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Total serum protein reference value as a clinical diagnostic index of equine proliferative enteropathy

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Equine proliferative enteropathy (EPE) caused by Lawsonia intracellularis is characterized by hypoproteinemia. There are currently no reliable reports that provide a reference value for the total serum protein (TP) concentration to clinically diagnose EPE. The objective of this study was to statistically determine the reference value. Feces and sera of 99 foals with EPE-like clinical signs and of 35 healthy foals were obtained. The samples were used for specific-gene detection of L. intracellularis, TP measurement, and specific-antibody detection against L. intracellularis. Based on these results, the optimal reference value for the TP concentration as a clinical diagnostic index of EPE was found to be ≤ 4.8 g/dl. This clinical diagnostic index will provide an effective approach for diagnosing EPE.

Key words: *clinical diagnostic index, equine proliferative enteropathy, Lawsonia intracellularis, total serum protein*

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Lawsonia intracellularis, an obligate intracellular organism, is the causative agent of proliferative enteropathy in domestic and wild animals [6]. This disease is characterized by proliferation of immature epithelial cells in the crypts of the distal small intestine and/or proximal large intestine [1], which leads to thickening of the intestinal mucosa, resulting in the enteric signs often associated with this disease [13]. Equine proliferative enteropathy (EPE) usually affects weanling horses and is rarely seen in older horses [2, 7]. Clinical signs of affected foals include anorexia, fever, lethargy, depression, peripheral edema, rapid weight loss, colic, and diarrhea, although they are nonspecific in the early stages [5]. A definitive diagnosis of EPE is based on the presence of histological lesions and the detection of *L. intracellularis* by immunohistochemistry of small intestinal sections obtained in necropsy [2, 7], but this gold standard diagnostic method is unrealistic in clinical practice.

The finding of hypoproteinemia caused by hypoalbuminemia is highly suggestive of EPE in weanlings [3]. Normal reference values (mean ± standard deviation) of total serum protein (TP) and albumin concentrations in 6-month-old foals are 6.2 ± 0.3 g/d*l* and 3.1 ± 0.3 g/d*l*, respectively [1]. However, no reliable reference value for the TP concentration specific to clinical EPE has been defined. Therefore, the objective of the present study was to use field data to assign a numerical reference value for the TP concentration that could be used as a clinical diagnostic index of EPE.

A total of 134 foals from 65 thoroughbred farms in the Hokkaido prefecture were examined in this study, from September 2013 to February 2016. Each farm had at least one suspected case of EPE in foals. Ninety-nine foals showed at least one clinical sign of EPE, such as anorexia, fever, lethargy, depression, peripheral edema, rapid weight loss, colic, or diarrhea. These foals were 4 to 12 months old, with most being 5 to 7 months old. In addition, many foals generally showed clinical signs between September and December. Additionally, 35 healthy foals that were

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housed with the foals showing clinical signs were examined. All 134 foals were less than a year old and had not been vaccinated.

Samples were collected when at least one foal at a farm showed clinical signs of EPE. Fresh feces samples were placed in conical tubes stored at 4°C and transported to the Equine Research Institute, Japan Racing Association. The samples were frozen at −20°C prior to DNA extraction for quantitative polymerase chain reaction (qPCR) [8]. Sera were separated from blood samples and were examined for TP concentration. Subsequently, they were frozen at −20°C and transported to a laboratory at the University of Miyazaki. *L. intracellularis*–specific antibodies were detected by using the indirect fluorescent antibody (IFA) technique [4]. The foals that tested positive with feces (qPCR) and/or serum (IFA) were considered to be infected by *L. intracellularis*.

Fecal samples were processed for DNA extraction using ZR Fecal DNA MiniPrep™ (Zymo Research, Irvine, CA, U.S.A.), following the manufacturer's recommendations. The extracted DNA was analyzed by qPCR, as previously reported [8]. Serum samples were analyzed to determine the TP concentration using a refractometer, followed by IFA to detect specific antibodies against *L. intracellularis*. The refractometer determined the TP concentration by measuring the refractive index of sera immediately after separation and before freezing. The IFA procedure used in this study was based on previously reported methods [4] with some modifications. Briefly, the wells of 12-well glass slides (TF1205A, Matsunami Glass Ind., Ltd., Osaka, Japan) were coated with the *L. intracellularis* vaccine antigen (Enterisol® Ileitis, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO, U.S.A.) at a concentration of $10^{4.29}$ to $10^{5.50}$ $TCID_{50}$/m*l* ($TCID_{50}$: 50% tissue culture infective dose) [12]. Sera and secondary antibodies were dropped into each well, in that order, after which the solutions were diluted at the ratio of 1:30. Samples were observed via fluorescence microscopy under ultraviolet excitation, and the samples showing fluorescence were confirmed as seropositive.

Foals were classified based on their EPE and/or *L. intracellularis* infection status. In *L. intracellularis* infection status-based classification, foals were divided into four categories (Table 1): positive for both qPCR and IFA (qPCR+/IFA+), only positive for qPCR (qPCR+/IFA−), only positive for IFA (qPCR−/IFA+), and negative for both tests (qPCR−/IFA−). Subsequently, foals were classified based on the presence or absence of clinical signs of EPE into three categories to describe their EPE/serological-*L. intracellularis* infection status (Table 2): presumptively clinical EPE (cEPE), subclinical *L. intracellularis*-infected (sLi), and seronegative (sNeg). The cEPE group included foals with the presence of clinical signs and a positive *L. intracellularis*-specific IFA result. The sLi group comprised of foals with a positive *L. intracellularis*-specific IFA result but no other indication of infection. The sNeg category included foals with no detectable *L. intracellularis*-specific antibodies. These clinical signs included at least one clinical sign of EPE, such as anorexia, fever, lethargy, depression, peripheral edema, rapid weight loss, colic, or diarrhea.

A statistical analysis was performed using the pairwise Wilcoxon rank-sum test (F) to assess the differences in TP concentration among the groups. A logistic regression analysis was performed three times to identify the best model for determining the reference value for the TP concentration (independent variable) as a clinical diagnostic index of EPE. Akaike's information criteria (AIC) were used to evaluate models (dependent variables), which included molecular and serological results, only molecular results, and only serological results (1, positive result; 0, negative result). For the category of molecular and serological results, both positive results were set to 1, and others were set to 0. Receiver operating characteristic (ROC) analyses with the area under the curve (AUC) calculated to provide the maximum sum of sensitivity and specificity were used to evaluate the reference value for the TP concentration and distinguish between cases with and without seropositive results and clinical signs. Furthermore, the reliability of the

Table 1. Total serum protein (TP) concentrations for the four categories in the *Lawsonia intracellularis* infection status-based classification

	qPCR+/IFA+[x]	qPCR+/IFA−[x]	qPCR−/IFA+[x]	qPCR−/IFA−[x]
Clinical signs				
Positive	53	1	28	17
Negative	6	3	14	12
Number of samples	59	4	42	29
Median (maximum–minimum) of total serum protein (g/d*l*)[y]	3.20[a] (7.50–1.80)	5.25[bc] (5.60–3.70)	4.15[b] (5.90–1.90)	5.70[c] (7.40–4.00)

[x]Groups were determined by results of quantitative polymerase chain reaction (qPCR) and indirect fluorescent antibody (IFA). qPCR+/IFA+, positive for both qPCR and IFA; qPCR+/IFA−, only positive for qPCR; qPCR−/IFA+, only positive for IFA; and qPCR−/IFA−, negative for both of tests. [y]These groups were compared by use of the pairwise Wilcoxon rank-sum test (F). Values indicated with different superscript letters (a–c) are significantly different ($P<0.05$).

Table 2. Total serum protein (TP) concentrations for the three categories in the equine proliferative enteropathy (EPE)/serological *Lawsonia intracellularis* infection status-based classification

	Presumptively clinical EPE affected (cEPE)[x]	Subclinical *L. intracellularis* infected (sLi)[x]	Seronegative (sNeg)[x]
Clinical signs	Positive	Negative	Positive/negative
IFA	Positive	Positive	Negative
Number of samples	81	20	33
Median (maximum–minimum) of total serum protein (g/dl)[y]	3.20[a] (5.90–1.80)	5.30[b] (7.50–2.80)	5.50[c] (7.40–3.70)

[x]Groups were determined by results of serological tests and presence or absence of clinical signs. cEPE, positive for indirect fluorescent antibody (IFA) with clinical signs; sLi, foals with only a positive IFA result and no other indication of infection; sNeg, negative for IFA. [y]These groups were compared by use of the pairwise Wilcoxon rank-sum test (F). Values indicated with different superscript letters (a–c) are significantly different ($P<0.05$).

new TP concentration reference value was assessed using a kappa score calculated for IFA results. Values of $P<0.05$ were considered to be statistically significant for all tests. All statistical analyses were conducted using R software version 3.2.1 (The R Project for Statistical Computing: https://www.r-project.org/).

Based on the sample analysis, 59 foals were qPCR+/IFA+ 4 foals were qPCR+/IFA−, 42 foals were qPCR−/IFA+, and 29 foals were qPCR−/IFA− (Table 1). The molecular-positive and seropositive ratios in foals with clinical signs were 54.5% (54/99) and 81.8% (81/99), respectively, while those in foals with no clinical signs were 25.7% (9/35) and 57.1% (20/35), respectively. In *L. intracellularis* infection status-based classification, the TP concentration of qPCR+/IFA+ foals was significantly lower than those of the other 3 groups, and the TP concentration of qPCR−/IFA+ foals was significantly lower than that of qPCR−/IFA− foals (Table 1). In EPE/serological *L. intracellularis* infection status-based classification, 81 foals were cEPE, 20 foals were sLi, and 33 foals were sNeg. Significant differences in the TP concentration were seen among all groups (Table 2).

In the logistic regression analysis, 23 subclinical *L. intracellularis*-infected foals in the first classification (6 qPCR+/IFA+, 3 qPCR+/IFA−, and 14 qPCR−/IFA+ without clinical signs) were eliminated, as the present study focused on determining the TP reference value as a clinical diagnostic index only for EPE. The logistic regression analysis revealed that the TP concentration was related to all dependent valuables ($P<0.05$), and only the serological test results category had a lower AIC (48.584) compared with the other dependent valuables (both molecular and serological results, 125.75; only molecular results, 125.61).

An ROC analysis was conducted to describe the ROC curve (Fig. 1) using 81 cEPE and 33 sNeg foals. As in the case of the logistic regression analysis in which 23 subclinically infected foals were eliminated from the analysis, 20 sLi foals in the second classification were eliminated from the ROC analysis. The best TP concentration reference value

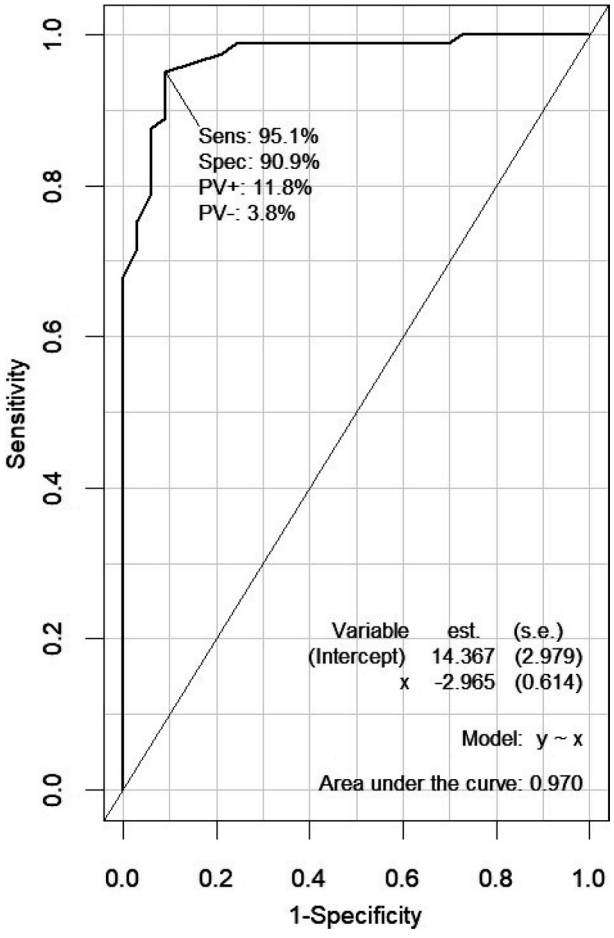

Fig. 1. Receiver operating characteristic (ROC) curve analysis. The ROC curve demonstrates that the optimal reference value of total serum protein for clinical diagnosis of equine proliferative enteropathy (EPE) is 4.8 with 95.1% sensitivity, 90.9% specificity, and an area under the curve of 0.970. Sens, sensitivity; spec: specificity, PV+, positive predictive value; PV−, negative predictive value; est., estimate; s.e., standard error.

was ≤ 4.8 g/d*l* (AUC=0.970). At this value, the sensitivity was 95.1%, and the specificity was 90.9%. In the foals showing clinical signs, the level of agreement between IFA and TP measurements using this reference value was 92.9%, and the kappa score was 0.77. The level of agreement and kappa score in all foals used in this study were 82.8% and 0.61, respectively.

We determined that the optimal reference value of the TP concentration was ≤ 4.8 g/d*l* for an effective diagnostic index in foals showing clinical signs of EPE. The usefulness of the TP value in the diagnosis of clinical EPE has been demonstrated previously [3]. However, an authoritative reference value had not been established for the clinical diagnosis of EPE until now. In this study, each score used to evaluate the reference value suggested that foals that show clinical signs and have a TP concentration ≤ 4.8 g/d*l* have a high probability of developing EPE. The kappa score and level of agreement between IFA and the new TP reference value for all foals were lower than those for symptomatic foals. This may suggest that the TP concentration is not an accurate screening method for EPE in presymptomatic foals. Most foals in this study presented with EPE, but there were a few foals without any clinical signs. Additionally, there is a difference in the number of foals used in this study and the natural population. Because of this, while TP measurement may become a screening tool for pre-EPE foals, further investigation is required.

Our results also revealed the possibility that serological examination may be more useful for clinical diagnosis than the molecular tests using feces. In this study, many qPCR−/IFA+ foals showed clinical signs, while very few qPCR+/IFA− foals presented with them. The main reason that qPCR+/IFA− foals were far less likely to show clinical signs than qPCR−/IFA+ foals is thought be due to a mismatch between the infectious periods and the antibody detection periods of *L. intracellularis* [9–11]. The qPCR+/IFA− foals were suggested for the early period of infection or passage time of the bacteria. Moreover, in this logistic regression analysis, the TP concentration was more associated with the serological status than the molecular status. This result may indicate that the serological test better reflects the pathological condition of EPE, that is hypoproteinemia, than the molecular test.

Some limitations exist in this study. First, as the horses in the study were less than 1 year of age, this reference value can apply only to foals. Second, there were some foals that did not match our reference value in this study, which means that it is not a perfect index. This TP value should be used as a reference value only. Finally, the clinical signs of each foal were observed by several veterinarians, so it is possible that there were differences in diagnosis of EPE, causing selection bias.

In conclusion, this is the first study to determine a reliable TP concentration reference value that is specific to clinical EPE in an on-site evaluation, although the confirmation about association of *L. intracellularis* should be examined by serological and/or molecular examinations, finally. Our clinical diagnostic index will prove to be an effective approach to the diagnosis and subsequent treatment of EPE.

References

1. Bauer, J.E., Harvey, J.W., Asquith, R.L., McNulty, P.K., and Kivipelto, J. 1985. Serum protein reference values in foals during the first year of life: comparison of chemical and electrophoretic methods. *Vet. Clin. Pathol.* **14**: 14–22. [Medline] [CrossRef]
2. Dauvillier, J., Picandet, V., Harel, J., Gottschalk, M., Desrosiers, R., Jean, D., and Lavoie, J.P. 2006. Diagnostic and epidemiological features of *Lawsonia intracellularis* enteropathy in 2 foals. *Can. Vet. J.* **47**: 689–691. [Medline]
3. Frazer, M.L. 2008. *Lawsonia intracellularis* infection in horses: 2005-2007. *J. Vet. Intern. Med.* **22**: 1243–1248. [Medline] [CrossRef]
4. Guedes, R.M.C., Gebhart, C.J., Winkelman, N.L., Mackie-Nuss, R.A.C., Marsteller, T.A., and Deen, J. 2002. Comparison of different methods for diagnosis of porcine proliferative enteropathy. *Can. J. Vet. Res.* **66**: 99–107. [Medline]
5. Lavoie, J.P., Drolet, R., Parsons, D., Leguillette, R., Sauvageau, R., Shapiro, J., Houle, L., Hallé, G., and Gebhart, C.J. 2000. Equine proliferative enteropathy: a cause of weight loss, colic, diarrhoea and hypoproteinaemia in foals on three breeding farms in Canada. *Equine Vet. J.* **32**: 418–425. [Medline] [CrossRef]
6. Lawson, G.H.K., and Gebhart, C.J. 2000. Proliferative enteropathy. *J. Comp. Pathol.* **122**: 77–100. [Medline] [CrossRef]
7. McGurrin, M.K., Vengust, M., Arroyo, L.G., and Baird, J.D. 2007. An outbreak of *Lawsonia intracellularis* infection in a standardbred herd in Ontario. *Can. Vet. J.* **48**: 927–930. [Medline]
8. Nathues, H., Holthaus, K., and grosse Beilage, E. 2009. Quantification of *Lawsonia intracellularis* in porcine faeces by real-time PCR. *J. Appl. Microbiol.* **107**: 2009–2016. [Medline] [CrossRef]
9. Pusterla, N., Jackson, R., Wilson, R., Collier, J., Mapes, S., and Gebhart, C. 2009. Temporal detection of *Lawsonia intracellularis* using serology and real-time PCR in Thoroughbred horses residing on a farm endemic for equine proliferative enteropathy. *Vet. Microbiol.* **136**: 173–176. [Medline] [CrossRef]
10. Pusterla, N., Vannucci, F.A., Mapes, S.M., Nogradi, N., Collier, J.R., Hill, J.A., Difrancesco, M., White, A.M., Akana, N.K., Simonek, G., and Gebhart, C.J. 2012. Efficacy of an avirulent live vaccine against *Lawsonia in-*

tracellularis in the prevention of proliferative enteropathy in experimentally infected weanling foals. *Am. J. Vet. Res.* **73**: 741–746. [Medline] [CrossRef]

11. Pusterla, N., Wattanaphansak, S., Mapes, S., Collier, J., Hill, J., Difrancesco, M., and Gebhart, C. 2010. Oral infection of weanling foals with an equine isolate of *Lawsonia intracellularis*, agent of equine proliferative enteropathy. *J. Vet. Intern. Med.* **24**: 622–627. [Medline] [CrossRef]
12. Sueyoshi, M., Miyayama, D., Nakamura, Y., Tsurita, M., Sasaki, Y., Uemura, R., Niwa, H., Katayama, Y., Oyama, T., and Harada, T. 2013. *Lawsonia intracellularis* antibodies in horses on breeding farms in Japan. *Proc. Conf. Res. Work. Anim. Dis. U.S.A.* 88 (December 8), Chicago.
13. Williams, N.M., Harrison, L.R., and Gebhart, C.J. 1996. Proliferative enteropathy in a foal caused by *Lawsonia intracellularis*-like bacterium. *J. Vet. Diagn. Invest.* **8**: 254–256. [Medline] [CrossRef]