Molecular Epidemiology of *Rhodococcus equi* Based on *traA*, *vapA*, and *vapB* Virulence Plasmid Markers

Alain A. Ocampo-Sosa,1,2 Deborah A. Lewis,1 Jesús Navas,4 Frances Quigley,3 Raquel Callejo,6 Mariela Scotto,1,5 Desmond P. Leadon,2 Ursula Fogarty,7 and José A. Vázquez-Boland1,5

1Bacterial Molecular Pathogenesis Group, Faculty of Medical and Veterinary Sciences, University of Bristol, Bristol, United Kingdom; 2Irish Equine Centre, Johnstown, Naas, and 3Central Veterinary Research Laboratory, Backweston Campus, Celbridge, Ireland; 4Departamento de Biología Molecular, Facultad de Medicina, Universidad de Cantabria, Cantabria, and 5Grupo de Patogénesis Molecular Bacteriana, Universidad de León, León, Spain; 6Servicio Bacteriología Especial, Instituto Nacional de Enfermedades Infecciosas “Dr. Carlos G. Malbrán,” Buenos Aires, Argentina

Molecular typing of the actinomycete *Rhodococcus equi* is insufficiently developed, and little is known about the epidemiology and transmission of this multihost pathogen. We report a simple, reliable polymerase chain reaction typing system for *R. equi* based on 3 plasmid gene markers: *traA* from the conserved conjugal transfer machinery and *vapA* and *vapB*, found in 2 different plasmid subpopulations. This “TRAVAP” typing scheme classifies *R. equi* into 4 categories: *traA*/ *vapA* B+, *traA*/ *vapA* B−, *traA*/ *vapAB*+, and *traA*/ *vapAB*− (plasmidless). A TRAVAP survey of 215 *R. equi* strains confirmed the strong link between *vapB* (*traA*/ *vapAB*+ plasmids) and horse isolates and revealed other host-related plasmid associations: between *traA*/ *vapAB*+ and pigs and between *traA*/ *vapAB*−—a new type of *R. equi* plasmid—and cattle. Plasmidless strains were more frequent among isolates from nonpathological specimens. All plasmid categories were common in human isolates, which possibly reflects the predominantly opportunistic nature of *R. equi* infection in this host and a zoonotic origin.

The soilborne actinomycete *Rhodococcus equi* is an opportunistic pathogen capable of infecting a wide range of animal hosts, including humans. *R. equi* pulmonary and bacteremic infections occur primarily in immunocompromised patients, particularly in patients with AIDS and organ transplant recipients [1–5]. In the veterinary field, this organism is typically isolated from young foals with suppurative bronchopneumonia or mesenteric lymphadenitis, but it is increasingly being identified as the causative agent of pulmonary and extrapulmonary infections in other animal species [6–12]. *R. equi* is ubiquitously distributed in the environment worldwide [13, 14].

The virulence of *R. equi* is associated with the 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 unknown mechanisms [6, 13–17]. Most equine isolates are *VapA*+ (or *vapA*+), whereas strains with this marker are much less frequent among isolates from humans and other animal species [18]. In nonhorse hosts, a variant plasmid encoding VapB, a VapA-related surface antigen of larger size (18–20 kDa), has been identified [19–21]. Lack of detection of VapA/B proteins (or *vapA/B* DNA sequences) is relatively common in nonhorse isolates [22] and is assumed to be due to an absence of virulence plasmids. However, these strains may carry other types of uncharacterized *R. equi* virulence-associated extrachromosomal replicons.

Although it is easy to distinguish between VapA and VapB plasmid types by current polymerase chain reaction (PCR)–based methods, the unambiguous dis-
crimination between the presence or absence of a plasmid in \(vapA^-\) and \(vapB^-\) (\(vapAB^-\)) \(R.\ equi\) isolates is less straightforward. This determination relies on plasmid DNA extraction and visualization [2, 10, 11, 15] using methods that are cumbersome, time-consuming, and in some cases unreliable unless very carefully standardized and optimized. Indeed, the extraction of virulence-associated plasmids from \(R.\ equi\) is difficult and practiced by only a few laboratories worldwide.

We report here a new “universal” plasmid marker for \(R.\ equi\) that facilitates the rapid discrimination between plasmid-positive and plasmid-negative strains by PCR. This PCR-based method targets the \(traA\) gene encoding a protein of the conserved conjugal transfer machinery of \(Rhodococcus\) species plasmids [23–25]. We incorporated this marker into a novel multiplex PCR assay with new \(vapA\) and \(vapB\) target sequences, making it possible to classify \(R.\ equi\) isolates into 4 major plasmid categories of epidemiological significance.

**MATERIALS AND METHODS**

**Bacterial strains, growth conditions, and chemicals.** We used a total of 215 \(R.\ equi\) isolates, including 4 reference strains and 211 field isolates from different sources (69 human, 59 horse, 30 pig, 25 bovine, 19 soil, 2 sheep, 2 goat, 1 cat, 1 dog, 1 pheasant, 1 iguana, and 1 primate) and countries (Argentina, Australia, Brazil, Canada, Czech Republic, Dominican Republic, France, Germany, Hungary, Ireland, Japan, Mexico, Slovenia, Spain, Thailand, the United Kingdom, and the United States) (table 1). The 4 reference isolates were the genome sequence strain 103S, ATCC 33701, and their corresponding isolates from early stationary-phase broth cultures of \(R.\ equi\) using the alkaline lysis method [13], as follows. Bacteria were grown with vigorous rotary shaking (250 rpm) in horizontally set 15-mL screw-cap tubes that contained 10 mL of BHI broth; they were harvested by centrifugation at 11,000 \(g\) for 10 min at 4°C, washed with 1× TBE (pH 7.5), transferred to 2-mL plastic microtubes, and centrifuged again under the same conditions. The bacterial pellets were resuspended in 0.2 mL of SETL solution (15% sucrose, 10 mmol/L EDTA, 25 mmol/L Tris-HCl [pH 8], and 5 mg/mL lysozyme) and incubated for 2–4 h at 37°C with shaking at 100 rpm, after which 0.4 mL of lysis solution (0.2 mol/L NaOH and 1% SDS) was added. The microtubes were inverted 250 times until a homogeneous viscous suspension was obtained; then, 0.3 mL of prechilled potassium acetate (5 mol/L) was added, and microtubes were inverted again 100 times and left on ice for 30 min. The mixtures were then centrifuged at 17,000 \(g\) for 10 min at 4°C, the supernatants were transferred to fresh 2-mL microtubes, and 10 \(\mu\)L of a 10 mg/mL solution of DNase-free RNase (Qiagen) was added. After incubation for 30 min at 37°C, the lysates were extracted with 0.5 mL of phenol-chloroform-isoamyl alcohol (25:24:1) by gently inverting 100 times and then centrifuged for 10 min at 17,000 \(g\) and 4°C. The upper phase was carefully transferred to a fresh tube, and the plasmid DNA was precipitated by the addition of 0.5 mL of isopropanol. The precipitates were pelleted by centrifugation under the same conditions, the supernatant was carefully decanted, and the plasmid DNA was washed twice with 1 mL of 70% ethanol. Excess ethanol was removed by centrifugation at 15,000 \(g\) and aspiration, and the plasmid DNA pellet was air dried for 15–20 min and resuspended in 20 \(\mu\)L of nuclease-free distilled water.

Plasmid DNA from \(R.\ equi\) strains was digested overnight with EcoRI endonuclease (New England Biolabs). Restriction fragments were resolved by electrophoresis in 1% agarose gels for 16 h at 40 V and were then transferred onto positively charged nylon membranes (Roche Diagnostics) by capillary action [28]. For Southern blotting, the \(traA\) probe was labeled and detected on blots by a luminescence-based method using the DIG-High Prime Labeling and Detection Kit (Roche Diagnostics) in accordance with the manufacturer’s instructions. PCR products were purified using the QIAquick purification kit (Qiagen). DNA was sequenced on both strands at AGOWA (Berlin). Sequences were assembled with Vector NTI (version 9.0.0; Invitrogen) and analyzed with BLAST software [29].

**R. equi plasmid minipreparation.** Plasmid DNA was isolated from early stationary-phase broth cultures of \(R.\ equi\) using the alkaline lysis method [13], as follows. Bacteria were grown with vigorous rotary shaking (250 rpm) in horizontally set 15-mL screw-cap tubes that contained 10 mL of BHI broth; they were harvested by centrifugation at 11,000 \(g\) for 10 min at 4°C, washed with 1× TBE (pH 7.5), transferred to 2-mL plastic microtubes, and centrifuged again under the same conditions. The bacterial pellets were resuspended in 0.2 mL of SETL solution (15% sucrose, 10 mmol/L EDTA, 25 mmol/L Tris-HCl [pH 8], and 5 mg/mL lysozyme) and incubated for 2–4 h at 37°C with shaking at 100 rpm, after which 0.4 mL of lysis solution (0.2 mol/L NaOH and 1% SDS) was added. The microtubes were inverted 250 times until a homogeneous viscous suspension was obtained; then, 0.3 mL of prechilled potassium acetate (5 mol/L) was added, and microtubes were inverted again 100 times and left on ice for 30 min. The mixtures were then centrifuged at 17,000 \(g\) for 10 min at 4°C, the supernatants were transferred to fresh 2-mL microtubes, and 10 \(\mu\)L of a 10 mg/mL solution of DNase-free RNase (Qiagen) was added. After incubation for 30 min at 37°C, the lysates were extracted with 0.5 mL of phenol-chloroform-isoamyl alcohol (25:24:1) by gently inverting 100 times and then centrifuged for 10 min at 17,000 \(g\) and 4°C. The upper phase was carefully transferred to a fresh tube, and the plasmid DNA was precipitated by the addition of 0.5 mL of isopropanol. The precipitates were pelleted by centrifugation under the same conditions, the supernatant was carefully decanted, and the plasmid DNA was washed twice with 1 mL of 70% ethanol. Excess ethanol was removed by centrifugation at 15,000 \(g\) and aspiration, and the plasmid DNA pellet was air dried for 15–20 min and resuspended in 20 \(\mu\)L of nuclease-free distilled water.

**Table 1. List of Rhodococcus equi strains used in the study and individual polymerase chain reaction (PCR) results.**

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases.*
**PCR primers and conditions.** The oligonucleotide primers used in the study are shown in table 2; they were purchased from Metabion. Primers were designed using CLUSTALW multiple alignments (European Bioinformatics Institute; http://www.ebi.ac.uk) of target gene homologs deposited in databases, and their specificity was determined in silico using BLAST software [29]. PCR reagents were purchased from Biotools. The reaction mixtures consisted of 1–5 μL of DNA preparation, 0.3 μmol/L each primer, 0.2 mmol/L dNTPs, 2.5 μL of 10× amplification buffer, 1 U of Taq DNA polymerase, and double-distilled water added to a final volume of 25 μL. PCR was performed in a PTC-200 thermocycler (MJ Research). The PCR mixture was subjected to initial denaturation for 5 min at 95°C, followed by 30 cycles of denaturation for 30 s at 95°C, primer annealing for 30 s at 56°C, and extension for 2 min at 72°C, with an additional extension step of 10 min at 72°C after the final cycle. The analytical sensitivity (detection limit) of each of the PCR assays was determined with 10-fold serial dilutions of total DNA from strains 103S and PAM 1220 (table 1).

**Statistical analysis.** The proportions of the different types of plasmid were compared using contingency tables and 2-tailed χ² tests or Fisher’s exact test if the expected cell value was <5 observations. The threshold for statistical significance was P < .05.

**RESULTS AND DISCUSSION**

**Design of the traA PCR.** Suitable targets for the development of a PCR assay for detecting plasmid-positive *R. equi* isolates should be present in all plasmid variants from this species. The only plasmid sequence available for *R. equi*, from the identical replicons p33701 and p103 [23], contains several conjugal transfer genes. We chose a target from among these genes, traA, that encodes a putative relaxase/helicase conserved in other conjugal extrachromosomal elements from *Rhodococcus* species [24, 25], on the assumption that all *R. equi* plasmids are related to p33701/p103 and share a similar conjugal transfer mechanism. From the p33701/p103 sequence (GenBank accession numbers AP001204 and AF116907), we designed the oligonucleotide primers TraA-F1 and TraA-R1, which amplify a 959-bp section of the traA gene. The TraA-F1 forward primer and the TraA-R1 reverse primer target nucleotide positions 1446–1465 and 2386–2405 of the traA gene, respectively. The primers were tested using total DNA from strain 103S and gave the expected amplicon with a detection limit of 10 pg. They were then pilot tested on a panel of 15 *R. equi* strains previously identified as vapA⁺ vapA⁻, vapA⁺ vapB⁻, or vapAB⁻ (n = 5 each) using established PCR assays (i.e., primers IP1/IP2 for vapA [30] and H1/H2 for vapB [21]; table 2). We previously determined, by alkaline extraction and agarose gel electrophoresis, that the 5 vapAB⁺ strains contained plasmid material (see Materials and Methods). The 15 strains yielded 959-bp amplicons, and the sequencing of these confirmed that they all corresponded to the same traA gene fragment. Sequence identity to p33701/103 traA was 98%–100%, which indicated that this gene is highly conserved in *R. equi* plasmids of different types.

We next tested the TraA-F1/R1 primer pair on 200 additional *R. equi* isolates from different sources. These isolates had previously been screened by the PCR tests for vapA and vapB described above. All strains giving a positive reaction for vapA or vapB (n = 124) were also positive by traA PCR, as determined by detection of the expected 959-bp amplicon. Of the 89 vapAB⁻ strains, 40 (44.9%) tested positive with traA, which indicated that they could also contain a plasmid.

**Validation of the traA PCR as a “presence-of-plasmid” marker in *R. equi.* We assessed the performance of the traA PCR using plasmid extraction and visualization as the reference

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method. All of the \textit{traA}+ strains were found to contain extrachromosomal elements on examination of EcoRI digests of plasmid minipreparations, equivalent to a specificity of 100%. Such elements were readily detectable for most \textit{vapA}+ or \textit{vapB}+ strains. However, the visualization of plasmid material from \textit{traA}/\textit{vapAB}+ strains was more problematic, given that, in most cases, only a faint banding pattern was observed in agarose gels (see profiles of strains PAM 1681 and 1682 in figure 1, top). We confirmed the presence of a plasmid in these strains by Southern-blot hybridization using a \textit{traA} probe (figure 1, bottom). No \textit{traA}+ strain was found to contain plasmid material by either agarose gel electrophoresis or Southern blot except in one case, corresponding to a sensitivity of 99.39%. The only \textit{traA}− (PCR) strain in which a plasmid was detected (PAM 1651) was a human isolate. This plasmid had an EcoRI pattern and estimated size (70–90 kb) similar to that of other \textit{R. equi} plasmids; it also gave a positive \textit{traA} signal on Southern hybridization, in an EcoRI fragment of the same size (≈23 kb) as that normally seen in \textit{traA}+ (PCR) isolates (figure 1).

**Design of a multiplex \textit{traA}-\textit{vapA}-\textit{vapB} (TRAVAP) PCR.** The \textit{traA} “universal” plasmid marker, together with the \textit{vapA}- and \textit{vapB}-specific plasmid markers, classified \textit{R. equi} isolates into 4 categories of potential diagnostic and/or epidemiological value: \textit{traA}/\textit{vapA}+/\textit{vapB}−, \textit{traA}/\textit{vapA}−/\textit{vapB}+, and \textit{traA}/\textit{vapAB}−, all of which are associated with the presence of a plasmid; and \textit{traA}/\textit{vapAB}−, which is indicative of the absence of a virulence plasmid. We developed a multiplex assay incorporating the 3 markers in a single reaction to facilitate the application of this typing method.

Initial attempts to combine the \textit{TraA}-F1/R1 primer pair with the previously described primers for \textit{vapA} (IP1/IP2) [30] and \textit{vapB} (H1/H2) [21] (see above) were unsuccessful because primers H1 and IP2 amplified both \textit{vapA} and \textit{vapB} when used together and because, in some cases, primer IP1 amplified \textit{vapB}.

![Figure 1.](https://academic.oup.com/jid/article-abstract/196/5/763/838806/19676383816)
Figure 2. *Rhodococcus equi* virulence plasmid typing with *traA*-vapA-vapB (TRAVAP) multiplex polymerase chain reaction. The 4 TRAVAP profiles (indicated below) are illustrated with a selection of the *R. equi* isolates used in the study (PAM collection nos. indicated above; see table 1); strains PAM 1126 (103S) and PAM 1272 (plasmid-cured ATCC 33701) were used as positive and negative controls, respectively. M, 100-bp DNA ladder (New England BioLabs); reference sizes are indicated on the left and the size of the TRAVAP amplicons on the right (in base pairs).

Survey of *R. equi* field isolates with TRAVAP. We tested the PvpAF/PvapBF/PABR/TraA-F1/TraA-R1 primer mixture with the complete *R. equi* isolate collection and found results identical to those obtained in simplex format (table 1). An example of the amplicon profiles of the 4 categories of isolate distinguished on the basis of TRAVAP plasmid markers is shown in figure 2; a summary of the results obtained according to the origin of the isolates is shown in figure 3.

Three clear patterns emerged from the analysis of the data. First, we confirmed previous observations [18, 21] that the vapA+ and vapB+ markers are never associated, which indicates that the 2 genes (or plasmid types, by extension) do not occur together in the same isolate. This mutual exclusivity suggests that vapA and vapB are allelic variants of a same locus that has divergently evolved in 2 different plasmid subpopulations. Second, although most (86.4%) equine isolates had plasmids, these were never associated with a vapB+ marker, which suggests host (horse)-driven (counter)selection of the vapB-type plasmid. Third, and consistent with host-driven selection of plasmid types in *R. equi*, bovine isolates mostly (84.0%) had a plasmid, but none were associated with a vapA+ marker. A previous study of bovine isolates reported no positive reactions for vapA or vapB, and this was interpreted as indicating the absence of virulence plasmids [9]. Our data clearly show that most bovine isolates do carry plasmids and that most of these (72%) are...
traA'/vapAB', reflecting the existence of a specific plasmid type associated with the bovine host. Almost all equine and bovine isolates derived from bona fide clinical samples associated with clear pathology: purulent bronchopneumonia or mesenteric abscesses in horses and tuberculous-like granulomatous lymphadenitis in various locations in cattle (table 1).

Consistent with previous observations [11, 31], pig isolates were predominantly associated (70.0%; P = .0001) with vapB-type plasmids (figure 3). This finding, while indicating an association between the vapB+ marker and pigs, is of unclear significance in terms of invasive infection, because most of these isolates were derived from submaxillary lymph nodes without macroscopic lesions (table 2). Interestingly, no vapA+ (“horse-type”) or vapAB+ (“bovine-type”) plasmids were identified in pigs, pointing again to host-driven selection of plasmid types in R. equi. A greater percentage of pig strains (30.0% vs. 13.6% in horse isolates and vs. 16.0% in bovine isolates) lacked plasmids according to traA PCR results, which possibly reflects their isolation from apparently healthy carriers (in which the selective pressure to maintain a virulence plasmid would be expected to be weaker).

Isolates from soil were either vapA+ (52.6%) or plasmidless (47.4%), consistent with these isolates being from horse-related environments and with the lack of importance of virulence plasmids for R. equi survival outside the host [6, 32]. Human strains, all of which had been isolated from pathological specimens (table 1), differed from the rest of the isolates in that all 4 TRA V AP plasmid categories were identified. This indicates that human hosts are susceptible to a variety of R. equi strains, with or without plasmids. The frequency of plasmids, as assessed from the traA+ marker, was lower than that in pathological specimens from horses and cattle (76.8% vs. 86.4% and 84.0%, respectively), consistent with the primarily opportunistic nature of R. equi infection in humans and the dispensability of virulence plasmids in these conditions [6, 21].

Conclusions. We have demonstrated the value of a new R. equi gene marker, traA, for rapid discrimination by PCR between plasmid-positive and plasmid-negative isolates. By integrating this marker with vapA and vapB markers, we have developed a useful PCR-based typing method. The application of this system to field isolates of R. equi suggested clear associations between specific plasmid types and animal hosts. Thus, plasmid type traA'/vapAB' is characteristic of horse isolates, traA'/vapA' B' is associated with pig isolates, and traA'/vapAB' is characteristic of bovine isolates, defining a novel host-specific virulence plasmid type in R. equi. Among plasmid-positive (traA+) strains, no vapB+ marker was found in horse isolates, no vapA+ marker was found in bovine isolates, and no vapAB+ marker was identified among pig isolates, which suggests host-driven exclusion of specific plasmid types. Humans, by contrast, appear to be susceptible to all R. equi TRA V AP categories. The presence in human isolates of plasmid types characteristic of specific animal hosts has possible public health implications, because it suggests that R. equi infection is zoonotic. Indeed, our preliminary analyses indicate that between 44% and 80% of human isolates have EcoRI plasmid restriction profiles identical to those found in animal isolates, consistent with a transmission flux of R. equi between animals and humans. TRA V AP is thus a useful entry-level tool for the molecular epidemiological analysis of R. equi.

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