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Invasive properties of south Indian strains of *Streptococcus pyogenes* in a HEp-2 cell model

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The objective of this study was to consider the invasive properties of *Streptococcus pyogenes* in human pharyngeal epithelial cells, and to correlate these with their clinical significance. Clinical isolates of *S. pyogenes* obtained from blood cultures over a period of 10 years, and throat and skin isolates from a community-based study, were used in this investigation. The *S. pyogenes* isolates were inoculated in HEp-2 cells and subsequently treated with antibiotics to kill the extracellular bacteria. The cells were then lyzed, and a colony count was carried out to check for invasion. The throat and skin isolates had 45.7%, 25.7% and 28.5% of low, intermediate and high invasion efficiencies, respectively, while 80%, 8.6% and 11.4% of the blood isolates had low, intermediate and high invasion efficiencies. We concluded that the throat and the skin isolates from superficial infections were more invasive than the blood isolates, which is an interesting and paradoxical feature.

Keywords *S. pyogenes*, invasion, HEp-2 cells, blood, throat and skin isolates

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INTRODUCTION

Streptococcus pyogenes is the causative agent of a variety of diseases, which include milder entities such as pharyngitis and impetigo, invasive forms such as toxic shock syndrome and necrotizing fascitis, and non-suppurative sequelae such as rheumatic fever and glomerulonephritis [1].

M-protein is considered to be the major virulence factor involved in the pathogenesis of streptococcal infections, based on over 80 M types currently identified [2]. The *emm* gene that codes for the M-protein has also been the basis for the newer molecular approaches for typing *S. pyogenes* [3]. Apart from the role of M-protein in adhesion and colonization, lipoteichoic acid, protein F/SfbI, fibronectin-binding protein, hyaluronic acid capsule and other adhesins have been studied. The extracellular pyrogenic exotoxins A, B and C and the newly described streptococcal superantigens and several other enzymes contribute to pathogenesis in streptococcal diseases [4].

Adhesion and colonization are considered to be the initial steps in establishing an infection: *S. pyogenes*, in addition to its ability to colonize the mucosal and skin surfaces, also has the ability to persist within the host cells [5]. This feature is thought to have various functions, such as: (1) escape from host defense mechanisms; (2) persistence in the presence of antibiotic; and (3) a significant role in serious invasive diseases [5,6].

The traditional concept of *S. pyogenes* being an extracellular pathogen that adheres to epithelial cells is no longer valid, as a result of newer findings that some strains can efficiently be internalized and survive within the epithelial cells [5–7].

There have been reports of an increased incidence of *S. pyogenes* associated with serious invasive diseases such as necrotitizing fascitis, toxic shock syndrome and septicemia over the past decade [8]. Organisms isolated from sterile sites such as blood and other fluids are generally designated invasive, and those from mucosal and skin surfaces as non-invasive.

In this study, we have investigated the invasive properties of *S. pyogenes* isolated from blood, throat and skin at a tertiary hospital in south India, using HEp-2 cells as a model.

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MATERIALS AND METHODS

Bacterial strains

This study was carried out at Christian Medical College & Hospital, Vellore, south India, from March 2001 to February 2002. The *S. pyogenes* isolates from throat ($n = 17$) and skin ($n = 18$) were obtained from a community-based study during this period. Blood isolates ($n = 35$) were recovered from blood cultures of patients with invasive streptococcal infections over the past 10 years; these had been stored in a lyophilized form.

Inoculum

Colonies on blood agar plates obtained after subculture were inoculated in Todd–Hewitt broth supplemented with 1% yeast extract and incubated for 6–8 h. The broth culture was then diluted 1 : 500 with Eagle's minimum essential medium without antibiotics.

Cell cultures

The human laryngeal epithelial cell line HEp-2 was grown in 25-cm^2 flasks (TPP Europe, Zurich, Switzerland) in Eagle's minimum essential medium (MEM; Life Technologies) containing antibiotics (penicillin 250 units/mL, streptomycin 250 mg/L, amphotericin B 1 mg/L) supplemented with 10% fetal calf serum (FCS; Life Technologies, Grand Island, New York, USA) at 37 °C in 5–10% CO_2. To carry out the invasion studies, the HEp-2 cells were grown in a 24-well plate (Falcon, Becton Dickinson, Lincoln Park, New Jersey, USA) and incubated for 2–3 days until a healthy and complete monolayer was formed. The monolayer was washed in MEM (free of antibiotics) three times before use.

Invasion assay

The invasion assay was carried out as described previously [5]. One hundred microliters of the diluted broth culture was added to the wells of the 24-well plate in duplicate and incubated for 2 h at 37 °C in an atmosphere of 5–10% CO_2. Ten microliters of the same broth was inoculated onto a blood agar plate and streaked for colony count, to check the number of bacteria inoculated per well. After incubation, the wells were treated with 5 mg/L penicillin (Sigma, St Louis, Missouri, USA) and 100 mg/L gentamicin (Sigma), and incubated for 2 h to kill the extracellular bacteria.

A bacteriologic loop of the MEM supernatant was streaked onto a blood agar plate to assess the ability of the antibiotics to kill the extracellular bacteria. The monolayer was then washed with phosphate-buffered saline (PBS) (pH 7.4), and the cells were detached by adding 200 μL of trypsin–versene solution (trypsin 0.25%; versene 0.02%). The cells were then lyzed by addition of 400 μL of Triton X-100 (Sigma, 0.25% in distilled water). Then, 10 μL of the lyzed material was inoculated onto a blood agar plate for colony counting. A standard reference strain (SS95) that was found to have a low invasion efficiency, and a blood isolate (B5294) that had a high invasion efficiency, were used as controls for every set of strains tested.

Statistical analysis

The invasion efficiency was calculated by use of the following formula: (bacteria inoculated/bacteria recovered) × 100. The data were analyzed using EPI Info Version 6.03 by the chi-square test.

RESULTS

There was no significant difference in the number of colony-forming units in the MEM supernatant before and after 2 h of initial incubation. Hence, the growth of the bacteria during the 2 h of incubation does not have any impact on the invasion rates. In all assays, the antibiotics were found to completely kill the extracellular bacteria. The invasion efficiencies of *S. pyogenes* isolated from blood, skin and throat specimens are shown in Table 1.

Intermediate to high invasion was seen in 20% of blood isolates, 64.7% of throat isolates, and

Table 1 Invasion efficiencies of *Streptococcus pyogenes* isolates from blood, throat and skin specimens

	Source of isolates		
Invasion efficiency	Blood ($n = 35$)	Throat ($n = 17$)	Skin ($n = 18$)
Low invasion (0–5%)	28 (80%)	6 (35.2%)	10 (55.5%)
Intermediate invasion (6–15%)	3 (8.6%)	5 (29.4%)	4 (22.2%)
High invasion (16–35%)	4 (11.4%)	6 (35.2%)	4 (22.2%)

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44.4% of skin isolates. The difference between the invasion efficiencies of throat and blood isolates was statistically significant ($P < 0.001$, χ^2). The difference between the invasion efficiencies of blood and skin isolates was not significant. The overall difference between blood isolates and skin and throat isolates together was significant ($P < 0.01$, χ^2).

DISCUSSION

Invasion and persistence of *S. pyogenes* within the respiratory epithelial cells has already been documented [5–7,10], but the bacterial components that mediate their entry into the epithelial cells have not been completely elucidated. However, most of the studies state that protein F/SfbI [10,11] is required for internalization. Apart from this, several other mechanisms are also thought to be involved in internalization [7,12–14].

Earlier reports have stated that strains from invasive diseases were internalized more efficiently than strains from non-invasive diseases [15]. However, most recent studies have now established that the strains from non-invasive diseases invade in vitro to a greater extent [5,10] than those from invasive diseases, which is in disagreement with the earlier studies.

However, a few studies have also demonstrated that *S. pyogenes* strains from invasive and non-invasive disease adhere to and penetrate HEp-2 cells equally well, and that many strains of *S. pyogenes* with various M-protein types have the ability to adhere to and penetrate HEp-2 cells [16].

This study used *S. pyogenes* isolates recovered form blood cultures of patients with septicemia, and throat and skin isolates from a community-based study. The isolates from skin and throat were internalized more efficiently than the blood isolates, which is in accordance with most of the recent studies; 80% of the blood isolates had low invasion efficiency, in contrast to only 45.7% of the throat and skin strains ($P < 0.001$, χ^2).

The results suggest that the epithelial cell internalization of *S. pyogenes* from superficial sites may prevent host immune defense mechanisms and antibiotics from gaining access to them. On the other hand, the blood isolates probably have a poor internalization ability due to the downregulation of the attachment, penetration and colonization processes, as these are not required once the organisms have reached the bloodstream, which is the last step of the infection process [5].

Though various hypothetical views based on the in vitro studies have evolved, in vivo studies are needed to substantiate them. One interesting piece of evidence came from Ostelund et al. [9], who showed *S. pyogenes* internalization in histopathologic sections taken from patients with recurrent pharyngotonsilitis. Immunohistochemistry, electron microscopy and culture were done on the surgically removed tonsils and cultured epithelial cells from the tonsillar and pharyngeal areas from these patients, to show the internalization of *S. pyogenes*. More in vivo studies are needed.

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