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Outbreaks of Strangles due to Capnophilic *Streptococcus equi* subsp *equi* in South Korea

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Abstract : We reported an outbreak of clinical strangles in thoroughbred horses due to capnophilic *Streptococcus equi* subsp *equi* in South Korea. On three different farms, we isolated 17 *S equi* subsp *equi* isolates from 29 horses with or without abscesses in their lymph nodes. Of the 17 isolates, two isolates from clinical cases grew well in aerobic conditions, whereas 7/7 isolates from clinical cases and 8/22 isolates from the nasal discharges of horses did not. The latter 15 isolates were capnophilic, oxygen-sensitive, and CO_2-requiring *S equi* subsp *equi,* which could not grow in aerobic conditions, but which grew well in a CO_2 incubator with 5% CO_2, in anaerobic conditions using a GasPak, and with reduced oxygen tension in a candle jar. This study is the first report of a strangles outbreak caused by capnophilic *S equi* subsp *equi* in South Korea.

Key words **:** Capnophilic, Strangles, *Streptococcus equi* subsp *equi.*

Introduction

Strangles is a well-known horse disease, which is caused by *Streptococcus equi* subsp *equi.* Typical signs of the disease are pyrexia, anorexia, soft coughing, a purulent nasal discharge, and swollen lymph nodes on the head that frequently form abscesses and discharge pus (4). *S equi* subsp *equi* is usually a facultative anaerobe, which grows on ordinary media containing defibrinated horse blood or serum in aerobic conditions. This disease has been reported since the 1910s and it is of great concern to equine populations throughout the world because it is highly contagious. We recently reported the first case of this disease in Korea, which has a relatively small horse population compared with other domestic or agricultural animals and there is a low level of surveillance of horse diseases (6). There are about 29,000 horses in South Korea and 22,000 (ca. 76%) of these horses are maintained on Jeju Island. There are three different horse breeds in South Korea, i.e., Jeju horses, Jeju-producing horses, and thoroughbreds. Jeju horses are ponies that are considered to be native horses, which are designated as a precious natural resource. Jeju-producing horses are usually produced by breeding a Jeju horse with a thoroughbred horse, for use by tourists. When we began work on strangles, the disease outbreak had started and a vaccine had been applied in some horse farms in South Korea. Our surveillance of the distribution of strangles in South Korea identified two distinct *S equi* subsp *equi* strains, i.e., one that grew well in aerobic conditions and another that grew well in anaerobic conditions.

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Materials and Methods

Sample collection

We collected nine clinical samples from seven sick horses with gross edema or ruptured lymph nodes on three farms (NA, GA, and DA), including six from purulent materials or abscesses, one from a nasal discharge, one from lung pus, and one from the trachea (Table 1). Nasal discharge samples were taken from 22 horses without superficial lymph node edema on farm NA. The samples were transported to the laboratory in insulated containers with ice.

Isolation and identification of bacterial isolates

Samples were inoculated onto tryptose-blood agar (TBA; Difco Laboratories, Detroit, MI) plates containing 5% (v/v) sheep blood within 3 h of collection and incubated for 15-24 h at 37°C. Plates were incubated at 37°C for 24 h in three different conditions: aerobic, reduced oxygen tension, and in a CO_2 incubator maintained at 5% CO_2. Five beta-hemolytic colonies were subcultured from each sample onto TBA medium and incubated in the same conditions as the first culture. Hemolytic colonies were identified using an API 20 STREP in a CO_2 incubator, according to the manufacturer's protocol with some modification. TBA cultures were suspended in 20% glycerol (Merck, Darmstadt, Germany) and stored at −70°C before use.

Six clearly separated hemolytic colonies were selected and inoculated onto two TBA plates to enumerate capnophilic *S equi* subsp *equi*. One plate was placed in an aerobic incuba-

Table 1. Properties of *Streptococcus equi* subsp *equi* isolated from the lesions of horses with grossly abscessed lymph nodes (LN)

Horse No.	Sampling point	Farm	No. of isolates	No. of aerobes	No. of anaerobes	Representative strains
1	Parotid LN[a)]	GA	5	0	5	*S. equi* B3D7
2	Parotid LN[a)]	GA	5	0	5	*S. equi* B3D8
3	Parotid LN[a)]	NA	5	0	5	*S. equi* B3E1
4	Nasal discharge	NA	5	0	5	*S. equi* B3E2
5	Submandibular LN[a)]	DA	5	0	5	*S. equi* B3E9
6[b)]	Submandibular LN[a)]	NA	5	0	5	*S. equi* B3E10
	Inguinal LN[a)]	NA	5	0	5	*S. equi* B3E11
7[c)]	Lung	DA	5	5	0	*S. equi* B3G6
	Trachea	DA	5	5	0	*S. equi* B3G7

[a)]Purulent discharges were taken from abscesses in each LN.
[b)]Horse No. 6 had abscesses in two positions.
[c)]Horse No. 7 was necropsied.

tor and the other in a CO_2 incubator, and both were incubated overnight at 37°C. Bacterial isolates that grew in the CO_2 incubator but not the aerobic incubator were designated as capnophilic *S equi* subsp *equi*. The growth of capnophiles was also observed in candle jars and anaerobic GasPak jars (Becton Dickinson, Franklin Lakes, MD).

Identification of *S equi* subsp *equi*

Polymerase chain reaction (PCR) was used to identify *S. equi* subsp. *equi*, according to a published method (7). Briefly, single colonies were suspended in 100 μL of deionized water (Milli-Q System; Millipore, Bedford, MA), before boiling for 1 min and then freezing until required. Each PCR tube contained 20 μL of reaction mix, which was composed of Tris-HCl (10 mM, pH 8.3), KCl (50 mM), $MgCl_2$ (2 mM), gelatin (100 μg mL^{-1}), glycerol (5%, v/v), dATP, dCTP, dGTP, and dTTP (200 μM each), Taq polymerase (Bioneer, Daejeon, Korea) (0.5 U 20 μL^{-1}), 1 μL of forward primer (PCR3FF 5′-GCATAAAGAAGTTCCTGTCATTAAAAT-3′), 1 μL of reverse primer (PCR3FR 5′-GATTCGGTAAGAGCTTGACGCTCA-3′), and 0.5 μL of bacterial cell lysate. The concentration of each primer was 25 pmol μL^{-1}. The thermocycling conditions for the first round of PCR were 95°C for 10 min followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min 30 sec, and 5 min at 72°C.

Results and Discussions

S equi subsp *equi* was isolated from the six purulent materials derived from abscesses and one nasal discharge from six horses with abscess (Table 1). These bacterial isolates did not grow in aerobic culture, whereas large hemolytic colonies were observed on the agar plates (horse Nos. 1 to 6) grown in candle jars, the CO_2 incubator, and anaerobic GasPak jars, indicating that these strains were capnophilic. In contrast, isolates from the lung and trachea samples of horse No. 7 grew in aerobic conditions. All the colonies, i.e., five from each sample, were identified as *S equi* subsp *equi* based on the bacteriological analysis and PCR amplification. Capnophilic *S equi* subsp *equi* produced large hemolytic colonies (Fig 1A) on blood agar plates in a CO_2 incubator, reduced

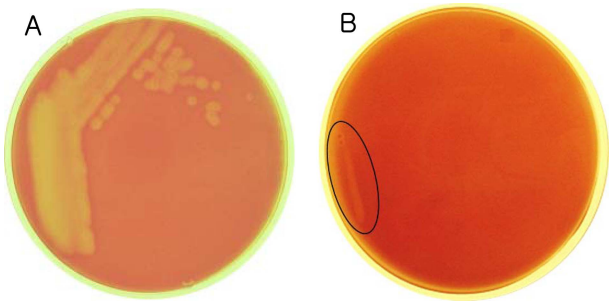

Fig 1. Colony morphologies of capnophilic *Streptococcus equi* subsp *equi* on blood agar with reduced oxygen tension (A) for 15 h, showing numerous colonies with a beta-hemolytic zone, and in aerobic conditions (B) for 72 h, showing only a few colonies with beta-hemolysis traces on the heavily inoculated agar surface (see the marked area).

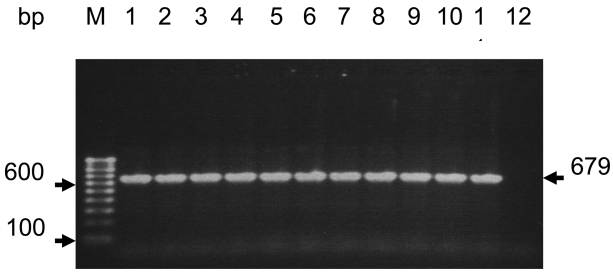

Fig 2. Representative PCR products of *Streptococcus equi* subsp *equi* isolated from horses with or without abscesses. Lane M, 100 bp DNA ladder; lane 1, *S equi* subsp *equi* NCTC9682; lanes 2 to 7, *S equi* subsp *equi* strain B3D7, B3D8, B3E1, B3E2, B3E9, B3E10 (capnophiles from clinical samples); lanes 8 to 10, capnophiles from horse nasal discharges on farm NA; lane 11, facultative anaerobic *S equi* subsp *equi* strain B3G6; lane 12, *S equi* subsp *zooepidemicus* isolate used as a negative control.

oxygen conditions, or anaerobic conditions, although lower growth was observed in aerobic conditions (Fig 1B). API 20 STREP was used to characterize the pathogenic *S equi* subsp *equi* strains. All bacterial isolates failed to ferment arabinose, lactose, trehalose, inulin, mannitol, ribose, or sorbitol. PCR using primers based on a previous report of the SeM

Table 2. Isolation rates of *Streptococcus equi* subspecies *equi* and subspecies *zooepidemicus* from nasal discharges of horses without gross abscesses on farm NA

No of horses tested	No of positive horses (%)		
	Capnophilic SEE	Capnophilic SEE + aerobic SEZ	Aerobic SEZ
22	1 (4.6)	7 (31.8)	9 (40.9)

SEE, *Streptococcus equi* subspecies *equi;* SEZ, *Streptococcus equi* subspecies *zooepidemicus*

gene from *S equi* subsp *equi* yielded a product with approximately 679 bp from the facultative anaerobic *S equi* subsp *equi* strain NTCT-9682, and the capnophilic *S equi* subsp *equi* strains B3D7, B3D8, B3E1, B3E2, B3E9, and B3E10 (Fig 2).

Nasal discharges from 22 horses without any gross abscesses on farm NA were examined bacteriologically to determine the distribution of capnophilic *S equi* subsp *equi* in horses (Table 2). Capnophilic *S equi* subsp *equi* was isolated from eight horses (36.4%), of which one was a pure culture and the remaining seven were co-cultures with facultative anaerobic *S equi* subsp *zooepidemicus*. Facultative anaerobic *S equi* subsp *zooepidemicus* were isolated from 16 (72.7%) horses, of which nine were pure cultures and the remaining seven were cocultures with capnophilic *S equi* subsp *equi* The 15 isolates were confirmed as *S equi* subsp *equi* by PCR (Fig 2).

Strangles has been recorded in horses for centuries, but there had been no reports in South Korea before our initial report in 2006 (6). The current study identified two biotypes, i.e., facultative anaerobic and capnophilic *S equi* subsp *equi,* which were isolated from sick horses on three farms that had problems with strangles.

The isolation of *S equi* subsp *equi* has been reported in horses with strangles (2,10,11,12,14), but the majority of these reports did not describe the precise culture conditions for facultative anaerobic *S. equi* subsp. *equi* isolates, i.e., aerobic conditions, reduced oxygen tension (candle jar), or 5% CO_2 in air (1,4,13). Therefore, this study tested three different culture conditions for isolating *S equi* subsp *equi*. It was notable that large (dia 3 mm) beta-hemolytic colonies were observed after 15 h of anaerobic culture (Fig 1A). All of the isolates also grew very well in CO_2 incubators containing 5% CO_2 and with reduced oxygen tension in a candle jar. In contrast, only a few hemolytic colonies were observed on blood agar in aerobic conditions, even after 72 h incubation with large amounts of pus material (Fig 1B). Heavy inocula were required for isolation under aerobic conditions. The results indicated that the pathogens isolated in this study were capnophilic or oxygen-sensitive *S equi* subsp *equi*, which required an increased CO_2 concentration.

Nasal discharge swabs were collected from 22 horses without any superficial abscesses on farm NA to determine the distribution of capnophilic *S equi* subsp *equi*. Eight (36%) of the 22 horses had capnophilic *S equi* subsp *equi* in the nasal cavity, with or without facultative anaerobic *S equi* subsp *zooepidemicus.* Therefore, we might have missed the opportunity to isolate the main agent of the strangles outbreak in previous samples, because we only used aerobic culture conditions.

Capnophilic *Streptococcus pneumoniae*, *Streptococcus thoraltensis*, and *Haemophilus influenzae* have been isolated from human patients and pigs (3,9). Atypical features of *S equi* subsp *equi* have been described in previous studies with respect to capsule maintenance and carbohydrate utilization, when compared with the type strains (5,8), but there have been no previous reports of capnophilic *S equi* subsp *equi.*

In conclusion, this is the first report of strangles outbreaks caused by capnophilic *S equi* subsp *equi,* which might be a major pathogen associated with strangles in South Korea. Our next concern is the differences between typical and capnophilic *S equi* subsp *equi*.

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호이산화탄소성 *Streptococcus equi* subspecies *equi*에 의한 선역의 발생

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요 약 : 이 연구는 더러브렛 말에서 호이산화탄소성 *Streptococcus equi* subsp *equi* 에 기인한 선역의 발생에 관한 보고이다. 3개의 다른 농장에서 선역의 전형적인 증상인 림프절 화농이 있거나 없는 29두의 말에서 17 균주의 *S equi* subsp *equi*를 분리하였다. 이들 중 임상증상이 있는 1두에서 분리한2균주는 호기배양에서 분리되었으나, 임상증상이 있는 6두에서 분리한 7균주와 임상증상이 없는 22두에서 분리한 8균주는 호기조건에서 배양되지 않았다. 이 15 분리균주는 일반 호기배양기에서는 증식하지 않았으며, 5% CO_2배양기, 산소분압을 낮추는 candle jar 및 GasPak을 이용한 혐기배양에서 증식하는 호이산화탄소성, 산소민감성 또는 이산화탄소 요구성 *S. equi* subsp. *equi*로 판명되었다. 이 연구는 호이산화탄소성 *S. equi* subsp. *equi* 에 의한 선역발생에 관한 최초의 보고이다.

주요어 : 호이산화탄소성, 선역, *Streptococcus equi* subsp *equi*