Serological Method Using Recombinant S2 Protein To Differentiate Equine Infectious Anemia Virus (EIAV)-Infected and EIAV-Vaccinated Horses

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We recently reported a highly protective attenuated live virus vaccine for equine infectious anemia virus (EIAV) based on a proviral construct (EIAVUKΔS2) with a genetically engineered mutation in the viral S2 gene that eliminates expression of this accessory protein. While the EIAVUKΔS2 vaccine provides protection from detectable infection by experimental challenge with highly virulent virus, the potential for commercial application of this vaccine is complicated by the fact that horses inoculated with the EIAVUKΔS2 vaccine strain become seropositive in various reference diagnostic assays based on detection of antibodies to virion core or envelope proteins. To address this issue, we describe here the development and optimization of a new serologic EIAV diagnostic enzyme-linked immunosorbent assay (ELISA) to detect serum antibodies to the EIAV S2 protein that are produced in infected horses but not in horses inoculated with the EIAVUKΔS2 vaccine virus. The test S2 protein antigen was developed using the S2 gene sequence from the EIAVUK strain of virus and a series of modifications to facilitate production and purification of the diagnostic antigen, designated HS2G. Using this HS2G as antigen, we describe the development of an affinity ELISA that provides a sensitive and specific detection of S2-specific serum antibodies in experimentally and field-infected horses (22 of 24), without detectable reactivity with immune serum from uninfected (12 of 12) or vaccinated (29 of 29) horses. These data indicate that the S2-based diagnostic ELISA has the potential to accurately differentiate horses infected with EIAV from horses inoculated with an attenuated EIAV vaccine strain with a mutant S2 gene.

The macrophage-tropic animal lentivirus equine infectious anemia virus (EIAV) causes a persistent infection in horses and has been recognized as an important veterinary infectious disease for over 150 years (14, 24, 29). EIAV, transmitted by blood-feeding insects and fomites such as contaminated needles, is characterized by recurring cycles of viremia and clinical episodes that include fever, anemia, thrombocytopenia, edema, and wasting. Following 6 to 12 months of this chronic disease stage, most animals progress to an inapparent stage absent overt clinical disease (9, 14, 24, 29). Because of the economic importance of controlling EIAV infections, combined with the successful use of EIAV as an animal lentivirus model for human immunodeficiency virus type 1, the development of an effective vaccine for EIAV has been aggressively pursued (8, 16, 19, 25, 26, 33).

This laboratory has previously described the development of a live attenuated EIAV vaccine based on an engineered proviral construct with a mutated S2 gene, EIAVUKΔS2 vaccine (19). The EIAV S2 gene is located in the pol-env intergenic region, overlaps the amino terminus of the EIAV envelope proteins, and is synthesized in the late phase of the viral replication cycle (4, 6). While the biological function of this 65-amino-acid accessory protein is unknown, previous studies have indicated that S2 is not essential for EIAV replication in vitro (21, 28) but that the absence of S2 severely reduces EIAV replication and virulence in experimentally infected horses (20). In vivo studies of EIAVUKΔS2 as a potential attenuated viral vaccine demonstrated protection from infection in vaccinated horses subjected to low-dose multixposure intravenous virulent virus challenge (to mimic field exposure) or to a single high-dose intravenous virus challenge (19).

In light of these promising initial observations on the efficacy of a live attenuated EIAV vaccine, the EIAVUKΔS2 vaccine may represent an effective vaccine for the control of EIAV infections. Thus, the development of a diagnostic assay that can effectively identify and differentiate EIAV-infected animals from EIAVUKΔS2-vaccinated horses has become a necessary advancement. In countries where veterinary regulatory policies are established, EIAV infections are currently controlled by detection and destruction or isolation of virus-infected horses. Approved diagnostic assays for EIAV infection are based on detection of serum antibodies to the capsid protein p26, transmembrane glycoprotein gp45, and surface glycoprotein gp90 in various assay formats (1, 3, 10, 15, 18, 22, 30–32). At present, the United States Department of Agriculture primary enzyme immunoassay (EIA) testing systems include the agar gel immunodiffusion assay (AGID or Coggins test) and enzyme-linked immunosorbent assay (ELISA), both of which are based on the detection of serum antibodies to EIAV p26 or gp45 (5, 13). Attenuated proviral vaccines containing a mutated S2 accessory gene still express the full complement of EIAV core and envelope proteins. Therefore, horses inoculated with EIAVUKΔS2 become seropositive in...
currently used diagnostic assays for EIAV infection. This is not a current problem but would become an issue if the EIAV UK/H9004 S2 vaccine were approved for use in horses, as it would complicate current regulatory procedures used to control infection and disease in horses.

To address this important regulatory issue, we sought to develop and evaluate the reliability of an EIAV S2-based serological assay in detecting EIAV-infected horses and differentiating them from vaccinated horses inoculated with EIAV UK/H9004 S2. We describe in the present report the engineering and production of a novel recombinant S2 protein antigen and the optimization of an S2 antigen-based ELISA for the detection of reactive antibodies in serum from experimentally and field-infected horses, without reactivity to antibodies in immune serum from horses inoculated with the attenuated EIAV vaccine. These data demonstrate for the first time the potential of an S2-based serological diagnostic assay to accurately identify and differentiate horses infected with wild-type EIAV from horses inoculated with S2 attenuated vaccine virus.

MATERIALS AND METHODS

Subcloning of S2 from EIAV UK. To achieve adequate levels of recombinant S2 protein production, a series of cloned S2 gene constructs were developed and evaluated for protein expression properties. These constructs are summarized in Table 1.

The pcDNAS2 construct (Table 1) was developed to express histidine peptide-tagged S2 protein from the native EIAV S2 gene sequences. For construction of pcDNAS2 expression vector (Table 1), the EIAV S2 gene sequence was amplified from EIAV UK (6) by PCR with Taq DNA polymerase (Invitrogen, Carlsbad, Calif.) with primers FS2 (GGATATTTGGTAAAGGG) and RS2 (TCATTCTCTTGGTCTCTTG). The PCR products were ligated into pcDNAHisMAX (Invitrogen) by TA cloning, and a six-histidine peptide was fused to the 5′ end of S2. This vector allows for expression of a His6-tagged S2 protein from the cytomegalovirus (CMV) and phage T7 promoters.

The pOptS2 construct was designed to express recombinant histidine peptide-tagged S2 from an S2 gene modified for codon optimization. For construction of pOptS2 expression vector, the Codon Usage Database (www.kazusa.or.jp) was used to optimize the codons of the EIAV UK S2 gene sequence for maximum expression in mammalian and Escherichia coli cells (Midland Reagent Company, Midland, Tex.). This synthesized codon-optimized S2 sequence (Fig. 1) was subcloned into the pcDNA3.1/mycHis vector (Invitrogen) by using the NheI and HindIII restriction enzyme sites, and a His6 peptide was fused to the 3′ end of S2. This vector allows for expression of the histidine peptide-tagged S2 from the CMV and phage T7 promoters.

The pOptS2GFP construct (Table 1) was designed to express recombinant histidine peptide-tagged S2 as a fusion protein with enhanced green fluorescent protein (EGFP). To construct the pOptS2GFP expression vector, the codon-optimized S2 gene sequence was amplified from the pOptS2 plasmid by PCR with the Pfu Turbo polymerase (Stratagene, La Jolla, Calif.) and primers FOptS2 (GGCCGTGCTAGCATGGGCCTGTTCGGCAAG) and ROptS2 (CTAGTGGTTACCCTTCTTGGTCTCCTGCTTGCGG). The digested fragments were ligated into the pEGFP-N3 plasmid (Clontech, Palo Alto, Calif.), which contained the EGFP, through the NheI and KpnI restriction enzyme sites. Expression from the pOptS2GFP vector was driven by the CMV promoter, and the EIAV S2-EGFP fusion protein was designated HS2G.

In addition to the expression plasmids described above for expression of mammalian cells, expression plasmids were also developed for production of recombinant proteins in E. coli. In the construction of the p80LOS2GFP expression vector for expressing the HS2G fusion protein in E. coli, the codon-optimized S2 gene sequence was amplified from the pOptS2 plasmid by PCR with primers FS2SacI (GTACCAGAAGCAGGCGCGGCGGCGGCAAG) and

| Antigen | Plasmid | Promoter | Primer | Length (bp) |
|---------|---------|----------|--------|-------------|
| EIAV UK S2 | pcDNAS2 | CMV | FS2 | 210 |
| EIAV UK S2 | pcDNAS2 | VV-T7 | FS2 | 210 |
| OptS2 | pOptS2 | CMV | FOptS2 | 210 |
| OptS2 | pOptS2 | VV-T7 | FOptS2 | 210 |
| OptS2GFP | pOptS2GFP | CMV | FOptS2 | 930 |
| EGFP | pEGFP-N3 | CMV | FKpGFP | 732 |

a F, forward primer; R, reverse primer.

b VV, vaccinia virus.
For analysis of intracellular S2 protein expression by Western blotting, cell lysates were first resolved on a precast 4% to 20% polyacrylamide gel (Bio-Rad) and then transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore, Billerica, Mass.) at 100 V for 1 h. The membrane was first blocked for 1 h with Tween-phosphate-buffered saline (PBS; 1.37 M NaCl; 27 mM KCl, 81 mM NaH2PO4·12H2O, 15 mM KH2PO4, and 0.1% Tween 20) containing 5% powdered nonfat milk and then incubated with primary antibodies (rabbit anti-S2 [1:200] or GFP monoclonal antibody [1:2,000; Promega, Madison, Wis.]) at room temperature for 1 h. Following three 5-min washes with Tween-PBS, membranes were incubated with secondary antibodies (horseradish peroxidase [HRP]-conjugated anti-rabbit IgG [1:10,000] or HRP-conjugated anti-mouse IgG [1:4,000]) (Sigma, St. Louis, Mo.) for 1 h and then treated with three final washes. Specific proteins were visualized with Western lightning chemiluminescence reagent plus substrate mixture (Perkin-Elmer,Boston, Mass.).

Quantification of S2-specific mRNA by RT-PCR assay. To evaluate the transcription levels of the various S2 genes constructs in transfected cells, COS7 cells were transfected with the S2 expression plasmids outlined in Table 1 and harvested 48 h posttransfection. The mRNA from transfected cells was then prepared using a one-step reverse transcription-PCR (RT-PCR) system (Pierce, Rockford, Ill.), and the assay was performed according to the manufacturer's instructions. Levels of β-actin mRNA were assayed separately as an internal reference. The β-actin primers used were: sense primer (5'-TCTAGGATATGCCTGCGG-3') and antisense primer (5'-GAGTCACCTACAGCTGCCA-3'). Levels of mRNA in the cell lysates were measured and quantified using Kodak 1-D gel electrophoresis analysis software.

Sources of immune sera. A panel of 65 immune serum samples was obtained from ELAV test-positive field horses and from horses experimentally infected with the attenuated ELAVgAdS2 vaccine strain (19). All field and experimental horses were shown to be seropositive in United States Department of Agriculture reference AGID (Coggins) assays. Control horse sera were obtained from uninfected horses and confirmed as seronegative in the Coggins AGID assay. Rabbit antisera to S2 protein was produced by immunization of rabbits with a synthetic S2 peptide preparation.

Detection of S2-specific serum antibodies by ELISA. Serum reactivity to the recombinant purified HS2G protein was assayed in a standard ELISA format and compared to reactivity in an affinity ELISA format. For the standard ELISA procedure, 96-well microplates (Immunolon 2; Dynatech Laboratories, Chantilly, Va.) were coated overnight with HS2G protein diluted in 0.05 M sodium bicarbonate buffer (pH 9.6) to a final concentration of 10 μg/ml. The following day the plates were washed using PBS-Tween (0.05%) and blocked with blocking buffer (PBS containing 5% calf serum, 5% nonfat dry milk, and 0.25% Tween 20) for 1 h at room temperature. After washing with PBS-Tween, 100 μl of a 1:2.5-diluted horse serum was added to the plates at room temperature for 1 h at room temperature. Following five washes with PBS-Tween, 100 μl of the substrate 3,3′,5′-tetramethylbenzidine (TMB) (Pierce) was added to each well, and color was allowed to develop for 10 min before the reaction was stopped by the addition of 100 μl of 2 M H2SO4. The extent of the reactions was determined spectrophotometrically with a microplate reader (MR5000; Dynatech Laboratories Inc.) at 450 nm with the software BIOLINX. In the analyses with rabbit anti-S2 antibody as the primary antibody in the ELISA, the rabbit immune serum was 1:200 diluted in blocking buffer, and anti-rabbit IgG HRP-conjugated antibody diluted 1:10,000 (Sigma) was used as the secondary antibody.

For the affinity ELISA procedure (NN-ELISA), HS2G protein was diluted in PBS-bovine serum albumin (BSA; 0.2%) buffer to a final concentration of 10 μg/ml. Ni-NTA HisSorb plates (Qiagen) were coated with 100 μl of diluted ELISA buffer by gentle shaking for 1 h at room temperature. Blocking buffer (0.1% BSA) was then added to the plates to block the nonspecific sites. The plates were washed five additional times with PBS-bovine serum albumin (BSA; 0.5%) buffer. To remove unbound material, the plates were washed with 0.1% BSA in PBS and dried at room temperature. The plates were then blocked with 1% BSA in PBS and incubated with 100 μl of diluted anti-rabbit IgG HRP-conjugated antibody (Sigma) for 1 h at room temperature. Following a final PBS-Tween wash, 100 μl of the substrate 3,3′,5′-tetramethylbenzidine (TMB) (Pierce) was added to each well, and color was allowed to develop for 10 min before the reaction was stopped by the addition of 100 μl of 2 M H2SO4. The extent of the reactions was determined spectrophotometrically with a microplate reader (MR5000; Dynatech Laboratories Inc.) at 450 nm with the software BIOLINX. In the analysis the rabbit anti-S2 antibody as the primary antibody in the ELISA, the rabbit immune serum was 1:200 diluted in blocking buffer, and anti-rabbit IgG HRP-conjugated antibody diluted 1:10,000 (Sigma) was used as the secondary antibody.

Horse sera known to be negative or positive for ELAV antibodies were tested in parallel in each assay as controls. Values for test serum samples were derived by subtracting the absorption values obtained at an optical density at 450 nm (OD450) of the negative control serum sample. The cutoffs of HS2G ELISA and p26 ELISA were 0.3 and 0.1, respectively. We defined these cutoff levels based on our preliminary ELISA study with a panel of serum samples known to be seropositive and seronegative. All data were representative of three independent experiments.
Production of recombinant EIAV p26. For the production of EIAV p26 capsid antigen, the p26 gene of EIAV was subcloned from EIAV UK, modified with a His6 tag for purification, and inserted into the pQE-80L expression plasmid as described above. E. coli cells were transformed with the p26 expression plasmid and precultured in 5 ml of LB medium supplemented with 2 g of glucose/liter and 100 μg of ampicillin/ml overnight at 37°C with vigorous shaking. The overnight culture was used to inoculate 100 ml of LB medium. The expression of p26 protein was induced by addition of 1 mM IPTG to the log-phase culture. The bacterial pellets were harvested at 4 h postinduction by centrifugation at 6,000 x g for 10 min. The cells were lysed with a bacterial protein extraction reagent (Pierce), filtered through 0.45-μm-pore-size filters, and applied to a column equilibrated with the binding buffer (10 mM Na2HPO4·2H2O, 10 mM NaH2PO4·H2O, and 0.5 M NaCl [pH 7.4]) according to the manufacturer’s directions (Amersham). The column was then washed with the wash buffer containing 10 mM Na2HPO4·2H2O, 10 mM NaH2PO4·H2O, 0.5 M NaCl, and 10 mM imidazole (pH 7.4) at a flow rate of 5 ml/min. The p26 protein was eluted using the elution buffer (10 mM Na2HPO4·2H2O, 10 mM NaH2PO4·H2O, 0.5 M NaCl, and 250 mM imidazole [pH 7.4]). To examine the quality of the purified p26 protein, fractions collected from the OD280 determinations were measured for their protein content and analyzed by SDS-PAGE and Western blot assays.

Detection of serum antibodies to p26. For p26-specific NN-ELISA procedures, purified recombinant p26 antigen was dissolved in PBS-BSA (0.2%) to a final concentration of 5 μg/ml and horse serum was diluted to 1:200 in PBS-BSA. The other procedures for the p26 assay were as described above for the NN-ELISA to HS2G.

RESULTS

Expression of recombinant S2 antigen in mammalian cells. To achieve reproducible and adequate expression of a recombinant S2 antigen, a series of S2 gene expression constructs were developed and evaluated. Comparative transcription and protein expression levels from this series of antigen expression constructs are summarized in Fig. 2. Our first attempts to express the EIAV S2 protein began with the pcDNAS2 expression plasmid containing the EIAVUK S2 sequence. This plasmid was transfected into the mammalian cell line COS7, and mRNA expression (Fig. 2A) and recombinant S2 protein production (Fig. 2B) were evaluated by RT-PCR and Western blot analyses, respectively. These assays indicated a lack of detectable S2 protein expression in the transfected cells, despite the presence of detectable S2 mRNA from both the T7 and CMV promoters.

The transcription and translation efficiencies of the pcDNAS2 expression plasmid were next evaluated as a possible reason for lack of expression. It was determined that the equine S2 arginine codons AGG and AGA are less frequently utilized in COS and E. coli cells, probably due to a lower abundance of corresponding tRNAs. Accordingly, we constructed the pOptS2 expression plasmid by using the optimized EIAVUK S2 (OptS2) codon sequence (Fig. 1), and following transfection of COS7 cells, S2 mRNA and protein expression were evaluated using both the T7 and CMV promoters (Fig. 2). The results of these assays indicated that the codon optimization enhanced S2 mRNA expression by the T7 or CMV promoters by 3.2-fold compared to the parental S2 gene but that this increased mRNA expression still failed to yield detectable S2 protein in the transfected cells.

These observations suggested a potential instability of the expressed S2 protein in transfected cells. Small peptides are often degraded in vivo, and as S2 has a molecular mass of only
The S2 protein in COS7 cells. The HS2G fusion protein successfully prevented degradation of EGFP. All combined, these data suggest that construction of verified using mouse anti-EGFP as a primary antibody (data not shown). These results indicate that the HS2G fusion protein is successfully recognized by anti-S2 antibodies and anti-EGFP. All combined, these data suggest that construction of the HS2G fusion protein successfully prevented degradation of the S2 protein in COS7 cells.

Production and purification of S2 antigen from E. coli. In parallel with our development of an S2 antigen expression system in mammalian COS7 cells as a basis for investigating S2 function in viral replication, we also examined the expression levels of the various recombinant antigen constructs in E. coli to provide a high-yield production system for a potential diagnostic antigen. For these purposes, the various engineered S2 genes were cloned into the prokaryotic expression plasmid pQE-80L, and their expression levels were evaluated in transformed E. coli. As observed with expression in COS7 cells, we observed in preliminary experiments that there was no detectable S2 antigen produced in E. coli cells transformed with either the parental S2 gene or the codon-optimized S2 gene, while substantial levels of the recombinant HS2G fusion protein were detected in E. coli transformed with the p80LOS2GFP expression plasmid (data not shown). Figure 3 summarizes representative data on HS2G expression in transformed E. coli cells. As described in Materials and Methods, p80LOS2GFP plasmid was transformed into E. coli XL cells. The expression of HS2G was verified by flow cytometric analysis as shown in Fig. 3A. Approximately 60% of recombinant cells expressed HS2G protein at 4 h postinduction with IPTG. This experimental study also demonstrated the optimal time frame for cell harvest to be 4 h post-IPTG induction because it presented the highest expression level of the fusion protein over the postinduction period 0 to 5.5 h.

To evaluate the possible methods for extracting the HS2G protein from the transformed bacteria, soluble proteins were extracted from a transformed E. coli cell pellet by lysis with a commercially available bacterial protein extraction reagent (Pierce), filtered, and evaluated by SDS-PAGE (lane 1, Fig. 3B) and Western blotting (lane 4, Fig. 3C), respectively. To extract insoluble proteins from the transformed cells, an identical E. coli cell pellet was lysed using 8 M urea (Materials and Methods) and the extracted proteins were evaluated by both SDS-PAGE (lane 2, Fig. 3B) and Western blotting (lane 5, Fig. 3C). The data in Fig. 3B and C indicate that the HS2G protein expressed in transformed E. coli was predominantly in the insoluble fraction of the cell pellet, thus requiring 8 M urea for extraction. The extracted HS2G protein was then purified from the total cellular extract by affinity chromatography on a nickel-charged IMAC column according to the manufacturer’s procedures. This purification procedure yielded a total of about 4.5 mg of HS2G protein from 500 ml of culture broth without detectable contaminating proteins (lane 3, Fig. 3B, and lane 6, Fig. 3C). To address the concern that denatured HS2G protein may be less reactive with serum antibodies and thus a less useful antigen in an ELISA, the affinity column-purified denatured HS2G protein was refolded by stepwise dialysis, as described in Materials and Methods. Typically, the final refolded HS2G protein yield was about 50% of the starting protein antigen recovered from the affinity column.

Development and optimization of an HS2G-based ELISA. Having achieved the necessary goal of S2 antigen production, we next sought to evaluate the utility of an S2-based serological assay to detect EIAV-infected horses and differentiate them from horses inoculated with attenuated vaccine virus lacking S2 expression. To address this issue, the reactivity of the purified HS2G protein was initially examined in a standard ELISA for reactivity with a reference rabbit immune serum and with a reference EIAV-positive horse serum. The data in Fig. 4A demonstrate that HS2G protein displayed significant reactivity to the rabbit anti-S2 polyclonal antibodies, but relatively low-level reactivity was observed with the reference horse serum. The relatively low level of S2-specific antibody reactivity was consistent with previous reports of low titers of antibody to S2-specific synthetic peptides in ELISA formats (2, 24).

To increase the sensitivity of the HS2G in vitro ELISA system, we modified the standard ELISA by employing Ni-NTA HisSorb 96-well ELISA plates to affinity adsorb the HS2G protein antigen through the His6 tag of the fusion protein. Thus, HS2G protein was applied as a coating to the Ni-NTA plates, and the reactivity of the adsorbed antigen with the reference rabbit and horse immune sera was evaluated. As shown in Fig. 4B, a level of reactivity of HS2G to rabbit anti-S2 antibodies was achieved in NN-ELISA similar to that in the standard ELISA above. In contrast, however, the reactivity of the horse immune serum in the NN-ELISA was increased by about threefold compared to the standard ELISA format, while the background reactivity with the negative control horse serum was reduced by about twofold in the NN-ELISA compared to the standard ELISA procedure. These data indicated the potential of the NN-ELISA as a format for further development and assessment.

To evaluate in more detail the sensitivity and specificity of HS2G protein and to validate the application of the NN-ELISA system for identifying EIAV-infected horses, we appraised the reactivity of a coded panel of reference sera from EIAV-negative horses, EIAV-infected horses, and EIAV(ΔS2)-vaccinated horses to HS2G in the NN-ELISA system and compared these results to those obtained from the standard ELISA system (Fig. 5). The results indicated that the NN-ELISA was superior to the standard ELISA procedure in both the sensitivity and the specificity of serum reactivity. For example, all five of the control serum samples from uninfected horses were seronegative in the NN-ELISA, while one of the uninfected horse serum samples displayed reactivity in the standard ELISA format. Similarly, all eight of the immune serum samples from EIAV(ΔS2)-vaccinated horses were seronegative in the NN-ELISA format, while
two of these vaccinated serum samples displayed low levels of seroreactivity in the standard ELISA. Taken together, these observations demonstrate the absence of nonspecific seroreactivity to the HS2G antigen in the context of the NN-ELISA format, providing the specificity required to avoid false indications of EIAV-infected horses. In addition to the superior specificity, the NN-ELISA also yielded substantially higher levels of reactivity of all six reference immune serum samples from EIAV-infected horses.
Having identified the NN-ELISA as the superior method for use in detecting EIAV-infected horses and distinguishing them from uninfected horses and EIAV \_UK\_ΔS2-vaccinated horses. All seven of the uninfected horse serum samples were found to be nonreactive in the NN-ELISA format. Similarly, all 21 of the immune serum samples from the vaccinated horses failed to display any detectable reactivity in the NN-ELISA, confirming the lack of S2-specific antibodies in these horses. In contrast to these seronegative sera, the NN-ELISA identified 16 of 18 EIAV-infected horses as seropositive, with two infected horse serum samples (samples 11 and 13) failing to display serum antibody reactivity to the HS2G antigen.

Combining the NN-ELISA serological data presented in Fig. 5 and 6, it can be calculated that the NN-ELISA was 100% accurate in identifying S2 antibody-negative serum from uninfected horses (12 of 12); there were no detectable false positives. The NN-ELISA achieved 92% sensitivity in identifying EIAV-infected horses (22 of 24) by S2 serum antibody reactivity. While the present studies utilize a relatively limited number of test samples, the data clearly indicate the sensitivity and specificity of the NN-ELISA as a potential diagnostic assay for detecting EIAV-infected horses and distinguishing them from horses inoculated with attenuated EIAV vaccine strains lacking S2 gene expression.

**DISCUSSION**

EIAV infection of horses is a global veterinary concern and a disease for which there is currently no vaccine. Therefore, control of this viral infection is achieved in certain countries by the destruction of EIAV-infected horses identified by serological diagnostic assays that detect antibodies to viral core or envelope proteins. The development of an effective vaccine to lentiviruses in general and EIAV in particular has been inhibited by a confounding array of immune evasion and persistence mechanisms intrinsic to these viruses, as frequently highlighted by AIDS vaccine efforts. The implementation of an effective EIAV vaccine, however, is even further complicated by present regulatory policies in certain countries that will require an ability to distinguish infected from vaccinated horses. We have previously described an experimental live attenuated EIAV vaccine, EIAV \_UK\_ΔS2, which appears to achieve a high level of protection from exposure to a closely related virulent virus challenge (19). Thus, the goal of the present study was to develop a companion serological diagnostic assay that is seropositive for infected horses while being seronegative for uninfected and vaccinated horses. The data presented here indicate that a diagnostic assay based on detecting serum antibody reactivity to EIAV S2 antigen has the required specificity and sensitivity for further development for application in detecting EIAV-infected horses and distinguishing them from vaccinated horses by using a practical and simple ELISA format that can be optimized for high throughput.

The first challenge in developing a satisfactory diagnostic test was to produce adequate quantities of EIAV S2 protein for use as antigen substrate. Previous studies from our lab indicated serum antibody reactivity with small synthetic pep-
tides derived from the S2 protein, but the level of peptide reactivity was very low, indicating the need to employ the whole protein as antigen (unpublished data). The S2 protein is not found in virus particles and is expressed at relatively low levels in infected cells. Therefore, the only practical approach to obtaining reasonable quantities was to clone the S2 gene and produce recombinant S2 protein. However, preliminary experiments in our lab indicated a frustrating inability to express S2 protein in various standard prokaryotic and eukaryotic protein expression systems. The studies described here exemplify the necessary evolution of S2 expression systems to finally achieve adequate levels of antigen production. These studies demonstrated that the lack of S2 expression was not due to deficiencies in transcription or translational efficiency,

FIG. 5. Comparison of HS2G reactivities to horse sera with the standard ELISA (Std-ELISA) and NN-ELISA (HS2G-NN) formats. Equal amounts of purified HS2G protein were analyzed in parallel in either the standard or affinity ELISA format against a panel of reference horse serum samples as described in Materials and Methods. Negative, uninfected horses shown to be seronegative in standard EIAV p26 diagnostic assays; positive, virus-infected horses shown to be seropositive in standard EIAV p26 diagnostic assays; vaccinated, horses experimentally inoculated with attenuated S2 vaccine strains of EIAV and seropositive in standard p26 diagnostic assays. All horse serum samples were used at a dilution of 1:25.

FIG. 6. Evaluation of HS2G NN-ELISA to identify serum samples from uninfected, infected, and vaccinated horses. The panel of reference serum samples included uninfected horses (samples 1 to 7), horses persistently infected with EIAV (samples 8 to 25), and horses vaccinated with attenuated S2 vaccine strains (samples 26 to 46). All horse sera were used at a dilution of 1:25 in HS2G NN-ELISA. The affinity ELISA procedures for detecting serum antibodies to the HS2G antigen or the EIAV p26 protein were as described in Materials and Methods.
as even codon-optimized constructs failed to produce detectable protein in transfected cells, despite substantial levels of S2-specific mRNA. Deducing that the lack of S2 protein expression may be due to rapid degradation of the recombinant protein, we achieved reproducible recombinant expression by fusing the S2 protein to the carrier EGFP, a fluorescent marker that can be employed in studies to elucidate S2 function in infected cells during viral replication. It seems feasible that other carrier proteins with advantageous properties for purification or assay development may be substituted for the EGFP employed here. In addition to the fusion to a carrier protein for stability, the final HS2G protein was also engineered to contain an oligonucleotide-histidine tag for purification and for affinity ELISA. We demonstrate here that the HS2G protein can be produced in either mammalian or prokaryotic cell expression systems. Thus, the HS2G protein construct provides a reasonable system for diagnostic antigen production.

The second challenge was to identify the optimal serological assay format for developing and evaluating a diagnostic assay based on serum antibody reactivity to the HS2G antigen. Towards this goal, we compared standard and affinity ELISA formats for their sensitivity and specificity of serum antibody reactivity to the HS2G antigen. An interesting observation from these studies was that the level of serum antibody reactivity was similar in both assay formats for an S2-specific rabbit antisera but markedly different in the level of antibody reactivity observed with immune serum from infected horses. With the equine serum, the affinity ELISA displayed substantially higher reactivity and lower backgrounds than did the standard ELISA, yielding the potential for a more sensitive and specific diagnostic assay format. Therefore, the affinity ELISA procedure was selected as the format for further evaluation. It seems reasonable that the HS2G antigen could be employed in other immunosassays, such as immunoblotting, AGID, competitive ELISA, or antigen capture (5, 11–13, 17, 23, 27).

To assess the accuracy of the affinity HS2G ELISA for detecting S2-specific serum antibodies in infected horses, we assayed a total of 65 reference serum samples from uninfected horses, from experimentally and field-infected horses, and experimentally vaccinated horses. The results of these experiments demonstrated that the affinity HS2G ELISA achieved a 92% accuracy (22 of 24 positive) in identifying EIAV-infected horses by S2-specific serum antibody reactivity. In contrast, there was no detectable S2-specific antibody reactivity in serum from uninfected horses and in horses inoculated with the attenuated EIAV vaccine. Based on these observations with this particular set of serum samples, we conclude that the affinity HS2G ELISA has the prerequisite sensitivity and specificity for further development and evaluation as a diagnostic assay that can accurately identify EIAV-infected horses and distinguish them from vaccinated horses. Future evaluations of the S2 diagnostic assay will need to determine specificity and sensitivity in large panels of horse serum samples taken from geographically diverse areas of the world, to determine the kinetics of the development and stability of S2-specific antibody responses during the course of EIAV infection, and to optimize assay formats to meet practical application.

It should be noted here that the diagnostic serological assays to detect serum antibodies to S2 may also be used to distinguish EIAV-infected horses from horses immunized with other EIAV vaccine formats, including inactivated viral particles, envelope or core protein subunit vaccines, or DNA or live vector vaccines expressing viral envelope or core proteins. None of these immunization regimens are expected to elicit host antibody responses to the S2 protein, thus providing a serological means of distinguishing infected from vaccinated horses. To increase the confidence levels of an S2 serological assay, it is possible to combine the detection of S2-specific antibodies with a complementary detection of specific antibodies to appropriate EIAV virion proteins (e.g., p26) for infected horses and detection of antibodies to an antigenic marker incorporated in the EIAV vaccine. However, all of these parameters are within the capabilities of present serological diagnostics technologies and readily adapted to the EIAV system.

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REFERENCES

1. Archambault, D., Z. M. Wang, J. C. Laca, A. Gazit, A. Yaniv, J. E. Dahlberg, and S. R. Tronick. 1989. Development of an enzyme-linked immunosorbent assay for equine infectious anemia virus detection using recombinant Pr55agp. J. Clin. Microbiol. 27:1167–1173.
2. Ball, J. M., K. E. Rushlow, C. J. Issel, and R. C. Montelaro. 1992. Detailed mapping of the antigenicity of the surface unit glycoprotein of equine infectious anemia virus by using synthetic peptide strategies. J. Virol. 66:732–742.
3. Birkelt, A. J., B. Yelamos, I. Rodriguez-Crespo, F. Gavilanes, and D. L. Peterson. 1997. Cloning, expression, purification, and characterization of the major core protein (p26) from equine infectious anemia virus. Biochim. Biophys. Acta 1339:62–73.
4. Carroll, R., and D. Derse. 1993. Translation of equine infectious anemia virus bicistronic tat-rev mRNA requires leaky ribosome scanning of the tat CUG initiation codon. J. Virol. 67:1433–1440.
5. Coggins, L., and N. L. Norcross. 1970. Immunodiffusion reaction in equine infectious anemia. Cornell Vet. 60:330–335.
6. Cook, R. F., C. Leroux, S. J. Cook, S. J. Cook, S. L. Berger, D. L. Lichtenstein, N. N. Ghabrial, R. C. Montelaro, and C. J. Issel. 1998. Development and characterization of an in vitro pathogenic molecular clone of equine infectious anemia virus. J. Virol. 72:1383–1393.
7. Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss. 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA 83:8122–8126.
8. Hammond, S. A., S. J. Cook, D. Falo, Sr., C. J. Issel, and R. C. Montelaro. 1999. A particulate viral protein vaccine reduces viral load and delays progression to disease in immunized ponies challenged with equine infectious anemia virus. Virology 254:47–49.
9. Hammond, S. A., F. Li, B. M. McKeon, Sr., J. J. Cook, C. J. Issel, and R. C. Montelaro. 2000. Immune responses and viral replication in long-term inapparent carrier ponies inoculated with equine infectious anemia virus. J. Virol. 74:5966–5981.
10. Henderson, L. E., R. C. Sowder, G. W. Smythers, and S. Oroszlan. 1987. Chemical and immunological characterizations of equine infectious anemia virus gag-encoded proteins. J. Virol. 61:1116–1124.
11. Hussain, K. A., C. J. Issel, K. L. Schnorr, P. M. Rwambo, and R. C. Montelaro. 1987. Antigenic analysis of equine infectious anemia virus (EIAV) variants by using monoclonal antibodies: epitopes of glycoprotein gag Pr55 of EIAV stimulate neutralizing antibodies. J. Virol. 61:2956–2961.
12. Hussain, K. A., C. J. Issel, K. L. Schnorr, P. M. Rwambo, M. West, and R. C. Montelaro. 1988. Antigenic mapping of the envelope proteins of equine infectious anemia virus: identification of a neutralization domain and a conserved region on glycoprotein 90. Arch. Virol. 98:213–224.
13. Issel, C. J., and W. V. Adams, Jr. 1982. Detection of equine infectious anemia virus in a horse with an equivocal agar gel immunodiffusion test reaction. J. Am. Vet. Med. Assoc. 180:276–278.
14. Issel, C. J., and L. Coggins. 1979. Equine infectious anemia: current knowledge. J. Am. Vet. Med. Assoc. 174:727–733.
15. Issel, C. J., and R. F. Cook. 1993. A review of techniques for the serologic diagnosis of equine infectious anemia. J. Vet. Diagnost. Invest. 5:137–141.
16. Issel, C. J., D. W. Hovhov, D. F. Lea, W. V. Adams, Jr., S. D. Hagius, J. M. McManus, A. C. Allison, and R. C. Montelaro. 1992. Efficacy of inactivated whole-virus and subunit vaccines in preventing infection and disease caused by equine infectious anemia virus. J. Virol. 66:3398–3408.
17. Knowles, D. P., Jr., and J. R. Gorham. 1993. Advances in the diagnosis of some parasitic diseases by monoclonal antibody-based enzyme-linked immunosorbent assays. Rev. Sci. Technol. 12:425–433.
18. Kong, X. G., H. Pang, T. Sugiura, H. Sentsui, T. Onodera, Y. Matsumoto, and H. Akashi. 1997. Application of equine infectious anemia virus core proteins produced in a baculovirus expression system to serological diagnosis. Microbiol. Immunol. 41:975–980.
19. Li, F., J. K. Craigo, L. Howe, J. D. Steckbeck, S. Cook, C. Issel, and R. C. Montelaro. 2003. A live attenuated equine infectious anemia virus proviral vaccine with a modified S2 gene provides protection from detectable infection by intravenous virulent virus challenge of experimentally inoculated horses. J. Virol. 77:7244–7253.
20. Li, F., C. Leroux, J. K. Craigo, S. J. Cook, C. J. Issel, and R. C. Montelaro. 2000. The S2 gene of equine infectious anemia virus is a highly conserved determinant of viral replication and virulence properties in experimentally infected ponies. J. Virol. 74:573–579.
21. Li, F., B. A. Puffer, and R. C. Montelaro. 1998. The S2 gene of equine infectious anemia virus is dispensable for viral replication in vitro. J. Virol. 72:8344–8348.
22. Mia, A. S., M. M. Tierney, and D. M. Krevvak. 1982. Detection of equine infectious anemia antibody by a rapid microenzyme linked immunosorbent assay (ELISA). Proc. Annu. Meet. Am. Assoc. Vet. Lab. Diagn. 25:159–166.
23. Montelaro, R. C., W. G. Robey, M. D. West, C. J. Issel, and P. J. Fischinger. 1988. Characterization of the serological cross-reactivity between glycoproteins of the human immunodeficiency virus and equine infectious anemia virus. J. Gen. Virol. 69:1711–1717.
24. Montelaro, R. C., W. G. Robey, M. D. West, C. J. Issel, and P. J. Fischinger. 1993. Equine retroviruses, vol. 2. Plenum Press, New York, N.Y.
25. Raabe, M. L., C. J. Issel, S. J. Cook, R. F. Cook, B. Woodson, and R. C. Montelaro. 1998. Immunization with a recombinant envelope protein (gp10) of EIAV produces a spectrum of vaccine efficacy ranging from lack of clinical disease to severe enhancement. Virology 245:151–162.
26. Raabe, M. L., C. J. Issel, and R. C. Montelaro. 1999. In vitro antibody-dependent enhancement assays are insensitive indicators of in vivo vaccine enhancement of equine infectious anemia virus. Virology 259:416–427.
27. Reischl, U. 1996. Application of molecular biology-based methods to the diagnosis of infectious diseases. Front. Biosci. 1:e72–e77.
28. Schiltz, R. L., D. S. Shih, S. Rasty, R. C. Montelaro, and K. E. Rushlow. 1992. Equine infectious anemia virus gene expression: characterization of the RNA splicing pattern and the protein products encoded by open reading frames S1 and S2. J. Virol. 66:3455–3465.
29. Sellon, D. C., F. J. Fuller, and T. C. McGuire. 1994. The immunopathogenesis of equine infectious anemia virus. Virus Res. 32:111–138.
30. Shane, B. S., C. J. Issel, and R. C. Montelaro. 1984. Enzyme-linked immunosorbent assay for detection of equine infectious anemia virus p26 antigen and antibody. J. Clin. Microbiol. 19:351–355.
31. Soutullo, A., V. Verwimp, M. Riveros, R. Pauli, and G. Tonarelli. 2001. Design and validation of an ELISA for equine infectious anemia (EIA) diagnosis using synthetic peptides. Vet. Microbiol. 79:111–121.
32. Suzuki, T., S. Ueda, and T. Samejima. 1982. Enzyme-linked immunosorbent assay for diagnosis of equine infectious anemia. Vet. Microbiol. 7:307–315.
33. Wang, S. Z., K. E. Rushlow, C. J. Issel, R. F. Cook, S. J. Cook, M. L. Raabe, Y. H. Chong, L. Costa, and R. C. Montelaro. 1994. Enhancement of EIAV replication and disease by immunization with a baculovirus-expressed recombinant envelope surface glycoprotein. Virology 199:247–251.