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Rhodococcus equi human clinical isolates enter and survive within human alveolar epithelial cells

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Abstract

Rhodococcus equi is an emerging opportunistic human pathogen associated with immunosuppressed people, especially those infected with the human immunodeficiency virus (HIV). This pathogen resides primarily within lung macrophages of infected patients, which may explain in part its ability to escape normal pulmonary defense mechanisms. Despite numerous studies as a pulmonary pathogen in foals, where a plasmid seems to play an important role in virulence, information on the pathogenesis of this pathogen in humans is still scarce. In this study, fluorescence microscopy and vancomycin protection assays were used to investigate the ability of *R. equi* human isolates to adhere to and to invade the human alveolar epithelial cell line A549. Our findings indicate that some *R. equi* clinical strains are capable of adhering, entering and surviving within the alveolar cell line, which may contribute to the pathogen persistence in lung tissues.

Keywords: *Rhodococcus equi*; Intracellular pathogen; Human alveolar cells

1. Introduction

The soil-dwelling actinomycete *Rhodococcus equi* is a multihost pathogen that causes pyogranulomatous infections in humans and in several animal species [1]. In immunocompromised persons, *R. equi* causes tuberculosis-like pneumonia associated with a high case-fatality rate, principally in HIV patients. Endocarditis, brain abscesses and other infections have also been reported [2]. *R. equi* infections are diagnosed by culturing and subsequent phenotyping analysis by morphological and biochemical tests, chemotaxonomic properties [3,4], and molecular methods [5,6]. Confirmation by 16S rDNA sequencing may be necessary to avoid misidentification with *Dietzia* spp [7].

R. equi is primarily a pathogen of foals. The virulence of this pathogen in foals is linked to presence of large plasmids. In horse isolates, these plasmids are 85–90 kb in size and encode virulence-associated protein A (VapA), a 15–17 kDa surface lipoprotein antigen important for intramacrophage survival, cytotoxicity, and horse pathogenicity via neutralization of phagosomes and lysosomes or their disruption [1]. Plasmid loss results in attenuation of virulence in both foals and mice [8,9]. In non-horse hosts, a plasmid variant encoding VapB, a VapA-related surface antigen of larger size has been identified [10,11]. Lack of detection of VapA/B proteins (or vapA/B DNA sequences) is relatively common in non-horse isolates and is assumed to be due to the absence of virulence plasmid. A molecular method (TRAVAP) based on traA, vapA and vapB virulence plasmid markers have been recently developed for *R. equi* typing [5]. A TRAVAP survey of 69 *R. equi* human isolates revealed that all plasmid categories

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were common, reflecting the opportunistic nature of *R. equi* infection in this host [5].

Both humoral and cellular immune responses can contribute to host defense against *R. equi* [12,13] with activated macrophages being the most important cellular type involved. The microorganism can inhibit macrophage phagosome–lysosome fusion, leading to non-specific lysosome degranulation [14,15]. Most *in vitro* studies have used use phagocytes to study the non-specific immunity against this pathogen. However, little is known about the survival of *R. equi* in non-phagocytic human cells. Epithelial cells play an important role as the interface between the host mucosal surfaces and the surrounding environment and are the initial site of colonization for bacterial pathogens. *R. equi* could entry in either epithelial or other type of non-phagocytic cells avoiding the host hostile environment and disseminating more easily like other intracellular pathogens do [16,17].

In this study we have investigated the interaction of *R. equi* human isolates with the human lung epithelial cell line A549. Our results show for the first time that *R. equi* invades and survives in human pulmonary cells. Invasion rates and surviving of *R. equi* did not depend on the presence of virulence plasmids.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Sixteen *R. equi* clinical strains isolated from human patients in several Spanish hospitals were used (Table 1). As control strains, we used *R. equi* ATCC 33701 and its plasmid-cured derivative strain ATCC 33701$^-$. Bacteria were cultured in brain heart infusion broth (BHIB) or blood agar (BA) at 37 °C, and frozen at −80 °C with 15% glycerol. All strains were confirmed as *R. equi* by 16S rRNA gene sequencing [7].

Table 1
Bacterial strains used in this study and their TRA/VAP characteristics.

Strain	*traA*[a]	*vapA*	*vapB*	Colony type[b]
RE6	+	+	−	B
RE7	+	−	+	B
RE9	+	+	−	B
RE23	+	−	−	A
RE37	+	−	−	C
RE38	+	+	−	B
RE39	+	+	−	A
RE40	+	+	−	B
RE55	−	−	−	C
RE56	−	−	−	B
RE61	+	−	+	B
RE62	+	−	+	A
RE70	+	+	−	B
RE156	+	−	+	A
RE158	+	+	−	B
RE163	+	+	−	B
ATCC 33701	+	+	−	B
ATCC 33701 (−)	−	−	−	B

[a] All *traA* positive strains also carry a virulence plasmid.
[b] Classification ABC: type A was a highly mucoid, coalescing colony; type B was less mucoid and non-coalescing, and type C was a non-mucoid, discrete and dry colony.

2.2. Molecular typing

Molecular typing of *R. equi* strains was performed using the TRAVAP system as described previously [5].

2.3. Morphological analysis of colonies

The morphologic description of *R. equi* isolates was based on that of Mutimer and Woolcock [18].

2.4. Immunofuorescence assays

We used the *R. equi* strain RE70 as bacterial antigen. RE70 was grown and inoculated into New Zealand rabbits for the production of anti-*Rhodococcus* polyclonal sera as described previously [19]. Antiserum was collected 8 weeks after the first boost, processed and stored using standard protocols [20]. Secondary antibodies conjugated to Alexa Fluor 594 and Alexa Fluor 488 goat anti-rabbit IgG were purchased from Invitrogen. For bacterial microscopic examinations, bacteria were stained as described previously [21]. Briefly, bacteria were grown on BHIB cultures at 37 °C. Ten microliters of each liquid bacterial culture was pipetted onto a glass slide, dried and fixed with 3.2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) pH 7.4 for 15 min at room temperature. Slides were washed three times in PBS. Samples were incubated for 20 min with the polyclonal antiserum (1:1000 in 0.1% Bovine Serum Albumin in PBS, BSA-PBS), and another 20 min with Alexa goat anti-rabbit antibodies (1:1000 in BSA-PBS).

2.5. Adherence and invasion experiments

Adherence and invasion experiments were based on a modification of the quantitative protection assay described by Isberg and Falkow [22]. The human lung epithelial A549 cell line was grown in RPMI-1640 supplemented with 10% fetal calf serum. Cells were plated at a density of 1.8×10^5 cells per well into 24-well tissue culture plates (Nunc). Strains of *R. equi* were cultured in 10 ml BHIB at 37 °C with shaking at 175 rpm. For infections, bacterial suspensions were prepared to a final concentration of $\sim 4 \times 10^9$ CFU ml^{-1}. Ten microliters of bacterial cultures were added to each well containing A549 cells and the mixtures centrifuged for 4 min at 200× *g* previous to the incubation to promote adherence of bacteria to cells. Our standard conditions (10 μl) accounted for a multiplicity of infection (MOI) of ∼200:1 (bacterium/eukaryotic cell ratio). Infected cells were incubated at 37 °C with 5% CO_2. The exact number of colony-forming units (CFUs) inoculated per well was determined by serial dilution in PBS and plating onto BA. External non-adhered bacteria were removed by washing the cells four times with PBS, after which the cells were incubated for 2 h with RPMI-1640 containing vancomycin (50 μg ml^{-1}), and then disrupted by addition of 100 μl of Triton X-100 (1% in PBS). Serial dilutions of the disrupted mixture were plated onto BA and incubated for 48 h at 37 °C. Control wells without A549 cells were also included to establish that the antibiotic treatment