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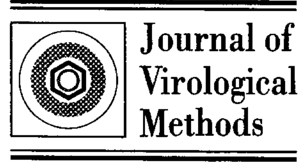
Journal of Virological Methods

# Development and evaluation of an ELISA using recombinant fusion protein to detect the presence of host antibody to equine arteritis virus

E.D. Chirnside [a,*], P.M. Francis [a], A.A.F. de Vries [b], R. Sinclair [a], J.A. Mumford [a]

[a] *Department of Infectious Diseases, The Animal Health Trust, Lanwades Park, Kennett, Newmarket, Suffolk CB8 7PN, UK*

[b] *Department of Virology, Institute of Infectious Diseases and Immunology, Veterinary Faculty, Utrecht University, Yalelaan 1, 3584 CL Utrecht, Netherlands*

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## Abstract

A recombinant glutathione-*S*-transferase fusion protein expressing amino acids 55–98 of equine arteritis virus (EAV) $G_L$ ($rG_L$55–98) was tested in an ELISA for its ability to detect serum antibodies to EAV. Host antibodies induced following EAV infection bound the recombinant antigen by ELISA. The ELISA specificity and sensitivity were determined with a panel of equine sera including postinfection and postvaccination samples. A good correlation existed between EAV neutralizing antibody titers and ELISA absorbance values ($r = 0.827$). The sensitivity and specificity of the ELISA were 99.6 and 90.1%, respectively, compared with the EAV neutralization test and the recombinant antigen did not crossreact in ELISA with equine sera directed against other common equine respiratory viruses. Three post-EAV infection equine sera raised against different EAV isolates reacted strongly in the ELISA, as did two equine sera raised against EAV vaccines, indicating that the viral epitope was conserved between the viruses tested. Following vaccination with an inactivated whole virus vaccine, antibody detected with the recombinant antigen ELISA preceded the development of a virus-neutralizing response. The study demonstrates the potential application of $rG_L$55–98 as a diagnostic antigen.

*Keywords:* Equine arteritis virus; Arterivirus; Recombinant protein; Enzyme-linked immunosorbent assay (ELISA); Equine antibody; Diagnosis

* Corresponding author. Fax: +31 (638) 750794.

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## 1. Introduction

Equine viral arteritis (EVA) has to date only been reported to infect horses (Doll et al., 1957; Chirnside, 1992) although seropositive donkeys have been found (Paweska and Barnard, 1993). The disease has been known for some 40 years and manifests itself with widely varying clinical signs. In its most severe form equine arteritis virus (EAV) infection causes abortion (Doll et al., 1957) and foal death (Golnik et al., 1981; Vaala et al., 1992), although it is more usual for this virus to cause mild respiratory disease (Chirnside, 1992). Disease outbreaks are identified infrequently due to clinically inapparent infection and field isolates of the virus are rare. The causative agent, EAV, is a small positive single-stranded RNA virus with a genome organization, mode of replication and gene expression strategy similar to that of corona- and toroviruses (de Vries et al., 1990, 1992; den Boon et al., 1991; Snijder et al., 1994). EAV has recently been classified in the genus arterivirus (Cavanagh et al., 1994) along with lactate dehydrogenase-elevating virus of mice (LDV) (Godeny et al., 1993; Kuo et al., 1992), simian hemorrhagic fever virus (SHFV) (Plagemann and Moennig, 1992), and the virus causing porcine respiratory and reproductive syndrome (PRRSV) (Conzelmann et al., 1993) Lelystad virus (LV) (Meulenberg et al., 1993).

EAV is transmitted by the respiratory and venereal routes, with a 30% carrier state existing in seropositive stallions (Timoney et al., 1986) making the latter route a particular cause for concern, as these stallions shed virus in their semen and may consequently infect broodmares (McCollum et al., 1988; Neu et al., 1988). EVA is geographically widespread (Chirnside, 1992) and most European and North American countries have EAV-seropositive animals. Some outbreaks have occurred following the importation of persistently infected animals causing primary transmission of EAV by the venereal route, followed by secondary respiratory spread (Wood et al., 1995). In the light of the potential economic importance of this disease to the horse breeding and racing industry, a requirement exists for both prophylactic treatment and reliable serodiagnosis of infection.

Laboratory-based serological tests include EAV virus neutralization test (NT), complement fixation (CF) and enzyme-linked immunosorbent assay (ELISA) (Senne et al., 1985; Fukunaga and McCollum, 1977; Cook et al, 1989). The ELISA has a relatively low specificity when applied to sera from horses previously vaccinated against equine influenza and herpesviruses; this is primarily due to host antibody induced by tissue culture contaminants of these vaccines reacting with cell culture-derived antigen present in the whole-virus ELISA antigen. The CF test has limited temporal sensitivity and is consequently only useful for serodiagnosis up to 6 weeks postinfection. At present, NT results from designated laboratories are internationally accepted for import/export testing.

Infection with EAV elicits a virus-neutralizing antibody response within 4–10 days. Stallions which have previously been exposed to EAV, and therefore may be shedders of infectious virus, can be detected by the NT as a discernible level of circulating neutralizing antibody persists for many years after recovery (Gerber et al., 1978). The NT is sensitive, but suffers from the disadvantage that it requires laboratory testing to maintain cell cultures and stocks of infectious virus. In addition, the NT takes several

days to complete. Laboratories use varied test protocols and different reagents which can lead to disparate results. Moreover, the NT does not differentiate between the host serological response induced by vaccination and that resulting from natural infection.

It has recently been demonstrated that neutralizing monoclonal antibodies recognize a determinant on the large envelope glycoprotein, $G_L$ (Balasuriya et al., 1993; Deregt et al., 1994). This paper describes an indirect ELISA using a recombinant glutathione-*S*-transferase fusion protein (Smith and Johnson, 1988) as an antigen to screen equine sera for the presence of antibodies to EAV, and its evaluation as a diagnostic test with large numbers of equine serum samples.

## 2. Materials and methods

### *2.1. Cloning, expression and purification of EAV $G_L$ fusion protein*

Plasmid DNA manipulations were carried out as described previously (Sambrook et al., 1989). Transformations with plasmid pGEX-3X (Smith and Johnson, 1988) were carried out in *Escherichia coli* TG1 and recombinant clones selected by antibiotic resistance. Clones were subsequently screened for fusion protein expression after IPTG induction by analysis in SDS–12.5% polyacrylamide gels, and the EAV-specific insert size, orientation and in-frame insertion confirmed by restriction endonuclease digestion analysis and dsDNA sequencing of purified plasmid DNA (Chirnside et al., 1995). The expression construct 5Rsa1, expressing $rG_L$ 55–98, comprised nucleotides 11,310–11,441 of EAV open reading frame (ORF) 5 fused in-frame to the carboxyl-terminus of the glutathione-*S*-transferase gene in the expression vector pGEX-3X.

Fusion protein was affinity purified using glutathione Sepharose 4B (Pharmacia). A 250-ml culture of bacterial cells expressing recombinant antigen was harvested by centrifugation for 15 min at 6000 *g* and 4°C, resuspended in 50 ml 10 mM Tris-HCl (pH 8.00), 1 mM EDTA, 100 mM NaCl (STE) and recentrifuged to pellet the cells. The supernatant was discarded and the cells resuspended in 3 ml STE, 50 μl lysozyme (50 mg/ml) added and the tube incubated at 37°C for 15 min. The tube was then frozen at −70°C overnight followed by a short immersion in a boiling water bath to thaw the frozen suspension. DNase 1 (20 μg) and 30 μl of 1 M $MgCl_2$ were then added to the thawed cells and the tube incubated at 37°C for 15 min. Triton X-100 was then added to a final concentration of 1%, the mixture kept on ice for 10 min and centrifuged at 10,000 *g* and 4°C for 10 min. Glutathione Sepharose 4B gel was added to the supernatant and the solution mixed by gentle agitation on a rotary mixer for 20 min. The gel was washed 3 times with PBS and the recombinant antigen eluted from the gel with reduced glutathione (5 mM glutathione, 50 mM Tris-HCl (pH 8.0)). Eluate was collected in 1-ml fractions and analyzed by SDS–polyacrylamide gel electrophoresis prior to pooling fractions containing purified protein. Pooled protein was dispensed into 500-μl aliquots and frozen at −20°C until used. Protein concentration was determined with a modified Lowry reagent solution from a Protein Assay Kit (Sigma).

### 2.2. Indirect ELISA

Optimal concentrations of reactants were determined by checkerboard titration. Immulon 3 microtiter plates (Dynatech) were coated with GST or fusion protein diluted in 50 mM carbonate buffer (pH 9.6) and kept overnight at 4°C. Plates were then washed 3 times with PBS containing 0.05% Tween 20 (PBST) and 100 $\mu$l of PBST containing 5% goat serum (PBSTG) added to each well. Following incubation at 37°C for 1 h and 3 washes with PBST, 100 $\mu$l of horse sera diluted $10^{-2}$ in PBSTG was added to each well and the plates incubated for 90 min at 37°C. Plates were then washed 3 times with PBST, 100 $\mu$l of a $10^{-3}$ dilution of affinity-purified biotin-labeled goat anti-$\gamma$ chain specific horse IgG (KPL) was added to each well and the plates incubated for 90 min at 37°C. The plates were then washed 3 times with PBST, and 100 $\mu$l of a $10^{-3}$ dilution of streptavidin-peroxidase (KPL) in PBSTG added to each well and the plate incubated at room temperature for 30 min. After a final 3 washes with PBST, substrate solution (0.5 mg/ ml *o*-phenylenediamine dihydrochloride dissolved in 50 mM phosphate-citrate buffer (pH 5.0), containing 0.03% sodium perborate) was added to each well and the plate incubated at room temperature for 10 min, after which the reaction was stopped by the addition of 50 $\mu$l 4 M $H_2SO_4$ to each well and the absorbance read at 490 nm. Each serum sample was assayed in duplicate wells against both purified GST and fusion protein and the mean absorbance value taken as the $A_{490}$ reading to each antigen. Each ELISA was validated by the inclusion of 8 $NT^-$/$ELISA^-$ control sera. The mean absorbance value for these control sera was $\leqslant 0.1$ in each individual ELISA test, with a grand assay mean over all tests of 0.085. In addition, 8 $NT^+$/$ELISA^+$ control sera were also run as standards in each test to confirm the sensitivity of the assay.

### 2.3. Immunoblotting

Immunoblots were carried out with equine sera diluted $10^{-3.4}$ using a modified version of the ELISA protocol after electrophoretic transfer of proteins from SDS–polyacrylamide gels onto nitrocellulose membrane. Non-specific binding of equine antibody to the nitrocellulose membrane was first blocked by overnight incubation at 4°C in PBSTG. The membrane was then washed 3 times in PBST and the ELISA protocol for binding and washing the components of the antibody sandwich performed as above with 3 washes, each of 5 min between steps. To develop a signal from the immunoblots the ECL detection system (Amersham) was employed using the manufacturer's protocol.

### 2.4. Virus neutralization test

The EAV neutralization test was carried out according to the method of Senne et al. (1985) with minor modifications. All sera were initially screened at a 1 : 4 dilution in 6 replicate wells of a microtiter plate. Virus neutralizing sera were subsequently titrated in 4 replicate wells from an initial dilution of 1 : 2 to 1 : 4096. Each individual NT included 3 positive control sera, a virus control to ensure that $100TCID_{50}$ of the Bucyrus strain of EAV was added to each well, and a series of RK-13 cell controls. Individual wells were scored for > 50% cytopathic effect (CPE) after 48 h incubation (37°C, 5% $CO_2$) and

titers calculated according to the formula of Karber (1931). A serum was considered seropositive when it had an NT titer $\geqslant 1:4$ ($\geqslant \log_{10} 0.6$) and a 4-fold rise in titer regarded as a seroconversion following virus infection or vaccination. Throughout the study, the same batch of the Bucyrus strain of EAV and of guinea pig complement was used, and RK-13 cells were used at passage numbers 85–99. This ensured excellent comparability between EAV neutralization tests.

### 2.5. Antisera

The field sera used for this study were submitted to the diagnostic service of the Animal Health Trust. The virus type-specific sera were provided by Dr. Yoshio Fukunaga of the Equine Research Institute, Japan, and the panels of European and American sera obtained from Dr. Margaret Lucas, Central Veterinary Laboratories, UK.

## 3. Results

### 3.1. ELISA development

#### 3.1.1. Fusion protein reactivity in Western blots

Fusion proteins derived from EAV $G_L$ have been shown to react with postinfection, EAV neutralizing horse sera (Chirnside et al., 1995). The glutathione-*S*-transferase

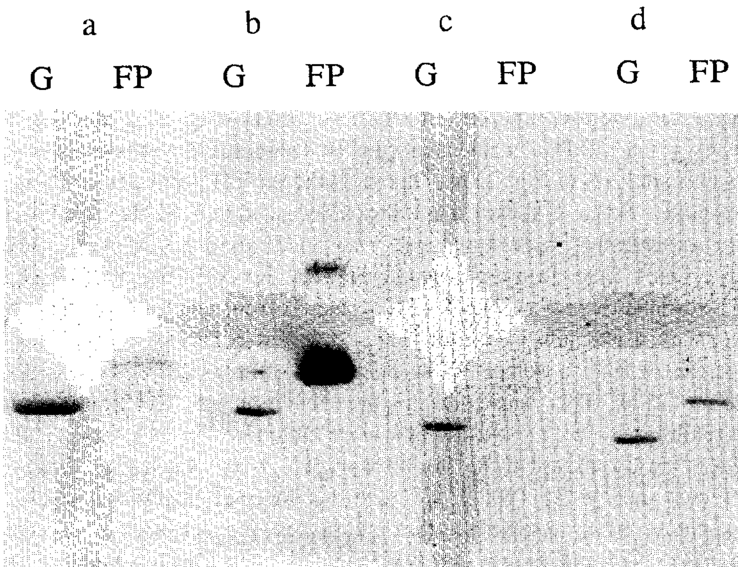


Fig. 1. Analysis of affinity purified glutathione-*S*-transferase (G) and purified $rG_L$55–98 (FP) stained with (a) Coomassie blue and immunoblotted with (b) an EAV neutralizing equine serum (c) a non-neutralizing equine serum and (d) an equine antiserum raised to glutathione-*S*-transferase.

fusion protein expressed by plasmid construct 5Rsa1 contains amino acid residues 55–98 of EAV $G_L$ derived from the Bucyrus isolate. This fusion protein ($rG_L$55–98) was expressed at high levels in IPTG-induced cells and purified readily by affinity chromatography on glutathione Sepharose 4B; the purified $rG_L$55–98 contained few contaminating bacterial proteins (Fig. 1a). In immunoblots to equine sera (Fig. 1b–d) $rG_L$55–98 was very strongly recognized by EAV neutralizing sera (Fig. 1b), faintly by the antiserum against GST (Fig. 1d) and only very slightly by the non-neutralizing equine serum tested (Fig. 1c). Purified GST was recognized by equine sera raised specifically to GST and also by both virus neutralizing and non-neutralizing equine sera.

*3.1.2. ELISA optimization*

The optimal antigen and antibody concentrations in ELISA were determined by checkerboard titration. A serum dilution of 1/100 and antigen concentration of 0.5 $\mu$g per well were selected to give maximum discrimination between $NT^+$ and $NT^-$ sera. The majority of the equine sera had some reactivity to GST, in the absorbance range 0–0.5, at these reagent concentrations. Consequently the absorbance value to GST was subtracted from the absorbance value to the recombinant protein to give an EAV-specific figure for each test sample. Equine sera raised specifically against equine herpesvirus types 1–4, equine rhinovirus types 1 and 2 and equine adenovirus did not recognize $rG_L$55–98 in ELISA; the recombinant antigen was only bound by EAV neutralizing equine sera.

*3.2. ELISA evaluation*

*3.2.1. ELISA sensitivity and specificity*

In order to evaluate the potential of $rG_L$55–98 as antigen for a diagnostic ELISA, and to compare results directly with the EAV neutralization test, the ELISA absorbances of 1500 equine sera were compared with their NT titers (Fig. 2). From the data plotted in Fig. 2 an $A_{490} > 0.113$ (the $y$ intercept plus two standard deviations as determined by linear regression analysis in Fig. 2) was taken as the cut-off point determining an ELISA seropositive value. Table 1 shows a numerical break-down of the results using these seropositive/seronegative cut-off values. Virus neutralizing sera were clearly distinguishable in ELISA from samples seronegative in the NT. The ELISA sensitivity in detecting NT seropositives was 99.6% (242 detected from 243). Only one virus-neutralizing sample, with a low antibody titer ($\log_{10}$ 0.75), was $ELISA^-$ ($A_{490} = 0.110$). The specificity of the ELISA to detect NT seronegative samples correctly was 90.1% (1133 detected from 1257). This was due to the detection of 124 samples which had absorbance readings $> 0.113$ but which were negative by NT ($\log_{10} \leqslant 0.6$). This $NT^-/ELISA^+$ group included 68 blood samples from 44 horses held on equine premises affected during the 1993 EAV outbreak in England (Wood et al., 1995), 40 diagnostic samples from assorted horses submitted for serological screening which included an EAV NT, and blood samples from 16 horses which had previously been vaccinated with either one or two doses of an inactivated EAV vaccine.

The 124 anomalous equine sera were subjected to further investigation by: (1) repeating the NT; (2) repeating the ELISA with a fresh batch of $rG_L$55–98; (3) ELISA

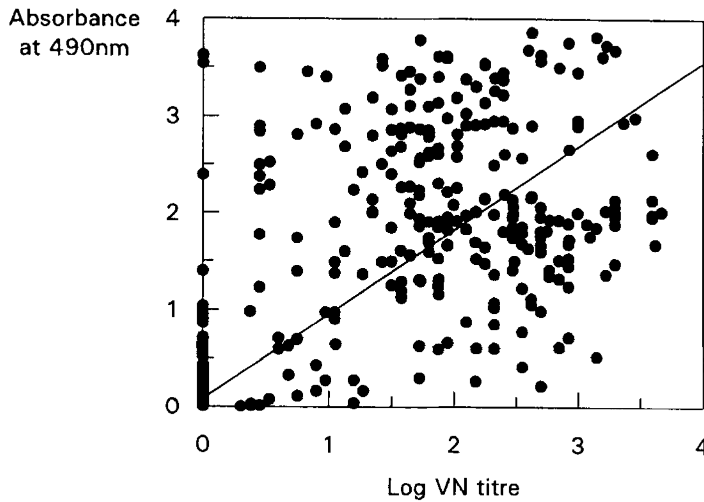


Fig. 2. Comparison of the ELISA absorbance value plotted against the EAV neutralizing titer for 1500 equine serum samples. The line shown was drawn by linear regression analysis; $r = 0.827$, $y$ intercept $0.085 \pm 0.014$.

to an EAV $G_L$-specific peptide (aa 75–97); and (4) by ELISA to another recombinant EAV protein. For 3 and 4 above, although the antigen differed, the ELISA was carried out as described for $rG_L$55–98. In these additional tests, all the sera remained $NT^-$, 108/124 sera remained $ELISA^+$ to $rG_L$55–98, 36/124 were $ELISA^+$ to the peptide and 29/124 $ELISA^+$ to the alternative EAV antigen. Sixteen $ELISA^+$ samples returned $ELISA^-$ results in the repeat ELISA to $rG_L$ 55–98; all 16 were borderline $ELISA^+$ ($0.3 \geqslant A_{490} > 0.113$) in the first $rG_L$ ELISA. In total 24/124 $NT^-$ samples were $ELISA^+$ to all 3 EAV-specific antigens; this number comprised 16 serum samples from vaccinated horses, 6 from outbreak-associated animals and two from sera submitted for general serodiagnosis.

Table 1
Analysis of 1500 sera by virus neutralization test (NT) and ELISA

| NT [a] | ELISA [b] | Number of sera | % |
|---|---|---|---|
| − | − | 1133 | 75.40 |
| − | + | 124 | 8.25 |
| Subtotal | | 1257 | |
| + | − | 1 | 0.06 |
| + | + | 242 | 16.24 |
| Subtotal | | 243 | |

[a] $Log_{10}$ NT titer $\geqslant 0.6$ taken as seropositive neutralization titer.
[b] $A_{490} > 0.113$ taken as seropositive by ELISA.

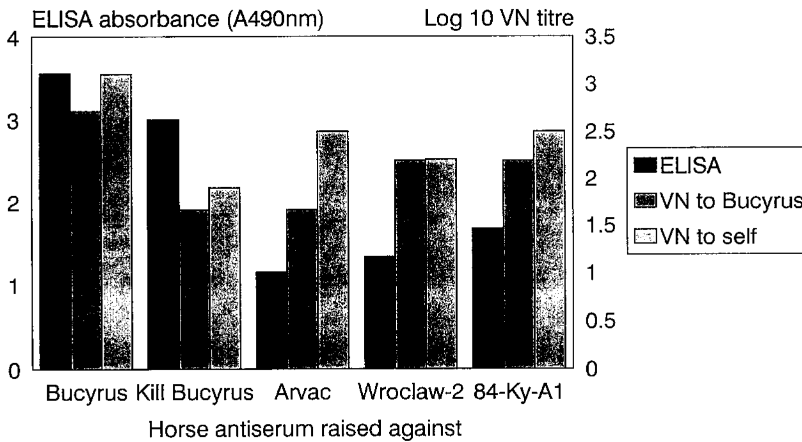


Fig. 3. Comparison in ELISA and virus neutralisation tests of equine sera raised to specific EAV isolates. The isolate specific equine antisera were raised to EAV Bucyrus (the prototype EAV) isolated USA 1953, Wroclaw-2 isolated Poland 1977 and 84-Ky-A1, isolated USA 1984. Arvac is a tissue culture live attenuated vaccine strain, and Kill Bucyrus a formalin-inactivated Bucyrus vaccine preparation.

### *3.2.2. Detection of antibody responses caused by infection or vaccination*

Paired sera originating from 26 horses during the course of the UK EAV outbreak (Wood et al., 1995) seroconverted in both the NT and ELISA tests from $NT^{-}/ELISA^{-}$ to $NT^{+}/ELISA^{+}$. A further 13 paired sera from horses associated with the EAV outbreak did not seroconvert in either diagnostic test.

Paired sera from 25 horses were tested by NT and ELISA both prior to, and 2–5 weeks after administration of two doses of an inactivated EAV vaccine. Among the vaccinees 1/25 (4%) was $NT^{-}/ELISA^{-}$, 15/25 (60%) were $NT^{+}/ELISA^{+}$ and 9/25 (36%) had an ELISA antibody response detectable in the absence of any vaccine-induced neutralizing antibody.

### *3.2.3. Effect of virus variation*

The possible effect of variation in $G_L$ between viruses was investigated by comparing ELISA absorbance readings and NT titers of two post vaccination sera raised against a live and killed vaccine derived from the Bucyrus strain, and 3 postinfection EAV isolate specific equine sera (Fig. 3). Although differences between absorbance values were evident between the sera, all were positive by ELISA. The homologous sera to $rG_L$55–98, derived from infection with the Bucyrus isolate (Bucyrus) or vaccination with inactivated virus (Kill Bucyrus) had higher absorbance values than equine sera raised to the heterologous virus isolates (84-Ky-A1, Wroclaw-2) and the live attenuated vaccine (Arvac; Fort Dodge Laboratories) although little variation in NT antibody titer to the Bucyrus isolate of EAV (from which the recombinant antigen is derived) was detectable between isolates.

To evaluate the utility of $rG_L$55–98 two further panels of equine sera were assayed by ELISA. The panels, one of European and one of American origin, comprised a

Table 2
European sera: virus neutralization test (NT) antibody titers and ELISA absorbance readings

| Serum no. | $Log_{10}$ NT [a] | | ELISA [b] | |
|---|---|---|---|---|
| | Titer | +/− | $A_{490}$ | +/− |
| 1 | 0 | − | 0 | − |
| 2 | 2.18 | + | 3.30 | + |
| 3 | 1.88 | + | 3.40 | + |
| 4 | 0 | − | 0.01 | − |
| 5 | 0.6 | + | 0.59 | + |
| 6 | 2.25 | + | 3.52 | + |
| 7 | 1.88 | + | 3.61 | + |
| 8 | 0 | − | 0.99 | + |
| 9 | 0.45 | − | 2.50 | + |
| 10 | 0 | − | 0.10 | − |
| 11 | 0.68 | + | 0.33 | + |
| 12 | 0 | − | 0.03 | − |
| 13 | 3.00 | + | 3.45 | + |
| 14 | 3.15 | + | 3.81 | + |
| 15 | 3.23 | + | 3.72 | + |
| 16 | 0 | − | 0 | − |
| 17 | 1.73 | + | 0.37 | + |
| 18 | 2.7 | + | 3.63 | + |
| 19 | 0.60 | + | 0.70 | + |
| 20 | 1.80 | + | 3.10 | + |
| 21 | 2.63 | + | 2.90 | + |
| 22 | 1.05 | + | 0.90 | + |
| 23 | 0 | − | 0.03 | − |
| 24 | 1.13 | + | 1.60 | + |
| 25 | 1.35 | + | 3.19 | + |
| 26 | 0 | − | 0 | − |
| 27 | 0 | − | 0.06 | − |

[a] $Log_{10}$ NT titer $\geqslant 0.6$ taken as seropositive neutralization titer.
[b] $A_{490} > 0.113$ taken as seropositive by ELISA.

mixture of postinfection and EAV-negative sera and have been used as reference sera to monitor the standard of virus neutralization testing between laboratories (unpublished). With these two serum panels (Tables 2 and 3) the ELISA sensitivity was 100% and the specificity 85%; 37/37 $NT^+$ sera tested $ELISA^+$, 17/20 $NT^-$ sera tested $ELISA^-$ and 3/20 $NT^-$ sera tested $ELISA^+$.

## 4. Discussion

The serological reactivity of a recombinant EAV $G_L$ fusion protein is reported and its use is described for the detection of equine antibodies to EAV in ELISA. The assay proved to be highly sensitive and specific for the detection of EAV antibodies; it correlated well with results from the EAV microneutralization test and detected postinfection seroconversions in horses following natural infection and vaccination.

Table 3
North American sera: virus neutralization test (NT) antibody titers and ELISA absorbance readings

| Serum no. | $Log_{10}$ NT [a] | | ELISA [b] | |
|---|---|---|---|---|
| | Titer | +/− | $A_{490}$ | +/− |
| 1 | 1.80 | + | 2.79 | + |
| 2 | 1.43 | + | 3.51 | + |
| 3 | 1.58 | + | 1.13 | + |
| 4 | 2.40 | + | 3.22 | + |
| 5 | 0 | − | 0.08 | − |
| 6 | 1.43 | + | 3.58 | + |
| 7 | 0 | − | 0 | − |
| 8 | 1.58 | + | 2.27 | + |
| 9 | 0 | − | 0.26 | + |
| 10 | 0 | − | 0 | − |
| 11 | 2.03 | + | 2.69 | + |
| 12 | 1.50 | + | 1.49 | + |
| 13 | 0 | − | 0.04 | − |
| 14 | 0 | − | 0.09 | − |
| 15 | 0 | − | 0.03 | − |
| 16 | 2.70 | + | 3.56 | + |
| 17 | 2.10 | + | 3.37 | + |
| 18 | 0 | − | 0 | − |
| 19 | 1.65 | + | 2.10 | + |
| 20 | 2.40 | + | 2.59 | + |
| 21 | 2.55 | + | 3.50 | + |
| 22 | 0 | − | 0 | − |
| 23 | 1.13 | + | 3.08 | + |
| 24 | 1.73 | + | 2.57 | + |
| 25 | 0.75 | + | 0.70 | + |
| 26 | 1.80 | + | 2.63 | + |
| 27 | 1.35 | + | 2.01 | + |
| 28 | 1.80 | + | 2.60 | + |
| 29 | 0 | − | 0 | − |
| 30 | 0 | − | 0 | − |
| 31 | 1.58 | + | 3.41 | + |

[a] $Log_{10}$ NT titer $\geq 0.6$ taken as seropositive neutralization titer.
[b] $A_{490nm} > 0.113$ taken as seropositive by ELISA.

In the ELISA, field sera diluted 1:100 reacted with GST causing a variable background absorbance of 0–0.5. It should be possible to reduce this background absorbance by cleaving the GST moiety from the $G_L$ fusion protein, or by cloning EAV $G_L$ 55–98 into a different expression vector. However in its present format, the correlation between the $rG_L$ 55–98 ELISA and NT is high ($r = 0.827$) with the ELISA detecting additional seropositives to the NT. The $ELISA^+/NT^-$ results could be due to differences in sensitivity between the two tests or because the ELISA detects equine antibodies that bind EAV $G_L$, of which those capable of neutralizing virus in a NT are a subpopulation. Alternatively, the detection of 124 $ELISA^+/NT^-$ samples, including 9 vaccinated horses, poses questions about the specificity of the ELISA and its biological relevance when compared with the NT. However, following one or two doses of an

inactivated vaccine, it was possible to demonstrate $ELISA^{+}/NT^{-}$ results in 39% of vaccinees. If a similar situation exists following natural infection with EAV, then the additional seropositive field samples detected by ELISA may have originated from horses previously infected with EAV, but in which a virus neutralizing antibody response was not induced or in which the response has dropped below detectable levels. This observation requires further investigation since the presence of circulating virus neutralizing antibody has to date been accepted as evidence of prior exposure to EAV, either through infection or vaccination. The $rG_L$55–98 ELISA results suggest this may not be the case.

The membrane topology of EAV $G_L$ remains to be established. However, the hydropathy profile (de Vries et al., 1992) and position of the sole *N*-glycosylation site (den Boon et al., 1991) both determine that the protein ectodomain is likely to encompass residues 19–115. By testing > 1500 equine sera in ELISA to $G_L$55–98, we have demonstrated that amino acid residues 55–98 of the Bucyrus strain of EAV $G_L$ encompass a highly immunoreactive antigen which correlates closely with the host virus neutralizing response. EAV $G_L$ has been shown to contain a major antigenic site (Chirnside et al., 1995) and virus neutralizing murine monoclonal antibodies react with a protein of a molecular size (29 kDa) equivalent to $G_L$, by Western blots (Balasuriya et al., 1993; Deregt et al., 1994).

EAV strain variation has been demonstrated at the nucleotide level by RNase T1 fingerprinting (Murphy et al., 1988, 1992) and by direct sequencing of the EAV N- and M-genes (Chirnside et al., 1994) and antigenic variation inferred by cross-neutralization studies (Fukunaga et al., 1994). The equine immune response to EAV induced by different EAV isolates was detectable in ELISA with $rG_L$55–98 derived from the Bucyrus isolate, implying a high degree of conservation of this epitope. To substantiate this finding sequencing studies of EAV $G_L$ from different isolates is currently being undertaken.

Changing from reliance solely on the NT, to an initial rapid screen by ELISA followed by neutralizing antibody testing of $ELISA^{+}$ samples would improve the speed of diagnosis for EAV seronegative horses, lower the cost of initial testing, and potentially eradicate differences in test results between laboratories. Additionally, the enhanced detection of seropositive animals allied to a vaccination policy and selective breeding practices would allow the better control and possible eradication of EAV from the breeding population. The expression and purification of $G_L$55–98 is simple and the ELISA is easily standardized between different testing laboratories. Since the recombinant protein is not infectious it affords the opportunity to undertake EAV serodiagnostic assays rapidly and safely without virus containment facilities. In addition, the use of a purified protein expressed in a bacterial system removes the problem of non-specific antibody crossreactivity to cell culture-derived antigen which has plagued previous EAV ELISA tests (Cook et al., 1989).

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