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Validation of an Enzyme-Linked Immunosorbent Assay Kit Using Herpesvirus Papio 2 (HVP2) Antigen for Detection of Herpesvirus Simiae (B virus) Infection in Rhesus Monkeys

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Serologic testing for antibody to monkey B virus (BV) in macaque sera is problematic due to the biohazardous nature of BV antigens. Herpesvirus papio 2 (HVP2), a herpesvirus of baboons, is nonpathogenic to humans and is genetically and antigenically more closely related to BV than is human herpes simplex virus 1. This paper describes the results of our in-house laboratory that compared a BV antigen-based enzyme-linked immunosorbent assay(ELISA) by commercial testing laboratory and an HVP2-based ELISA in our laboratory by using 447 sera from 290 rhesus monkeys. The HVP2-based ELISA identified as positive 99.11% of the sera identified as BV-positive by the BV ELISA. The BV antigen-based ELISA identified as positive 98.21% of the sera identified as BV-positive by the HVP2-based ELISA. The HVP2 ELISA also identified two BV-negative and six BV-equivocal sera as positive. Both ELISAs identified the same 85 negative and three equivocal samples as negative and equivocal, respectively. The high degree of correlation (weighted kappa coefficient, 0.94) between the two tests indicates that the HVP2 ELISA is a sensitive and reliable assay for in-house testing of the BV status of rhesus monkeys.

Herpesvirus simiae (cercopithecine herpesvirus 1, monkey B virus) of old world monkeys is highly pathogenic for humans. Human infection with B virus (BV) occurs primarily as a result of bites or scratches by macaque monkeys kept as laboratory animals (9, 13-15). It is necessary to establish a production resource of specific-pathogen-free (SPF) rhesus monkeys free of BV to use in biomedical and especially AIDS-related research. Towards this end, a strategy of test-and-cull has been used widely for developing SPF breeding colonies from conventional BV-infected animals (4, 11, 20, 27). Healthy, productive SPF breeding colonies will help both long-range national research and testing requirements for newly developed biologics (6, 22, 23). In this context, better serological analyses to identify BV infection in humans and particularly nonhuman primates are required. At present, serological analyses for BV include enzyme-linked immunosorbent assays (ELISA) and Western blotting.

BV is an alpha-herpesvirus that is endemic in macaques (5, 12, 14, 18, 24). In its natural hosts, BV can produce localized self-limiting herpetic lesions and establish latency in sensory nerve ganglia. In humans, BV can cause lethal encephalitis and infects an average of one animal handler annually in the United States. The mortality is 60 to 70% in the absence of treatment and perhaps as low as 20% with appropriate treatment (14). The National Center for Research Resources, a component of the National Institutes of Health, has funded programs to develop SPF colonies of rhesus macaques at U.S. primate breeding facilities (4). In U.S. primate facilities, the seroprevalence of BV has been reported to be approximately 80 to 100% in adult animals (12, 25, 26). Sato and colleagues (19) reported the prevalence of BV antibodies in nonhuman primates reared at a national university in Japan. An antibody prevalence of 40% (384 of 961) was demonstrated in Japanese monkeys (*Macaca fuscata*). These data indicate that nonhuman primates reared in animal facilities do pose an occupational health danger for persons working with or around macaques.

At present, serological testing for BV in macaques housed at the Wisconsin National Primate Research Center (WNPRC) is being performed by a commercial testing laboratory, and confirmatory testing is done by the National Resource B Virus Reference Laboratory (1). Serologic testing for antibodies to BV in macaque sera is problematic due to the biohazards associated with the preparation and use of BV antigens. For this reason, the human herpes simplex virus 1 (HSV1) commonly has been used as an alternative test antigen for detection of BV antibodies at WNPRC. However, herpesvirus papio 2 (HVP2), an alpha-herpesvirus of baboons, is related genetically and antigenically much more closely to BV than is HSV1 (3, 8). The aim of the present investigation was to establish a standardized BV screening test system that can be performed in-house. Sensitive and reliable screening tests that can be performed in-house generally are more convenient and confidential than is testing at commercial testing laboratories and so can help to establish SPF colonies and identify BV infection in monkeys to be assigned to research

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protocols at primate centers.

In this study, we evaluated an HVP2 antigen-based ELISA kit in screening for BV infection in rhesus macaques at the WNPRC. The sensitivity and specificity of the assay were compared with those of the BV antigen-based ELISA used by commercial testing laboratories. The HVP2-based ELISA kit, which we are developing, has been distributed free of charge for several years under the condition that test data and a corresponding number of 0.1-ml serum samples are sent to us. We have been improving the sensitivity and shelf life of the ELISA. The kit is prepared by using HVP2 antigen that was dried after it had been inactivated with a detergent (we refer to it as a "dry antigen"), and its shelf life is about 6 months. Therefore, our ELISA is an excellent candidate for a ready-to-use kit for in-house testing. We hope that the convenience of this kit will become widely known.

Materials and Methods

Sera. A total of 447 sera collected from 290 rhesus monkeys maintained at the WNPRC were used in this study. Among them, 393 serum samples were collected mainly in February and August 2000 during regular medical examination of 289 rhesus monkeys. Of these samples, 104 were serial samples (collected in February and August from the same monkey). Another 54 serum samples were collected in 2001 from 54 rhesus monkeys and of these, 53 were from monkeys whose serum samples had also been collected in 2000. All sera were stored at –80°C until use. The WNPRC is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, and the institutional animal care and use committee of WNPRC approved all procedures for the handling and treatment of the animals.

Viruses and cells. HVP2 strain OU1-76 originally was isolated from oral ulcerations of an infant baboon at the Oklahoma University Health Sciences Center, Oklahoma City (7). HVP2 was propagated in Vero cells (10) under Biosafety Level 2 (BSL2) conditions.

BV ELISA. The BV antibody ELISA was performed by a commercial testing laboratory using authentic BV antigen. In this assay, sera were classified as positive or negative according to the optical density (OD) values from the ELISA. Sera giving OD values of 0.00 to 0.16, 0.17 to 0.39, or ≥ 0.40 were defined as negative, equivocal, and positive, respectively, for the presence of BV.

HVP2 ELISA. Substrate antigens for HVP2 ELISA were prepared in Vero cells as described previously (17). For ELISA, 96-well round-bottom plates (Immulon II, Dynatech Laboratories, Chantilly, Va.) were coated with HVP2 and control antigens prepared at Nagasaki University as described previously (17), dried, and transferred to the WNPRC for use. The ELISA plates were stored at 4°C until use (within 6 months of preparation).

At the WNPRC, sera were assayed using the HVP2 ELISA to detect rhesus monkeys with BV IgG antibody as described previously (17). All sera were tested using both HVP2-infected and uninfected cell antigens. The OD of each well was measured using an ELISA plate reader (Dynatech MR5000, Dynatech Laboratories, Inc.) set at a wavelength of 490 nm. The OD values of duplicate wells of HVP2 antigen and controls were averaged for each test sample as described (17). The BV antigen ELISA was designated as the reference test for comparing the performance of the alternative HVP2 ELISA. Sera that were known to be negative for BV antibody (–), to have low levels of anti-BV IgG (+), or high levels of anti-BV IgG (+++) were used as controls.

ELISA testing was conducted in the conventional manner as described previously (17). Test and control (the three BV antibody controls described earlier as well as HVP2 and Vero antigens) sera were diluted to 1:200 with antibody diluent before use; 50-μl aliquots of each were placed in each of two wells (17). The plates were incubated for 1 h at room temperature covered with the film to prevent drying. Wells were washed once with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS/T), primary antibody diluted in PBS containing 0.01% gelatin (PBS/G) was added (50 μl/well), and the plates were incubated at room temperature for 1 h. Wells were washed five times with PBS/T, biotinylated anti-human IgG (Vector Laboratories, Burlingame, Calif.) diluted 1:5000 in PBS/G was added (50 μl/well), and plates were incubated for 1 h at room temperature. After plates were washed five times with PBS/T, a complex of avidin–biotinylated peroxidase (Vector Laboratories) that was prepared in PBS/T according to the manufacturer's instructions and diluted 1:32 was added (50 μl/well), and plates were incubated at room temperature for 1 h. The wells were washed five times with PBS/T, substrate solution (200 μg/ml o-phenylenediamine, 0.003% H_2O_2 in 0.1M sodium citrate, pH 5.0) was added (100 μl/well), and plates were incubated in the dark at room temperature for 12 to 15 min. The reaction was stopped by addition of 2 M H_2SO_4 (50 μl/well), and the OD at 490 nm was read on an ELISA plate reader as described earlier. All samples were tested in duplicate, and the mean was used for all calculations. The criteria for classification of results were as previously described (17).

Statistical analysis. A weighted kappa coefficient was computed to evaluate the agreement of BV antibody-positive percentages between the two ELISA tests. The Wilcoxon rank-sum test was used to compare the distribution of B virus antibody-positive monkeys between sexes. A P value of < 0.05 was regarded as statistically significant. All computations were completed using SAS software release 8.2 (SAS Institute Inc., Cary, N.C.).

Results

Comparison of the two tests. Testing was done on 447 sera collected from 290 rhesus monkeys at the WNPRC between February and April 2000. The 447 samples consisted of 393 serum samples from 289 monkeys that had been collected for medical examinations in 2000 and 54 samples collected in 2001. Among the 289 monkeys that were bled in 2000, 104 were bled twice in February and again in August. All 447 sera were sent to a commercial BV testing laboratory and also were tested at the WNPRC using the HVP2 ELISA. Table 1 shows the comparison of the results obtained by the commercial testing laboratory using a BV antigen-based ELISA and those obtained using the HVP2 ELISA. From the results of these two tests, 349 of 447 samples (78.08%) were classified as positive by the commercial laboratory, whereas 353 (78.97%) were positive according to the HVP2 ELISA; therefore the results of the two methods were similar. Statistical analysis of the BV-positive results between the two methods yielded a weighted kappa coefficient of 0.94 and a 95% confidence interval of 0.91 to 0.97, suggesting that the HVP2 ELISA can reliably be used in-house to monitor BV infection in rhesus monkey colonies.

Three sera that were classified as positive by the commercial laboratory were identified as equivocal by the HVP2 ELISA, as was one sample that were classified as positive by the commercial laboratory deemed negative by the HVP2 ELISA, giv-

Table 1. Sensitivity comparison between HVP2 ELISA (HVP2 antigen) and BV ELISA (B virus antigen)

	Positive (%)	Equivocal (%)	Negative (%)
HVP2 ELISA	353[a,b] (78.97)	8[c,d] (1.79)	86[e] (19.24)
BV ELISA	349[c,e] (78.08)	9[b] (2.01)	89[a,d] (19.91)

A total of 447 samples were tested with each type of ELISA. The BV ELISA testing was done by submitting samples to a commercial laboratory. BV antibody-positive percentages did not differ significantly (weighted kappa coefficient, 0.94; 95% confidence interval, 0.91 to 0.97) between the two methods.
[a]Value includes two samples that had been negative for B virus antibody according to the BV ELISA but tested positive in the HVP2 ELISA.
[b]Value includes six samples that were equivocal for B virus antibody according to the BV ELISA but tested positive in the HVP2 ELISA
[c]Value includes three samples that had been positive for B virus antibody according to the BV ELISA but were equivocal in the HVP2 ELISA.
[d]Value includes two samples that were negative for B virus antibody according to the BV ELISA but were equivocal in the HVP2 ELISA.
[e]Value includes one sample that was positive for B virus antibody according to the BV ELISA but tested negative in the HVP2-ELISA.

ing a false-negative rate of 0.2% for the HVP2 ELISA. A total of eight samples that were classified as BV-negative (2 of 447) or equivocal (6 of 447) by the commercial testing were identified as positive by using the HVP2 ELISA, giving a false-positive rate of 1.8%. All of these inconsistent samples are summarized in Table 2. Almost all false-positive sera were from older (> 10 years) monkeys.

Sex and age distribution of BV infection. The BV infection status of the rhesus monkey colony in the WNPRC according to the results of the HVP2 ELISA is summarized in Tables 3 and 4. No significant difference (two-tailed $P = 0.17$) in BV antibody-positive percentages was identified between sexes (Table 3).

When 3-year age groups were compared, the percentage of BV antibody-positive monkeys was about 50% by 3 years of age, 64% by 6 years, and gradually rose to 75 to 91% thereafter, accompanying sexual maturation (Table 4).

Discussion

The sensitivity and specificity of an HVP2 ELISA were evaluated relative to those of a BV antigen-based assay by using a total of 447 sera from the rhesus monkey colony of 290 animals at the WNPRC. High correlation was demonstrated between the results obtained by a commercial testing laboratory using a BV antigen-based ELISA and those from the HVP2 ELISA. This similarity suggests that it is possible to test for BV infection of rhesus monkeys by using ELISA plates with HVP2 antigens. We also confirmed that in-house BV testing could be done as a routine service by using the HVP2 ELISA. However, as shown in Tables 1 and 2, eight samples that were classified as negative (–) or equivocal (±) by the BV-antigen ELISA were classified as positive (+) with HVP2 ELISA. In contrast, one sample that was positive (+) with the BV ELISA was classified as negative (–) by the HVP2 ELISA, which also deemed three BV ELISA-positive samples as equivocal (±). In the Abstract, we showed the 99.11% and 98.21% coincidence of the antibody positive samples of the BV-based and HVP-2 based ELISA assay systems, respectively, with the corresponding antibody positive samples identified by the other system. In the Results section and Table 1, the positive percentages of the test samples identified by the respective test systems are shown. That is to say, these are different expressions of the same results. The inconsistency rate is not particularly high, but we still need to examine the cause of this discrepancy. We will further examine these few inconsistent samples (Table 2) through analysis of comparative tests using ELISA tests with "non-dried antigen" or using a lower dilution of test samples. Most of the monkeys whose serum samples showed inconsistency are rather old (Table 2) and may have been used experimentally for multiple purposes. The differences in the ELISA results for these samples between the two ELISA tests were minimal (negative, equivocal, and very low positive). Thus, this inconsistency seems to be due to nonspecific reaction of serum samples because of animal age and/or multiple stimulations of the immune system from prior experimental use.

Zwartouw and colleagues (27) reported that total exclusion of animals with any trace of antibody enabled the establishment of new breeding colonies which are free of BV. In establishing an SPF colony, it is best to exclude any monkey suspected of possible BV infection (e.g., monkeys with equivocal serological test results) from addition to the colony. However, it is not advantageous to decrease the number of candidate monkeys as a result of excluding monkeys that are actually negative. On the basis of the outside testing results, we believe that use of the HVP2 ELISA to screen rhesus monkeys for BV antibody is equally sensitive to the BV antigen ELISA performed by the commercial testing laboratory. Reliability can be increased by retesting any animals giving equivocal results through sampling at different times and by confirming by Western blotting.

Of the 14 sera that gave disparate results using the two ELISAs, 10 gave higher OD values (stronger positive reactions) with the HVP2 ELISA. Therefore, one might consider it safer or more reliable (fewer potential false negatives) to use the HVP2 ELISA plates in establishing a SPF colony. The HVP2 ELISA appears to have no great problems in specificity or sensitivity in detecting

Table 2. All of the samples that showed inconsistent results between the HVP2 and BV ELISA

Sample no.	Monkey	BV ELISA[a]	HVP2 ELISA[b]	Sex	Age (years) at sampling	Date of sampling
77	r95010	–	+	female	5	08.02.00
119	rhan25	–	+	female	14	07.31.00
122	r80048	±	+	male	20	08.09.00
162	r89129	±	+	female	10	02.07.00
209	r80048	–	±	male	19	02.16.00
219	r84062	±	+	female	15	02.10.00
234	r89051	+	±	female	10	02.14.00
267	rh1752	+	±	female	26	02.11.00
275	rhaa73	+	–	female	23	02.15.00
279	rhan25	+	±	female	13	02.17.00
121	r78146	±	+	female	21	07.25.00
296	r81126	±	+	male	18	03.10.00
303	r83129	±	+	male	16	03.08.00
317	r87107	±	+	female	12	02.09.00

[a]Sera giving OD_{490} values of 0.00 to 0.16 were classified as negative, 0.17 to 0.39 as equivocal, and 0.40 as positive by a commercial testing laboratory.
[b]Sera giving OD_{490} values of 0.00 to 0.099 were classified as negative, 0.100 to 0.122 as equivocal, and 0.123 as positive as described previously (17).

Table 3. Comparison of the sex distribution of B virus antibody-positive monkeys

Sex	No. of animals tested	Age (years)	No. (%) of animals		
			Positive	Negative	Equivocal
Male	83	1–31	59 (71.08)	24 (28.92)	0 (0)
Female	179	1–33	140 (78.21)	35 (19.55)	4 (2.23)
Unknown	28	2–29	20 (71.42)	5 (17.85)	3 (10.71)
Total	290		219 (75.52)	64 (22.07)	7 (2.41)

Data shown are those of the latest serum sample available from multiply sampled animals; the HVP2 ELISA was used.
There was no significant difference in BV antibody-positive percentages between sexes (Wilcoxon rank sum test, two-tailed $P = 0.17$).

Table 4. Age distribution of B virus-positive rhesus monkeys

Age (years)	No. of animals tested	No. (%) of animals		
		Positive	Negative	Equivocal
0–3	59	31 (52.50)	28 (47.5)	0
4–6	45	29 (64.4)	16 (35.6)	0
7–9	40	34 (85.0)	6 (15.0)	0
10–12	67	50 (74.62)	15 (22.38)	2 (2.98)
13–15	48	42 (87.50)	5 (10.41)	1 (2.08)
16–18	36	32 (88.88)	3 (8.33)	1 (2.77)
19–21	35	30 (85.71)	4 (11.43)	1 (2.86)
≥ 22	32	29 (90.625)	1 (3.13)	2 (6.25)
Total	362	277 (76.52)	78 (21.55)	7 (1.93)

Data are those from the HVP2 ELISA and include samples from the same animals at different time points. When the 3-year age groups were compared, the percentage of BV antibody-positive monkeys became about 50% by 3 years of age and remained at 80 to 90% thereafter.

BV antibody. The ELISA plates with dry HVP2 antigen used in this study are by no means inferior to those with the "non-dried antigen" described previously (17). The plates can be stored for about 6 months and therefore can easily be supplied to various facilities. Consequently, it becomes possible to do in-house BV testing and to apply this test for safety management in individual facilities. Furthermore, the HVP2 ELISA kits prepared with dried antigen can be shipped to foreign countries as well as domestic areas and used by distant investigators. Further studies will be necessary to determine the stability of HVP2 ELISA plates during storage at 4°C for prolonged time periods. The existence of several different genotypes of BV associated with macaques has been reported (2, 16, 21). Therefore, whether the serological pattern of reactivity with HVP2 ELISA kits observed for rhesus macaques will apply to other macaque species has to be examined in the future.

The modified HVP2 ELISA method detected and measured BV antibodies with high sensitivity and reproducibility. The results indicate that in-house BV testing could be incorporated as a routine medical examination procedure by using ELISA plates prepared in other laboratories. In addition, the BV-positive rate was found to increase considerably in monkeys older than 3 years in the WNPRC colony and that all sera giving equivocal results were from animals older than 5 years. Therefore, we recommend that antibody-negative monkeys younger than 3 years are the best candidates to establish SPF colonies.

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