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RESEARCH ARTICLE

Molecular Epidemiology of *Streptococcus equi subsp.zooepidemicus* Isolated from Thoroughbred Horses Using Multi Locus Sequence Typing (MLST) in Korea

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Abstract:

Background:

Multi Locus Sequence Typing (MLST) is a new global molecular typing method used for analyzing the DNA polymorphisms in bacteria. In this study, using MLST, we analyzed the sequence profiles of *Streptococcus (S.) zooepidemicus* isolates from the Jeju and Jangsu provinces in South Korea.

Objective:

This study characterized the molecular epidemiology of *S. zooepidemicus* infection in Thoroughbred horses using MLST with an aim to control and prevent bacterial endometritis in mares.

Methods:

A total of 79 *S. zooepidemicus* isolates were included in this study. Sequencing of the 7 loci for the MLST analysis was performed as described in the MLST website manual (http:// pubmlst.org/szooepidemicus/) with some modifications. For each locus, every unique sequence was assigned a distinct allele number, and each Sequence Type (ST) was defined by a series of 7 integers (the allelic profile) corresponding to the alleles at the 7 loci (*arcC*, *nrdE*, *proS*, *spi*, *tdk*, *tpi*, and *yqiL)* using the MLST module in the Main Workbench.

Results:

Among the 79 isolates, 73 different MLST patterns were identified, each corresponding to 1-3 strains. The genetic relationships between the 79 isolates are presented in a dendrogram, and they diverged by up to 11% homology. At 11% homology, three MLST groups, M1, M2, and M3, were identified, and at 26% homology, five subgroups, L1-L5, were observed.

We observed various MLST patterns in the strains isolated from Jeju and Jangsu. In addition, by estimating the epidemiological relationships among the strains isolated from Jangsu in 2007 and Jeju in 2009, which had similar MLST patterns, we determined that some strains from Jangsu may have been transported to Jeju.

Conclusion:

MLST can be used as a framework for determining the epidemiological relationships that form the genetic basis of host and infection site selection.

Keywords: MLST, *Streptococcus zooepidemicus*, South Korea, Thoroughbred horse, Molecular epidemiology, Endometritis in mares.

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1. INTRODUCTION

Bacterial infections are a frequent cause of infertility in mares, which is responsible for significant losses in the equine industry. A relatively wide range of microorganisms has been identified in endometrial swabs from mares with endometritis, and *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) is the most frequently isolated pathogen [1 - 3].

S. zooepidemicus belongs to the β-hemolytic group C *streptococci,* which can cause disease in animals and humans [4]. *S. zooepidemicus* can be found in the nasopharynx, tonsils,

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respiratory tract, and on the genital mucous membrane of healthy horses and cattle. It is an important cause of respiratory tract infections in foals and young horses, and it is associated with uterine infections in mares [2]. It has also been associated with a wide variety of infections, including mastitis, in pigs, sheep, cows, goats, and several other mammalian species. Human infections are rare; however, the clinical signs in humans include pharyngitis, septicemia, meningitis, purulent arthritis, and endocarditis [2, 4 - 7].

Bain [8] reported that β-hemolytic *Streptococcus* was the most severe pathogen in the first year of infection, and only 40% of infected horses could deliver healthy foals and 35% of all infected horses could not achieve a successful pregnancy for 3 years. *S. zooepidemicus* causes 75% of vaginal infection and most septic abortions [9]. Furthermore, *Escherichia coli*, coagulase-positive *Staphylococcus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Corynebacterium equi*, and fungal infections have also been shown to be severe pathogens resulting in difficult recoveries [10, 11]. Eliminating such pathogens from the genital tract is difficult, and the infections have been proven to be difficult to treat in some cases. However, the normal flora inhabiting the prepuce of horses and the external genitalia and vaginal vestibulum of mares can typically inhibit the growth of these virulent bacteria [8, 12].

The unambiguous characterization of the strains is crucial for addressing questions related to its epidemiology, population, and evolutionary biology. Multi Locus Sequence Typing (MLST), which characterizes strains using the sequences of 7 housekeeping loci, has become the method of choice for molecular typing of many bacterial and fungal pathogens as well as non-pathogens, and MLST schemes and strain databases are available for a growing number of prokaryotic and eukaryotic organisms [13]. Sequence data are ideal for strain characterization as they are unambiguous, meaning that strains can readily be compared between laboratories via the internet [13]. Laboratories undertaking MLST can quickly progress from sequencing the 7 gene fragments to charac-terizing the strains and determining their relationship to strains submitted by others and to the population as a whole. The MLST website provides a gateway to a number of MLST schemes, each of which contains a set of tools for the initial characterization and methods for relating query strains to other strains of the species, including clustering based on differences in allelic profiles, phylogenetic trees based on concatenated sequences, and a recently developed method, called eBURST, for identifying clonal complexes within a species and dis-playing the overall structure of the population. The address of the MLST website is http://www.mlst.net [14].

This study was prompted by our desire to determine the pathway of the disease that led to an abortion in an experimental mare at 5 months of pregnancy. Both the stallion and impregnated mare were imported into the Jeju province at the same time (in 2006) after following the quarantine procedures required to introduce a stallion from other countries. The disease inspection showed no unusual cause for the abortion in the mare or fetus. Finally, we confirmed the cause of the abortion to be an *S. zooepidemicus* infection.

The subtyping of *S. zooepidemicus* isolates is epidemiologically important for monitoring *S. zooepidemicus* infectious outbreaks in Thoroughbred horses. MLST is a new global molecular typing method used for analyzing the DNA polymorphisms in bacteria. In this study, using MLST, we analyzed the sequence profiles of *S. zooepidemicus* isolates from the Jeju and Jangsu provinces.

2. MATERIALS AND METHODS

2.1. Bacterial Isolates

A total of 79 *S. zooepidemicus* isolates were included in this study. All the isolates were recovered from the vaginal and uterine swabs of 78 mares with clinical signs of endometritis and 1 aborted fetus. The bacteria were isolated from clinical specimens on blood agar supplemented with nalidixic acid (15 mg/L). Molecular identification of the *S. zooepidemicus* isolates was performed by *sodA–seeI* specific multiplex PCR using the following primer pairs: *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′) [15]. Each PCR mixture contained 2 μL of DNA, 2 μL (10 pmol/μL) of each primer, 12.5 μL of 10× reaction buffer (Qiagen, Germany), and 6.5 μL of distilled water in a total volume of 25 μL. The PCR amplification program for *sodA-seeI* was as follows: an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 59°C for 30 sec, and 72°C for 40 sec. After identification, the bacteria were stored at -70°C until use [15].

2.2. Preparation of Chromosomal DNA

Overnight cultures of the bacteria were suspended in 300 μL of sterile, ultra-high quality water, heated on a heat block (Biometra, Germany) at 100°C for 10 min, and centrifuged at 10,000 × *g* (Thermo, USA) for 1 min. An aliquot of the supernatant (200 μL) containing genomic DNA was removed and placed into a fresh tube. The DNA was extracted from this sample using the Mag DNA extraction kit (Toyobo, Japan) according to the manufacturer's instructions and then resuspended in 100 μL of sterile, ultra-high quality water. The DNA yield was approximately 2 μg/mL [16].

2.3. MLST Analysis

Sequencing of the 7 loci for the MLST analysis was performed as described in the MLST website manual (http://pubmlst.org/szooepidemicus/) with some modifications. Internal fragments of carbamate kinase (*arcC*), ribonucleoside-diphosphate reductase (*nrdE*), prolyl-tRNA synthetase (*proS*), signal peptidase I (*spi*), thymidylate kinase (*tdk*), triose-phosphate isomerase (*tpi*), and acetyl-CoA acetyltransferase (*yqiL*) were amplified by PCR from chromosomal DNA. The sequences of the oligonucleotide primers and the conditions for the PCR amplification and sequencing are presented in Tables **1** and **2**.

Table 1. Oligonucleotide primers and PCR program used in present study for amplification

Target Gene	Primers Name	Sequence (5'-3')	PCR Program
arcC	*arcC1* *arcC4*	AGC CAT CTA CCG ACT AAC AC TCT GAA AGG GTT TGG CTA GC	34×(94°C 30 s, 52°C 30 s, 72°C 1 min)
nrdE	*nrdE1* *nrdE4*	TTC TCC TTC AGG TGA CAG ATG AGA CTA GGC GTT TGA ACC TG	34×(94°C 30 s, 54°C 30 s, 72°C 1 min)
proS	*proS1* *proS4*	TTG GTT GGA AAT GAC CAG ATC CCT GAT CCT TGA CAT TAA CGG	34×(94°C 30 s, 51°C 30 s, 72°C 1 min)
spi	*spi1* spi4	CTT AGA GCA TTG CGC TAA GC TTG CCT GCT ATC TAG GGA AG	34×(94°C 30 s, 50°C 30 s, 72°C 1 min)
tdk	*tdk1* *tdk4*	GAA TTC AGG AAA GAC CAT TG TAA TGC TTG CGA CAA ACT GG	34×(94°C 30 s, 54°C 30 s, 72°C 1 min)
tpi	*tpi1* *tpi4*	GGC AGT AGT AAG CAA ATT ACC AGC AAG GCA AGG AAG CTA TC	34×(94°C 30 s, 51°C 30 s, 72°C 1 min)
yqiL	*yqiL1* *yqiL4*	CCA CAT GGG AAT TAC AGC AG TCA ATA CAG ACG TCC CTT GAC C	34×(94°C 30 s, 53°C 30 s, 72°C 1 min)

Table 2. Oligonucleotide primers and sequencing cycle program used in present study for sequencing

Target Gene	Primers Name	Sequence (5'-3')	PCR Program
arcC	*arcC2* *arcC3*	TGT CGC ACT TGG AGG AAA TG CAC CAC AAC ACC AGA ATC AAC	1× (96 1min), 25×(96°C 10s, 50°C 5s, 72°C 4min)
nrdE	*nrdE2* *nrdE3*	CGC TCT ATT AAT TCT GCC TTG C GCA TAG GTT GCT CAT GAT GAT G	
pro*S*	*proS2* *proS3*	GAC TTC TTA GAG CCA GCT AG AAC GGA GCT AGC TCT TTA GG	
sp*i*	*spi2* *spi3*	CCT ACT TTT TGG ACT CTC ACG GAT TTT ATT GAG TGG CCA AAA GCG	
td*k*	*tdk2* *tdk3*	CTT GAA GGT AGC ACA CAA TTA TG GGT CTC ATT GCC ACC AAT TTG	
tp*i*	*tpi2* *tpi3*	GGT CAC TAC AAT TGA GGC TG TTC AGG CTT CAC AGA ACC AC	
yq*iL*	*yqiL2* *yqiL3*	AGT ACC GCA ACG TAA AGG TG GCC TGA CGC TTT TCC ATC TC	

The reactions were performed in a total volume of 20 µL using *Pfu* DNA polymerase (Qiagen, Germany), and the amplification conditions were 34 cycles of 94°C for 30 s, 50–55°C for 30 s, and 72°C for 1 min. The amplified DNA fragments were purified using the PCR purification kit (Qiagen), and the amplicons were sequenced in reactions containing 1 µL of PCR product (containing 3-10 ng/µL DNA), 1 µL of BigDye v3.1 (Applied Biosystems, USA), 2 µL of 5× buffer (Applied Biosystems), 5 µL of sterile water, and 1 µL of primer (3 pmol/µL). For each gene fragment, both strands were sequenced. The cycling protocol and sequencing reaction product clean-up are described in Table **2**. The sequencing was performed using an ABI 3130xl DNA Analyzer (Applied Biosystems).

Sequence data were assembled using the Main Workbench (CLC bio, USA), and high quality double-stranded sequence data were used for further analysis. For each locus, every unique sequence was assigned a distinct allele number, and each Sequence Type (ST) was defined by a series of 7 integers (the allelic profile) corresponding to the alleles at the 7 loci, in the following order (alphabetical) *arcC*, *nrdE*, *proS*, *spi*, *tdk*, *tpi*, and *yqiL* using the MLST module in the Main Workbench. A dendrogram representing the genetic relationship between the 79 *S. zooepidemicus* strains was drawn using the InfoQuest Character Types Module (Bio-Rad) as previously described.

3. RESULTS

3.1. *S. zooepidemicus* Strains

The species identification of the 79 strains was confirmed by a positive *sodA* (235 bp) reaction and a negative *seeI* (520 bp) reaction in the *sodA-seeI* multiplex PCR. The multiplex PCR used in this study has been previously shown to be a reliable molecular method to identify and differentiate *S. zooepidemicus* and *S. equi* (Fig. **1**).

3.2. MLST

All 79 strains were positive for the 7 MLST alleles, with amplicon sizes of 500-800 bp. An agarose gel illustrating the typical amplicons for the 7 loci is shown in Fig. (**2**). The sequence data were assembled using Main Workbench, and high-quality double-stranded sequence data were used for further analysis (Fig. **3**). All 73 of the STs found in the MLST analysis of the 79 isolates were new assignments. Of the new STs, 25 strains represented novel combinations of known alleles and new alleles (237, 243, 244, 246, 247, 250, 253, 254,

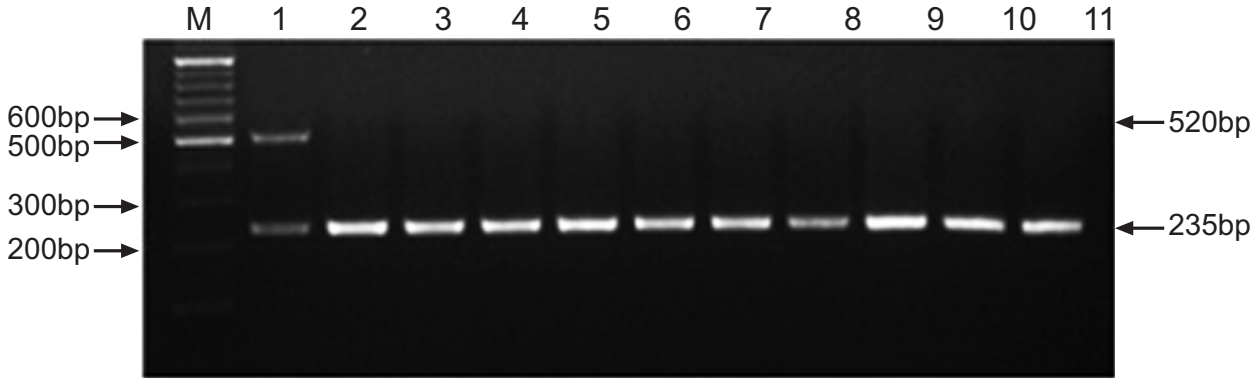

Fig. (1). PCR amplification of sodA-seeI for the identification of *Streptococcus zooepidemicus*. Lane M, molecular size marker (100 bp DNA ladder; Takara, Japan); Lane 1, *S. equi* isolates; Lanes 2–11: *S. zooepidemicus* isolates.

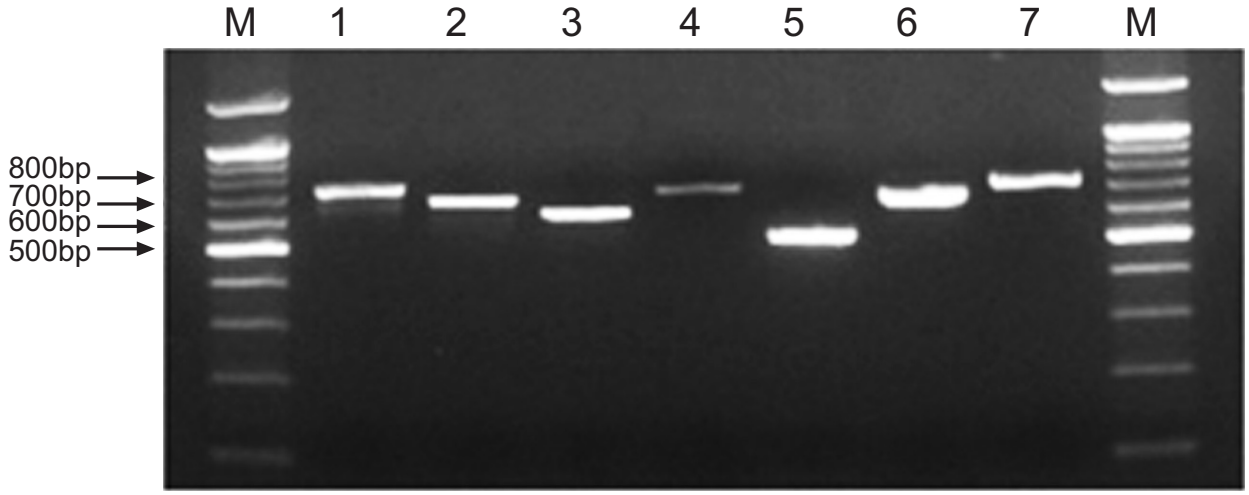

Fig. (2). PCR amplification of the 7 loci for the MLST of *Streptococcus zooepidemicus*. Lane M, molecular size marker (100 bp DNA ladder; Takara, Japan); Lane 1, arcC; Lane 2, nrdE; Lane 3, proS; Lane 4, spi; Lane 5, tdk; Lane 6, tpi; Lane 7, yqiL.

257, 258, 259, 264, 266, 268, 269, 270, 272, 284, 285, 295, 300, 301, 302, and 307). The new alleles included *arcC* 46-102, *nrdE* 48-107, *proS* 46-119, *spi* 46-120, *tpi* 46-110, *tdk* 46-114, and *yqiL* 44-121.

Among the 79 *S. zooepidemicus* isolates, 73 presented with different MLST patterns (Fig. **3**), each corresponding to 1–3 strains. The genetic relationships between the 79 *S. zooepidemicus* isolates are presented in the dendrogram, and they diverged by up to 10% homology. At 10% homology, 3 MLST groups, M1, M2, and M3, were identified, and at 26% homology, 5 subgroups, L1-L5, were observed (Fig. **3**).

The results of the MLST analysis to compare the epidemiological properties of the *S. zooepidemicus* isolates from the abortion case (L5 subgroup) to other isolates from mares showed that the *S. zooepidemicus* isolates from both the mare that had the abortion and the aborted fetus exhibited the same pattern; however, these strains were not genetically similar to the strains isolated from other mares in Jeju or Jangsu (Fig. **4**).

4. DISCUSSION

MLST is a well-established molecular typing method that has been used successfully to determine the population structure of many bacterial species [17 - 19]. Here, we report the development of an unambiguous and discriminatory MLST scheme for *S. zooepidemicus*. The data generated from the *S. zooepidemicus* isolates in this study have been used to construct a database on the main MLST website that permits direct comparison with data generated in other laboratories (http://pubmlst.org/szooepidemicus/).

PFGE is the gold standard for the subtyping of bacteria [20, 21]. However, in PFGE, various patterns can be observed in the analysis of the same strains depending on variations in the analysis, including the type of equipment, standard size marker, restriction enzyme, agarose concentration, and buffer concentration. Therefore, to overcome this weakness, world standardization is induced by organizing a global network; however, the established protocol is also limited to specific species, such as *E. coli* and *Salmonella* spp. Therefore, it is

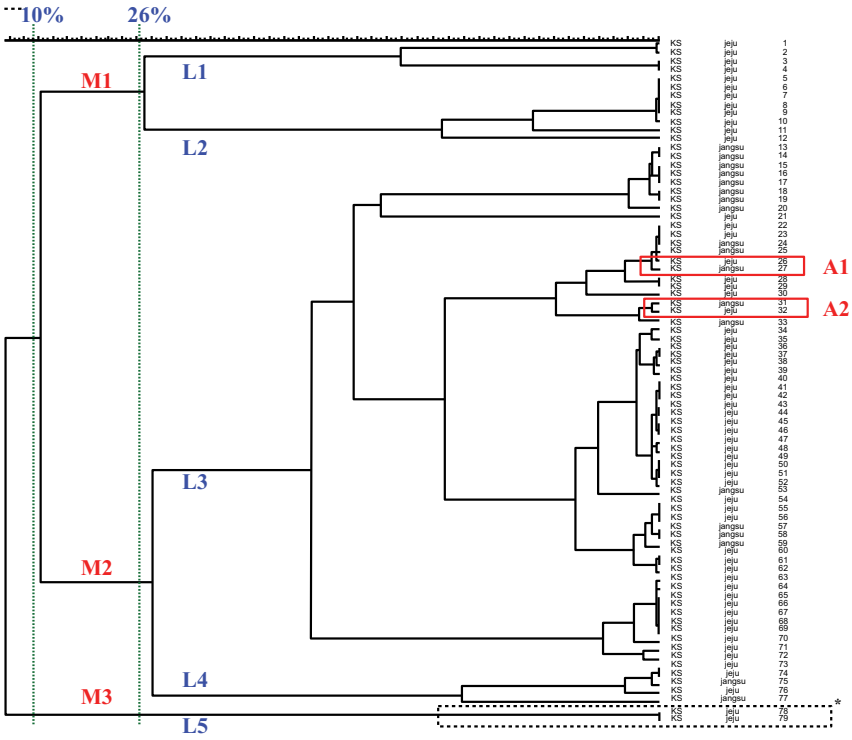

Fig. (3). Dendrogram of 79 *Streptococcus zooepidemicus* isolates from Thoroughbred horses in Korea. The dendrogram was constructed with the InfoQuest Character type module software (Ver. 4.5; Bio-Rad) using the UPGMA algorithm with the Dice similarity coefficient. In general, different MLST patterns were observed by year and by region. However, the A1 and A2 subgroups, the 2009 Jeju isolates and the 2007 Jangsu isolates, respectively, showed similar patterns with 94% and 96% homology. * The interrupted line represents an abortion case (mare and aborted fetus).

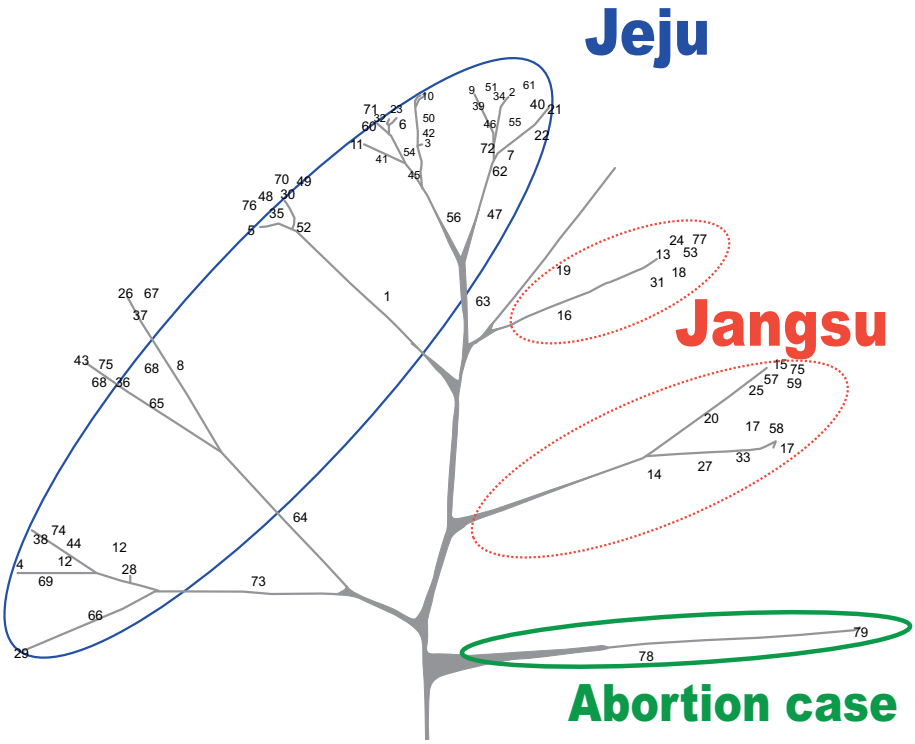

Fig. (4). Phylogenetic tree of the 79 *Streptococcus zooepidemicus* isolates obtained from Thoroughbred horses in Korea. The phylogenetic tree was constructed with InfoQuest software Ver. 4.5 (Bio-Rad). Clusters of individual unlinked patterns within the study sample are displayed as a single unrooted branch. Analysis of the genetic origins of the *S. zooepidemicus* strains isolated from Jeju and Jangsu showed that the isolated strains have different genetic origins.

difficult to compare and analyze experimental data from unstandardized strains generated in different laboratories. For this reason, a new technique based on a standardized method that makes it possible to compare and analyze data between laboratories is greatly needed. Based on this need, the MLST technique was developed.

Data generated by MLST can not only be compared and analyzed to experimental data from the same laboratory but can also easily be compared to results from laboratories in other countries. Because the import and export of horses with predominant genetic traits are more common than that of other industrial animals, diseases occur more frequently in horses possibly because of disease spread as a result of extensive trade between countries. Therefore, analysis of the epidemiological characteristics of bacterial infectious diseases in horses must be accompanied by a comparison and analysis of the experimental data generated both within the same country and among countries.

Our analysis of the genetic features of *S. zooepidemicus* isolates from horse mares using MLST showed that in domestic isolates, allele types in the existing MLST database were identified; however, strains with identical genetic traits were not observed. In addition, in our analysis of 79 strains, 73 different MLST patterns were obtained, and efficient subgrouping was possible by analyzing the correlation among the 73 patterns. Based on these results, we judged that MLST of *S. zooepidemicus* strains is very efficient for identifying strains and is suitable for comparing and analyzing the epidemiological characteristics of strains isolated both within a single country and among different countries. However, the current *S. zooepidemicus* database has some weaknesses compared to those for *Staphylococcus aureus*, *Yersinia* spp., and *Salmonella* spp. strains; namely, the number of strains deposited in the database is lacking. If many more strains are added, it should be possible to compare and analyze worldwide experimental data.

The genetic relationship between *S. zooepidemicus* isolates based on the sequence patterns of 7 loci after the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering is shown in Fig. (**4**). The vast majority of the MLST patterns (93.7%) were represented by a single isolate, and except for a cluster of 2 isolates with a similarity of 100%, the MLST patterns of the *S. zooepidemicus* isolates shared a low level of genetic similarity between them. These results showed the high genetic heterogeneity amongst the isolates studied, which may be related to either the wide distribution of this microorganism in the equine population or the fact that the *S. zooepidemicus* strains were isolated from individual mares, most of them belonging to single owners without any epidemiological or geographical relationship between them. These data demons-trate that many different strains of *S. zooepidemicus*, even from the same animal, could be isolated from uterine infections in mares. These findings correlate well with the fact that *S. zooepidemicus* is a mucosal commensal organism in the equine population, with the opportunistic character of this micro-organism or possibility of the influx of disease from other areas, and the presence of isolated strains in a specific area for a long period of time.

Our genetic analysis using MLST to analyze the epidemiological characteristics of the *S. zooepidemicus* strains isolated from an abortion case showed that the *S. zooepidemicus* isolates from both the mare and aborted fetus had the same pattern; however, they were not genetically similar to other strains isolated from mares in Jeju or Jangsu (Fig. **4**). Therefore, there is a good possibility that the strains isolated from the mare and aborted fetus were already present outside the country and that even if we exclude that possibility, the strains were not the same as other strains present in Jeju.

Different MLST patterns were observed by year and by region. However, the A1 and A2 subgroups, which showed similar patterns, included 2009 Jeju isolates and 2007 Jangsu isolates with 94% and 96% homology (Fig. **3**). The phylogenetic analysis of the A1 and A2 subgroups showed that they have different genetic origins, with 85% homology, and the 4 isolates in the A1 and A2 groups have similar genetic origins. In addition, by estimating the epidemiological relationships among isolates from Jangsu in 2007 and Jeju in 2009, which had similar MLST patterns, we determined that some strains from Jangsu might have been imported into Jeju.

In our analysis of the genetic origins of the *S. zooepidemicus* strains isolated in Jeju from 2006 and 2009, we observed that the strains from 2006 and 2009 had different genetic origins. Based on the results, which showed that the strains from Jeju and Jangsu caused infections due to annual breeding, we believe that *S. zooepidemicus* strains come from many different areas and once established, can cause breeding-related diseases. As the breeding season ends, these strains disappear.

Studies on *S. zooepidemicus* isolates from horses in Asia are very limited compared with those in Europe and America. According to studies from these regions, *S. zooepidemicus* infection typically occurs on horse rearing farms and stud farms. Most infections occur through genital and respiratory infection and lead to fatal pneumonia, endometritis, and abortion.

CONCLUSION

There were no *S. zooepidemicus* studies specific to Asia. Therefore, to the best of our knowledge, this is the first study on *S. zooepidemicus* infection patterns and molecular epidemiological characteristics in Asia. Our report of the epidemiological characteristics of *S. zooepidemicus* isolates in Korea will be helpful for similar future studies. Furthermore, it will help not only the horses themselves but also the people who work on horse farms and at equestrian parks and animal hospitals by establishing an *S. zooepidemicus* screening system.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study received ethical approval from the College of Veterinary Medicine, Kyungpook National University.

HUMAN AND ANIMAL RIGHTS

The reported experiments were in accordance with the standards set forth in the 8th Edition of Guide for the Care and

Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/Guide-for-thecare-and-use-of-laboratory-animals.pdf) published by the National Academy of Sciences, The National Academics Press, Washington DC, United States of America.

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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