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TYPING OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* USING WHOLE CELL POLYPEPTIDE TECHNIQUE

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ABSTRACT

The methicillin resistant *Staphylococcus aureus* (MRSA) isolated from various clinicalspecimens was typed so as to identify the common clone of this region and the typing accuracy of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Twenty three isolates of MRSA isolated from Doon Valley hospitals were typed by analyses of whole cell proteins. The average percentage similarity (%S) among the whole cell protein profiles was calculated based on Dice co-efficient. An average percentage similarity of 83.3% amongst the MRSA isolates was determined based on whole cell proteins. SDS-PAGE of whole cell extracts was not much helpful in distinguishing different isolates of MRSA. However, as the dissimilarity was very meagre within each group, the precise typing of such isolates was found to be difficult using this technique.

KEYWORDS: MRSA, SDS PAGE, Doon Valley.

INTRODUCTION

MRSA is the terms used to refer to the strains of Staphylococcus aureus which possess intrinsic resistance to methicillin and all beta lactam antibiotics. These strains are also resistant to macrolides, lincosamides, aminoglycosides, quinolones and rifamycins. The glycopeptide antibiotics seem to be the only agents active against MRSA. Besides being multiple resistant to common anti-staphylococcal agents, some MRSA strains spread more readily than others once introduced into hospitals and are often difficult to eradicate once established (Udo *et al*., 1996). They are known to cause both nosocomial and community acquired infections (Shanson, 1986). 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 mechanisms (Barber, 1961). Also, emergence of multidrug resistant MRSA is reported frequently and glycopeptide antibiotics seem to be the drugs of choice (Lacy and Kruczenyk, 1986). 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 study the whole cell protein profiles of MRSA strains collected from Doon Valley hospitals based on SDS-PAGE analysis as to type these isolates.

MATRIALS AND METHODS

(A) Collection and Enrichment of Samples

Patients admitted in the hospitals were examined for MRSA infection. Nasal samples were taken from each patient. A total of 200 patients were monitored for the proposed study of nasal carrier state of Methicillin Resistant *Staphylococcus aureus.*Autoclaved cotton swabs (dipped in normal saline 0.9%) were used for nasal swabbing of the anterior nares of the patients. The nasal swabs collected were cultured on Mannitol Salt agar (selective medium for *Staphylococcus aureus*) within one hour after collection by spreading as per the conventional technique. The culture plates were incubated at 37^{0}C for 24-48 hours in the incubator.

(B) Identification of *Staphylococcus aureus*:

The suspected *Staphylococcus aureus* yellow colour colonies showing Mannitol fermentation were selected and subjected to **Gram staining** and sub-cultured into nutrient agar slopes.

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The isolates showing gram-positive cocci in clusters were subjected to Catalase test, DNase, Coagulase test by slide and test tube technique using undiluted and 1:5 diluted human plasma respectively.

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(D) Identification of MRSA by "Crome agar" plate method

For the identification of the MRSA among the isolates of *Staphylococcus aureus*, the Hi-Media (India) made HIMEDIA Hi Crome MeReSa Agar Base (M1674) was used. The medium is cooled to around 50-55^{0}C and MeReSa Selective Supplement (FD229) was added and mixed very thoroughly.

Soon after that the medium was poured into Petri plates and cooled. The MRSA only grew on this Hi Crome Me Re Sa agar, while the MSSA was inhibited on the same agar plate. All cultures showing bright blue colored growth were taken as MRSA positive strains, while all others are recorded as MSSA strains.

(E) Antibiotic susceptibility testing

All nasal isolates of *Staphylococcus aureus* were subjected to in vitro anti-microbial testing method on Muller-Hinton agar containing 2-3% NaCl, using 2-hour-old nutrient broth culture and HIMEDIA make antibiotic discs as per the method described by Kirby and Bauer (1966). The zone of inhibition around the discs were measured and interpreted as sensitive, moderately sensitive and resistant using the interpretation chart supplied by the antibiotic disc manufacturers (HIMEDIA, Mumbai).

(F) Protein profiling

- **Extraction of Whole Cell Proteins**

Clinical specimens were inoculated to brain heart infusion (BHI) agar (supplemented with 5% sheep blood). After overnight incubation at 35°C, a single colony was taken and transferred to 3 ml BHI broth and after incubation for 48 hrs at 35°C, centrifuged for 3 minutes at 12100 rpm.

Collected cells were washed 3 times with sterile distilled water. Washed cells were stirred after adding 25 µl SDS sample buffer (0.06 M Tris, 2.5 % glycerol, 0.5 % SDS, 1.25 % ß-mercaptoethanol and bromophenol blue 0.001% (w/v)) and the proteins were denatured in boiling water for 5 min. They were centrifuged again for 3 min at 12100 rpm, collected in a microfuge tube and stored at -50°C until the electrophoresis process was carried out.

- **SDS-PAGE**

Following overnight incubation at 35°C in BHI agar, bacterial cells were washed 3 times with distilled water. After suspending the bacterial cells in sample buffer (pH 6.8) (0.06 M Tris, 2.5 % glycerol, bromophenol blue 0.001 % (w/v)), proteins were extracted by multiple freezing and thawing (37-70°C) and centrifuged again for 3 min at 12100 rpm. Whole cell proteins were analysed by Native-PAGE according to Laemmle. Proteins were loaded to wells in a 4 % stacking gel over a 7.5 % acrylamide separating gel. The gel was run at a constant current of 30 mA until the Comassive Brilliant blue marker reached the bottom. Whole cell protein gels were stained with Comassive Brilliant Blue.

OBSERVATIONS AND RESULTS

Out of 200 nasal samples, 80*Staphylococcus aureus* were recovered and were further subjected to biochemical testing. The detection of MRSA among the isolates of the *Staphylococcus aureus* was carried out using the Hi-Crome MeReSa agar medium.

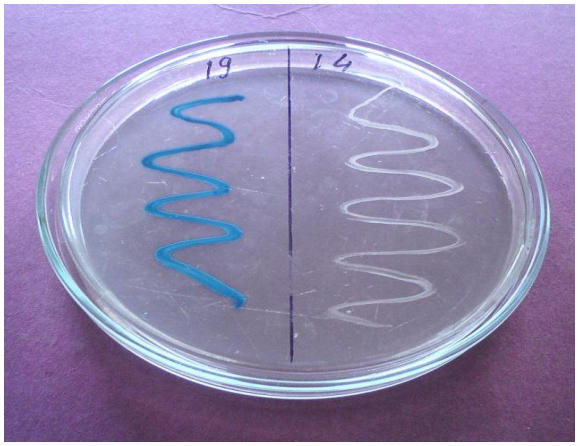

Figure 1: MRSA positive (on left) and negative (on right) on Hi-Crome MeReSa agar medium

A total of 23 *Staphylococcus aureus* were found to be MRSA. The prevalence of MRSA from nasal samples of Doon Valley hospitals was 28.75%. The details of the results are given in Table 1.

Table 1. The prevalence of MRSA from nasal samples of Doon Valley hospitals

No. of samples	*Staphylococcus aureus*	MRSA
200	80	23

Maximum MRSA positive strains were found among the females than the males (60.86% and 39.13 %, respectively).The details of the results are given in Table- 2.

Table 2. Prevalence of the MRSA in different sexes

Gender	MRSA	Percentage (%)
Male	9	39.13
Female	14	60.86

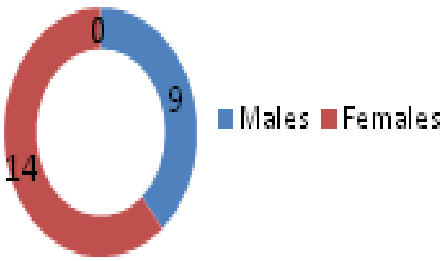

Figure 2. Prevalence of the MRSA in different sexes

Antibiogram

We have tested 14 different types of antibiotics for the susceptibility pattern of MRSA isolates on Mueller-Hinton agar (MHA) plates(Bauer, Kirby, Sherris and Turck, 1966).The drug resistance patterns of MRSA isolated from clinical specimens and carrier screening samples were found to be highly variable. Almost all the MRSA strains (91.3%) screened from nasal samples were resistant to Amikacin, 86.95% to kanamycin and Cloxacillin, 78.26% to ciprofloxacin, 56.52% to erythromycin, 52.17% to chloramphenicol, and 34.78% to both tetracycline and gentamycin. In, general all MRSA provided were multidrug resistant (as shown in Table 3).

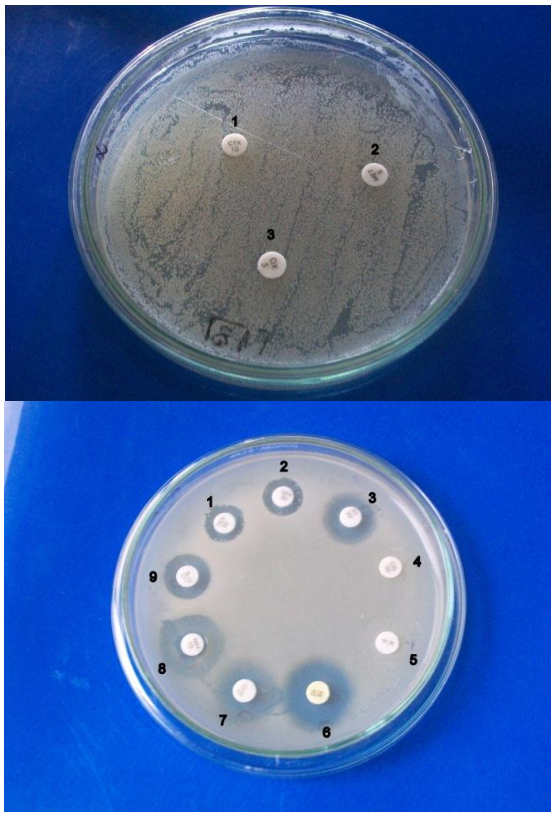

Figure 3. Antibiogram of MRSA

Table 3. Antibiogram of MRSA

Antimicrobial Agent	Disc Potency (µg)	Zone Diameter (mm)		
		Resistant	Intermediate	Susceptible
Ciprofloxacin	10	78.26%	21.73%	0.01%
Amoxicillin	10	34.78%	4.34%	60.86%
Cephalexin	30	60.86%	4.34%	34.78%
Cloxacillin	5	73.91%	17.39%	8.69%
Methicillin	5	100%	-	-
Cefotaxime	10	100%	-	-
Oxacillin	5	86.95%	-	13.05
Gentamicin	50	34.78%	39.13%	26.09%
Kanamycin	5	86.95%	13.04%	0.01%
Tetracycline	10	34.78%	52.17%	13.04%
Chloramphenicol	10	52.17%	43.47%	4.36%
Amikacin	10	91.30%	4.34%	4.36%
Vancomycin	30	-	4.34%	95.65%
Erythromycin	15	56.52%	43.47%	-

Phenotypic study

The whole cell protein profiles of clinical MRSA (only 6 strains) are shown in slide 5.12. A represents the protein marker whereas B to G are MRSA strains. The visual inspection of these protein profiles showed 15 to 20 bands with an average of 16 bands. Strain C and D

have maximum number of bands which shows that these strains are highly virulent. The average similarity percentage (%S) amongst the MRSA isolates was found to be 83.3%. The least degree of similarity occurred between MRSA strain E and G(74.1%). The average percentage similarity (%S) of whole-cell protein profiles (calculated based on Dice coefficients) is shown for only six strains in Table 5.

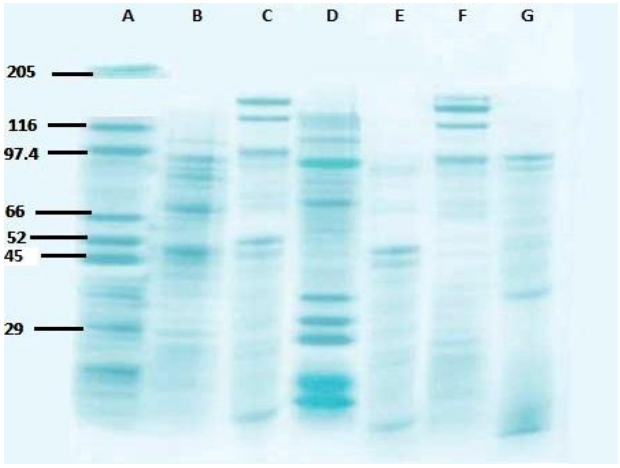

Figure 4. Whole cell protein profile of MRSA strains

Table 4. Percentage similarity between MRSA isolates based on whole cell proteins

Isolate name	No. of bands	B	C	D	E	F	G
		15	18	18	16	17	14
B	15		77.8%	77.8%	90.3%	87.5%	85.7%
C	18			77.8%	88.2%	85.7%	82.8%
D	18				88.2%	85.7%	82.8%
E	16					90.9%	74.1%
F	17						78.6%
G	14						0.0%

DISCUSSION

Methicillin resistant *Staphylococcus aureus* has become an enormous problem for health care providers because it is hard to treat and is sometimes called super bug. Multiple studies have been carried out on growing concern over multidrug resistance including India. MRSA is becoming a problem in paediatric population including hospital setting. The previous inclination of MRSA is in high intensity in the surgical and intensive care services, where antibiotic usage is the greatest. According to our study, there is high occurrence of MRSA in surgical wound infection, due to overcrowding, workload, and understaffing of wards.

The MRSA could be prevented by identifying and screening MRSA carriers inside high-risk wards.A study from Eritrea revealed low MRSA (9%) prevalence(Teclu and Nazik, 2009) which is less than the prevalence observed in our study. The rate of prevalence of MRSA isolates have increased over the years as reported by a study where they found 85.9% MRSA in 2003, decreasing to 57.8% in 2005 and again increasing to 90.8% in 2006(CLSI). In our study the frequency of MRSA is 23% which is less than that reported from Karnataka (77.9%), Delhi (44%) and Uttar Pradesh (38.44%) (Naseer*et al.,* 2010, Tyagi*et al.,* 2008 and Tiwari*et al.,* 2008).Frequency of MRSA in our study is comparable with another study done in Kashmir, where MRSA prevalence was 23.9% (Ahmed, 2009).

Regarding the carriage rate in relation to the age group, the prevalence of the MRSA carriage in our study was different than reported by other workers (Chaudhary*et al.,* 2007). A much higher prevalence rate was seen in (52%) old persons, i.e. more than 55 years than (30%) those aged 35-55 years.

Table 5. MRSA prevalence in different age groups

Age group	MRSA	Percentage
0-35	4	18%
35-55	7	30%
55 and above	12	52%

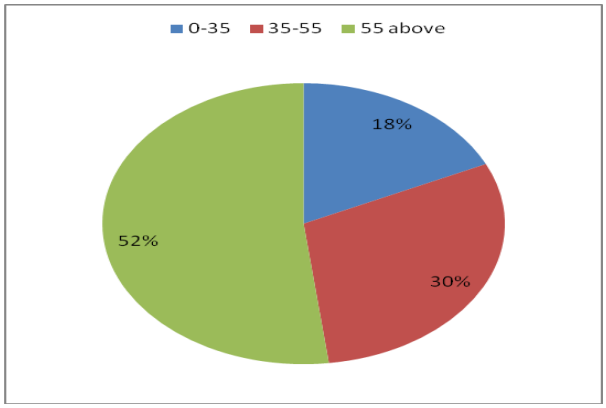

Figure 5. Prevalence of the MRSA in different Age groups

A total of 23 patients who developed MRSA infection/colonization were evaluated in our study. Out of which 9 were males (39.13%) and 14 were females (68.9%). A higher prevalence rate was seen in females than in the males which are similar to studies conducted by other workers (Chaudhary*et al.,* 2007).

A majority of the MRSA isolates showed multiple drug resistance and were fully sensitive to vancomycin only. A majority of the strains were resistant to Oxacillin, Ciprofloxacin, Kanamycin and Amikacin.

Similarly, the β-lactamase production rate observed was 83% in MRSA which have been shown in other studies (Paradisi , Corti and Messeri, 2001).

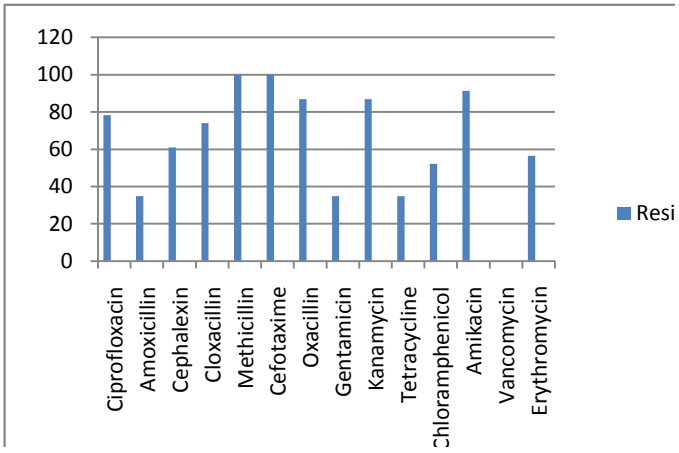

Figure 6. Bar graph showing different Antibiotic resistant pattern of MRSA

In this study the epidemiological typing tools such SDS PAGE was 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 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 some profiles. These findings were also supported and confirmed by Fiona 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.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.

CONCLUSION

MRSA is a persistent and ever growing problem for healthcare institutions, and HA-MRSA adds another degree of complexity. Minimizing the emergence of this organism and its spread remain to be the challenges that need to be addressed. In conclusion, a high rate of the carriage of *Staphylococcus aureus*in this hospital, with a large proportion of strains being resistant to penicillin and the isolation of MRSA strains from these carriers calls for the periodic surveillance of nosocomial infections due to *Staphylococcus aureus* and other important bacterial pathogens. The usual hygienic methods such as hand disinfection after each contact with patients, and the use of masks when is appropriate, must be performed by all workers in hospitals to protect the patients from nosocomial infections. Alcohol hand rub must be placed at every bedside in hospitals and promotional materials must be used to remind health workers and visitors to use the hand rub.In this study, 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. Additionally, as the SDS PAGE results show phenotypic rather than genotypic characters, the more developed and sensitive tools need to be incorporated for typing clinical MRSA.

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