Prevalence of virulent *Rhodococcus equi* in isolates from soil collected from two horse farms in South Africa and restriction fragment length polymorphisms of virulence plasmids in the isolates from infected foals, a dog and a monkey

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ABSTRACT

TAKAI, S., HENTON, M.M., PICARD, G.A., GUTHRIE, A.G., FUKUSHI, H. & SUGIMOTO, C. 2001. Prevalence of virulent *Rhodococcus equi* in isolates from soil collected from two horse farms in South Africa and restriction fragment length polymorphisms of virulence plasmids in the isolates from infected foals, a dog and a monkey. *Onderstepoort Journal of Veterinary Research*, 68:105–110

The prevalence of virulent *Rhodococcus equi* in soil isolates from two horse farms in South Africa and nine clinical isolates from six foals, a foal foetus, a dog, and a monkey was investigated. The isolates were tested for the presence of virulence plasmid DNA and 15- to 17-kDa antigens by immunoblotting. *Rhodococcus equi* was isolated from almost all of the soil samples obtained from the two farms with 5.0×10^1 to 3.3×10^4 colony forming units per gram of soil. Virulent *R. equi* was isolated from three soil samples from one of the farms and appeared in 3.8 % (three of 80 isolates), but not in any of the 182 isolates from the other farm. Of the three virulent *R. equi* isolates, one contained an 85-kb type I plasmid and two an 87-kb type I plasmid. Of nine clinical isolates from the foals, foal foetus, dog and monkey, five from the foals were virulent *R. equi* which expressed the virulence-associated antigens and contained a virulence plasmid 85-kb type I, and were all isolated from cases of pneumonia typical of that induced by *R. equi* in young foals living in widely separated areas in South Africa. The isolates from the other four foals, the dog and the monkey were avirulent *R. equi*.

Keywords: Dogs, horse, monkeys, plasmid, *Rhodococcus equi*, South Africa, virulence

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Accepted for publication 30 January 2001—Editor

INTRODUCTION

Rhodococcus equi is one of the most important bacterial pathogens of young foals. Infections caused by this organism are characterized by chronic, suppurative bronchopneumonia and enteritis, and are associated with a high mortality rate (Barton & Hughes 1980; Prescott 1991; Takai 1997).

The disease is worldwide in distribution. Recently, *R. equi* has emerged as an important pulmonary pathogen in immunosuppressed human patients, especially those with human immunodeficiency virus (HIV) infection (Prescott 1991). We have reported that virulence associated 15- to 17-kDa antigens (VapA) of *R. equi* were associated with virulence in mice and foals (Takai, Koike, Ohbushi, Izumi & Tsubaki 1991a; Wada, Kamada, Anzai, Nakanishi, Kanemaru, Takai & Tsubaki 1997), and that the presence

of a large virulence plasmid is essential for virulence and expression of VapA (Takai, Sekizaki, Ozawa, Sugawara, Watanabe & Tsubaki 1991c; Tkachuk-Saad and Prescott 1991). The virulence-associated antigens and plasmids have been applied as epidemiological markers to detect virulent *R. equi* in horses and their environment in Japan and other countries (Takai, Ohbushi, Koike, Tsubaki, Oishi & Kamada 1991b; Tkachuk-Saad & Prescott 1991; Takai, Morishita, Nishio, Sasaki, Tsubaki, Higuchi, Hagiwara, Senba, Kato, Seno, Anzai & Kamada 1994a; Takai, Takahagi, Sato, Yamaguchi, Kakizaki, Takehara, Matsukura, Tamada, Tani, Sasaki, Tsubaki, & Kamada 1994c; Haites, Muscatello, Begg & Browning 1997; Martens, Takai, Cohen, Chaffin, Liu, Sakurai, Sugimoto & Lingsweiler 2000). More recently, restriction enzyme digestion patterns of virulence plasmids in human and foal isolates from several countries have been examined (Nicholson & Prescott 1997; Rahal, Kodjo, Gretzel & Richard 1999; Takai, Shoda, Sasaki, Tsubaki, Fortier, Pronost, Rahal, Becu, Begg, Browining, Nicholson & Prescott 1999). The digestion patterns divided the plasmids of virulent isolates into five closely related types:

- 85-kb type I
- 85-kb type II
- 87-kb type I
- 87-kb type II
- 90-kb plasmids

The 85-kb type I plasmid was found in isolates from Argentina, Australia, Canada and France. Plasmid 87-kb type I was isolated in specimens from Argentina, Canada and France. The 85-kb type II plasmid appeared in isolates from France. On the other hand, plasmids 87-kb type II and 90-kb were found only in isolates from Japan. These results revealed geographic differences in the distribution of the virulence plasmids found in the five countries, and suggest that the restriction fragment length polymorphism of virulence plasmids might be useful to elucidate the molecular epidemiology of virulent *R. equi* in the world (Takai *et al.* 1999).

In Africa, there are few reports of outbreaks of *R. equi* infection in horses; one of these concerned a thoroughbred stud farm in Zimbabwe (Knottenbelt 1993) and another the disease in South Africa (Grosskopf, Tustin & Muir 1957). Clinical cases of *R. equi* infection in humans suffering from acquired immunodeficiency syndrome (AIDS) have occurred in Africa (Sonnet, Wauters, Zech & Gigi 1987; Colebunders, De Roo, Verstraeten, Van Den Abbeele, Ieven, Hauben, Van Marck, Schaal & Portaels 1996; Nathoo, Chigonde, Nhembe, Ali & Mason 1996). However, the pathogenicities and plasmid profiles of these isolates remain uncertain or were not determined. The purpose of this study was to investigate plasmid profiles of virulent isolates of *R. equi* from clinical cases in foals and the soil of horse farms in South Africa and to compare the genotypic variation among virulence plasmids of the isolates.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used as reference strains in this study were *R. equi* ATCC 33701 (85kb-type I), 96E35 (85kb-type II), 222 (87kb-type I), 96B6 (87kb-type II), and L1 (90kb) (Takai, Watanabe, Ikeda, Tsubaki, Ozawa, Matsukura, Tamada & Sekizaki 1993b; Takai *et al.* 1999). Nine clinical isolates of *R. equi* obtained from infected foals, and one isolate each from a foal foetus, a dog and a vervet monkey (*Cercopithecus aethiops pygerythrus*) which had been harvested from lesions in organs submitted over a period of 25 years to the Onderstepoort Veterinary Institute or the Faculty of Veterinary Science, University of Pretoria, were examined (Table 2). No information is available about the isolate from a foal made in 1974, but the isolate made in 1979 was from an aborted foetus, and *R. equi* was only isolated from the kidney, and not from any of the other organs. *R. equi* was isolated from the dog with chronic tracheitis and the 6-month-old monkey with interstitial pneumonitis.

Collection and isolation of *R. equi* from soil

Soil samples were collected from one or two sites in small paddocks that were used for mares of thoroughbred and standardbred breeds with a foal at foot on farm A and from paddocks that were used for adult thoroughbred horses on farm B. The soil was scraped from the ground surface with a small spoon and poured into sterile glass tubes. One gram of soil from each sample was diluted serially with a 10-fold volume of sterile saline. Each dilution was inoculated onto two plates of nalidixic acid-novobiocin-actidione (cycloheximide)-potassium tellurite (NANAT) medium for selective isolation of *R. equi* (Woolcock, Farmer & Mutimer 1979), and the plates were incubated at 30 °C for 2 or 3 days. All suspected colonies of *R. equi* were counted and the number of viable organisms per gram of soil was calculated. Three to ten colonies of *R. equi* per specimen were subcultured and examined for VapA by colony blot enzyme-linked immunosorbent assay with MAb 10G5 (Takai, Iie, Kobayashi, Morishita, Nishio, Ishida, Fujimura, Sasaki & Tsubaki 1993a). 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 h. A sheet of nitrocellulose filter (pore size; 0.45 mm, BAS 85; Schleicher & Schuell, Dassel, Germany) was then placed on 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 the same as those described previously by Takai *et al.* (1994a) for the immunoblot.

Isolation of plasmid DNA

Plasmid DNA was isolated from *R. equi* by the alkaline lysis method (Birnboim & Doly 1979), with some modifications, as described previously (Takai *et al.* 1993a). Plasmid DNAs were analyzed by digestion with restriction endonucleases *Bam*HI, *Eco*RI, *Eco*T22I and *Hin*dIII for detailed comparison and estimation of the plasmid size. Samples of the plasmid preparations were separated in 0.7% or 1.0% agarose gels at approximately 5 V/cm for 2 h.

RESULTS

Isolation of virulent *R. equi* from soil samples collected from the farms

Soil from both the farms was culture-positive for *R. equi* (Table 1), the number of organisms ranging from 5.0×10^1 to 3.3×10^4 per gram of soil. Three to 18

TABLE 1 Isolation of *R. equi* from soil samples collected from two horse farms in South Africa

Farm	Sample no.	Number of *R. equi*	No. of isolates	No. of virulent *R. equi*	Plasmid type
A	1	4.5×10^3	10	0	
	2	9.3×10^2	10	1	85 kb type I
	3	2.2×10^3	10	0	
	4	6.0×10^3	10	0	
	5	8.0×10^2	10	1	87 kb type I
	6	7.5×10^2	10	0	
	7	1.0×10^2	10	1	87 kb type I
	8	1.6×10^3	10	0	
B	1	3.0×10^3	18	0	
	2	1.0×10^3	8	0	
	3	3.0×10^4	14	0	
	4	2.5×10^3	8	0	
	5	5.5×10^3	10	0	
	6	1.6×10^4	8	0	
	7	5.5×10^3	11	0	
	8	1.0×10^4	8	0	
	9	1.0×10^3	10	0	
	10	1.3×10^2	8	0	
	11	5.0×10^2	3	0	
	12	1.2×10^4	8	0	
	13	5.0×10^2	5	0	
	14	3.3×10^4	8	0	
	15	4.8×10^3	16	0	
	16	3.8×10^2	8	0	
	17	5.0×10^1	3	0	
	18	1.8×10^3	8	0	
	19	1.0×10^3	10	0	
	20	5.7×10^3	10	0	

TABLE 2 Identification of clinical isolates of *R. equi* in South Africa

OVI number	Date	Specimen (animal)	Expression of 15- to 17-kDa antigens	Plasmid type	Reference
037/74	1974	Foal	–	–	Dr Henton, OVI[1]
7067	6/7/78	Vervet monkey	–	–	Dr Henton, OVI[1]
7818	19/2/79	Foal (6ws)	+	85 kb type I	Dr Henton, OVI[1]
8280	2/7/79	Foetus of horse (> 6 months)	–	–	Dr Henton, OVI[1]
5318	12/1/89	Foal	+	85 kb type I	Dr Henton, OVI[1]
9426	29/1/91	Foal	+	85 kb type I	Dr Henton, OVI[1]
4527	3/12/97	Dog	–	–	Dr Henton, OVI[1]
9714	15/11/99	Foal (2 months)	+	85 kb type I	Dr Henton, OVI[1]
48100	16/1/00	Foal	+	85 kb type I	Dr Picard, UP[2]

[1] OVI = Onderstepoort Veterinary Institute
[2] UP = Faculty of Veterinary Science, University of Pretoria

colonies (averaging 10 colonies) per sample were subcultured. Identification of 262 colonies was performed, and these were tested for the presence of 15- to 17-kDa antigens by colony blotting. As shown in Table 1, virulent *R. equi* from soil appeared in 0–10 % of the isolates from the two farms. Positive colonies were then tested for the presence of virulence plasmids. Of the three virulent isolates, one isolate contained an 85-kb type I and the remaining two contained an 87-kb type I plasmid.

Plasmid profiles of virulent *R. equi* from clinical samples

Of nine clinical isolates from the foals, and those from the dog and monkey, five from the foals were virulent *R. equi*, which expressed the virulence-associated antigens and contained a virulence plasmid (Table 2). The five foal isolates contained an 85-kb type I plasmid. On the other hand, the one isolate each from the dog, that from the monkey, one from the foal feotus and one from a foal were avirulent *R. equi*, which did not show any virulence-associated antigens and did not contain virulence plasmids.

DISCUSSION

The present study revealed that virulent isolates of *R. equi* in South Africa contain an 85-kb type I or an 87-kb type I plasmid, which have been found in clinical and environmental isolates in Europe, South and North America, and Australia (Haites *et al.* 1997; Nicholson & Prescott 1997; Takai *et al.* 1999; Martens *et al.* 2000), and the five virulent strains were all isolated from cases of pneumonia in young foals living in widely separated areas in South Africa. There were no indigenous horses in South Africa before the arrival of Europeans with horses being imported from the island of Java in 1653 (Burman 1993). Then, further imports were made from European countries when horses of Arab, Spanish, French and English breeds were introduced into the country (Burman 1993). In a previous study, it was considered that the majority of virulent *R. equi* strains isolated in Japan were probably imported in carrier animals originating from European countries, since for the last 100 years, Japan has introduced European horses into the country for crossbreeding to improve Japanese stock for racing and military purposes (Nozawa 1992). Patterns of restriction enzyme fragments of plasmid DNAs of Japanese isolates from Thoroughbred and Anglo-Arab breeds revealed the existence of an 87-kb type II or a 90-kb plasmid, which were found only in the Japanese isolates (Takai *et al.* 1999). However, Australian isolates, which contain an 85-kb type I plasmid, are likely to have been imported, since no equids were present in Australia before 1788 when seven horses arrived in the country (Hendericks 1995).

In this study, certain *R.equi* isolates from the two foals and those from the dog and monkey were considered avirulent because they did not contain virulence-associated plasmids nor express VapA. Previous studies have revealed that *R. equi* isolates from diseased foals manifesting lesions typical of those of *R. equi* infection were virulent, and that infections in foals are caused principally by virulent *R. equi*, but not avirulent *R. equi* (Takai *et al.* 1993b; Takai 1997). The two avirulent isolates from foals were isolated in 1974 and 1979. Strain ATCC 6939, the type strain of *R. equi*, which was isolated from a foal with fatal pneumonia in 1923 by Magnusson, has been passaged repeatedly since it was isolated. This strain does not possess the virulence plasmid (Takai *et al.* 1991c), and does not induce disease in foals (Takai, Kawazu & Tsubaki 1987). Attenuation of strain ATCC 6939 might have occurred as a result of plasmid curing of the strain (Takai *et al.* 1991c). It has been demonstrated that repeated passage of virulent strains, such as ATCC 33701 and L1, at 38 °C results in attenuation of the strains as a result of curing the virulence plasmid; at 30 °C, repeated passage has no such effect (Takai, Sugawara, Watanabe, Sasaki, Tsubaki & Sekizaki 1994b). A similar phenomenon of virulence plasmid-curring in ATCC 6939 might have occurred.

In contrast, the clinical isolates of *R.equi* from the dog and monkey were avirulent. This organism has been reported sporadically as a cause of lymphadenitis in pigs, cats, goats, sheep and cattle, and of abscesses in dogs (Cotchin 1943; Barton & Hughes 1980, Prescott 1991; Takai, Fukunaga, Ochiai, Imai, Sasaki, Tsubaki & Sekizaki 1996; Cantor, Byrne, Hines & Richards 1998; Tkachuk-Saad, Lusis, Welsh & Prescott 1998; Davis, Steficek, Watson, Yamini, Madarame, Takai & Render 1999). Recent reports have indicated that the isolates from these animals were avirulent *R. equi*, which did not express VapA (Cantor *et al.* 1998; Tkachuk-Saad *et al.* 1998; Davis *et al.* 1999). It is probable that these infections were opportunistic; but the immunocompetency of these diseased animals remains unclear. Further studies are needed to determine the pathogenicity of avirulent *R.equi* for dogs, cats, goats and cattle.

In conclusion, the present study revealed that the soil of a horse-breeding farm in South Africa was contaminated with virulent *R. equi* containing an 85-kb type I or an 87-kb type I plasmid. In Africa, zebras (*Equus burchelli*) are abundant in certain areas; it would be very interesting to investigate the virulence of isolates from zebras.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research (11691164) from the Ministry of Edu-

cation, Science, Sports and Culture, Japan (C. Sugimoto, H. Fukushi & S. Takai).

The authors thank Ms Chie Sasaki of Kitasato University for technical support.

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