Prevalence of the Virulence-Associated Gene of *Rhodococcus equi* in Isolates from Infected Foals

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*Rhodococcus equi* is a major cause of disease in foals worldwide, and the incidence of pneumonia due to *R. equi* infection appears to be increasing in all breeds (3). Morbidity rates between 5 and 17% have been reported, with mortality rates between 40 and 80% (1).

Strains of *R. equi* which are virulent for mice possess a plasmid of 80 to 90 kb and can be cured of these plasmids by growth at 38°C (9, 10). Such cured strains are less virulent for mice (9), and an isolate lacking the plasmid has been shown to be less able to survive and replicate in murine and equine macrophages than a strain possessing the plasmid (2). The similarity of 85-kbp plasmids in 20 isolates of *R. equi* from horses has been shown by restriction endonuclease digestion (12). These plasmids carry the gene *vapA*, which encodes a virulence-associated lipoprotein of 15 to 17 kDa (4, 11). This lipoprotein is a prominent antigen recognized by antibodies from diseased foals, and in limited studies a high proportion of isolates from infected foals have been found to express it (5, 12). Thus, there is circumstantial evidence for the involvement of this lipoprotein in virulence. Recent work resulted in the development of a PCR assay for rapid identification of *vapA* in isolates of *R. equi* (6), and studies performed on environmental isolates from horse farms have suggested an association between the proportion of environmental isolates carrying the virulence plasmid and the incidence of disease (6).

The purpose of this study was to use the PCR assay reported previously (6) to determine the prevalence of *vapA* in a large collection of clinical isolates of *R. equi* from foals, enabling the significance of this gene in virulence to be better established, and to establish the utility of the published assay for the detection of clinically significant strains. In addition, the degree of sequence conservation of this gene among isolates from several different horse farms over 5 years was examined.

**Bacterial isolates.** *R. equi* was isolated from tracheal washings showing signs of purulent bronchopneumonia by cytological examination or from pus samples collected from abscesses. The isolates were collected over the period 1991 to 1996 and came from foals on 29 different Australian Thoroughbred horse farms (Table 1). Isolates were identified as *R. equi* if the colonies were irregular, 3 to 5 mm in diameter, smooth, and very mucoid and translucent after 48 h at 37°C, if they developed a salmon-pink color with age, and if the organisms from these colonies were gram-positive pleomorphic rods. Isolates were stored at 4°C for up to 5 years as nutrient agar stab cultures. The capsular types of the isolates were not determined.

**PCR assays.** PCR was performed on emulsified bacterial colonies with the primers and under the conditions described by Takai and colleagues (6). The primers (5′ to 3′) were GAC TCTTCACAAGACGGT and TAGGCGTTGTGCCAGCTA, corresponding to bases 6 to 23 and 569 to 552, respectively, of the *vapA* gene sequence. An individual colony of each isolate was gathered with a sterile toothpick and emulsified in 25 μl of reaction mixture containing a 400 μM concentration of each dNTP, 0.4 U of *Taq* polymerase (Promega), a 2.5 μM concentration of each primer, and 3 mM MgCl₂ in the buffer supplied by the manufacturer. PCR was performed in an Omnogene thermal cycler (Hybaid Ltd., Teddington, United Kingdom) programmed for 40 cycles of 94°C for 1.5 min, 57°C for 1 min, and 72°C for 2 min with a tube temperature control.

Each reaction was examined for the production of a DNA fragment of approximately 563 bp by electrophoresis through a 1% agarose gel followed by staining with ethidium bromide. A negative control was performed in parallel with each group of samples.

**Restriction endonuclease digestion of PCR products.** Sixty representative isolates were chosen for analysis by restriction endonuclease digestion of the *vapA* gene. At least one isolate was chosen from each horse farm, and where there were isolates over multiple years from cases on a single horse farm, at least one isolate was chosen from each year. PCR products were digested with *Hin*FI restriction endonuclease in a 10-μl reaction mixture by adding 2 μl of the PCR mixture to a tube containing 0.25 μl of enzyme, 1 μl of the 10× buffer supplied by the manufacturer, and 6.75 μl of water, and then incubating the mixture at 37°C for 2 h. The fragments generated by digestion were examined by silver staining after separation by electrophoresis through a 20% polyacrylamide gel.

The results from the examination of a selection of *R. equi* isolates for the presence of *vapA* by PCR are shown in Fig. 1. Of 154 isolates of *R. equi* examined by PCR for the presence of the *vapA* gene, 151 (98%) gave a product of approximately 560 bp, as would be expected for the amplification of the *vapA* gene. No negative control reactions generated a detectable product. The three isolates which did not give a product were characterized biochemically. They produced catalase and urease, and neither fermented lactose or glucose, thus supporting their characterization as *R. equi*.

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Hinfl restriction endonuclease digestion of the *vapA* gene PCR product are shown in Fig. 2. There was no evidence of sequence variation, either between isolates from the 29 different horse farms, or between isolates from the same horse farms over 5 consecutive years. The predicted sizes of the fragments generated by Hinfl digestion based on an examination of the published sequence of the *vapA* gene (4, 11) were 224, 189, 63, 34, 34, and 20 bp. This agrees closely with the estimated sizes of the fragments seen in Fig. 2, which were 246, 189, 55, and 27 bp.

In previous studies Takai and colleagues (5) found that 23 of 23 clinical isolates of *R. equi* possessed either an 85-kbp plasmid or a 105-kbp plasmid and all expressed the 17-kDa virulence-associated antigen. Similarly Tkachuk-Saad and Prescott (12) found that 21 of 22 clinical isolates from foals possessed an 80-kbp plasmid and that most of these isolates also expressed the 17-kDa antigen. Using a large series of clinical isolates collected over a number of years, this study has demonstrated that most isolates of *R. equi* from cases of pneumonia in foals possess *vapA*, thus confirming its role in virulence in horses. Furthermore, we have demonstrated the conserved nature of *vapA* genes among equine isolates. It is possible that the small proportion of isolates which did not possess the *vapA* gene were cured of the plasmid by growth at 37°C during isolation, particularly given the fact that a single colony was chosen for storage of all isolates. In addition, limited studies in our laboratory have suggested that the copy number of the virulence plasmid may be significantly reduced in less nutritious media. Use of agar stabs for storage may also result in some plasmid curing.

The strength of the association between possession of the *vapA* gene and virulence in foals can be best appreciated by a comparison of the prevalence of the gene found in the virulent strains examined in this study with the prevalence of this gene found in environmental isolates in other studies. While the prevalence of the gene was 98% in isolates from cases of disease, only 21.2% of 1,725 equine fecal isolates and 10.2% of 2,200 soil isolates of *R. equi* were found to carry virulence plasmids, as determined by possession of a plasmid of 85 to 90 kb and production of the 15- to 17-kDa virulence-associated antigens (6). It is notable that while the association between possession of a large plasmid and virulence is shared by isolates from pigs, this association is not apparent in isolates from humans. In a study of isolates from immunocompromised human patients, only 8 of 39 were found to possess plasmids (8). This finding suggests that there may be other virulence genes carried in the chromosome of *R. equi*, which are particularly important in immunocompromised humans. Such genes may also play a role in virulence in other species, albeit a less obvious one than that played by the virulence-associated plasmid. In addition, the significance of other genes encoded on the virulence-associated plasmid remains to be determined.

The sequence conservation of the *vapA* gene is remarkable. Given the prominence of the immune response against its product in foals (7) and the enhanced clearance of *R. equi* induced by vaccination of mice with the 17-kDa lipoprotein (11), it might be supposed that this gene would be subjected to immune selection during infection. However, as the population of *R. equi* is predominantly maintained by cycling between gastrointestinal tract infections of foals and growth in fecally contaminated environments, it must be assumed that within
this cycle there is a significant selective pressure against variation. Any explanation for this conservation will thus depend on establishing the role of the virulence-associated gene in the normal habitat of *R. equi*.

The close association between the vapA gene and virulence in horses has three important consequences. First, it establishes that detection of the vapA gene by PCR may have potential for the rapid diagnosis of infection in horses, as suggested by Takai and colleagues (6). Second, it confirms the value of examining environmental isolates from horse farms and discriminating between those which are carrying vapA and those which are not, with the aim of establishing the factors which result in the greater challenges of virulent strains. Finally, the findings of Tan and colleagues (11) that the antibody against the 17-kDa protein is protective in mice, combined with the lack of variation in the gene for this protein and its association with disease, suggest that this protein should be investigated for its ability to induce protective immunity in horses.

REFERENCES