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emm 81, The Predominant Group a Streptococcus from North India in Year 2003 in Context to Adhesion, Invasion and Antimicrobial Susceptibility Pattern

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Abstract

Heterogeneity exists among Group a Streptococcus *emm* types worldwide. In 2003, we observed 11 circulating *emm* types in northern region of India, of which *emm* 81 was found to be the predominant type (17.5%). As *emm* 81 has been reported to be associated with invasive diseases in western countries, hence, in the present study, attempts were made to study the virulence potential of these isolates from skin and throat samples. Isolates were screened for nine fibronectin binding protein (FBP) genes, evaluated for adherence & invasion potential along with drug resistance to various commonly prescribed antibiotics for treatment. Throat isolates showed higher distribution of FBP genes as compared to skin isolates. All the isolates were found to be positive for *sciB* and *prtF15;* 92.9% for *sfb* and *fba*, 78.6% for *sciA*; 35.2% for *prtF1*; 7.1% for *prtF2* but none for *sfb2* and *pfbp*. Isolates showed low (8.5%) to moderate (27.7%) adherence and negligible invasion potential in the experimental A549 cell line which was confirmed by immunofluorescent confocal microscopy. Drug resistance profiling showed isolates to be highly resistant to macrolides, tetracycline, co-trimaxazole but all susceptible to penicillin. The study shows *emm* 81 strains from northern India to be of less virulent nature with respect to adherence/invasion potential, highlighting that same *emm* type in different geographical regions may have a different clinical outcome, the latter being dependent on number of factors like ethnicity, geographical, socioeconomic factors besides its molecular type and source.

Keywords: Gas; *emm* 81; Invasion; Adherence; Fibronectin binding protein; Antibiotic susceptibility

Introduction

Streptococcus pyogenes, (GAS) is an etiological agent causing wide range of human disorders including pharyngitis, pyoderma and post infection sequela like rheumatic fever/ rheumatic heart disease (RF/RHD). The incidence of such disorders is high in India [1]. M protein, the major virulence antigen of GAS has been found to be highly heterogeneous in India as reported earlier by our laboratory [2]. Beside M proteins, there are other virulence factors like Streptolysin O & S, C5a peptidase, streptococcal protective antigen (spa), streptococcal pyrogenic exotoxin (spe) etc. which have been known to play a key role in pathogenesis of this bacterium. The earlier studies carried out in our laboratory have demonstrated low frequency of spe A gene within Indian isolates which is indicative of less virulent nature of the isolates [3]. However, recently, the presence of virulent streptococcal inhibitor of complement (sic) protein and its encoding genes (closely related *sic*: crs and distantly related *sic*: drs) have been documented in Indian isolates [4].

Like other bacteria, the initial step for establishment of streptococcal infection is bacterial adherence and colonization to host tissue for which *Streptococcus pyogenes* genome encodes multiple adhesin genes for various adherence determinants, out of which fibronectin binding proteins (FBP) are the most important contributors [5]. FBPs like *sciA, sciB, sfb,sfb2, prt*F1, *prt*F2,*prt*F15, *fbaA, fbaB , pfbp, sfb*II, *fbp-54* and *sof* not only enhance the adherence of GAS to epithelial cells but also facilitate the bacteria for invasion and persistence within the host cells, reflecting its virulence [6]. The drug of choice for prevention of GAS infection remains penicillin till date as no penicillin resistant isolate has been reported [7]. Moreover, other drugs like macrolides are being more commonly used for the treatment of GAS infection in penicillin allergic patients. However, there have been worldwide reports of resistance of GAS to commonly used antibiotics with persistent increase in drug resistance [8,9,10].

In the present scenario of changing epidemiology and emergence of new *emm* types during different seasons and years, it becomes necessary to look into regional prevalence of *emm* types in a community along with their virulence credentials. However, such data is lacking in Indian context. Apart from this, another challenge being encountered with GAS isolates is increasing resistance towards commonly prescribed antibiotics. Therefore, in the present prospective study the most predominant GAS type obtained from the community during the year 2003 was evaluated for virulence traits in terms of presence of FBP genes, their adherence and invasion potential along with susceptibility to commonly used antibiotics used for their treatment.

Materials and Methods

Isolation of GAS and emm typing

Approximately 400 children in the age group of seven to eleven

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years from different clinics and hospitals in and around urban slum area near Chandigarh, North India with defined GAS disorders like pharyngitis and pyoderma were registered for community survey in the year 2003. Swabs were taken from symptomatic patients by rubbing over both the tonsils and the posterior pharyngeal walls (pharyngitis cases) and skin lesions (pyoderma cases) by a physician after consent from their parents. Throat and skin swabs were streaked on sheep blood agar plates. The plates were screened for beta haemolysis after 24 hours of incubation at 37 °C in presence of 5% CO_2. The grouping of pure cultures was then done using streptex kit (Murex Biotech, UK). The *emm* typing of GAS isolates was done using PCR and sequencing [11,12]. The *emm* gene sequence of GAS isolates was subjected to homology search against CDC reference strains (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm) as well as by BLAST search analysis (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi).

PCR amplification of fibronectin binding protein genes

Genomic DNA of *emm* 81 isolates was extracted and amplification of nine sequence specific FBP genes: *sciA, sciB, sfb, sfb2, prt*F1*, prt*F2*, prt*F15*, fba* and *pfbp* were carried out with published primers at standardized conditions [13]. The experiments were repeated twice to confirm the results.

Adherence and invasion assay

A549 cells (ATCC CCL 185), a human alveolar carcinoma cell line was used to study cell adherence and internalization of group A streptococci, which is a prerequisite for establishment of infection in the host and reflects on its virulence potential. Streptococcal adherence was done by Giemsa staining and further checked by fluorescence activated cell sorter (FACS). Briefly, 100 µl aliquots of fluorescein isothiocyanate (FITC, Sigma chemicals Co, USA) labeled streptococcal suspension (10^8 bacteria) was added in each well of multi-well plate containing confluent monolayers of cell line (1:100). After 30 minutes at 37°C under 5% CO_2 atmosphere, the wells were washed thrice with 0.15M NaCl to remove non-adherent bacteria. The fluorescence associated with adherent streptococci was measured in a flowcytometer (Becton Dickinson, FACS Calibur and USA). Percent adherence was calculated as the number of adherent streptococci, which was calculated from the absolute fluorescence value of the total number of streptococci in each assay. Streptococcal internalization was evaluated by antibiotic protection assay [14]. Immunofluroscent confocal microscopy was also performed [15] for further confirmation. Standard reference strain M1 obtained from ATCC, USA having high adherence and invasion efficiency was run in parallel as control.

Antibiotic sensitivity pattern

The sensitivity of *emm* 81 isolates towards the generally prescribed drugs for the GAS treatment was evaluated. Apart from penicillin, the obvious drug of choice for treatment of GAS infection, macrolides including erythromycin and azithromycin as well as other drugs like tetracycline and co-trimaxazole were included in the study due to their wide usage in developing countries. Kirby Bauer disk diffusion method on Muller Hinton agar medium according to National Committee for Clinical Laboratory Standards guidelines for penicillin G (10 µg), co-trimoxazole (25 µg), tetracycline (30 µg), erythromycin (15 µg) and azithromycin (15 µg) procured from BD Biosystem was used [16].

Results

After confirmation of eighty GAS isolates from throat and skin of children screened in this study, *emm* type analysis was performed. GAS isolates revealed *emm* 81 (17.5%, 14/80) to be the most predominant type from our region in the year 2003, while frequency of other *emm* types 11 (11.2%, 9/80), 15 (8.7%, 7/80), 42 (7.5%, 6/80) , 49 (10%, 8/80), 55 (6.2%, 5/80), 57 (7.5%, 6/80), 66 (6.2%, 5/80), 68 (7.5%, 6/80), 103 (7.5%, 6/80) and 112 (10%, 8/80) (unpublished data) were found to be low. Out of total 14 isolates of *emm* 81 type, there were seven isolates from patients with pharyngitis & seven from impetigo. Thirteen isolates were typed as *emm* 81.1 while a single isolate was typed as *emm* 81.2. *emm* 81 being the most predominant type and as reported to be invasive in western countries [16] prompted us to further characterize this type. The PCR analysis of nine FBP genes showed all isolates (100%; n=14) positive for *sciB* and *prtF*15 gene, 92.9% (n=13) for *sfb* & *fba;* 78.6% (n=11) for *sciA* genes; 35.2% (n=5) for *prtF1;* 7.1% (n=1) for *prtF2* and none positive for *sfb2* and *pfbp* gene. All throat isolates were positive for *sciA, sciB, sfb, prtF15 & fba* genes while *sciB, sfb* and *prtF*15 were present in most of the skin isolates (Table 1).

The adherence pattern of *emm* 81 isolates to A549 cells was evaluated and the results showed varying degree of binding. Giemsa staining showed low (1+, n=9) and moderate adherence (2+, n=5), as compared to standard M1 isolate, a positive control, which showed sufficient adherence to A549 cell (Figure 1 A-C, Table 1). The qualitative % adherence calculated by FACS analysis indicated adherence range from 8.5% to 27.7%. The invasive capacity of *emm* 81 GAS isolates was evaluated by infecting A549 cells and monitoring the number of viable intracellular bacteria at two, four and twenty hours post infection. To our knowledge, out of the nine genes considered in present study, *prtF* is known to have well established correlation with invasive potential. No viable count was observed for *prtF1*$^-$ isolates, however low counts were obtained for *prtF1*$^+$strains indicating very low cell invasion efficiency which was statistically insignificant. Further immunoflorescent confocal microscopy confirmed moderate adherence with internalization of few GAS isolates to A549 cells.

Further the isolates showed resistance towards all the drugs including tetracycline (92.9% {R+I}: 78.6%R, 14.28%I), co-trimoxazole (78.6%R), erythromycin (50% {R+I}: 14.3%R, 35.7%I) and azithromycin (64.3% {R+I}: 7.1%R, 57.1%I). However, no resistance was found for penicillin G. Interestingly, the *emm* 81.2 isolate from skin was sensitive to both tetracycline and co-trimoxazole whereas most of emm 81.1 strains were resistant.

Discussion

The heterogeneous *emm* type prevalence at different time intervals has been reported from India [2,10]. However, there is a lack of information regarding invasive/non-invasive nature of these isolates from the region. Our earlier study has shown a low frequency

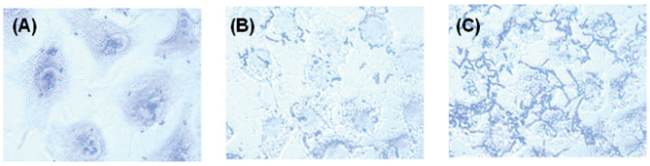

Figure 1: Adherence of GAS *emm* 81 isolates to A549 cell line by Gimsa staining, **(A)** *prt* F1 negative isolates (+1); **(B)** *prt* F1 positive isolates (+2); **(C)** Control (M1, reference strain).

Sr. No.	Source	*Emm* type	Fibronectin binding protein genes						Adherence				
			sci A	*sci B*	*sfb*	*sfb2*	*prtF1*	*prtF2*	*prtF15*	*fba*	*pfbp*	*Giemsa*	*FACS*
1	Throat	81.1	+	+	+	-	+	-	+	+	-	2+	26.70%
2	Throat	81.1	+	+	+	-	-	-	+	+	-	1+	13.80%
3	Throat	81.1	+	+	+	-	-	-	+	+	-	1+	13.60%
4	Throat	81.1	+	+	+	-	+	-	+	+	-	2+	27.73%
5	Throat	81.1	+	+	+	-	+	-	+	+	-	2+	23.40%
6	Throat	81.1	+	+	+	-	-	-	+	+	-	1+	12.60%
7	Throat	81.1	+	+	+	-	-	-	+	+	-	1+	13.40%
8	Skin	81.1	-	+	+	-	-	-	+	+	-	1+	17.75%
9	Skin	81.1	+	+	+	-	-	+	+	-	-	1+	9.40%
10	Skin	81.1	+	+	+	-	+	-	+	+	-	2+	24.40%
11	Skin	81.1	+	+	+	-	+	-	+	+	-	2+	26.70%
12	Skin	81.1	+	+	+	-	-	-	+	+	-	1+	17.70%
13	Skin	81.1	-	+	-	-	-	-	+	+	-	1+	8.50%
14	Skin	81.2	-	+	+	-	-	-	+	+	-	1+	22.68%
15	Control	1	+	+	-	-	-	-	-	+	-		
	%	81	**78.6**	**100**	**92.9**	**0**	**35.2**	**7.1**	**100**	**92.9**	**0**		

Note: Fbp genes +: presence; - : absence 2+: moderate adherence; 1+: low adherence when compared to standard reference M1 GAS strain

Table 1: Distribution of fibronectin binding protein genes and adherence potential of isolated *emm* 81 GAS isolates.

of the speA gene, a gene usually associated with scarlet fever or toxic shock–like syndrome, within Indian isolates [3] suggestive of less virulent nature of isolates in Indian community, though the presence of sic genes, a streptococcal virulence factor, has been documented by us in Indian isolates [4].

In the year 2003, the study conducted at our laboratory showed predominance of *emm* 81 type in North Indian community, which has also been reported from other countries [8,16,18,19,20]. Report of association of *emm* 81 with invasive disease in Sweden and Romania [17,20] prompted us to look for a virulent potential of the circulating predominant *emm* 81 isolates from our community. We focused on adherence and invasion potential of these isolates towards host cell, the process resulting in infection or disease outcome. FBP genes, the major contributors of adherence/invasion were screened in isolated *emm* 81 strains, which showed these strains to possess nine known FBP's to a variable extent, indicating their highly heterogeneous nature. The study conducted by Luca-Harari et al [20] explored the presence of only one FBP gene i.e. *prt*F1 gene in its isolated *emm* 81 strains and found all the isolates to possess the same. To best of our knowledge, none of the reports in literature has investigated such elaborate distribution of FBP genes among *emm* 81 GAS isolates, making our study as first of its kind.

As regards adherence-invasion assays, these isolates showed low to moderate adherence. Maximum adherence (22.7– 27.7%) was observed in *sfb*$^+$ and *prtF1*$^+$ isolates, which decreased to 13.8% - 17.75% for *sfb*$^+$ *and prtF1* $^-$ isolates, as compared to lowest value of 8.45% for ones lacking both genes. On the other hand a single strain with *sfb*$^+$ and *prtF2*$^+$ character showed adherence to the value of 9.40%, suggesting the possible role of all these adhesins in adherence potential. The other studies have also suggested that strains possessing two or more of the genes for these FBPs have more adhering capacity as compared to strains possessing none or one of the genes. Further, the negligible/lower internalization efficiency of *emm* 81, especially *prtF1*$^+$ isolates may be attributed to lower adherence recorded (8.5-27.7%) in spite of presence of s*fb1, prt*F1 genes in isolates.

We also looked in for drug susceptibility of these isolates to commonly prescribed drugs in country. No penicillin resistant isolate have ever been reported from any region of world in spite of its extensive and indiscriminate usage [7] and our results also showed susceptibility of all *emm*81 strains to penicillin. As regards macrolides, insight into Indian literature till 2002 indicated less than 1% erythromycin resistance rates among GAS which increased to 9.04% in 2006 [9], while the present study indicated 50% resistance among *emm* 81 isolates, comparable to the reports of increasing resistance from other countries of the world [8,19]. A new macrolide azithromycin being used extensively in the country for past few years alongwith co-trimaxazole also exhibited high resistance among isolates. Similarly to reports of tetracycline resistance in GAS [9], we found all thirteen *emm* 81.1 isolates showed resistance to tetracycline while a single isolate, *emm* 81.2, a skin isolate was susceptible to it. The interesting feature observed was that *prtF1*$^+$ isolates from throat showed resistance to tetracycline, erythromycin and azithromycin but were sensitive to co-trimaxazole, in spite of high resistance shown towards cotrimaxazole by *prtF1*$^-$ isolates. We understand that our study represents only limited number of *emm* 81 types from north India and the resistance may be only the result of spreading of one or few strains in this limited area. Further work is required with more number of strains to confirm these observations.

The present study reflected predominance of *emm* 81 during year 2003 in northern region of India with capability of superficial colonization and negligible invasion capacity, an indicative of its less virulent nature as compared to the prevalent GAS isolates reported from other countries. It seems that same *emm* types in different geographical regions may have a complete different clinical outcome and suggesting that besides the *emm* type, the disease outcome also depends on ethnicity, geographical and socioeconomic factors.

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