Two Cases of Dead Foals Associated with *Rhodococcus equi* Pneumonia in Turkey

Yakut OZGUR^{1*}, Serkan IKIZ¹, Babur CARIOGLU², Atilla ILGAZ¹ and Shinji TAKAI³

¹Department of Microbiology, Veterinary Faculty, University of Istanbul, Avcilar, Istanbul, ²Izmit Stud farm of Turkey Jockey Club, Izmit, TURKEY and ³School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan

Rhodococcus equi has been recognized as an equine pathogen since 1923 and is a worldwide pathogen in foals. Up to now, there has been one report on *R. equi* infection in an Arabian foal in Turkey. We report here two cases of *R. equi* pneumonia diagnosed in Turkish foals. *R. equi* was isolated from pure cultures obtained from lung abscesses of two dead foals which were sent to the laboratory from distinct stud-farms in the Izmit district, Turkey, due to the increasing mortality of foals with pneumonia. These isolates were analyzed by plasmid and protein profiles: one isolate had an 85-kb type I virulence plasmid and the other was considered avirulent. Virulent *R. equi* strains possessing an 85-kb type I or an 87-kb type I were isolated from one of the stud-farms with the problem. These results indicate that the stud-farm was contaminated with virulent *R. equi*.

Key words: *Rhodococcus equi*, foal, Turkey

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Rhodococcus equi causes suppurative pneumonia and enteritis associated with lymphadenitis in 1 to 6 month old foals. The pathogen was first isolated from a foal and considered to be the cause of purulent bronchopneumonia by Magnusson in Sweden in 1923 [8]. The disease particularly affects foals aged 1 to 3 months old with high mortality [1, 9, 10, 19]. Generally, the infection occurs sporadically even on farms endemically affected, which might be attributable to differences in the contamination of virulent *R. equi* and environmental factors such as temperature, dust, soil, pH and feeding [2, 4, 7, 16].

R. equi has two different virulence levels, virulent and avirulent [15]. An 85 or a 90 kb virulence plasmid was determined in a virulent *R. equi* strain with 15- to 17-kDa antigens [20]. A strain which has either an 85 or a 90 kb virulence plasmid causes natural infections in foals [22]. More recently, Takai *et al.* [21] examined restriction enzyme digestion patterns of virulence plasmids in foal isolates from several countries. The

digestion patterns divided the plasmids of virulent isolates into 5 closely related types [21]. The agent is found in horse feces and in areas with fecal contamination [2, 18]. Recent studies, however, have demonstrated that virulent *R. equi* contamination is more common in endemically infected farms [10, 12]. Inhalation of dust contaminated with virulent *R. equi* is the main route of infection, although foals also can be infected by ingestion [1, 6, 9, 24]. Three clinical types of the disease have been reported: acute pneumonic type, chronic pneumonic type with pyogranulomatous abscesses and intestinal type associated with mesenteric lymphadenitis, which is the least common [6, 9, 10, 24].

For economic reasons, racehorses comprise the most important segment of horse populations in Turkey and most racehorses are maintained in the Marmara region. Recently, the problem of infectious diseases has accelerated with the increase in racehorse breeding. Even though *R. equi* pneumonia is one of the most important diseases in foals, no detailed articles associated with *R. equi* infection in foals in Turkey have been published. We have recently observed two cases of *R. equi* infection in Turkish foals, and herein report our findings.

Materials and Methods

Foals

In 1998, many foal deaths were observed in stud-farms in the Izmit district from April to June, a period of very high humidity during which foals had to remain in their stalls for long periods of time. The Izmit district has many stud-farms which lie in close proximity to each other. The two foals studied were Thoroughbreds. Foal 1 was a three-month-old male from a private stud-farm, while foal 2 was two-month-old male owned by a Turkey Jockey Club stud-farm. Two lungs and swabs from lung abscesses from the foals were sent to our laboratory for bacteriological examination in May and June.

Bacteriological examination

Lung abscess samples sent to our laboratory were investigated macroscopically and Gram staining was performed for bacterioscopy. Samples from the lung abscesses were inoculated onto horse blood agar, MacConkey agar and nutrient broth with serum and incubated at 37°C for 48 hr aerobically and microaerobically. The colonies that formed on the blood agar were observed macroscopically. Gram stain was applied to the colonies and broth culture, and routine bacteriological methods were used for identification. CAMP with β -hemolysis, which produced *Staphylococcus aureus*, also was performed [9]. *R. equi* ATCC 6939 was used as the reference strain for these identification tests.

Soil samples

Soil samples were collected from 6 sites at the small paddock that was used for a mare with a foal at foot on one farm. The soil was scraped from the ground surface with a small spoon and poured into sterile tubes. One gram of soil was diluted serially with a 10-fold volume of sterile saline. Each dilution was inoculated onto 2 plates of NANAT medium [23], 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.

Colony blot analysis

For the detection of 15- to 17-kDa virulence associated antigens by colony blot analysis, bacterial strains were injected onto brain heart infusion agar plates with an inoculation needle and incubated at

38°C for 24 hr [17]. 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 for the immunoblot [14, 17].

Plasmid profiling

Plasmid DNA was isolated from *R. equi* isolates by the alkaline lysis method, with some modifications, as described previously [20, 22]. Samples of the plasmid preparations were separated along with the reference plasmids of *R. equi* ATCC 33701 and strain L1 in 0.7% agarose gel at approximately 5 v/cm for 2 hr. The bacterial strains used as reference strains in this study were *R. equi* ATCC 33701 (85 kb-type I), 96E35 (85 kb-type II), 222 (87 kb-type I), 96B6 (87 kb-type II), and L1 (90 kb-type I) [21].

Results

Clinical symptoms and postmortem examination of foals

Foals 1 and 2 had similar clinical signs, which included increased respiration rate and fever, abnormal pulmonary sounds on auscultation and cough without nasal discharge. Extensive pyogranulomatous lung abscesses were observed at postmortem examinations.

The lungs from the two foals studied showed similar lesions consisting of numerous, diffuse, 1–4 cm thin-walled pyogranulomatous abscesses containing concentrated exudate. The lung from foal 1 demonstrated more extensive abscesses.

*Isolation of *R. equi* from foals*

Extensive suppurative abscesses on the lungs were observed at postmortem examinations. Gram positive bacilli were seen on the bacterioscopy. After aerobic incubation, non-hemolytic, white, mucoid and transparent colonies began to grow as pure culture on the blood agar plates from the samples of both foals and turbidity was seen in the broth cultures. No growth was seen on the MacConkey agar plates.

Gram positive pleomorphic bacilli were seen on microscopy of the cultures. The strains were non-fermentative, oxidase negative and catalase positive. CAMP test was positive for shovel-shaped hemolysis.

Table 1. Physical, biochemical and immunological properties of *R. equi* from foals

Characteristics	Isolate 1 (y1)	Isolate 2 (y2)	ATCC 6939 (types train)
Motility	-	-	-
VP	-	-	-
Nitrate reduction	+	+	+
Citrate utilisation	-	-	-
Urease	+	+	+
Lysin Decarboxylase	-	-	-
Gelatinase	-	-	-
Aesculin hydrolysis	-	-	-
Lactose	-	-	-
Glucose	-	-	-
Sucrose	-	-	-
Rhamnose	-	-	-
Xylose	-	-	-
Maltose	-	-	-
Mannitol	-	-	-
Inositol	-	-	-
Sorbitol	-	-	-
15- to 17-kDa antigens	+	-	-

Table 2. Isolation of *R. equi* from soil samples collected from a horse-breeding farm in Turkey

Paddock plasmid No.	<i>R. equi</i> /g of soil	Prevalence (%) (No. of positive/ No. of tested)	Virulence type
1	4.3×10^4	5.3 (1/19)	85 kb type I
2	3.6×10^4	10.5 (2/19)	85 kb type I
3	3.5×10^4	15.8 (3/19)	85 kb type I (2 isolates) and 87 kb type I
4	5.1×10^4	0 (0/19)	-
5	2.7×10^4	5.3 (1/19)	85 kb type I
6	2.3×10^4	0 (0/19)	-
Total		6.1 (7/114)	

The other properties are shown in Table 1. *R. equi* was identified positively as the infecting organism. As a result of colony blot analysis 15-to 17-kDa antigens were detected from isolate 1 but not from isolate 2.

Isolation of *R. equi* from soil samples collected from a horse-breeding farm

Quantitative culture of *R. equi* from the soil samples collected from paddocks on the farm was done (Table 2). Soil from 6 paddocks was culture-positive for *R. equi*. The mean number of *R. equi* from soil on the farm ranged from 2.3×10^4 to 5.1×10^4 per gram of soil. Nineteen colonies per sample were subcultured and

Fig. 1. *EcoRI* restriction fragments of the plasmids of isolates and reference strains of *R. equi*. Lanes: 1, ATCC 33701 (85-kb type I); 2, isolate 1 (y1); 3, 222 (87-kb type I); 4 soil isolate 3-3.

were tested for the presence of 15- to 17-kDa antigens by colony blotting. As shown in Table 2, virulent *R. equi* from soil appeared in 0 to 15.8% of the isolates from the 6 sites.

Plasmid profiles of *R. equi* isolates

Two isolates from the infected foals and 7 isolates from the soil samples were then tested for the presence of virulence plasmid by agarose gel electrophoresis. As these similar plasmids were not easily distinguished by migration on the gel, all of the plasmids were analyzed by digestion with restriction endonucleases *Bam*HI, *Eco*T221, *Eco*RI, and *Hind*III for comparison in detail. Isolate 1 contained an 85-kb type I virulence plasmid (Fig. 1) and isolate 2 had no virulence plasmids. As shown in Table 2, of the 7 virulent isolates from the soil samples, 6 isolates contained an 85-kb type I and one isolate contained an 87-kb type I plasmid (Fig. 1).

Discussion

R. equi is a well-described pathogen in foals in the world. However, in Turkey there has been only one report on this disease, and it did not contain plasmid analysis [3]. The reason for there being only one report is that horse owners tend to treat illness among their horses without the help of veterinarians and post-mortem investigation. Recently, Turkish breeders have

begun to seek help with clinical matters by consulting our laboratory, which led to the finding of *R. equi* infection among Turkish foals. The clinical and autopsy findings were similar to those previously reported in the other areas of the world [1, 4, 9].

Generally, the majority of pneumonia cases associated with *R. equi* occur in the late spring to summer [1, 4, 9, 10, 19]. The two foals of this study died in May and June, and the infection apparently occurred after March and April during a period of high humidity when the foals were confined to their stud boxes for long periods. These poor conditions are thought to be factors predisposing the foals to *R. equi* infection. It has been reported that *R. equi* pneumonia is generally sporadic but endemic on some farms [7]. Many similar clinical cases were reported in the Marmara region in the same season. The mortality rate of foals quartered in studs has increased over the last few years, however, *R. equi* infection in Turkey has not been investigated.

Unfortunately, our laboratory has received only two dead Turkish foals for analysis. One of the isolates (isolate 2) was found to be avirulent, which cannot be explained fully at this time. One explanation may be related to the number of isolates obtained from one abscess. Takai *et al.* [11, 13] reported the prevalence of virulent *R. equi* in clinical isolates. The majority of clinical isolates demonstrated virulent *R. equi* containing a virulence plasmid; however, avirulent *R. equi* was isolated from lesions with low prevalence. Another possibility is that at the end stage of infection, foals might become immunodeficient. Avirulent *R. equi* is widespread in the environment of horse-breeding farms [10, 12], which enables exposure to avirulent *R. equi*. The other possibility is that the isolates cure the virulence plasmid by repeated passage [20].

The present study demonstrated that one horse farm in Turkey was contaminated with virulent *R. equi*, and that virulent *R. equi* isolates from foals and soil contained an 85-kb type I plasmid. And 87-kb type I was also found in one of the soil isolates. This means that farms in Turkey might be contaminated with both an 85-kb type I and an 87-kb type I plasmid. These types were also found in European and American isolates [21]. Further epidemiological studies are needed to clarify contamination of virulent *R. equi* on farms in Turkey.

As in many cases, the Turkish foals were diagnosed only by postmortem examinations of lesions with

bacteriologic confirmation, because the disease tends to progress insidiously and no other diagnostic means were available. As we mentioned above, it is likely that foal deaths associated with *R. equi* pneumonia will increase in the next season. Therefore, we are planning to perform an ELISA with blood sera of 35 to 40-day-old foals using the method of Higuchi *et al.* [5] for early diagnosis of the disease. These studies will give us more information on the epidemiology of *R. equi* pneumonia in foals in Turkey.

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