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Development of Immuno-PCR for Diagnosis of Bovine Herpesvirus 1 Infection

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A highly sensitive immuno-PCR method specific for the detection of antigens of and antibodies to bovine herpesvirus 1 was established. This assay attained a high sensitivity of up to $10^{7.0}$ times higher than that of enzyme-linked immunosorbent assay (ELISA) or PCR for antigen detection and $10^{5.0}$ times higher than that of ELISA for antibody detection.

Bovine herpesvirus 1 (BHV-1) generally causes latent infections. Unless reactivated, latent infections are not detectable by virus isolation (2), hence the need for the development of alternative sensitive and more rapid diagnostic methods. Sano et al. (9) developed a highly sensitive antigen detection system that combines enzyme-linked immunosorbent assay (ELISA) and PCR (immuno-PCR). Immuno-PCR-based systems have been described elsewhere (4, 6, 8, 13). We developed a highly sensitive and specific immuno-PCR assay for the detection of the antigens (Immuno-PCR/Ag) of and antibodies (Immuno-PCR/Ab) to BHV-1. We assessed its sensitivity and specificity and compared it with those of virus isolation, ELISA, and PCR. The applicability of the assay was tested with experimentally infected animals.

MDBK cells were used for virus isolation (1). Eight holstein-friesian, seronegative calves, 3 to 5 months of age, were housed in separate pens. The calves were experimentally infected with $10^{8.3}$ 50% tissue culture infective doses of the Los Angeles strain of BHV-1 by intranasal inoculation. Nasal swab samples, taken daily up to 21 days postinfection (dpi) were examined for the presence of antigens and antibodies. The ELISA was performed by the method of Kida et al. (3). The PCR assay was performed essentially by the method of Vilcek (10).

The procedure of immuno-PCR (9) was modified as shown in Fig. 1. The *Hind*III-*Acc*I fragment of plasmid DNA, pUC19 (12), was selected for amplification as a marker. Any DNA with known nucleotide sequence and easily amplifiable by PCR can be used as a marker DNA. One biotin molecule was incorporated at its 3' terminus by a filling-in reaction with Klenow enzyme (New England Biolabs) in the presence of biotin-14-dATP (Gibco BRL). Free nucleotides were removed by using Sephadex G-50 (Boehringer Mannheim). Synthetic oligonucleotides PC1:1070 (5'-GCTGTAGGTATCTCAGTTCG GTGTAGGTCG-3') and PC2:1261 (5'-CACCGCCTACATA CCTCGCTCTGCTAATCC-3') of the *ori* gene sequence of pUC19 were used as primers. The ELISA part of immuno-PCR was performed as described previously (3), except that free streptavidin (Wako) diluted 1/3,000 in phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA) fraction V and 0.5% Tween 20 (PBS5T) was added in place of the conjugate and incubated for 1 h at room temper-

ature. After the wells were washed with PBS supplemented with 0.5% Tween 20 (PBST), 50 μ l of 1.5×10^{-21} mol of the marker DNA in PBS5T was added to each well and incubated. The plates were washed 10 times and subjected to PCR (7). One microliter each of PC1 and PC2 (50 pmol), 4 μ l of deoxynucleoside triphosphates (10 mM), 5 μ l of 10 \times reaction buffer (Perkin-Elmer), 5 μ l of MgCl₂ (25 mM), and 1 μ l (1 U) of AmpliTaq DNA polymerase (Perkin-Elmer) were added to each well, mixed, and overlaid with 40 μ l of mineral oil. Thirty-five amplification cycles were carried out in a PTC-100 programmable thermal controller (MJ Research, Inc.); 95°C, 1 min of denaturation, 58°C, 1 min of annealing, and 72°C, 2 min of extension, and final extension, 72°C for 5 min. The product size, 191 bp, predicted upon the published gene sequence (12), was visualized by electrophoresis in 2% agarose gels containing ethidium bromide. No amplification product was detected when one of the following materials was omitted: antigen

ELISA PLATE COATED WITH BHV-1 ANTIGEN

↓ a

MONOCLONAL ANTIBODY TO BHV-1

↓ a

BIOTINYLATED GOAT ANTI-MOUSE ANTIBODY

↓ a

STREPTAVIDIN

↓ a

BIOTINYLATED MARKER DNA

↓ b

PCR AMPLIFICATION

↓

AGAROSE GEL DETECTION OF AMPLIFIED PRODUCTS

FIG. 1. Schematic presentation of immuno-PCR for the detection of BHV-1. The ELISA plate was coated with antigen. BHV-1-specific monoclonal antibody, biotin-labeled goat anti-mouse antibody, streptavidin, and biotin-labeled marker DNA were added sequentially. Blocking with 1% BSA was done after the plate was coated with antigen. The plates were washed with PBST 5 (a) or 10 (b) times. Finally, the plates were subjected to PCR. For antibody detection, the test serum was added in place of monoclonal antibody against BHV-1.

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TABLE 1. Comparison of the sensitivities of the four antigen detection assays on culture supernatants of BHV-1-infected cells^a

Assay	Titer ^b
PFU.....	7.2 (7.0, 7.6, 7.0)
ELISA.....	3.9 (3.6, 3.6, 4.6)
PCR.....	4.0 (4.0, 4.0, 4.0)
Immuno-PCR.....	10.9 (9.9, 11.9, 10.9)

^a The BHV-1 infectious cell culture supernatant was diluted as described in the text.

^b The results are shown as log₁₀ reciprocal of the highest dilution that was positive for BHV-1. Numbers in parentheses are the results of a set of experiments performed on the same sample by each assay.

(BHV-1), primary antibody (anti-gB of BHV-1), secondary antibody (biotinylated goat anti-mouse), streptavidin, marker DNA, *Taq* polymerase, or primers (data not shown).

For antigen detection, the culture supernatant of MDBK cells infected with BHV-1 or nasal secretions of the calves were centrifuged at low speed and serial 10-fold dilutions in PBS were prepared. Each dilution was treated with disruption buffer (0.05 M Tris-HCl [pH 7.8], 0.5% Triton X-100, 0.6 M KCl). Each well of 96-well plates (Falcon 3911; Becton Dickinson) was coated with 50 µl of each dilution at 4°C overnight. The plates were blocked with 1% BSA in PBS. Fifty microliters of monoclonal antibody 2G6-51 against the gB of BHV-1 (5),

diluted 1/2,000 in PBS5T, was added to each well. Biotin-labeled goat antibodies specific to mouse immunoglobulin G Fc (EY Laboratories, Inc.) diluted 1/2,000 in PBS5T were then added. Immuno-PCR/Ag detected the antigen in the culture supernatant (10^{7.0} PFU/ml) up to a dilution of 1:10^{9.9} (Fig. 2a). ELISA detected the antigen up to a dilution of 1:10^{4.9} (Fig. 2b) of the same sample. Results obtained from three independent tests with the same sample are summarized in Table 1. Immuno-PCR/Ag was up to 10^{3.7}, 10^{7.0}, and 10^{6.9} more sensitive than plaque-forming assay, ELISA, and PCR, respectively.

For the detection of antibodies in the rabbit anti-BHV-1 immune serum or sera obtained from the experimentally infected calves, serial twofold dilutions in PBS5T were added to the BHV-1-coated plates. Biotinylated goat anti-rabbit or anti-bovine immunoglobulin G Fc (EY Laboratories, Inc.) antibodies diluted 1/3,000 in PBS5T, respectively, were then added. The results showed that immuno-PCR/Ab was 10⁵ times more sensitive than ELISA (data not shown).

All calves infected with BHV-1 developed various degrees of the clinical signs (Table 2). On 1 dpi, virus was recovered from 6 of the 8 calves while viral antigen was detected in all the calves by immuno-PCR/Ag. No more virus shedding was detected by the virus isolation assay after 10 dpi, while viral antigen continued to be detected by immuno-PCR/Ag. All samples negative by immuno-PCR/Ag were negative by virus isolation as well. A large number of those samples negative by

TABLE 2. Clinical signs and results of virus isolation and immuno-PCR/Ag to detect BHV-1 in nasal secretion samples^a

Calf no.	Assay	Result ^b on dpi:																					
		-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	Virus isolation	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	Immuno-PCR	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Clinical signs	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
2	Virus isolation	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	Immuno-PCR	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Clinical signs	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Virus isolation	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	Immuno-PCR	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+
	Clinical signs	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
4	Virus isolation	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	Immuno-PCR	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+
	Clinical signs	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	Virus isolation	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	Immuno-PCR	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
	Clinical signs	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
6	Virus isolation	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	Immuno-PCR	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+
	Clinical signs	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
7	Virus isolation	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
	Immuno-PCR	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Clinical signs	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
8	Virus isolation	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
	Immuno-PCR	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Clinical signs	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

^a Calves 1 to 8 were intranasally inoculated with BHV-1.

^b The symbols + and - indicate positive and negative results, respectively. For clinical signs, a positive result indicates the presence of at least one of the following: fever, nasal mucosal lesions, nasal discharge, dyspnea, conjunctivitis, and depression. In all cases, the severe clinical signs were observed only up to 10 dpi.

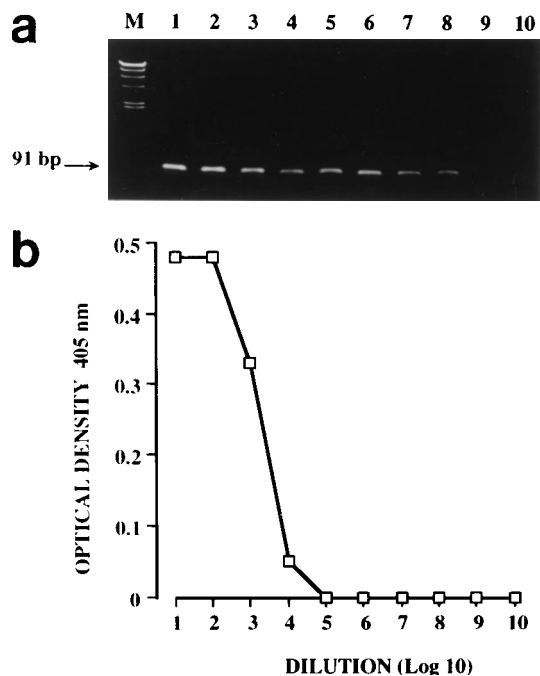


FIG. 2. (a) Sensitivity of immuno-PCR on the culture supernatants of the cells infected with BHV-1. Lane 1, PCR amplification product from a dilution of 4.0×10^{-1} of the sample; lanes 2 through 9, 50 μ l of 10-fold serial dilutions of antigen starting with 4.0×10^{-2} up to 4.0×10^{-9} , respectively. No product was visible from the antigen-negative well (lane 10). Lane M shows the migration of marker fragments of *Hind*III-digested lambda DNA of 23,130, 9,416, 6,557, 4,361, 2,322, 2,027, 564, and 125 bp. (b) ELISA sensitivity on the same sample as shown in panel a. Detection of the BHV-1 antigen immobilized on a microtiter plate by ELISA with anti-BHV-1 monoclonal antibody, biotinylated goat anti-mouse immunoglobulin G antibody, and avidin-conjugated alkaline phosphatase. Wells 1 through 9 contained 50 μ l of 10-fold serial dilutions of the sample starting with 4.0×10^{-1} . Well 10, antigen negative. After addition of the substrate, color development was observed at 4.0×10^{-3} or more of the immobilized antigen.

virus isolation were positive by immuno-PCR/Ag. From 14 to 21 dpi, some of the samples were negative probably because of reduced antigen presence. ELISA on the test sera of the calves 7 days prior to vaccination gave antibody-negative results. Immuno-PCR/Ab performed on the same samples detected two positive samples, with a titer of $1:10^{4.6}$ (data not shown), probably because of the presence of colostrally acquired maternal antibodies (2).

An immuno-PCR assay based on biotinylated anti-species antibodies and the primers from the *ori* gene of pUC19 provided a practical means of detecting BHV-1 infection with a sensitivity higher than those of plaque-forming assay, ELISA,

and PCR. The sensitivity and specificity of the present modified method compare favorably with those reported previously (4, 6, 8, 9, 13). Whereas the plaque-forming assay detects infectious BHV-1 particles only, immuno-PCR/Ag detects the physical particles and free gB antigen. The sensitivity of a PCR assay on BHV-1 detection in nasal swabs was found to be at least as high as that of virus isolation (11) with extensive purification of the template DNA from the sample.

We conclude that immuno-PCR/Ag and immuno-PCR/Ab are more sensitive than any existing tests for antigen and antibody detection, respectively, and thus may become the assays of choice for the routine diagnosis of not only BHV-1 infection but also other infections.

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