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Parasitology

Setaria digitata was the main cause of equine neurological ataxia in Korea: 50 cases (2015–2016)

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ABSTRACT. This study was performed to examine and clarify the cause of hindlimb ataxia and neuropathy seen in the South Korean horse population. Fifty horses diagnosed with hindlimb ataxia and neuropathy were referred for this study. Neurological examination was performed on 47 horses while necropsy was performed in all 50 animals. The occurrence of neurological diseases increased rapidly in the summer and 47 out of 50 horses were referred after the end of July. The incidence of neurological diseases started from the southern part of Korea in July and proceeded northward in August and September. Although there was no correlation with age, Thoroughbred and Warmblood horses showed a higher incidence rate than Halla and Jeju horses. The incidence rate was 5 times higher in geldings than in mares and stallions. Of the 20 cases, 16 were diagnosed with eosinophilic meningoencephalomyelitis in 2015. The most common lesions observed in 2016 were parasitic meningoencephalomyelitis (10 cases, 33%) and eosinophilic meningomyelitis (7 cases, 23%). Histopathological analysis of the brain and spinal cord revealed nematodes of approximately 100–200 µm in diameter, microcavitation and infiltrates of eosinophils, and brown pigmented macrophage infiltrates. The nematodes were identified as *Setaria digitata* via DNA sequencing, performed subsequent to polymerase chain reaction using DNA isolated from formalin-fixed paraffin-embedded tissue sections of the spinal cord. These results show that aberrant migration of *Setaria digitata* larva in the brain and spinal cord was a major cause for neurological signs in horses.

KEY WORDS: aberrant parasite migration, acute hindlimb ataxia, horse, necropsy, *Setaria digitata*

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In recent years, the outbreak of acute hindlimb ataxia in horses has been increasing in South Korea. The progression of symptoms is rapid and treatment has been reported to be difficult, costing not only individual horse ranches but also the horse industry as a whole. However, due to the high expenses associated with examinations and transportation, the number of cases that have undergone necropsy and received an official diagnosis has been low. This has resulted in a lack of systematic studies on the overall prevalence, causes, epidemiologic investigation, treatment and prevention to accurately investigate equine neurological diseases.

Neurological diseases with acute hindlimb ataxia can be caused by various etiologies. Non-infectious agents that can cause neurological symptoms in horses are Wobbler disease, trauma, and fungi, and infectious agents include bacteria, viruses, and parasites. Some of which show distinct clinical symptoms. In most cases, however, it is difficult to confirm the diagnosis solely based on the patient's history, clinical symptoms, physical examination, or neurological examination. Laboratory tests and pathologic examinations must also be performed in order to increase the probability of confirming a diagnosis [3, 15, 20]. Therefore, in order to identify the cause of hindlimb ataxia and neurological symptoms, test samples must be secured and a differential diagnosis must be established based on the test results.

This study was performed to investigate the cause of acute hindlimb ataxia cases that has been recently increasing in South Korea.

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MATERIALS AND METHODS

Horses with related symptoms were recruited nationwide for two years, from 2015 to 2016. Fifty horses consisting of 44 Thoroughbred, 3 Warmblood, 2 Jeju crossbred (Halla horse), and 1 Jeju horse were referred to this study. Of the 50 horses, 2 horses were less than 3 years old, 34 were between 4 to 9 years old and 14 were over 10 years old. Neurological examination, laboratory tests and pathologic examinations were performed on the recruited horses in line with the examination procedure established for differential diagnosis of neurological symptoms.

Horses displaying neurological symptoms were referred to the study after a primary diagnosis by local veterinarians. Signalment and history were collected, then physical and neurological examinations were performed to differentiate from musculoskeletal diseases.

The study used previously reported neurological examination charts [6, 13, 17] revised to be applied in clinical practice. The neurological examination was conducted in 4 steps: step 1 (evaluation of mental status and behavior), step 2 (spinal reflexes and muscle evaluation (tone and size)), step 3 (cranial nerve examination), and step 4 (gait and posture examination plus postural reactions).

Upon completion of both examinations, horses were transported to the Animal and Plant Quarantine Agency, where samples needed for testing were obtained through blood, feces, nasal swab and CSF tapping. The animals were then euthanized for necropsy and histopathological examination. These were followed by bacterial, viral, and parasite tests as necessary. In the necropsy, gross lesions of the nervous system and internal organs were observed, and tissue samples were taken for additional exams. The following tests were conducted using the collected samples.

Blood tests

Complete blood cell count (CBC) and serum chemistry tests were performed through routine procedures. The serum chemistry panel consists of a number of sub-tests: total protein (TP), albumin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), glucose, gamma-glutamyl transpeptidase (γ-GTP), uric acid (UA), blood urea nitrogen (BUN), creatinine, calcium (Ca), phosphate (P), magnesium (Mg^{2+}), sodium (Na^{+}), potassium (K^{+}), and chloride (Cl^{-}).

Histopathological examinations

Tissues collected from the nervous system and internal organs were fixed and stabilized in 10% neutral buffered formalin solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Coronal sections of the brain and spinal cord were made at every 2–3 cm level to embed the entire nervous system for section cutting. In addition, if the causative agents were found under a microscope, genomic DNA was isolated and DNA sequencing was conducted [16].

Polymerase chain reaction (PCR) and DNA sequencing

Genomic DNA was isolated from formalin-fixed paraffin-embedded (FFPE) tissue curls of the spinal cord from a horse. Eight 5 µm thick sections were cut from FFPE tissues, placed in 1.5 ml tubes, deparaffinized with xylene, and washed with ethanol. Genomic DNA was extracted following the manufacturer's instructions (QIAamp® DNA FFPE Tissue Kit, Qiagen, Germany). PCR, targeting the 12S rDNA conserved among the nematode species *Onchocerca volvulus*, *Ascaris suum*, and *Caenorhabditis elegans*, was performed with the DNA template using a primer set (F: 5′-GTT CCA GAA TAA TCG GCT A-3′, R: 5′-ATT GAC GGA TG (AG) TTT GTA CC-3;) designed by Casiraghi *et al*. [1]. PCR was performed at a final volume of 50 µl under the following conditions: 2 × EmeraldAmp® PCR Master Mix, 0.2 µM of each primer, 50–100 ng genomic DNA, and H_2O up to 50 µl (EmeraldAmp® PCR Master Mix, Kusatsu, Japan). The following thermal profile was used: 40 cycles of 94°C for 45 sec, 50°C for 45 sec, and 72°C for 90 sec. The 450 bp amplicon was submitted to Macrogen (Daejeon, Korea), where DNA sequencing was conducted using an Applied Biosystems DNA sequencing system.

Virus tests

PCR, neutralization test of serum samples, or enzyme-linked immunosorbent assay (ELISA) were performed to detect the following viruses that could cause neurological diseases in horses: equine herpesvirus-1 & -4 (EHV-1, EHV-4), equine influenza virus (EIV), Japanese encephalitis virus (JEV), West Nile virus (WNV), eastern equine encephalomyelitis virus (EEE), western equine encephalomyelitis virus (WEE), Venezuelan equine encephalomyelitis virus (VEE) and rabies virus [11, 12].

Parasite tests

Fecal flotation tests were performed to detect parasite burdens in feces. *Halicephalobus gingivalis*, *Setaria* spp., *Strongylus* spp., *Angiostrongylus cantonensis*, *Parelaphostrongylus tenuis* and *Habroneoma* spp. were tested by whole blood PCR. Antibodies in the CSF and serum were tested for *Sarcocystis neurona* [4].

RESULTS

Occurrence: Prevalence, time period, and regional distribution

In 2015 and 2016, 20 and 30 horses were referred respectively. Forty-seven of the 50 horses included in this study were referred from the end of July. The number of horses displaying neurological symptoms significantly increased from the beginning of August

and lasted until October every year.

Equine neurological diseases occurred mainly in the northwest (Gyeonggi-do) and southeast (Gyeongsang-do) region, with 23 and 20 horses, respectively. In 2015, it was observed in the south (Gyeongsang-do) around July and in the north (Gyeonggi-do) in September. In 2016, cases significantly increased in the Gyeonggi-do and Gyeongsang-do region after the beginning of August and persisted until the beginning of October.

Patient information: Breed, age, and gender specificity

The study was conducted on 50 horses consisting of 44 Thoroughbred, 3 Warmblood, 2 Jeju crossbred (Halla horse), and 1 Jeju horse. A wide range of age was observed, and no correlation with age was made: 2 horses were less than 3 years old, 34 were between 4 to 9 years old and 14 were over 10 years old. As a result of investigating the gender distribution of horses with neurological symptoms, there were 17 mares, 8 stallions, and 25 geldings.

Physical and neurological examination

Physical examination in all 50 horses showed vital signs to be within normal ranges. Neurological examination was performed in 47 out of 50 horses. The remaining three horses were excluded from the neurological exam due to their inability to stand. All horses showed a staggering gait of different severity and ataxia was markedly severe on one or both sides of the hindlimb. Behavioral and mental changes, and cranial nerve abnormalities were not observed; however, some horses showed weak reactions during the skin response test. Pathology examination showed that horses with weak reactions to the skin response test possessed lesions in the spinal cord responsible for the nerves innervating that particular skin region. Three horses whose symptoms had been persisting for a longer period of time due to the late reference to the study showed urinary incontinence (urine dribbling). No horses displayed symptoms such as trembling, muscle atrophy, sudden collapse, or dysphagia.

Blood tests

Blood chemistry showed that aspartate aminotransferase (AST), glucose and creatine kinase (CK) were high in the majority of horses. Electrolyte abnormalities (hyponatremia, hypochloremia, and hyperkalemia) were also observed. However, these disturbances were found to be clinically insignificant due to their minute deviation from normal values.

Virus tests

Nervous system tissues, nasal swabs and whole blood were examined by PCR for viruses that can cause neurological diseases. Nasal swabs were used to test EHV-1, EHV-4 and EIV, while whole blood was used for EHV-1, EHV-4, EIV, JEV, WNV, EEE, WEE and VEE. All tests were negative, as confirmed by neutralization tests or ELISA using serum samples.

Parasite tests

The results of fecal flotation tests showed eight horses with Strongyle eggs in their feces. The eggs of *Parelaphostrongylus tenuis*, *Drachia megastoma* and *Habronema microstoma* were not found.

Whole blood PCR, which was performed to detect *Halicephalobus gingivalis*, *Setaria* spp., *Strongylus* spp., *Angiostrongylus cantonensis*, *Parelaphostrongylus tenuis* and *Habroneoma* spp., was negative in all samples.

Cerebrospinal fluid (CSF) and serum were examined by antibody titration test for detecting *Sarcocystis neurona*. One horse was suspected of having equine protozoal myeloencephalitis (EPM).

Gross lesions and histopathology

On gross examination, significant lesions were not observed. Histopathological analysis revealed microcavitation, infiltrates of macrophages containing brown pigment, and infiltrates of eosinophils and lymphocytes in the leptomeninges of the brain. In the spinal cord, microcavitation, infiltrates of macrophages containing brown pigment, eosinophils, lymphocytes, axonal degeneration, fibrosis in the parenchyma, and infiltrates of eosinophils and lymphocytes in the dura mater and leptomeninges were observed (Fig. 1). Cross sections of parasites ranging from 100 to 200 µm in diameter were observed in the brain or spinal cord in 13 horses (Fig. 1). In three samples, cross sections of denatured parasites were observed.

In 2015, 16 horses (80%) were diagnosed with eosinophilic meningoencephalomyelitis (Table 1), while the remaining 4 horses showed parasitic meningoencephalomyelitis, granulomatous meningoencephalomyelitis, encephalomalacia, and myelomalacia, respectively. In 2016, the most common lesions were parasitic meningoencephalomyelitis (10 cases, 33%) and eosinophilic meningomyelitis (7 cases, 23%). Other lesions observed were eosinophilic meningoencephalomyelitis, parasitic meningomyelitis, eosinophilic meningitis, eosinophilic myelomeningitis, nonsuppurative myelomeningitis, eosinophilic spinal meningitis, encephalomalacia, and myelomalacia.

PCR and DNA sequencing

From a case in 2016, the parasites in the FFPE tissue sections of the spinal cord were identified as *Setaria digitata* (*S. digitata*) by DNA sequencing. The 12S rDNA sequence of the amplicons (GenBank accession number: MW647953) showed 99% nucleotide similarity with reference sequences of *S. digitata* (Table 2).

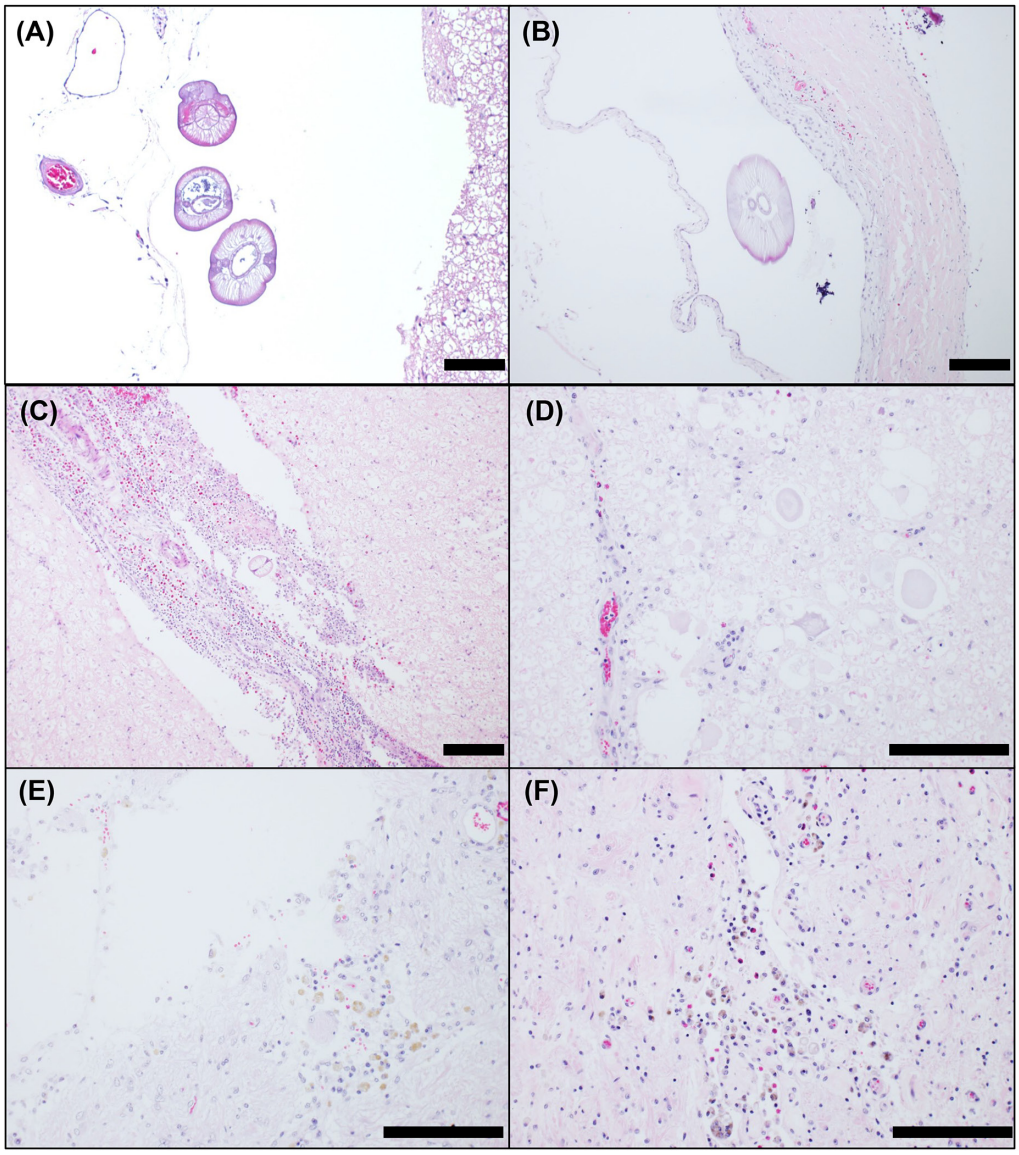

Fig. 1. Histopathological lesions. (A, B) Cross section of nematodes in the leptomeninges of the cervical and lumbar spinal cord. Hematoxylin and eosin (H&E). Bar=100 µm. (C) Cross section of nematode and infiltration of eosinophils in the leptomeninges of the thoracic spinal cord. H&E. Bar=100 µm. (D) Axonal degeneration and infiltration of eosinophils in the parenchyma of the thoracic spinal cord. H&E. Bar=100 µm. (E) Microcavitation and infiltration of brown pigmented macrophages in the parenchyma of the brain. H&E. Bar=100 µm. (F) Microcavitation, infiltration of brown-pigmented macrophages and eosinophils, and fibrosis in the parenchyma of the spinal cord. H&E. Bar=100 µm.

DISCUSSION

Neurological diseases characterized by acute hindlimb ataxia in horses have been increasing in South Korea since the mid-2000s. In 2015 and 2016, 20 and 30 horses were referred respectively in this study. According to a survey on the status of horse industries conducted in 2015 and 2016 [9], 26,330 and 27,116 horses were being raised in South Korea, respectively, making the incidence rate approximately 0.094%. This study investigated the cause of acute hindlimb ataxia in the Korean horse population by conducting neurological examination, laboratory tests, and histopathological examinations on horses with acute hindlimb ataxia recruited nationwide for 2 years. The characteristics of the neurological symptoms that have been increasing recently in Korean horses were as follows were as follows: first, the onset of symptoms was acute; second, although there were differences in severity, all horses showed hindlimb ataxia; and third, there was an explosive seasonal outbreak from late July to early October.

Since the onset of symptoms was acute, chronic diseases such as degenerative changes of the spine were excluded from the differential diagnosis. Cranial nerve reflexes were all normal with no changes in mentation and behavior, and clinical symptoms such as scoliosis, head tilt, head turn, postural change, muscle atrophy, trembling, and dysphagia were not observed, thus eliminating the possibility for rabies, equine motor neuron disease (EMND), vestibular disease and botulism [3]. Based on the fact

Table 1. Histopathological results of the horses with neurological symptom with hindlimb ataxia in 2015 and 2016

Histopathologic findings[†]	Number of horses (2015)	Number of horses (2016)
Eosinophilic meningoencephalomyelitis	16	3
Parasitic meningoencephalomyelitis	1	10
Granulomatous meningoencephalomyelitis	1	-
Eosinophilic meningomyelitis	-	7
Parasitic meningomyelitis	-	2
Eosinophilic meningitis	-	1
Eosinophilic myelomeningitis	-	2
Nonsuppurative myelomeningitis	-	1
Eosinophilic spinal meningitis	-	1
Encephalomalacia	3[ab]	6[de]
Myelomalacia	2[c]	1[f]
Total	23 (20 horses)	34 (30 horses)

[†]: Department of Diagnostics, Animal and Plant Quarantine Agency. [a]: Concurrence with eosinophilic meningoencephalomyelitis: 2 cases, [b]: concurrence with myelomalacia: 1 case, [c]: concurrence with encephalomalacia: 1 case, [d]: concurrence with eosinophilic meningomyelitis: 2 cases, [e]: concurrence with myelomeningitis: 1 case, [f]: concurrence with eosinophilic meningitis: 1 case.

Table 2. Sequence analysis following PCR using formalin-fixed paraffin embedded tissue sections of the spinal cord from a case in 2016

GenBank		% identity
Species	No.	
Setaria digitata	GU138699.1	99
Setaria digitata	EF179382.1	97
Setaria labiatopapillosa	AJ544833.1	85
Setaria equina	AJ544835.1	88
Setaria tundra	JN228376.1	88

that there was seasonal specificity, infectious diseases were more likely to be the causative agents than noninfectious diseases.

Non-infectious agents were ruled out for the following reasons. In Wobbler disease, cervical vertebral instability (CVI) usually occurs at 4 to 12 months of age and cervical static stenosis (CSS) occurs at 12 to 36 months of age [14]. The horses involved in this study were not age specific, thus excluding the possibility of Wobbler disease. In addition, since there were no trauma-related histories or injuries noted in physical examination, diseases related to trauma were excluded. Fungal infection was also disregarded due to the lack of suppurative or granulomatous lesions in histopathological examination. Furthermore, the manifestation of clinical symptoms is rarely acute unless exposed to large amounts of fungi at once. Fever is usually present and it mainly shows clinical symptoms such as mentation and behavior change, head pressing, decreased appetite, weight loss, kidney damage and liver damage.

Infectious agents that can cause neurological symptoms in horses can be divided into bacteria, viruses and parasites [10]. In bacterial infections, histopathologic findings can reveal suppurative or granulomatous lesions. In this study, suppurative lesions were not observed in any of the 50 horses, while granulomatous lesions were observed in only one horse. In addition, since inflammatory changes could not be confirmed on blood tests, the possibility of bacterial infection was excluded.

Nervous system tissues, nasal swabs and whole blood were examined by PCR to identify viral infections that may cause neurological symptoms in horses. The viruses to be examined were EHV-1, EHV-4, EIV, JEV, WNV, EEE, WEE, VEE and rabies virus. The results of PCR, neutralization test and ELISA were negative in all samples, thus excluding virus infection.

Diagnosis of parasites can be made when microfilaria is detected in the blood, and the CNS lesions are seen in histopathologic examination with clinical signs [18]. Although microfilaria circulating in the bloodstream of cattle can be detected by filtering a large amount of blood using an isopore filter membrane having a 3 µm filter hole [8], this method was not used in this study. Instead, whole blood PCR, histopathological examination and PCR for isolated parasites were conducted. Whole blood PCR was conducted to examine parasite antigens, but negative results were obtained in all samples.

In histopathologic examination of animals infected with *Setaria* spp., migrating larvae can cause gross lesions seen as brown foci or streaks, as well as microcavitation and variable haemorrhage [18]. There is loss of myelin and fragmentation of axons locally with eosinophils, neutrophils and macrophages present along with a mild meningitis and vascular cuffing [18, 19, 21]. In this study, several of the affected horses had lesions corresponding to a *Setaria* spp. infection, such as eosinophilic infiltration with microcavitation, cerebral hemorrhage and eosinophilic infiltration with axonal degeneration in the spinal cord. In 2015, eosinophilic meningoencephalomyelitis was the most common (16 cases, 80%) and parasitic meningoencephalomyelitis was confirmed in only one case. In 2016, 14 cases of eosinophilic lesions, 10 cases of parasitic meningoencephalomyelitis and 2 cases of parasitic meningomyelitis accounted for 86.7%. The frequency of observations of parasites increased significantly in 2016 compared to 2015. This is presumed to be due to the fact that the majority of the animals in 2016 were referred to the study with minimal previous treatment attempts. Because specimens from 2015 were exposed to a variable degree of diagnostic and treatment procedures prior to their enrollment in this study, it is possible that the parasites may have been eliminated by the use of those drugs. However, symptoms persist due to the permanent damage to the brain and spinal cord.

In the 13 cases where parasite bodies were observed, the cross sections of larvae were between 100 to 200 µm in diameter and had an eosinophilic hyaline cuticle, well-developed longitudinal muscles, voluminous lateral hypodermal chords, and blood in the intestines (Fig. 1), all consistent with the phenotypic characteristics of *Setaria* spp. fourth-stage larvae (L4). Results of DNA sequencing and sequence similarity searches using BLASTN analysis (https://blast.ncbi.nlm.nih.gov) demonstrated that the 12S

Table 3. The incidence rate of neurological diseases by age, breed and gender in 2015 and 2016

		Population of horses	No. of affected horses	Incidence rate (%)
Age				
	Under 3 years old	17,484	2	0.011
	3 to 9 years old	24,299	34	0.140
	Over 10 years old	10,485	14	0.134
	Unidentified	1,178	0	0.000
	Total	53,446	50	0.094
Breed				
	Thoroughbred	24,246	44	0.181
	Warmblood	1,419	3	0.211
	Halla horse	17,272	2	0.012
	Jeju horse	5,113	1	0.020
	Etc.	5,396	0	0.000
	Total	53,446	50	0.094
Gender				
	Mare	30,408	17	0.056
	Stallion	13,281	8	0.060
	Gelding	9,131	25	0.274
	Unidentified	626	0	0.000
	Total	53,446	50	0.094

rDNA sequence of the amplicons showed 99% nucleotide similarity with reference sequences of *S. digitata* (Table 2). Based on their morphologic and genotypic characteristics, the L4 was identified as *S. digitata*.

Aberrant parasite migration can occur anywhere in the CNS. Previous studies have shown that the larvae of *S. digitata* have been detected in the central nervous systems of horses, goats and sheep [5, 22]. Spinal cord lesions are common, while aberrant migration into the eye also occurs in horses [2]. Invasion into the anterior chamber causes corneal opacity and, in severe cases, can lead to blindness. When the central nervous system is invaded, it can cause cerebrospinal nematodiasis, resulting in torticollis and hindlimb gait disturbances without systemic symptoms. In severe cases, the affected horse may be unable to stand or will stay in a dog-sitting posture. They may also show symptoms of brain disorders such as obsessive-compulsive disorder, clonic convulsions and nystagmus [7, 18].

In this study, it was confirmed that the outbreak of the disease was from late July to early October, coinciding with the occurrence of mosquitoes, the intermediate host of *S. digitata*. According to the Korea Meteorological Administration, the summer of South Korea has the highest rainfall in early and mid-July, followed by a heat wave from late July to August. As a result, the population density of mosquitoes increases sharply from July to August. Therefore, it is possible that the sudden increase in intermediate hosts leads to a high number of horses showing neurological symptoms from summer to early autumn.

Table 3 shows the incidence rate of neurological diseases by age, breed and gender. There is no specificity for the age at onset, but additional studies are needed on the breed and gender specificity. The incidence rate of Halla (0.012%) and Jeju horses (0.020%) were lower than those of Thoroughbred (0.181%) and Warmblood (0.211%) and it was confirmed that the incidence rate was 5 times higher in geldings (0.274%) than in mares (0.056%) and stallions (0.060%)

Of the 50 horses that were necropsied over a period of 2 years, eosinophilic infiltration in the brain and spinal cord were observed in 30 horses. Among these 30 specimens, parasites or denatured parasites were observed in 13 of them. Therefore, it is estimated that the main cause of acute hindlimb ataxia, which prevails in the summer and early autumn in South Korea, is due to aberrant parasite migration of *S. digitata*. However, an additional epidemiologic investigation is necessary regarding the sudden increase in the frequency of horses showing hindlimb ataxia due to aberrant parasite migration, which was not common in the past. Further studies are also needed for horses whose cause of the symptoms has not been found.

Upon onset of symptoms, recovery to a normal performance is difficult due to permanent physical damage in the brain and spinal cord. Therefore, preventive management is of great importance to decrease incidence. To establish an effective prevention management program, identifying the causative parasites and confirming the lifecycle of the parasite is necessary. With the identification of *S. digitata* larvae in the brain and spinal cord as a major cause for neurological signs in horses in South Korea, establishment of an appropriate prevention program may now be feasible with further research.

CONFLICT OF INTEREST. All authors do not have any potential conflicts-of-interest.

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