[SUMMARY]

Studies on the characterization of *Rhodococcus equi* derived from horse

Muneo NAKAZAWA

National Institute of Animal Health, Tsukuba, Ibaraki 305

Rhodococcus equi is associated with several diseases of domestic animals, particulary purulent pneumonia, mesenteric lymphadenitis, ulcerative enteritis, cellulitis, and arthritis in foals, and tuberculosis-like lesions in porcine cervical lymph nodes. On the other hand, it has been isolated not only from the lesions of foals and swine but also from the feces of different domestic animals and from soil. Although it has also been found in the feces of healthy foals and mares, the pathogenic significance of its fecal isolates in foals has remained to be elucidated. Likewise, little attention has been paid to the epizootiological significance of *R. equi* harbored in the feces of healthy foals and mares.

On the other hand, the clinical and pathological findings of *R. equi* infection in foals have been clarified by numerous case reports in many countries, including Japan. It has not always been successful, however, to reproduce this disease experimentally. Little is known about the virulence of *R. equi* for domestic animals, including the horse itself and laboratory animals.

The author, therefore, made an attempt to settle the

important problems as mentioned above.

First, as a part of the epizootiological studies on *R. equi* infection, investigation was carried out to clarify the distribution of the organism in the feces of foals and mares. In addition, an effort was made to establish the biotyping or serogrouping of *R. equi* in order to develop a suitable method to discriminate or characterize this organism isolated from the feces and lesions of horses. Another effort was made to elucidate the virulence of *R. equi* by using laboratory animal models and macrophages cultured *in vitro*.

The results obtained are summarized as follows.

1. Quantitative culture of *R. equi* from the feces of horses.

In a preliminary examination with Woolcock's NANAT medium for selective isolation of *R. equi*, the medium was found to be partially inhibitory on the growth of *R. equi*. Therefore, it was modified to improve its efficiency by excluding potassium tellurite, which inhibits the growth of *R. equi*, from its original composition. Hereafter,

the modified medium is referred to as NANC. The composition of NANC medium is as follows: 30 g of tryptone soya broth(Oxoid), 1 g of yeast extract(Oxoid), 15 g of bacto-agar (Difco) and 1,000 ml of distilled water with an addition of 10.0 μg/ml of nalidixic acid, 12.5 μg/ml of novobiocine and 20.0 μg/ml of cycloheximide. The inoculated plates were incubated in aerobic condition at 37°C for 40 hours. *R. equi* could be recognized as an isolated colony showing mucous and slimy character on NANC medium with some other bacteria.

The selective isolation of *R. equi* in NANC medium was made by quantitative culture of the organism in the feces. The appearance of *R. equi* in the feces was noted in all the 91 mares and 36 foals investigated, showing a 100 % positive isolation rate. The mean viable count of *R. equi* organisms in 1 gram of feces was 8.42×10^2 CFU in the mares and 7.57×10^2 CFU in the foals. No statistically significant difference was found between the mares and foals in the number of colonies isolated from the feces ($P>0.05$).

On the other hand, 5 apparently healthy mares were

examined quantitatively for the presence of *R. equi* in the feces during a period from January to July, 1980. The monthly incidence of the organism in the feces was fairly constant during the experimental period. No seasonal variation was found in any mare.

These results indicate that *R. equi* is a member of the normal intestinal flora in the horse.

2. Physiological and biochemical characteristics of *R. equi*.

R. equi has been isolated not only from the lesions of foals but also from the feces of healthy horses. It is impossible to neglect the presence of the organism in the feces of horses in *R. equi* infection. Therefore, a question was raised whether the organism isolated from the feces were the same as that isolated from the lesions or not. Then, an attempt was made to discriminate both isolates from each other by using the standard biochemical tests and the API ZYM system.

A total of 80 isolates derived from both groups examined had almost the same physiological and biochemical characteristics as 8 reference strains of *R. equi* supplied by the

ATCC and NCTC. That is, in the standard biochemical tests, they were positive for Gram staining, Kinyoun's acid-fast staining, catalase, urease, and the reduction of nitrate and tellurite, but negative for the fermentation of any sugar. When they were subjected to the API ZYM system to examine the activity of 19 bacterial enzymes, six enzymes, esterase-lipase, leucine arylamidase, valine arylamidase, phosphatase acid, phosphoamidase, and α-glucosidase, were detected from them, as well as the reference strains.

The results obtained indicate that there was no clear difference in physiological or biochemical characteristics or enzyme profiles between the fecal and clinical isolates.

3. Serogrouping of *R. equi*.

a. Establishment of a serogrouping method for *R. equi*.

The serological relationship of 27 isolates of *R. equi* selected from a total of 1,195 isolates was investigated by cross-agglutination and absorption tests. The presence of a capsular material was demonstrated in all the 27 isolates by electron microscopic observation. Antisera were prepared by employing formalized antigen of each isolate. In the

cross-agglutination test with formalized antigen, 13 antisera reacted with homologous antigens alone, but the remaining 14 antisera not only with homologous antigens but also with 1 to 4 heterologous antigens.

In the cross-agglutination test with autoclaved antigen, all the antisera reacted not only with homologous antigens but also with 1 to 8 heterologous antigens, except one which reacted with homologous antigen alone. Like this, the strains tended to remove their type or group specific antigens after treatment by autoclaving. It was proved that formalized antigen was more suitable for serogrouping than the autoclaved one.

On the other hand, when 14 antisera possessing heterologous agglutinins to several formalized antigens were absorbed with each of the cross-reacting ones, 14 specific antisera were obtained. The cross-agglutination test with these 27 antisera revealed that the 27 strains examined were serologically distinct from one another. These strains were designated serogroups 1 to 27, respectively. Thus the same number of grouping antisera was prepared.

In the present study, serogrouping was made by the slide agglutination test. The test was performed on a glass slide with grouping antiserum diluted 1:4 to 1:10. For it, formalized antigen was adjusted to the density of McFarland opacity tube No. 10. It was proved that this test was a simple and rapid one, yielding the same results as the tube agglutination test.

b. Serogroups of *R. equi* derived from feces and lesions.

Serogrouping of a total of 1,195 isolates from horses was performed with the 27 grouping antisera. There was no tendency for these isolates to show a cross-reaction to one another. All the isolates could be divided into groups. Of them, 821 isolates were derived from the feces of healthy horses. Of them, 204 belonged to serogroup 4, 107 to 2, 93 to 11, 82 to 3, 59 to 15, 42 to 8, 36 to 7, 35 to 1, and 34 to 14. The remaining 129 isolates belonged to the other 18 serogroups.

On the other hand, 374 isolates were derived from the lesions of diseased foals. Of them, 122 isolates belonged to serogroup 4, 112 to 2, 89 to 8, 16 to 11, 13 to 9, 10 to 3,

6 to 1, 4 to 5, 1 to 6, and 1 to 7.

It should be noted that serogroups 1, 2, 3, 4, 7, 8, and 11 were found in common to both groups of isolates. This result indicates that no separation can be made between the fecal and clinical isolates on the basis of serological properties; that is, both groups of isolates may be closely related to each other.

c. Relationship between Prescott's serovars and the author's serogroups.

Prescott studied 97 strains derived from horses, cattle, pigs, dogs, cats and human beings and divided them into 7 serovars. So, an attempt was made to elucidate the serological relationships between Prescott's serovars and the serogroups designated by the author.

As a result, a close antigenic relationship existed between Prescott's serovars 1, 2, 3, 4, 5, 6, and 7 and the author's serogroups 4, 16, 2, 12, 21, 1, and 9, respectively. Likewise, serogroups of the eight reference strains received from ATCC and NCTC were also determined by the slide agglutination test. From these results, it is considered that

the serogrouping scheme established by the author is useful for the determination of serogroups of isolates from human beings and various animals, and that it may be a useful tool for epizootiological studies on *R. equi* infections in animals.

4. Virulence of *R. equi*.

A study was conducted to clarify the relationship between the virulence of *R. equi* for mice and the ability of the organism to multiply *in vivo*, or its resistance to intracellular killing by peritoneal macrophages and monocytes *in vitro*.

Comparison was made on the virulence of *R. equi* for mice between strains by calculating LD_{50} value. The virulence of *R. equi* for mice differed from one strain to another. The LD_{50} of the least virulent strain exceeded 10^8 CFU, whereas those of moderately and the most highly virulent strains were 10^7 and 10^6 CFU, respectively. For example, the LD_{50} of strain NCTC 1621 exceeded 10^8 CFU, indicating that the strain was avirulent for mice, and that of strain CE220 was 10^6 CFU, indicating that the strain

was the most virulent. So far as the virulence for mice is concerned, more of virulent strains were found among the clinical isolates than among the fecal ones ($P<0.01$).

So, multiplication in mice was compared between virulent strain CE220 and avirulent strain NCTC 1621 by examining curves of growth *in vivo*. Histopathological examination revealed that *R. equi* was phagocytosed by macrophages of the reticuloendothelial system in the spleen and liver. Then, the number of viable organisms in the spleen was counted.

An apparent difference was noticed in the number of organisms and bacterial retention *in vivo* between the two inoculated groups. That is, the organisms were isolated continually from mice inoculated with strain CE220 in the order of more than 10^4 CFU per spleen for as long as 14 days. On the other hand, strain NCTC 1621 was rapidly eliminated from the spleen. It disappeared from this organ within 7 days after inoculation.

The result of this study indicate that there was a close correlation between the virulence of *R. equi* for

mice and its ability to multiply *in vivo*.

The interaction between *R. equi* and mouse peritoneal macrophages or equine monocytes was further investigated to clarify the difference between virulent and avirulent strains *in vitro*. There was no significant difference in the rate of phagocytosis between both strains. On the other hand, the survival rate of *R. equi* organisms against intracellular killing action of cultured mouse peritoneal macrophages at 24 hours of incubation was 298.8 % for virulent strain CE220 and 44.8 % for avirulent strain NCTC 1621. The mean rate of organisms surviving within equine monocytes at 72 hours of incubation was 226.8 % for CE220 and 0.48 % for NCTC 1621.

From the results obtained it was suggested that the ability of *R. equi* to multiply within macrophages might be dependent on the virulence of this organism.