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Anaplasma bovis infection in a horse: First clinical report and molecular analysis

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ABSTRACT

A 23-year-old male Thoroughbred horse at the Korean Military Academy appeared thin with visible rib bones and presented clinical signs of fever, anorexia, lethargy, and severe dehydration. To determine the presence of various febrile disease-causing agents, the 23 cohabiting horses at the academy, including this horse, were subjected to hematology, blood chemistry, and molecular analysis using whole blood samples collected during regular medical check-ups. On the basis of clinical history, physical examination, hematology, blood chemistry, and fecal examination, differential diagnosis using molecular analyses was performed for various febrile disease-causing agents, including Lyme borreliae, *Coxiella*, piroplasms (*Babesia* and *Theileria*), Rickettsiales (*Anaplasma*, *Ehrlichia*, and *Rickettsia*), equine herpesvirus, equine infectious anemia virus, and equine arteritis virus. While other pathogens were not detected, PCR and phylogenetic analysis targeting the *Anaplasma* 16S rRNA gene revealed that the horse was infected with *Anaplasma bovis*. Although PCR targeting the *groEL* and *gltA* genes of *A. bovis* was not successful, the restriction enzyme fragment length polymorphism assay for differential diagnosis and determination of coinfectivity between *Anaplasma phagocytophilum* and *A. bovis* confirmed the pathogen as *A. bovis*. To the best of our knowledge, this is the first clinical report of *A. bovis* infection in a horse, suggesting a new reservoir host.

1. Introduction

Anaplasma phagocytophilum causes febrile disease in animals and humans and tick-borne fever in domestic ruminants (Stuen et al., 2013). Equine granulocytic anaplasmosis (EGA) is an infectious multisystemic disease in humans and animals, particularly horses (Chen et al., 1994). Typical EGA clinical signs include fever, petechia, anorexia, icterus, depression, lower limb edema, reluctance to move, and ataxia (Madigan and Pusterla, 2000). Many cases of *A. phagocytophilum* infection are perhaps unrecognized because subclinical infections are common, and many of the clinical signs are relatively nonspecific (Madigan and Pusterla, 2000). Thus, recognition of the disease is critical for the differential diagnosis of EGA from various other febrile diseases.

To date, several *Anaplasma* spp. have been detected in Korea by molecular methods: *A. phagocytophilum* in shelter dogs (Lee et al., 2016); *A. phagocytophilum* and *A. phagocytophilum*-like *Anaplasma* spp. (APL) in cattle (Seo et al., 2018a); *A. capra* and *A. bovis* in cattle (Seo et al., 2018b); and *A. phagocytophilum* in horse (Seo et al., 2018c).

Clinical anaplasmosis in horses is perhaps underdiagnosed as many horses recover naturally, and clinical signs are similar to those caused by various other febrile disease-causing agents such as Lyme borreliae (*Borrelia* spp.), *Coxiella*, equine piroplasms (*Babesia caballi* and *Theileria equi*), *Ehrlichia*, *Rickettsia*, equine herpesvirus, equine infectious anemia virus, and equine arteritis virus (Butler et al., 2008). The total number of horses reared in Korea was reported to be 27,676 in 2016, and the horse industry is gradually growing (Ministry of Agriculture, Food and Rural Affairs, South Korea (MAFRA, 2017). EGA is known to be a potentially zoonotic disease but has not been reported in Korea. In this view, we detail the first clinical report and molecular analysis of *A. bovis* infection in a horse.

2. Materials and methods

2.1. Ethics approval

Korean Military Academy (KMA), located in Seoul, Korea, raises military horses to educate Korean soldiers and citizens in horse racing. In this facility, a total of 23 horses are raised in January 2017, and practicing veterinarians conduct regular medical check-ups quarterly (January, April, July, and October). This study, conducted in January

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2017, did not receive approval from the Institutional Animal Care and Use Committee (IACUC) of KMA because the data analyzed were collected during regular medical check-ups. In addition, the IACUC of Kyungpook National University (KNU) evaluates the use of laboratory animals maintained in indoor facilities and does not regulate research involving outdoor animals. Blood samples were collected by practicing veterinarians at a veterinary clinic in the KMA during regular medical check-ups.

2.2. Case history and sample collection

During a visual examination in January 2017, among 23 horses cohabiting at the KMA, a 23-year-old male Thoroughbred horse appeared thin with visible rib bones. Furthermore, this horse showed clinical signs of fever (39–40 °C), anorexia, lethargy, and severe dehydration. However, the pulse rate (32–36 beats/min) and respiratory rate (10–24 breaths/min) were within normal ranges. The horse had been raised in the KMA for 5 years without any prior clinical signs. Following a physical examination, whole blood samples collected from the 23 horses during regular medical check-ups were kept in an ice-pack container and transported to the Laboratory of Veterinary Parasitology, KNU, Daegu, Korea, for hematology, blood chemistry, and molecular analysis to detect various febrile disease-causing agents. To assess the potential involvement of gastrointestinal parasites, a fresh fecal sample was obtained directly from the rectum. Data on age, sex, and breed were recorded for data analysis.

2.3. DNA extraction and PCR

Genomic DNA was extracted from the whole blood using a commercial DNeasy Blood and Tissue kit (Qiagen, Melbourne, Australia) according to the manufacturer's instructions. The extracted DNA was stored at −20 °C until use. The AccuPower HotStart PCR Premix kit (Bioneer, Daejeon, Korea) was used for PCR amplification. Differential diagnosis using molecular analyses was performed for various other febrile disease-causing agents, including Lyme borreliae (*Borrelia* spp.), *Coxiella*, piroplasms (genera *Babesia* and *Theileria*), Rickettsiales (genera *Anaplasma*, *Ehrlichia*, and *Rickettsia*), equine herpesvirus, equine infectious anemia virus, and equine arteritis virus.

Lyme borreliae were screened by nested PCR (nPCR). The 5S–23S intergenic spacer and outer surface protein A (*ospA*) gene fragments of *Borrelia* spp. were amplified by nPCR (VanBik et al., 2017). A sample of *B. afzelii* isolated from a tick (VanBik et al., 2017) was included as a positive control.

Multiple primer sets described in a previous study (Seo et al., 2016) were used to amplify the 16S rRNA gene of the genus *Coxiella*, including *C. burnetii* and *Coxiella*-like bacteria, using nPCR. A sample of *C. burnetii* isolated from a horse (Seo et al., 2016) was included as a positive control.

Piroplasms were screened by amplifying the 18S rRNA gene fragments with PCR (Seo et al., 2013). Samples of *T. equi* isolated from a horse (Seo et al., 2013) and *Babesia gibsoni* isolated from a dog were included as positive controls.

Rickettsiales infection was first screened by PCR using a commercial AccuPower Rickettsiales 3-Plex PCR kit (Bioneer), which detects the 16S rRNA genes of three genera of Rickettsiales and includes positive controls of Rickettsiales. Next, a positive sample was further amplified for species identification. For *Anaplasma* spp., two primer sets, EE1/EE2 and EE3/EE4, were re-amplified to identify the 16S rRNA genes of *Anaplasma* spp., producing an expected amplicon of 924–926 bp (Seo et al., 2018a, b, c). For species identification, PCR-positive samples were re-amplified to identify the 16S rRNA genes of *A. phagocytophilum* and *A. bovis* by speciesspecific primer sets (Seo et al., 2018b). For multilocus genotyping, heat shock protein (*groEL*) and complete citrate synthase (*gltA*) gene fragments of *A. bovis* were amplified by nPCR (Seo et al., 2018b).

For the detection of viral pathogens, the equine arteritis virus *ORF1b* gene (Gilbert et al., 1997) and the *gag* gene from the equine infectious anemia virus (Nagarajan and Simard, 2001) were amplified by nPCR as described. Because Korea is free of these two viruses, positive controls were not included in this analysis. The gB genes of equine herpesvirus types 1, 2, 4, and 5 were amplified by PCR (Negussie et al., 2017). Samples of equine herpesvirus types 2 and 5 isolated from horses were included as positive controls.

A sample without DNA template was used as a negative control for each PCR reaction.

2.4. DNA cloning

Positive PCR products from primers EE3/EE4 of the 16S rRNA gene were purified using the QIAquick Gel Extraction kit (Qiagen). Purified products were ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA), following the manufacturer's instructions. The ligation product was transformed into *Escherichia coli* DH5α-competent cells and then incubated at 37 °C overnight. Plasmid DNA extraction was conducted using a plasmid miniprep kit (Qiagen) following the manufacturer's instructions.

2.5. DNA sequencing and phylogenetic analysis

Recombinant clones in the plasmids were selected and sent to Macrogen (Seoul, Korea) for nucleotide sequencing. The sequences were aligned and analyzed with the multiple sequence alignment program CLUSTAL Omega (v. 1.2.1). The alignment was corrected with BioEdit (v. 7.2.5). Phylogenetic analysis was performed based on the maximum likelihood method in MEGA (v. 6.0). The aligned sequences were analyzed with a similarity matrix. The stability of the trees obtained was estimated by bootstrap analysis for 1000 replicates.

2.6. Restriction enzyme fragment length polymorphism (RFLP) assay

For differential diagnosis and analysis of coinfectivity of *A. phagocytophilum* and *A. bovis*, the 16S rRNA nPCR products of 868–870 bp (PCR amplicon of 924–926 bp without primer sequences) were aligned to select two restriction enzymes for the RFLP assay (Seo et al., 2018b). Two restriction enzymes *AleI* and *BtgZI* were selected for RFLP assay using CLC Main Workbench 6.7.2 (CLC Bio, Qiagen). Restriction reactions were performed in a final volume of 50 µl containing 10 µl PCR product, 5 µl buffer (10×), 1 µl *AleI* (10,000 U/ml; New England Biolabs, Hitchin, UK) or 2 µl *BtgZI* (5000 U/ml; New England Biolabs), and distilled water to the final volume. The reactions were incubated for 1 h at 30 °C for *AleI* or at 60 °C for *BtgZI*. The restricted fragments were separated on a 3% agarose gel by electrophoresis in TAE buffer at 100 V for 60 min and visualized under UV illumination after staining with ethidium bromide.

2.7. Fecal microscopy

Qualitative and quantitative parasitological examinations were performed by the fecal flotation method following standard measures that used a saturated solution of sodium nitrate to evaluate the presence of parasite eggs and oocysts (Gebeyehu et al., 2013).

3. Results

3.1. Blood chemistry and hematology

The results of blood chemistry and hematology for the horse with clinical signs of febrile disease revealed increased values of albumin, glucose, phosphorous, total bilirubin, total protein, white blood cells (WBC), lymphocytes, hemoglobin, globular volume, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean

Table 1
Blood chemistry and hematology data of a horse with clinical signs of febrile disease.

Parameter*		Unit	Value	Reference range
Blood chemistry	Albumin	g/dl	> 6.0	2.5–4.2[a]
	Calcium	mg/dl	10.4	10.7–13.4[a]
	Cholesterol	mg/dl	18	51–109[a]
	Creatinine	mg/dl	1	0.6–1.8[a]
	Glucose	mg/dl	138	72–114[a]
	Phosphorous	mg/dl	7	1.9–5.4[a]
	Total bilirubin	mg/dl	> 27.9	0.1–1.9[a]
	Total protein	g/dl	> 12	4.6–6.9[a]
	BUN	mg/dl	14	8–27[a]
Hematology	WBC	$\times 10^3$ cells/μl	14.4	4.9–10.3[a]
	Lymphocytes	$\times 10^3$ cells/μl	11	1.7–5.8[a]
	Neutrophils	$\times 10^3$ cells/μl	2.8	2.2–8.1[a]
	RBC	$\times 10^6$ cells/μl	9.74	6.2–10.2[a]
	Hemoglobin	g/dl	21.9	11.4–17.3[a]
	Globular volume	%	82	31–50[a]
	MCV	fL	84.2	37–53[b]
	MCH	pg	22.4	14–20[b]
	MCHC	g/dl	42.7	36–39[b]
	Thrombocytes	$\times 10^3$ cells/μl	32	72–183[a]
	MPV	fL	7.7	5.4–9.3[b]
	PDW	%	7.9	24–72[b]
	PCT	%	0.2	0.07–0.21[b]

* BUN, blood urea nitrogen; WBC, white blood cells; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MPV, mean platelet volume; PDW, platelet distribution width; PCT, platelet crit.

[a] Southwood, L.L., 2013. Normal ranges for haematology and plasma chemistry and conversion table for units. In: Practical Guide to Equine Colic, 1 st ed., John Wiley & Sons, Inc., Hoboken, NJ, pp. 339–340.

[b] UCDAVIS Veterinary Medicine, 2005. Clinical diagnostic laboratory CBC reference intervals. https://www.vetmed.ucdavis.edu/sites/g/files/dgvnsk491/files/local_resources/pdfs/lab_pdfs/UC_Davis_VMTH_Hematology_Reference_Intervals.pdf (accessed 01 June 2017).

corpuscular hemoglobin concentration (MCHC), whereas those of calcium, cholesterol, thrombocytes, and platelet distribution width (PDW) decreased (Table 1). These abnormalities in blood chemistry and hematology were mainly induced by severe dehydration, nutrient imbalance, and inflammation, as indicated by the approximately twofold increase of the value of globular volume (82%) compared with the reference range (31–50%). The values of total bilirubin (> 27.9 mg/dl; reference range: 0.1–1.9 mg/dl) and hemoglobin (21.9 g/dl; reference range: 11.4–17.3 g/dl) appeared to be increased by hemolysis as well as systemic dysfunction accompanied with dehydration. Among the 23 horses cohabiting the KMA, the 22 nonsymptomatic horses showed normal ranges of blood chemistry and hematology.

3.2. nPCR

While Lyme borreliae, *Coxiella*, piroplasms, *Ehrlichia*, *Rickettsia*, equine herpesvirus, equine infectious anemia virus, and equine arteritis virus were negative according to PCR, in this study, nPCR amplification of 16S rRNA gene fragments using the EE1/EE2 and EE3/EE4 primer sets revealed that the one horse (4.3%, 1/23) with clinical signs of febrile disease among 23 horses cohabiting the KMA was positive for *Anaplasma* spp. For species identification, additional nPCR for 16S rRNA gene fragments using species-specific primer sets indicated that the horse was infected with *A. bovis* (Table 2). Furthermore, *A. phagocytophilum* was undetected in all tested horses. No samples were positive for amplified *groEL* and *gltA* gene fragments of *A. bovis*.

Table 2
PCR detection of *Anaplasma bovis* in horses from Korean Military Academy.

Group		No. tested	No. positive (%)
Sex	Female	9	0
	Male	14	1 (7.1)
Age group (years)	5–10	6	0
	11–20	11	0
	> 20	6	1 (16.7)
Breed	Thoroughbred	15	1 (6.7)
	Warmblood	3	0
	Pony	1	0
	Mixed	2	0
Total		23	1 (4.3)

3.3. Sequencing and phylogeny

The *A. bovis* 16S rRNA nucleotide sequence (H-SE-10) from the horse with clinical signs of febrile disease has been submitted to GenBank with accession number MH794247. Phylogenetic analysis showed that the sequence was clustered with previously deposited *A. bovis* 16S rRNA gene sequences (Fig. 1). The *A. bovis* sequence, in this case, belonged to clade B with 99.3–100% identity with those detected in China, Japan, Korea, and the USA, including in deer from Japan (100%, AB196475), sika deer from China (99.8%, KJ659040), leopard cat from Japan (99.4%, AB723716), rabbit from the USA (99.3%, AY144729), and tick from Korea (99.3%, AF470698). In contrast, the *A. bovis* sequence obtained in this study shared 99.5–99.9% identity with clade A isolated from East Asia (China and Korea), including from goat from China (99.5%, JN558829) and tick from Korea (99.9%, GU064901).

3.4. RFLP

The 16S rRNA gene sequencing data were confirmed by RFLP assay. Further discrimination and determination of coinfectivity of *A. phagocytophilum* and *A. bovis* were accomplished by digesting 16S rRNA amplicons (924–926 bp) with *AleI*. *A. bovis* amplicons were cut to generate 660 and 264 bp fragments, while *A. phagocytophilum* amplicons were not cut by the same enzyme (Fig. 2A, lanes 3 and 5). Digestion of the same amplicons with *BtgZI* also distinguished *A. phagocytophilum* and *A. bovis*. *A. phagocytophilum* amplicons generated two fragments of 707 and 222 bp, while *A. bovis* amplicons were not digested by the same enzyme (Fig. 2B, lanes 3 and 5). RFLP revealed that the horse was infected with *A. bovis* but not *A. phagocytophilum*.

3.5. Fecal microscopy

No eggs and oocysts were observed in fecal samples from the 23 horses cohabiting the KMA by microscopy.

4. Discussion

In the present clinical case study, we have fortuitously identified *A. bovis* infection in a horse with clinical signs of febrile disease for the first time. Recently in Korea, EGA was identified for the first time by molecular analysis (Seo et al., 2018c). In Japan, a survey to detect *Anaplasma* spp. identified *A. phagocytophilum* antibodies in horses using an indirect immunofluorescent antibody test, and APL and *A. bovis* were detected in ticks obtained from horses by PCR (Ybañez et al., 2013). In that study, although DNA obtained from horses did not correspond to any *Anaplasma* spp., it is possible that APL or *A. bovis* may opportunistically infect horses via tick bites. *A. bovis*, a monocytotropic species, is widespread in tropical and subtropical areas (Dong et al., 2014). While *A. bovis* is a general ruminant pathogen infecting cattle and buffalo in Africa, Asia (Parola et al., 2005), and South America (Santos

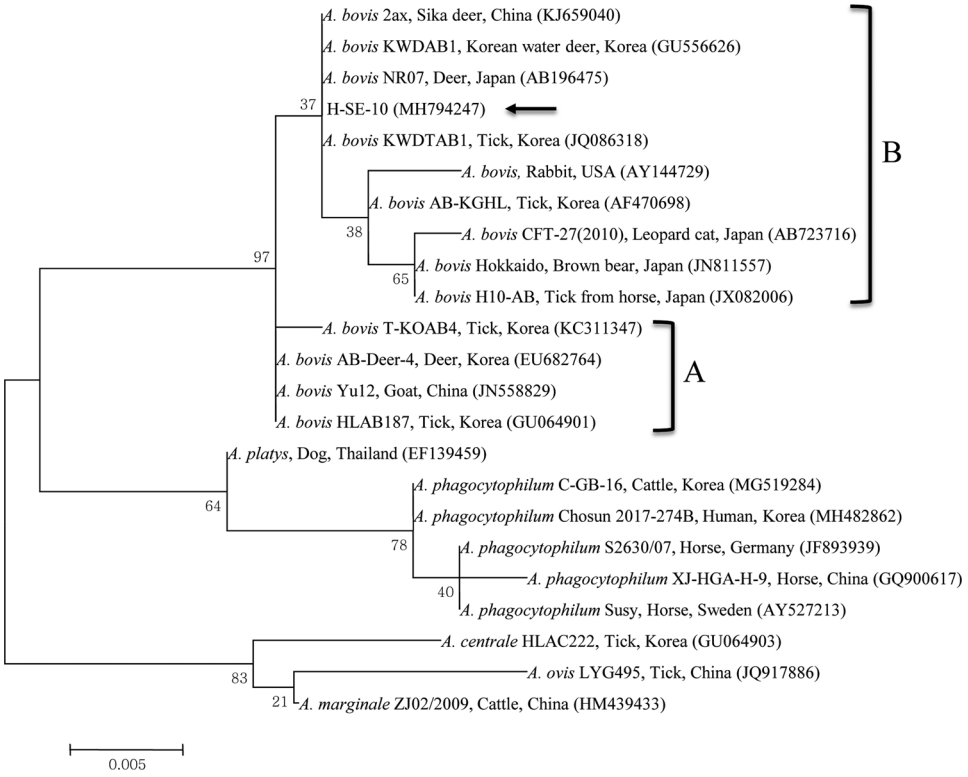

Fig. 1. Phylogenetic tree constructed using the maximum likelihood method, based on the 16S rRNA gene nucleotide sequences of *Anaplasma bovis*. The sequence analyzed in this study is marked by an arrow. Accession numbers of other sequences in GenBank are shown with the sequence name. Branch numbers indicate bootstrap support (1000 replicates). Scale bar represents the phylogenetic distance of the sequences. A and B, two clades of *A. bovis*.

and Carvalho, 2006), infection thereof has also been detected in other hosts of different species: eastern rock sengi (*Elephantulus myurus*), goat (*Capra aegagrus hircus*), sika deer (*Cervus nippon*), Brazilian brown brocket deer (*Mazama gouazoubira*), roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), Korean water deer (*H. inermis argyropus*), march deer (*Blastocerus dichotomus*), dog (*Canis lupus familiaris*), Mongolian gazelle (*Procapra gutturosa*), cottontail rabbit (*Sylvilagus*), leopard cat (*Prionailurus bengalensis*), raccoon (*Procyon lotor*) (Uilenberg, 1993; Atif, 2016), and deer (*Mazama americana*) (Soares et al., 2017).

Mild anemia, leukocytosis or leukopenia, and thrombocytopenia are normally reported hematological abnormalities in EGA affected horses (Dzięgiel et al., 2013). In the present study, the horse demonstrated thrombocytopenia and leukocytosis, but other abnormal values of blood chemistry and hematology were observed; these abnormalities have not been previously reported in other cases of EGA. Additionally, because of hemolysis and severe dehydration, levels of blood chemistry and hematology were overall increased. This horse was in good health at previous medical check-ups conducted in 2016. Among the 23 horses, however, only this horse appeared thin with rib bones visible to the naked eye and displayed clinical signs at the medical check-up in January 2017. The results of blood chemistry and hematology were abnormal, indicating poor health. The other horses aged more than 20 years showed normal results and were in good health. Thus, it is likely that *A. bovis* infection caused the clinical signs in this case. Indeed, *A. bovis* and *A. phagocytophilum* cause different clinical signs and show different virulence in several hosts. *A. bovis* frequently infects

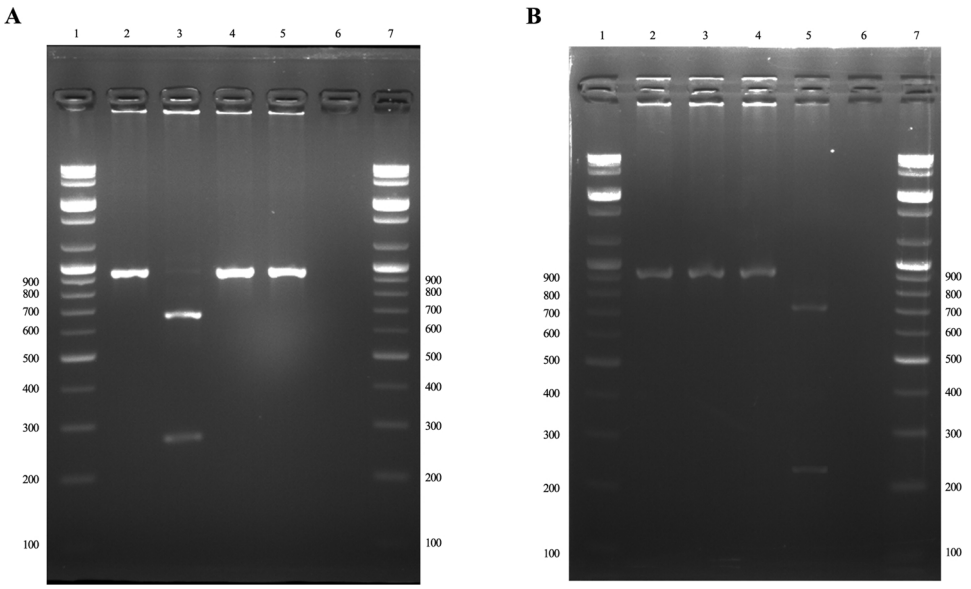

Fig. 2. Restriction enzyme fragment length polymorphism (RFLP) assay strategy and *Anaplasma* spp. identification. A previously described RFLP strategy (Seo et al., 2018b) was used for the detection of and differentiation between *Anaplasma phagocytophilum* and *A. bovis*. (A) RFLP analysis of PCR products using *AleI* enzyme. Lines 1 and 7: 100 bp ladder; lines 2 and 3: PCR products of the horse with clinical signs of febrile disease before (924 bp) and after (660 and 264 bp) *AleI* restriction; lines 4 and 5: PCR products of *A. phagocytophilum* detected in a dog (positive control) before (924 bp) and after (924 bp) *AleI* restriction; and line 6: negative control. (B) RFLP analysis of PCR products using *BtgZI* enzyme. Lines 1 and 7: 100 bp ladder; lines 2 and 3: PCR products of the horse with clinical signs of febrile disease before (924 bp) and after (924 bp) *BtgZI* restriction; lines 4 and 5: PCR products of *A. phagocytophilum* detected in a dog (positive control) before (924 bp) and after (707 and 222 bp) *BtgZI* restriction; and line 6: negative control.

monocytes, and leukopenia and thrombocytopenia may occur (Brouqui and Matsumoto, 2007). Some cattle positive for *A. bovis* in Japan demonstrated thrombocytopenia, mild to severe anemia, and mild leukocytosis; however, co-infections of *Theileria* sp. and *Anaplasma* sp. were confounding factors that may have also caused the hematological results (Ooshiro et al., 2008). The relationship between *A. bovis* infection in other host species including horse and clinical signs has yet to be clarified.

Vector control is important in controlling vector-borne diseases. The KMA horses were usually allowed to graze freely in the nearby grassland area, thereby allowing easy access and longer exposure to tick vectors. Thus, acaricides were regularly administered during tick season (May to October) at this facility. However, the average temperature in Korea during winter (December to February) is relatively low (−5 to 0 °C), which is unfavorable for tick distribution. During the medical check-up in January 2017, ticks were not seen on any horses or in any breeding areas at this facility. Nonetheless, the horses may have come in contact with ticks at some point, as they are prevalent in Korea during tick season. After the check-ups conducted in January 2017 and following the detection of *A. bovis* infection, antibiotic treatment was scheduled. However, the horse suddenly broke his leg while roaming in a nearby grassland area and was euthanized because of poor prognosis at that time. Unfortunately, additional check-ups and post-mortem study were not performed in this horse. The other 22 horses raised together were not infected with Lyme borreliae, *Coxiella*, piroplasms, Rickettsiales, equine herpesvirus, equine infectious anemia virus, and equine arteritis virus at additional check-ups.

Classical nPCR analysis can routinely identify *Anaplasma* species. However, an additional method, such as RFLP assay, is needed to differentiate single- and co-infection. RFLP analysis does not require sequencing or cloning to evaluate strain prevalence and co-infection rates and minimizes the time and cost of molecular diagnosis (Seo et al., 2018b). In the current study, RFLP was used to discriminate *Anaplasma* species, and no co-infection was observed in this case.

Phylogenetic analysis with 16S rRNA gene sequences classified *A. bovis* into two clusters. The *A. bovis* sequence obtained in the present study belongs to clade B, including those from Korea, Japan, the USA, and China. *A. bovis* sequences belonging to clade A include those from East Asia. The genetic variety of genus *Anaplasma* has been characterized by analyzing nucleotide sequences of different genes (Battilani et al., 2017). Though we attempted to amplify the *groEL* and *gltA* genes of *A. bovis*, PCR failed to amplify these genes, suggesting that gene sequence methods cannot yet entirely clarify the role of genotypes and ecotypes in epidemiological cycles (Battilani et al., 2017). Even if the *groEL* and *gltA* genes could differentiate *Anaplasma* spp., *Anaplasma* 16S rRNA gene nucleotide sequences were sufficient to identify the *Anaplasma* species in this study.

5. Conclusion

Although *A. bovis* is not considered a zoonotic pathogen compared with *A. phagocytophilum*, this clinical case demonstrates that horses may also be infected by the pathogen *A. bovis*. Because of the growing horse industry in Korea, horse diseases are important; in addition, humans are exposed to a constant health threat because of the zoonotic potential of several diseases, including EGA. In this horse, the illness was likely caused by infection with *A. bovis*. Therefore, further experimental studies should shed light on whether this was an isolated case, or if *A. bovis* can successfully infect horses and cause illness.

Declarations of interest

None.

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