***Rhodococcus equi* Infection in Foals —Current Concepts and Implication for Future Research—**

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I. Introduction

Rhodococcus equi is a gram-positive coccobacillus which causes suppurative pneumonia and ulcerative enteritis and is associated with lymphadenitis in foals [4, 18, 31, 49, 68, 70]. Magnusson [49] described the first isolation of the bacterium from the lesions of an infected foal in 1923 and named it *Corynebacterium equi*. In the last decade, *R. equi* has been increasingly recognized as an important

pathogen of immunocompromised hosts such as AIDS patients [17, 26, 41, 48, 68, 106, 131].

The pathogenesis of *R. equi* infection in foals is not fully understood, and the diagnosis, treatment and a preventative method for this disease have not been fully elucidated [2, 4, 31, 68, 70, 124]. It is thought that the characteristics of *R. equi* infection have delayed research efforts on the disease. That is, the infection occurs sporadically on some farms, affecting foals of 1–3 months of

age, when the immune system is still immature and maternal antibodies have disappeared [22, 51, 130]. This suggests that *R. equi* infection is an opportunistic infection in foals. Moreover, the disease progresses insidiously without any clinical symptoms in the early stage of infection, making early diagnosis difficult and reliance on postmortem examinations for a definitive diagnosis in some cases [70]. A type strain of *R. equi*, ATCC6939, cannot reproduce the disease experimentally in foals [52, 96], and there is no experimental infection model except in foals. These problems have complicated efforts to clarify the pathogenesis of the disease.

In the last two decades, studies on the development of a selective medium [118], capsular serotyping of *R. equi* [40, 54, 60, 65], and the application of an enzyme-linked immunosorbent assay for serological diagnosis [20, 29, 94] have revealed data on the ecology of *R. equi* in horse-breeding farms [5, 34, 78, 88, 92, 102, 104, 112, 122] and humoral immune responses of foals to *R. equi* [79, 95, 96]. Studies on the chemical composition of the bacterial cell wall have led to a change in the name of this organism from *Corynebacterium equi* to *Rhodococcus equi* [3, 23, 24] but scant information is available on the virulence of *R. equi*.

We recently obtained a new finding concerning the antigens and plasmids related to the virulence of the bacterium [98, 108]. There are two virulence levels in *R. equi*: virulent *R. equi* has 15- to 17-kDa antigens and a large plasmid, and it kills mice, but avirulent *R. equi* does not have these antigens and plasmids, and it does not kill mice [98, 108]. These findings led to a breakthrough in studies on the pathogenesis of *R. equi* infection in foals [86, 87, 90, 91, 93, 99, 101, 105, 106, 109, 110, 114].

We here will review the current state of knowledge of the pathogenicity of *R. equi* and its molecular epidemiology obtained at our laboratory.

II. *R. equi* Infection in Foals

1. Characteristics of the Disease

Although a majority of equine infectious diseases are controlled by vaccination, *R. equi* infection is exceptional as an intractable disease that causes high mortality in newborn foals. Morbidity rates of 5 to 17% worldwide and mortality rates of up to 80% have been reported [1, 31, 85]. In the United States, about 3% of foals' deaths are caused by the disease [47].

The main routes of infection are the respiratory and alimentary tracts [36, 37, 68, 72, 124]. Inhalation of the organism is probably the main route of exposure in all foals. There are three clinical types: suppurative bronchopneumonia with abscesses (pneumonia type), ulcerative enteritis associated with lymphadenitis and abdominal abscesses (enteritis type), and both pneumonia and enteritis (mixed type) [14, 129]. In Australia, 40 cases were classified into 3 types: nine with pneumonia (22.5%), eight with enteritis (20%), and 23 having mixed types (57.5%) by postmortem examination [33]. On the other hand, 125 Canadian foals were classified into 4 types: 60 with pneumonia (48%), 5 with enteritis (4%), 55 having mixed types (44%), and 5 having other types (4%) [129]. The former 3 types of the disease were also observed in postmortem examinations of foals in Japan (Table 1). Intratracheal and intragastric challenge can produce pneumonia and enteritis, but most cases of the enteritis type are thought to follow respiratory infection [36, 37].

2. Clinical Diagnosis

Naturally infected foals primarily demonstrate pyrexia, cough and an increase in white blood cells in the early stage of infection [70]. The disease progresses insidiously in many cases. Delay in diagnosis and treatment tends to increase the morbidity rate. It is thought that the symptoms and conditions of the infection are influenced by

Table 1. Postmortem examination of 70 infected foals at Hidaka during 1992 to 1995

Year	No. of foals examined	Pneumonia type	Mixed type	Enteritis type
1992	18	13	5	0
1993	16	11	5	0
1994	17	11	6	0
1995	19	12	5	2

various factors, such as the host, route of infection, amount of bacteria, period of infection, and treatment time, etc. [124]. Bacterial cultures of tracheal washings are effective in diagnosing the disease definitively [70].

Enzyme-linked immunosorbent assay (ELISA) has been used as a sensitive, convenient, serological diagnostic method in the laboratory [20, 29, 79, 94], but its value at actual clinical sites has not been fully assessed. During a two-year study (1993 and 1994), we measured the ELISA antibody titer in serum extracted from a total of 878 foals in Hidaka that demonstrated symptoms of *R. equi* disease [30]. Of these, 138 foals showed an OD value of 0.3 (the positive limit of OD 0.3 is the average OD for healthy foals plus 3 standard deviations), or higher, and were identified as "Foals suspected of having *R. equi* infection." The suspected foals had been treated with antibiotics just before and after the serodiagnosis, and 126 foals (91%) recovered from the disease. The morbidity rate for this disease was 9% (12 of 138 foals), which was significantly lower than those in previous reports [1, 31, 85]. These results suggest that the detection of an antibody to *R. equi* by ELISA offers the clinician the advantage of early diagnosis of *R. equi* infection and the opportunity for early treatment of the disease [30].

3. Treatment with Antibiotics

It is well known that an effective antibiotic should be lipid soluble to enable infiltration into macrophages [32, 66, 71, 75, 77], since *R. equi* is a facultative intracellular pathogen of macrophages. The combined use of rifampin and erythromycin in infected foals has proved to be effective [32, 85]: 88% of the infected foals in Sweeney's study recovered with this treatment [85] but in Japan, gentamycin and cephem compounds are used more generally, as the side effects of erythromycin can be problematic. Gentamycin acts as an inhibitor when used with erythromycin or rifampin together, though it shows a synergistic action when combined with penicillin [62, 75]. The combination of vancomycin and imipenem or rifampin has been shown to be more effective than that of erythromycin and rifampin in the experimental infection model of nude mice [61]. The effect of these antibiotics on naturally and experimentally infected foals is a challenging subject for future studies.

4. Sensitivity and Predisposing Factors in Foals

R. equi exists widely in the environment of horse-breeding farms and in herbivorous animals [16, 55, 67, 78, 111, 112, 120, 122, 123]. Few clinical cases of *R. equi* infection are known among domestic and wild animals, with the exception of foals [4, 68]. The characteristics of the disease are age dependency and host-specificity [10]. It is unknown why foals less than 3 months old are more predisposed to infection than older foals, and factors such as immaturity, impairment of the immune system, absence of maternal antibodies, viral infection and parasite infection have been considered [31, 68, 113, 124]. But the recent discovery of virulence-associated antigens and plasmids has shed new light on the role of cellular immunity in the disease process.

5. Immune Response and Experimental Infection in Foals

Since *R. equi* is thought to be an intracellular bacterium, cellular immunity plays a major role in *R. equi* infection in foals [19, 74, 76, 121, 124]. Alveolar macrophages collected from experimentally infected foals were activated to the same level as those obtained from adult horses [28]. Foals immunized orally with live *R. equi* did not show any clinical signs after intratracheal inoculation [13]. These results suggest that cellular immunity plays a major role in protecting against *R. equi* infection in foals. On the other hand, humoral immunity has been identified as important protective mechanism in studies such as one in which *in vitro* killing activity of alveolar macrophages was significantly increased by opsonization of the bacteria with immune serum [27, 53, 128]. Moreover, the foals which received immune plasma immune to live *R. equi* did not become sick in the experimental infection, and the mortality rate for this group of foals was significantly lower than that for the non-receiving group [50]. Pregnant dams were immunized with killed bacteria; however their foals, which received raised maternal antibodies, were not completely infection free [47]. Plasma immune to the bacterium was made available in the United States in 1990, and since then, has been used to prevent outbreaks of the disease [47].

III. Ecology of *R. equi*

R. equi is thought to be a soil organism; but until the development of a selective NANAT medium by Woolcock and Mutimer [119], there was no effective way to isolate it from the soil. This medium has led to advanced epidemiological and ecological studies, in which *R. equi* has been shown to multiply in soil and the feces of foals and to be widespread in horses and their environment [5, 16, 67, 88, 102, 120, 122].

Barton and Hughes [5] pointed out that dung was important as a source of infection, since *R. equi* multiplies to 1,000 times or more in the affected horses' dung which is left on the paddock or the pasturing ground. Prescott *et al.* [78] investigated *R. equi* contamination of the loafing paddocks and pasture areas on 6 farms in Ontario. They pointed out the danger of progressive development of infection in affected soil on horse farms with prolonged use, because conditions favorable to the proliferation of *R. equi* were provided in the environment of a horse-breeding farm which had been established for 30 years. Takai *et al.* [102] studied the ecology of *R. equi* in soil on a horse-breeding farm and indicated that *R. equi* could multiply in the soil and flourish in the cycle existing between horses and their soil environment. The highest numbers of *R. equi* were found in surface soil, whereas almost no bacteria were found in soil observed 30 cm or more underground (Table 2). In addition, *R. equi* grows as well in soil exudate broth as in nutrient broth, so that it is able to proliferate in soil when growth conditions such as temperature and humidity are suitable for growth [102]. Moreover, *R. equi* was isolated from air in the stalls at the end of March, and the number of *R. equi* in the air increased

particularly on dry and windy days [88]. Prevalence of the disease appears to increase in dusty environments and dry weather [21].

R. equi was also isolated from the feces of dams at various isolation rates [16, 104, 122]. The mean numbers of *R. equi* obtained from the feces ranged from 10^2 to 10^3 per gram of feces (Table 2). On the other hand, *R. equi* was isolated first from the feces of foals at 1 to 2 weeks of age, and then it was isolated from all of the foals by 4 weeks of age. The mean number of *R. equi* increased to 10^4 to 10^5 /g of feces, was maintained up to the age of 8 to 10 weeks, and then gradually decreased to the level of the dams. The greatest numbers of *R. equi*, which were 100- to 1,000-fold greater than those of the dams, were present in the intestinal tract during the first 8 weeks of life, when foals are most liable to the infection [104]. In addition, the infected foals shed a large number of *R. equi* (10^6 – 10^8 /g of feces) in the feces, and the quantitative culture of *R. equi* in the feces was useful for making a supplementary diagnosis of *R. equi* infection [92]. The excretion of this huge number of *R. equi* may play an important role in contamination and expansion of the source of infection.

IV. New Findings Concerning the Pathogenicity of *R. equi*

1. Past Research Concerning the Pathogenicity of *R. equi*

As promising candidates for the virulence factors of *R. equi*, capsular antigens, equi factors and glycolipids of the cell wall were examined [12, 25, 44, 45, 58, 125, 127] but these components exist in all *R. equi* strains and were not able to be identified

Table 2. Distribution of virulent *Rhodococcus equi* in horses and their environment

Isolated from	Number of <i>R. equi</i> /g	Prevalence of virulent <i>R. equi</i>	References
Soil			
At surface	10^2 – 10^4	0–23.3%	87, 88, 101, 102, 103
30 cm underground	0 – 10^1	NT	102
Feces			
Healthy foals	10^3 – 10^5	0–17.7%	87, 88, 92, 101, 103, 104
Infected foals	10^6 – 10^8	–80%	92, 110
Adult horses	10^2 – 10^3	0–14.6%	87, 88, 101
Lesions in infected foal	10^8	–100%	86, 110

as virulence factors.

Capsular antigens: Prescott [65] reported that there were seven capsular serotypes in *R. equi* and Nakazawa *et al.* [60] showed the existence of 27 serotypes by means of the agglutination test. Although the distribution of serotypes seems to be dependent upon the isolates according to their origin, there is probably no relation between serotypes and virulence [60, 65]. On the other hand, Yager *et al.* [125] observed that capsular antigens of *R. equi* prevented a phagosome-lysosome fusion in foal macrophages by electron microscopy, and concluded that the capsule may be one of the virulence factors in *R. equi*. No quantitative difference in capsular antigens among *R. equi* isolates was observed with ruthenium staining [119]. Recently, the chemical structure of Prescott's capsular serotypes 1 and 2 were shown to consist of a repetitious structure of three to four sugars: mannose, glucose, rhamnose and glucuronic acid [43, 81].

Extracellular enzymes (equi factors): *R. equi* produces equi factors which interact with phospholipase D of *Corynebacterium pseudotuberculosis*, and the beta-toxin of *Staphylococcus aureus* completely hemolyses with sheep, horse and cattle erythrocytes, which are characteristics used for the identification of *R. equi* [6, 73]. Equi factors consist of cholesterol oxidase and phospholipase C exoenzymes, and have high antigenicity [45]. The equi factor neutralization test was developed to reveal the antibodies equi factors in infected foals [59, 69, 82, 83, 84]. The immunoprophylactic capacity of specific sera immune to equi factors was tested in foals experimentally induced with *R. equi* pneumonia, but the foals were not protected against the infection and later developed clinical signs of the disease [44]. These results suggest that antibodies to equi factors may not be involved in protective immunity.

Mycolic acid-containing glycolipid: The genus *Rhodococcus* belongs to the phylogenetic group described as nocardioform *Actinomycetales*, which contains the genera *Caseobacter*, *Corynebacterium*, *Mycobacterium*, and *Nocardia* [3]. *R. equi* causes chronic granulomatous pneumonia in foals [14, 35–37, 46]. The pathological change elicited by *R. equi* is similar to that of some other intracellular bacteria within the group *Mycobacterium-Nocardia*, with which it shares lipid-rich cell wall components [35–37, 67]. Gotoh *et al.* [25] showed that the

carbon chain length of the mycolic acid of *R. equi* is critical in granuloma formation in the murine liver. The mycolic acid content and type may be important to the intracellular survival of the organism and in the formation of granulomatous lesions in *R. equi* infection [25].

2. Discovery of Virulence-Associated Antigens and Virulence Plasmids in *R. equi*

Attempts have been made to elucidate the virulence of *R. equi*, and it has long been recognized that variations in the outcome of experimental infections of foals are related to differences in bacterial virulence [7, 56, 58, 100]. For instance, fresh isolates from the lesion of an infected foal reproduced the disease experimentally in foals, though strain ATCC 6939 could not reproduce the disease [52, 96]. Several investigators have reported differences in virulence between equine lung isolates and soil isolates in mouse models [7], and have showed that the lethality of *R. equi* is related to the ability of the organisms to resist phagocytosis and intracellular killing by murine macrophages [56, 58, 100]. Nevertheless little is known of markers and/or factors associated with the virulence of *R. equi*. Chirino-Trejo and Prescott [11, 12] reported the presence of a prominent and somewhat diffuse protein of 17.5 kDa and a less prominent protein of 15.1 kDa in a clinical isolate from the lungs of an infected foal. These antigens were strongly

Fig. 1. Immunoblot profiles of *R. equi* ATCC 33701 incubated at different growth pH at 38°C. Whole-cell preparations were analyzed by immunoblotting with serum from a naturally infected foal. The arrow on the left indicates the band for the 15- to 17-kDa antigen. Molecular markers are indicated by bars on the right.

Table 3. Pathogenicity of *Rhodococcus equi* for mouse and foal

<i>R. equi</i> strain	Virulence-associated antigens	Virulence plasmids	Pathogenicity for mice (LD ₅₀)	Pathogenicity for foals (Minimum infectious dose)
Virulent	15–17 kDa	85 kb, 90 kb	10 ⁶	10 ⁴
Avirulent	–	–	>10 ⁸	>10 ⁹
ATCC 6939 ^a	–	–	>10 ⁸	>10 ⁹

^aType strain, which was isolated from a infected foal by Magnusson [49].

recognized by rabbit hyperimmune serum. We compared the seroreactivities of antigens prepared from culture supernatants and whole cells of *R. equi* ATCC 33701 with sera from five infected foals by immunoblotting analysis to identify the proteins associated with infection [98, 105]. Infected-foal sera recognized several major antigens in culture supernatants and whole cells of *R. equi*. The most intense and consistent reactions occurred with the diffuse proteins having approximate molecular masses of 15 to 17 kDa (Fig. 1). These antigens were common to all of the clinical isolates of *R. equi* tested [86, 98, 114]. Then we examined the mouse pathogenicity of the clinical isolates to clarify the relationship between mouse pathogenicity and the presence of 15- to 17-kDa antigens [98]. All of the clinical isolates killed mice at a concentration of 10⁶ cells and were identified as being virulent in mice (Table 3). These antigens have also been found in environmental isolates which are virulent in mice [98] but environmental isolates without the antigens did not kill mice with more than 10⁸ cells. These results demonstrated that 15- to 17-kDa antigens are present in epidemiologically important strains, making them potentially useful as virulence markers [87, 98, 101, 103, 110].

3. Discovery of Virulence Plasmids

Nothing has been reported on the molecular mechanisms involved in the virulence of *R. equi* but it is well established that extrachromosomal elements are responsible for virulence factors of bacterial cells. With this in mind, we examined the plasmid profiles of *R. equi* strain and demonstrated the presence of a large plasmid of approximately 85 kb in virulent *R. equi* strains showing 15- to 17-kDa antigens in immunoblots [90, 108]. We also demonstrated the absence of plasmids in an

avirulent *R. equi* strain which lacked the antigen. We first showed that a large plasmid in *R. equi* strains correlated with virulence in mice and the expression of 15- to 17-kDa antigens [39, 108, 114]. In order to study the association of plasmids with the virulence of *R. equi*, mutants lacking the plasmid were obtained by repeated passages at 38°C. The mutants lacked the 15- to 17-kDa antigens and showed a dramatic decrease in lethality in mice (Table 3). These results suggested that the presence of an 85-kb plasmid may be essential for virulence and expression of the antigens of *R. equi* [39, 80, 108].

Tkachuk-Saad and Prescott [116] isolated and partially characterized *R. equi* plasmids. Six plasmids of different sizes were isolated from clinical material but no information on the relationship between the presence of the six plasmids and virulence was obtained because they did not examine the virulence of the strains by means of the mouse pathogenicity test. In later studies, we also found 20 different cryptic plasmids of various sizes in isolates from horses and their environment [93]. There were two different-sized virulence plasmids of 85 and 90 kb in these isolates (Fig. 2). Restriction enzyme and Southern blot analysis

Fig. 2. Plasmid profiles of *R. equi* strains ATCC 33701 (85 kb plasmid) and L1 (90 kb plasmid). Plasmid DNA was extracted, resolved in a 0.7% (wt/vol) agarose gel, and stained with ethidium bromide. Lanes: 1, strain ATCC 33701; 2, strain L1. chr, chromosomal DNA.

showed large regions of DNA homology between the 85- and 90-kb virulence plasmids [114]. The nucleotide sequence of the gene encoding 15- to 17-kDa antigens, located on plasmid pREAT701, has been determined [80]. The gene encodes a 19-kDa protein of 189 amino acids having an Alanine-rich leader signal sequence. At least five SS peptidase cleavage sites were found in this region, suggesting that the molecular diversity of 15- to 17-kDa antigens may be attributed to the multiple SS peptidase cleavage sites [80]. Further studies are needed to define the function and structure of the virulence-associated antigens involved in the pathogenesis of *R. equi* infection.

4. Attenuation of Clinical Isolate ATCC 6939

Strain ATCC 6939, which was isolated from a foal with fatal pneumonia in 1923 by Magnusson [49], could not experimentally reproduce *R. equi* infection in foals (Table 3). The type strain has been passaged repeatedly since it was isolated. It is very likely that the attenuation of strain ATCC 6939 occurred as a result of curing the plasmid in the strain by repeated passage [108] but the phenomenon of attenuation is not fully understood [11, 12]. We demonstrated that attenuation of the virulent strains of *R. equi* by repeated passage at 38°C is attributable to the selection of spontaneous plasmid-cured derivatives which exhibit a higher growth rate than do their virulent parents [109]. And the growth rate of neither strain was affected at 30°C by the presence or absence of the plasmid [109]. Workers who intend to examine the virulence of isolates therefore need to be aware that growth temperature is an important factor in maintaining the virulence of *R. equi*.

5. Expression of Virulence-Associated Antigens is Regulated by Temperature and pH

We have recently revealed that the expression of virulence-associated antigens is regulated by two environmental signals, pH and temperature (Table 4 and Fig. 1). The expression of these antigens was temperature regulated: when cells were grown at a low temperature (25 to 32°C), they did not express them, but when they were grown at higher temperatures (34 to 41°C), they expressed them in large amounts [91]. More recently, we found that the expression of the virulence-associated antigens of *R. equi* was regulated by pH, the antigen being produced maximally when the

Table 4. Regulation of the expression of 15- to 17-kDa antigens by temperature and pH

Temp. (°C)	pH	Expression of 15- to 17-kDa antigens
30	6.5	—
30	8.0	—
38	6.5	+
38	8.0	—

isolates were grown at 38°C and pH 6.5, but not produced when grown at 38°C and pH 8, or at temperatures below 30°C [89]. Our study also revealed that the virulence-associated antigens were expressed on the cell surface, as evidenced by their susceptibility to proteolysis by a trypsin and by the biotin-avidin protein blotting technique [91]. These results revealed that the regulatory systems of the virulence-associated antigens of *R. equi* respond to combinations of environmental signals, and that the antigens are expressed on the cell surface. These antigens may play important roles in bacterial virulence.

6. Experimental Infection in Foals and Mice

Many differences among bacterial strains, doses, routes of infection, breeds and age of foals in experimental infections, have been reported [2, 36, 37, 52, 72, 96, 117]. *R. equi* infection could not be reproduced in foals by strain ATCC6939, though the strain had been isolated from lesions of an infected foal by Magnusson [49]. Moreover, the dose used in the experimental infection was thought to be larger than that of natural infections [2, 36, 37, 44, 52, 72, 96], which might have influenced the host defense factors. In order to reevaluate the conditions of experimental infection in foals, we devised an experimental infection model with support from the Equine Research Institute of the Japan Racing Association. Foals aged 30 to 80 days old, which had not received colostrum for the first 24 hours after birth, were infected with 10⁴ to 10⁹ ATCC 33701 by intra-tracheal inoculation (Table 3). All of the experimentally infected foals showed clinical signs at 1–2 weeks after inoculation, and then these foals were autopsied to confirm the presence of lung lesions and to recover the bacteria. A plasmid-cured derivative was also inoculated into a group of foals, but they did not show any clinical signs.

Mice have been used for the pathogenicity test [7–9, 56, 58, 100, 107, 115] and for analysis of

immune responses to *R. equi* [38, 63, 64, 78, 99, 126]. Yager *et al.* [126] investigated pulmonary clearance of *R. equi* in immunodeficient mice. Recently, Nordmann *et al.* [61] used nude mice with disseminated *R. equi* infection to study the efficacy of antibiotics alone or in combination to reduce the number of bacteria in organs after treatment.

Because most previous studies on experimental infection in foals and mice did not investigate virulence-associated antigens and plasmids, some of them should be reevaluated.

V. Application of Virulence Markers to the Epidemiology of *R. equi* Infection in Foals

1. Contamination of a Farm with Virulent *R. equi*

It has been thought that endemic farms have a greater number of *R. equi* in stable soil than non-endemic ones, and that the number of cases of *R. equi* pneumonia tends to correlate with the number of *R. equi* in soil [68, 78]. However, our recent studies revealed that the difference in the prevalence of the disease on the farms is related not only to the number of *R. equi* in the environment, but also to the prevalence of virulent *R. equi* in the environment [87, 101, 103]. We recently showed that foals bred on a farm with endemic disease were exposed more frequently than those from a farm without the problem [103, 110]. An infected foal on a farm with the disease could have constantly shed virulent *R. equi* in its feces at over 10^6 /g because its intestinal contents contained a very high percentage of virulent *R. equi* [110]. This observation indicates that infected foals are the major source of virulent *R. equi* and that they contaminate the environment of farms by shedding large quantities of *R. equi* in their feces (Table 2). This may be the mechanism of progressive development of infection on stud farms with a history of the disease [110]. These findings dem-

onstrate the usefulness of virulence markers such as 15- to 17-kDa antigens and virulence plasmids in epidemiological studies of *R. equi* contamination on horse-breeding farms.

2. Virulent *R. equi* Isolated from Lesions of Infected Foals

Pathological and bacteriological examinations of infected foals were undertaken at Hidaka, Hokkaido, where *R. equi* was obtained from pure culture from lung abscesses of infected foals. *R. equi* was also isolated in large numbers from the intestinal contents of the infected foals [110]. One or other virulence plasmids were isolated from independent clinical cases in foals; the clinical isolates from Hokkaido predominantly harbored an 85-kb plasmid. In contrast, clinical isolates from Aomori predominantly harbored a 90-kb plasmid [87, 114]. Virulence plasmids may be useful markers for understanding the pathogenesis of the infection in foals. These studies suggest that natural infections in foals are caused principally by virulent *R. equi* but not by avirulent organisms.

One characteristic of *R. equi* infection is that it occurs endemically on some farms and sporadically on others, but is not found on the majority of farms [67, 122]. Before the finding of virulence markers of *R. equi*, there were numerous explanations for these observations (Table 5). The results of molecular epidemiological studies led to the proposal that the incidence of infection depends on the extent of virulent *R. equi* contamination in horses and their environment [87, 110].

3. Development of Rapid Diagnosis by Polymerase Chain Reaction (PCR)

During the last decade, various methods have been developed for the rapid diagnosis of *R. equi* infection in foals [11, 20, 42, 57, 59, 69, 76, 83, 84, 94, 95, 97]. The immunologic tests developed for rapid diagnosis are enzyme-linked immunosorbent assays (ELISA) [20, 29, 94]. An ELISA has been shown experimentally to be useful in early

Table 5. The difference in the prevalence of *R. equi* infection in foals is related to the prevalence of virulent *R. equi* in horses and their environment

Farms	Incidence of the disease	Contamination with virulent <i>R. equi</i>
Some	Endemic	Heavy
Some	Sporadic	Slight
Majority	None	None

diagnosis of infection relative to agar gel diffusion [59], synergistic hemolysis inhibition [83, 84] and radial immunodiffusion tests [69], which detect the cholesterol oxidase enzyme of *R. equi*. One rapid method leading to definitive diagnosis is thought to be a bacteriologic culture of tracheo-bronchial exudate of suspected foals [15, 70]. However, it takes several days to identify *R. equi* by culture. As an improvement, the PCR has the potential for identifying virulent *R. equi* rapidly by amplification of gene sequences unique to virulence plasmids [93], and its usefulness in the detection and identification of virulent *R. equi* in clinical specimens cannot be denied. The application of PCR to the detection of virulent *R. equi* in bronchial lavage fluid from suspected foals is now in progress.

VI. Conclusion

Recent interest in the virulence mechanisms of *R. equi* has undoubtedly been stimulated by the discovery of virulence plasmids. A series of studies in our laboratory on the virulence markers of *R. equi* has clarified some of the inconsistent findings on the pathogenicity of *R. equi* and confirmed some of the empirical observations concerning *R. equi* infection in foals. Reexamination of the pathogenesis and immunologic and epidemiologic factors of *R. equi* infection in foals in light of the virulence plasmids may lead to further insights into the molecular pathogenesis of this infection.

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