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Short communication

Infection of human keratinocytes by *Streptococcus dysgalactiae* subspecies *dysgalactiae* isolated from milk of the bovine udder

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Abstract

Streptococcus dysgalactiae subsp. *dysgalactiae* (SDSD) are considered exclusive animal pathogens; however, a putative zoonotic upper limb cellulitis, a prosthetic joint infection and an infective endocarditis were described in humans. To unravel if bovine SDSD isolates are able to infect human cells, the adherence and internalization to human primary keratinocytes of two bovine SDSD strains isolated from milk collected from udder were analyzed. Bacterial adhesion assays and confocal microscopy indicate a high adherence and internalization of SDSD isolates to human cells, suggesting for the first time the ability of bovine isolates to infect human cells.

Keywords: Adhesion; Bovine; Host; Human keratinocytes; Internalization; *Streptococcus dysgalactiae* subspecies *dysgalactiae*

1. Introduction

Streptococcus dysgalactiae subspecies dysgalactiae (SDSD) are alpha-hemolytic or non-hemolytic streptococci belonging to the Lancefield group C [1,2]. They are considered as exclusive animal pathogens, despite three sporadic cases of infection in humans being reported, namely an ascending upper limb cellulitis in a woman that contacted raw fish [3], a prosthetic joint infection after total knee arthroplasty [4], and very recently, an infective endocarditis in a male patient [5].

Previous investigations in our group evidenced that SDSD strains recovered from subclinical bovine mastitis carried and expressed phage-encoded virulence genes including genes that code for the exotoxins SpeK, SpeC, SpeL and SpeM, genes coding for DNase I (Spd1), and streptodornase gene (*sdn*) of the strictly human pathogen *Streptococcus pyogenes* [6,7]. Despite these reported cases of human infections caused by SDSD, it has never been reported that animal SDSD isolates, particularly bovine mastitis subclinical strains, are capable to infect human cells. Nevertheless, the presence of *S. pyogenes* virulence factors in the genome of bovine SDSD has lead to the hypothesis of SDSD dissemination to other hosts. Hence, here the possibility of SDSD strains to adhere to and invade human cells was investigated. Adherence and invasion of SDSD isolates in epithelial cells has been described to occur in mammary cell lines and fish epithelial cell lines cultured *in vitro* [8–10]. The virulence of SDSD seems to be related to cell surface properties, such as high hemagglutination and

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List of abbreviations

CFUs	colony forming units
DMEM	Dulbecco's modified eagle medium supplemented with 10% (v/v) foetal bovine serum
PBS	phosphate buffer saline
SDSD	*Streptococcus dysgalactiae* subps. *dysgalactiae*
THB-5YE	Todd Hewitt Broth supplemented with 5% (w/v) yeast extract

hydrophobic properties that determine the main adhesion and invasive pathogenic mechanism of the species [8,11–13].

2. Materials and methods

2.1. Bacterial species and culture conditions

S. dysgalactiae subsp. *dysgalactiae* strains VSD5 and VSD13 were isolated from milk collected from the udder of cows with mastitis [6,7]. Strain identified as GCS-Si was responsible for an ascending upper limb cellulitis in a woman that was pricked by the fins and scales of a raw fish [3]. *S. pyogenes* GAP58 is an invasive strain isolated from the blood a patient and GAP8 is a strain isolated from the tonsils of an asymptomatic patient [14]. The characterization of each strain based on 16S sequencing, *emm* subtype and MLST was previously performed [7,14]. Strains were cultivated in 20 mL Todd Hewitt broth supplemented with 5% (w/v) yeast extract (THB-5YE) in 100 mL Erlenmeyer-flasks and grown at 37 °C until reach a standardized optical density at 600 nm (OD600) of 0.3–04 ($5 \times 10^7 - 1 \times 10^8$ cells/mL). For each strain, an aliquot of 1 mL of cell suspension was collected and cells were washed 3 times in fresh THB-5YE.

2.2. Human cells and culture conditions

Human primary epidermal keratinocytes (ATCC-PCS-200-010, ATCC, Manassas, USA) were grown in keratinocyte growth media according to ATCC (Manassas, USA) instructions. For infection assays, cells were seeded in a 96-well culture plate at a density of 3×10^4 cells/well and incubated for 24 h at 37 °C, 5% (v/v) CO_2 and 99% (v/v) relative humidity. For confocal microscopy analysis 1×10^5 cells were placed over a Polylysine treated microscope slide and incubated for 24 h at 37 °C, 5% (v/v) CO_2 and 99% (v/v) humidity.

2.3. Bacterial internalization and adherence assays

The adhesion and internalization assays were based on previously described protocols [15,16] with few modifications. Bacteria were grown at 37 °C in Todd Hewitt Broth supplemented with 5% (w/v) yeast extract (THB-5YE) until the middle of the exponential phase. The infection was started by adding 10^6 bacterial cells in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) Foetal Bovine Serum (DMEM) to 10^4 human primary epidermal keratinocytes (ATCC-PCS-200-010, ATCC, Manassas, USA). After 2 h of incubation at 37 °C, 5% (v/v) CO_2 and 99% (v/v) relative humidity, cell monolayers were washed with Phosphate Buffer Saline (PBS) to remove unbound bacteria. To obtain the number of bacteria that adhere and internalize to keratinocytes [$(Adh + Int)_{value}$] and the number of bacteria that internalize keratinocytes (Int_{value}), infected keratinocyte cell monolayers were incubated for 30 min with DMEM or DMEM supplemented with Penicillin 100 units/mL, respectively. Keratinocytes were detached from the well, disrupted with Triton X-100 0.01% (v/v) (Sigma), and the bacterial colony-forming units (CFUs) were calculated using standard plate counting techniques. In parallel, the exact same procedure was performed in keratinocyte free wells in order to obtain procedural negative control values. The values obtained in the procedural control is subtracted from Int_{value} and $(Adh + Int)_{value}$. Int_{value} was the averaged CFUs counted for the keratinocytes infected cells treated with Penicillin minus the averaged CFUs obtained in Int_{ctrl}. The $(Adh + Int)_{value}$ was the averaged CFUs counted for the keratinocytes infected cells minus the averaged CFUs obtained in $(Adh + Int)_{ctrl.}$ The Adh_{value} of each strain was obtained by subtracting the Int_{value} to the $(Adh + Int)_{value}$. Every experiment was repeated at least 4 times.

2.4. Confocal laser scanning microscopy

For confocal laser scanning microscopy analysis, pelleted bacteria were resuspended in THB-5YE supplemented with 0.2 mg/mL Hoechst 33258 (LifeTechnologies), incubated at 37 °C for 45 min, washed with PBS and resuspended in DMEM. Keratinocytes in microscope slides were washed 3 times with PBS and bacterial cells placed over the human cells. After 2 h of incubation at 37 °C, 5% (v/v) CO_2 and 99% (v/v) relative humidity, cells were fixed with 2% (w/v) paraformaldehyde, lysed with 0.1% (v/v) Triton X-100 and animal cells were stained with AlexaFluor 488 Phalloidin (LifeTechnologies) according to the manufacturer's instructions. After washing 3 times with PBS, a drop of ProLong Diamond antifade mountant (LifeTechnologies) was placed on top of the cells, covered with a cover slip and sealed. A confocal laser scanning microscope (Carl Zeiss, LSM 700), using the 488 nm laser for visualization of cells stained with AlexaFluor 488 Phalloidin, and the 405 nm laser for visualization of bacterial cells stained with Hoechst 33258, and respective software (ZEN Black, 2011), was used for image acquisition. This approach only used the bovine SDSD VSD13 and the human *S. pyogenes* GAP58. A control sample was also prepared, consisting in keratinocytes stained for 2 h with 20 μg/mL Hoechst 33258.

3. Results

To unravel if bovine SDSD isolates were able to interact with human cells, human keratinocytes were infected for 2 h with two bovine SDSD strains, VSD5 and VSD13, both isolated from milk collected from udder [6,7], and the adherence