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G．知的所有権の出願・登録状況

1. 特許取得

なし。

2. 実用新案登録

なし。

3. その他

なし。

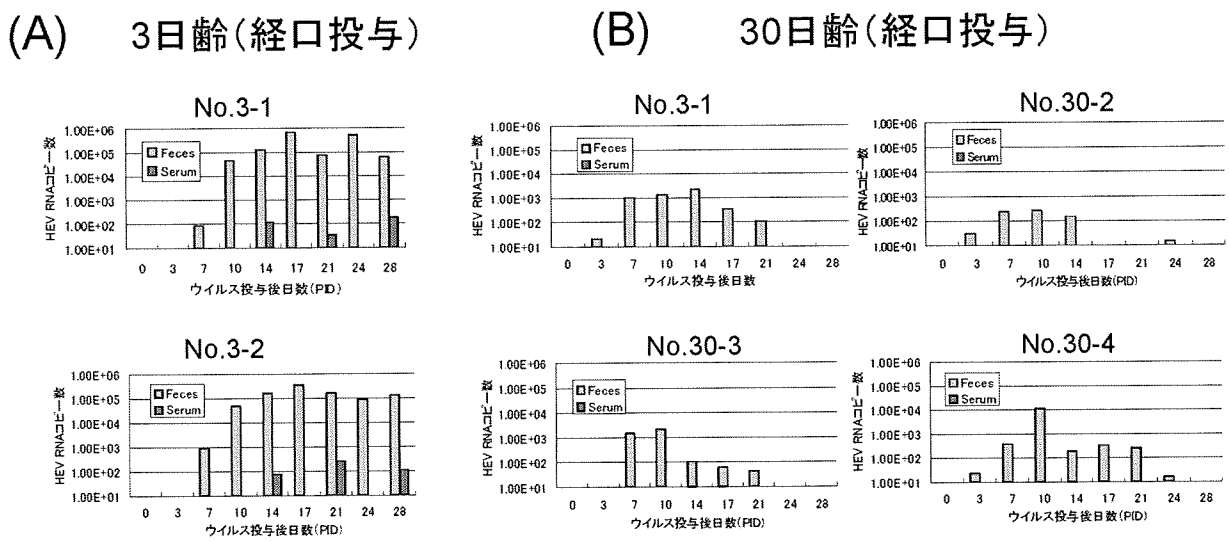

図１．HEV 経口接種豚［(A)3 日例；(B)30 日齢］での糞便ならびに血清中の HEV RNA 量

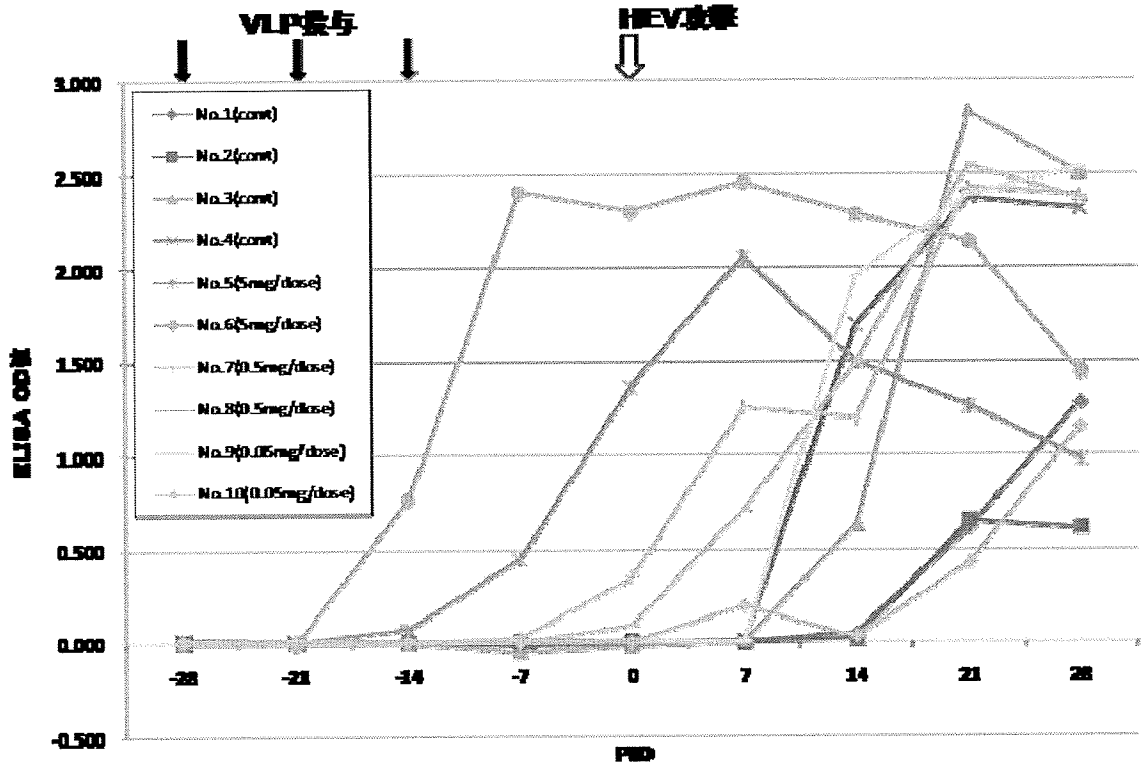

図２．血清中 HEV IgG 抗体の推移（VLP の経口投与）

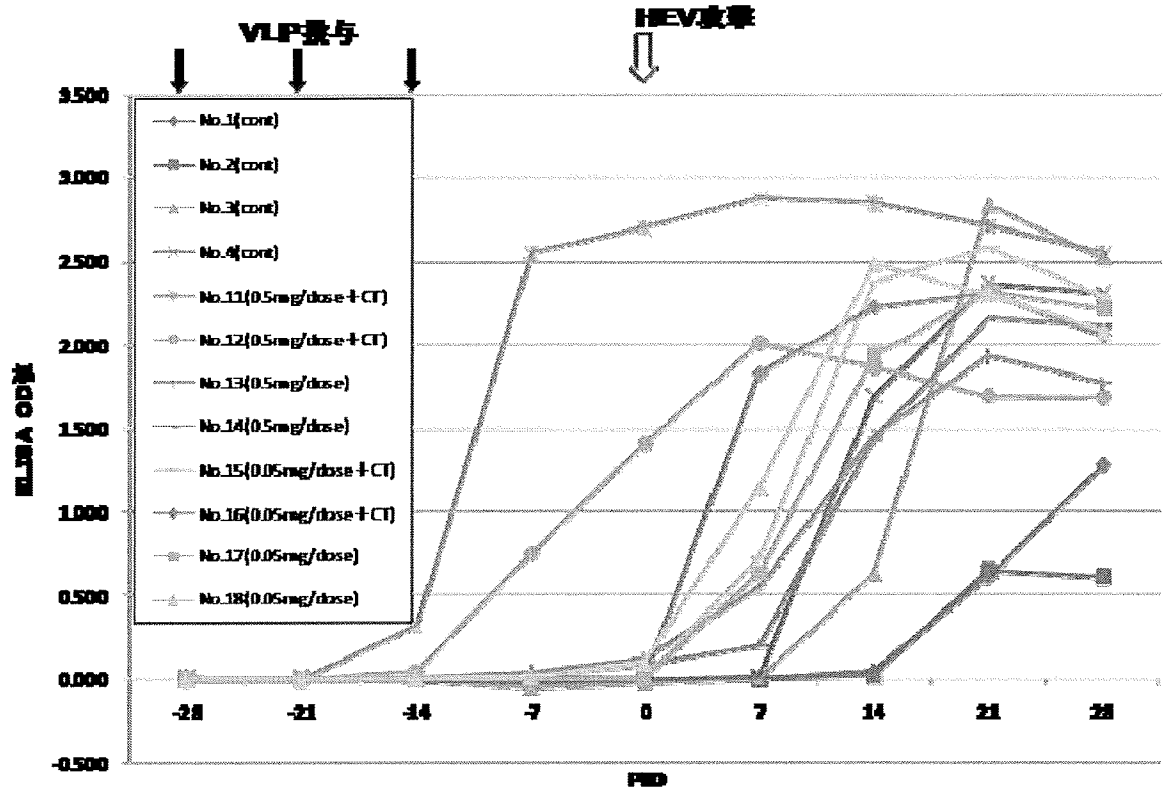

図３．血清中 HEV IgG 抗体の推移（VLP の経鼻投与）

表１．nested RT-PCR 法による糞便ならびに血清中 HEV RNA 検出成績

処 理		HEV 攻撃後日数											
		-21	-14	-7	0	3	7	10	14	17	21	24	28
対 照	糞便	0/2	0/2	0/	0/2	2/2	2/2	2/2	2/2	2/2	0/2	0/2	0/2
	血清	0/2	0/2	0/2	0/2	NT	1/2	N/T	0/2	N/T	0/2	N/T	0/2
VLP 経鼻1回	糞便	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
投与	血清	0/2	0/2	0/2	0/2	N/T	0/2	N/T	0/2	N/T	0/2	N/T	0/2
VLP 経鼻2回	糞便	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
投与	血清	0/2	0/2	0/2	0/2	0/2	0/2	N/T	0/2	N/T	0/2	N/T	0/2
VLP 経口1回	糞便	0/2	0/2	0/2	0/2	0/2	0/2	1/2	1/2	1/2	0/2	0/2	0/2
投与	血清	0/2	0/2	0/2	0/2	NT	0/2	N/T	1/2	N/T	0/2	N/T	0/2
VLP 経口2回	糞便	0/2	0/2	0/2	0/2	0/2	1/2	1/2	1/2	0/2	0/2	0/2	0/2
投与	血清	0/2	0/2	0/2	0/2	N/T	0/2	N/T	0/2	N/T	0/2	N/T	0/2

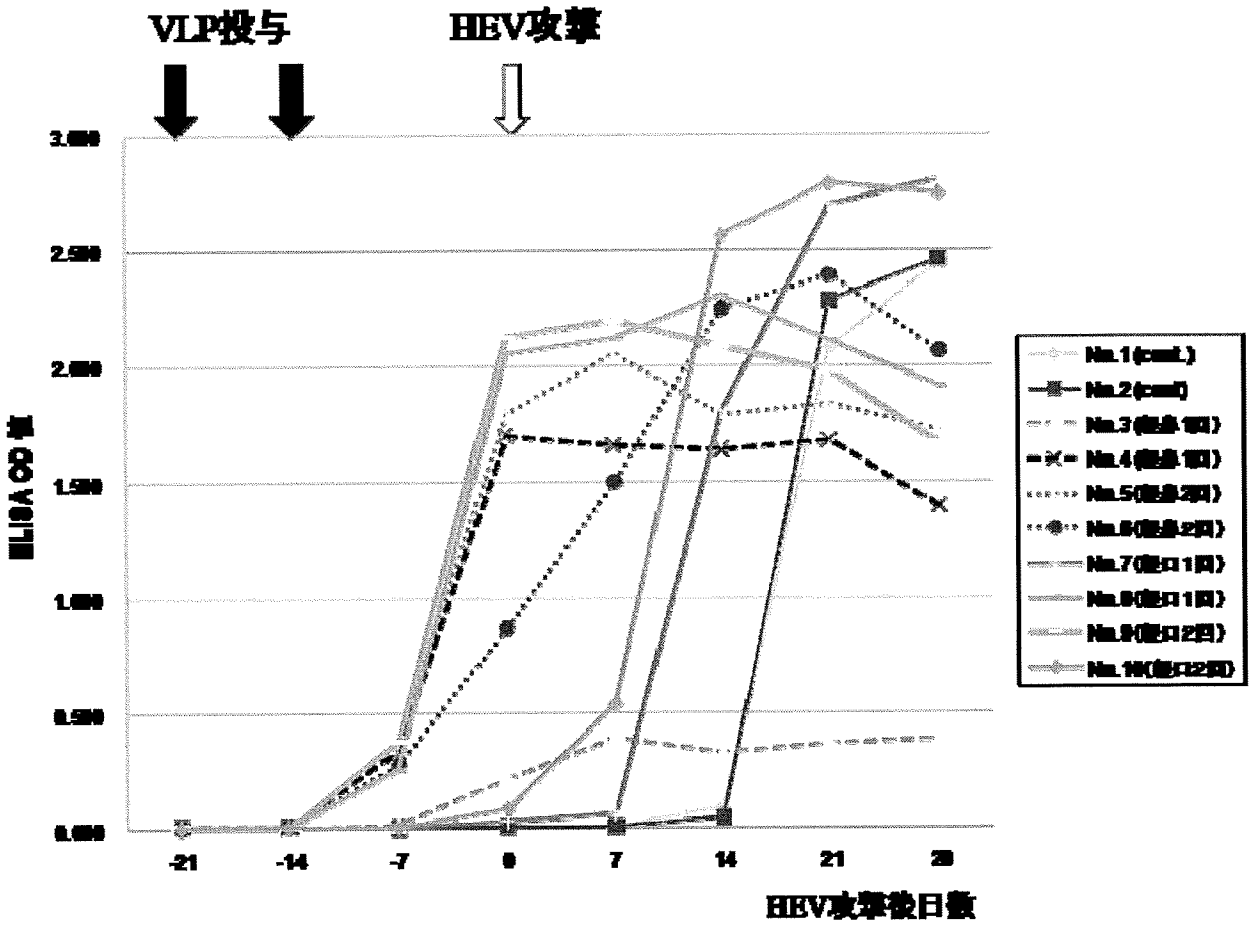

図４．血清中 HEV IgG 抗体の推移（VLP の少回数投与）

表 2. 平成 15 年度～平成 21 年度　大阪府南部～紀伊半島における HEV 保有野生動物

年度	動物種	狩猟地域	検体数	HEV遺伝子陽性	抗体陽性
H15年度	イノシシ	A	4	1	1
		B	5	–	–
	シカ	A	–	–	–
		B	2	–	–
H16年度	イノシシ	A	19	1	2
		B	2	–	–
	シカ	A	–	–	–
		B	1	–	–
H17年度	イノシシ	A	17	–	–
		B	9	–	–
		C	10	–	–
	シカ	A	–	–	–
		B	7	–	–
		C	7	–	–
H18年度	イノシシ	A	2	–	–
		B	18	–	–
		C	1	–	–
	シカ	A	–	–	–
		B	6	–	–
		C	2	–	–
H19年度	イノシシ	A	9	3	5
		B	–	–	–
		C	–	–	–
	シカ	A	–	–	–
		B	–	–	–
		C	–	–	–
H20年度	イノシシ	A	23	0	9
H21年度	イノシシ	A	30	0	0

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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無し

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Ⅲ. 研究成果の刊行物・別冊

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Brief Report

Prevalence of antibody to hepatitis E virus among wild sika deer, *Cervus nippon*, in Japan

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Summary

We examined 976 sika deer serum samples, 159 liver tissue samples and 88 stool samples collected from 16 prefectures in Japan, and performed ELISA and RT-PCR assays to detect antibodies to HEV and HEV RNA, respectively. Although 25 (2.6%) of 976 samples were positive for anti-HEV IgG, the antibody titers were very low. The OD values ranged between 0.018 and 0.486, forming a single distribution rather than a bimodal distribution, suggesting that the antibody detected in this study was not induced by HEV infection, or that deer have low sensitivity to HEV. HEV RNA was not detected in these samples, also suggesting that deer may not play a role as an HEV reservoir.

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Hepatitis E virus (HEV), the sole member of the genus *Hepevirus*, is the causative agent of type E

acute hepatitis in humans [3]. HEV does not have an envelope and is likely to have icosahedral symmetry. The genome is a positive-sense single-stranded polyadenylated RNA molecule, and the 5′ end is capped [11]. The genome of HEV contains three open reading frames, ORF1, ORF2, and ORF3. ORF1 encodes 1693 amino acids (aa) encompassing nonstructural proteins involved in viral replication. ORF2 encodes a 660-aa capsid protein. ORF3 encodes a 123- or 114-aa protein of unknown function [23, 28].

To date, at least four major genotypes of HEV have been identified by phylogenetic analyses. Genotype 1 (G1) HEV was isolated from Asia and Africa [16, 18], genotype 2 (G2) from Mexico [26], Namibia and Nigeria [2, 12], and genotypes 3 (G3) and 4 (G4) from the United States, European countries, China, Taiwan, Japan and Vietnam [4, 13, 17, 19, 27–29]. These viruses are thought to comprise a single serotype [16].

Transmission of human HEV occurs primarily by the fecal-oral route through contaminated water in developing countries [1, 5]. Since 1997, when the first animal strain of HEV was isolated from swine in the United States, there has been much indirect and direct evidence indicating that hepatitis E is a zoonosis and that humans appear to be at risk of infection with swine HEV by cross-species infection [13–15]. Recently, direct evidence of HEV transmission from wild boar (*Sus scrofa*) to humans was provided in Japan, suggesting that these animals are the main zoonotic reservoir of HEV in this country [9]. Indirect evidence of HEV transmission from swine to humans has also been accumulated [22, 30].

Because a case of HEV infection from sika deer meat was reported by Tei et al., sika deer have been considered a possible reservoir in Japan [24, 25]. However, there is only limited surveillance data of HEV infection in deer. In this study, we collected serum samples from wild deer and examined them for the presence of anti-HEV IgG by an antibody ELISA using recombinant virus-like particles (VLPs) as the antigen. We also attempted to detect HEV RNA in serum, stool, and liver samples from the wild deer by RT-PCR analysis.

Between 2003 and 2006, 866 serum samples were collected from wild deer captured in Hokkaido, Iwate, Tochigi, Chiba, Nagano, Aichi, Mie, Hyogo, Shimane, Hiroshima, Oita, Fukuoka, Kumamoto, Miyazaki, and Kagoshima prefectures, and 110 serum samples were collected in 1991–1993 from a deer farm, where the deer were introduced from the habitat at Miyagi prefecture (Fig. 1). In Hyogo Prefecture, an estimated age of 0–10 years was assigned by the tooth replacements and counting cementum annuli of the fist incisors [6]. A total of 88 stool samples were collected from deer captured in Hokkaido, Iwate, Tochigi, Chiba, Nagano, Mie, Hyogo, Hiroshima, Oita, Fukuoka, Kumamoto, Miyazaki, and Kagoshima from 2004 to 2006. They were resuspended in 10 mM phosphate-buffered saline (PBS) to prepare a 10% suspension, shaken at 4 °C for 1 h, and clarified by centrifugation at $10,000 \times g$ for 20 min. A total of 159 deer liver tissue were collected from Hyogo (50), Iwate (11) and Hokkaido (98) from 2003 to 2006. The tissue was resuspended in lysis buffer (Qiagen, Inc.) and homogenized. All of the specimens were stored at −20 °C until use.

Serum anti-HEV IgG antibody was detected by ELISA by the method described previously with slight modification [8]. Briefly, a flat-bottom 96-well polystyrene microplate (Immulon 2; Dynex Technologies, Inc. Chantilly, VA) was coated with the purified VLPs (1 μg/ml, 100 μl/well) derived from the G1 Myanmar strain [7]. The plates were incubated at 4 °C overnight. Unbound VLPs were removed, and the wells were washed twice with 10 mM phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), and then blocked at 37 °C for 1 h with 200 μl of 5% skim milk (Difco Laboratories, Detroit, MI) in PBS-T. After the plates were washed 4 times with PBS-T, deer serum (100 μl/well) was added in duplicate at a dilution of 1:200 in PBS-T containing 1% skim milk. The plates were incubated at 37 °C for 1 h and then washed 4 times as described above. The wells were incubated with 100 μl of peroxidase-conjugated rabbit anti-deer IgG (H + L) (1:1000 dilution) (KPL, Guildford, UK) in PBS-T containing 1% skim milk. The plates were incubated at 37 °C for 1 h and washed 4 times with PBS-T. Then, 100 μl of the substrate orthophenylenediamine (Sigma Chemical Co., St. Louis, MO) and H_2O_2 was added to each well. The plates were incubated in a dark room at room temperature for 30 min, then

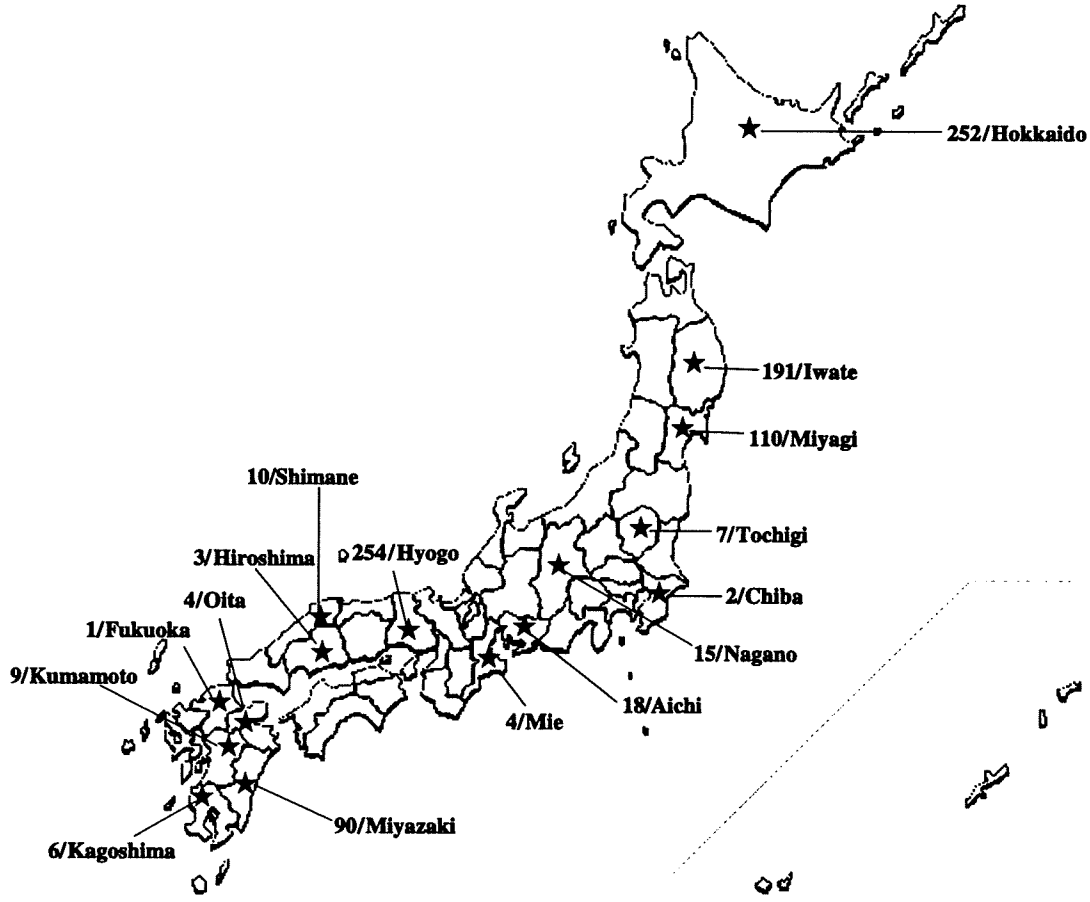

Fig. 1. Numbers and prefecture of captured wild sika deer

50 µl of 4N H_2SO_4 was added to each well. After the plates had stood at room temperature for 10 min, the absorbance at 492 nm was measured.

Anti-HEV IgG-positive serum was obtained from experimentally immunized captive sika deer that had been shown to be negative for HEV IgG by ELISA. The first and second immunizations were performed with purified VLPs (100 µg) in Freund's complete adjuvant by intramuscular injection at intervals of 2 week. After 2 weeks, the deer received booster injections of the same amount of VLPs in Freund's incomplete adjuvant. The deer was bled one week after the last booster injection. Pre-immunization serum was collected before administration and used as the negative control. Anti-HEV IgG-positive serum and pre-inoculation serum were stored at −30 °C. The anti-HEV IgG titer of the positive serum was 1:3,276,800.

Deer serum samples were tested for anti-HEV IgG at a dilution of 1:200 by ELISA. The distribution of the optical density (OD) values is shown in Fig. 2. The OD values of anti-HEV IgG ranged from 0.018 to 0.486 with the highest antibody titers being 1:400, and formed a single distribution. To determine whether the IgG antibody detected in deer sera was specific for HEV, the positive control serum and negative control serum, and the sera whose OD values were higher than 0.150 were selected and examined by Western blot assay. Approximately 1 µg of the VLPs derived from G1, G3, and G4 HEV was separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane.

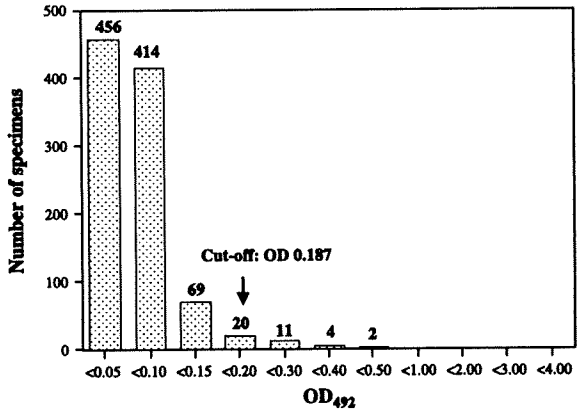

Fig. 2. Distribution of OD values of IgG antibodies. Serum samples from 976 deer were tested by ELISA. The arrows indicate the cutoff values

The membrane was then blocked with 5% skim milk in 50 mM Tris–HCl (pH 7.4) and 150 mM NaCl, and incubated with deer serum (1:200 dilution). Detection of deer IgG antibody was achieved by using phosphatase-labeled rabbit anti-deer IgG (H + L) (1:1000 dilution) (KPL, Gaithersburg, MD). Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate P-toluidine were used as coloring agents (Bio-Rad Laboratories, Hercules, CA). As shown in Fig. 3, strong bands with a molecular weight of 53 k corresponding to the G1, G3 and G4 VLPs were detected with positive control sera. Weak bands were detected with Hyogo 0588, Hyogo 0409, and Miyagi 1, whose OD values were 0.486, 0.358, and 0.287, respectively, whereas no band was detected with Iwate 137, D0505, or the negative control serum, which had low OD values of 0.205, 0.152, and 0.051. These results indicated that the anti-HEV IgG detected in deer serum by ELISA was specific for HEV.

After eliminating 17 serum samples found to be positive by Western blot assay, 959 deer serum samples were used to evaluate the cutoff value of IgG. The OD values of these sera were between 0.018 and 0.248, and the mean value was 0.058 with a standard deviation (SD) of 0.043. Therefore, the cutoff value, the mean value + 3SD, was calculated to be 0.187 (Fig. 2). When this value was employed, the prevalence of anti-HEV IgG appeared to be

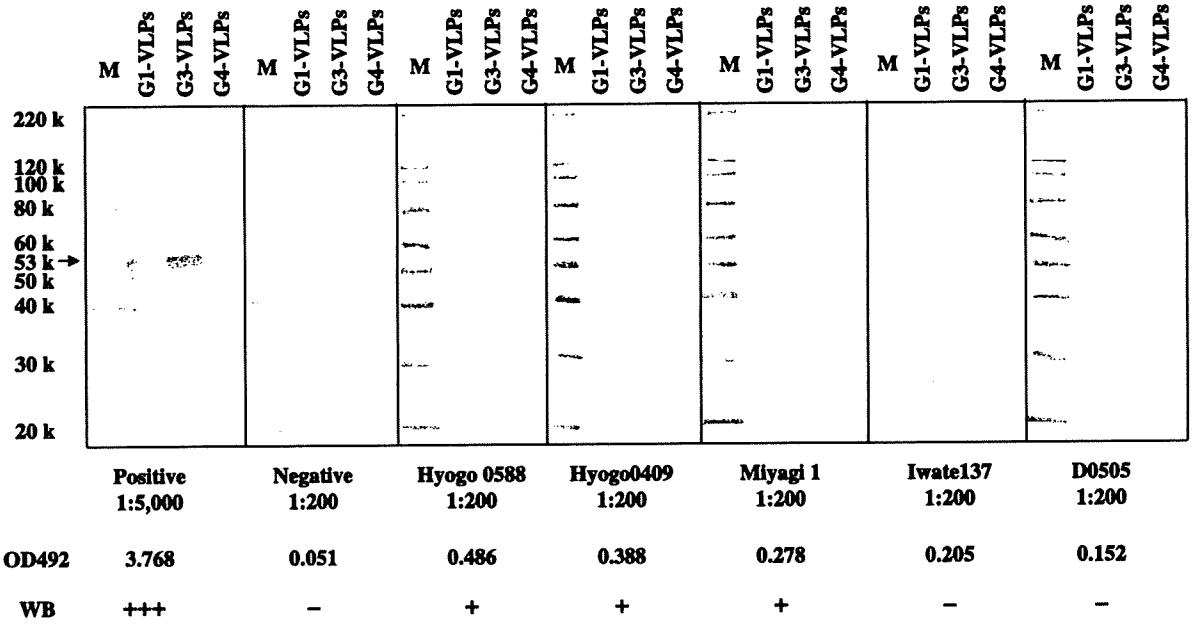

Fig. 3. Specificity of the IgG antibody determined by Western blot assay. The G1, G3, and G4 VLPs were used as the antigens, and 7 deer sera with different OD values were evaluated. The results of the Western blot assay are indicated as +++ (strong band), + (weak band), or – (no band). *M* Molecular weight marker