

Evaluation of two enzyme-linked immunosorbent assays for the detection of antibodies against equine arteritis virus

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In order to establish an efficient system for serological diagnosis of equine viral arteritis in Japan, we compared enzyme-linked immunosorbent assays (ELISAs) provided by two manufacturers (Nisseiken Co., Ltd., Tokyo, Japan, and VMRD Inc., Pullman, WA, U.S.A.) by testing a series of horse sera. The results revealed that 159 of 160 virus-neutralizing (VN) antibody-positive serum samples were positive in both the Nisseiken-ELISA and VMRD-ELISA. Of the VN-negative sera (n=157), 134 and 154 samples were negative in the Nisseiken-ELISA and VMRD-ELISA, respectively. Sensitivity was 99.4% for both the Nisseiken-ELISA and VMRD-ELISA. The specificity of the VMRD-ELISA (98.1%) was significantly higher than that of the Nisseiken-ELISA (85.4%, $P<0.05$). The diagnostic performance of the VMRD-ELISA was superior to that of the Nisseiken-ELISA because of this greater specificity.

Key words: ELISA, EVA, sensitivity, specificity

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Equine arteritis virus (EAV) is the causative agent of equine viral arteritis (EVA), an acute contagious disease of horses with a range of signs including respiratory and reproductive pathology [1, 13]. Despite the virus's worldwide distribution, no EVA outbreaks have occurred in Japan. However, five cases of EAV infection have been detected during quarantine inspections of horses imported into Japan over the period 2004 to 2011 (Annual Report of the Animal Quarantine Service, Ministry of Agriculture, Forestry and Fisheries). Appropriate quarantine measures are therefore needed to ensure that domestic horse populations are protected from EVA.

The virus-neutralization (VN) test is highly sensitive and specific for the detection of EAV-infected horses and is prescribed by the World Organization for Animal Health (OIE) for international trade purposes [11]. However, the VN test is labor-intensive and time-consuming, and it is sometimes subject to interference by cytotoxicity caused by tested horse sera containing anticellular antibodies to

rabbit kidney-13 (RK-13) cells [9]. Injection of horses with inactivated equine herpesvirus vaccine (Duvaxyn EHV 1,4, Zoetis, Parsippany, NJ, U.S.A.) that contains antigens grown on continuous tissue culture cell lines was most likely the cause of such cytotoxicity [9]. To overcome these disadvantages, many research groups have developed enzyme-linked immunosorbent assays (ELISAs) for EAV [2–7, 9, 10, 12, 15]. In Japan, although there is no commercial ELISA kit for EVA that is licensed under the Pharmaceutical Affairs Act, a chimeric antigen composed of EAV gp5 and N proteins was developed by Nisseiken Co., Ltd. (Tokyo, Japan) as a coating antigen for the ELISA. In this conventional ELISA (Nisseiken-ELISA), horse antibodies to multiple epitopes on the chimeric antigen are detected by a secondary antibody against horse IgG. It had 98.3 to 100.0% sensitivity and 74.2 to 84.6% specificity to the VN test, and its use has been authorized by Japan's Council for Equine Disease Prevention (CEDP) (Report of the 3rd Expert Meeting on Diagnosis of Equine Viral Arteritis, 2000). Since its introduction it has been used on imported horses as a screening test and for serological surveillance for EVA in Japan. A research group in Turkey confirmed the usefulness of the Nisseiken-ELISA in a serosurveillance study [14]. However, a high frequency of false positives (74.2–84.6% specificity as described above) resulted in a requirement for confirmation by VN testing. Thus, an alternative ELISA kit with higher specificity was required to establish a more

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efficient system for the diagnosis of EVA in Japan.

Currently, several commercial ELISA kits for EVA are available worldwide, namely, Equine Arteritis Virus cELISA (VMRD Inc., Pullman, WA, U.S.A.), ID Screen Equine Viral Arteritis Indirect (IDvet Genetics, Grabels, France) [7], and INgezim Arteritis ELISA (Ingenasa, Madrid, Spain) [5]. However, the last two kits are not available in Japan, and only the one from VMRD Inc. (VMRD-ELISA) can be purchased for research use through a distributor at this time. The VMRD-ELISA is a competitive ELISA detecting serum antibodies to a single epitope of EAV gp5 which is recognized by a monoclonal antibody 17B7 [4]. Purified EAV antigens are coated on the wells, and if the tested sera contain antibodies to the epitope, the binding of a monoclonal antibody 17B7 to the gp5 antigen is inhibited. The horse antibody level to EAV is expressed as an inhibition index. Pfahl *et al.* [12] reported that, because of its high sensitivity (98.9 to 99.6%) and specificity (98.3 to 98.7%), the VMRD-ELISA is the most promising ELISA kit as an alternative to the VN test. Although the diagnostic performances of the Nisseiken-ELISA and VMRD-ELISA have been evaluated independently as described above, the sources of sera evaluated can markedly influence the results. Thus, our current study aimed to compare the two ELISA kits directly by using the same series of serum samples. Here, we examined VN-positive/-negative horse sera by using the two different ELISAs for EVA to evaluate their usefulness in regard to sensitivity and specificity.

The sources of the seropositive samples in the VN test were as follows: 1) naturally infected horses (n=109) that were imported from Canada in 2008 and tested positive for EAV at a quarantine facility in Hyogo Prefecture, Japan; 2) experimentally infected horses (n=28) that were inoculated with EAV (84KY-A1 strain [n=14], Bucyrus strain [n=10], Bibuna strain [n=1], Red Mile strain [n=1], Vienna strain [n=1], or Wroclaw strain [n=1]) at the Equine Research Institute of the Japan Racing Association from 1977 to 1997; and 3) EAV-vaccinated stallions (n=23, the information for the vaccine products was not available) kept in Hokkaido Prefecture, Japan, from 2013 to 2016. The sources of the seronegative samples in the VN test were as follows: 1) unvaccinated stallions (n=97) located in Hokkaido Prefecture from 2013 to 2015 and 2) unvaccinated racehorses (n=60) kept at a training facility in Ibaraki Prefecture, Japan, in 2015. The serum samples from naturally infected horses were kindly provided by the Animal Quarantine Service, Ministry of Agriculture, Forestry and Fisheries. The serum samples from stallions were kindly provided by the Hokkaido Hidaka Livestock Hygiene Service Center. Experimental infection with EAV was approved by the Animal Care Committee of the Equine Research Institute of the Japan Racing Association. VN testing was performed in

accordance with the OIE's *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2013*, using the modified Bucyrus CVL strain of EAV and CVL-RK-13 cells [11].

The Nisseiken-ELISA is a recombinant antigen-based conventional ELISA, and it was performed as described previously [14]. Briefly, the antigen was coated onto flat-bottomed 96-well plates (Nunc MaxiSorp, Thermo Scientific, Roskilde, Denmark), which were then incubated at 4°C overnight. In each subsequent step, the plates were incubated at room temperature for 1 hr. They were washed between the steps with phosphate-buffered saline containing 0.05% (w/v) Tween 20 (PBST). The plates were first treated with a blocking solution supplemented with 1% (w/v) gelatin, subsequently treated with the sera (diluted 1/100 with diluent) in triplicate wells, and finally treated with a horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1/100 with diluent). After the final washing, color development was performed with SIGMAFAST OPD (Sigma Aldrich Inc., St. Louis, MO, U.S.A.), and the reaction was stopped by adding 5N sulfuric acid. Optical density (OD) at a wavelength of 492 nm was measured, and if the mean OD of triplicate wells was higher than a cutoff point calculated from standard samples, the result was regarded as positive. The sample/cutoff ratio was calculated by dividing the OD of the sample by that of the cutoff point. The reagents described above were provided by Nisseiken Co., Ltd., with the exception of the PBST, gelatin, SIGMAFAST OPD, and sulfuric acid.

The VMRD-ELISA is a competitive ELISA that detects antibodies to a single epitope of EAV antigen, and it was performed in accordance with the manufacturer's instructions. Plates pre-coated with antigen extract of EAV [12] were provided by the manufacturer. They were inoculated with undiluted sera in duplicate wells and then incubated at room temperature for 2 hr. After being washed with a washing buffer, the plates were treated with a primary antibody (monoclonal antibody 17B7 [12], diluted 1/10 with a diluent) for 30 min, followed by treatment with an HRP-conjugated secondary antibody (1/100 with the diluent) for 30 min. After a final washing, color development was performed by using the solutions provided by the manufacturer. OD at a wavelength of 450 nm was measured, and the inhibition index was calculated from the mean OD values of tested sera and those of negative control serum. If the inhibition index was 35% or higher, it was regarded as positive. All reagents described above were provided by VMRD Inc.

The statistical significance of the sensitivities and specificities of the ELISAs was analyzed by using a χ^2 test. A level of $P < 0.05$ (Bonferroni corrected) was considered significant.

The results of the ELISAs and their agreement/disagree-

ment with the VN test are summarized in Table 1. Detailed results for the false-negative and false-positive samples are summarized in Table 2. Among the VN-positive horse sera (n=160), 159 were positive in both the Nisseiken-ELISA and VMRD-ELISA. One sample from a vaccinated stallion (horse #1 in Table 2) yielded false-negative results in both ELISAs, although it had a high VN titer of 1:512. Among

the VN-negative sera (n=157), 154 were negative in the VMRD-ELISA, with the remaining three samples (horses #25–27) being false-positive. In contrast, the Nisseiken-ELISA determined 134 samples to be negative and provided false-positive results for the remaining 23 samples (from 21 stallions [horses #2–22] and two racehorses [horses #23–24]). The false-positive samples in each ELISA were

Table 1. Concordance between VN test, Nisseiken-ELISA, and VMRD-ELISA results

		VN test							Total
		Positive				Negative			
		Naturally infected horses	Experimentally infected horses	Vaccinated stallions	Subtotal	Unvaccinated stallions	Unvaccinated racehorses	Subtotal	
Nisseiken-ELISA	Positive	109	28	22	159	21	2	23	182
	Negative	0	0	1	1	76	58	134	135
	Total	109	28	23	160	97	60	157	317
VMRD-ELISA	Positive	109	28	22	159	1 ^b	2 ^b	3	162
	Negative	0	0	1 ^a	1	96	58	154	155
	Total	109	28	23	160	97	60	157	317

^aThe same sample as the false negative in the Nisseiken-ELISA. ^bDifferent samples from the false positives in the Nisseiken-ELISA.

Table 2. Results of the VN test and two ELISAs for the samples with false-negative or false-positive results

Category	Horse #	VN titer	Nisseiken-ELISA (sample/cutoff ratio ^a)	VMRD-ELISA (inhibition index ^b)
False negative in both ELISAs	1	≥1:512	– (0.45)	– (6.8)
False positive in the Nisseiken-ELISA	2	<1:4	+ (1.14)	– (12.0)
	3	<1:4	+ (1.05)	– (5.3)
	4	<1:4	+ (1.17)	– (9.9)
	5	<1:4	+ (1.26)	– (–6.5)
	6	<1:4	+ (1.12)	– (13.4)
	7	<1:4	+ (1.62)	– (–29.7)
	8	<1:4	+ (1.67)	– (12.7)
	9	<1:4	+ (1.32)	– (4.6)
	10	<1:4	+ (1.96)	– (–22.4)
	11	<1:4	+ (3.95)	– (4.3)
	12	<1:4	+ (1.00)	– (7.0)
	13	<1:4	+ (2.27)	– (–1.7)
	14	<1:4	+ (1.53)	– (25.6)
	15	<1:4	+ (4.38)	– (9.9)
	16	<1:4	+ (2.36)	– (–17.9)
	17	<1:4	+ (1.01)	– (–3.7)
	18	<1:4	+ (1.00)	– (16.6)
	19	<1:4	+ (1.16)	– (5.9)
	20	<1:4	+ (1.04)	– (11.6)
	21	<1:4	+ (1.25)	– (–30.7)
	22	<1:4	+ (1.09)	– (–7.6)
	23	<1:4	+ (1.22)	– (–3.1)
	24	<1:4	+ (4.01)	– (14.4)
	False positive in the VMRD-ELISA	25	<1:4	– (0.39)
26		<1:4	– (0.34)	+ (39.1)
27		<1:4	– (0.42)	+ (39.8)

^aSample/cutoff ratio=sample OD/cutoff OD. A ratio ≥1.00 was regarded as positive. ^bInhibition index=100[1–(sample OD/negative control OD)]. An index ≥35.0 was regarded as positive.

independent; that is, they were not the same set of samples.

For the samples with true-positive results, the sample/cutoff ratios of the Nisseiken-ELISA and the inhibition indexes of the VMRD-ELISA are shown in Table 3. In the Nisseiken-ELISA, the sample/cutoff ratios were in the range of 1.83–8.67 (5.02, mean) in the naturally-infected horses, 2.18–10.78 (6.80, mean) in the experimentally-infected horses, and 1.27–5.53 (2.84, mean) in the vaccinated stallions. For the 23 false-positive samples in the Nisseiken-ELISA (horses #2–24), the sample/cutoff ratios were in the range of 1.00–4.22 (1.72, mean), and therefore, some samples in the infected/vaccinated horses and fell in the same range as the false-positive samples. As a result, the Nisseiken-ELISA yielded a high frequency of false positive results. In the VMRD-ELISA, the inhibition indexes were in the range of 66.1–89.2% (81.0%, mean) in the naturally-infected horses, 59.7–90.2% (78.6%, mean) in the experimentally-infected horses, and 72.2–85.3% (78.1%, mean) in the vaccinated stallions. The inhibition indexes of the three false-positive horses (horses #25–27) ranged from 39.1 to 46.4%, which was distant from the indexes of infected/vaccinated horses. Such a distant distribution of indexes in infected/vaccinated horses compared with the false-positive horses led us to consider a possible employment of a higher cutoff point to reduce the number of false-positive results. However, in a previous study by Pfahl *et al.*, a much wider range of inhibition indexes (35.8–100.0%) was shown among VN antibody-positive horses [12]. Thus, the current cutoff point for the inhibition index (35%) is likely essential to ensure sufficient specificity of the assay.

The sensitivities of the two ELISAs were almost the same: 99.4% (95% confidence interval [CI], 97.0 to 99.9%) for the Nisseiken-ELISA and 99.4% (95% CI, 97.5 to 99.9%) for the VMRD-ELISA (Table 4). In contrast, the specificity

of the VMRD-ELISA (98.1% [95% CI, 96.2 to 98.6%]) was significantly higher than that of the Nisseiken-ELISA (85.4% [95% CI, 82.9 to 85.9%], $P < 0.05$) (Table 4). The high frequency of false positive results in the Nisseiken-ELISA was consistent with a previous report by the CEDP indicating that the specificity of this test was 74.2 to 84.6% (Report of the 3rd Expert Meeting on Diagnosis of Equine Viral Arteritis, 2000). In the present study, the majority of false-positive samples in the Nisseiken-ELISA (21 out of 23 sera) were from the unvaccinated stallions, and this might have been associated with the ages of the horses: stallion populations are generally older than active racehorses. Stallions are also more likely to have been exposed to various pathogens and therefore have a variety of antibodies. In the Nisseiken-ELISA, the horse antibodies against all epitopes on the chimeric antigen consisting of gp5 and N proteins are detected, which is likely to be beneficial in regard to its sensitivity. However, the high frequency of false-positive results in this ELISA suggests the presence of epitopes which may bind nonspecifically to the antibodies against other pathogens or to unknown components in horse sera. In contrast, the significantly higher specificity in the VMRD-ELISA might be attributed to the use of a single epitope for antibody detection, because use of a monoclonal antibody, whether direct or indirect, generally enhances the specificity of the ELISA [8].

In this study, we did not have the chance to evaluate ELISA kits for EVA other than the VMRD-ELISA. A previous report showed that ID Screen Equine Viral Arteritis Indirect (IDvet Genetics), which is mainly used in France, had 98.0% specificity, although information for its sensitivity was not provided [7]. The INgezim Arteritis ELISA (Ingenasa) has 98.8% sensitivity and 96.6% specificity according to its data sheet. However, Duthie *et al.* [5]

Table 3. The sample/cutoff ratio in the Nisseiken-ELISA and the inhibition index in the VMRD-ELISA for the samples with true-positive results

		Naturally infected horses	Experimentally infected horses	Vaccinated stallions
Nisseiken-ELISA sample/cutoff ratio ^a	Mean	5.02	6.80	2.84
	Range	1.83–8.67	2.18–10.78	1.27–5.53
VMRD-ELISA inhibition index ^b	Mean	81.0	78.6	78.1
	Range	66.1–89.2	59.7–90.2	72.2–85.3

^aSample/cutoff ratio=sample OD/cutoff OD. A ratio ≥ 1.00 was regarded as positive. ^bInhibition index= $100[1 - (\text{sample OD}/\text{negative control OD})]$. An index ≥ 35.0 was regarded as positive.

Table 4. Sensitivities and specificities of the Nisseiken-ELISA and VMRD-ELISA

	Sensitivity (95% CI)	Specificity (95% CI)
Nisseiken-ELISA	99.4 (97.0–99.9)	85.4 (82.9–85.9)
VMRD-ELISA	99.4 (97.5–99.9)	98.1 (96.2–98.6)

reported that the same kit showed 96.3% sensitivity and 26.3% specificity. Therefore, the diagnostic performances of each ELISA kit are not definitive, and in some cases, they have not been validated sufficiently. In contrast, the VMRD-ELISA has been well validated using sufficient numbers of both experimental (n=1,235) and field samples (n=1,851) and has shown excellent performance with high sensitivity (98.9 to 99.6%) and specificity (98.3 to 98.7%) [12]. In this study, direct comparison of the two ELISA kits using the same series of sera clearly showed the usefulness of the VMRD-ELISA as a replacement for the Nisseiken-ELISA for an efficient diagnosis of EVA in Japan.

In conclusion, the diagnostic performance of the VMRD-ELISA was superior to that of the Nisseiken-ELISA in terms of specificity. The potential reduction of false-positive samples requiring confirmation by VN testing will be highly advantageous, particularly in terms of quarantine surveillance for EVA in Japan.

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