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Analysis of antibody responses by commercial western blot assay in horses with alvelolar echinococcosis

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WESTERN BLOT IN EQUINE ECHINOCOCCOSIS

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ABSTRACT.

Commercial western blot (WB) assay was used to detect serum antibodies specific to *Echinococcus multilocularis* in 23 horses in which infection was confirmed by postmortem inspection at a slaughterhouse. Livers contained from 1 to >20 nodular lesions; foci diameter ranged from 1 to 25 mm. Antibody tests of serum from all 23 animals were negative for antigen bands at 7, 16, 18, and 26-28 kDa, which show specificity in the serum of human patients. However, sera from two infected horses with the largest nodules (diameter, 25 mm) showed positive response to one of the 22-kDa and 30-kDa antigen bands. It may be possible to diagnose *E. multilocularis* infection in horses based on the detection of these bands on commercial WB assay.

Key words

Echinococcus, PCR, horse, western blot

The world's first occurrence of equine alveolar echinococcosis was reported in eastern Hokkaido in 1984 [7]. Recently, during inspections at Yonezawa Municipal Slaughterhouse in Yamagata Prefecture, which is in the northeast of mainland Japan, we detected *Echinococcus multilocularis* in horses for the first time outside Hokkaido, and subsequently, an infection rate as high as 20% was found in racehorses sent to the same slaughterhouse as part of ongoing inspections [4]. This infection rate is thought to be due to the recent expansion of the highly endemic area of alveolar echinococcosis from eastern Hokkaido to the whole Hokkaido, including the Hidaka and Iburi regions, which are Japan's main areas of racehorse production. Consequently, the breeding environments of these horses, including at stock farms, are becoming contaminated with the eggs of *E. multilocularis*. Cases of equine cystic echinococcosis caused by *Echinococcus granulosus* or *Echinococcus equinus* have been reported from Europe, the Middle East, South and East Africa, North America, and Southeast Asia [2]. However, since Japan is the only country to have reported equine infection by *E. multilocularis*, the clinical picture of this disease is unclear and further study is needed. It is also important to understand the current status of *E. multilocularis* infection in horses from a public health standpoint, since this is a zoonotic infection and further transmission within Japan needs to be prevented [5]. Therefore, we investigated the potential for using western blot (WB) assay to conduct serodiagnosis of *E. multilocularis* infection in horses with infection confirmed by liver examination during slaughterhouse inspection.

Gross inspection of 158 horses at the Yonezawa Municipal Slaughterhouse between May 2010 and March 2011, revealed nodular lesions in the livers of 97 animals. The number and sizes of the lesions were recorded, and the lesions were removed and fixed in 10% formalin for histopathological examination. Using routine methods,

samples were embedded in paraffin, sliced in 5- to 10-μm sections, stained by hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) staining, and examined under the microscope. Using the same paraffin-embedded sections, DNA was extracted from the tissue with DEXPAT (TaKaRa Bio, Japan) and subjected to PCR, the obtained PCR products were then applied to PCR-RFLP [3, 9]. A total of 23 animals were diagnosed with alveolar echinococcosis based on the histopathological findings and/or molecular analysis of the lesions. Serum collected from the infected horses at time of slaughter was also tested for antibodies, using the *Echinococcus* Western Blot IgG (LDBIO Diagnostics, France) commercial assay for diagnosis of echinococcosis in humans [6]. Test procedures adhered to the assay kit instructions in all respects except for the addition of alkaline phosphatase-labeled goat anti-horse IgG [H+L] (Jackson Immuno Research Labs, USA) as a labeled secondary antibody. This assay using a whole *E. multilocularis* larval antigen is designed to detect human echinococcosis by probing specific antigen bands which occur in the relatively low molecular weight region of ≤30 kDa. Specifically, the pattern of responses to antigen bands at 7, 16, 18 and/or 26-28 kDa form the basis of diagnosis. In keeping with this approach, our evaluation was based on antigen bands in the relatively low molecular weight region.

Table 1 shows the sex and age of the 23 horses infected with *E. multilocularis*, the number and size of liver nodules, histological and genetic test data, and interpretation of the WB response bands shown in Figure 1. The infected horses were 4 to 9 years old and had 1 to >20 nodules per animal with foci ranging in diameters from 1 to 25 mm. A PAS-positive cuticular layer characteristic of *E. multilocularis* cysts was confirmed in 12 animals, infection was confirmed by genetic identification in 17 animals, and infection was confirmed by both methods in 6 animals. Figure 1 shows the banding patterns

obtained from the commercial assay of sera of the 23 animals (Lane Nos. 1 to 23). Lane No. 24 is the banding pattern in serum from a human patient with alveolar echinococcosis. It is evident that none of the serum samples from the horses infected with *E. multilocularis* showed a positive response for antigens at 7, 16, 18 and 26-28 kDa as seen in the infected human serum sample. However, a positive response at 30 kDa was seen for No. 2, which had a 25-mm nodule, and a very clear positive band at 22 kDa was seen for No. 9, which also had a 25-mm nodule. The 30-kDa band was also observed for Nos. 1, 3, 15, 18 and 19. The response at the 22-kDa was strong for Nos. 5 and 7, in addition to No. 9, and somewhat weaker for Nos. 10, 11, 12 and 13. In total, the 30-kDa band was positive for 6 of 23 animals (26%), and the 22-kDa band was positive for 7 of 23 animals (30%). As no animals were positive for both antigen bands, 13 of 23 animals (56%) showed a positive response on the commercial WB assay. In the remaining 10 animals (Nos. 4, 6, 8, 14, 16, 17, 20, 21, 22, 23), no positive response was seen for the molecular weight regions targeted in this diagnostic.

It is interesting that none of the 23 *E. multilocularis*-infected horses in this study showed positive responses to any of the commercial assay antigen bands thought to be specific to *E. multilocularis* in humans. Like humans, horses are non-suitable intermediate hosts in the life cycle of *E. multilocularis*. However, development of the larval stage (metacestode) of *E. multilocularis* as far as protoscolex formation has been known in humans on rare occasions, although in horses it is currently unknown whether development to the brood capsule or protoscolex stage occurs. In humans, there is an asymptomatic incubation period of at least 5 to 15 years after infection [8]. This makes it difficult to identify the timing of egg ingestion, and consequently the time needed for antibodies to appear in the serum after infection is still unknown. It is

generally thought that in most cases, either larval development is suspended in the early stage of *E. multilocularis* infection, or if the larvae undergo regressive change, the host's immune response is too weak to detect any resulting antibodies. Aoki [1] conducted a clinicopathological study of 70 Hokkaido patients with alveolar echinococcosis who underwent hepatic resection and investigated the progression of the lesions and elevated antibody titers by ELISA. The patients were 48.7 ± 16.6 years (mean ± SD) and the size of the hepatic lesions were 62 ± 37 mm (diameter, mean ± SD). Antibody titers were found to correlate positively with foci diameter, confirming that elevated antibody titer was due to the host's strengthened immune response as foci grew larger. Furuya et al. [3] found a positive response in sera collected preoperatively using the same commercial WB assay as used here in 60 of 64 patients (93.7%) in the Hokkaido groups. Considering that the infected horses in our study were 4 to 9 years of age and had nodular lesions of 1 to 25 mm in diameter, one possible explanation for the lack of response against the antigen bands thought to be specific to *E. multilocularis* in humans for the equine sera was that the infection was still in an early stage or in a mild phase. It would be productive to examine the correlation between the extent of antibody production in horses judged to be in such phases of infection by *E. multilocularis* and the size of nodular lesions. In this regard, it is noteworthy that sera from the two infected horses with the largest nodule (diameter, 25 mm) showed a positive response to one of the 22-kDa and 30-kDa antigen bands in our study. If an WB assay using a whole *E. multilocularis* larval antigen like the commercial WB assay used here could be applied for testing horse serum, it may be possible to diagnose *E. multilocularis* infection in horses judged to have 'early stage or mild infection' through the detection of these bands. Further study is needed to determine whether these responses were

actually specific to *E. multilocularis* infection.

Since cases of equine infection with *E. multilocularis* are unknown outside Japan, we have no clear clinical picture of the disease, and thus an ongoing study of the relationship between antibody detection and clinical symptoms or imaging diagnostics is necessary. Since alveolar echinococcosis is a zoonotic infection, further transmission needs to be prevented. Surveys of the current state of *E. multilocularis* in horses by postmortem inspection at slaughterhouses will be of great importance from a public health standpoint.

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REFERENCES

1) Aoki, S. 1996. Clinicopathological study on alveolar echinococcosis of the liver. *Kanzo* 37: 88-95 (in Japanese).

2) Blutke, A., Hamel, D., Huttner, M., Gehlen, H., Roming, T., Pfister, K. and Hermanns, W. 2010. Cystic echinococcosis due to *Echinococcus equines* in a horse from southern Germany. *J. Vet. Diagn. Invest.* 22: 458-462.

3) Furuya, K., Kawanaka, M., Yamano, K., Sato, N. and Honma, H. 2004. Laboratory Evaluation of commercial immunoblot assay kit for serodiagnosis of Echinococcus infections using sera from patients with alveolar hydatidosis in Hokkaido. *Kansenshougaku Zasshi.* 78: 320-326 (in Japanese)

4) Goto Y, Sato K, Yahagi K, Komatu O, Hoshina H, Abiko C, Yamasaki, H. and . Kawanaka M. 2010. Frequent isolation of *Echinococcus multilocularis* from the liver of racehorses slaughtered in Yamagata, Japan. *Jpn. J. Infect. Dis.* 63: 80-81

5) Kaji, Y., Taniyama,H., Matsukawa, K., Okada, H., Tsunoda, S., Tagami, M. and Akita, H. 1993. First incidence of multilocular echinococcosis in a race horse in Japan. *J. Vet.. Med. Sci.* 55: 869-870

6) Liance, M., Janin, V., Bresson-Hadni, S., Vuitton, D., Houin, R. and Piarroux, R. 2000. Immunodiagnosis of *Echinococcus* infections: Confirmatory testing and species differentiation by a new commercial western blot. *J. Clin. Microbiol.* 38: , 3718-3721

7) Miyauchi, T., Sakui, M., Ishige, M., Fukumoto, S., Ueda, A., Ito, M. and Ohbayashi, M. 1984. A case of multilocular echinococcosis in a horse. *Jpn. J. Vet. Res.* 32: , 171-173

8) WHO Informal Working Group on Echinococcosis. 1996. Guidelines for treatment of cystic and alveolar echinococcosis in humans. *Bull. World Health Org.* 74: 231–242

9) Yamasaki H, Nakao M, Nakaya K., Schantz, P. and Ito, A. 2008. Genetic analysis of *Echinococcus multilocularis* originating from a patient with alveolar echinococcosis occurring in Minnesota, in 1977. *Am. J. Trop. Med. Hyg.* 79: 245-247

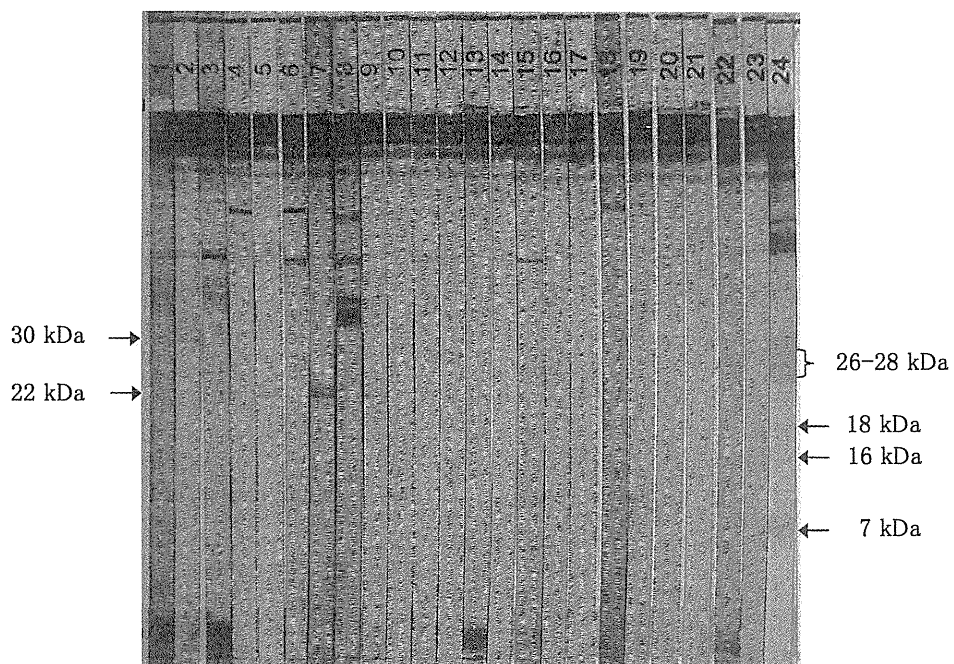

Fig. 1 Western blot profiles of sera from inspected horses.

Nos. 1–23: Serum samples from horses infected with *E. multilocularis*.

No. 24: Positive control (serum from human patient with alveolar echinococcosis).

Table 1. Western blot of 23 horses infected with *E. multilocularis*

No.	Sex	Age	No. of liver nodules	Nodule size (mm)	PAS positive cuticular layer	PCR-RFLP	Bands detected 30kDa	22kDa
1	F	5	1	5	−	+	+	
2	F	5	2	2, 25	+	−	+	
3	F	6	1	2	+	+	+	
4	F	6	2	2	+	−		
5	F	4	1	2	+	−		++
6	M	6	1	2	+	−		
7	F	6	4	1〜5	+	+		++
8	M	5	6	2〜7	+	−		
9	M	5	1	25	+	+		++
10	F	5	10	1〜3	−	+		+
11	M	5	4	2〜5	−	+		+
12	F	4	3	2〜5	−	+		+
13	F	6	3	5〜10	−	+		+
14	F	5	6	3〜10	−	+		
15	F	7	1	10	+	+	+	
16	F	6	12	2〜5	−	+		
17	F	7	3	5	−	+		
18	M	6	3	2〜6	−	+	+	
19	F	8	3	5〜10	+	+	+	
20	M	5	4	1	+	−		
21	F	9	> 20	1〜7	−	+		
22	F	6	2	1〜5	−	+		
23	F	6	11	1〜7	+	+		