Detection of Antibodies against Equine Herpesvirus Types 1 and 4 by Using Recombinant Protein Derived from an Immunodominant Region of Glycoprotein B

ROBERT SINCLAIR,* MATTHEW M. BINNS, EWAN D. CHIRNSIDE, AND JENNIFER A. MUMFORD
Department of Infectious Diseases, Animal Health Trust, P.O. Box 5, Newmarket, Suffolk, England CB8 7DW

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The N-terminal fragment comprising residues +1 to +50 (gB1-50) of equine herpesvirus type 1 (EHV-1) glycoprotein B was expressed as a glutathione S-transferase fusion protein in *Escherichia coli*. Recombinant gB1-50 (rgB1-50) was recognized in immunoblots by sera from rabbits immunized with EHV-1 and by convalescent-phase sera from horses with natural EHV-1 infections. An enzyme-linked immunosorbent assay (ELISA) for monitoring antibody levels against EHV-1 was developed by using rgB1-50, and its specificity was assessed with a panel of reference antisera against other equine viruses. A specific cross-reaction was detected with EHV-4, which was confirmed by inhibition ELISA. Convalescent-phase sera from horses with natural EHV-1 or EHV-4 infections possessed antibody titers against rgB1-50 ranging from 1:2,000 to 1:64,000, indicating the presence of an immunodominant antigenic site. The study demonstrated the potential application of rgB1-50 as a diagnostic antigen and highlights the glutathione S-transferase fusion system as a simple and effective method of producing purified milligram quantities of antigen.

Equine herpesvirus type 1 (EHV-1) and EHV-4 are antigenically related viruses (1). Electrophoretic analysis of their structural proteins has revealed polypeptides with a comparable range of molecular weights, with similarities and differences between the electrophoretic mobilities of individual counterpart polypeptides (50, 51). Studies with polyclonal and monoclonal antibodies have revealed the presence of both type-cross-reactive and type-specific antigenic determinants among these proteins (3, 7, 10, 14, 30, 30–41, 49). Both viruses possess some similarities in their pathogenic potentials, causing respiratory disease, abortion, lethargy, and loss of performance (1, 11, 13, 32). Additionally, EHV-1 can cause neurological disease and multiple abortions, from which mortality rates can be as high as 50% of individuals on an affected premise (12, 31, 33, 37). In contrast, EHV-4 is not associated with outbreaks of neurological disease, and its potential for causing abortion is usually restricted to small numbers of animals or individual cases. The similarities between these two viruses was reflected in their classification as EHV-1 subtypes (subtypes 1 and 2) until significant differences between their genomes were demonstrated by restriction endonuclease analysis and DNA homology studies (2, 47, 52), resulting in their current taxonomic separation into two distinct types.

Although direct detection of virus antigen is the definitive means of diagnosis, the complexity of disease caused by EHV-1 and EHV-4 necessitates a multiple-test approach. Virus may be isolated from nasopharyngeal swabs, the leukocyte fraction of blood, or fetal tissues such as liver, lung, thymus, and spleen (32). Viral antigen can also be detected in fetal tissue by histological examination and by reaction with polyclonal or monoclonal antibody (34, 40, 43). Most recently, the polymerase chain reaction (PCR) has been applied to the detection of viral DNA in nasopharyngeal swabs and fetal tissue, although possible interference from latent virus requires thorough investigation (4, 38). For

serological diagnosis, the complement fixation test is widely used (49) and recent field studies have highlighted the practical use of this test for management of natural outbreaks (33). Antibody levels can be compared in acute- and convalescent-phase serum samples, and a fourfold rise in titer is used to identify infected animals. Additionally, the short duration of complement fixation antibody responses (2 to 3 months) enables high levels of complement fixation antibody in single serum samples to be used as an indicator of recent exposure to the virus (49). Despite this advantage, the complement fixation test nevertheless possesses certain drawbacks. Distinct differentiation between EHV-1 and EHV-4 is difficult because of the antigenic cross-reactivities of these viruses, and diagnosis is further hampered by requirements for standardized batches of complement-, hemolysin-, and tissue culture-derived viral antigen.

We report here the cloning and expression of 50 amino acids that make up the N terminus of glycoprotein B (gB1-50) of EHV-1. We demonstrated the presence of an immunodominant antigenic determinant with homology to EHV-4 glycoprotein B and assessed the serological cross-reaction. An enzyme-linked immunosorbent assay (ELISA) was developed for monitoring antibody levels to EHV-1 and EHV-4 in serum, which may have applications for the diagnosis of acute infections or monitoring the immune status of individual animals.

MATERIALS AND METHODS

Expression plasmid. The bacterial plasmid pGEX-3X (Pharmacia LKB Biotechnology Limited, Uppsala, Sweden) which directs the expression of cloned DNA as a glutathione S-transferase (GST) fusion protein was selected for these studies (Fig. 1). Viral DNA amplified by PCR was inserted into the multiple cloning site located at the carboxy terminus of the GST gene under the control of the *tac* promoter.

Primers for PCR. DNA primers were synthesized by using phosphoramidite chemistry on an automated oligonucleotide synthesizer (391 PCR-Mate; Applied Biosystems, San Jose,

* Corresponding author.

FIG. 1. Linear representation of the bacterial expression plasmid pGEX-3X (4,952 bp). A DNA segment encoding amino acid residues +1 to +50 of the EHV-1 glycoprotein B gene product was inserted into the *Bam*HI site located at the terminus of the GST gene.

Calif.). Primer sequences were selected within the gene encoding glycoprotein B (gp14) of EHV-1 strain HVS 25A (53), and the primer sequences were designed to amplify the region which encodes the 50 N-terminal amino acids (+1 to +50 [gB1-50]) of the mature glycoprotein. The primers were designed with internal *Bam*HI restriction endonuclease sites to enable insertion of the amplified DNA into the pGEX-3X linker in frame with the gene encoding GST. The nucleotide sequences of the primers were 5'-AAGGATCCAAGTAC CCACCACGCCAAGCC-3' for the upstream primer and 5'-TGAGGATCCATCCTCAGCGAGTATACC-3' for the downstream primer (underscores indicate restriction endonuclease sites). After synthesis, the primers were cleaved from the solid support and were deprotected by treatment with ammonium hydroxide, precipitated with ethanol, resuspended in distilled water, and stored at -20°C .

Amplification of DNA. Viral DNA was extracted from EHV-1 strain Ab4 (15). Tissue culture supernatants containing approximately 10^7 PFU/ml were treated with 1% Nonidet P-40 and 200 μg of proteinase K per ml for 1 h at 37°C ; this was followed by a 10-min incubation at 95°C . PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus) with 10- μl aliquots of extracted DNA in a total reaction volume of 100 μl . Reaction mixtures contained 200 μM (each) dATP, dCTP, dGTP, and dTTP, 0.5 μM each primer, 50 mM KCl, 1.5 mM MgCl_2 , 0.01% gelatin, and 10 mM Tris-Cl (pH 8.3). After heating the mixture to 95°C for 5 min, 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus) was added and the mixture was overlaid with 50 μl of mineral oil. Thirty-five thermal cycles were performed, each consisting of an incubation at 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min. The extension time during the final cycle was increased to 7 min in order to complete DNA synthesis.

Cloning of viral DNA and expression of fusion protein. Products of the PCR were extracted with phenol-chloroform and then ether; this was followed by precipitation with ethanol. They were then digested with *Bam*HI for 3 h at 37°C and subjected to electrophoresis through 3% NuSieve agarose. DNA fragments were excised from the gel and purified

by using a Mermaid Kit (Bio 101 Incorporated, La Jolla, Calif.) before ligation to dephosphorylated *Bam*HI pGEX-3X. The ligated vector was then used to transform competent *Escherichia coli* TG1 cells. Transformed colonies were grown in Luria broth containing 50 μg of ampicillin medium per ml for 3 h at 37°C . Protein expression was induced by the addition of isopropyl- β -D-thiogalactoside to give a 0.1 mM final concentration. Cultures were incubated for a further 2 h at 37°C and were then screened for expression of fusion proteins by electrophoresis through sodium dodecyl sulfate (SDS)-12% polyacrylamide gels.

Purification of fusion protein. For use as antigen in ELISAs, the recombinant fusion protein (rgB1-50) was affinity purified by using glutathione Sepharose 4B (Pharmacia LKB Biotechnology). A 250-ml culture of *E. coli* cells expressing recombinant antigen was harvested by centrifugation at $4,000 \times g$ for 15 min at 4°C , resuspended in STE buffer (10 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA [pH 8.0]), and recentrifuged to pellet the cells. The supernatant was discarded and the cells were resuspended in 20 ml of GTE buffer (50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA [pH 8.0]) containing 0.5 mg of lysozyme per ml. After incubation on ice for 30 min, 2 ml of 10% Triton X-100 was added and the reaction mixture was incubated for 15 min. Insoluble cell debris was removed by centrifugation at $12,000 \times g$ for 15 min, and the supernatant was added to 1 ml of a 50% glutathione Sepharose 4B gel slurry prepared according to the manufacturer's instructions (Pharmacia LKB Biotechnology). After gentle agitation on a rotary mixer for 15 min, the gel was washed three times in phosphate-buffered saline (PBS). Recombinant antigen was eluted from the gel by the addition of reduced glutathione (5 mM glutathione, 50 mM Tris-Cl [pH 8.0]).

Antisera. Primary screening of fusion proteins by immunoblotting was performed by using rabbit and horse anti-serum directed against EHV-1. Rabbit antiserum was prepared by immunization of rabbits with 200 μg of gradient-purified whole virus (41) emulsified in Freund's complete adjuvant. Three booster injections each of 200 μg prepared in Freund's incomplete adjuvant were administered at 1-month intervals, and serum was collected 14 days after the final booster injection. Field sera from naturally occurring outbreaks of EHV-1 and EHV-4 were obtained from the Animal Health Trust's diagnostic laboratory. Sera were selected from outbreaks in which virus was isolated and typed by using pools of monoclonal antibodies (54).

Reference antisera directed against EHV-1, EHV-2, EHV-3, EHV-4, equine rhinovirus types 1 and 2, and equine adenovirus were also obtained from the Animal Health Trust's diagnostic laboratory. Preinfection (day 0) and postinfection (day 14) horse anti-EHV-1 and anti-EHV-4 sera were obtained from experimentally infected ponies by previously described methods (22).

DNA sequencing. Sequencing of cloned DNA was performed by the chain termination method (48) by using Sequenase Version 2.0 (United States Biochemical Corporation, Cleveland, Ohio). Double-stranded template DNA was prepared according to the manufacturer's instructions, and sequencing was performed by using the primer 5'-GGCGACCATCCTCCAAA-3' located approximately 40 bp upstream of the pGEX-3X *Bam*HI cloning site. The DNA sequence of the cloned fragment of strain AB4 used in the present study possessed 100% homology with that of the published strain HVS 25A (53).

Polyacrylamide gel electrophoresis and immunoblotting. Electrophoresis through SDS-12% polyacrylamide gels was

FIG. 2. Electrophoretic analysis of total *E. coli* cell proteins in SDS-polyacrylamide gels. (a) Staining with Coomassie blue. (b and c) Immunoblots with rabbit (b) and horse (c) anti-EHV-1 sera. Lanes 1, *E. coli*; lanes 2, *E. coli* expressing GST; lanes 3 to 5, *E. coli* clones expressing gB1-50; lanes 6, EHV-1 whole virus antigen. Molecular mass markers (in kilodaltons) are indicated to the left of the gel.

performed as described previously (41) by using low-molecular-weight protein markers (Sigma Chemical Company, St. Louis, Mo.) to determine relative molecular masses. Samples were prepared by dilution of bacterial cells 1:1 in sample buffer; this was followed by heating at 95°C for 3 min. The separated proteins were then transferred electrophoretically to nitrocellulose in buffer containing 0.025 M Tris (hydroxymethyl)methylene, 0.2 M glycine, and 20% methanol. Nitrocellulose strips were incubated in PBS containing 5% bovine serum albumin for 3 h to block nonspecific binding. They were then incubated with a 1:500 dilution of rabbit (or horse) anti-EHV-1 serum for 3 h at 37°C and then a 1:500 dilution of goat anti-rabbit (or rabbit anti-horse) immunoglobulin-peroxidase conjugate for 3 h at 37°C. Bound antibody was visualized by incubation with substrate solution (0.3 mg of diaminobenzidine per ml, 0.05 M Tris-Cl, 0.15 M sodium chloride, 0.0001% H_2O_2 [pH 7.6]) for 5 min at room temperature. Antisera were diluted in PBS containing 5% bovine serum albumin and 0.1% Nonidet P-40. Nitrocellulose strips were washed twice with PBS and twice with PBS containing 0.1% Nonidet P-40 each time between incubations.

Indirect ELISA. The optimal concentrations of the reactants were predetermined by checkerboard titration. Microtiter plates were coated with a 1- μ g/ml solution of rgB1-50 or GST antigen diluted in 0.1 M carbonate buffer (pH 9.6) for 16 h at 4°C. Plates were then washed with PBS containing 0.05% Tween 20 (PBST) and blocked by the addition of PBST containing 1% bovine serum albumin and 5% normal rabbit serum for 1 h at room temperature. Twofold serial dilutions (triplicate) of horse antisera were added to the plates and incubated for 90 min at 37°C. Plates were washed again, and a 1:2,000 dilution of goat anti-horse immunoglobulin-peroxidase conjugate was added for 90 min at 37°C. After their final wash, substrate solution (as described above) was added for 30 min at room temperature, the reaction was stopped with 4 M H_2SO_4 , and the A_{492} was read.

Inhibition ELISA. Optimal concentrations of reactants were predetermined by checkerboard titration. Inhibition assays were performed by using a limiting concentration of gB1-50 antigen passively adsorbed to 96-well microtiter plates (Dynatech Laboratories, Billingham, United King-

dom) for 16 h at 4°C in 0.1 M carbonate buffer (pH 9.6). The plates were washed with PBST and blocked by the addition of 1% bovine serum albumin and 5% normal rabbit serum in PBST for 1 h at room temperature. Twofold serial dilutions of gradient-purified EHV-1 or EHV-4 (41) were added; this was followed immediately by the addition of a limiting dilution of horse antisera, and then the mixture was incubated for a further 90 min at 37°C. Plates were then washed with PBST, and a 1:4,000 dilution of goat anti-horse immunoglobulin-peroxidase conjugate was added for 90 min at 37°C. Plates were washed again with PBST, substrate (0.04 mg of *o*-phenylenediamine per ml in 0.05 M sodium citrate–0.15 M sodium phosphate–0.01% hydrogen peroxide [pH 5.5]) was added, and the plate was incubated for 30 min at room temperature. The reaction was stopped by the addition of 4 M H_2SO_4 acid, and the A_{492} was read. The dilution buffer used throughout was PBST containing 1% bovine serum albumin and 5% normal rabbit serum.

RESULTS

Expression screening for rgB1-50. Total cell proteins from transformed *E. coli* were screened for expression by electrophoresis in SDS–12% polyacrylamide gels (Fig. 2). Identification of clones expressing potential recombinant protein was based on reductions in their electrophoretic mobilities when compared with the electrophoretic mobility of the unaltered 27.5-kDa GST protein (Fig. 2a). A major 27.5-kDa band was visible in a clone expressing GST without the insert (lane 2), but was not visible in untransformed *E. coli* (lane 1). A major 32.7-kDa band was visible in three clones transformed with vector ligated to insert DNA (lanes 3 to 5), which was consistent with the predicted molecular weight of the gB1-50 fusion protein. The serological reactivity of rgB1-50 was assessed in immunoblotting experiments (Fig. 2b and c). Antiserum from rabbits immunized with whole virus antigen and serum from a horse naturally infected with EHV-1 both reacted specifically with recombinant gB1-50 (lanes 3 to 5) and whole purified virus (lane 6) but did not react with GST (lane 2). Antibody reactivities to several *E. coli* proteins were also detected in both antisera (lanes 1 to 5).

Reactivity of affinity-purified rgB1-50 by ELISA. In order

FIG. 3. Titration of day 0 postinfection (acute-phase sample [A]) and day 14 postinfection (convalescent-phase sample [C]) sera from ponies experimentally infected with EHV-1 (a) or EHV-4 (b). Sera were titrated against affinity-purified GST (□), affinity-purified rgB1-50 (▨), and gradient-purified homologous virus (■).

to test rgB1-50 as a potential diagnostic antigen, its reactivity with a panel of sera raised against different equine viruses was assessed by an indirect ELISA. The rgB1-50 antigen reacted strongly with sera raised against EHV-1 and also with sera raised against EHV-4; EHV-1 and EHV-4 are known to possess some common antigenic determinants. No cross-reactions were detected with EHV-2, EHV-3, equine rhinovirus types 1 and 2, or equine adenovirus.

The serological cross-reaction between EHV-1 and EHV-4 was examined further by using sera from ponies which were infected with either EHV-1 or EHV-4 under experimental conditions (Fig. 3). Four two-year-old ponies with no serological evidence of infection prior to challenge were selected for each experiment. Serum samples taken on day 0 postinfection (acute-phase sample) and day 14 postinfection (convalescent-phase sample) were titrated on GST, rgB1-50, and homologous whole virus antigen. No reaction with GST was detected with the lowest dilution (1:1,000) of either acute- or convalescent-phase samples. The mean A_{492} value for these sera was 0.039 with a standard deviation ($n = 1$) of 0.021. All sera from the four animals infected with EHV-1 or EHV-4 demonstrated a specific antibody response to rgB1-50 and whole virus antigen. For sera from animals infected with EHV-1, end point titers to rgB1-50 were comparable to those against homologous virus and possessed geometric mean titers (GMTs) of 1:80,000 and 1:140,000, respectively (Fig. 3a). For animals infected with EHV-4, GMTs against recombinant gB1-50 and homologous virus were 1:8,000 and 1:40,000, respectively (Fig. 3b).

The reactivities of field sera with rgB1-50 were also evaluated by ELISA (data not shown). Thirty convalescent-phase serum samples from horses confirmed to be involved in EHV-1 outbreaks were found to possess titers ranging from 1:8,000 to 1:64,000, with a GMT of 40,000. Convalescent-phase sera from nine animals with confirmed EHV-4 infections possessed titers ranging from 1:2,000 to 1:8,000,

with a GMT of 1:4,000. Normal control sera from 20 different horses showed no reactivity with rgB1-50 throughout the dilution range tested (1:1,000 to 1:100,000).

The presence of common antigenic determinants within the 50 N-terminal amino acids of EHV-1 and EHV-4 glycoprotein B was confirmed by inhibition ELISA (Fig. 4). Gradient-purified EHV-1 and EHV-4 antigens were both found to specifically inhibit the binding of horse anti-EHV-1 sera to rgB1-50.

FIG. 4. Specificity of rgB1-50 determined by inhibition ELISA. Antibody binding to rgB1-50 in the absence of viral antigen (■) was compared with antibody binding in the presence of serial twofold dilutions of gradient-purified EHV-1 (●) and EHV-4 (▲) antigen. Error bars indicate mean absorbance values $\pm$ 2 standard deviations ($n = 1$).

DISCUSSION

Here we reported the bacterial expression and serological reactivity of the 50 N-terminal amino acids of EHV-1 glycoprotein B (gB1-50). We also described its use for detection of antibodies to EHV-1 and EHV-4 and its potential application in monitoring the immune status of individual animals or for diagnosis of acute infections. The mature form of this protein exists as an 895-amino-acid glycosylated heterodimer located in the viral envelope (20, 30, 53). It is known to be a target of the equine humoral immune system and can stimulate the production of monoclonal antibodies which neutralize virus in vitro and mediate passive protection in vivo (5, 39, 41, 46). Vaccinia virus recombinants expressing EHV-1 gB elicit the production of neutralizing antibody in guinea pigs and, when inoculated into hamsters, can protect the animals against subsequent lethal challenge (21). These studies have led several investigators to propose that this glycoprotein should be considered a potential component of future subunit vaccines (5, 20, 21, 30, 39, 41, 46, 53).

Several criteria were used for selection of an appropriate region of gB for expression in *E. coli*. Studies of several other herpesvirus glycoproteins have demonstrated antigenic sites located at their N termini. Herpes simplex virus type 1 (HSV-1) gB possesses an antigenic site located between residues +32 and +47 of the mature glycoprotein, HSV-1 glycoprotein D possesses an antigenic site located between amino acids -5 and +55 (6, 9, 16, 19, 26-28), and pseudorabies virus glycoprotein I possesses an antigenic site located between residues +33 and +100 (18, 23). Like the N terminus of EHV-1 gB, these regions possess characteristics typical of sequential epitopes, including long stretches of hydrophilic amino acids and predicted β turns in their secondary structures (16). Although the epitopes synthesized in *E. coli* are likely to be sequential, evidence suggests that local conformations in peptide sequence can be important in protein antigenicity. Denaturation of bacterially expressed sequential epitopes of infectious bronchitis virus and the G2 protein of Rift Valley virus are known to adversely affect antibody binding (25, 29). We therefore selected *E. coli* expression vector pGEX-3X, which facilitates the production of affinity-purified fusion proteins under nondenaturing conditions (24, 42).

Results of the present study demonstrate the presence of an immunodominant antigenic site located within the 50 N-terminal amino acids of EHV-1 gB. The successful expression of a 32.5-kDa rgB1-50 was demonstrated in immunoblots by reaction with sera from a rabbit immunized with EHV-1 and with sera from a horse naturally infected with EHV-1. Antibody reactivity to *E. coli* proteins was detected in both antiserum samples, although this reaction was predominant in the equine serum sample, which reacted with at least eight distinct bands with molecular masses ranging from 10 to 100 kDa. This phenomenon has previously been documented (8) between bacterial-derived recombinant antigens of human immunodeficiency virus and human sera. For this reason, all ELISAs in the present study were performed with affinity-purified antigens, resulting in highly specific assays with very low backgrounds. Reactions between equine sera ($n = 60$) and affinity-purified GST were not detected, suggesting efficient separation from *E. coli* proteins. The absence of host antibody to GST is likely to enhance the use of this expression system for future production of equine diagnostic antigens. It possesses a significant advantage over the use of β -galactosidase fusions, since specific antibody to this protein has been identified in equine

sera (8a) and sera from other species, resulting in high backgrounds and/or false-positive results (23).

The cross-reaction of sera from EHV-4-infected ponies with rgB1-50 was not unexpected since the amino acid sequences of mature gB1 and gB4 are 88% conserved (36, 53). However, the GMT of sera from EHV-4-infected ponies (1:8,000) was 10-fold lower than the GMT of sera from EHV-1-infected ponies (1:80,000). This may reflect diversity in the amino acid sequence between the 50 N-terminal amino acids of gB1 and gB4, or alternatively, this region of EHV-4 may be inherently less immunogenic. In comparable studies between HSV-1 and HSV-2 gB, in which antibody responses to a 690-amino-acid eukaryotic expression product were assessed, it was noted that sera from HSV-2-infected patients reacted with gB1 less markedly than sera from patients with HSV-1 infection did (35). This was attributed to the presence of type-specific epitopes which have been identified in the gB molecule (17, 27, 28).

A high degree of amino acid homology exists between gB of EHV-1 and gBs of other herpesviruses. Common epitopes have been identified on EHV-1 gB, EHV-4 gB, HSV-1 gB, HSV-2 gB, and bovine herpesvirus gII by immunoprecipitations with polyclonal rabbit antisera (45). A conserved epitope has also been mapped to amino acids +107 to +172 of EHV-1 gB by using a murine monoclonal antibody and the lambda gt11 expression system (20). However, this may not reflect the distribution of epitopes which are immunogenic in the natural host. In the present study we did not detect any cross-reaction with two other EHV-1s, EHV-2 and EHV-3, although we have yet to test cross-reaction with human or bovine herpesviruses. It is likely that selection of a 50-amino-acid region of gB, which makes up only 6% of the entire molecule, has reduced the potential for serological cross-reactions with these viruses. The use of rgB1-50 as a diagnostic antigen is being assessed with large numbers of serum samples from natural outbreaks. Of those tested to date, 100% of sera from animals involved in both EHV-1 or EHV-4 outbreaks reacted with rgB1-50. Further studies to develop recombinant antigens which differentiate between EHV-1 and EHV-4 infections are in progress. This remains an important objective because of differences in the pathogenicities between these viruses.

In conclusion, our studies demonstrated the presence of an immunodominant antigenic site located within the 50 N-terminal amino acids of EHV-1 gB. We also demonstrated the potential application of recombinant antigen for monitoring antibody levels to EHV-1 and EHV-4, although a more rigorous examination of antibody responses to this antigen is in progress.

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