IDENTIFICATION OF VIRULENCE-ASSOCIATED ANTIGENS AND PLASMIDS IN RHODOCOCCUS EQUI FROM PATIENTS WITH ACQUIRED IMMUNE DEFICIENCY SYNDROME AND PREVALENCE OF VIRULENT R. EQUI IN SOIL COLLECTED FROM DOMESTIC ANIMAL FARMS IN CHIANG MAI, THAILAND

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Abstract. The prevalence of virulent Rhodococcus equi in soil collected from 17 domestic animal farms (from 12 cattle, 1 pig, and 4 horse farms) and in 6 clinical specimens from patients with acquired immune deficiency syndrome (AIDS) in Chiang Mai, Thailand, was investigated. The isolates were tested for the presence of 15–17-kDa antigens (VapA) and a 20-kDa antigen (VapB) by immunoblotting and for the presence of virulence plasmid DNA. Rhodococcus equi was isolated from most soil samples (68 of 80) obtained from the 17 farms, with 2.0 × 10^5 to 6.0 × 10^7 colony-forming units per gram of soil. We detected VapA in none of the 537 isolates from the soil samples. In one isolate from a pig farm, both VapB and virulence plasmid DNA were detected. Of the 6 clinical isolates from patients with AIDS, however, 4 isolates contained both VapB and virulence plasmid DNA. The remaining 2 isolates were avirulent.

INTRODUCTION

Rhodococcus equi is a facultative, intracellular, gram-positive coccobacillus that causes suppurative pneumonia and ulcerative enteritis in foals aged 1–3 months. Rhodococcus equi has been identified as an increasingly common opportunistic pathogen of immunocompromised hosts such as people infected with the human immunodeficiency virus (HIV): the reported incidence of R. equi pneumonia in patients with acquired immune deficiency syndrome (AIDS) is on the rise. At least 3 virulence levels of R. equi strains have been identified: virulence, intermediate virulence, and avirulence. Virulent R. equi is characterized by the presence of virulence-associated 15- to 17-kDa antigens (VapA), virulence plasmid DNA of 85–90 kb, and suppurative pneumonia in foals (murine 50% lethal dose [LD50] = 10^6). Rhodococcus equi strains of intermediate virulence are identified by a virulence-associated 20-kDa antigen (VapB) and virulence plasmid DNA of 79–100 kb and are found in the submaxillary lymph nodes of pigs (murine LD50 = 10^7). In comparison, avirulent R. equi shows evidence of neither virulence-associated antigens nor plasmid DNA (murine LD50 > 10^9).

Inhalation of R. equi is probably the main route of exposure in foals. The primary source of infection is the soil. The route of infection in humans remains unknown, although contact with farm animals and manure has been reported in a third of cases. The majority of R. equi isolates from patients with AIDS are virulent (VapA or VapB positive), whereas most isolates from immunocompromised patients without AIDS are avirulent.

Thailand has one of the highest rates of HIV-AIDS in the world. It is estimated that 1 million Thais have already been infected with HIV. Pulmonary infection is a major cause of morbidity and mortality in HIV-infected patients. In Thailand, Mycobacterium tuberculosis, Pneumocystis carinii, and Penicillium marneffei are major etiological agents of such pulmonary infection. Infection by R. equi has also been reported. Little is known, however, about the pathogenicity and the source of R. equi in Thailand.

The purpose of the present study was to investigate the virulence-associated antigens and plasmids in R. equi isolated from patients with AIDS and the prevalence of virulent R. equi isolated from soil collected from domestic animal farms in Chiang Mai, Thailand, to substantiate a possible zoonotic route of infection of R. equi in AIDS.

MATERIALS AND METHODS

Bacterial strains. Strains ATCC 33701 (equine origin, virulent strain) and strain 5 (human origin, strain of intermediate virulence) were used as reference strains because some of the protein profiles, plasmid characteristics, and virulence levels of these strains have been described. In addition, 6 clinical isolates obtained from patients with AIDS were examined. These bacteria were harvested from various lesions examined in the Department of Microbiology, Faculty of Medicine, Chiang Mai University.

Collection and isolation of R. equi from soil. Eighty soil samples were collected from 4–12 sites from 17 farms (12 cattle, 1 pig, and 4 horse farms) in Chiang Mai in March 2000. These farms were randomly selected and were not related in any obvious way to HIV-infected patients with R. equi infection. Soil was scraped from the ground with a small spoon and poured into sterile tubes. One gram of soil was diluted serially with a 10-fold volume of sterile saline. Each dilution was inoculated onto 2 plates of nalidixic acid-novobiocin-actidione (cycloheximide)-potassium tellurite medium, as described by Woolcock and others: the plates were incubated at 30°C for 2–3 days. Rhodococcus equi colonies were counted, and the number of viable organisms per
gram of soil was calculated. Three to 10 colonies of *R. equi* per specimen were subcultured and examined for VapA and VapB by colony blot enzyme-linked immunosorbent assay with monoclonal antibodies.\textsuperscript{8,18} For colony blot analysis, bacterial strains were injected onto brain-heart infusion agar plates with an inoculation needle and incubated at 38°C for 24 hr. A nitrocellulose filter (pore size 0.45 mm, BAS 85; Schleicher and Schuell, Dassel, Germany) was then placed over the cultures for a few minutes to wet them completely. The membrane was removed, air dried, and treated by autoclaving at 105°C for 1 min. All buffers, antibodies, and conjugate dilutions were as described previously for the immunoblot.\textsuperscript{8,18}

**Isolation of plasmid DNA.** Plasmid DNA was isolated from *R. equi* by the alkaline lysis method,\textsuperscript{20} with some modifications as described previously.\textsuperscript{18} Plasmid DNAs were analyzed by digestion with restriction endonucleases *Eco*RI and *Eco*T22.\textsuperscript{4,21} Samples of the plasmid preparations were separated in 0.7 or 1.0% agarose gels at 5 V/cm for 2 hr.

**Virulence-associated gene.** The target DNAs for polymerase chain reaction (PCR) amplification were the published sequences of the 15- to 17-kDa antigen (VapA) gene and a 20-kDa antigen (VapB) gene (GenBank accession numbers D21236l and D44469) from *R. equi* strains ATCC 33701 and 5, respectively. Primer 1 (5′-GACTCTTTCACAA-GACGGTTGTGCCAGCTA-3′) corresponded to the sense strand at position 6 to 23, and primer 2 (5′-TAGGCCTTGGTGCCAGCTA-3′) corresponded to the antisense strand at position 569 to 552 in the sequence of the 15- to 17-kDa antigen gene.\textsuperscript{22} Primer 3 (5′-AACGGTACTGGCGGTGAGAA-3′) corresponded to the sense strand at position 240 to 258, and primer 4 (5′-ACGGAGCTTGGACCGACTA-3′) corresponded to the antisense strand at position 1066 to 1048 in the sequence of the cloned fragment containing the 20-kDa antigen gene.

Polymerase chain reaction amplification was performed with 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 MgCl\textsubscript{2}, 0.2 mM (each) deoxynucleotide triphosphates, 1 mM of each primer, and 2.5 U of Taq DNA polymerase, (Takara, Tokyo, Japan), as described previously.\textsuperscript{23} The samples were subjected to 30 cycles of amplification in a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT). The cycling conditions were as follows: denaturation for 90 sec at 94°C; primer annealing for 1 min at 55°C; and extension for 2 min at 72°C.\textsuperscript{23}

**RESULTS**

**Isolation of virulent *R. equi* from soil samples collected from 17 farms in Chiang Mai.** Quantitative culture of *R. equi* was achieved for the 80 soil samples from 17 farms, and results are shown in Table 1. Sixty-eight (85%) of 80 soil samples were culture-positive for *R. equi*. The number of *R. equi* from these soil samples ranged from $2.0 \times 10^2$ to $6.0 \times 10^3$ per gram of soil. Three to 10 colonies per positive sample were subcultured, yielding 537 colonies for analysis. Colony blotting revealed VapB in 1 (1.1%) of 91 isolates from a pig farm. The other isolates did not express any virulence-associated antigen (Table 2).

**Protein and plasmid profiles of *R. equi* isolates from patients with AIDS and soil.** Of 6 clinical isolates from patients with AIDS, 4 were positive for VapB and virulent plasmid DNA. The remaining 2 isolates were negative for both virulence-associated antigens and plasmid.

The 6 clinical isolates (isolates 32, 48, 60, 62, 63, and 64) and the colony blot-positive isolate (isolate 70) from soil were then analyzed by PCR. Five strains expressing VapB and a positive control, strain 5, gave positive results, showing an 827-bp product of the expected size in the PCR amplification (Figure 1). Plasmid DNA preparations of the 5 isolates were analyzed further by restriction enzyme digestion with *Eco*RI and *Eco*T22 (results shown in part in Figure 2). The 4 clinical isolates showed 3 restriction enzyme digestion patterns, which have been previously reported in AIDS and pig isolates,\textsuperscript{4,21} 79 to 88.5 kb in size (Table 3).

**DISCUSSION**

Domestic animals are involved in the epidemiology of many AIDS-associated infectious diseases and several other

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### Table 1

<table>
<thead>
<tr>
<th>Farm</th>
<th>No. farms</th>
<th>No. samples</th>
<th>Positive cultures, n (%)</th>
<th>No. <em>R. equi</em> g of soil (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>12</td>
<td>39</td>
<td>33 (84.6)</td>
<td>$2.0 \times 10^2$–$6.0 \times 10^3$</td>
</tr>
<tr>
<td>Pig</td>
<td>1</td>
<td>12</td>
<td>10 (83.3)</td>
<td>$7.5 \times 10^2$–$4.7 \times 10^3$</td>
</tr>
<tr>
<td>Horse</td>
<td>4</td>
<td>29</td>
<td>25 (86.2)</td>
<td>$1.3 \times 10^2$–$1.8 \times 10^3$</td>
</tr>
</tbody>
</table>

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### Table 2

Monoclonal-based colony blotting detection of VapA and VapB in *Rhodococcus equi* isolates from animal farm soil samples and from patients with AIDS

<table>
<thead>
<tr>
<th>Source</th>
<th>No. isolates</th>
<th>No. VapA-positive isolates</th>
<th>No. VapB-positive isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle farms</td>
<td>280</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pig farm</td>
<td>71</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Horse farms</td>
<td>166</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Patients with AIDS</td>
<td>6</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

* AIDS = acquired immune deficiency syndrome; VapA = 15–17-kDa antigens; VapB = 20-kDa antigen.

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**FIGURE 1.** Agarose gel electrophoresis of polymerase chain reaction products obtained after amplification of DNAs from 6 clinical isolates and 1 soil isolates of *Rhodococcus equi* with primers. Lane 1, isolate 32; lane 2, isolate 48; lane 3, isolate 60; lane 4, isolate 62; lane 5, isolate 63; lane 6, isolate 64; lane 7, isolate 70; lane 8, strain 5. The markers (lane M) are *Hae*III digestion products of bacteriophage ΦX174 DNA.
opportunistic infections in HIV-infected people.24 However, the role of domestic animals in the transmission of most of these diseases has not been fully clarified.5,11,12 In the present study, a majority (4 of 6) of the clinical isolates of \textit{R. equi} from the patients with AIDS expressed VapB and were of intermediate virulence. Soil from one pig farm in Chiang Mai was contaminated with \textit{R. equi} of intermediate virulence. Our results suggest contaminated soil to be a possible source of \textit{R. equi} infection in patients in Chiang Mai because the majority of the patients of Chiang Mai University Hospital were laborers or farmers living in rural northern Thailand.15

The natural habitat of \textit{R. equi} is soil, particularly soil associated with the manure of herbivores.25 Exposure to soil contaminated with herbivore manure is likely the major route for both animal and human infection.1 People exposed to horses, livestock farmers, and gardeners might have a higher risk of infection.1,5 Our previous study demonstrated that the majority (21 of 29, 72%) of \textit{R. equi} isolates from patients with AIDS in 5 countries (Australia, France, Italy, Denmark, and the United States) were of virulent and intermediate virulent strains (7 of 29, 24%) and expressed VapA, and 14 (48%) of 29 expressed VapB.8,13 Contact with farm animals or manure has been reported in about a third of human cases.8,11–13 In the present study, VapA was not detected in the clinical or environmental isolates. Four (67%) of the 6 clinical isolates and 1 of the 91 environmental isolates were \textit{R. equi} of intermediate virulence and were VapB positive. To determine whether pig \textit{R. equi} is the source of infection in patients with AIDS with \textit{R. equi}–associated bacteremia or pneumonia, we are now investigating the environment of patients with AIDS who live or work near pigs.

There are at least 10 distinct plasmids, 79 to 100 kb in size, from human and pig isolates that are associated with expression of the 20-kDa antigen (VapB).8,21,26 We previously demonstrated that human isolates contain 1 of 4 distinct plasmids (79-, 87-, 95- and 100-kb plasmids).3 In the present study, 2 of 4 human isolates contained a 79-kb plasmid; the remaining 2 isolates contained an 88- or 88.5-kb plasmid found in Japanese pig isolates.21,26 Furthermore, we found a new plasmid in a soil isolate from a pig farm in Chiang Mai. Surveillance studies are needed to determine the prevalence of \textit{R. equi} in the submaxillary lymph nodes of pigs and the incidence of environmental contamination of virulent \textit{R. equi} in Chiang Mai.

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REFERENCES


<table>
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<tr>
<th>Isolation no.</th>
<th>Original no.</th>
<th>Source</th>
<th>Plasmid size and type</th>
<th>Reference</th>
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<tbody>
<tr>
<td>32</td>
<td>HIS0606596</td>
<td>Blood</td>
<td>79 kb</td>
<td>Takai and others8</td>
</tr>
<tr>
<td>62</td>
<td>Sp1441099</td>
<td>Blood</td>
<td>88.5 kb</td>
<td>Takai and others21</td>
</tr>
<tr>
<td>63</td>
<td>Sp10812998</td>
<td>Sputum</td>
<td>79 kb</td>
<td>Takai and others21</td>
</tr>
<tr>
<td>64</td>
<td>HIS121299</td>
<td>Blood</td>
<td>88 kb</td>
<td>Takai and others21</td>
</tr>
<tr>
<td>70</td>
<td>Pig farm no. 7</td>
<td>Soil</td>
<td>New type</td>
<td>Present study</td>
</tr>
</tbody>
</table>

**TABLE 3**

Plasmid profiles of \textit{Rhodococcus equi} isolates expressing VapB.


