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**Clinical and microbiologic characteristics of invasive Streptococcus pyogenes infections in north and south India**

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# Clinical and Microbiologic Characteristics of invasive *Streptococcus pyogenes* Infections in North and South India

*Running title:* Invasive *S. pyogenes* infections in India

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**Abstract**

The lack of epidemiologic data of invasive *Streptococcus pyogen*es infections in many developing countries is concerning as *S. pyogenes* infections are commonly endemic in these areas. Here we present the results of the first prospective surveillance study of invasive *Streptococcus pyogenes* infections in India.

Fifty four patients with invasive *S. pyogenes* infections were prospectively enrolled at two study sites, one in the north and one in the south of India. Sterile site isolates were collected and clinical information was documented using a standardized questionnaire. Available acute phase sera were tested for ability to inhibit superantigens produced by their own isolate using a cell-based neutralizing assay.

The most common clinical presentations were bacteremia without focus (30%), pneumonia (28%) and cellulitis (17%). Only two cases of streptococcal toxic shock syndrome and no cases of necrotizing fasciitis were identified. Characterization of the isolates revealed great heterogeneity with 32 different *emm* subtypes and 29 different superantigen gene profiles being represented among the 49 sterile site isolates. Analyses of acute phase sera showed that only 20% of the cases in the north cohort had superantigen-neutralizing activity in their sera, whereas 50% of the cases from the south site had neutralizing activity.

The results demonstrate that there are important differences in invasive *S. pyogenes* infections in India, both in clinical presentation and strain characteristics, as compared to invasive *S. pyogenes* infections in Western countries. The findings underscore the importance of epidemiologic studies on streptococcal infections in India and have direct implications for current vaccine developments.

## Introduction

*Streptococcus pyogenes* is a significant human pathogen capable of causing a wide spectrum of diseases ranging from uncomplicated infections of the throat and skin to severe life-threatening diseases and post-streptococcal sequela. Streptococcal pharyngitis is one of the most common childhood diseases worldwide, accounting for several millions of cases each year. Inadequate treatment of *S. pyogenes* infections, predominantly throat infections, can result in the serious post-infectious sequela acute rheumatic fever that may lead to rheumatic heart disease. The burden of post-streptococcal sequela is great in developing countries but relatively rare in developed countries, although isolated outbreaks have been reported (5, 12). Furthermore, *S. pyogenes* cause invasive infections, including the two most severe invasive manifestations Streptococcal Toxic Shock Syndrome (STSS) and necrotizing fasciitis associated with high morbidity and mortality.

*S. pyogenes* invasive disease in North America and Europe has been an area of intense research since their reemergence in the late 1980s (1, 9). In contrast, these infections have not received much attention in developing countries. In an attempt to estimate the global burden of *S. pyogenes* infections, Carapetis et al. (5) reviewed available databases and estimated that more than 18 million people currently suffer from a serious *S. pyogenes* disease, about 2 million new cases occurring each year, with an annual mortality of more than hundred thousand. Added to this are 111 million cases of streptococcal pyoderma and 616 million new cases of *S. pyogenes* pharyngitis each year. It was noteworthy that the vast majority of cases were in resource-limited countries. This report especially highlighted the fact that epidemiologic data from less developed countries are scarce; thus, emphasizing the need for studies in these regions (5).

In India, the disease burden of streptococcal infections is considerable (26) and the incidence of acute rheumatic fever and rheumatic heart disease range from 0.3–5.4 per 1000 children (24). *Streptococcus pyogenes* pharyngitis has a high prevalence in North India (14), whereas pyoderma is more frequent in South India (4). In light of invasive infection, this is a completely neglected field in India and the only data available in the literature is one retrospective study of invasive β-hemolytic streptococcal infections (19). To advance our understanding of *S. pyogenes* infections in India and to obtain epidemiologic data that could direct the design of an effective vaccine, an European Commission funded project, ASSIST, was launched in 2007. The project included surveillance of acute rheumatic fever/rheumatic heart disease as well as invasive *S. pyogenes* infections at study sites in North and South India. Here we present the results of the first prospective surveillance study of invasive *S. pyogenes* infections conducted in India, which show important differences as related to surveillance data from Western countries.

## Material and Methods

*Setting and study population*

Surveillance of invasive *S. pyogenes* infections was established at the two ASSIST study sites, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, in the north of India and the Christian Medical College (CMC), Vellore in the south of India. All consenting patients with invasive *S. pyogenes* infection (n=56) identified at the two sites during the study period January 2007 and February 2010 were enrolled in the study. Invasive cases were defined as isolation of *S. pyogenes* from normally sterile sites, and cases of STSS was defined according to published definition criteria (29). Identification of cases was achieved by daily contact with the microbiology laboratory serving the hospital. Isolates were collected (n=49) and clinical information obtained from the patients' medical records was documented in a standardized questionnaire designed to harmonize with data collected in the recently published StrepEuro study of invasive *S. pyogenes* infections in Europe (15). Acute phase sera were obtained from 10 and 22 invasive cases from the north and south sites, respectively.

The study was ethically approved by Human Research Ethics committees at respective hospital in India, as well as in Stockholm, Sweden. Written informed consent was obtained from all enrolled patients.

*Strain characterization: emm sequencing and superantigen gene profile*

Clinical isolates were confirmed as *S. pyogenes* by use of conventional bacteriologic techniques. Genomic DNA was isolated from strains grown overnight in Todd Hewitt medium plus yeast extract (THY) using zirconia beads in combination with the DNeasy kit (Qiagen, Hilden, Germany). Direct sequencing of the 5'-end of the *emm* gene was done as detailed http://www.cdc.gov/ncidod/biotech/strep/M-ProteinGene_typing.htm, using the oligos *emm*_fwd

5'-TATTCGCTTAGAAAATTAA-3' and *emm*_rev 5'-GCAAGTTCTTCAGCTTGTTT-3'. The *emm* type was obtained as described at http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm by BLAST comparison to *emm* sequences in the database.

Superantigen genes were detected by PCR using the primers listed in supplementary Table 1. As positive controls for respective gene, the sequenced strains MGAS315, MGAS6708, and MGAS8232 were used.

*Proliferation and neutralization assays*

Supernatants were prepared from overnight bacterial cultures in Todd Hewitt Broth supplemented with yeast extract (THY) and tested for mitogenic activity in proliferation assays using peripheral blood mononuclear cells (PBMC) from healthy donor as detailed (22).

Patient sera were tested for ability to inhibit superantigens produced by the patients' own isolates by use of a cell-based neutralizing assay described in detail in (10, 22, 30). In brief, PBMC from healthy donors were stimulated with superantigen-containing bacterial culture supernatants (SUP)(1:50 dilution) in the presence of 2.5% heat-inactivated patient serum (PS) supplemented with 2.5% fetal calf serum (FCS), or 5% FCS. After 72 hours [$^3$H]thymidine uptake was determined. Phytohemagglutinin-L (1 μg/ml)(Sigma) was included as a control for non-specific toxicity and PBMC quality. All samples were assayed in triplicates, and the data presented as mean counts per minutes (CPM) of [$^3$H]thymidine uptake±SD. Serum-neutralizing activity was calculated by the equation: $[1-(cpm_{PS+SUP} - cpm_{PS+medium})/(cpm_{FCS+SUP}-cpm_{FCS+medium})]x100$. Significant neutralizing activity was defined as 50% inhibition of proliferative responses, as previously established (22). The analyses also included sera from 58 healthy controls (median age 18 years; provided by PGIMER), which were tested for neutralizing

activity against SUPs from two selected isolates (01-1400-01 and 01-1400-08), both from the north Indian study site as that was the region of the controls. Intravenous polyspecific immunoglobulin (IVIG; 2.5mg/ml))(Baxter) were tested for neutralizing activity against bacterial culture supernatants using medium supplemented with 5% FCS.

*Statistical analyses*

Data were analyzed using Prism software 5.01 (Graphpad). D'Agostino & Pearson omnibus normality test was used to assess Gaussian distribution. Student's *t* test or Mann-Whitney U test were used to compare the two patient cohorts, and the $\chi 2$ test or Fisher's exact test to compare the distribution of categorical data.

## Results

A total of 56 invasive cases were enrolled at the two Indian study sites, including 26 and 30 patients from the north and south sites, respectively. Sterile sites isolates were obtained from blood (n=41,73%), pleural fluid (n=4), synovial fluid (n=3), tissue aspirates (n=3), peritoneal fluid (n=2), bile (n=1) and vitreous fluid (n=2). Upon further analyses, two isolates were reclassified as group G streptococcus and consequently, these two cases were excluded from the study. The median age of all invasive cases was 30 years old (range 1 day – 80 years), with 24% being ≤4 years old (Table 1 and Figure 1). The two cohorts differed significantly with respect to age as children and adolescents were more common among patients enrolled at the north site as compared to the south site (Table 1 and Figure 1).

*Clinical characteristics of invasive cases*

Forty six percent of the invasive cases reported underlying conditions, including cancer (13%), chronic liver disease (7.4%), malnourishment (7.4%), surgery (5.6%) and burns (5.6%). The most common clinical diagnoses were bacteremia with no identified focus (30%) and pneumonia (28%), followed by cellulitis (17%). Septic arthritis cases were only found in the south site patient cohort, whereas meningitis cases were represented exclusively in the north cohort (Table 1). No necrotizing fasciitis cases and only two cases of STSS were identified. The two STSS cases were both children who presented with cellulitis respectively meningitis and pneumonia. Nine patients were treated at the intensive care unit (16.7%). Fatal outcome was recorded in eight of the 54 invasive cases, including one of the STSS cases, accounting to a fatality rate of 14.8%. However, it should be noted that the study did not include a Day 28 follow-up and many patients declined hospitalization due to the associated cost; hence, the recorded fatalities are likely underestimated.

*Microbiologic findings*

From the 54 invasive *S. pyogenes* cases, 49 isolates were retrieved and further characterized with respect to *emm* type and toxin-gene profiling (Table 2). The *emm* sequencing revealed a great heterogeneity among the isolates with 32 different *emm* types being represented in the cohort. The most common subtypes were *emm*49.0 (n=4), *emm*74.0 (n=4), *emm*80.0 (n=4), *emm*12.0 (n=3), and *emm*28.5 (n=3). The fatal cases were caused by varying subtypes, including the two *emm*1-2.2 isolates and one each of *emm*28.5, *emm*74.0, and *emm*80.0. The type distribution differed between isolates from the north and south sites with all isolates of *emm*12.0 (n=3), *emm*28.5 (n=3), and *emm*49.0 (n=4) subtypes identified in the south cohort, whereas *emm*74.0 (n=3), *emm*80.0 (n=3) and *emm*1-2.2 (n=2) predominantly in the north isolate cohort.

Exotoxin-gene profiling of the 49 invasive isolates identified 29 distinct profiles with unique combinations of superantigen genes, evenly distributed amongst the 32 different *emm*-subtypes (Table 2). The 29 profiles contained between 1-9 superantigen genes per profile with the greatest variation in prophage-encoded genes. There were five profiles that harboured only one or two exotoxin genes. Profile No. 5 harboured only *sme*Z and was exclusively represented by *emm*28.0 strains. *spe*G and *sme*Z were the most common superantigen genes being present in 86% and 83% of the profiles, respectively.

Superantigen-containing culture supernatants from all isolates were potent triggers of proliferative responses and there was no statistical difference between the cohorts (Figure 2). There was no relation between degree of proliferative response and *emm* subtype, number of superantigen genes harboured or superantigen profile.

*Superantigen neutralizing activity in acute phase sera*

As lack of protective humoral immunity against superantigens has been shown to be a risk factor for invasive *S. pyogenes* infections in Europe and in Canada (2, 18, 22), it was of interest to assess protective antibody levels among Indian patients. Available acute phase sera were tested for ability to inhibit superantigens produced by the patient's own isolate using a cell-based neutralizing assay previously employed in epidemiologic studies (2, 18, 22, 30). Only 20% of the cases in the north cohort had superantigen-neutralizing activity in their sera (Figure 3A), whereas 50% of the cases from the south cohort showed neutralizing activity (Figure 3B). Equally low neutralizing activities were seen in the healthy controls with 26% and 40% showing neutralizing activity against the isolates 01-1400-01 and 01-1400-08, respectively (Figure 3C). IVIG was included as a source of neutralizing superantigen antibodies that completely inhibits superantigenic activity of invasive *S. pyogenes* isolates in Europe and in Canada (7, 13, 21). Similarly, superantigens produced by Indian invasive isolates were completely neutralized by IVIG (Figure 3).

## Discussion

Here we present the results of the first prospective study of invasive *S. pyogenes* infections in India including two study sites, one in the north and one in the south. At both sites, the most common clinical presentations were bacteremia without focus, pneumonia, and cellulitis. Three meningitis cases were reported, all from the north site, consistent with children being more prevalent in this patient cohort as compared to the south site. In contrast, four septic arthritis cases were found in the south patient cohort, whereas none in the north. It was noteworthy that only two STSS cases and no necrotizing fasciitis were identified, which is considerably less than what has been reported from Western countries (8, 15). Equally low numbers were reported in a retrospective study of β-haemolytic streptococcal infections in India, in which 3 cases of STSS were found among 225 invasive cases and there was no mention of necrotizing fasciitis cases (19). In contrast, results from the 2003-2004 European surveillance covering 11 countries reported 13% and 8% of cases with STSS and necrotizing fasciitis, respectively (15), and similar figures have been observed in Canada (8, 11) and the US (23). Two recent prospective studies from developing countries, including one from New Caledonia reported 43% of necrotizing fasciitis but only low frequencies of STSS (3%) (16), and one from Fiji where 5% and 7% of STSS and necrotizing fasciitis, respectively, was noted (27).

The lack of necrotizing fasciitis cases was particularly unexpected considering that the south of India, as many other tropical settings, is known to have a high prevalence of streptococcal skin infections. It is recognized that different *S. pyogenes emm* types are associated with specific tissue tropism (3), and particular disease manifestations, such as the association between *emm*1 and *emm*3 with STSS and necrotizing fasciitis cases (reviewed in (9). *emm*

subtyping of the Indian invasive isolates revealed a great heterogeneity with a total of 32 different subtypes being represented among the 49 isolates. Importantly, there were no *emm*1 or *emm*3 types among the invasive isolates, nor were there any *emm*15 that was predominant in the study from New Caledonia and responsible for 19% of the necrotizing fasciitis cases (16). Thus, the *emm* distribution indicates a lack of types previously found to be associated with STSS and necrotizing fasciitis, which might explain the low number of cases with these severe manifestations in this material. However the small size of the patient material does not allow for robust conclusions and future studies are warranted to confirm these data.

The heterogeneity in *S. pyogenes emm* types is in agreement with other studies from developing countries and indigenous populations, which all report highly diverse *emm* types among throat, skin and invasive isolates (16, 25, 27). Not only are the isolates more heterogenic, but the *emm* types are also strikingly different from the Western population (17, 28). This has implications for the coverage of the 26-valent vaccine that has completed clinical phase I and II trials (20), inasmuch as only 22% of the Indian isolates in this study are accounted for in the vaccine as compared to 69% and 76% for isolates in Europe and the US, respectively (17, 23). However, it should be noted that a recent report of a 30-valent M-protein based vaccine showed that vaccine elicited bactericidal antibodies cross-reacted to some extent with non-vaccine serotypes, indicating that the efficacy may extend beyond the included serotypes (6).

Similar heterogeneity was seen in the superantigen-gene profile with 29 distinct profiles identified. Despite different profiles all isolates were prominent triggers of T cell activation and the degree of response was not associated with either number of superantigen genes, presence of *sme*Z or with particular profiles or *emm* types. Lack of superantigen-neutralizing antibodies has

been identified as a significant risk factor for invasive *S. pyogenes* infections in Western countries (2, 18, 22, 30). We hypothesized that the antibody levels should be higher in India considering the endemic nature and thus likely greater exposure to streptococcal infections in this area. However, analyses of acute phase sera and sera from healthy controls revealed surprisingly low levels of superantigen-neutralizing activity in both patients and controls. Thus, despite an expected high exposure to streptococcal infections, the Indian cases were equally susceptible to streptococcal superantigens as the Western population. Here we only assessed neutralizing activity in a functional cell-based assay, but in future studies it will be of interest to investigate ELISA antibody-titers against superantigens as well as other streptococcal factors to address how neutralization relates to prior exposure. It has previously been shown that ELISA antibody titers to streptococcal superantigens frequently do not correlate with neutralizing activity (22), but the underlying rationale for this remains as of yet unknown.

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## Figure Legends

**Figure 1.** Age distribution among invasive *S. pyogenes* cases enrolled at the north and south study sites in India.

**Figure 2.** Mitogenic activity induced by overnight bacterial culture supernatants. Peripheral blood mononuclear cells from healthy donors were stimulated with bacterial supernatants prepared from invasive isolates collected at the north site (A) and south site (B), respectively. Proliferative responses were determined after 72 hours of culture by measurement of [$^3$H]-thymidine uptake. The data are shown as mean counts per minute (CPM) ± SD of triplicate for unstimulated cells (unst.), and cells stimulated with supernatants of all invasive isolates (indicated by patient ID) or PHA as positive control.

**Figure 3.** Superantigen-neutralizing activity in acute phase patient sera and in healthy controls. Peripheral blood mononuclear cells from healthy donors were stimulated with bacterial culture supernatants in the presence or absence of patient sera, and proliferative responses were assessed. The data are presented as percent inhibition of bacterial supernatant in the presence of patient's serum (2.5%) or IVIG (2.5 mg/ml) as related to the response obtained in the presence of fetal calf serum. Each patient was tested for neutralizing activity against its own isolate whereas healthy control sera were analyzed using two selected isolates, as indicated. The dashed lines indicate 50% inhibition of mitogenic activity, which is considered significant neutralizing activity. Samples from the north site are shown in (A) and from the south site in (B). (C) shows compiled data of controls and patients. The horizontal lines denote median values in each group. PHA was included as a control for non-specific toxicity, and induced equally high responses in the presence of patient sera as with FCS.

**Table 1.** Demographics and characteristics of invasive cases at the two Indian study sites

| Characteristic | Invasive cases from | | *P* value[a] |
|---|---|---|---|
| | North India (n=24) | South India (n=30) | |
| Age (years), median (range) | 8.5 (4 days – 60 yr) | 51 (1 day – 80 yr) | 0.0001 |
| Male sex, % | 54.0 | 66.7 | NS |
| Fatal outcome, n (%) | 4 (16) | 2 (6.7) | NS |
| Underlying condition, % | 37.5 | 53.3 | NS |
| *Clinical diagnosis; n (%):* | | | |
| Bacteremia, no focal infection | 9 (37.5) | 7 (23.3) | NS |
| Pneumonia | 8 (33.3) | 7 (23.3) | NS |
| Cellulitis | 3 (12.5) | 6 (20) | NS |
| Septic arthritis | 0 | 4 (13.3) | NS |
| Meningitis | 3 (12.5) | 0 | NS |
| STSS | 2 (8.3) | 0 | NS |
| Necrotizing fasciitis | 0 | 0 | NS |
| Other[b] | 3 (12.5) | 3 (10) | NS |

[a]NS, not significant, significant, P<0.05 using Mann-Whitney U test or Fisher's exact test.

[b]Including empyema, endopthalmitis, puerperal sepsis, nephrotic syndrome, peritonitis, follicular cholecystitis

**Table 2.** Superantigen gene profiles as related to *emm* subtypes of invasive *S. pyogenes* isolates

| No | *emm* type | No of isolates | Superantigen | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *spe*A | *spe*C | *spe*G | *spe*H | *spe*I | *spe*J | *spe*K | *spe*L | *spe*M | *ssa* | *sme*Z |
| 1 | 76.0, 80.0 (3), 105.0, 112.2 | 6 (12) | - | - | + | - | - | + | - | - | - | - | + |
| 2 | 63.0, 78.3, 113.0 (2), st6735.0 | 5 (10) | - | - | + | - | - | - | - | - | - | - | + |
| 3 | 42.0 (2), 58.8, stNS554.0 | 4 (8) | - | + | + | - | - | - | - | - | - | - | + |
| 4 | 12.0 (2), 118.0 | 3 (6) | - | + | + | - | - | - | - | - | - | + | + |
| 5 | 28.5 (3) | 3 (6) | - | - | - | - | - | - | - | - | - | - | + |
| 6 | 6.35, 74.0 (2) | 3 (6) | + | + | + | - | - | - | - | - | - | - | + |
| 7 | 11.0, 43.3 | 2 (4) | - | + | + | - | - | - | - | + | - | - | + |
| 8 | 49.0 (2) | 2 (4) | + | - | + | + | + | - | - | - | - | - | - |
| 9 | 1-2.2 | 1 (2) | + | - | + | + | + | + | - | + | + | + | + |
| 10 | 1-2.2 | 1 (2) | + | - | + | - | - | + | - | - | - | - | + |
| 11 | 4.5 | 1 (2) | - | - | - | - | - | - | + | - | - | + | + |
| 12 | 4.5 | 1 (2) | - | - | - | - | - | - | - | + | + | + | + |
| 13 | 9.0 | 1 (2) | - | - | + | - | - | - | + | - | - | - | + |
| 14 | 9.0 | 1 (2) | - | + | + | - | - | - | + | - | - | - | + |
| 15 | 12.0 | 1 (2) | - | + | + | + | + | - | - | - | - | + | + |
| 16 | 18.8 | 1 (2) | + | - | + | - | - | - | - | - | - | - | + |
| 17 | 44.0 | 1 (2) | - | + | + | + | - | + | - | - | - | - | - |
| 18 | 49.0 | 1 (2) | - | - | + | - | - | - | - | + | - | - | - |
| 19 | 49.0 | 1 (2) | - | - | + | + | + | - | - | + | + | - | - |
| 20 | 71.1 | 1 (2) | + | - | + | - | - | + | - | - | + | - | + |
| 21 | 74.0 | 1 (2) | + | + | + | + | + | - | - | + | + | + | + |
| 22 | 74.0 | 1 (2) | + | + | + | + | + | - | - | + | - | - | + |
| 23 | 79.1 | 1 (2) | - | + | + | - | - | - | - | - | - | - | - |
| 24 | 80.0 | 1 (2) | - | - | + | - | - | + | + | - | - | - | + |
| 25 | 85.0 | 1 (2) | - | - | + | + | + | - | - | - | - | - | + |
| 26 | 91.0 | 1 (2) | - | + | + | + | + | + | - | - | - | - | + |
| 27 | 103.0 | 1 (2) | - | - | + | + | - | + | + | - | - | + | + |
| 28 | 109.1 | 1 (2) | + | - | - | - | - | - | - | - | - | - | + |
| 29 | st2147.0 | 1 (2) | + | - | + | - | - | - | + | - | - | + | + |
| | No. of isolates | 49 | 34 | 38 | 86 | 34 | 28 | 28 | 21 | 24 | 17 | 28 | 83 |
| | Gene frequency,%[a] | | | | | | | | | | | | |

[a]Percentage related to number of profiles