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Phenotypic characterization of nasal Methicllin resistant staphylococcus aureus isolates from HIV infected and HIV non infected individuals

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Abstract
Introduction: The incidence of MRSA continues to rise and it has become a worldwide phenomenon now. This makes it imperative to control the spread of MRSA keeping in view the implications on morbidity and cost.
Material and methods: Fifty HIV infected and 50 HIV non infected patients were screened for nasal carriage of methicillin resistant Staphylococcus aureus in Delhi, North India. The isolates were characterized phenotypically by phage typing by the conventional set of phages, MRSA typing by supplementary Phages, Biotyping and antibiogram. Strains were devided into two groups typable or non typable by conventional phage typing.
Result: Three isolates of Methicllin resistant Staphylococcus aureus (MRSA) were from HIV infected individuals while, a single isolate was from HIV non infected individuals. While using the MRSA supplementary phages, all were typable. Using the biotyping scheme, strains belonged to two groups A and D. Using the antibiotic sensitivity pattern, all the 4 strains were typed by the scheme called antibiogram. By this method the 4 different isolates showed 4 distinct mnemonic codes known as Resistant Phenotypes.

Keywords: MRSA, phage typing, biotyping, Antibiogram, mnemonic code

Introduction
Methicillin resistant strains of *Staphylococcus aureus* (MRSA) appeared as early as in 1961, just two years after the introduction of Methicillin into clinical practice [1]. Since then MRSA continues to be a global problem and the last two decades in particular have seen a great increase in the infections caused by MRSA [2-5]. Although MRSA is a well known nosocomial pathogen, community acquired infection by this organism have also been well documented [6, 7] Although their pathogenic potential is similar to that of their methicillin sensitive counterparts, their tendency to multiple antibiotic resistance often complicates treatment [8].
Colonization of the skin and mucous membrane with MRSA is a known predisposing factor for MRSA infection [9]. This tenet had been well illustrated among the population of individuals infected with human immunodeficiency virus (HIV), who have an increased rate of both MRSA carriage and infection [10, 11]. Because of high morbidity and mortality associated with staphylococcus infections in HIV infected patients [13], it needs to track down these strains to their phenotypic levels to get better picture of their origin.
The incidence of MRSA continues to rise and it has become a worldwide phenomenon now. This makes it imperative to control the spread of MRSA keeping in view the implications on morbidity and cost. For this purpose, it is important to fully understand the epidemiology of the organism. A reliable indicator of the relationship between isolated organisms is a prerequisite for successful epidemiological investigation and is called as a typing scheme [14].
The study of origin and spread of micro-organisms requires appropriate typing methods. Phage typing have long been the standard typing method for S. aureus, but many current MRSA strains are not typable by the current International Set of phages. The use of supplementary phages has sometimes been successful [15]. Newer approaches include the use of various biochemical and morphological characteristics [16].

In this study we have done phenotypic characterization of the four nasal MRSA isolates obtained. Also, comparison of the four phenotypic methods namely phage typing by conventional set of phages, typing of MRSA by supplementary set of phages, biotyping [14] and antibiogram [17] was done using Numerical Index of discriminatory ability.

Materials and Methods

Bacterial strains

A total of four isolates of MRSA were isolated from the nasal swabs of 50 HIV infected and 50 HIV non infected individuals. Methicillin resistance was determined using the cefoxitin disc diffusion method using Muller Hinton agar plates as recommended by CLSI[18] as well as VITEK 2 system (Biomeuriex,).

Phage typing

All the strains of MRSA were phage typed employing the conventional set of phages as described by Blair and Williams [20]. Phage typing was done at the National Phage Typing Centre, Maulana Azad Medical College, New Delhi, India. The propagating strain was first sub-cultured onto a blood agar plate. A single colony was picked up and the phage pattern was checked using the 23 phages of the basic set at 1 and 100 RTD (routine test dilution). Strains that were non-typable at 1 RTD were typed at 100 RTD. Non-typability was recorded when the strains were non-typable at 100 RTD and were classified accordingly – Group I (29, 52, 52A, 79, 80), Group II (3A, 3C, 55, 71), Group III (6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85) and Non allocated group (81, 94,95,96)

Phage typing using supplementary MRSA phages

All MRSA strains were additionally phage typed using 9 supplementary phages for MRSA. The 9 phages used were M3, M5, M12, M8, MR25, 622, C30, C33, C38. Biotyping
All MRSA were biotyped as described by Coia *et al.* [14] into 4 groups in the following way (Table 1). Tween 80 1% v/v was incorporated in Nutrient agar and the test organism was spread over an area of 1cm in diameter. Plates were incubated at 37 ºC for 2-3 days. A positive test was denoted by a halo of fatty acids around the inoculum. This medium enhanced pigmentation of S. aureus and were described as gold, buff or cream. Urease production was tested in Brain heart infusion broth with 2% of urea and 0.0012% of phenol red. A heavy inoculum was used. After 18-48 hours incubation at 37 ºC, a positive test was indicated by red colour of the medium [14].

Table 1: The MRSA isolates were classified into 4 biotypes in the following manner [14]:

Test	Biotype			
	A	B	C	D
Tween 80 hydrolysis	---	---	+	+
Urease production	---	+	---	+
Pigment production	Cream	Buff	Variable	Gold
Gentamicin sensitivity/resistance	S	R	S	R

Typing by Antibiogram

The antimicrobial susceptibility was performed by the disc diffusion method using the Stokes technique[19] against Tetracycline (30µg), Chloramphenicol (5µg), Erythromycin (15µg), Amikacin (30µg), Ciprofloxacin (5µg) and Rifampicin (5µg), Readings were taken as per standard recommendations.
The typing by antibiogram of the MRSA isolates was done by the scheme described by Krishna Prakash S *et al.* [17]. This typing scheme was designed using two panels of antimicrobials each containing three agents. The "left" panel contained three conventional agents: Tetracycline (TET), Chloramphenicol (CM), and Erythromycin (EM). The "right panel" contained three higher agents *viz*. Amikacin (AK), Ciprofloxacin (CIP) and Rifampicin (RF). [Table 2].
Scoring was done by the binary system. Resistance was given a score of '1' and sensitive a score of '0' for each agent included. A mnemonic code was thus developed wherein the triplet code of the binary system was converted into an alphabet. By a process of permutation and combination eight alphabets were arrived at. On the "left" panel these were designated by capital letters and an identical pattern on the "right" panel by small letters [Figure 1]. Table 2:

Table 2: Typing Scheme Employing Antibiogram [17]

Left Panel				Right Panel			
Antimicrobial agent			Mnemonic coding	Antimicrobial agent			Mnemonic coding
TET	CM	EM		AK	CIP	RF	
0	0	0	A	0	0	0	a
1	0	0	B	1	0	0	b
0	1	0	C	0	1	0	c
0	0	1	D	0	0	1	d
1	1	0	E	1	1	0	e
1	0	1	F	1	0	1	f
0	1	1	G	0	1	1	g
1	1	1	H	1	1	1	h

Statistical analysis was performed using chi square test. A p value <0,05 was taken as statistically significant. Numerical Index of discriminatory ability was calculated using Simpsons index of diversity.

Results

A total of 100 nasal swabs (50 from HIV infected and 50 from HIV non infected) who attended the ICTC centre were taken. Amongst these 4 (8%) were MRSA. Three (6%) were from HIV infected and 1 (2%) from HIV non infected cases.
Phage typing When all the four strains were phage typed by the conventional set of phages it was found that two of the MRSA isolates were typable (50% typability) while the other two were non typable. The two strains that were typable were from HIV infected group and belonged to group III (47/54, 75). The sole MRSA from HIV non infected group was non typable.
All 04 MRSA when additionally typed using 9 supplementary phages for MRSA, were found to be typable (100% typability). The four strains that were typable showed two different typing patterns, either 622 or 622/C22 phage type. (Table 3) (Figure 1)Biotyping
The strains when subjected to biotyping were divided into two groups (A and D). 75% of the strains belonged to biotype A while one strain (25%) belonged to group D. One of the HIV infected strain was resistant to gentamicin, produced golden coloured pigment, produce a halo on Tween 80 hydrolysis hence grouped in group D. Rest of the three strains were resistant to gentamicin and produced no hydrolysis on Tween 80 agar. (Table 3)

Table 3: Distribution of strains in the various groups of conventional groups, by supplementary MRSA phages and biotyping,

S. No.	Strain identity	Biotype	Phage pattern by Conventional phages	Phage pattern by supplementary MRSA phages
1.	N12[a]	A	III: 47/54	622/C33
2.	N14[a]	A	NT	622
3.	N43[a]	D	III 75	622/C33
4.	NN14[b]	A	NT	622

A= HIV infected cases b = HIV non infected cases

Antibiogram

Using the antibiotic sensitivity pattern, all the 04 strains of MRSA were typed by the scheme described by Krishna Prakash S *et al.* [17]. By this method the 04 different isolates showed 4 distinct mnemonic codes (Resistant Phenotypes). This is shown in Table 4.

Table 4: Resistant phenotype pattern of the four strains

S. No	Left Panel				Right Panel				
	Antimicrobial agent			Mnemonic coding	Antimicrobial agent			Mnemonic coding	Resistant Phenotype
	TET	CM	EM		AK	CIP	RF		
N12	0	0	1	D	0	0	0	A	Da
N14	1	0	1	F	0	1	1	G	Fg
N43	1	0	0	B	0	1	0	C	Bc
NN14	0	0	1	D	0	1	0	C	Dc

Numerical Indices of Discriminatory Ability

Numerical Indices of Discriminatory Ability of the 04 phenotypic methods employed applying the Simpson's index of diversity[22] are shown in Table 5.

Table 5: Comparison of the discrimination Indices of Different Phenotypic Typing Methods used

S. No	Typing method	No. of Types seen	Numerical Index of Discrimination	Size of the largest group
1.	Biotyping	02	0.50	03
2.	Resistant Phenotype typing	04	1.00	01
3.	Phage typing by the Conventional set of phages	03	0.833	02
4.	Typing by supplementary MRSA phages	02	0.633	02

The Numerical Index of Discriminatory ability was least for the Biotyping techniques (0.50). Between the 02 phage typing techniques the use of conventional phages was shown to have a better Numerical Index of 0.833, albeit non typability being taken as one group. In case of Resistant phenotype typing, the Numerical Index of Discrimination was found to be 1.

Discussion

Although many studies have been carried out in relation to nasal carriage of MRSA but few studies have done phenotypic characterization of the isolates by phage typing, MRSA typing by supplementary phages, biotyping and Antibiogram. The purpose of this study was to characterize our MRSA nasal isolates by the four different phenotypic methods that can be used collectively for epidemiological purposes.

Our phage typing results revealed 50% typability as compared to 43.4% by Krishna Prakash *et al.* [21]. Only 2 of our 4 MRSA isolates could be typed by the basic set of phages. All the 4 strains could be typed by the supplementary MRSA phages. However, the discrimination appeared to be much less since the 02 strains showing different phage patterns by the conventional set of phages, displayed an identical pattern when typed by the MRSA phages, phage pattern 622 / C33. Again, both the strains un-typable by the conventional set of phages (NT) appeared to show an identical phage pattern with the supplementary phage pattern 622. Though phage typing is considered to be standardized, reproducible and easy to perform [14], but it could not clearly differentiate our four strains.

On the other hand, Biotyping results showed that **t**hree of our strains (75%), were sensitive to gentamicin and failed to hydrolyze Tween 80, thus placing them in biotype A and the single gentamicin resistant strain that hydrolysed Tween 80 was placed in biotype D. However, it was interesting to note that none of our isolates belonged to biotype C or B, in which gentamicin sensitive and gentamicin resistant strains are respectively and 03 of our MRSA were sensitive to gentamicin. By using biotyping our strain discrimination was limited only to two groups. Though simple, quick and reproducible and can be incorporated easily into daily bench routine, such a simple scheme may not be of value with broader based collection of S. aureus strains [14].

On the contrary, Antibiogram divided the four strains into four different mnemonic types known as resistant phenotypes. Antibiogram described by Krishna Prakash *et al.* [17] is a much more easier process which could further discriminate the strains into smaller types.

Numerical indices of discriminatory ability of the four phenotypic methods was also calculated to type MRSA applying the Simpson's index of diversity [22], and revealed that the discriminatory index for the biotyping to be 0.50. Phage typing by the conventional set of phages had a much higher discriminatory index at 0.833, as compared to the typing by the supplementary MRSA set of phages as 0.663. Typing by the antibiogram yielded a numerical index of 1.0, which seemed to have an excellent numerical index of discrimination. Though our strains were less in number but still we came to the conclusion that Antibiogram was the best amongst the four methods in discriminating the strains. It was followed by the phage typing by basic set of phages, typing by the MRSA supplementary phages, and the method which had the least discriminatory ability was the biotyping. Subdividing the strains of MRSA into as many types as

possible, is more informative for epidemiological surveillance and infection control

The present study had few pitfalls, firstly that the number of nasal MRSA isolates in this study were very less. Secondly, that we used only phenotypic methods and no genotypic methods were used. But still we conclude that a combination of techniques yielded a small, if not large epidemiological information regarding the phenotypic characters of the nasal MRSA isolates.

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