한국가축위생학회지 제34권 제3호 (2011)
Korean J Vet Serv 34(3) : 201~208 (2011)

The biochemical and molecular characteristics of *Streptococcus equi* subsp. *zooepidemicus* isolated from the genital tract of Thoroughbred mares in Korea

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(Received 1 April 2011; revised 22 August 2011; accepted 26 August 2011)

Abstract

Streptococcus equi subsp. *zooepidemicus* (*S. zooepidemicus*) is a pathogen of a variety of infections in horse. We studied biochemical and molecular characteristics of *S. zooepidemicus* isolated from the genital tract of Thoroughbred mares in Korea. Seventy-nine isolates were identified as *S. zooepidemicus* by biochemical and PCR method from 374 horses. The biochemical characteristics of *S. zooepidemicus* isolates were positive reaction of lactose and sorbitol. However, *S. zooepidemicus* isoltes were negative reaction of inulin, mannitol, raffinose, trehalose, aesculin hydrolysis, growth in 6.5% NaCl and variable reaction of maltose. Epidemiological investigations of *S. zooepidemicus* isolates were performed by fragment analysis of *SzP* (*S. zooepidemicus* protective protein) gene, *CNE* (collagen binding protein) gene and *ISR* (16s rRNA intergenic spacer region) gene using ABI Prism 3,130 ×l Genetic Analyzer System. All isolates were shown single amplification size of 906 bp in *CNE* gene, but *SzP* and *ISR* gene were shown variable patterns of fragment size. The characteristics of *S. zooepidemicus* investigated in this study will be very useful for the prevention of infection and the studies of epidemiologic characteristics of *S. zooepidemicus*, causing the severe economic losses due to reproductive failures.

Key words : Thoroughbred horse, Genital tract, *Streptococcus equi* subsp. *zooepidemicus*

INTRODUCTION

Bain (1966) reported that β-hemolytic Streptoccocus was the most severe pathogen in the first year of infection, so only 40% of the infected horses could deliver the healthy foals and 35% of all horses could not achieve the successful pregnancy for 3 years. Furthermore *Escherichia coli*, coagulase positive *Staphylococcus* spp, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Corynebacterium equi suppurative*, and fungal infection were also shown to be severe pathogens which make hard recover. Eliminating such bacteria from genital tract was difficult, and proven hard to be treated in cases. The normal flora inhabiting in the prepuce of horses and the external genitalia and vaginal vestibulum of mares inhibit the growth of the virulent bacteria (Harkness and Wagner, 1983) Among them, *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) caused 75% of vaginal infection and most of the septic abortion (Roszel and Freeman, 1988). *S. zooepidemicus* is often isolated from secondary bacterial infections in horse. It can invade the upper respiratory mucosa and lymph nodes after a viral infection, sometimes mimicking strangles. In susceptible mares, it can cause cervicitis, metritis and placentitis (as an ascending infection from the vagina) resulting in abortion. It is the most common cause of mastitis in horses and has been associated with numerous other infections including wound infections, septicemia in colts, lower airway inflammation in young horses. Lower airway inflammation can occur repeatedly in young horses

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and adversely affect subsequent racing performance (Bain, 1966; Choi et al, 2007).

Thoroughbred and Anglo-Arab horse that were imported from Japan and Australia have been used as racing horse in Korea since 1970s. Since early 1980s, Thoroughbred horse breeding has been produced within country for national qualified horse racing and developed domestic animal industry. Now a day, about 26,000 domestic horses were raised including 10,000 improved breed horses and 16,000 native breed horses. Among them, 2,300 Thoroughbred mares and 70 stallions were raised for breeding and about 1,200 foals were bred every years (Choi et al, 2007). The study of the propagation features related to the improvement in productivity Thoroughbred mares raised within the country has been reported. Cho et al (2007) reported the necropsy and histological characterisitics of fibrinopurulent peritonitis and abdominal abscesses associated with *S. zooepidemicus* in a 1 year old Thoroughbred horses in Jeju. However, there are no report the *S. zooepidemicus* isolated in the genital tract of horse in Korea. At this point, this research will be the first one based on *S. zooepidemicus* infection clinical patterns and characteristics. In particular, the biochemical and genetic features of *S. zooepidemicus* will help out many other parts of similar researches. Furthermore, it will help not only the horses themselves but also people in horse farm, horse riding club and animal hospitals through *S. zooepidemicus* screening system. Therefore, this study performed the biochemical and molecular characteristics of *S. zooepidemicus* isolated from the genital tract of Thoroughbred mares.

MATERIALS AND METHODS

Animals

A total of 374 Thoroughbred horses from equine stud farm and raising farm at Jeju and Jangsu were examined for 3 years from June 2006 to July 2009. Samples were collected from vaginal discharge, uterine washing, aborted fetus and uterine of abortion mare in the fifth month of pregnancy. In this study 79 *S. zooepidemicus* isolated from 374 Thoroughbred horses (230 healthy horses, 143 clinical endometritis horses and 1 abortion fetus) from Jeju and Jeonbuk region in Republic of Korea were investigated. Culture for isolation of *S. zooepidemicus* was performed according to previously described techniques (Anzai et al, 2002). The vaginal swabs were taken from clitorial fossa of healthy horses, uterine flushing fluids from clinical endometritis horses only. The swabs were transported in modified Stuart's medium (BD, USA) in a portable fridge. Samples were transported to the laboratory in Liquid Stuart Medium (BD, USA) on ice packs and refrigerated until used (Quinn et al, 1993).

Isolation and identification of *S. zooepidemicus*

Culture for isolation of *S. zooepidemicus* from samples was performed according to previously described techniques (Widders et al, 1984; Bannerman et al, 2003). Following vortexing of swabs in the sample buffer, the swabs were transferred using sterile technique to individual tubes containing 2 ml of Bacto Todd-Hewitt broth (BD, USA), and incubated in ambient air at 37°C overnight. Approximately 10 μl of broth was then inoculated on Bloods agar at 37°C for 24 h. *S. zooepidemicus* suspected strains were stored at −70°C in freezer vials containing 15% sterile glycerol for further analysis. The isolates were identified on the basis of colonial and cellular morphology, gram staining, catalase test, oxidase test, OF-test, and were also identified with VITEK 2 instrument (BioMerieux, France) with Gram Positive (GP) identification card (Eigner et al, 2005).

Identification of *S. zooepidemicus* was performed by using multiplex-PCR with *S. zooepidemicus* suspected strains obtained from biochemistry test. Molecular identification of *S. zooepidemicus* isolates was performed by using *sod*A-*see*I specific multiplex PCR using following primer pairs (Anzai et al, 2002): *SodA*-F (5'- CAG CAT TCC TGC TGA CAT TCG TCA GG-3') and *SodA*-R (5'- CTG ACC AGC CTT ATT CAC AAC CAG CC-3'); *SeeI*-F (5'- GAA GGT CCG CCA TTT TCA GGT ACT TTG-3') and *SeeI*-R (5'- GCA TAC TCT CTC TGT CAC CAT GTC CTG-3'). The species identification of *S. zooepidemicus* was confirmed by *sod*A-*see*I multiplex

Table 1. Oligonucleotide primers and PCR programs used for fragment analysis in this study

Target gene	Sequence (5'→3'), 5'-Fluorescent label	PCR program	Size of product
CNE	F-(6-FAM*) GCAACTAATCTTAGTGACAACAT R-AAAGCTGGTATAGCGACTGCCA	1†	906 bp
ISR	F-(6-FAM)TTGTACACACCGCCCGTCA R-GGTACCTTAGATGTTTCAGTT	2‡	Size polymorphism
SzP	F-(6-FAM)ACAAAAGGGGAATAAAATGGC R-TTTACCACTGGGGTATAAGGCT	3§	Size polymorphism

*Carboxyfluorescein, †1× (95°C 180 s), 20× (94°C 30 s, 54°C 40 s, 72°C 50 s), 1× (72°C 180 s), ‡1× (95°C 180 s), 20× (94°C 60 s, 58°C 60 s, 72°C 60 s), 1× (72°C 180 s), §1× (95°C 180 s), 20× (94°C 30 s, 50°C 60 s, 72°C 90 s), 1× (72°C 180 s).

Fig. 1. PCR amplification of *sodA-seeI* for identifi-cation of *S. zooepidemicus*. Lane M: molecular size marker (100 bp DNA ladder, Elpis, Korea), lane 1: *S. equi* isolates, lane 2 to 11: *S. zooepidemicus* isolates. *S. zooepidemicus* were identified by PCR products of only 235 bp in the *SodA* gene. as it was compared with PCR products of both 235 bp and 520 bp in *S. equi*.

PCR giving a positive *sodA* gene (235 bp) and negative *seeI* gene (520 bp) reaction. PCR assays were accomplished in a total volume of 25 μl of the following mixtures: 2 μl DNA, 2 μl (10 pmol/μl) of each primer, 12.5 μl of 10× reaction buffer (Qiagen, Germany), and 6.5 μl distilled water. PCR amplification was as follows: first step was performed by initial denaturation for 3 min at 94°C, followed by 30 cycles at 94°C for 30 sec, 59°C for 30 sec and 72°C for 40 sec for *sodA-seeI*. After identification, bacteria were stored at −70°C until use (Bannerman et al, 2003; Bienert et al, 2003).

Fragment analysis of *S. zooepidemicus*

PCR amplification (Hirota et al, 2010) was performed in a reaction mixture of 10 μl containing 5 μl of Multiplex Master Mix (Qiagen, Germany), 30 ng of DNA template, and *SzP* (*S. zooepidemicus* protective protein) gene, *CNE* (collagen binding protein) gene and *ISR* (16s rRNA intergenic spacer region) specific primers, respectively (Younan et al, 2005). Concentration, primer sequences, PCR programme and respective dyes used are shown in the Table 1. PCRs were performed and prepared for subsequent analysis on sequencers in accordance with the manufacturer's instructions. Amplification products were diluted 1:10 with water purified by high performance liquid chromatography, and 0.5 μl of diluted DNA was mixed with 9.5 μl of Hi-Di formamide (Applied Biosystems, USA) and 0.5 μl of GeneScan-1200 LIZ Size Standard (Applied Biosystems, USA) as internal lane size standard. Before fragment sizing in ABI Prism 3,130 xl Genetic Analyzer System (Applied Biosystems, USA), samples were incubated for 2 min at 95°C and immediately frozen at 20°C for >3 min to denature the DNA. If a *SzP* and *ISR* were not detected during fragment analysis, reactions were repeated by using singleplex reactions with minor modifications to amplify the specific locus. In that particular instance, the primer concentration was increased to 0.2 μmole/ml, annealing temperatures were reduced to 49°C, and the extension time was tripled to amplify larger fragments because of possible insertion sequence element transposition or other genetic events (Lee and Cho, 2006; Lindstedt et al, 2007; Hirota et al, 2010; Hyytia-Trees et al, 2010). After fragment analysis, corresponding peak data were examined by using GeneMapper Ver. 4.0 software (Applied Biosystems, USA).

RESULTS

Isolation and identification of *S. zooepidemicus*

Seventy-nine of *S. zooepidemicus* isolates from 374 samples in this study grew as small, grey to white colo-

Table 2. The isolation frequency of *S. zooepidemicus* isolates isolated from Thoroughbred horses

Specimen	No. of positive/test samples (%)		Total
	Jeju	Jangsu	
Aborted fetus	1/1 (100)	–	1/1 (100)
Uterine of abortion mare	1/1 (100)	–	1/1 (100)
Healthy horse (Vaginal discharge)	16/82 (19.5)	4/148 (2.7)	20/230 (8.7)
Endometritis (Uterine washing)	42/102 (41.8)	15/40 (37.5)	57/142 (40.1)
Total	60/186 (32.3)	19/188 (10.1)	79/374 (21.2)

Table 3. Biochemical characteristics of 79 *S. zooepidemicus*

Substrates	No. of positive isolates (%)	Substrates	No. of positive isolates (%)
D-amygdalin	0 (0.0)	Lactose	76 (96.2)
Ala-Phe-Pro arylamidase	79 (100.0)	D-mannitol	52 (65.8)
Leucine arylamidase	79 (100.0)	Salicin	71 (89.9)
Alanine arylamidase	79 (100.0)	Urease	0 (0.0)
D-ribose	16 (20.3)	D-mannose	79 (100.0)
Novobiocin resistance	67 (84.8)	Saccharose/sucrose	72 (91.1)
D-raffinose	0 (0.0)	Beta galactopyranosidase	29 (36.7)
Optochin resistance	79 (100.0)	Alpha mannosidase	0 (0.0)
Cyclodextrin	58 (73.4)	Polymixin b resistance	56 (70.9)
L-proline arylamidase	3 (3.8)	D-maltose	79 (100.0)
Tyrosine arylamidase	79 (100.0)	D-trehalose	0 (0.0)
L-lactate alkalinization	0 (0.0)	Alpha glucosidase	79 (100.0)
Growth in 6.5% NaCl	0 (0.0)	Phosphatase	79 (100.0)
D-xylose	0 (0.0)	D-galactose	79 (100.0)
Beta glucuronidase	31 (39.2)	Bacitracin resistance	53 (67.1)
D-sorbitol	13 (16.5)	Pullulan	79 (100.0)

nies on 5% sheep blood agar plates surrounded by a wide zone of complete hemolysis. *S. zooepidemicus* species-specific *sod*A-*see*I gene generated by 235 bp fragments (Fig. 1). It was isolated 60 (32.3%) samples from Jeju and 19 (10.1%) from Jangsu, respectively (Table 2). *S. zooepidemicus* isolates was isolated from uterine washing fluid (40.1%) and vaginal discharge (8.7%).

Biochemical characteristics of *S. zooepidemicus*

The biochemical characteristics of *S. zooepidemicus* isolates are shown in Table 3. The *S. zooepidemicus* isolates were showed Gram positive cocci that occur in pairs, facultative anaerobes, catalase-negative, oxidase-negative, non- motile. A total of 79 isolates shown the same pattern in the majority of biochemical properties such as Ala-Phe-Pro arylamidase (100.00%), Leucine arylamidase (100.00%), Alanine arylamidase (100.00%), Optochin resistance (100.00%), Tyrosine arylamidase (100.00%), D-mannose (100.00%), Alpha glucosidase (100.00%), D-maltose (100.00%), D-galactose (100.00%), Phosphatase (100.00%). But, D-ribose (20.3%), Cyclodextrin (73.4%), Beta glucuronidase (39.2%), D-sorbitol (16.5%), Lactose (96.2%), D-mannitol (65.8%), Salicin (89.9%) showed the diversity by isolates.

Molecular characteristics of *S. zooepidemicus*

Fragment analysis of *S. zooepidemicus* isolates is shown in Fig. 2～4. All isolates were shown single amplification size of 906 bp in *CNE* gene, but *SzP* gene was shown variable patterns, 1,158 bp (n=2) and 1,118 bp (n=77). Fragment size of *ISR* gene were observed 769 bp (n=33), 783 bp (n=4), 787 bp (n=25), 933 bp n=3), 940 bp (n=10), 953 bp (n=3), and 1,074 bp (n=1).

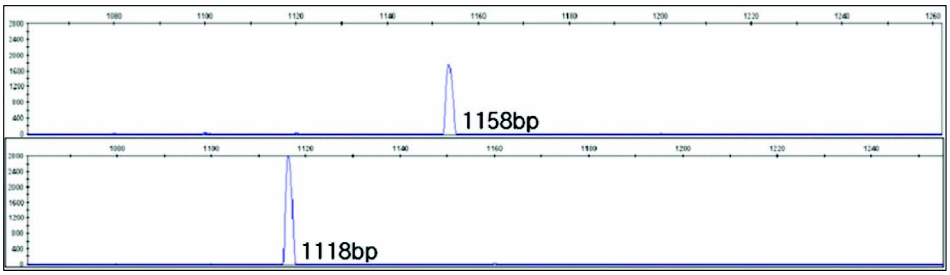

Fig. 2. Peak data of *SzP* fragment analysis. After fragment analysis, *SzP* peak data were examined by using GeneMapper Ver. 4.0 software (Applied Bio-systems, USA). *S. zooepidemicus* isolates with sizes of 1,158 bp, 1,118 bp.

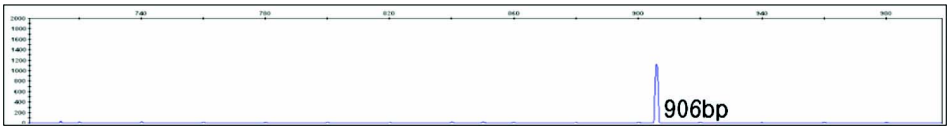

Fig. 3. Peak data of *CNE* fragment analysis. After fragment analysis, *CNE* peak data were examined by using GeneMapper Ver. 4.0 software (Applied Bio- systems, USA). *S. zooepidemicus* isolates with sizes of 906 bp.

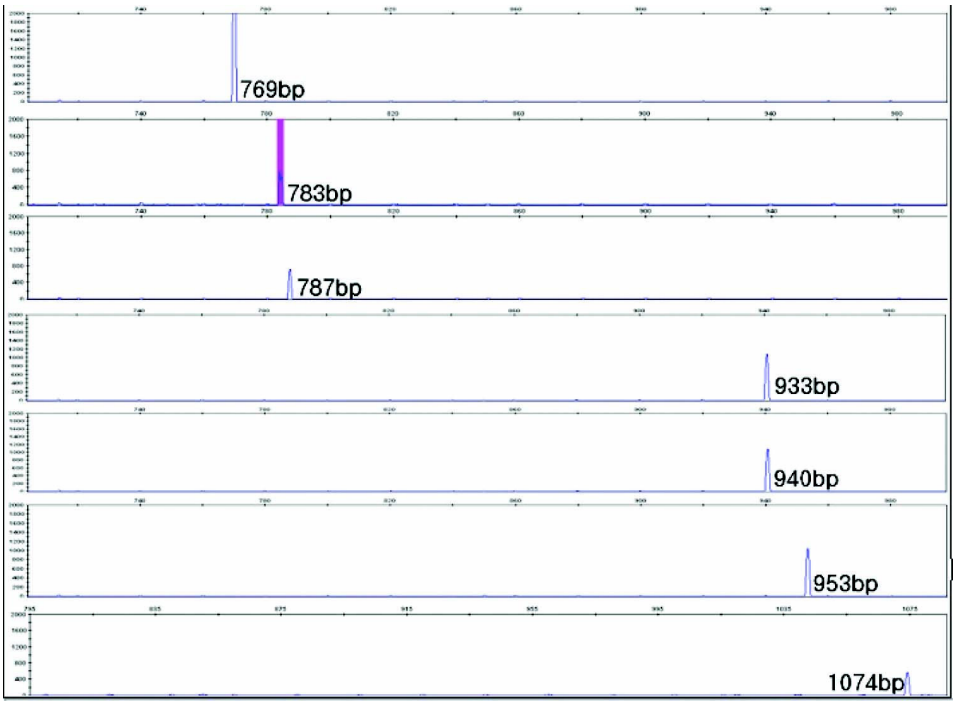

Fig. 4. Peak data of *ISR* fragment analysis. After fragment analysis, *ISR* peak data were examined by using GeneMapper Ver. 4.0 software (Applied Bio- systems, USA). *S. zooepidemicus* isolates with sizes of 769 bp, 783 bp, 787 bp, 933 bp, 940 bp, 953 bp, 1,074 bp.

DISCUSSION

Genital infections are a significant source of reproductive failure and economic loss to the horse breeding industry (Jeffcott et al, 1982; Pycock and Allen, 1990; Miragaya et al, 1997; Ozgur et al, 2001). The principal cause of endometritis in mare is *S. zooepidemicus*, the isolation rate of which range from 22 to 54% of affected mares (Bain, 1966; Asbury et al, 1980; Wingfield Digby and Ricketts, 1982; Widders et al, 1985). *S. zooepidemicus* has also been identified as the primary etiological agent in 15 to 20% of equine abortions (Rober, 1971; Widders et al, 1984).

Frontoso et al (2008) reported that samples were collected from the uterine of 75 mares and tested with rectal palpation and ultrasonography for foal heat. As a re-

sult of the bacterial test from the samples, 49 horses (65.3%) in total showed positive reaction and beta hemolytic *Streptococcus* (25.3%) and *E. coli* (24.0%) were the most frequently detected bacteria. It is primarily required not only to select the horses being of good line, but also to establish and improve the technique of horse breeding in order to meet the needs of Korean horse market and secure nicer race horses and riding horses.

The samples in this study were collected from Jangsu and Jeju. But the selection for sampling done intensively from the certain regions including Jangsu and Jeju is attributed to the geographical nature peculiar to horse raising and reproducing. As to the domestic horse breeding, more than 95% is confined to Jeju and Jangsu. With this condition considered, it is preferred that other types of diseases be examined at the same time as well as the survey over S. zooepidemicus infection rate. The isolation rate of S. zooepidemicus in this study was indicated comparatively higher than that of Bain with 22% (Bain, 1966) and lower than those like Welsh with 54% (Welsh, 1984). It is a reality that there has not been any survey practically about S. zooepidemicus infection rate in the country. It is hard to mention any other studies available and simple comparison. However, the comparatively high rate of S. zooepidemicus in this study is sure to appear domestically when compared to such foreign case study by Roszel and Freeman (1988) and McKinnon and Voss (1993).

Although there were some differences in sorbitol, lactose, salicin, maltose among 79 *S. zooepidemicus* tested in this study, the results obtained were almost in accordance with the traditionally biochemical characteristics of *S. zooepidemicus* isolates. Interestingly, our results showed that the fermentation rate for sorbitol (16.46%) in this study was indicated comparatively lower than traditionally biochemical characteristics of *S. zooepidemicus* (Quinn et al, 1993). As a result of fragment analysis about *CNE*, it was confirmed that all Korean isolates were of same amplification size due to the observation of amplification size of 906 bp in all isolated strains.

Table 4. Molecular characteristics of 79 *S. zooepidemicus*

Gene	Fragment size	Region		Total (%)
		Jeju (%)	Jangsu (%)	
CNE	906 bp	60*/60† (100)	19/19 (100)	79/79 (100)
SzP	1,118 bp	58/60 (96.7)	19/19 (100)	77/79 (100)
	1,158 bp	2/60 (3.3)	–	2/79 (100)
ISR	769 bp	30/60 (50.0)	3/19 (15.8)	33/79 (41.8)
	783 bp	4/60 (6.7)	–	4/79 (5.1)
	787 bp	20/60 (33.3)	5/19 (26.3)	25/79 (31.6)
	933 bp	–	3 (15.8)	3/79 (3.8)
	940 bp	6/60 (10.0)	4/19 (21.1)	10/79 (12.7)
	953 bp	–	3 (15.8)	3/79 (3.8)
	1,074 bp	–	1 (5.5)	1/79 (1.3)

*No. of positive, †Test samples (%).

However, analyzing epidemiological properties and characteristics of strains using *CNE* fragment analysis was difficult. But in the case of *SzP* virulence factors, 1,158 bp (n=2), 1,118 bp (n=77) and *ISR* gene region, 769 bp (n=33), 783 bp (n=4), 787 bp (n=25), 933 bp (n=3), 940 bp (n=10), 953 bp (n=3), 1,074 bp (n=1) amplicon size observed. Based on fragment analysis of *SzP* results, we observed that most of the 60 strains isolated from Jeju had amplification sizes of 1,158 bp (n=2), 1,118 bp (n=58) and the 19 strains isolated from Jangsu had amplification size of 1,118 bp (n=19). Isolates from Jeju were classified into two groups, while those of Jangsu were categorized into one group by fragment analysis results of *SzP* gene.

Also, based on fragment analysis of *ISR* results, we observed that most of the 60 *S. zooepidemicus* strains isolated from Jeju had amplification sizes of 769 bp (n=30), 783 bp (n=4), 787 bp (n=20), 940 bp (n=6) and the 19 *S. zooepidemicus* strains isolated from Jangsu had amplification sizes of 769 bp (n=3), 787 bp (n=5), 933 bp (n=3), 940 bp (n=4), 953 bp (n=3), 1,074 bp (n=1) (Table 4). *S. zooepidemicus* strains isolated from Jeju were classified into five groups, while those of Jangsu were categorized into six groups by fragment analysis of *ISR* gene.

It is difficult to confirm the characteristics of isolates from each area merely based on *SzP* and *ISR* fragment analysis. But, it is highly correlated with the fact that various *SzP* and *ISR* patterns were shown in Jeju and Jangsu. These results indicate a high genetic heterogeneity among the isolates which would be related either with the wide distribution of this microorganism in equine population, or with the fact that *S. zooepidemicus*

strains were isolated from individual mares, most of them belonging to single owners without any epidemiological or geographical relationship between them and possibility of influx of diseases from other areas.

ACKNOWLEDGMENTS

This study was supported by the Technology Development Program for Agriculture and Forestry (108175-03-1-CG000), Ministry for Agriculture, Forestry and Fisheries, Republic of Korea.

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