

## RAPD Analysis and Antimicrobial Susceptibility of *Streptococcus equi* subsp. *zooepidemicus* Isolated from Thoroughbred Horses

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A total of 68 samples were collected including vaginal mucosa (n=66) from Jangsu stud farm, an equine aborted fetus (n=1), and uterine contents (n=1) from Jeju island. Seventeen *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) strains isolated from horses in Korea were identified as *S. zooepidemicus* by biochemical tests and *sodA* - *sed* specific multiplex PCR. All isolated strains were divided into 4 clusters: group 1 (No. 2, 3, 5, 6, 7, 11, 12, 13, 14, 15), group 2 (No. 4, 9), group 3 (No. 10, 16, 17), and group 4 (No. 1, 8) by RAPD typing. In group 3, No. 10 isolate that was isolated from vaginal mucosa was indistinguishable from No. 16 and 17 isolates, which were isolated from the equine uterine contents and the equine aborted fetus, respectively. The results of this study suggest that a limited epidemiological relationship exists between the strains from Jangsu (No. 10) and Jeju (No. 16 and No. 17). All isolates showed a high susceptibility to ampicillin, cefoxitin, ceftiofur, cephalothin, florfenicol, gentamicin, nalidixic acid, oxacillin, penicillin, tiamulin, tylosin and vancomycin in antimicrobial susceptibility tests. These results may provide the basic information needed to establish strategies for the treatment and prevention of reproductive diseases in mares in Korea.

**Key words** : Antimicrobial susceptibility, equine, *Streptococcus zooepidemicus*

### Introduction

*Streptococcus equi* (*S. equi*) which belongs to the beta-hemolytic Lancefield group C, is an important pathogen in equine respiratory disease. Originally regarded as a single species, *S. equi* have recently been divided into two subspecies, *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* [2]. These two subspecies share 98% DNA homology but exhibit distinctly different phenotypes. *S. equi* subsp. *equi* (*S. equi*) is a cause of the equine disease known as strangles and is considered far more pathogenic than *S. equi* subsp. *zooepidemicus* (*S. zooepidemicus*) [28]. *S. zooepidemicus* is a normal commensal organism and an opportunistic pathogen in the equids. Also *S. zooepidemicus* is commonly found in colonizing the mucous membranes of healthy equids. It is associated with respiratory tract infections in foals and with uterine infections in mares [28].

While *S. equi* is essentially confined to equids, *S. zooepidemicus* can cause disease both in animals and humans [17]. Human infections are rare and the clinical presentations include pharyngitis, septicemia, meningitis, purulent arthritis,

and endocarditis [6,7]. The source of human infection was often traced back to contact with domestic animals, especially horses, or ingestion of unpasteurized milk or milk products [10,21]. *S. zooepidemicus* is a bacterium the most frequently isolated from cases of equine pneumonia and pleuropneumonia [33] and has generally been regarded as the most important secondary pathogen of the respiratory tract of horses following primary viral infection [3] and it is associated in uterine infections in mares. Streptococcal infection of the uterus has long been recognized as a leading cause of infertility in horses [17]. Several species of bacteria have been incriminated as causative agents of equine abortion. Of these bacteria, *S. zooepidemicus* is one of the frequent causes of infectious abortion in mares [16,18].

There have been several typing techniques described for isolates of *S. zooepidemicus* using various technologies that have been available at different times [1,25,32]. The recently developed technique of random amplified polymorphic DNA (RAPD) analysis generates DNA fingerprints that can be used to compare strains within a species or from closely related species [9,31].

The objective of the study was to investigate the RAPD analysis and antimicrobial susceptibility of *S. zooepidemicus* isolates isolated from horses.

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## Materials and Methods

### Samples and Bacterial isolation

During the breeding season (March to June) from 2008 to 2009, 68 samples were collected from an equine fetus aborted in the fifth month and uterine contents of mare, and 66 vaginal mucosa of mares suspicious of the genital disease. The isolates were identified on the basis of colonial and cellular morphology, Gram staining, and biochemical characteristics using standard bacteriological techniques [13], MicroLog (BIOLOG, USA) and molecular identification. Molecular identification of *S. zooepidemicus* isolates was performed by using *saxA* - *saeI* specific multiplex PCR [2]. The SzP and the CNE encoding gene were then performed with the oligonucleotide primers described by Anzai *et al.* [4,5] and Lannergard *et al.* [22], respectively. The sequences of the oligonucleotide primer is given in Table 1.

### Random Amplification of Polymorphic DNA

The 17 strains (No. 1~15 isolates isolated from equine vaginal mucous, No. 16 uterine contents and No. 17 equine aborted fetus) were typed by using RAPD analysis. The sequences of the oligonucleotide primer is given in Table 1. PCR was carried out in a 50 ul reaction mixture containing 50 ng of template DNA, 25 ul of master mix, 50 pM of each primer, and 23 ul distilled water. PCR amplification was as follows: first step was performed by initial denaturation for 5 min at 94°C, followed by 35 cycles at 94°C for 1 min, 36°C for 1 min and 72°C for 3 min [9]. A negative control incorporating all PCR components except genomic DNA was carried out for each RAPD-PCR conducted. The presence PCR products was determined by electrophoresis of 20 ul of the reaction product in a 1% agarose gel (SeaKem, USA)

Table 1. Oligonucleotide primers used in this study

Primers	Sequence (5'-3')
<i>SaxA</i>	F-CAGCATTCTGCTGACATTCGTCAGG R-CTGACCAGCCTTATTCACAACCAGCC
<i>SaeI</i>	F-GAAGGTCCGCCATTTTCAGGTAATTTG R-GCATACTCTCTCTGTCACCATGTCCTG
<i>SzP</i>	F-ACAAAAGGGGAATAAAATGGC R-TTTACCACTGGGGTATAAGGCTT
<i>CNE</i>	F-GCAACTAATCTTAGTGACAACAT R-AAAGCTGGTATAGCGACTGCCAT
<i>RAPD</i>	F-AAGTAAGTGACTGGGGTGAGCG R-TACATTCGAGGACCCCTAAGTG

with Tris-acetate electrophoresis buffer (Bioneer, Korea). After electrophoresis, the gel was stained with ethidium bromide. RAPD patterns with the same number and size of DNA fragments were considered to belong to the same strain, regardless of band intensity.

### Antimicrobial susceptibility test

All *S. zooepidemicus* tested were investigated for their antimicrobial susceptibility by an agar diffusion disk method using the following disks (Difco, USA) [8] : amikacin (30 µg), ampicillin (10 µg), cefoxitin (30 µg), ceftiofur (30 µg), cefoxitin (30 µg), cephalothin (30 µg), ciprofloxacin (5 µg), enrofloxacin (10 µg), florfenicol (10 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), nitrofurantoin (30 µg), oxacillin (30 µg), penicillin (30 µg), trimethoprim/sulphamethoxazole (1.25/23.75 µg), tetracyclin (30 µg), tiamulin (30 µg), tylosin (150 µg), and vancomycin (19 µg). The results were evaluated according to the document M31-A2 of the clinical and laboratory standards institute.

## Results and Discussion

A total of 17 (25%) isolates were identified as *S. zooepidemicus* after negative aesculin and sodium hippurate hydrolysis reactions; fermentation of lactose, salicin, and sorbitol but not mannitol, raffinose and trehalose; negative CAMP reaction and other biochemical characteristics determined by using MicroLog (BIOLOG, USA) (Table 2). All isolates were confirmed by *saxA*-*saeI* PCR giving a positive *saxA* (235 bp) and a negative *saeI* reaction (Fig. 1), also positive for the SzP and the CNE encoding gene with amplicon sizes of approximately 1,200 bp and 906 bp, respectively (Fig. 2). Younan *et al.* [29] reported that the *saxA* and CNE encoding gene sizes of *S. zooepidemicus* isolated from camels and camel milk were 235 bp and 900 bp, respectively, and the SzP encoding gene sizes ranged from 1,075 bp to 1,225 bp. Alber *et al.* [2] reported that the sizes of PCR product using *saxA*-

Table 2. Frequencies of *S. equi* subsp. *zooepidemicus* isolated from horse

Sources	No. of horse	No. of <i>S. equi</i> subsp. <i>zooepidemicus</i> (%)
Fetus and uterine contents	2	2 (100.00)
Vaginal mucosa	66	15 ( 22.72)
Total	68	17 (25.00)

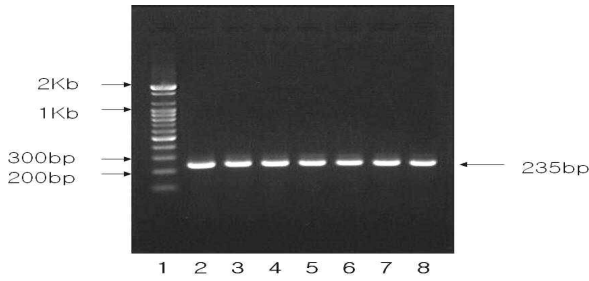


Fig. 1. Gel electrophoresis of PCR products of *S. equi* subsp. *zooepidemicus* with sizes of approximately 235 bp using *sodA* - *sef* specific multiplex PCR. Lane 1: 100 bp ladder marker (Bioneer, Korea), Lane 2-7: vaginal mucosa, Lane 8: aborted equine fetus.

*sef* oligonucleotide primers for identification of *S. equi* subsp. *equi* and *S. zooepidemicus* were approximately 230 bp and 520 bp, respectively. These results were almost consistent with results of this study. These molecular analysis are potentially useful in identifying and characterizing *S. zooepidemicus* strains, although an epidemiological relationship could not be confirmed with molecular methods.

RAPD has been used for typing a number of medically important bacteria, fungi and parasites [30]. It is a simple tool for the identification of closely related species and as-

essment of their genetic relationships. In this study, all isolates were divided into 4 cluster by RAPD typing (Fig. 3). Group 1 (No. 2, 3, 5, 6, 7, 11, 12, 13, 14, 15), group 2 (No. 4, 9), group 3 (No. 10, 16, 17), and group 4 (No. 1, 8). In group 3, No. 16 and 17 isolates isolated from equine uterine contents and equine aborted fetus, respectively, were indistinguishable from No. 10, but an epidemiological relationship could not be confirmed with RAPD typing. The results of this study suggest that a limited epidemiological relationship between the strains from Jangsu (No. 10) and Jeju (No. 16 and No. 17). Further studies are required to determine the epidemiological relationships of *S. zooepidemicus* infection and the opportunistic pathogenic character in horses.

All isolates showed high susceptible to ampicillin, ceftiofur, cephalothin, florofenicol, gentamicin, nalidixic acid, oxacillin, penicillin, tiamulin, tylosin and vancomycin in antimicrobial susceptibility tests (Table 3). These results show that  $\beta$ -lactams, enrofloxacin and gentamicin could be recommended as empiric antimicrobial therapy in cases of endometritis caused by *S. zooepidemicus* in horses [24]. The common infectious causes of equine abortions are EHV-1, *S. zooepidemicus*, *Escherichia coli*, and *Leptospira* spp. [16,18]. *S. zooepidemicus* is an important pathogen of the horse being

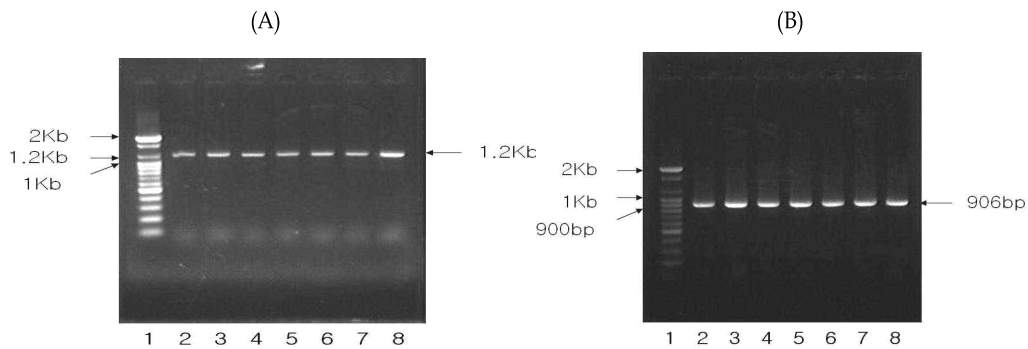


Fig. 2. PCR products of 1200 bp (A) and 906 bp (B) fragments of SzP and CNE encoding gene of *S. equi* subsp. *zooepidemicus*, respectively. Lane 1: 100 bp ladder marker (Bioneer, Korea), Lane 2-7: vaginal mucosa, Lane 8: aborted equine fetus.

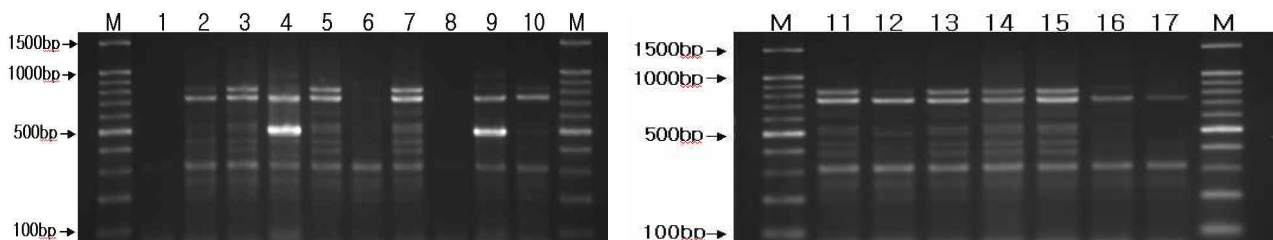


Fig. 3. Random amplified polymorphic DNA-PCR banding patterns of *S. equi* subsp. *zooepidemicus* isolates from horses. Lane M: 100 bp ladder marker (Elpisbiotech, Korea), Lane 1-15: vaginal mucosa, Lane 16: uterine contents, Lane 17: aborted equine fetus.

Table 3. Results of antimicrobial susceptibility tests of 17 *S. equi* subsp. *zooeidemicus* isolates

Drugs	Susceptible (%)	Drugs	Susceptible (%)
Amikacin	13 (76.47)	Ampicillin	17 (100.00)
Cefoxitin	17 (100.00)	Ceftiofur	17 (100.00)
Cephalothin	17 (100.00)	Ciprofloxacin	11 (64.71)
Enrofloxacin	13 (76.47)	Florfenicol	17 (100.00)
Gentamicin	17 (100.00)	Kanamycin	14 (82.36)
Nalidixic acid	17 (100.00)	Nitrofurantoin	16 (94.12)
Oxacillin	17 (100.00)	Penicillin	17 (100.00)
Tetracycline	9 (52.94)	Tiamulin	17 (100.00)
Trimethoprim/Sulfamethoxazole	12 (70.59)	Tylosin	17 (100.00)
Vancomycin	17 (100.00)		

associated with respiratory tract infections of foals and with uterine infections in mares. Also, this bacterium can be isolated from a wide variety of animals including pigs, sheep, cows, goats, dogs, foxs, birds, rabbits and guinea pigs [11,23,27]. *S. zooeidemicus* has been indicated as the most frequent bacterium isolated from the aborted equine fetus according to the several reviews [14,16,18-20,26]. Several species such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were isolated from the genital tract in Thoroughbred mares [12]. Although it was reported to be isolated from genital organs of normal and subfertile mares [15], no investigation has reported isolation from an aborted equine fetus in Korea. These results may provide the basic information to establish strategies for the treatment and prevention of reproductive disease in mares in Korea, and further studies will be needed for much more profound understanding of the role of microbiologic agents associated with reproductive disease.

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초록 : 더러브렛 말에서 분리한 *Streptococcus equi* subsp. *zooepidemicus*의 RAPD 분석 및 약제 감수성

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*Streptococcus equi* subsp. *zooepidemicus*는 말의 생식기 질환을 유발하는 중요한 병원체 중 하나로서 국내 말의 생산성 향상을 도모할 목적으로 제주도에서 사육중인 더러브렛 암말의 유산태아 및 그 말의 자궁내용물과 장수목장에서 암말의 교배전 생식기 질환이 의심되는 말의 질 내용물로부터 시료를 채취하여 *Streptococcus equi* subsp. *zooepidemicus*를 분리하여 그 분리균의 특성 및 항균제 감수성 검사를 실시한 결과, RAPD typing에서는 크게 4개의 cluster로 구분되었다. 특히 제주도에서 분리한 균주와 장수목장에서 분리한 하나의 균주가 동일한 pattern을 나타내었다. 항균제 감수성 검사에서는 대부분의 균주가 ampicillin 등의 약제에 감수성이 있음을 확인하였다. 본 연구의 결과는 국내에서 사육중인 암말의 생식기 질환의 예방 및 치료에 유용할 것으로 생각된다.