Evaluation of a Monoclonal Antibody-Based Colony Blot Test for Rapid Identification of Virulent *Rhodococcus equi*

Shinji TAKAI, Taiyo MORISHITA, Yasushi NISHIO, Yukako SASAKI, Shiro TSUBAKI, Tohru HIGUCHI¹⁾, Shintaro HAGIWARA¹⁾, Hiroyuki SENBA¹⁾, Masakatu KATO²⁾, Noboru SENO²⁾, Tohru ANZAI³⁾, and Masanobu KAMADA³⁾

Department of Animal Hygiene, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034,
¹⁾Mitsuiishi Regional Veterinary Clinical Center, Mitsuiishi, Hokkaido 059-32, ²⁾Hidaka Livestock Hygiene Service Center, Shizunai,
Hokkaido 056, ³⁾Epizootic Research Station, Equine Research Institute, Japan Racing Association, Kokubunji, Tochigi 329-04, Japan

(Received 28 January 1994/Accepted 15 March 1994)

ABSTRACT. We recently generated a monoclonal antibody immunoglobulin G1 (MAb 10G5), which can recognize 15- to 17-kDa antigens, virulence-associated antigens of *Rhodococcus equi*, and developed a colony blot enzyme-linked immunosorbent assay with MAb 10G5 for the rapid identification of virulent *R. equi*. In this epidemiologic study, we evaluated the results of the colony blot test in the identification of virulent isolates of *R. equi* from feces of horses and soil and compared them with those from a conventional procedure (plasmid profiles of isolates by agarose gel electrophoresis). Environmental isolates (778 isolates from feces of foals, 170 isolates from feces of dams, and 1,267 isolates from soil on horse-breeding farms in Hokkaido) were tested by the colony immunoblot test, and 238 of the 778 isolates, 6 of the 170 isolates, and 85 of the 1,267 isolates showed positive signals. Positive isolates were then analyzed for the presence of virulence plasmid DNA, and 235 (98.7%) of the 238 isolates from foals, 6 (100%) of the 6 isolates from dams, and 75 (88.2%) of the 85 isolates from soil showed the presence of virulence plasmids. On the other hand, 50 isolates from each source, which were randomly selected from the isolates that showed negative signals by colony immunoblot, did not contain virulence plasmids. These results demonstrated that the colony blot test that uses a monoclonal antibody specific for virulence-associated antigens is a rapid and reliable test for the identification of virulent *R. equi*.—**KEY WORDS:** monoclonal antibody, *Rhodococcus equi*, virulent.

J. Vet. Med. Sci. 56(4): 681-684, 1994

Rhodococcus equi is one of the most important bacterial pathogens in 1- to 3-month-old foals, and infections caused by this organism are characterized by chronic, suppurative bronchopneumonia that is often accompanied by ulcerative enteritis and mesenteric lymphadenitis [1, 3, 6, 19]. The disease is worldwide in distribution and reportedly accounts for more than 3% of all foal deaths [3]. *R. equi* multiplies dramatically in fecal contaminated soil and within the intestines of foals less than 3 months old [13]. The natural reservoir for *R. equi* is soil, and natural infection in foals seems to be acquired by inhalation or ingestion of soil-borne organisms. Despite the importance of the disease, which is virtually limited to foals, its pathogenesis and epidemiology are not well understood [1, 3, 6, 19].

We have recently reported that the 15- to 17-kDa antigens of *R. equi* are associated with virulence for mice [11] and that the presence of an 85-kb or 90-kb plasmid is essential for virulence and the expression of the 15- to 17-kDa antigens [4, 7, 15, 17]. In previous epidemiologic studies, a definitive identification of virulent *R. equi* depended on the identification of virulence-associated antigens by Western immunoblotting with naturally infected foal serum [11, 12] or isolation of virulence plasmid DNA by agarose gel electrophoresis, or both [7, 8]. However, these conventional procedures were time-consuming and not suitable for a large number of isolates. Therefore a reliable and accurate assay was needed to detect virulence-associated antigens in isolates without the need for further identification procedures. With this in

mind, we produced an MAb specific to the 15- to 17-kDa antigens and attempted to identify virulent *R. equi* by immunoblotting assays [9, 14].

The purpose of this study was to assess the reliability of the colony blot enzyme-linked immunosorbent assay for the identification of virulent *R. equi* by comparing it with conventional methods, and to evaluate its applicability for routine use in epidemiological surveillance studies of *R. equi* infection in horse-breeding farms.

MATERIALS AND METHODS

Bacteria: *R. equi* ATCC 33701 (pREAT701), its plasmid-cured derivative (ATCC 33701P⁻), and L1 (pREL1) were used as reference strains [15, 17].

Soil and feces of foals and dams: Soil sample (236 sites) were collected from 6 to 10 sites from small paddocks used for a mare with a foal at foot from 15 horse-breeding farms in Hidaka from June to July, 1993. The soil was scraped from the surface of the ground with a small spoon and poured into sterile tubes. Fecal samples were collected from 232 foals and 89 dams on the 15 farms. Fecal samples were also taken from freshly passed materials in the stables.

Isolation of *R. equi* from soil and feces: For the selective isolation of *R. equi*, nalidixic acid-novobiocin-actidione (cycloheximide)-potassium tellurite (NANAT) medium, previously described by Woolcock *et al.* [18], was used. One gram of soil or fecal materials was diluted serially with a 10-fold volume of sterile saline. Each dilution was

inoculated onto two plates of NANAT medium. The plates were incubated at 30°C for 2 or 3 days. All suspectable colonies of *R. equi* were counted and the number of viable organisms per gram of soil was calculated. Two to ten colonies per specimen were subcultured and identified in our laboratory and then tested for the presence of 15- to 17-kDa antigens by colony blotting and Western blotting, and for the presence of virulence plasmids by agarose gel electrophoresis.

Colony blot enzyme-linked immunosorbent assay with MAb 10G5: For colony blot analysis, bacterial strains were injected onto brain heart infusion agar plates with an inoculation needle and were incubated at 38°C for 24 hr. A sheet of nitrocellulose filter (pore size; 0.45 µm, BAS 85; Schleicher & Schuell, Dassel, Germany) was then placed on the cultures for a few minutes to wet them completely. The membrane was removed and air dried. In the previous study, chloroform treatment was used to bind the proteins of isolates to the nitrocellulose. In the present study, we treated the membrane by autoclaving at 105°C for 1 min instead of using chloroform, since the intensity of the colored blots was better than in the blots treated with chloroform. All buffers, antibodies, and conjugate dilutions were the same as those used for the immunoblot described previously [11].

Gel electrophoresis and immunoblot analysis: Sodium dodecyl sulfate (SDS)-gel electrophoresis and immunoblotting analysis were performed as described previously [5, 11].

Isolation of plasmid DNA: Plasmid DNA was isolated from *R. equi* by an alkaline lysis method [2], with some modifications, as described previously [15]. Samples of the plasmid preparations were separated along with the plasmids of *R. equi* ATCC 33701 (pREAT701) and L1 (pREATL1) in 0.7% agarose gels at approximately 5 V/cm for 2 hr.

RESULTS

In previous studies [8, 9, 17], the isolates were examined for the presence of 15- to 17-kDa antigens by Western blot and the positive isolates were then analyzed for the presence of virulence plasmids, and vice versa. These procedures were generally laborious and not practical for routine use. Our preliminary study [9] showed a perfect correlation between the colony blot in

the 23 clinical and 102 environmental isolates and the plasmid profiles of these strains, although the number of isolates was small. Therefore, we applied the colony blot test to a rapid screening for a large number of isolates in this surveillance study.

Results of quantitative culture of *R. equi* from feces of foals and dams and from soil samples collected in 15 farms is shown in Table 1. The mean numbers of *R. equi* from the feces of foals and dams on the 15 farms examined ranged from 5.0×10^1 to 2.6×10^6 , and 2.5×10^1 to 9.5×10^3 per gram of feces, respectively, and the isolation rate of *R. equi* from the feces of foals and dams was 60.8% (141 of 232 samples) and 50.6% (45 of 89 samples), respectively. The mean numbers of *R. equi* from the soil samples ranged from 5.0×10^1 to 8.3×10^5 per gram of soil, and the isolation rate of *R. equi* from the soil samples was 95.3% (225 of 236 samples).

The 2,215 *R. equi* isolates were then tested for the presence of 15- to 17-kDa antigens by colony blot enzyme-linked immunosorbent assay. Of the 778 isolates from feces of foals, 238 isolates showed positive signals, and 6 of the 170 isolates from feces of dams and 85 of the 1,267 isolates from soil samples showed positive signals (Table 2 and Fig. 1). Positive isolates were then analyzed for the presence of virulence plasmid DNA by agarose gel electrophoresis, and 235 (98.7%) of the 238 isolates from foals, 6 (100%) of the 6 isolates from dams, and 75 (88.2%) of the 85 isolates from soil showed the presence of virulence plasmids (some of the results are shown in Fig. 2). Three of the 238 isolates and 10 of the 85 isolates did not contain any plasmids. On the other hand, 50 isolates from each source, which were randomly selected from the isolates showing negative signals by colony immunoblot, did not contain virulence plasmids. The ratio of 85-kb plasmids and 90-kb plasmids in the 316 virulent isolates was 303:13.

DISCUSSION

The present study revealed that the rapid identification of virulent *R. equi* in the environmental isolates could be achieved by the monoclonal antibody-based colony blot test. There was a good correlation between the presence of the virulence plasmids and the positive signals shown by the monoclonal antibody-based colony blot test in the fecal isolates of *R. equi*: the identification rates in the

Table 1. Isolation of *R. equi* from feces of foals and dams and soil from 15 horse-breeding farms

Sample	No. of samples	No. of positive cultures(%)	Mean number of bacteria	Range (/g)	No. of isolates
Feces of foals	232	141(60.8)	3.4×10^4	5.0×10^1 – 2.6×10^6	778
Feces of dams	89	45(50.6)	1.1×10^3	5.0×10^1 – 9.5×10^3	170
Soil	236	225(95.3)	7.2×10^3	5.0×10^1 – 8.3×10^5	1,267

Table 2. Comparison of colony blots with plasmid profiles for identification of virulent *R. equi*

Isolates from	No. of isolates	No. of positive isolates by colony blot	No. of isolates with virulence plasmids (%)	Prevalence of virulence plasmids (%)
Feces of foals	778	238	235(98.7)	30.6
Feces of dams	170	6	6(100)	3.5
Soil	1,267	85	75(86.2)	5.9

Fig. 1. Colony blot profiles of *R. equi* environmental isolates. Dots: 1a, isolate LF2150; 2a, isolate LF2151; 3a, isolate LF2218; 4a, isolate LF2219; 5a, isolate LF2441; 1b, isolate LF2242; 2b, isolate LF2444; 3b, isolate LF2245; 4b, isolate LF2446; 5b, isolate LF2447; 1c, strain ATCC 33701; 2c, strain ATCC 33701P⁻. Fig. 2. Plasmid profiles of *R. equi* environmental isolates. Plasmid DNA was extracted, resolved in 0.7% (wt/vol) agarose gel, and stained with ethidium bromide. Lanes: 1, isolate LF2151; 2, isolate LF2218; 3, isolate LF2219; 4, isolate LF2242; 5, isolate LF2245; 6, isolate LF762; 7, isolate LF763; 8, isolate LF764; M, strains ATCC 33701 and L1. The arrowheads on the right indicate plasmids ATCC 33701 and L1.

isolates from the feces of foals and dams are 98.7% and 100% and that from the soil is 88.2%. At present, we could not explain why a relatively large number (11.8%) of false-positive reactions was observed in the soil isolates compared with that in the fecal isolates by the colony blot. However, no false-negative reactions by the colony blot were observed in the 50 isolates from each source. Therefore, the colony blot test seems to be a very practical and rapid procedure for epidemiological surveillance of virulent *R. equi* in the environment of horse-breeding farms.

Our previous study revealed that the environment of horse-breeding farms endemic for *R. equi* was heavily contaminated with virulent *R. equi*, whereas the environment of a farm with no history of *R. equi* infection was only slightly contaminated with the virulent organism [12]. Natural infections in foals might be caused principally by virulent *R. equi*, but not by avirulent organisms, since all of the isolates from the lesions of infected foals were virulent *R. equi* [7, 17]. Therefore, it is important to know whether the environment of horse-breeding farms is contaminated by virulent *R. equi*, which cast pose a high risk for infection. For prevention of progressive contamination of virulent *R. equi* in horse-breeding farms, a seasonal examination by these procedures seems to be extremely useful.

The prevalence of virulent *R. equi* in isolates from feces of foals on the 15 farms was significantly higher ($P < 0.05$ by the chi-square test) than those in the isolates from the feces of dams and soil. Details of results demonstrating the prevalence of virulent *R. equi* in isolates from feces of foals in the farms will be described elsewhere.

One of most important points to consider in examining the virulence of clinical and environmental isolates is the effect of growth temperature to maintain the virulence of *R. equi* [15, 16]. Recent studies demonstrated that repeated passage of virulent *R. equi* at 38°C resulted in attenuation of the organism as a result of plasmid curing; whereas at 30°C, repeated passage gave no effect [16]. Therefore, during isolation and identification procedures, growth temperature of the isolates should be kept at 30°C. On the other hand, the expression of the 15- to 17-kDa antigens is regulated by growth temperature; the antigens are expressed during growth at 34 to 41°C but not below 32°C [10], so in the colony blot test, growth temperature should be kept at 38°C.

In conclusion, this new monoclonal antibody-based colony blot test revealed good sensitivity and specificity and was time-saving. Moreover, it was particularly suitable and useful for epidemiological studies to investigate the contamination of virulent *R. equi* in horse-breeding farms with endemic infections.

ACKNOWLEDGEMENTS. This study was supported by a grant-in-aid from the Equine Research Institute, Japan Racing Association, and by a grant-in-aid for All Kitasato Project Study from the Kitasato University.

REFERENCES

1. Barton, M. D. and Hughes, K. L. 1980. *Corynebacterium equi*: a review. *Vet. Bull.* 50: 65–80.
2. Birnboim, H. C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7: 1513–1523.
3. Elissalde, G. S. and Renshaw, H. W. 1980. *Corynebacterium equi*: an interhost review with emphasis on the foal. *Comp. Immunol. Microbiol. Infect. Dis.* 3: 433–455.
4. Kanno, T., Asawa, T., Ito, H., Takai, S., Tsubaki, S., and Sekizaki, T. 1993. Restriction map of a virulence-associated plasmid of *Rhodococcus equi*. *Plasmid* 30: 309–311.
5. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227: 680–685.
6. Prescott, J. F. 1991. *Rhodococcus equi*: an animal and human pathogen. *Clin. Microbiol. Rev.* 4: 20–34.
7. Takai, S., Anzai, T., Sasaki, Y., Tsubaki, S., and Kamada, M. 1993. Virulence of *Rhodococcus equi* isolated from lesions of infected foals. *Bull. Equine Res. Inst.* 30: 9–14.
8. Takai, S., Anzai, T., Yamaguchi, Y., Kakizaki, K., Takahagi, J., Sato, Y., Takehara, F., Tamada, Y., Matsukura, S., Tani, A., Kato, M., Seno, N., Sasaki, Y., Tsubaki, S., and Kamada, M. 1994. Prevalence of virulence plasmids in environmental isolates of *Rhodococcus equi* from horse-breeding farms in Hidaka, Hokkaido. *J. Equine Sci.* (in press)
9. Takai, S., Iie, M., Kobayashi, C., Morishita, T., Nishio, Y., Ishida, T., Fujimura, T., Sasaki, Y., and Tsubaki, S. 1993. Monoclonal antibody specific to virulence-associated 15- to 17-kilodalton antigens of *Rhodococcus equi*. *J. Clin. Microbiol.* 31: 2780–2782.
10. Takai, S., Iie, M., Watanabe, Y., Tsubaki, S., and Sekizaki, T. 1992. Virulence-associated 15- to 17-kilodalton antigens in *Rhodococcus equi*: temperature-dependent expression and location of the antigens. *Infect. Immun.* 60: 2995–2997.
11. Takai, S., Koike, K., Ohbushi, S., Izumi, C., and Tsubaki, S. 1991. Identification of 15- to 17-kilodalton antigens associated with virulent *Rhodococcus equi*. *J. Clin. Microbiol.* 29: 439–443.
12. Takai, S., Ohbushi, S., Koike, K., Tsubaki, S., Oishi, H., and Kamada, M. 1991. Prevalence of virulent *Rhodococcus equi* in isolates from soil and feces of horses from horse-breeding farms with and without endemic infections. *J. Clin. Microbiol.* 29: 2887–2889.
13. Takai, S., Ohkura, H., Watanabe, Y., and Tsubaki, S. 1986. Quantitative aspects of fecal *Rhodococcus (Corynebacterium) equi* in foals. *J. Clin. Microbiol.* 23: 794–796.
14. Takai, S., Sasaki, Y., Ikeda, T., Uchida, Y., Tsubaki, S., and Sekizaki, T. 1994. Virulence of *Rhodococcus equi* isolates from patients with and without AIDS. *J. Clin. Microbiol.* 32: 457–460.
15. Takai, S., Sekizaki, T., Ozawa, T., Sugawara, T., Watanabe, Y., and Tsubaki, S. 1991. Association between a large plasmid and 15- to 17-kilodalton antigens in virulent *Rhodococcus equi*. *Infect. Immun.* 59: 4056–4060.
16. Takai, S., Sugawara, T., Watanabe, Y., Sasaki, Y., Tsubaki, S., and Sekizaki, T. 1994. Effect of growth temperature on maintenance of virulent *Rhodococcus equi*. *Vet. Microbiol.* 39: 187–192.
17. Takai, S., Watanabe, Y., Ikeda, T., Tsubaki, S., Ozawa, T., Matsukura, S., Tamada, Y., and Sekizaki, T. 1993. Virulence-associated plasmids in *Rhodococcus equi*. *J. Clin. Microbiol.* 31: 1726–1729.
18. Woolcock, J. B., Farmer, A.-M. T., and Mutimer, M. D. 1979. Selective medium for *Corynebacterium equi* isolation. *J. Clin. Microbiol.* 9: 640–642.
19. Yager, J. A. 1987. The pathogenesis of *Rhodococcus equi* pneumonia in foals. *Vet. Microbiol.* 14: 225–232.