Short Report: Identification of Virulence-Associated Plasmids in *Rhodococcus equi* in Humans with and without Acquired Immunodeficiency Syndrome in Brazil

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Abstract. Virulence of *Rhodococcus equi* strains from 20 humans in Brazil was investigated by using a polymerase chain reaction to characterize isolates as virulent (VapA), intermediately virulent (VapB), and avirulent. Nine isolates were obtained from human immunodeficiency virus (HIV)-positive patients, six from HIV-negative patients, and five from patients of unknown status. Five isolates were VapB positive, four were VapA positive, and eleven were avirulent. Among the nine isolates from HIV-positive patients, five contained VapB plasmids and two contained VapA plasmids. Five VapB-positive isolates had the type 8 virulence plasmid. Eleven of the patients had a history of contact with livestock and/or a farm environment, and none had contact with pigs.

Rhodococcus equi is a well-recognized, gram-positive, intracellular bacterium associated with pyogranulomatous infections in humans, domestic animals, and wildlife.¹ Currently, *R. equi* has emerged as an increasingly common opportunistic pathogen among immunocompromised human patients, particularly those infected with human immunodeficiency (HIV).² Approximately two-thirds of patients with rhodococcosis are co-infected with HIV.³

Rhodococcus equi is widespread in soil and manure on farms, mainly in feces of foals and other herbivores.⁴ Inhalation and consumption of contaminated water and contact with pastures appear to be the major source of transmission this microorganism in livestock.¹ However, routes of *R. equi* transmission for humans remain controversial, although a history of contact with livestock and farms is considered to be a risk factor.⁵

The virulence of *R. equi* has been attributed to the presence of virulence-associated antigens and plasmids. Three virulence levels of isolates are recognized: virulent, intermediately virulent, and avirulent. Virulent strains contain a large 80–90-kb plasmid that contains the gene encoding virulence-associated 15–17-kD antigen (VapA).⁴ These virulent isolates are recognized as the major causes of suppurative pneumonia and ulcerative enteritis in foals.⁶ Intermediately virulent isolates are characterized by the presence of 20-kD antigens (VapB), which are encoded by 79–100-kb plasmids. These intermediately virulent strains are commonly found in lymph nodes of pigs with and without lymphadenitis^{4,7} and in humans with immune system dysfunction, especially HIV-positive patients.^{2,3} Avirulent isolates show no evidence of *vapA* or *vapB* genes and are found in HIV-positive patients, in environments containing livestock, and in soil or sand from parks and household yards.⁴

The purpose of this study was to investigate virulence-associated genes (*vapA* and *vapB*) and plasmid profiles of

R. equi isolates from 20 humans with and without acquired immune deficiency syndrome (AIDS) in Brazil.

Eighteen *R. equi* isolates were obtained from 18 patients admitted to hospitals in four states in Brazil over a 10-year period (1997–2007). We reviewed records concerning epidemiologic factors (sex, age, and history of contact with domestic animals and/or farms), primary site of infection, major clinical signs, and other laboratory diagnoses (particularly antibodies against HIV and other debilitating diseases). Two strains were isolated from asymptomatic farm workers. Unfortunately, some epidemiologic details and outcomes were not available or were not identified in certain cases, especially for HIV-positive patients. All strains were isolated by culturing on sheep blood agar incubated aerobically for 3 days at 37°C. *Rhodococcus equi* isolates were classified according to conventional methods⁸ and analyzed by using a polymerase chain reaction (PCR).²

Isolation of plasmid DNA was obtained by using an alkaline lysis method with some modifications as described elsewhere.² Specific DNAs for PCR amplification were determined according to reported sequences of the *vapA* (15–17-kD antigen) and *vapB* (20-kD antigen) genes (GenBank accession nos. D212361 and D44469).

Plasmid DNA was digested with restriction endonucleases *Eco* RI, *Eco* T22I, and *Hind* III.^{7,9} Samples of plasmid were separated by electrophoresis on a 1.0% agarose gel, stained with ethidium bromide, and examined under ultraviolet light. Primer 1 (5'-GACTCTTCAAGACGGT-3') and primer 2 (5'-TAGGCGTTGTGCCAGCTA-3') were used to identify virulent isolates (*vapA* gene) on the basis of amplification of the expected 569–552-basepair product. Primer 3 (5'-AACGTAGTCGCGGTGAGAA-3') and primer 4 (5'-ACCGAGACTTGAGCGACTA-3') were used to identify intermediately virulent (*vapB* gene) isolates by using the expected 1,066–1,048-basepair product.⁹ The PCR amplification was carried out using 10 µL of the DNA preparation in a 50-µL reaction containing 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each deoxynucleotide triphosphate, 1 mM primer (each), and 2.5 units of Taq DNA polymerase.² Samples were subjected to 30 cycles of amplification: denaturation for 90 seconds at 94°C, annealing for 1 minute at 55°C, and extension for 2 minutes at 72°C.⁹

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The virulent ATCC 33701 strain (equine origin), intermediately virulent strains (human and pig origin), and other representative *R. equi* strains for various plasmid types were used as reference strains. Corresponding plasmid profiles and virulence levels of reference strains have been described.^{2,6,7,9,10} Statistical analysis was conducted by using the chi-square test (Epi Info version 6.4; Centers for Disease Control and Prevention, Atlanta, GA), and $P < 0.05$ was considered significant.

Epidemiologic, diagnostic, and virulence plasmid profile findings for the 20 *R. equi* strains isolated from symptomatic and asymptomatic patients are shown in Table 1. Of 20 patients, 6 were men (patients 5, 16, 17, 18, 19, and 20), and the sexes of the remaining patients were unknown. Three patients (patients 17, 19, and 20) were 31–40 years of age, 4 (patients 5, 14, 16, and 18) were 41–50 years of age, and the ages of other patients were unknown.

Nine patients were HIV positive, which was defined as the presence of antibodies against HIV in serum samples. Among HIV-positive patients, one had also hepatitis. Six patients were not infected with HIV, and the HIV status of the five remaining patients was unknown. One HIV-negative patient had a history of alcoholism and hepatitis, and another HIV-negative patient had a history of alcoholism as an underlying condition.

The major clinical sign identified in 15 (75.0%) patients was pneumonia. *Rhodococcus equi* was isolated from bronchial washes (14 patients), nasal mucosa (2 patients), lung fragment (1 patient), hepatic fragment (1 patient), blood and cutaneous fragments (1 patient), and the fingernail region (1 patient).

Eleven patients (55.0%) had history of contact with livestock and/or farms. Contact exclusively with a farm environment was reported by four (20.0%) patients, and contact with only equines or bovines were reported by two (10.0%) patients and one (5.0%) patient, respectively. For nine patients, the date

of contact with a farm environment or livestock was unknown. No patients had a history of contact with pigs or pig breeders.

Virulence plasmid analysis of the 20 *R. equi* isolates showed the presence of 5 (25.0%) intermediately virulent, 4 (20.0%) virulent, and 11 (55.5%) avirulent isolates. Among the four VapA isolates, three had the 87-kb type I plasmid and 1 had the 85-kb type I plasmid. For two isolates with a VapA plasmid profile from patients who had a history of equine contact, one isolate contained the 85-kb type I plasmid and the other isolate contained the 87-kb type I plasmid.

Of nine strains isolated from HIV-positive patients, five contained the *vapB* gene, two contained the *vapA* gene, and two did not contain either the *vapA* or the *vapB* gene. Plasmid profiles of the five intermediately virulent isolates were characterized as type 8, and plasmids with intermediate virulence was significantly associated with an HIV-positive diagnosis ($P < 0.001$). The presence of the *vapA* or *vapB* genes was also significantly associated with HIV-positive patients ($P = 0.0124$). Among the 20 patients, 11 had avirulent isolates, accounting for 2 HIV-positive patients, 2 HIV-negative patients, and 2 HIV-negative patients with a history of alcoholism and/or hepatitis. The five remaining patients had an unknown history of HIV infection or other debilitating diseases.

Among the 20 patients, clinical outcome was known only for four patients. Among these patients, cure was observed in 2 patients (patients 5 and 16), and disease was fatal in 2 patients (patients 2 and 19).

The first recognized case of human rhodococcosis was reported in 1967. Only 12 new cases were described in 15 years after this initial report.⁵ In contrast, a substantial increase in the number of reported human cases has occurred in recent decades worldwide, mainly as co-infection with HIV.^{2,11,12}

In Latin America, approximately 1.6 million persons are infected with HIV. The Brazilian Ministry of Health reported 474,273 cases of AIDS during 1980–2007. The disease was

TABLE 1

Epidemiologic, diagnostic, and virulence plasmid profile findings for *Rhodococcus equi* strains isolated from 20 symptomatic and asymptomatic humans, Brazil, 1997–2007*

Patient no.	Animal or farm contact	Major clinical sign	Diagnostic culture	Underlying condition	Virulence plasmid profile (type)
1	Unknown	Pneumonia	Bronchial wash	HIV positive	VapB (type 8)
2	Farm	Pneumonia	Lung fragment	HIV positive	VapA (87-kb type I)
3	Equine and farm	Pneumonia	Bronchial wash	HIV positive	VapB (type 8)
4	Unknown	Pneumonia	Bronchial wash	HIV positive	VapB (type 8)
5	Equine	Hepatitis, enteritis, and weight loss	Hepatic fragment	HIV negative, alcoholism, and hepatitis	Avirulent
6	Unknown	Pneumonia	Bronchial wash	Unknown	Avirulent
7	Unknown	Pneumonia	Bronchial wash	HIV negative	Avirulent
8	Equine, bovine, and farm	Pneumonia	Bronchial wash	HIV positive	VapA (85-kb type I)
9	Unknown	Pneumonia	Bronchial wash	Unknown	Avirulent
10	Equine	Pneumonia	Bronchial wash	Unknown	Avirulent
11	Unknown	Pneumonia	Bronchial wash	Unknown	Avirulent
12	Unknown	Pneumonia	Bronchial wash	Unknown	Avirulent
13	Bovine	Pneumonia	Bronchial wash	HIV positive	Avirulent
14	Unknown	Pneumonia	Bronchial wash	HIV positive	VapB (type 8)
15	Farm	Pneumonia	Bronchial wash	HIV positive	Avirulent
16	Equine, bovine, and farm	Pneumonia	Bronchial wash	HIV negative	VapA (87-kb type I)
17	Equine, bovine, and farm	Weight loss and gastroenteritis	Swab under a fingernail	HIV negative and alcoholism	Avirulent
18	Unknown	Hepatitis and cutaneous lesion	Blood and cutaneous fragment	HIV positive and hepatitis	VapB (type 8)
19	Farm	Asymptomatic	Nasal swab	HIV negative	VapA (87-kb type I)
20	Farm	Asymptomatic	Nasal swab	HIV negative	Avirulent

* HIV = human immunodeficiency virus; Vap = virulence-associated protein; VapB = intermediately virulent; VapA = virulent.

fatal in 28,609 persons in Brazil during 1983–2003.¹³ Despite implementation of control strategies and treatment protocols against HIV/AIDS in Brazil, deaths of HIV-positive patients co-infected with *R. equi* have been observed.¹⁴ However, little information is available in Brazil about virulence plasmid profiles of *R. equi* isolates from humans.^{15–17}

Intermediately virulent strains of *R. equi* are predominantly identified in HIV-positive patients,² the lymph nodes of pigs with and without lymphadenitis,^{7,12} and, more recently, in lymph nodes of wild boars (*Sus scrofa*) in Hungary¹⁰ and Brazil.¹⁵ Furthermore, virulent *R. equi* strains also have been described in HIV-negative human patients.¹¹ Despite similarities of virulence-associated plasmid profiles of *R. equi* reported for humans, pigs, and wild boars, the role of these animal species in transmission of the microorganism to humans is controversial.^{10,12} In addition, the history of contact between persons infected with *R. equi* and domestic pig breeders, or an environment in which pigs are present is unclear.¹⁵ Evidence supports the hypothesis that consumption of pork products or undercooked pork may be a probable route of *R. equi* infection in humans in some countries.²

Rhodococcus equi infections in pigs and wild boars are restricted mainly to the lymphatic system, and are predominantly found in submaxillary lymph nodes.^{7,10} A recent investigation of *R. equi* strains from lymph nodes of domestic pigs and wild boars in Brazil showed that the VapB type 8 plasmid is the most common virulence type.¹⁵ Distinct 79–100 kb plasmids associated with expression of VapB in *R. equi* isolated from humans have been described in different countries and involve mainly types 1, 4, 7,² and 5.¹² Ours results indicate the exclusive presence of the type 8 plasmid among intermediately virulent isolates from humans. Interestingly, this predominant type 8 VapB plasmid has not been reported in cases of human rhodococcosis. Thus, to our knowledge, the present report is the first description of HIV-positive patients infected with *R. equi* containing the VapB type 8 plasmid. None of the patients in our study had a history of contact with pigs or pig products. However, the fact that the type 8 virulence plasmid is found in humans, pigs, and wild boars in Brazil provides information about the source and route of *R. equi* infections in humans in this country.

For humans, exposure to soil contaminated with equine manure has been suggested as a route of transmission of virulent strains.^{3,11} The virulence of 41 *R. equi* isolates from foals in Brazil showed that all isolates were VapA positive: 33 strains contained the 87-kb type I plasmid, 6 contained the 85-kb type I plasmid, and 2 contained proposed new variants.⁶ Our results showed the same types of VapA (87-kb type I and 85-kb type I) in four patients, including those with and without AIDS. In addition, some of these patients had a history of contact with equines and/or farm environments. This circumstantial evidence suggests that exposure to equines and their environment may play an important role in cases of human rhodococcosis in Brazil.

Although our study focused on virulence-associated plasmid profiles of *R. equi*, some epidemiologic aspects of the cases were evaluated, in addition to contact between persons and livestock and/or farm environments. No sex or age predispositions have been found to be determinants of human rhodococcosis.¹ In the present study, six patients were men 30–50 years of age. This finding is consistent with results of other studies, which have also identified similar frequencies

of rhodococcosis in men 30–50 years of age,^{5,14} indicating an occupational risk of human infection by *R. equi*, particularly to immunocompromised patients exposed to livestock or farm environments.^{1,11}

The clinical manifestation of *R. equi* in humans is diverse, although pulmonary infections are present in approximately 80% of cases.³ In the current study, *R. equi* was also isolated predominantly from pulmonary infections. Interestingly, two of our field strains were isolated from asymptomatic farm workers, one from the nasal mucosa and one from the finger-nail region. This result provides further evidence that contact with soil or manure in contaminated farms or inhalation of the pathogen in dry or warm regions that facilitate aerosolization represent a risk factor for *R. equi* infections in humans.^{1,4,5,11}

Surveillance studies concerning virulence plasmid profiles of *R. equi* isolates from humans and domestic animals in different geographic areas are needed to determine the role of animals in transmission of the pathogen to humans, particularly because of the opportunistic behavior of this bacterium in immunocompromised patients.

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