

Typing of methicillin resistant *Staphylococcus aureus* using whole cell polypeptide and immunoblotting techniques

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Abstract

The methicillin resistant *Staphylococcus aureus* (MRSA) isolated from various clinical specimens were typed so as to identify the common clone of this region and the typing accuracy of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot techniques was assessed.

Fifty two isolates of MRSA isolated from Coimbatore, Tamilnadu were typed by analyses of whole cell and exported proteins. The banding patterns of whole cell protein profiles obtained by SDS-PAGE and the immunoblots of exported proteins were compared. The average percentage similarity (%S) among the whole cell protein profiles and immunoblots was calculated based on Dice co-efficient.

An average percentage similarity of 72.2% amongst the MRSA isolates was determined based on whole cell proteins. Where as the average percentage similarity based on excretory protein profiles was found to be 84.6%. SDS-PAGE of whole cell extracts was not much helpful in distinguishing different isolates of MRSA. But, patterns obtained by immunoblot could be divided into 3 types based on Dice coefficient. However, as the dissimilarity was very meager within each group, the precise typing of such isolates was found to be difficult using either one of these techniques.

Introduction

Staphylococcus aureus infections are important causes of morbidity and mortality in hospitals and the communities¹. Remarkably, methicillin resistant *S. aureus* strains having intrinsic resistance to methicillin and all beta-lactam antibiotics are significant pathogens and have emerged over the past 30 years². They are known to cause both nosocomial and community acquired infections³. The spectrum of the diseases produced by these organisms and the ranges of pathogenic processes are very wide and include toxin production, direct tissue

damage and secondary immune mechanisms⁴. Also, emergence of multidrug resistant MRSA is reported frequently and glycopeptide antibiotics seem to be the drugs of choice⁵. Treatment and eradication of diseases caused by MRSA and multidrug resistant MRSA require precise typing of various pathogenic strains so as to find out the common clones, their origins, sources and routes of transmission. The study of origins and spread of microorganisms needs appropriate typing methods. However, no single typing method considered as a best choice and the method of typing employed in each region varies depending upon the access

and availability of a particular technique and other facilities. It is now understood that only combined application of various typing schemes allows accurate analysis of clonal relatedness among MRSA isolates. The major objective of this study was to compare the whole cell as well as exported protein profiles of MRSA strains collected from Coimbatore, India based on SDS-PAGE and western blot analysis as to type these isolates and to evaluate the accuracy of these techniques in typing MRSA.

Materials and Methods

MRSA isolates

Various clinical specimens (n=7172) such as urine, pus, sputum, throat swab, blood, plural and synovial fluid, semen, catheter tip, bedsore and tissue exudates were obtained from microbiological laboratories (Micro labs), Coimbatore, Tamilnadu and were subjected to standard microbiological investigations to isolate *S. aureus*⁶. Initially the isolates were characterized as gram-positive, catalase-positive cocci. Further, they were tested for coagulase, DNase, thermonuclease production. Subsequently, antibiotic sensitivity pattern was determined based on Kirby-Bauer disc diffusion method in accordance with NCCLS (National committee for clinical laboratory standards) and the isolates were identified as methicillin resistant strains of *S. aureus* using 1µg oxacillin discs⁷. The other antibiotics used were Penicillin-G (10 unit); Ampicillin (10 µg); Cloxacillin (30µg); Cephalexin (30µg);

Cephalexin (30µg); Erythromycin (15µg); Gentamycin (10µg); Amikacin (30µg); Netillin (30µg); Ciprofloxacin (5µg); Ofloxacin (5µg); Norfloxacin (10µg); Co-trimoxazole (25µg); Vancomycin (30µg); Linezolid (30µg). *S. aureus* ATCC 25923 was used as control. Based on the common antibiotic susceptibility patterns, 52 isolates of MRSA were taken and subjected to typing using SDS-PAGE of whole cell protein (WCP) and western blot of exported proteins (EP) analyses.

Preparation of WCP and EPs

The test isolates were inoculated in to 3 ml Brain heart infusion broth and incubated for 48 hours at 35°C, centrifuged for 3 minutes at 12000 rpm. The supernatants were stored for studying exported proteins. The cell pellets were washed 3 times with sterile distilled water and washed cells were stirred after adding 25 µl SDS sample buffer (0.06 M Tris, 2.5% glycerol, 0.5% SDS, 1.25% β-mercaptoethanol and bromophenol blue 0.001% (w/v)) and the proteins were denatured in boiling water for 5 min. After centrifugation the protein samples were stored at -80°C until separation by SDS-PAGE. Similarly, the supernatant samples were concentrated based on ammonium sulphate precipitation (90%) and dialyzed. Subsequently the concentration of the desalted protein was estimated by standard method⁸ and used in SDS-PAGE separation for further analysis by western blot.

Protein separation by SDS-PAGE

The denatured whole cell and exported proteins were fractioned separately using 10% separating gels based on the recommendations of Laemmli⁹ and the gel used for immunoblot analysis was not stained.

Western blot analysis

Transfer of separated supernatant proteins from SDS-PAGE to nitrocellulose membrane was achieved by the modified technique¹⁰. The Bio-tech transblot cell was used for transfer and the sandwich was prepared. The nitrocellulose membrane was placed between the SDS-PAGE gel carrying the separated

proteins and filter papers. Transfer was effected overnight at 80 mA, with the buffer¹¹. The nitrocellulose paper was washed in blocking buffer (phosphate-buffered saline, pH 7.4, containing newborn calf serum (10%v/v) and Triton X-100 (0.2%) for 30 min, followed by incubation with pooled human plasma diluted 1 in 50 in blocking buffer for 1 hour. Several samples of human plasma known to contain antibodies against large number of staphylococcal polypeptides were pooled to provide test plasma¹². After washing three times for 15 min each in phosphate-buffered saline pH 7.4, Triton X 100 (0.2% v/v) the nitrocellulose membrane was incubated with horseradish peroxidase-linked sheep antihuman IgG serum (Bangalore Genei Pvt. Ltd), diluted 1 in 1000 in blocking buffer, for 45 min. After three further washes of 15 min each in phosphate-buffer saline, pH 7.4, Triton X 100 (0.2% v/v) the nitrocellulose membrane was rinsed in phosphate- buffered saline alone and stained with di amino-benzidine tetrahydrochloride (0.05 %w/v) in 0.1M Tris-Hcl, pH 7.6, containing hydrogen peroxide (0.01%v/v).

Finally the protein profiles of MRSA whole cell as well as exported proteins were recorded and compared. Further, average percentage similarity (%S) for these two profiles was calculated and analysed.

Results And Discussion

The whole cell and exported protein profiles of clinical MRSA (only 7 strains) are shown in



Figure 1. Whole cell protein profiles of clinical MRSA (only 7 strains)

figure 1 and 2 respectively. The visual inspection of these protein profiles was found to show 15 to 20 bands with an average of 17

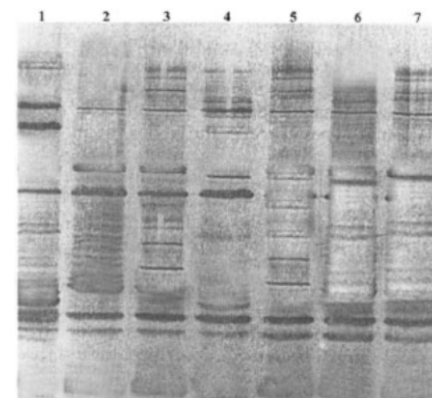


Figure 2. Exported protein profiles of clinical MRSA (only 7 strains)

bands. The average similarity percentage (%S) amongst the MRSA isolates was found to be 72.2%. The least degree of similarity occurred between MRSA strain nos.2 and 21 (72.2%). The average percentage similarity (%S) of whole-cell protein profiles (calculated based on Dice coefficients) is shown for only twenty five strains in table 1. Immunoblot analysis of exported proteins proved reproducible banding patterns and in each track, blot showed between 12 and 20 bands, with an average of 14. The least degree of similarity (69%) was observed between strain nos.13 and 20. An average similarity of 84.6% was calculated among MRSA isolates. The average percentage similarity (calculated based on Dice coefficients) among MRSA for twenty five isolates is shown in table 2.

Many different typing methods have been used for typing MRSA isolates. Antibiotyping, bacteriophage typing, analysis of different cellular protein profiles, genomic DNA restriction polymorphism and random amplification of polymorphic DNA (RAPD) are some of them. However, even with techniques such as pulsed field gel electrophoresis (PFGE) and polymerase chain reaction (PCR) problems of interpretation and inter laboratory reproducibility are evident. In this study the epidemiological typing tools such SDS-PAGE and immunoblot were used to study the diversity of MRSA isolates using their protein profiles. The visual inspection of whole-cell extract profiles by SDS-PAGE showed minor differences between isolates and was suspected that the isolates of MRSA could be closely related to each other. However, both similar and contrasting reports have been

Table 1. Percentage similarity (%S) among MRSA isolates based on whole cell proteins

Isolate no.	No. of bands	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
		15	18	15	17	16	17	17	16	17	16	17	16	16	18	18	16	17	18	17	16	16	17	17	18	14
1	15		85	93.3	81.3	96.8	87.5	93.8	90.3	87.5	96.8	87.5	90.3	96.8	84.8	84.8	83.9	87.5	78.8	87.5	83.9	90.3	81.3	87.5	78.8	85.7
2	18			84.8	85.7	88.2	85.7	85.7	88.2	80.0	88.2	85.7	82.4	82.4	77.8	77.8	76.5	85.7	72.2	85.7	76.5	82.4	74.3	85.7	72.2	82.8
3	15				87.5	90.3	81.3	87.5	90.3	87.5	83.9	81.3	90.3	83.9	78.8	84.8	90.3	87.5	78.8	87.5	83.9	90.3	81.3	87.5	78.8	92.9
4	17					90.9	94.1	88.2	90.9	94.1	90.9	94.1	78.8	90.9	80.0	91.4	84.8	94.1	80.0	94.1	90.9	90.9	82.4	82.4	80.0	78.6
5	16						97.0	90.9	87.5	90.9	93.8	90.9	87.5	93.8	82.4	88.2	87.5	90.9	94.1	90.9	100.0	93.8	97.0	90.9	94.1	89.7
6	17							94.1	90.9	100.0	90.9	88.2	100.0	97.0	85.7	97.1	90.9	100.0	91.4	100.0	100.0	90.9	94.1	88.2	91.4	96.6
7	17								90.9	94.1	90.9	82.4	84.8	90.9	91.4	85.7	84.8	88.2	80.0	88.2	78.8	90.9	82.4	76.5	91.4	76.9
8	16									90.9	93.8	97.0	93.8	93.8	82.4	82.4	87.5	90.9	94.1	90.9	100.0	87.5	84.8	90.9	94.1	100.0
9	17										90.9	94.1	90.9	97.0	80.0	85.7	97.0	82.4	85.7	94.1	90.9	97.0	88.2	94.1	85.7	73.3
10	16											90.9	100.0	93.8	94.1	88.2	93.8	84.8	94.1	97.0	93.8	93.8	97.0	90.9	82.4	74.1
11	17												90.9	97.0	85.7	91.4	84.8	88.2	85.7	82.4	84.8	90.9	94.1	88.2	80.0	96.3
12	16													93.8	94.1	88.2	87.5	84.8	94.1	84.8	100.0	87.5	90.9	97.0	94.1	92.9
13	16														76.5	88.2	81.3	90.9	76.5	90.9	81.3	93.8	78.8	90.9	76.5	85.7
14	18															94.4	100.0	100.0	88.9	97.1	100.0	100.0	91.4	97.1	100.0	80.0
15	18																100.0	91.4	100.0	97.1	94.1	100.0	100.0	97.1	100.0	75.9
16	16																	84.8	88.2	84.8	93.8	93.8	97.0	84.8	88.2	96.6
17	17																		97.1	94.1	97.0	100.0	94.1	100.0	91.4	78.6
18	18																			97.1	100.0	100.0	100.0	91.4	94.4	89.7
19	17																				97.0	90.9	82.4	88.2	91.4	78.6
20	16																					93.8	97.0	90.9	82.4	89.7
21	16																						97.0	90.9	94.1	81.5
22	17																							94.1	97.1	96.6
23	17																								97.1	85.7
24	18																									89.7
25	17																									0.0

made on the utility of SDS-PAGE for typing by other researchers. According to Krikler and Clink SDS-PAGE of polypeptides of whole cell extract could not readily provide data suitable for the establishment of typing schemes as only minor differences were noted between profiles 12 & 13. These findings were also supported and confirmed by Fiona 14 suggesting that SDS-PAGE analysis of whole-cell extract may not distinguish between MRSA isolates more precisely as more than 90% of the bands detected were present in similar amount in all strains of this species investigated.

In this study, although differences were noted between protein profiles, most of them should be said as virtually indistinguishable as the differences could also be due to variations in band intensity, rather than presence or absence of bands between isolates.

Therefore, it was determined that SDS-PAGE of whole-cell extracts will not readily provide the basis for typing different isolates of MRSA. Since, the results by SDS-PAGE are generally ambiguous and different workers have followed varying approaches for typing MRSA, we studied excretory proteins (culture supernatants) using immunoblotting. It can provide rapid, reproducible and comparatively sensitive results for characterization of MRSA isolates 15. It also permits detection of products present in amounts too small to be identified by conventional staining methods and because the detection and characterization of extra cellular products, which elicit antibodies in human subjects, may be of value in furthering an understanding on the typing of these isolates. Furthermore, all the antigenic polypeptides of each isolate can be identified and the immunoblot profiles could be grouped

consistently according to inter-group dissimilarity and intra-group similarity.

In our evaluation, the MRSA isolates using culture supernatants were divided into 3 immunoblot types (2 major and a minor) based on Dice coefficient. However, as the dissimilarity was very meager within each group, the precise typing of such isolates was found to be difficult. Additionally, as the immunoblotting results reproducibility is linked to a standardized antibody probe and it types phenotypic rather than genotypic characters, the more developed and sensitive tools need to be incorporated for typing clinical MRSA.

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Table 2. Percentage similarity (%S) among MRSA isolates based on exported proteins

Isolate No.	No. of bands	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25		
		14	15	14	14	15	15	12	15	16	13	13	14	14	16	15	15	14	15	14	15	13	15	14	15	14		
1	14		76	92.9	85.7	82.8	89.7	92.3	89.7	93.3	81.5	88.9	78.6	92.9	93.3	89.7	89.7	14.3	89.7	85.7	89.7	88.9	89.7	85.7	89.7	85.7		
2	15			82.8	75.9	80.0	80.0	81.5	80.0	83.9	78.6	78.6	75.9	82.8	83.9	80.0	80.0	82.8	80.0	75.9	86.7	92.9	73.3	82.8	80.0	82.8		
3	14				85.7	89.7	89.7	100.0	89.7	80.0	88.9	96.3	85.7	92.9	93.3	82.8	89.7	85.7	89.7	100.0	82.8	81.5	89.7	100.0	75.9	92.9		
4	14					89.7	82.8	84.6	82.8	86.7	96.3	88.9	78.6	78.6	80.0	75.9	82.8	85.7	75.9	92.9	75.9	88.9	82.8	85.7	89.7	78.6		
5	15						73.3	96.3	93.3	90.3	85.7	78.6	89.7	75.9	83.9	86.7	86.7	96.6	93.3	96.6	86.7	100.0	86.7	96.6	80.0	89.7		
6	15								81.5	93.3	77.4	92.9	92.9	96.6	100.0	83.9	93.3	86.7	96.6	100.0	89.7	93.3	78.6	73.3	82.8	86.7	96.6	
7	12									74.1	78.6	96.0	88.0	76.9	76.9	78.6	88.9	88.9	76.9	74.1	84.6	81.5	88.0	88.9	76.9	88.9	76.9	
8	15										90.3	78.6	85.7	96.6	82.8	83.9	73.3	86.7	82.8	86.7	100.0	80.0	100.0	86.7	96.6	73.3	100.0	
9	16											89.7	82.8	73.3	86.7	68.8	71.0	77.4	73.3	83.9	80.0	71.0	82.8	77.4	86.7	83.9	73.3	
10	13												76.9	81.5	74.1	69.0	85.7	85.7	88.9	92.9	74.1	71.4	92.3	78.6	74.1	92.9	74.1	
11	13														81.5	88.9	89.7	85.7	92.9	96.3	71.4	81.5	92.9	76.9	92.9	81.5	85.7	96.3
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