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Short communication

Acinetobacter baumannii and *A. pittii* clinical isolates lack adherence and cytotoxicity to lung epithelial cells *in vitro*

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Abstract

The molecular and genetic basis of *Acinetobacter baumannii* and *Acinetobacter pittii* virulence remains poorly understood, and there is still lack of knowledge in host cell response to these bacteria. In this study, we have used eleven clinical *Acinetobacter* strains (*A. baumannii* $n = 5$; *A. pittii* $n = 6$) to unravel bacterial adherence, invasion and cytotoxicity to human lung epithelial cells. Our results showed that adherence to epithelial cells by *Acinetobacter* strains is scarce and cellular invasion was not truly detected. In addition, all *Acinetobacter* strains failed to induce any cytotoxic effect on A549 cells.

Keywords: *Acinetobacter baumannii*; *Acinetobacter pittii*; Adherence; Cytotoxicity; Epithelial cells

1. Introduction

Acinetobacter baumannii and *Acinetobacter pittii* cause various types of human infections, including pneumonia, bacteraemia, wound infections, meningitis and urinary tract infections [1,2]. *Acinetobacter* species are inherently resistant to several antibiotics or capable of readily acquiring resistance and clinical isolates are able to rapidly spread among patients and survive in the hospital environment [3,4].

Since adherence of bacteria to cells is considered as an essential first step in bacterial pathogenesis, *in vitro* cell culture methods provide a useful tool to investigate the interactions between pathogens and the human epithelium that occurs during infections. After adhesion, invasion of host non-phagocytic cells may be exploited by pathogens to avoid the hostile environment, therefore facilitating their dissemination. In this regard, attempts have been made to elucidate the mechanisms by which *A. baumannii* promotes adherence to host cells. Moreover, whether *Acinetobacter* spp. are facultative intracellular pathogens or not has been a subject of debate because both the host cells surface factors or bacterial adhesins that mediate adherence of *A. baumannii* are largely uncharacterized, the intracellular trafficking of the bacteria is not clear and there is a lack of evidence for intracellular replication or long term intracellular survival inside cells.

In this work, eleven *Acinetobacter* strains isolated from human patients were analysed for adherence, internalization and cytotoxic capacity in the A549 cell line in order to better understand their mechanisms of pathogenicity.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

Eleven *Acinetobacter* strains were used (Supplementary Table 1). All strains were isolated as pure cultures from clinical samples at the Hospital Universitario Marqués de Valdecilla (HUMV). The strains were routinely cultured on blood agar (BA) plates, brain hearth infusion broth (BHIB) or Luria Bertani broth (LB) at 37 °C, and frozen at −80 °C with 20% glycerol. As control for specific adherence experiments, one strain of *Haemophilus influenzae* (HiNT 375) was used [5]. As a control for bacterial cytotoxicity, *Serratia liquefaciens* strain (HUMV-3250) was used [6].

2.2. Transmission electron microscopy

All *Acinetobacter* strains were examined by transmission electron microscopy (TEM) after growth at 37 °C in LB, BHIB or BA to confirm the presence of fimbriae. Bacteria were applied to Formvar-coated grids, negatively stained with 1% phosphotungstic acid and finally examined with a JEM-1011 transmission electron microscope (JEOL) operating at 80 kV.

2.3. Scanning electron microscopy

Coverslips containing infected cultures (*Acinetobacter*, *Haemophilus* or *Serratia*) or cells challenged with bacterial extracellular products (ECPs) were fixed in ice-cold 3% glutaraldehyde for 20 min at 4 °C. Samples were dehydrated in a series of graded acetone, dried by the critical point method, coated with gold in a Fine coat ion sputter JFC-1100 226 (JEOL, Ltd) and observed with an Inspect S microscope (FEI Company) working at 15 kV or 20 kV.

2.4. Adherence and invasion experiments

Adherence and invasion experiments by all strains were performed as previously described [7]. The human lung epithelial A549 (ATCC CCL185) cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum and 2 mM L-glutamine.

In order to clarify if *Acinetobacter* strains prefer the cell surface or the inert surface for adherence inside cell culture plates, we perform the following experiment. A549 cells were plated at a density of 1.9×10^5 cells/well (~100% confluence) or at one half, 9.5×10^4 cells/well (~50% confluence), into 24-well tissue culture plates. In the case that *Acinetobacter* prefer the cellular surface for adherence, we will expected to obtain more CFUs in the wells with more cells/less inert surface exposed (~100% confluence). However, if *Acinetobacter* prefer the inert surface instead the cell surface (plastic, or glass coverslips in IF experiments), we will expect more CFUs after adherence experiments in the wells with less cells/more inert surface exposed (~50% confluence).

Strains of *Acinetobacter* were cultured overnight in 10 ml of BHIB at 37 °C with moderate shaking at 175 rpm. Bacterial suspensions were washed in phosphate-buffered saline (PBS) and adjusted to approximately 5.5×10^9 CFU ml^{-1} ($OD_{620} = 0.5$). A549 cells were infected with bacteria at a multiplicity of infection (MOI, bacterium: eukaryotic cell ratio) of ~100:1 (~100% cell confluence) or ~200:1 (~50% cell confluence). The number of CFUs inoculated per well was determined by serial dilution in PBS and plating on BA and incubated for 48 h. The infected plates were centrifuged for 4 min at 200 × *g* prior to the incubation to promote adherence of bacteria to cells and to synchronize infections. Infected monolayers were then incubated at 37 °C with 5% CO_2 for 90 min. For quantification of adherent bacteria, external non-adherent bacteria were removed by washing the cells four times with PBS, and then disrupted by addition of 100 µl Triton X-100 (1% in PBS) per well. Serial dilutions of the disrupted mixture were plated onto BA and incubated for 48 h at 37 °C. Adherence of *Acinetobacter* strains was calculated as the average of the total number of CFUs per total initial inoculum and expressed as a percentage. Adherence experiments were repeated five times.

For prolonged intracellular survival experiments, four strains (two *A. baumannii* and two *A. pittii*) were arbitrarily selected. The MIC of gentamicin for these was determined by using the microdilution method and results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI). After infections with these four strains, cells were incubated for 2 h and after extracellular killing of bacteria by gentamicin (75–300 µg ml^{-1}), cells were washed and culture medium was replaced by medium containing 200 µg ml^{-1} gentamicin, incubated for a further 2 h, and lysed as described before. After this time, number of putative viable intracellular bacteria was counted. Parallel samples were processed for immunofluorescence microscopy.

As positive control for bacterial adherence to epithelial cells or cytotoxicity, A549 cell cultures were infected with, *H. influenzae* or *S. liquefaciens* strains at a MOI of ~100:1 for 90 min, and processed for SEM. Control cultures were incubated with the same volumes using fresh bacterial culture medium or cell culture medium.

2.5. Immunofluorescence

Adherence, invasion and differential double immunofluorescent labelling experiments were performed as previously described [7]. For double immunofluorescence assays, strains *A. baumannii* ATCC 19606^T and *A. pittii* LMG 10559 were used to produce polyclonal sera as previously described [7]. Cells were infected for 3–4 h and all preparations were examined with a Nikon A1R confocal scanning laser microscope equipped with 403 nm, 488 nm and 561 nm lasers. Images were captured at random with a ×20 Plan-Apo 0.75 NA, ×40 Plan-Fluor 1,3 NA or ×100 Apo-TIRF 1,49 NA objectives, and processed using the NIS-Elements 3.2 software.