Comparison of antibody detection assays for the diagnosis of equine herpesvirus 1 and 4 infections in horses

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Objective—To compare methods of detecting equine herpesvirus type 1 (EHV1)- and EHV4-specific antibodies in horse sera.

Sample Population—33 acute and convalescent serum samples from experimentally or naturally infected horses after confirmed EHV1 or EHV4 infection.

Procedure—For each sample, serum antibody titers against EHV1 and EHV4 were determined by use of virus neutralization (VN) and complement fixation (CF) assays. The ELISA absorbance values for each serum sample were determined against the EHV1 and EHV4 recombinant ELISA antigens. Values obtained for acute and convalescent sera in each assay were compared.

Results—Following experimental infection of foals, EHV1 or EHV4 antibodies that were specific for the inoculating virus were detected only by use of the ELISA. Results of VN and CF assays indicated that the foals seroconverted to EHV1 and EHV4 following infection with EHV4 only. After EHV1-induced abortion, myeloencephalitis, or respiratory tract disease, the VN and CF assay results revealed seroconversion to EHV1 and EHV4, whereas results of the ELISA revealed seroconversion to EHV1 only. Similarly, after confirmed EHV4-induced respiratory tract disease, increases in EHV4-specific antibodies were detected only by use of the ELISA with no indication of an increase in EHV1 antibodies. The CF and, to a lesser degree, VN assays revealed that seroconversion to EHV1 and EHV4 occurred between the time of obtaining acute and convalescent serum samples.

Conclusions and Clinical Relevance—The EHV1/EHV4 type-specific antibody ELISA clearly identifies horses that have been infected with EHV1 or EHV4 by use of acute and convalescent sera. Results of VN and CF assays indicate that cross-reactive antibodies greatly limit their use. (*Am J Vet Res* 2005;66:921–928)

Equine herpesvirus 1 (EHV1) and **EHV4** are alphaherpesviruses that are substantial causes of economic loss to the horse industry worldwide. Equine herpesvirus 1 causes abortion, whereas EHV4 is a major cause of respiratory tract disease in young horses.[1,2] Equine herpesvirus 1 also causes respiratory tract disease on primary infection and myeloencephalitis, whereas EHV4 has only occasionally been associated with abortions.[2-7] Equine herpesvirus 1 and EHV4 are closely related alphaherpesviruses that share extensive genomic sequence homology[8,9] and have broad antigenic cross-reactivity.[1,10-13]

Diagnosis of viral infections occurs either directly through the detection of virus in clinical samples or indirectly through the demonstration of an increase in virus-specific antibody between acute and convalescent sera obtained 10 to 14 days apart. Direct detection of EHV1 or EHV4 in samples has evolved over time from traditional virus isolation in cell culture to the development of many different type-specific **polymerase chain reaction (PCR)** assays.[14-19] Similarly, the detection of EHV1 or EHV4 antibody has developed from the detection of viral-neutralizing or complement-fixing antibodies to the development of an EHV1/EHV4 type-specific ELISA that is based on recombinant antigens of the variable region at the C terminus of **glycoprotein G (gG)** of EHV1 and EHV4.[20,21]

Diagnostic technologies continue to develop with the aim of providing more rapid, sensitive, and specific tests. Clearly, the development of EHV1- and EHV4-specific PCR assays for virus detection enables rapid identification (within 24 hours) of the specific virus causing disease (EHV1 or EHV4), compared with virus isolation that may take upwards of 2 days to determine the presence or absence of an untyped virus. The relative merits of technologies for the detection of EHV1- or EHV4-specific antibodies are perhaps less obvious, given that there has not been any direct comparison of the different serologic tests published to date. A decade has elapsed since the development of a type-specific ELISA that has been used in numerous epidemiologic studies to differentiate horses infected with EHV1 from those with serum antibodies against EHV4. In the study reported here, we directly compared the 3 most commonly used diagnostic tests for the detection of serum antibodies against EHV1 or EHV4 in which acute and convalescent sera from experimentally and naturally infected horses were compared by use of **virus neutralization (VN)** and **complement fixation (CF)** assays[22-24] and an EHV1/EHV4 type-specific ELISA.[21]

Materials and Methods

Viruses and cells—Equine fetal kidney (EFK) cells were used at the fifth passage. Equine herpesvirus 1.438/77

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was isolated from the lung of an aborted fetus[7] and had been passaged 8 times on EFK monolayer cell cultures. Equine herpesvirus 4.405/76 was isolated from a horse with acute respiratory tract disease[25] and had been passaged 5 times on EFK cells. Supernatant from uninfected EFK monolayer cell cultures that had been frozen and thawed was used as the negative control in the CF assay.

Sera—Many of the serum samples used in this study were selected from previously published investigations. Serum samples from the following studies were used in our study: Fitzpatrick and Studdert's[12] study of experimental infection in specific pathogen-free foals (serum samples from foals 1 and 3 of that study), Drummer et al's[26] investigation of an EHV1 abortion outbreak (serum samples from horses 2, 24, and 28 of that study), and Studdert et al's[5] investigation of an EHV1 myeloencephalitis and respiratory disease outbreak (serum samples from mares 3 and 4 and foals 4, 5, and 6 of that study).

VN assay—A complement-independent VN assay was performed in sterile 96-well, flat-bottom, polyvinyl chloride microtiter plates. Sera were diluted 1:2 in Dulbecco minimal essential media supplemented with 1% fetal bovine serum, 45mM $NaHCO_3$, and ampicillin (50 µg/mL) and heat inactivated at 56°C for 30 minutes prior to use. Serial 2-fold dilutions were incubated for an hour at 37°C with an equal volume (50 µL) containing 100 $TCID_{50}$ of virus. Approximately 2.4×10^4 EFK cells in 50 µL were added to each well, and the plates were incubated for 5 days at 37°C in a humidified atmosphere of 5% CO_2 in air. The viral-neutralizing antibody titer of each serum sample was determined as the reciprocal of the highest dilution of serum that neutralized virus by 50%, and the final titers were calculated by use of the methods of Reed and Muench.[27] Titrations were performed in quadruplicate.

CF assay—A CF assay was performed essentially as described by Thomson[22] with modifications[23,24] in U-bottomed microtiter wells. Sera were heat inactivated at 60°C for 30 minutes after an initial 1:5 dilution in veronal buffer (145mM NaCl, 3.1mM diethylbarbituric acid, 1.8mM sodium barbiturate, 0.8mM MgCl, and 0.25mM CaCl, pH 7.3), which was used as the diluent for all components of the CF assay. Heat-inactivated serum was further diluted such that a 1:20 dilution of serum was the lowest dilution tested in the assay. Serial 2-fold dilutions of serum were incubated with an equal volume (25 µL) of the same EHV1 and EHV4 antigens used in the VN assay as well as with supernatant from an uninfected EFK cell lysate as a negative control antigen. Equine herpesvirus 1 and EHV4 antigens were diluted to contain 4 U of antigen/25 µL, which was a 1:4 dilution in veronal buffer for these supernatants. The uninfected EFK supernatant was similarly diluted. After the addition of 25 µL of 3 CH50 units of guinea pig serum,[a] plates were incubated overnight at 4°C. Prior to the preparation of the haemolytic system, rabbit antiserum-to-sheep RBCs was titrated with complement and sheep RBCs to determine the dilution of rabbit antiserum to use in the CF assays; 1 AMB unit is defined as the highest dilution of rabbit antiserum-to-sheep RBCs that provides complete hemolysis.[24] To prepare the hemolytic system, an equal volume of 2% (vol/vol) sheep RBCs was incubated with 6 AMB units[24] of rabbit antiserum to RBCs for 1 hour at 37°C before the addition of 50 µL to each well. All tests were accompanied by a titration of complement, RBC controls (zero lysis; veronal buffer plus hemolytic system only), and 100% lysis controls (hemolytic system in 0.1% Triton X-100 in veronal buffer). Plates were incubated at 37°C for a further 30 minutes prior to centrifugation at 1,000 × *g* for 5 minutes at 4°C. The primary difference between our CF method and that described by others [22-24] was that the titer was not read by scoring RBC buttons. Instead, 80 µL of supernatant was removed from each well after centrifugation and the absorbance read at 414 nm. The absorbance values were expressed as a percentage of the 100% lysis control after subtraction of the absorbance value of the zero lysis controls from both values. The 50% end point was taken as the dilution of serum that resulted in the closest value to 50% lysis by absorbance. None of the sera had any reactivity to the negative (EFK) antigen control. The CF assay was performed on 3 separate occasions, and all sera were tested simultaneously.

ELISA—An EHV1/EHV4 type-specific ELISA that is based on gG recombinant antigen from EHV1 and EHV4 was performed as described by Crabb et al.[21] Sera were tested on 3 separate *Escherichia coli*–expressed recombinant antigens in the ELISA consisting of the following: 1) the variable region of EHV1 gG fused to glutathione S-transferase, 2) the variable region of EHV4 gG fused to glutathione S-transferase, and 3) glutathione S-transferase alone. Purified antigen diluted in 0.1M carbonate-bicarbonate buffer was used to coat wells of a 96-well plate[b] before unoccupied sites were blocked by incubation for 1 hour at 37°C with PBS solution that contained bovine serum albumin (10 mg/mL) and 5% sheep serum. Polyclonal horse sera were diluted 1:100 in PBS solution containing bovine serum albumin (5 mg/mL), 0.05% Tween 20, and 2.5% sheep serum as diluent and added to the microtiter wells. Bound antibody was detected with affinity-purified goat anti-horse IgG conjugated to horseradish peroxidase[c] and tetramethylbenzidine dihydrochloride substrate.[d] The reaction was stopped with 1M HCl, and the absorbance of each well was determined at 450 nm.[e]

ELISA data presentation—Results were expressed as absorbance values only after the absorbance value for each serum sample against glutathione S-transferase had been subtracted. Positive values were taken from a cutoff of 0.09 as determined by Crabb and Studdert.[28] Sera were tested in triplicate against each antigen.

Results

Foals inoculated with EHV1, EHV4, or both—To compare test results for the detection of EHV1- and EHV4-specific antibody, we first examined serum samples from 2 specific pathogen-free foals that had been experimentally infected with EHV1 or EHV4. Sera were prepared as described by Fitzpatrick and Studdert.[12] Sera used in this study were from 2 specific pathogen-free foals prior to and after inoculation with EHV1, EHV4, or both viruses.

Sera from the first foal were obtained prior to inoculation (day 0), and on post-EHV1 (only) inoculation day 71 (after IM inoculations with inactivated EHV1.438/77 on days 0 and 14 and intranasal challenge with live EHV1.438/77 on days 38 and 60), and on post-EHV1 and -EHV4 inoculation day 120 (39 days after cross challenge with intranasal EHV4.405/76). Sera from the second foal were obtained on post-EHV4 (only) inoculation day 46 (after IM inoculations with inactivated EHV4.405/76 on days 0 and 14 and intranasal challenge with live EHV4.405/76 on day 35) and on post-EHV4 and -EHV1 inoculation day 108 (45 days after cross challenge with intranasal EHV1.438/77).

Sera were evaluated in an ELISA and CF assay, and results were compared with published antibody titers

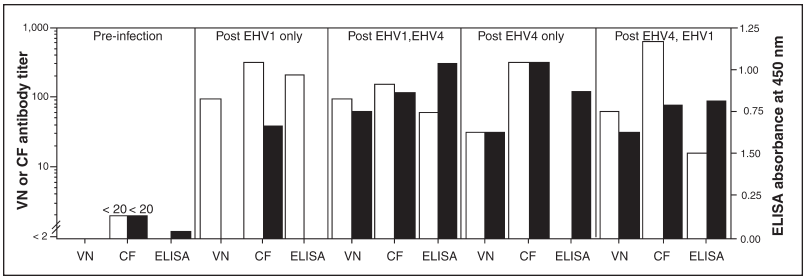

Figure 1—Serologic results of 2 specific-pathogen-free foals before (preinfection) or after inoculation with equine herpesvirus 1 (EHV1; post-EHV1 only) or EHV4 (post-EHV4 only) or after cross challenge of each foal with the heterologous viruses (post-EHV1, -EHV4 and post-EHV4, -EHV1).[12] Serum samples were tested in virus neutralization (VN) and complement fixation (CF) assays to determine antibody titers against EHV1 and EHV4. Serum samples were also tested in the EHV1/EHV4 type-specific ELISA to determine absorbance values for EHV1 and EHV4 recombinant antigens after a 1:100 dilution. Antibody titers that were determined by use of a VN assay are those reported by Fitzpatrick and Studdert.[12] Cutoff values of < 20 or < 2 indicate results below detection limits of CF or VN assays, respectively. Open bars = Antibody titers against or absorbance values for EHV1. Closed bars = Antibody titers against or absorbance values for EHV4.

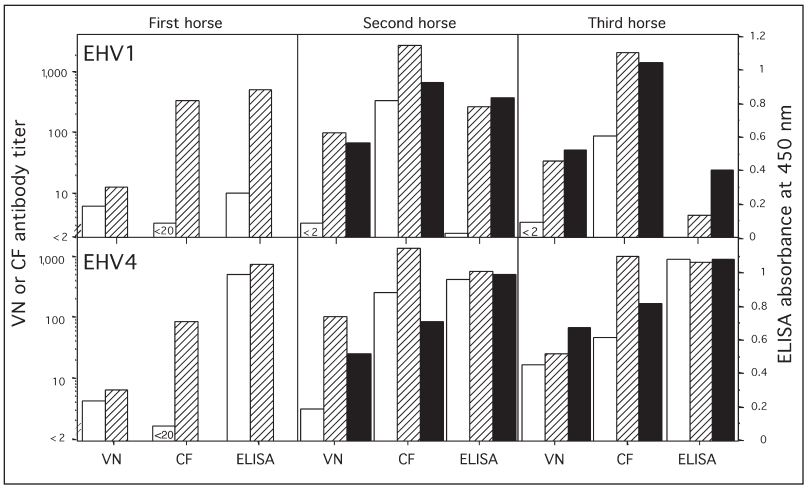

Figure 2—Serologic results of 3 mares with confirmed EHV1 infections associated with abortion (first and second mare, days 17 and 7 of outbreak, respectively) or perinatal foal death (third mare, day 13 of outbreak). Sera were evaluated in VN and CF assays to determine antibody titers against EHV1 and EHV4. Serum samples were also tested in the EHV1/EHV4 type-specific ELISA to determine absorbance values for EHV1 and EHV4 recombinant antigens. Sera were obtained from mares at days 3, 13, and 67 after the first abortion on the property. No serum was available from the first horse on day 67. Open bar = Day 3. Hatched bars = Day 13. Closed bar = Day 67.

that were determined by use of a VN assay for these sera[12] (**Figure 1**). Sera from the first foal prior to inoculation with any virus did not react with either EHV1 or EHV4 in any of the tests. After inoculation with EHV1, the first foal seroconverted to EHV1 as measured in all 3 assays. No reactivity with EHV4 was observed in the VN assay or ELISA; however, EHV4-reactive antibodies were detected in the CF assay, albeit at an 8-fold lower titer than that obtained for the same serum against EHV1. After cross challenge with EHV4, each test detected EHV1- and EHV4-reactive antibodies.

After inoculation and challenge of the second foal with EHV4, EHV4-reactive antibodies were detected in all tests. However, EHV1-reactive antibodies were also detected in the VN and CF assays to the same titer as EHV4. In contrast, only EHV4-reactive antibodies, not EHV1-reactive antibodies, were detected by use of an ELISA. After cross challenge with EHV1, the EHV1- and EHV4-reactive antibodies detected in the VN and CF assays remained essentially unchanged, compared with titers determined prior to cross challenge. The major difference after cross challenge in this instance was the detection of EHV1-reactive antibodies by use of an ELISA, which were not detected before cross challenge with this virus.

Horses with EHV1 infections—Acute and convalescent sera of naturally infected horses from several EHV1 outbreaks were investigated to compare the results obtained by the 3 assays. Serum samples were collected from 33 mares on a stud farm during an EHV1 abortion outbreak in 1994.[26] Sera were collected on days 3, 13, and 67 after the first abortion on the property. For this study, serum samples from 3 mares were evaluated in VN and CF assays and the ELISA (**Figure 2**). The first 2 mares aborted on days 17 and 7, respectively, and the foal of the third mare was born

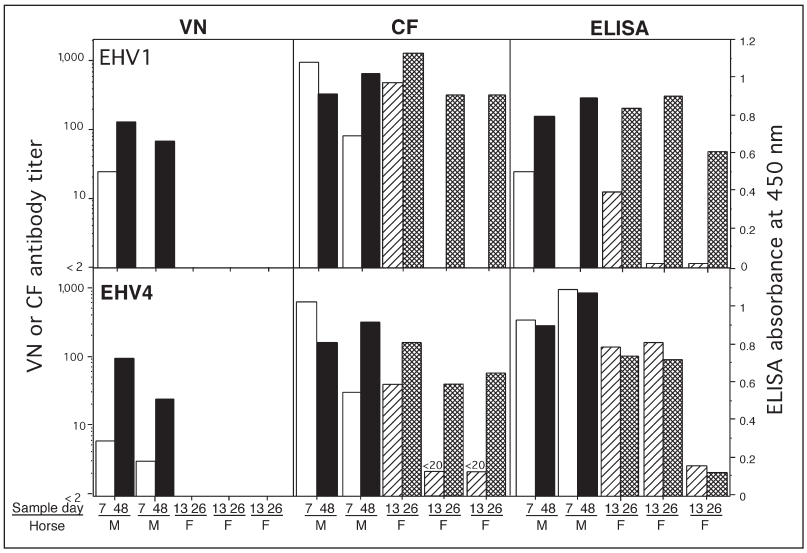

Figure 3—Comparison of VN and CF antibody titers with the EHV1/EHV4 type-specific ELISA absorbance values of sera obtained from 2 mares and 3 foals during an outbreak of EHV1-induced myeloencephalitis and respiratory tract disease.[5] The EHV1/EHV4 type-specific ELISA absorbance values were determined at 450 nm after reaction with a 1:100 dilution of serum. Both mares had EHV1-induced myeloencephalitis, whereas the 3 foals had signs of respiratory tract disease. M = Mare. F = Foal. Open bar = Day 7. Closed bar = Day 48. Hatched bar = Day 13. Cross-hatched bar = Day 26.

alive but died 12 hours after birth on day 13 of the outbreak.

Results of all assays revealed a substantial increase in EHV1-reactive antibodies between days 3 and 13. Results of the ELISA revealed no change in EHV4-reactive antibody titers between days 3 and 67, and results of the VN assay revealed no substantial change in antibody titers against EHV4 in 2 of the 3 mares between days 3 and 13. In contrast, antibody titers obtained by use of the CF assay against EHV4 mirrored the EHV1 titers for the same sera; this assay was therefore unable to discern the immunologic specificity of antibodies induced after infection.

Similar comparisons were performed by use of sera obtained during the acute and convalescent phase of illness in an outbreak of EHV1-induced myeloencephalitis and respiratory tract disease[5] **(Figure 3)**. Results of the ELISA revealed an increase in EHV1-reactive antibody titers between each of the acute and convalescent sera and did not reveal any substantial change in the EHV4-reactive antibody titer. The VN assay results revealed an increase in EHV1- and EHV4-reactive antibodies in 2 mares and were unable to detect EHV1- or EHV4-reactive antibodies in serum samples from any of the foals. The CF assay results revealed a substantial increase in the EHV1-reactive antibody titer for 1 mare and 2 foals; however, this increase in antibody titer was also detected against the EHV4 antigen.

Horses with EHV4 infections—Acute and convalescent sera were taken from horses with respiratory tract disease to compare the ability of each of the tests to detect EHV4-specific antibody after infection. Results of testing sequential sera obtained from a Thoroughbred weanling before and after detection of EHV4 shedding were determined[29] **(Figure 4)**. Nasal

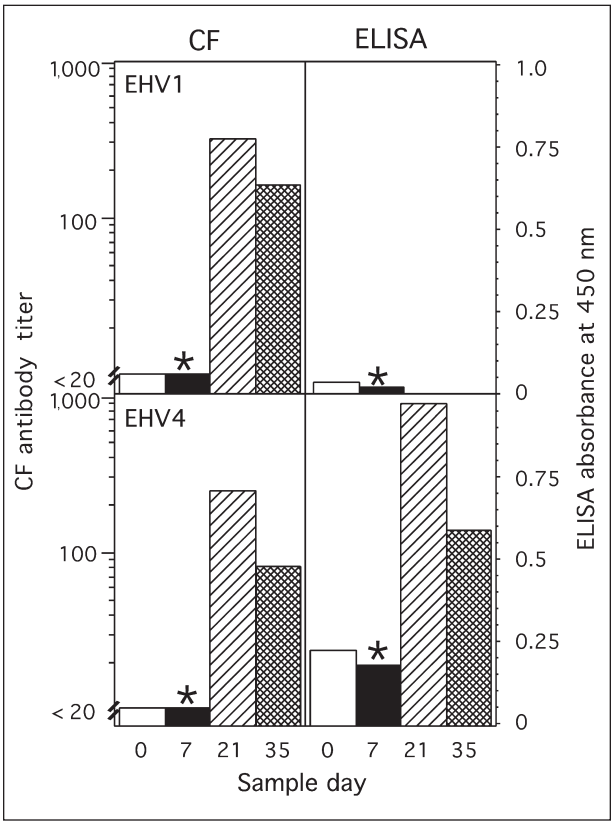

Figure 4—Comparison of CF antibody titers against EHV1 (top panels) and EHV4 (bottom panels) to EHV1/EHV4 type-specific ELISA absorbance values for EHV1 and EHV4 in sequential serum samples of a weanling foal. *Nasal swab taken on day 7 had positive polymerase chain reaction (PCR) assay and virus isolation results for EHV4. Open bar = Day 0. Closed bar = Day 7. Hatched bar = Day 21. Cross-hatched bar = Day 35.

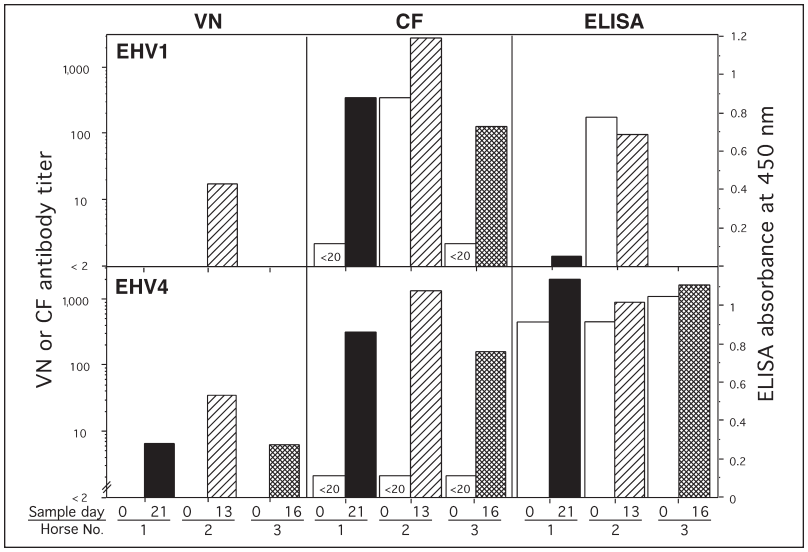

Figure 5—Comparison of the EHV1/EHV4 type-specific ELISA absorbance values with antibody titers against EHV1 (top panel) and EHV4 (lower panel) that were determined by use of VN and CF assays of acute and convalescent serum samples of 3 horses after EHV4-induced respiratory tract disease. Equine herpesvirus 4 was detected by use of PCR assay and virus isolation from nasal swab specimens taken with the acute-phase (day 0) serum samples. Open bar = Day 0. Closed bar = Day 21 after EHV4-positive nasal swab specimen. Hatched bar = Day 13 after EHV4-positive nasal swab specimen. Cross-hatched bar = Day 16 after EHV4-positive nasal swab specimen.

swab specimens were taken from the weanling at the same time as sera, and the nasal swab taken on day 7 had EHV4-positive PCR assay results. Each of the 4 sera from the foal had negative ELISA results for EHV1-reactive antibodies, whereas a sharp increase in EHV4-reactive antibodies was detected in serum samples collected 14 days after the foal was found to be shedding EHV4. Results of the CF assay revealed the same increase in EHV4-reactive antibodies between days 7 and 14; however, the EHV1-reactive antibody titers that were determined by use of a CF assay also increased to mirror the CF assay results for EHV4. No virus-neutralizing antibodies were detected against EHV1 or EHV4 in any of these sera.

Results of the 3 assays with acute and convalescent sera obtained from 3 older horses that had respiratory tract disease were determined (**Figure 5**). Nasal swab specimens were taken from these horses at the same time as the acute-phase sera, and the presence of EHV4 was confirmed in all affected horses on the basis of EHV4-specific PCR assay[18] and virus isolation results (data not shown). Antibody titers as detected by use of the VN assay for each of these sera were low, but an increase in antibody titers against EHV4 was detected between the acute and convalescent sera of all horses. An increase in EHV1 virus-neutralizing antibodies between the acute and convalescent sera was detected for 1 horse, whereas no EHV1 viral-neutralizing antibody was detected in sera from the other 2 horses. Antibody titers of these sera that were determined by use of the CF assay were much higher than those determined by use of the VN assay, and an increase in EHV4-reactive antibodies was clearly detected between the acute and convalescent sera. Again, however, results of the CF assay also revealed an increase in EHV1-reactive antibodies in these sera even though there was no evidence of EHV1 infection. In contrast to results of the VN and CF assays, results of the ELISA revealed only a small difference in the EHV4-reactive antibody titer between the acute and convalescent sera, whereas the EHV1-reactive antibody titers were substantially unchanged. Differences between absorbance values of the acute and convalescent sera for these 3 horses were 0.229, 0.101, and 0.063. Although there was an increase in the EHV4-reactive antibody titer for 1 horse, differences for the other 2 horses were not obvious.

The ELISA used in our study has been developed to test large numbers of sera; hence, all sera were tested at a single dilution (1:100) rather than with serial dilutions, as is done for the VN and CF assays. By use of a single serum dilution, differences between antibody titers in each serum sample can be detected only if the concentration of primary antibody in the chosen dilution is not saturating in both sera. To better illustrate this phenomenon, acute and convalescent sera from the 3 horses were titrated in the ELISA (**Figure 6**). The first dilution of the titration was a 1:100 dilution (ie, the same dilution of serum used for the single dilution format of the test). Despite small differences in absorbance values from the 1:100 dilution of sera against the EHV4 antigen, titration results revealed a 53-, 56-, and 16-fold difference in the EHV4-reactive antibody titers of the acute and convalescent sera from the 3 horses. Only 1 horse had detectable EHV1-reactive antibody titers, and the EHV1-reactive antibody titers remained unchanged between the acute and convalescent sera from this horse. Clearly, it would be less economically feasible to titrate horse sera in the ELISA, rather than use the single dilution format, to

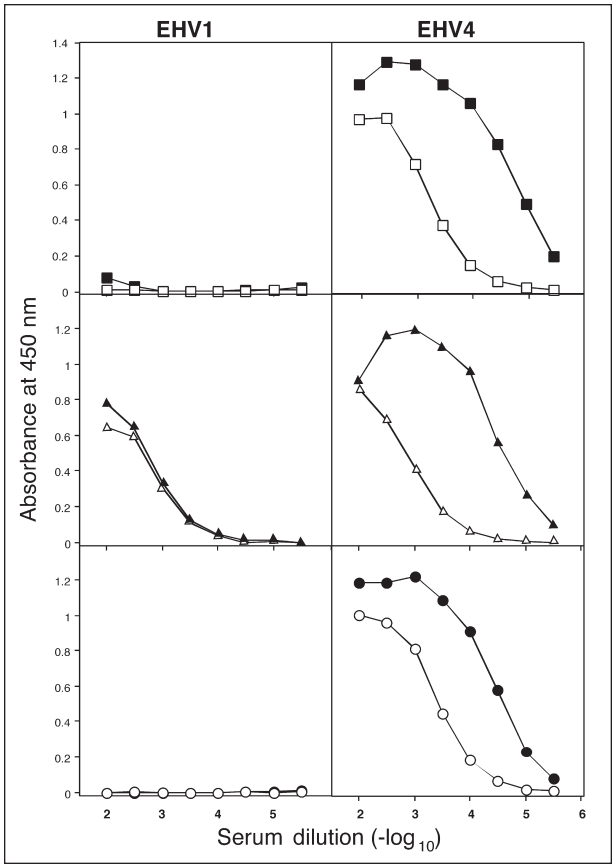

Figure 6—Titration of acute (open shapes) and convalescent (closed shapes) sera from 3 horses with confirmed EHV4 in the EHV1/EHV4 type-specific ELISA for the detection of serum antibodies against EHV1 (left panels) and EHV4 (right panels) antigens.

detect differences in EHV4-reactive antibody titers. Nevertheless, because the seroprevalence of EHV4-reactive antibodies approaches 100% in older horses,[28,30-32] most acute-phase sera will contain a substantial EHV4-reactive antibody titer that may be at saturating concentrations for this ELISA. These results suggest that a 1:1,000 or 1:3,000 dilution of serum may be more appropriate dilutions to use to detect any increase in EHV4-reactive antibody titers between acute and convalescent sera.

Discussion

Our study directly compares the 3 most commonly used diagnostic tests for the detection of antibody specific for EHV1 or EHV4. Serum samples from specific pathogen-free foals that were challenged with EHV1 and EHV4, sera from mares that aborted EHV1-positive foals, and sera from horses with EHV1-induced myeloencephalitis and EHV4-induced respiratory tract disease were tested, and the results unequivocally demonstrate the capacity of the gG antibody detection ELISA to differentiate antibody against EHV1 from antibody against EHV4. Our study also highlights the difficulties associated with interpreting results of VN and CF assays because cross-reactive antibodies confound the results of these assays. The ability to unequivocally differentiate between antibody responses to EHV1 and EHV4 is essential for diagnostic laboratories. The results presented here illustrate that the EHV1/EHV4 type-specific ELISA based on gG recombinant antigens clearly identifies horses infected with EHV1 or EHV4 when acute and convalescent sera are available. However, results from the VN and CF assays indicate that cross-reactive antibodies detected by use of these tests greatly limit the interpretation of results with acute and convalescent serum samples from horses naturally or experimentally infected with EHV1 or EHV4.

Equine herpesvirus 1 and EHV4 are closely related alphaherpesviruses that were thought to be the same virus until a correlation between differences in pathogenesis and restriction fragment-length polymorphisms was established.[13,33,34] Since then, the complete nucleotide sequences of the genomes of EHV1[8] and EHV4[9] have been determined, confirming the high degree of DNA sequence homology between these 2 viruses and also identifying differences throughout the entire 150,000 nucleotides of the 2 genomes. These DNA sequencing studies have shown that all of the **open reading frames (ORFs)** of EHV1 have a homologous ORF in EHV4. Both genomes contain 77 distinct putative protein-coding ORFs. The degree of amino acid identity between individual EHV1 and EHV4 proteins ranges from 55% (gene 76, tegument protein) to 96% (gene 42, major capsid protein). Given the close relationship of the 2 viruses, it is not surprising to find that the antigens of EHV1 and EHV4, including most of the surface glycoproteins, possess both type-specific and cross-reactive epitopes and differentiation of the antibody response to these viruses has proven challenging. Virus neutralization and CF assays are widely accepted methods of detecting antibody to EHV1 or EHV4. Both of these methods use whole-virus preparations as the viral antigen and thus have the inherent problem of cross-reactivity of EHV1 antibody with EHV4 antigens, and vice versa. The development of EHV1/EHV4 type-specific ELISAs by different groups[21,35] has allowed serologic differentiation of the type-specific antibodies and has overcome the difficulties associated with the cross-reactive antibodies that are produced following infection with these viruses.

The nature of assays used in our study determines the type of antibodies that each assay detects. The VN and CF assays use whole-virus preparations as the antigen and, as such, present type-specific and cross-reactive epitopes for antibody binding. The VN assay detects only that subset of antiviral antibodies that bind to neutralizing epitopes, and these are likely to be present on the envelope gB, gC, and gD. Type-specific and cross-neutralizing epitopes have been defined on these antigens.[36] Only low titers of virus-neutralizing antibody are generally detected against EHV1 or EHV4, and our results indicate that this assay is unable to detect any antibody in some sera that had positive ELISA and CF assay results. The CF assay detects bound antibodies that fix complement and, as such, discriminates on the basis of antibody subclass rather than the type specificity of the epitope to which that antibody binds. It has been reported that IgM as well as IgGa and IgGb can fix complement, whereas IgGc and IgG(T) do not.[37] The IgG and IgM antibodies are

induced after primary and secondary infection with EHV1[38,39]; IgGa and IgGb antibodies are induced in EHV4-infected horses; and IgGb, but not IgGa, is long-lasting.[40] Other tests that use whole-virus antigens, such as immunofluorescence or western blotting, are also likely to be clouded by the difficulties associated with cross-reactive antibodies. The EHV1/EHV4 type-specific ELISA used in our study also detects only a subset of the total antibody titer produced following infection with EHV1 or EHV4. This ELISA was designed to detect only those antibodies that bind to the variable region of the gG of EHV1 or EHV4 and thus avoid the difficulties associated with cross-reactive antibodies. Evidence of recent infection requires demonstration of an increase in specific antibody titers between acute and convalescent serum, regardless of the type of antibody detection assay used. The presence or absence of virus-neutralizing, complement-fixing, or ELISA-detectable antibodies in a single serum sample is not sufficient to unequivocally diagnose recent infection with either virus.

Several studies[22,41-43] have shown contradictory results with respect to the type specificity of antibody detection by use of VN and CF assays. It has been reported that assay conditions and the virus strains used in these assays can markedly influence the sensitivity and type specificity of the VN and CF tests.[44] The nature of both assays is such that assay variation between laboratories may be inherent in the strains of viruses used and the antigenic relatedness of EHV1 and EHV4 strains. Furthermore, the susceptibility of different cell types or even the same cell type in different laboratories may influence the VN assay results of antibody titers against EHV1 or EHV4. It may be more straightforward to standardize the components of the ELISA as a means of normalizing testing for EHV1- and EHV4-specific antibody, rather than identify the precise reasons for published variations in the VN and CF assay results.

The results presented here for the experimental infection of the specific pathogen-free foals indicate that the virus-neutralizing antibody response of these foals is type-specific for foals challenged only with EHV1 but cross-reactive after they receive EHV4.[12] In the CF assay, some cross-reactivity is observed in both directions but is present most strongly in foals after receiving EHV4. No such cross-reactivity was observed in the EHV1/EHV4 type-specific ELISA, which only detected antibodies specific for the viruses that had been inoculated. This clear differentiation was maintained for each of the paired sera tested in the remainder of our study. Given the high seroprevalence of EHV4-specific antibodies in adult horses[28,30-32] and the cross-reactive nature of antibodies elicited to EHV4 in VN and CF assays,[1,2,22] it appears that the use of the EHV1/EHV4 type-specific ELISA with acute and convalescent sera provides the most definitive information for a diagnosis of EHV1 or EHV4 infection in horses.

a. Institute of Medical and Veterinary Science (IMVS), Gilles Plains, Australia.
b. Nunc Immuno-plate, Maxisorp, Roskilde, Denmark.
c. Horseradish peroxidase-labeled goat anti-horse IgG, Kirkegaard & Perry Laboratories Inc, Gaithersburg, Md.
d. TMB, Sigma Chemical Co, St Louis, Mo.
e. Titertek Multiscan MC3, Flow Laboratories, Inglewood, Calif.

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