was used as a gel reference standard. PFGE typing of *Sma*I (Gibco, BRL, UK) digested DNA was performed by a modification of a previously described method (Bannerman *et al.* 1995). A colony was inoculated into brain heart infusion broth and incubated overnight at 37 °C without agitation. The pellet from 0.4 ml of this culture was washed in 0.8 ml NET buffer (10 mM Tris, 1 mM EDTA, 10 mM NaCl), resuspended in 0.25 ml NET buffer and mixed with 200 units of achromopeptidase (Sigma) (Leonard *et al.* 1995) and an equal volume of 2% SeaPlaque agarose (Flowgen) at 50 °C. The cell/agarose suspension was loaded into block molds (BioRad) and allowed to solidify at 4 °C. Cells were lysed by incubation at 50 °C for 60 min in lysis buffer (6 mM Trizma base, 100 mM EDTA, 1 M NaCl, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% lauroyl sarcosine). The blocks were washed three times for 10 min each at room temperature in TE buffer (10 mM Trizma base, 1 mM EDTA). One quarter of each agarose block was digested with 30 units of *Sma*I for 3 h according to the manufacturer's instructions and loaded into the wells of a 1% PFGE certified agarose gel

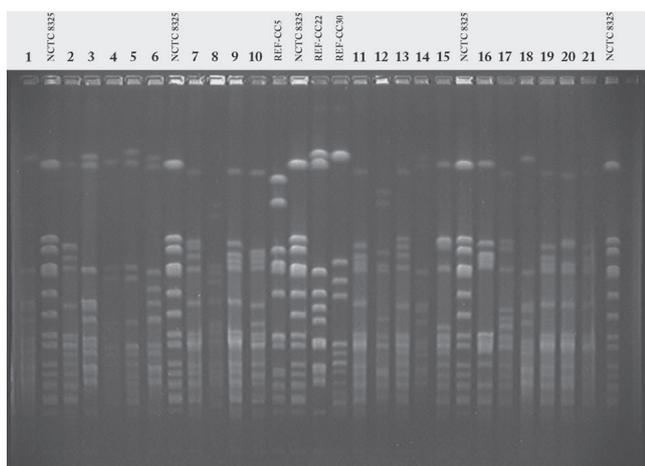


Figure 1. *Sma*I digested genomic DNA fingerprints of MRSA and MSSA isolates by pulsed field gel electrophoresis (PFGE).

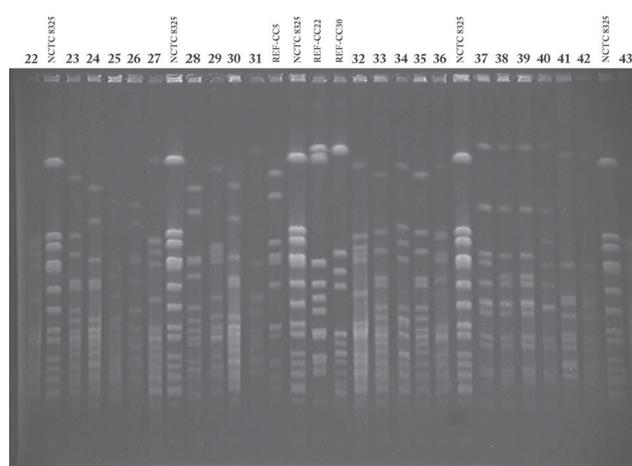


Figure 2. *Sma*I digested genomic DNA fingerprints of MRSA and MSSA isolates by pulsed field gel electrophoresis (PFGE).

(BioRad). Electrophoresis was performed in 0.5×TBE buffer (44.5 mM Trizma base, 44.5 mM boric acid, 1 mM EDTA) (BioWhittaker) by the contour-clamped homogenous electric field method with a CHEF Mapper system (BioRad). The fragments were separated with a linear ramped pulse time of 6.8 to 63.8 seconds over a period of 23 h at 14 °C and the gels were stained with 1 µg/mL ethidium bromide (Sigma) solution for 30 min, visualized under UV and photographed. Differences between isolates were recorded by visual comparison of DNA fingerprints and their PFGE patterns were determined. First, the patterns were examined to identify the common pattern (usually recognized as type A). Subsequently, the size and number of the fragments in the common pattern were compared with the fragments that made up the patterns of the other isolates. On the basis of fragment-for-fragment comparisons, each isolate's pattern was then classified. Patterns that were different from common patterns by six or more band differences were considered unrelated types (as type B, type C etc.). Patterns that differ from the common pattern (i.e type A) by two or three fragment differences were considered to be subtypes (such as A1, A2 etc.) of the common pattern (Tenover et al. 1995).

Statistical Analysis

Data are represented in figures, descriptive as mean ± SD, 95% Confidence Interval with normal approximation has been calculated around estimates of prevalence. Chi Square test in case of proportions and Student's *t*-test in case of means were used for analyses.

Results

The PFGE restriction patterns of the 43 isolates as well as known reference and control strains were visually examined.

Presence of 12-15 well resolved bands of approx. 670 to <80 kb were confirmed. The reproducibility of banding patterns was confirmed using reference strains and control strains on different gels. Upon PFGE typing of 19 MRSA (47.5% of 250), a total of 11 different PFGE patterns were identified and were further grouped under 9 types (Table 1).

Upon PFGE typing among 19 of 250 MRSA, a total number of 11 different PFGE patterns were identified in which 9 types were confirmed (Table 1). It was found that each type was differing from others by 6 to 7 bands. For example, PFGE types O and H; A and F differed from each other by 6 bands with a similarity between 90% and 95%. Types such as T and O, K and L were found to be 95% related. Further, these MRSA strains were observed to represent different geographical areas of this region. PFGE type O was isolated in sputum specimen from Erode (western part of Tamil Nadu state) and type H in synovial fluid collected from a hilly area called Coonoor (a town in the Nilgiris district, Tamil Nadu state, located at an altitude of 1,850 m above sea level). The two PFGE types A and F carried sub-types/variants such as A1, A2 and F1, F2 respectively and among subtypes A and F only one band difference was noticed. The most frequent pattern A2 represented 26% of the 19 MRSA isolates and all of these were isolated from a single (Salem; a western district of Tamil Nadu state) center. Other subtypes were found to represent varying geographical areas. Subtypes A1, F1 and F2 were isolated from Trichy (central part of Tamilnadu state), Erode and Coimbatore (western part of Tamilnadu state) and the dendrogram-based analysis estimated 95% similarity among the subtypes of each type (Fig. 3).

Similarly, of 24 MSSA, 16 PFGE patterns were identified and further categorized in to 10 PFGE types (Table 1). Types D and Q, C and B were observed to be 95% related and all were geographically found to be from different places

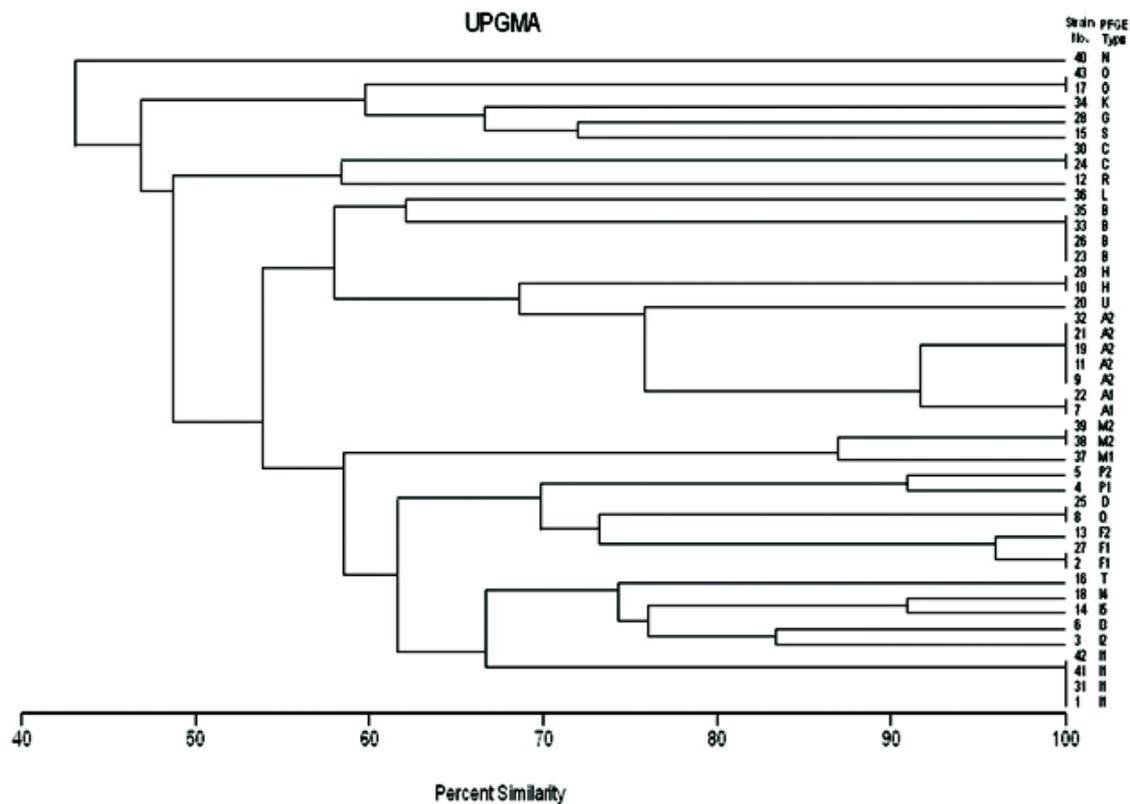


Figure 3. Dendrogram showing the percentage similarity among PFGE patterns (*SmaI*) of MRSA and MSSA genomic DNA.

(type Q and B from Coimbatore; type C from Palayam, type D from Salem). Furthermore, types I, M and P in MSSA carried varying number of sub types and the most frequent PFGE subtype I-1 made up 16% of the 24 MSSA isolates. All the strains of I-1 subtypes were isolated from pus specimens of a single hospital in Coimbatore. However, subtypes I-2 through I-5 were from another geographical area (Trichy). Subtypes I-2 and I-3, and sub types I-4 and I-5 were 95% related, whereas, subtype I-1 was only 90% related to other types. Similarly, subtypes P-1 and P-2, and M1 and M2 shared 95% similarity.

The PFGE types of all the *S. aureus* strains tested were compared with three recognized major international types/clones. Three of the major international clonal complexes of *S. aureus* were found among 24 MSSA isolates analyzed using PFGE. PFGE type I is a variant of EMRSA – 15, which belongs to the MLST clonal complex - 22. PFGE type P is a MSSA variant of EMRSA-16, which belongs to the MSST clonal complex - 30 and, PFGE type R belongs to the MLST clonal complex 5.

Discussion

Bacterial pathogens with antimicrobial resistance are more difficult and expensive to treat as they may increase other

complications and could lengthen hospital stay (Elixhauser and Steiner 2007). In this respect, emergence of hospital and community associated MRSA is causing worldwide concern (Mehta et al. 1998; Maree et al. 2007; Ray et al. 2011; Sujatha, 2014). Many tertiary care hospitals refer MRSA as an important nosocomial pathogen (Anupurba et al. 2003; Thati et al. 2011; Ahmed et al. 2012; Mir and Srikanth 2013; Kali et al. 2013) and therefore frequent surveillance and accurate epidemiological typing is indispensable to understand and identify the sources and routes of transmission so as to control its further spread (Fujino et al. 2004; Adesida et al. 2007).

Epidemiological studies involve different typing methods to explore the epidemiology of MRSA and pulsed field gel electrophoresis (PFGE) has been recommended as a 'gold standard' (Mehndiratta and Balla 2012) for typing MRSA isolates because it can distinguish among several concurrent epidemic strains. As a result of the rich diversity of the chromosomal digestion profiles and clarity, this typing method has been used in several epidemiological studies of MRSA infections (Erdenizmenli et al. 2004; Wolter et al. 2008; Tenover and Goering, 2009; Shabir et al. 2010; Pereira et al. 2014).

In this study, PFGE played a vital role in typing the MRSA and MSSA isolates obtained from various parts of Tamilnadu and also in understanding the most predominant

and potential strains of this region. This was a multicenter study, in which PFGE patterns of the test strains (both MRSA and MSSA) collected from different districts of Tamilnadu state were studied and their patterns were compared among themselves as well as with the PFGE patterns of major international reference clones. A total of 9 and 10 PFGE types were identified from 19 MRSA and 24 MSSA isolates, respectively. Ichijama et al. (1991) had proposed PFGE as a useful method for investigating the source, transmission and spread of nosocomial MRSA infections and reported 31 distinctive fragment patterns among 111 infecting and colonizing MRSA isolates from inpatients of six different hospitals under Nagoya University in Japan. Based on the PFGE restriction patterns, their study confirmed the involvement of 4 types of test strains causing epidemic infections among patients. In a similar type of study, Wei and Grubb (1992) classified 26 clinical isolates of MRSA collected from six Australian hospitals into 17 groups based on RFLPs of chromosomal DNA using PFGE and also highlighted the sensitivity of PFGE in typing MRSA both within and between hospitals.

A multicenter study assessed the optimal resolution and interlaboratory reproducibility of results from genotyping *S. aureus* by PFGE (van Belkum et al. 1997) and it was observed that several isolates did not give a 100% match at different centers, indicating that the standardization of PFGE depends on controlling a variety of experimental factors. However, in comparison with conventional typing methods such as phage typing and antibiotyping, PFGE's potential in discriminating MRSA during epidemiological studies is still significant (Loureiro et al. 2000). This has also been acknowledged by several workers (Shopsin and Kreiswirth 2001; Sasaki et al. 2002; Fujino et al. 2004). Struelens et al. (1992) also demonstrated the discriminatory ability of PFGE with typing ability index of 0.982 when compared to the indices of 0.959 and 0.947 for antibiotyping and phage typing, respectively. The first report on EMRSA - 15 variant from the United States employed PFGE as the only epidemiological tool (Wolter et al. 2008) and identified the pathogen. In this study, neither MRSA nor MSSA isolates can be more heterogeneous, because the difference in diversity among MRSA and MSSA isolates may not be significant and statistically valid, especially when we look at types rather than patterns, as 9 PFGE types were determined among MRSA and only 10 PFGE types in MSSA. However, Carles-Nurit et al. (1992) found that the *SmaI* restriction pattern in each of 20 MSSA strains collected from a single hospital over a period of 4 months was unique, whereas only seven RFLP patterns were seen among 27 test MRSA strains. The authors also confirmed these findings through nearly similar *ApaI* restriction patterns supporting low diversity among the MRSA isolates tested. This proved that MSSA strains were more diverse than MRSA.

Although MRSA PFGE subtype A2 was found to be the commonest pattern, it may not be widespread geographically

as all these isolates were obtained from a single center. Other MRSA subtypes were also confined to a particular center, and no isolates of a single subtype were found in varying centers of this region. The MRSA isolates of subtype A2 were isolated at different time intervals from the same center during the study, suggesting the continued presence of these strains in that area.

All the MSSA PFGE subtype I-1 strains were isolated from patients' pus specimens of a single orthopedic hospital in Coimbatore. Large interval was noticed between the isolation time of each of these isolates and the patients were found to form pus only after their admission, showing the dissemination of this strain locally. The identification of subtypes is becoming possible, as there are alterations in *SmaI* macro-restriction digestion patterns resulting from gain or loss of restriction sites due to point mutation, insertions or deletions. Generally, a number of subtypes are generated from a well-studied MRSA clone and the DNA profiles of the test strains generated by PFGE techniques appear stable and reproducible even after several times of subculturing. Among the sub-types of major MSSA clones such as P and M and MRSA PFGE subtypes of clones A and F of this study, more than 95% similarity was calculated and it was found that the strains were genetically related and had evolved and disseminated within this area. However, one of the MSSA subtypes I-1 of clone I shared only 90% similarity with the rest of the subtypes (I-2 through I-5) and this should be a distantly related strain of this group. Stewart et al. (1993) estimated the genetic distances between MRSA with unusual methicillin resistance by analyzing DNA polymorphisms. The authors observed that the majority of the isolates were belonging to one group with only minor genetic differences between the isolates that showed varying resistance to methicillin, and suggested that this was the evidence of the development of resistant variants from a particular clonal type during outbreaks.

More interestingly, in this study we compared the PFGE types of MRSA and MSSA with representatives of three major international clonal complexes of *S. aureus* and identified the presence of all three of them in South India.

No data were available about international travel or contact in the case of the patients involved. This fact suggests that these three clones may also be important causes of *S. aureus* infections in India. EMRSA-15 is the dominant MRSA in the UK, followed by EMRSA-16 (Ellington et al. 2010). In Scotland, EMRSA-16 and EMRSA-15, represented 80.0% and 15.4% of all MRSA, respectively (Monnet et al. 2004; Goering et al. 2008). EMRSA-16 and other clones within Clonal Complex 30 are found worldwide (Chen et al. 2013). EMRSA-16 is the second most common MRSA in the USA and is a common cause of community acquired MRSA in both the USA and Australia. USA100, also known as the New York/Tokyo clone was the most common pulse-field type (PFT) in the USA (44%) and Japan and is found in many

other countries worldwide. However, a first report appeared in the literature about the incidence of an EMRSA-15 variant in the United States in 2008 (McDougal et al. 2003; Wolter et al. 2008). MSSA variants of these three MRSA clones have been widely reported. Although none of the MRSA clones found in this study belonged to these three clones, it is clear that the mobile genetic element known as staphylococcal cassette chromosome mec (SCCmec) which confers methicillin resistance is able to transfer into these clones and given the right selective pressure it is possible that this may happen in this region.

As MRSA infection is alarming particularly in hospital set ups/community and in unique cases such as diabetic and immunocompromised patients, quick, efficient and reliable characterization of strains and identification of clonal spread within a region need to be done. The PFGE-based DNA fingerprinting is a useful method for investigating the transmission patterns of nosocomial MRSA infections. The understanding of MRSA epidemiology in the respective regions and identification of the DNA restriction patterns of the most infecting/prevalent strain(s) by PFGE could assist the monitoring of the spread of these strains. Also, infected patients can be isolated and provided with better treatment so as to bring out effective control and prevention strategies. We undertook this study to determine the type or clonality of MRSA and MSSA in a specific region and also to identify the level of diversity among the tested isolates.

MRSA subtype A2 alone represented 26% of the 19 MRSA, while MSSA type I represented 33% of the 24 MSSA examined. The predominance of certain types/subtypes in the studied area could be due to the effective spread of particular strains. Although the probable reason for the speedy transmission is ambiguous, it may be related with the virulence factors of the particular strain. A clear clinical understanding of predominant and widespread endemic MRSA/MSSA could be derived by analysing their virulence factors as well as by typing isolates representing other regions of state Tamilnadu. When epidemiological surveys of this kind are carried out continuously, effective control measures can be implemented by identifying specific MRSA clones that are responsible for frequent infections.

Acknowledgement

We would like to thank the Scottish MRSA reference Laboratory, Microbiology Department, Stobhill Hospital, Glasgow, UK G21 3UW for the part of PFGE.

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