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Rhodococcus equi pneumonia in foals in Gyeonggi-do and characterization of the isolates from lesions and environment

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Abstract : We report here two cases of *Rhodococcus* (*R.*) *equi*-causing pneumonia of Throughbred foals in Gyeonggi-do in 2006. *R. equi* was isolated from the lung lesions of the dead foals, and from the feces and soils on the farms where the clinical cases of *R. equi* infection occurred. The isolates were characterized by biochemical properties, polymerase chain reaction for *vapA* gene and antimicrobial susceptibility. In drug susceptibility test, erythromycin, gentamycin, vancomycin, and rifampin were found to be the most susceptible for all isolates. These results suggest that *R. equi* pneumonia may be endemic in the horse-breeding farms in inland Korea and the farm environment may be widely contaminated with virulent *R. equi*.

Keywords : *R. equi* pneumonia, *vapA* gene, virulent *R. equi*

Introduction

Rhodococcus (*R.*) *equi* is a facultative intracellular, Gram-positive coccobacillus that causes suppurative pneumonia and ulcerative enteritis, and is associated with high mortality in one- to three-month-old foals [7, 11]. *R. equi* infection has worldwide distribution, and generally the infection occurs sporadically even on farms endemically affected [7, 17]. *R. equi* pneumonia has a long-term detrimental effect on the race horse breeding farms, because foals that recovered from the disease are less likely to race as the unaffected adults [2]. However, the pathogenesis of *R. equi* infection in foals has not been fully understood, and the diagnosis, treatment, and a preventive method for this disease have not been completely established yet [17].

It has been reported that the presence of a large plasmid of an 85 or a 90 kb is essential for virulence and these plasmids carry the gene *vapA*, which encodes a virulence-associated lipoprotein of 15- to 17-kDa [3, 4, 17]. Almost clinical isolates from lesions of infected foals contained one or the other virulence plasmid [16, 18]. Therefore, the virulence-associated antigens and plasmids have been used as epidemiological markers to identify virulent *R. equi* in horses and their environments [14, 18, 19].

In Korea, distribution of *R. equi* in Jeju native horses and their environments was investigated by polymerase chain reaction (PCR) for *vapA* gene, and the genetic diversity of the isolates was analyzed by genotyping of plasmid and chromosomal DNA [12]. However, *R. equi* infection in the horse farms in inland Korea has not been reported yet. We have recently observed two cases of *R. equi* pneumonia in foals in two different horse-breeding farms located in Icheon city, Gyeonggi-do, and herein report our findings on characterizaton of *R. equi* isolated from lung lesions of dead foals, feces, and soil in the horse-breeding farms in inland Korea.

Cases

In June to August 2006, two of three-month-old foals on two different farms located in Icheon city,

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Gyeonggi-do, were dead due to the severe respiratory syndromes. Before death, the foals were treated with erythromycin and rifampin for a week by a veterinarian. The samples of lung abscesses, mandibular and bronchial lymph nodes were collected aseptically from the dead foals, and were sent to the laboratory for bacteriological examination. Twenty samples of feces and soil were collected at the paddocks of the farms by the methods as described previously [12].

The swabs from the lung abscess and lymph nodes were inoculated directly onto the selective nalidixic acid-novobiocin-actidione (cycloheximide)-potassium tellurite (NANAT) medium, and the non-selective media such as blood agar and MacConkey agar, as previously described by Woolcock *et al.* [21]. The plates were aerobically incubated at 37°C for 48 h. Gram stain was applied to the colonies, and routine bacteriological methods were used to characterize the isolates.

For calculating the mean number (± SD) of colony-forming unit (CFU/g), the diluted samples were inoculated onto NANAT medium, and the specific colonies for *R. equi* were counted. [12, 18, 21]. The representative strains isolated from the lung lesions and environment were further tested by API Coryne kit (bioMerieux, France), PCR, and antimicrobial susceptibility test. *R. equi* (KCTC 9082) obtained from the Korean Research Institute of Bioscience and Biotechnology was used as a reference strain. Among the isolates, two strains from lung lesion, and four strains from the fecal and soil samples were examined for *vapA* gene by PCR. Plasmid DNA was isolated from *R. equi* by an alkaline lysis method with some modification as described previously [15, 18]. Primer 1 (5'-GACTCTTCACAAG ACGGT-3') corresponds to the sense strand at positions 6 to 23, and primer 2 (5'-TAGGCGTT GTGCCAGCTA-3') corresponds to the antisense strand at positions 569 to 552 in the sequence of *vapA* gene. PCR amplification was performed by the method with some modification as described previously [15, 16, 18]. Antimicrobial susceptibility test was performed by disk diffusion method as recommended by Wiker [20]. BBL Sensi-Disc (Becton Dickinson, USA) was used according to the manufacturer's instructions.

Foals 1 and 2 showed the similar clinical signs, such as high fever (up to 41°C), increased respiratory rate, abnormal pulmonary sounds, and cough without nasal discharge. Extensive pyogranulomatous lung abscesses were observed at postmortem examination. The sizes of lung abscesses were various from hen's egg (7 cm × 4 cm) to pea-sized (1.5 cm × 1 cm), and contained inspissated pus (Fig. 1).

In bacterial examination, a specific salmon-pink colored colonies were observed in 72-h cultures on blood agar, and specific grey colonies were shown on NANAT medium. Gram-positive pleomorphic bacilli were observed on microscopy for the colonies. None of Rhodococcus was isolated from the lymph nodes.

Quantitative cultures of *R. equi* from the fecal and soil were carried out. High prevalence (86% to 100%) of positive cultures were observed in the samples (Table 1). The mean number of *R. equi* in feces from farm 1 and 2 was $1.8\ (\pm 1.12) \times 10^2$ CFU/g and $1.0\ (\pm 0.58) \times 10^3$

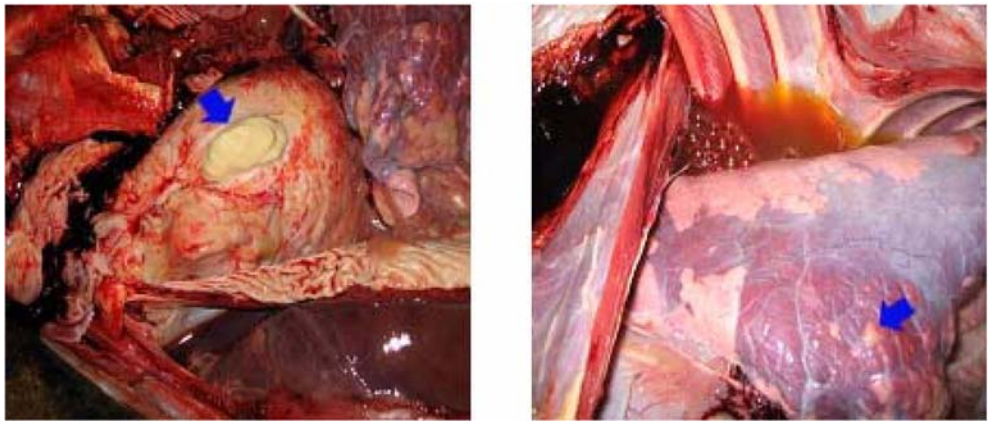

Fig. 1. Lungs of the foals with extensive pyogranulomatous abscesses typical of the lesions of *Rhodococcus equi* pneumonia. The arrows indicate the lesions.

Table 1. Isolation of *Rhodococcus equi* from the samples from lung lesions, feces, and farm soil

Specimens	Sources	No. of samples	No. of positive cultures (%)	Mean (± SD) (CFU/g) of *R. equi* (range)	Representative strains
Lung lesion	Foal-1	3	3 (100)	NT*	CNK-1
	Foal-2	3	3 (100)	NT*	CNK-2
Feces	Farm-1	13	11 (85)	$1.8 (\pm 1.12) \times 10^2$ $(1.9 \times 10^1 - 4.4 \times 10^2)$	CNK-3
	Farm-2	7	6 (86)	$1.0 (\pm 0.58) \times 10^3$ $(6.8 \times 10^1 - 2.0 \times 10^3)$	CNK-4
Soil	Farm-1	13	12 (92)	$4.4 (\pm 1.49) \times 10^2$ $(2.5 \times 10^2 - 8.2 \times 10^2)$	CNK-5
	Farm-2	7	7 (100)	$4.9 (\pm 2.14) \times 10^2$ $(2.3 \times 10^2 - 1.2 \times 10^3)$	CNK-6

*NT; not tested.

Table 2. Antimicrobial susceptibility by disk diffusion methods of 6 strains of *Rhodococcus equi* isolates

Strains	Response to																
	AN	AM	Amc	C	CZ	CIP	E	GM	K	N	NOR	P	S	SXT	T	RD	VA
CNK-1	S	R	R	I	R	S	S	S	R	S	S	R	R	R	S	S	S
CNK-2	S	I	R	I	R	S	R	S	R	R	R	R	R	R	R	S	S
CNK-3	R	R	R	S	R	R	S	S	I	R	S	R	R	I	I	S	S
CNK-4	R	S	S	R	R	S	S	S	R	R	S	R	R	S	S	I	S
CNK-5	I	R	S	S	R	S	S	S	S	R	I	R	R	R	R	S	S
CNK-6	R	S	R	R	R	R	S	R	I	S	R	R	S	R	R	S	S

R, resistant; I, intermediate; S, sensitive; AN, amikacin; AM, ampicillin; Amc, amoxycillin + clavulanic acid; C, chloramphenicol; CZ, cefazoline; CIP, ciprofloxacin; E, erythromycin; GM, gentamicin; K, kanamycin; N, neomycin; NOR, norfloxacin; P, penicillin; S, streptomycin; SXT, sulfamethoxazol-trimethoprim; T, tetracycline; RD, rifampin; VA, vancomycin.

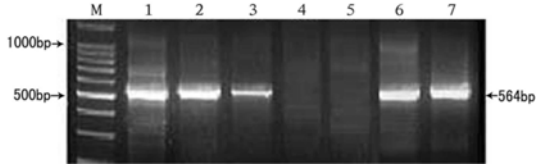

Fig. 2. Patterns of PCR amplication of virulence-associated *vapA* gene in *R. equi*. Lane M, 100 bp ladder (Invitrogen); Lane 1, CNK-1; Lane 2, CNK-2; Lane 3, CNK-3; Lane 4, CNK-4; Lane 5, CNK-5; Lane 6, CNK-6; Lane 7, *R. equi* (KCTC 9082).

CFU/g, respectively, and the mean number of *R. equi* in the soils from farm 1 and 2 was 4.4 ($\pm$ 1.49) $\times 10^2$ CFU/g, and 4.9 ($\pm$ 2.14) $\times 10^2$ CFU/g, respectively.

Among the isolates, two strains from lung lesion were named as CNK-1 and CNK-2, and four strains from the fecal and soil samples were designated as CNK-3, CNK-4, CNK-5, and CNK-6 (Table 1). All of six representative strains showed the identical properties as the reference strain in biochemical tests. By PCR, the presence of *vapA* gene was detected in CNK-1, CNK-2, CNK-3, and CNK-6, and the reference strain (KCTC 9082) (Fig. 2). The antimicrobial susceptibility of six representative strains to 17 antimicrobial agents was summarized in Table 2. Overall, erythromycin, gentamycin, vancomycin, and rifampin were the most susceptible for all isolates.

Discussion

R. equi is an important pathogen of foals worldwide, causing pneumonia mainly in 1-3 months of age, when immune system is still immature and maternal antibodies have disappeared [17]. In Korea, the reports on the cases of *R. equi* infecton in foals have been limited to Jeju Island, where it is a major horse-breeding region in Korea. Takai *et al.* [18], and Son *et al.* [12] have reported that virulent *R. equi* containing the 90

kb type II plasmid has been isolated from fecal and environmental samples of the native Jeju horse and Throughbred farms on Jeju Island. However, no report on *R. equi* infecton in foals has been published yet in inland Korea, where the horse-breeding industry is actively expanding in recent years. In this region, the respiratory diseases in foals have been considered as an important hindrance for development of the horse industry. We speculated that the *R. equi* infection might be major cause of outbreaks of respiratory diseases in foals in inland Korea.

The present study described two clinical cases of *R. equi* pneumonia in foals in Throughbred farms located in Icheon city, Gyeonggi-do, where the horse farms were contaminated with *R. equi*. It was confirmed by means of colony characteristics, biochemical tests [5], and subsequent analysis by PCR that four of six isolates had the typical properties of virulent strains of *R. equi*. Two isolates, CNK-4 and CNK-5, that showed the negative results in PCR were considered as avirulent strains of *R. equi* [16, 17].

Prevalence of virulent *R. equi* isolated from soil samples on a horse farm has been studied in various countries [3, 10, 19]. In Jeju Island, the virulent *R. equi* in soil, feces, and lung lesions was prevalent by 7% (7 of 98 samples), 15% (13 of 89 samples), and 88% (14 of 16 samples), respectively [12]. In this report, we could not present the overall prevalence of virulent *R. equi* for the samples tested, because only six strains from the isolates were examined for the biochemical properties and the virulence gene by PCR. However, we can draw a conclusion that the virulent *R. equi* are closely associated with the clinical cases of pneumonia, and that the horse-breeding farms are widely contaminated with virulent *R. equi*. This pathogen is a soil saprophyte that is most commonly found in the superficial soil layer at concentrations of up to $10^{4\text{-}6}$ CFU/gram at horse-breeding farms in Japan and other countries where *R. equi* infecton endemically occurs [3, 10, 19]. In this study, the concentration of *R. equi* in the feces and soil in the farms was much lower than the previous reports [12, 17, 18]. It has been indicated that various factors such as seasonal and geographical conditions, farm history of outbreaks of *R. equi* infection could influence the prevalence of *R. equi* isolation [7, 8, 13]. More intensive and nationwide studies are needed to elucidate the epidemiological relationships of *R. equi* infection between inland Korea and Jeju Island.

Since *R. equi* is a facultative intracellular pathogen of macrophages, it is well known that an effective antibiotic for treatment should be lipid soluble to enable infiltration into macrophage [6, 17], Administration of erythromycin in combination with rifampin is frequently used in practice, because the survival rate of *R. equi*-infected foals using this treatment is higher than that achieved by other antimicrobial therapies [6]. However, two foals with Rhodococcosis pneumonia in this study died, despite treatment with erythromycin and rifampin that were found susceptible *in vitro* for the isolates (Table 2). With present data, it is difficult to interpret these observations. However, it is conjectured that the antibiotics might be administered too late to cure the illness of foals.

In antimicrobial susceptibility test, the representative strains were found resistant to cefazoline, kanamycin, penicillin, streptomycin, and sulfamethoxazol-trimethoprim, and were the most sensitive to erythromycin, gentamycin, vancomycin, and rifampin. These antimicrobial spectrums were more or less similar to the patterns of the isolates from Jeju Island [1]. However, the effect of various antibiotics on the foals infected with *R. equi* may be a challenging subject for future studies [9, 17].

This is the first report on *R. equi* infection in foals and contamination of *R. equi* in the horse-breeding farms in inland Korea. We hope that this report will lead to nationwide investigations on the molecular epidemiology of *R. equi* in horse-breeding farms in Korea, and on the development of the effective measures for diagnosis and treatment for the disease.

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