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Prevalence of equine proliferative enteropathy in Hidaka district, Hokkaido, over five seasons

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Equine proliferative enteropathy (EPE) is an equine infectious disease that can lead to severe weight loss and hyperplasia of the intestinal mucosa due to infection with Lawsonia intracellularis*. In this study, we investigated the prevalence of EPE in a major Thoroughbred breeding area: Hidaka district, Hokkaido, Japan. Of the 252 symptomatic horses that we tested, 192 EPE cases (76.2%), including 8 fatal cases, were confirmed from April 2015 to March 2020 by etiological and/or serological investigation. Most of the EPE cases were observed in foals (88.5%), with fewer cases in yearlings (7.3%) and adults (4.2%). Asymptomatic infection was observed in 62.9% of the horses kept with affected horses. These results suggest that EPE is an enzootic disease in Hidaka district.*

Key words: *asymptomatic infection, equine proliferative enteropathy, Hidaka, Lawsonia intracellularis*

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Equine proliferative enteropathy (EPE) is an infection caused by the bacterium *Lawsonia intracellularis* that causes severe weight loss and hyperplasia of the intestinal mucosa in horses [9]. When appropriate antimicrobial agents are used, the prognosis for EPE is typically good; however, infection can affect the sales price of yearlings [2]. Hidaka district, Hokkaido, is the most important region for Thoroughbred breeding in Japan, with approximately 85% of Japanese Thoroughbreds and approximately 5,000 foals born per year in the district. EPE has been reported in this district previously [4, 12]; however, the prevalence of infection is not known. In the present study, we investigated the prevalence of EPE in Hidaka district across five seasons as well as EPE exposure in asymptomatic horses kept in the same stables as affected horses over the same period.

The fecal and serum samples used in this study were collected from symptomatic horses in Hidaka district from April 2015 to March 2020. The annual research period ran from April to March the next year because EPE generally occurs in autumn and winter. Samples from horses were submitted to the Equine Research Institute by the local livestock hygiene service center or clinics of the agricultural mutual aid association in Hidaka district to diagnose EPE, with diagnosis performed by equine practitioners. Symptomatic horses mainly presented with symptoms such as pyrexia, hypoproteinemia, anorexia, and edema. Intestinal membrane thickness and diarrhea also occurred simultaneously in some cases. In postmortem examinations for EPE, the intestinal mucosa was evaluated as detailed below. At several farms at which EPE cases were confirmed, fecal and serum samples from apparently unaffected horses in the same stables as infected horses were also collected to determine the degree of intra-farm infection spread. For the collection of samples from asymptomatic horses, informed consent was obtained from the horse owners by the equine practitioners. Fecal, serum, and mucosal samples were kept at −20°C before examinations. Additionally, the history of administration of a live attenuated *L. intracellularis* vaccine approved in pigs (Enterisol Ileitis, Boehringer Ingelheim, Ingelheim, Germany) was obtained by the practitioners.

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Lawsonia intracellularis DNA was detected using quantitative PCR (qPCR) as described by Nathues *et al.* [5]. Whole DNA from the feces of symptomatic and asymptomatic horses and intestinal mucosa of autopsied horses was extracted using a Quick-DNA Fecal/Soil Microbe Kit (Zymo Research, Irvine, CA, USA) and DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), respectively, in accordance with the manufacturer's protocols. To perform qPCR, 2 μl of DNA extract was added to 18 μl of qPCR mixture comprising 10 μl of EagleTaq Universal Master Mix with ROX (Merck, Darmstadt, Germany), 0.4 μl of 10-μM probe (Li-ubi-probe), (FAM)-TAGCCACATCAAGT-GTTCCAGCTGCAAG-(BHQ1); 0.5 μl of 20-μM forward primer (Li-ubi-F), GCTCATACCGATTGTGTAATGCA); 0.5 μl of 20-μM reverse primer (Li-ubi-R), GAAAAACAG-GCCGTATCCTTGA); and 6.6 μl of distilled water. PCR programs, performed using a StepOnePlus Real-time PCR instrument (Thermo Fisher Scientific, Waltham, MA, USA), included the following steps: preincubation at 95°C for 10 min followed by 45 incubation cycles at 95°C for 15 sec and 68°C for 20 sec. When exponential amplification was observed in the sample within 40 cycles, the sample was considered to be *L. intracellularis* positive.

Anti-L. intracellularis antibody was detected using the slide-streptavidin biotin (s-SAB) method as follows. Vaccine strain (B3903) cells of *L. intracellularis* were used as an antigen. Enterisol Ileitis (Boehringer Ingelheim) was dissolved using the solution contained in the package. The dissolved solution was washed using phosphate-buffered saline (PBS) and resuspended using PBS with a 2-fold concentration relative to that of the initial concentration. The antigen solution was dropped in 12 spots on a fluorine-coated glass slide (Matsunami Glass Ind., Ltd., Osaka, Japan), and the spots were fixed using acetone (stored at −20°C before use). The test serum was diluted 29-fold using PBS and dropped onto a slide, which was incubated at 37°C for 30 min in a moist chamber. After washing the slide three times for 5 min per wash in a staining vat using PBS, it was incubated with a secondary antibody, anti-horse IgG (G+L)-conjugated biotin (Aviva Systems Biology, San Diego, CA, USA), at 37°C for 30 min. The slide was again washed three times for 5 min per wash in a staining vat using PBS, after which a drop of peroxidase-labeled streptavidin solution (Histofine Simple Stain, Nichirei Biosciences Inc., Osaka, Japan) was poured onto each spot; the slide was then incubated at room temperature for 20 min. After three PBS washes for 5 min per wash, a drop of aminoethyl carbazole solution (Histofine Simple Stain, Nichirei Biosciences Inc.) was overlayed as an enzyme substrate, and the slide was then incubated at room temperature for 5 min. Following a 5-min wash using PBS and a 1-min wash using distilled water, the slide was dried and sealed. Subsequently, it was examined using a light microscope, and when the red-brown color of a small comma-shaped bacillus was observed, the sample serum was considered positive for L. intracellularis-specific antibodies.

Although all the submitted cases were suspected by practitioners to involve EPE based on the presence of symptoms such as pyrexia and hypoproteinemia, the results of qPCR and/or s-SAB tests were used for the definitive diagnosis of EPE. In postmortem investigations, positive qPCR results in the intestinal mucosa were considered to indicate EPE-positive cases. If asymptomatic horses kept with the affected horses were positive on qPCR and/or s-SAB, they were considered to have an asymptomatic infection.

The annual number of EPE cases and farms with confirmed EPE cases across five seasons are shown in Fig. 1. From April 2015 to March 2020, 192 of 252 tested symptomatic horses (76.2%) were confirmed to be EPE positive, including 8 of 13 autopsy cases (Table 1). At least 10 cases of EPE were confirmed annually, and the highest number of cases was observed in 2017. Over the study period, 125 EPE-positive farms, comprising 117 single-incident farms, 7 double-incident farms, and 1 triple-incident farm, were identified. Interestingly, only a small number of farms had confirmed cases of EPE in multiple years over the study period. Although it is unclear why multiple-year incidences of EPE were uncommon in this district, farm-level immunity of mares against EPE may have been established after *L. intracellularis* invasion at the affected farms, or EPE may have been eradicated temporally from the farms by the next epidemic season. On average, there were 712 farms in Hidaka district in 2015–2019 (data available in an online database provided by the Japan Bloodhorse Breeder's Association: https://jbba.jp/data/statistics.html); thus, approximately 20% of the farms in the district were affected by EPE over this period. These results suggest that EPE is widely spread in Hidaka district.

The ecology of *L. intracellularis* is not yet known, although it is possible that the invasion and transmission of EPE at farms is associated with wildlife, as reported in

Table 1. Outline of the equine proliferative enteropathy (EPE) cases in this study

Horses		Number of cases (number of autopsies)
Symptomatic		252 (13)
	EPE	192 (8)
	Non-EPE	60 (5)
Asymptomatic*		264 (NA)
	EPE	166 (NA)
	Non-EPE	98 (NA)

*The horses were kept in the same stable as the affected horses. NA: not applicable.

previous studies [3, 10]. Using multilocus variable-number tandem repeat analysis, Kinoshita *et al.* found that the genotypes of *L. intracellularis* obtained from horses in Hidaka district included an equine genetic cluster and miscellaneous (including horses and wildlife) genetic clusters [4]. Thus, the occurrence of EPE might be associated with the introduction of carrier horses and/or the invasion of wildlife into farms in this district.

Most EPE cases were observed in foals (n=170; 88.5%), with fewer cases in yearlings (n=14; 7.3%) and adults (n=8; 4.2%). The number of cases per month is shown in Fig. 2. All cases were observed from July to April the next year, with most cases (n=161; 83.9%) observed from September to December. These EPE characteristics are consistent with those reported previously [1, 2]. Because the clinical signs of EPE are usually nonspecific [8], it is not easy to diagnosis EPE presumptively only on the basis of symptoms. This epidemiological information could help practitioners in the clinical diagnosis of EPE in Japan.

In asymptomatic horses kept in the same stables (n=44) as affected horses, *L. intracellularis*-specific genes were found in 11.7% (range of the positive rate at each farm: 0%–100%) of fecal samples, and *L. intracellularis*-specific antibodies were detected in 61.0% (range of the positive rate at each farm: 0%–100%) of serum samples. Overall, 62.9% (166 of 264) of horses were considered to have an asymptomatic infection (Table 1). Thus, the cases submitted for diagnosis were not always the index cases at farms, and it is possible that EPE was transmitted to the affected horses from asymptomatic horses and vice versa. On a few farms, hypoproteinemia was detected among asymptomatically infected horses (data not shown). The degree of severity of EPE varied from subclinical to fatal [7, 10]. Thus, to ensure that foals grow appropriately, farmers and practitioners that encounter EPE cases must focus on not only clinical cases but also apparently healthy horses at the same farm.

Among the 93 EPE-affected horses for which vaccination records were available for the porcine *L. intracellularis* vaccine, 92 horses had not received the vaccine, and the remaining horse had received the first of two injections about a month before the onset of EPE symptoms. The experimental effectiveness of the porcine live attenuated *L. intracellularis* vaccine has been reported previously [11]. However, the field efficacy of the vaccine was not confirmed in a previous field study [6]. Although off-label use of the vaccine in Japanese Thoroughbred breeding has been gradually spreading, our finding that almost all EPE-affected horses were unvaccinated indirectly indicates the field efficacy of the porcine vaccine in terms of EPE control.

In conclusion, EPE appears to be an enzootic disease in Hidaka district. Most EPE cases occurred in foals from September to December. On many farms, asymptomatic infection was also observed in horses kept with EPE-affected horses. Almost all of the EPE-affected horses had not been vaccinated with the porcine vaccine. Although the vaccine has not been approved for horses, the porcine vaccine might be effective for the control of EPE

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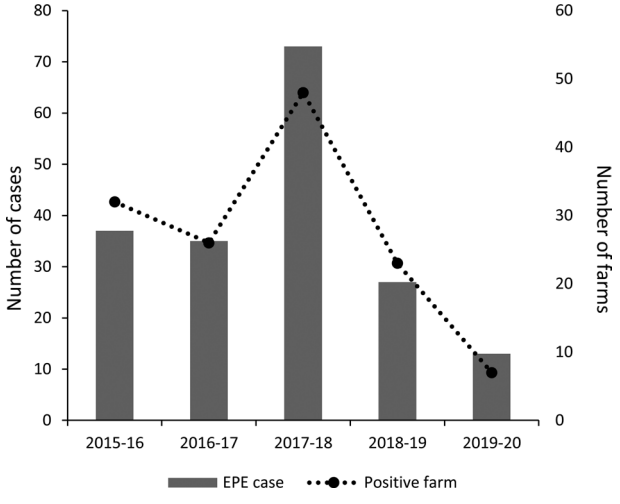

Fig. 1. Annual number of equine proliferative enteropathy (EPE) cases and farms on which infections were confirmed at the Hidaka district across five seasons.

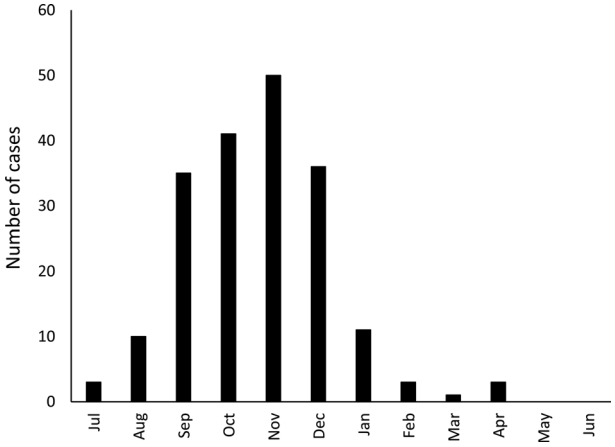

Fig. 2. Total number of equine proliferative enteropathy cases per month in Hidaka district across five seasons.

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