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In vitro characterization of representative clinical South African *Staphylococcus aureus* isolates from various clonal lineages

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Abstract

Data concerning the virulence and pathogenesis of South African strains of *Staphylococcus aureus* are limited. We investigated host–pathogen interactions of randomly selected clinical *S. aureus* isolates representing various clones. We characterized the ability of isolates to adhere to fibronectin, fibrinogen, collagens IV and VI, to invade host cells and to induce cell death *in vitro*. We analysed the possible association of these results with characteristics such as methicillin resistance, Panton–Valentine leucocidin (PVL) positivity and clonality. The *S. aureus* isolates displayed diversity in their abilities to adhere to various human ligands. All isolates were highly invasive except for ST121. PVL-negative isolates were significantly more invasive than the PVL-positive isolates (p 0.004). Isolates of CC5, CC30 and CC121 were non-cytotoxic, whereas isolates of CC22, CC8, CC15, CC45 and CC88 were very cytotoxic. No statistical association was identified between cell death and methicillin resistance, bacterial PVL status, clonality or patient HIV status. The vast majority of isolates were invasive and induced significant cell death. PVL-negative isolates were more invasive than PVL-positive isolates, while methicillin-resistant isolates were not found to be more invasive or cytotoxic than methicillin-susceptible isolates.

Keywords: Cellular invasiveness, cytotoxic, methicillin-resistance, Panton–Valentine leucocidin, *Staphylococcus aureus*, South Africa
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Introduction

Staphylococcus aureus is a facultative intracellular bacterium and a significant human pathogen. It possesses many surface factors that aid with host colonization and cellular invasion as well as secreted virulence factors involved in host cell death induction [1].

Fibronectin, fibrinogen and collagen are three of many extracellular matrix molecules found in macromolecular structures. Both fibronectin and fibrinogen play significant roles in the adherence of *S. aureus* during infections associated with skin diseases such as atopic dermatitis [2]. Fibronectin is also a component of human plasma and connective tissue [3]. Fibrinogen-binding is commonly associated with infective endocarditis [4], whereas collagen binding is commonly required for the colonization of cartilage [5]. Numerous bacterial surface proteins can be used during the process of adherence to host ligands and are called 'microbial surface components recognizing adhesive matrix molecules' or MSCRAMM, such as fibronectin-binding proteins A and B [6], staphylococcal protein A and clumping factors. Another group of bacterial proteins which is involved in this process are the SERAM molecules, or the 'secreted expanded repertoire adhesive molecules' [7], such as the extracellular adherence protein (Eap). Adherence to fibronectin by *S. aureus* can be mediated by fibronectin-binding proteins A and B (FnbA/B), which also aid in the binding of the organism to plasma clots [8]. Both genes are fundamental for the invasion of eukaryotic cells [7]. *Staphylococcus aureus* possess two distinct fibrinogen-binding proteins, namely clumping-factor A and B, of which clumping factor A is mainly used to adhere to substances containing fibrinogen [8].

Many groups have clearly demonstrated the role of Fnb proteins as the main invasin of *S. aureus* and identified a

fibronectin-dependent bridging mechanism to the host cellular integrin $\alpha_5\beta_1$ [9]. Fibronectin-binding proteins do not require any other *S. aureus*-specific co-receptors to confer invasiveness and this function can be accomplished by either FnbA or FnbB [10], which must be anchored into the bacterial cell wall, as truncation of these proteins results in deficient adherence and cellular invasiveness [11]. It has been shown that the extracellular adherence protein, with its broad binding capacity, can play a role in the cellular invasion of host cells [12].

In any given *S. aureus* strain, host cell death induction is difficult to predict and depends on many factors [13]. Various bacterial virulence factors are involved, of which α-toxin [14] is described as the most prominent. Intracellular *S. aureus*, if viable, can exist free in the cytoplasm and kill endothelial cells, partly by apoptosis [15]. Metabolically active intracellular staphylococci are required for the induction of apoptosis in endothelial cells, which is dependent on *agr* and *sigB* [16]. Strains with invasive and haemolytic phenotypes are normally associated with caspase-dependent induction of apoptosis, while non-invasive haemolytic or non-haemolytic invasive isolates are not [16].

Another well-characterized virulence factor of *S. aureus* that can be responsible for host cell death induction, especially of human neutrophils [17], is the two-component leucotoxin, Panton–Valentine leucocidin (PVL). This toxin has been associated with necrotizing pneumonia [18], skin-and-soft tissue infections (SSTI) [19] and necrotizing lesions of the skin and subcutaneous tissues [20] and is very common among diverse genetic backgrounds associated with community-acquired methicillin-resistant *S. aureus* (MRSA), especially the USA300 clone [21].

The aim of this research was to investigate the abilities of *S. aureus* isolates representative of clones causing infection in our patient population to adhere to immobilized ligands, to investigate their cellular invasiveness and host cell death induction abilities, and to identify any associations between adherence, invasiveness or cell death induction and bacterial characteristics, such as methicillin resistance, PVL positivity and clonality.

Materials and Methods

Selection of representative isolates (n = 25)

From a collection of 367 well-characterized clinical *S. aureus* isolates originating from patients in the Western Cape [22], South Africa, a representative isolate was randomly selected from each major and intermediate pulsed-field gel electrophoresis clone. Two isolates from minor clones statistically associated with HIV infection and two isolates selected from the HIV-positive patients from the dominant MRSA and methicillin-susceptible *S. aureus* (MSSA) clones were also included to investigate any specific associations with HIV infection. An MRSA isolate with a non-typeable SCC*mec* element was also included as representative of a unique local clone.

Bacterial strains

All bacterial isolates were stored at −80°C until further testing. The following isolates were used as controls: NCTC8325-4 (adherence), Cowan I (invasive control isolate), *Staphylococcus carnosus* TM300 (non-invasive control and non-cytotoxic control isolate) and 6850 (cytotoxic control isolate).

Adherence assay

Adherence was tested first in uncoated plates to establish a baseline. Then, 96-well plates (Sarstedt, Nümbrecht, Germany) were coated with a specific ligand using a modified method of Peacock *et al.* [23]. Standardized bacterial cultures were used at an OD_{600} = 1 in triplicate with three independent experiments. The plate was inoculated with bacterial culture, incubated at 37°C/5% CO_2 overnight, washed with PBS and stained using 0.1% crystal violet solution. After this, the plate was washed with PBS, eluted with 1% SDS at room temperature overnight, and subsequently measured with an ELISA reader (TECAN Infinite Pro 200, Männedorf, Switzerland) at 620 nm. Adherence to a specific ligand was expressed as a percentage relative to the positive control after subtraction of the PBS negative control. The mean of the means and standard error of mean (SEM) were determined.

Mammalian cell culture

293 cells (www.atcc.org) were used to investigate the cellular invasiveness. 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (PAA Laboratories, Pasching, Austria) and 1× Pen/strep mix (100 U/mL penicillin and 100 μg/mL streptomycin) (Cambrex Bio Science, Verviers, Belgium) and maintained in humidified air (37°C/5% CO_2). Ea.hy926 cells (www.atcc.org) were used to investigate cell death induction. Ea.hy926 cells were maintained in DMEM/F-12 supplemented with 10% fetal calf serum and 1× penicillin/streptomycin mix (100 U/mL penicillin and 100 μg/mL streptomycin) and maintained in humidified air (37°C/5% CO_2).

FACS invasion assay

The cellular invasiveness was determined by adapting a previously published FACS-based invasion assay [10]. Briefly, 293 cells were plated in 24-well plates at 3×10^5 cells/well the day before the assay. The day of the assay, the cells were washed once with 500 μL invasion medium (1% human serum