



Challenges and proposed solutions for more accurate serological diagnosis of equine infectious anaemia

C. J. Issel, M. T. Scicluna, S. J. Cook, R. F. Cook, A. Caprioli, I. Ricci, F. Rosone, J. K. Craigo, R. C. Montelaro, G. L. Autorino

Serological diagnosis of equine infectious anaemia virus (EIAV) infections has depended mainly on the agar gel immunodiffusion test (AGIDT). This study documents the presence of EIAV genetic sequences in a number of persistently infected horses and mules whose serums were interpreted as negative/equivocal on AGIDT, but positive on more than one ELISA test and in immunoblot tests. Strategies designed to take advantage of the combined strengths of the ELISA and AGIDT are shown effective in a national surveillance program for EIA in Italy where 17 per cent (25/149) of the equids considered to be infected with EIAV on combined/comparative serological data had reactions in the AGIDT that were interpreted as negative or equivocal. These data document the benefits of using a three-tiered laboratory system for the diagnosis of EIA. Although the ELISA-first strategy introduces some confusing results, the discovery of up to 20 per cent more cases of EIA makes it compelling. In our opinion, it is better and more defensible to find two samples in 1000 with resolvable but falsely positive ELISA tests for EIA than to release two to three horses in 10,000 with falsely negative test results for EIA (the rates seen in the Italian surveillance presented here).

Introduction

The control of equine infectious anaemia (EIA) was possible when a positive correlation was made between virus presence as measured in the horse inoculation test, and the presence of antibodies in the agar gel immunodiffusion test (AGIDT) (Coggins and Norcross 1970, Coggins and Patten 1971, Coggins and others 1972a, b). Since that time, the AGIDT (known colloquially as the Coggins test), using the major core protein (p26) of equine infectious anaemia virus (EIAV), gained wide international acceptance. Control of the infection, known to occur only in equid host species, has in many countries progressed to a point where the infection is rarely diagnosed in the tested population. It is unusual to find reliable data on the true prevalence of the infection, because testing in most countries is not mandatory.

In the USA, testing of equids is often required for movement and the infection is virtually eradicated in that group. Each year in the

USA, new cases are found, and frequently represent premises where testing has occurred for the first time, often when required for change of ownership. Testing in the USA has expanded to about two million samples each year, with fewer than 200 cases reported from 2008 to 2010. When these statistics are compared with the number of positive samples reported in 1976 (>10,000), the success of the control programme is clear and evident. In the USA today, it is rare to find equids with clinical signs associated with the infection; the vast majority of positives found are inapparent carriers of the virus.

It has been recognised for over 30 years that a low percentage of equids exposed to EIAV may become persistent carriers of the virus and pose diagnostic challenges when examined by the AGIDT (Toma, 1980, Issel and Adams 1982, McConnell and others 1983). Since the late 1980s, a number of ELISA-based serologic tests have been approved for the serodiagnosis of EIA, and the vast majority of equid serum samples have identical results in AGIDT and ELISA tests (Matsushita and others 1989). As ELISA tests are inherently more sensitive than AGIDTs for detecting antibody, the ELISA tests facilitate the detection of EIAV-infected equids with serum reported as negative in the AGIDT because of low antibody levels against the p26 antigen of EIAV.

The use of a combination of tests to more accurately diagnose infections with EIAV has been espoused (Issel and Cook 1993, Issel and others 1987, 1999, McConnico and others 1998). The strategy uses the increased power of ELISA tests on negative samples, the increased power of the AGIDT for positive samples, and the use of immunoblot tests on the rare samples that produce positive ELISA and negative AGIDTs. The majority of such samples have proven to be 'falsely positive' in the ELISA tests, because they either recognise none of the major proteins of EIAV (gp90, gp45 and p26), or recognise only the one EIAV antigen used in the ELISA procedure (p26). In a minority of cases, these 'Positive ELISA/Negative AGID' samples represent true EIAV infections, as the serum reacts against all three major

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C. J. Issel, DVM, PhD,

S. J. Cook, MSc,

R. F. Cook, PhD,

Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington KY, USA

M. T. Scicluna, DVM,

A. Caprioli, DVM,

I. Ricci, DVM,

F. Rosone, DVM,

G. L. Autorino, DVM,

Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Rome, Italy

J. K. Craigo, PhD,

R. C. Montelaro, PhD,

Center for Vaccine Research, University of Pittsburgh, Pittsburgh PA, USA;

E-mail for correspondence:

e-mail: cissel@uky.edu

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proteins in the immunoblot test. In some of these cases, the samples were reported as negative by AGIDT, but were interpreted incorrectly. In others, the samples have such a low level of antibodies against the p26 protein that a positive interpretation in the AGIDT is not possible.

This paper documents a number of these ‘false-negative’ AGIDT reactors in a group of equids intentionally exposed to relatively high doses of EIAV, and in a group of mules assembled to study the responses of such equids following drug-induced immunosuppression. These data, together with results from a national surveillance programme in Italy, provide compelling evidence for the utility of a system using ELISA tests first, followed by AGIDT, and immunoblot tests where indicated, that is, a ‘three-tiered’ diagnostic system.

Materials and methods

Experimental subjects and samples

Reference weak positive serums from University of Kentucky and United States Department of Agriculture

Serum from a horse named Flicker, from Louisiana, referred to in this paper as University of Kentucky reference weak positive serum, was used for all aspects of this study, and has been used extensively by our research programme since 1976. (Issel and Adams 1982) For comparison, in 2011, we purchased and performed the same range of serology on the United States Department of Agriculture (USDA) marketed reference weak positive serum for AGID testing. The serum is available from the USDA National Veterinary Services Laboratory (the OIE

Reference Laboratory for the Americas for EIA) as equine-infectious anaemia-weak positive serum, Reagent code 903-EDV. The USDA requires this serum to be interpreted as positive in the AGIDT, or none of the tests that day would be considered valid. The comparisons of the two weak positive samples are presented in Table 1 and Fig 2.

Horses immunised with EIAV

During a series of vaccine trials using EIAV as a model for HIV, several strains were created lacking functional S2 gene expression that replicated well in equid cell cultures in vitro, but which replicated poorly when inoculated into equid hosts (virus strains ΔS2 and D9) (Li and others 1998, 2003, Craigo and others 2007b). Horses were immunised with the EIAV strains designated ΔS2 (BT 15, C50 and B62) or D9 (all others) at intravenous doses of 10³ TCID₅₀ or intramuscular doses of 10³ (C9, C16) or 10⁵ TCID₅₀ (C15). Methods used to detect viral RNA sequences for the experimentally infected group have been reported earlier (Craigo and others 2007a). The work with horses was conducted in compliance with University of Kentucky Institutional Animal Care and Use Committee approved protocols.

Mules

A group of five mules from Italy are included in this report, and were assembled as a result of their detection in a national surveillance programme for EIA in Italy starting in 2007. They were selected because their serologic reactions in AGIDT, ELISA and immunoblot tests were not in agreement. The animals were gathered and treated with the

Table 1. Comparative virologic and serological results of samples from equids selected because of AGID test-reaction interpretations ranging from very weak positive (reaction 1), or negative (NEG).*

Animal		Virus†	AGID	ELISAs Tests US kits‡	IT§	Immunoblot p26	gp45	gp90
Experimental infections [∞]				1/2/3				
C9		Yes	NEG	+/+	1:24			
C15		Yes	NEG	+/-	<1:6			
C16		Yes	NEG	+/-	<1:6			
C22		Yes	NEG	+/-	<1:6			
C23		Yes	NEG	+/+	<1:6			
B62		Yes	NEG	-/-	<1:6			
BT210		Yes	NEG	+/+	1:12			
C50		Yes	NEG	-/-	<1:6			
H46		Yes	NEG	+/-	<1:6			
H32		Yes	NEG	+/-	1:6			
Mules before and after immunosuppression ⁷								
Mule 3	4/10	No	NEG	+/-	<1:6			
	5/10	Yes	1	+++	1:6			
Mule 5	4/10	No	1	+++	1:6			
	5/10	Yes	1	+++	1:24			
Mule 6	4/10	No	NEG	+++	<1:6			
	5/10	No	NEG	+++	<1:6			
Mule 7	4/10	No	NEG	+++	1:6			
	5/10	No	1	+++	1:24			
Mule 8	4/10	No	1	+++	1:24			
	5/10	No	1	+++	1:24			
Reference Positive Serums								
Flicker W+		Yes	NEG	+++	1:6			
USDA W+		ND	1	+++	1:48			

*Please refer to Fig [f1] for visual guide for interpretation of agar gel immunodiffusion test reactions as measured by detection of equine infectious anaemia virus-RNA or proviral DNA, or in horse inoculation test (Flicker only).

Virus RNA levels in the experimentally infected group determined by QRT-PCR ranged from 1×10¹ to 1.7×10⁵ per ml

†The majority of the samples listed as ‘-’ in this figure had measurable activity above controls, but not sufficient to warrant a ‘+’ by the manufacturer’s recommendations

‡For the ELISA test developed in Italy, samples were initially tested at 1 : 6 dilutions; positive samples were retested and diluted to determine endpoint titres

§Samples collected 180-210 days after infection were tested for virus RNA and for antibody, in comparative serological testing

∞Viral sequences were cloned only from assays for proviral DNA; assays for viral RNA in plasma were negative

AGID Agar gel immunodiffusion, ND Not done, NEG Negative, USDA, United States Department of Agriculture

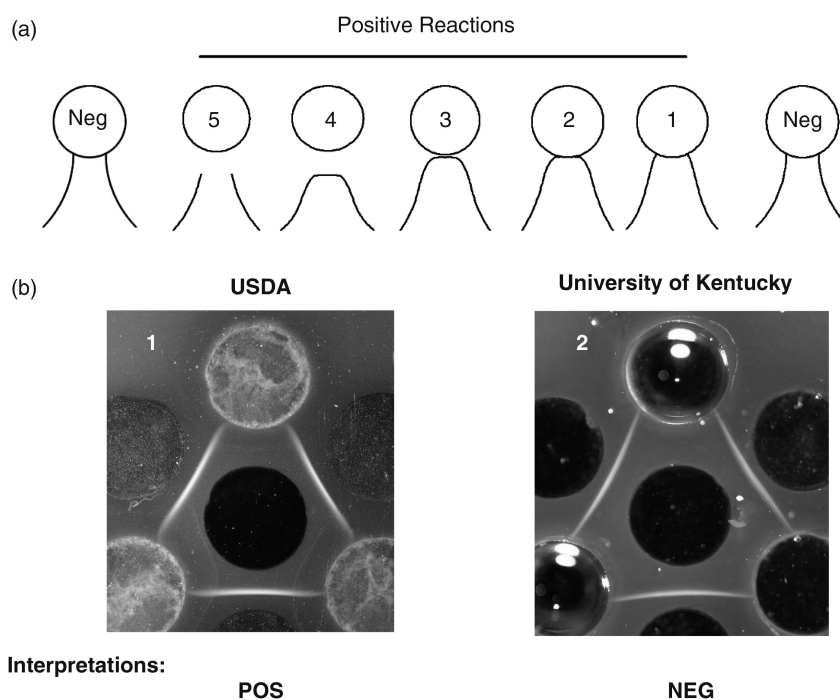


FIG 1: Standards used for reporting agar gel immunodiffusion test (AGIDT) reactions and challenges with accurate interpretations in the AGIDT: (a) graphical representation of standardised numerical interpretations by intensity and (b) photographs of reactions of weak positive reference serums from United States Department of Agriculture (USDA) (Panel 1) and University of Kentucky (Panel 2). All three test wells in each panel are the subject serum. Legend: Samples in (b) are the USDA (Panel 1) and the University of Kentucky (Panel 2) weak positive reference serums. Serum was washed from wells to facilitate photography. Both digital photographs receive identical editing

immunosuppressive drug, dexamethasone, at 0.11 mg/kg intramuscular (Craig and others 2002, 2007a) for nine days, and repetitively sampled for anti-EIAV antibodies in serologic tests using serum from clotted blood, or for viral genome in PCR tests (see below) in plasma and leucocytes obtained from blood collected in EDTA. Results from samples collected immediately prior to (April 6, 2010) and 28 days after initiation of dexamethasone treatment (May 4, 2010) are reported here. For detection of EIAV nucleic acid sequences, RNA was extracted from plasma (50 μ l) using the MagMAX Viral RNA Isolation Kit in conjunction with the automated MagMAX Express apparatus (Applied Biosystems/Ambion Inc, Austin, Texas, USA). A GenElute blood DNA kit (Sigma, St Louis, Missouri, USA) was used to extract genomic DNA from buffy coat preparations (100 μ l) according to the manufacturer's recommendations. All extracted nucleic acids were stored at -80°C prior to analysis. EIAV sequences were amplified using nested PCR as described by Cappelli and others (2011), except that annealing was conducted at 52°C . Prior to PCR amplification, RNA was converted to cDNA using Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics, Indianapolis, Indiana, USA) with EIAV DNAITAR as a specific primer (Cappelli and others 2011).

Serological tests

Samples from horses in experimental studies at the University of Kentucky were tested in AGIDT and ELISA test formats using commercially available test kits and following approved protocols, all of which use undiluted serum. The AGIDT kits all utilise the p26 antigen of EIAV, and kits in 2011 are marketed by three manufacturers; two use only recombinant p26, the other uses a 'blend' of viral and recombinant p26. In this paper, the kit using the 'blend' was used for results presented in Table 1. For the photographs of AGIDT results presented in Fig 1, a kit using only recombinant p26 was used. Samples were tested in the AGIDT by the current USDA approved standards, which includes verifying appropriate sensitivity of the assay using the USDA-reference weak positive serum as a standard. The AGIDT reactions were judged by the intensity of the reaction (see Fig 1a). Experimental subjects in this report had AGIDT reactions of one or less to qualify for inclusion in this study.

ELISA test kits in 2011 are available from four manufacturers in the USA, and all utilise the p26 antigen; one also incorporates an envelope determinant in each well, but there is no discrimination between

the two antigen reactions. Three ELISA test kits are included in this analysis, and are referred to as 1, 2 and 3 for this report. All test kits are licensed by the USDA and must meet the same standards of performance for release; thus, no identification of test kits is included in this report, to intentionally try to reproduce what a variety of laboratories would experience. Visual and spectrophotometric readings of ELISA test reactions were done and final results are presented in Table 1 as + or -, according to the manufacturers recommendations.

All samples tested with kits manufactured in the USA and presented in Table 1, including those from the mules, were tested using the same AGID kit and in three ELISA test kits at the same time, with the exception of the ELISA readings of the reference weak positive samples in Table 1, which were tested at a different time using the same kits.

The immunoblot test antigens of EIAV were derived from a cell-adapted strain of EIAV (EIAV-PV), which had been produced in cultures of fetal equine kidney cell cultures, and purified through multiple filtration and centrifugation steps including a final separation in glycerol gradients followed by pelleting. Details of these procedures have been published (Issel and Cook 1993, Issel and others 1999). Although the envelope glycoproteins are highly diverse in different strains of EIAV, the gp90 and gp45 appear to contain some highly conserved determinants. Preparation of the membranes and procedures used for immunoblotting were as described previously, with one major modification. In earlier studies, immunoblotting was primarily used on multiple sequential samples from experimental subjects using the membrane in a miniblotting apparatus. In the current analyses, individual strips of the membrane were cut and used for each sample, as they have proven more sensitive than the miniblotting technique because of increased surface and sample volume used. All serum samples are tested at 1 : 20 dilutions using a final volume of 450 μ l for the Immunitics Miniblott system, or 1.5 ml when the BioRad incubation trays are used. Rabbit antihorse IgG conjugate (Sigma) is used at a 1 : 1500 dilution and the same final volumes. Samples were referred to as positive by immunoblot if they recognised at least two of the three major proteins of EIAV at the same or more intense staining as seen with the University of Kentucky reference weak positive serum from the horse named Flicker who was proven to be a carrier of EIAV by horse inoculation. The same immunoblot protocol was used for the selected samples during the National surveillance programme in Italy.

Serological survey and testing for EIA in Italy

The data presented here refers to the activity conducted in the Latium Region within the EIA Italian national surveillance effort from 2007 to 2010. All 96,468 blood samples submitted to the laboratory as part of the surveillance programme were tested in the AGIDT as well as in a Competition-ELISA (C-ELISA), both using the recombinant p26. The test kits are standardised using the same standards of performance as the USDA-approved kits. The AGIDT is performed according to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE 2008), and the samples are tested undiluted. The in-house C-ELISA (IT C-ELISA) was developed in collaboration with the Istituto Zooprofilattico Sperimentale delle Regioni Lombardia ed Emilia Romagna. A brief description of the method follows: Nunc Maxisorp plates are sensitised overnight at 4°C using as catcher an anti-p26 Mab diluted in phosphate buffer solution. In time for the end of the adsorption incubation period, serum samples examined at a final dilution of 1:6 are initially diluted on inert microplates, in PBS pH 7.2–7.4 containing yeast extract (0.05 per cent) and mouse serum (1 per cent). The following internal controls are also included in each plate: an antigen control, a positive and negative serum control and a blank reaction control. The recombinant p26 antigen is then added to all samples and controls. At the end of a 75 minute incubation at 37°C, the samples and controls are transferred onto the previously sensitised plate together with the horseradish-conjugated tracer Mab, and the plates are again incubated under the same conditions as before. At the end of this second incubation, *o*-Phenylenediamine dihydrochloride substrate is added and after 10 minutes, the reaction is stopped using 1M sulphuric acid. The sample's reactivity is read at an optical density (OD) of 492 nm using a spectrophotometer. The results are interpreted using the following algorithm: Percentage Inhibition (PI) = 100 – (OD mean of sample / OD mean of negative control) × 100. The sample is considered negative if the PI is <30 per cent, positive if >50 per cent, and equivocal if between 30 and 50 per cent. The test has been validated according to the recommendations reported by the OIE (2008). The results will be published in a separate paper. If samples had positive/equivocal results in the C-ELISA test, they were tested by AGIDT and, if negative, then examined in immunoblot using the same method as described above. The in-house C-ELISA test was used on samples presented in Table 1 (column labelled IT); samples were tested initially at a dilution of 1 : 6, and positive samples were diluted further to determine endpoint titres.

Results

Italian competition ELISA assay (IT C-ELISA)

Validation of the IT C-ELISA test was carried out according to standards in the OIE manual (OIE 2008) and, in particular, performed against the AGIDT by comparison of results from a group of 857 AGIDT-positive and 238 AGIDT-negative serum samples collected from routine surveillance testing in Italy using reagents and kits developed internally in Italy. The C-ELISA results agreed at a 100 per cent level with AGIDT-positive samples. Eighty per cent of the C-ELISA-positive samples gave positive AGIDT interpretations, an apparent specificity of the ELISA test of 80 per cent. Because of the good correlation of results between tests, the C-ELISA was utilised further by combining ELISA, AGIDT and immunoblot tests in a portion of a National surveillance for EIA in Italy starting in 2007.

During 2007–2010, a total of 923,299 samples from across Italy were tested for EIA. Of the total, 96,468 are included in this study and were tested first in the C-ELISA test. Overall, 331 of the 96,468 samples proved positive in C-ELISA, but only 124 of those were interpreted as positive by AGIDT. The 207 samples positive by C-ELISA, but negative by AGIDT, were tested further by immunoblot. Of the 207 samples, 25 recognised multiple EIAV proteins in immunoblot; of the other 182, the majority (146) was not reactive, and 36 (20 per cent) only recognised the p26 protein in immunoblot tests which corresponds to 0.037 per cent of the total samples examined in the ELISA. The 25 met the criteria established for this study as demonstrating evidence of exposure to EIAV. Of the 25 equid cases found through the comparative serology, 22 were able to be investigated further, and 17 of them (77 per cent) had been exposed to risk-related factors, for example, history of EIA on the premises. The other 182 were judged

to be falsely positive on the C-ELISA test. Thus, serologic evidence of infection was found at a 17–20 per cent higher rate if the comparative serology system was used. The apparent false negative rate for AGIDT for the entire 96,468 samples was 0.026 per cent; stated another way, 17 per cent (25/149) of the equids judged exposed to EIAV using comparative serology were interpreted/reported falsely as negative by AGIDT testing. Only 12 per cent of the ELISA-positive samples judged to be negative on AGIDT were also positive on immunoblot tests. Stated another way, an apparent false positive rate of 0.19 per cent (182/96,468) was noted in the C-ELISA test.

Reference weak positive reference serums from University of Kentucky and USDA

Serologic test results from the University of Kentucky and the USDA weak positive serums for EIA are presented at the bottom of Table 1 and Fig 2. Both reference serums produce positive results in all ELISA test kits from the USA, with the USDA serum having twofold to fourfold higher endpoint titres in all three ELISA formats used in this study (data not shown). In the IT C-ELISA protocol, the UK and USDA serums have endpoint titres of 1 : 6 and 1 : 48, respectively. When the samples were diluted 1 : 20, they clearly recognised the three major EIAV structural proteins gp90, gp45 and p26 in immunoblot tests (Fig 2).

The AGIDT kit used for graphic representations of these weak positive samples uses recombinant p26 antigen only and produces a bright line of reaction (Fig 1b). Reactions were photographed 48 hours after plating (panels 1 and 2), because reading was more problematic at 24 hours. The reaction of the USDA weak positive reference serum was interpreted as positive in the AGIDT (Fig 1b, panel 1). The reaction of the University of Kentucky serum was interpreted as negative by AGIDT when read as an unknown without bias at 48 hours (Fig 1b, panel 2). When the AGIDT kit antigen was diluted by 20 per cent, the reaction of the UK serum could be more easily interpreted as equivocal, but was subjectively less reactive than the USDA reference serum (data not shown).

Horses intentionally infected with EIAV

A number of horses inoculated with the ΔS2 or D9 strain of EIAV and held 180–210 days until challenge with a pathogenic strain of EIAV, maintained low levels of antibody against EIAV. At time of challenge, all these subjects (top panels in Table 1) were positive for serologic evidence of exposure to EIAV in immunoblot tests, and positive for EIAV virus signal in quantitative RT-PCR assays of plasma for viral RNA (Table 1) in quantities ranging from 10 to 100,000 copies per millilitre (data not shown). Serum samples from these individuals, however, were interpreted to be negative by AGIDT, and gave variable results in the three ELISA kits from the USA and in the IT C-ELISA. Results for US kits in Table 1 are posted as + or – based on the manufacturer's recommendations, while results using the IT C-ELISA test are posted as endpoint titres with <1 : 6 being interpreted as negative. Although a sample in this group may be posted as '–', in fact 12 of the 16 reactions posted as – or <1 : 6 were reactive in the specific ELISA test and would have demanded further study.

Mules from Italy

These five mules were gathered and immunosuppressed with dexamethasone. This study documents the results of comparative serology and analysis of plasma and leucocyte samples for the presence of the RNA or proviral DNA of EIAV on samples collected at the initiation of the study and 28 days later (Table 1 and Fig 2). On the first samples, only two of the five mules (mules 5 and 8) had AGIDT reactions that should be interpreted as positive. After immunosuppression, an additional two (mules 3 and 7) had unequivocal positive AGIDT interpretations. In ELISA tests, all mules were positive or reactive on all ELISA tests with the exception of mule 7 which remained negative on one US ELISA test kit, and mule 6 whose first sample in the IT C-ELISA remained below the equivocal cut-off line (26 compared with 30 per cent), and whose second sample was slightly above that line (36 per cent), but below the positive cut-off of 50 per cent. By using titre data from the IT C-ELISA test, two mules had significant increases in titre after immunosuppression (mules 5 and 7). On immunoblot tests, all mule samples, except those from mule 7,

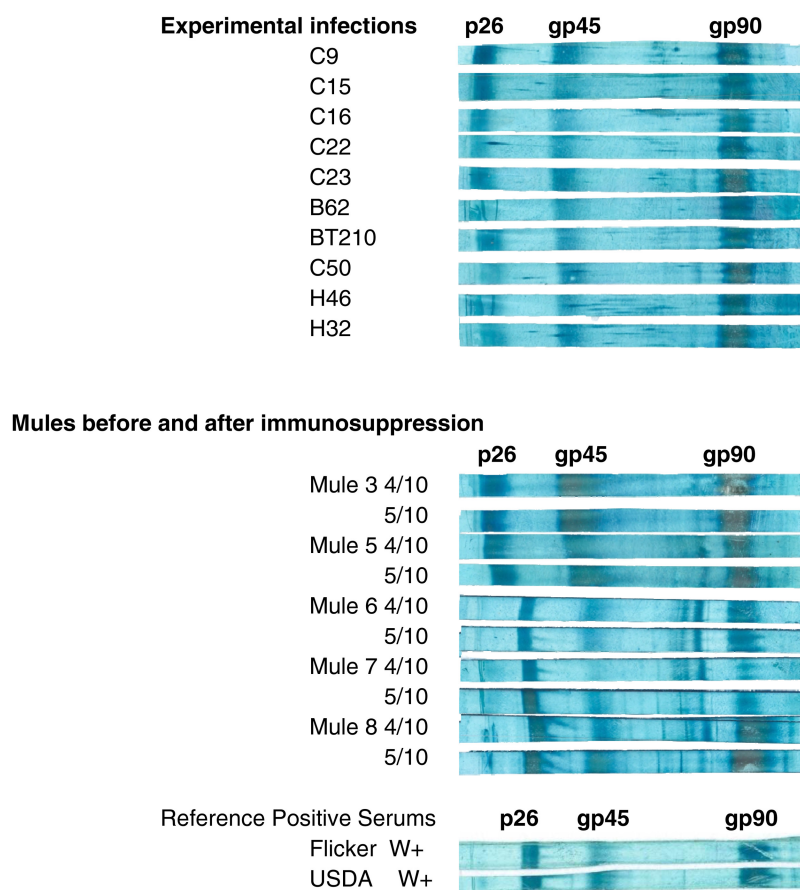


FIG 2: Immunoblot reactions of horses and mules to complement Table 1. (Please refer Table 1 for details)

had equal or higher activity than our reference weak positive reactor, Flicker. Mule 7 had qualitative increases in activity against gp45 and gp90 after immunosuppression. In total, comparison of pre- and postimmunosuppression samples in these mules indicated serologic evidence of active infection with EIAV in three of these five mules.

Using the primers described by Cappelli and others (2011), viral RNA could not be visualised in any of the plasma samples tested by nested PCR. EIAV proviral DNA, however, was amplified by these primers in leucocyte DNA samples collected from mules 3 and 5 following dexamethasone treatment; sequence data indicated identity with EIAV (data not shown). Interestingly, and in contrast with the other three mules, both mules 3 and 5 experienced clinical episodes consistent with EIA after immunosuppression (G L Autorino, unpublished observations). Additional studies on samples from the mules collected during and after immunosuppression were analysed, and all five mules proved to have detectable EIAV signals by nested PCR at more than one time (G L Autorino, unpublished observations).

Discussion

After the AGIDT for EIA was described and validated, its use was immediately embraced by the international veterinary community. Since the late 1980s, there have been a series of test kits produced using ELISA test formats that have also received wide acceptance. The AGIDT, however, remains the gold standard serologic test for EIA because of its proven correlation with results in horse inoculation tests for EIAV and the high accuracy (extremely low rate of false positive AGIDT reactions). Any movements toward alternate standards have received skeptical receptions because of the successes achieved by regulatory bodies using the AGIDT alone. Data presented earlier and here indicate a greater need for use of ELISA tests today for reasons mainly centred on human error and technical issues. Our explanations and arguments follow.

ELISA test kits

For these comparisons, this study used only three of the four available ELISA test kit formats from different manufacturers in the USA.

A direct comparison of sensitivity/specificity was avoided, as each has been licensed by the USDA, and has been formulated according to proprietary standards to produce results that are judged to be comparable with those in AGIDTs. Thus, the vast majority of ELISA test results in all the four available kits are in alignment with those of the AGIDT. The authors are aware that the kits have been formulated to permit the alignment, but not to reach the maximum sensitivity possible with the assay. Each of the manufacturers has approached this differently, and so a direct comparison of available kits was not considered appropriate for this report. The authors are confident that if the standards are readjusted, ELISA test kits could be packaged with increased sensitivity as a goal if desired. Another consideration with the ELISA test kits in the USA is the fact that they are packaged and distributed to about 500 laboratories approved by the USDA to conduct tests for EIA. The kits have been licensed to permit direct visual comparisons of sample reactions against controls, and results reported without the use of a spectrophotometer, in part, to benefit smaller laboratories without such equipment. In our laboratories, visual and spectrophotometric results are recorded before reporting results, a practice that is recommended as a routine.

In this study, the focus was on those samples that are reactive/positive in multiple ELISA test kits but judged negative or equivocal in AGIDT. In these cases, it is imperative to use the power of the immunoblot test to clarify the status of these equids. When the majority of equids with samples that are reactive/positive in multiple ELISA tests, and interpreted as negative in AGIDT are tested by immunoblot, the samples react with both the envelope glycoproteins of EIAV and the major core protein. Similar reactions in ELISA kits, however, can be observed in equids that recognise only the p26 antigen of EIAV, and are judged as not specific for EIA. The only way to differentiate these is with the power of the immunoblot test. Fortunately today, equids with these types of reactions are very rarely encountered in equid populations.

Each of the ELISA kits has historically had some minimal 'false-positive' background, and when samples judged to be true 'false-positives' are analysed, reactivity to components are usually found in only one kit. In other samples, reactivity in multiple ELISA kits

is detected and is determined to be specific for the p26 antigen, but is not deemed to be raised following infection with EIAV, as no reactivity against the envelope glycoproteins is found. This type of reaction might be explained as a cross-reaction to a related antigen, such as the recognised interspecies determinant of the lentivirus major core protein, most notably demonstrated with EIAV and HIV (Montagnier and others 1984, Egberink and others 1990, Grund and others 1994).

The ELISA test developed in Italy is currently used only by a limited number of official state laboratories, and is designed to follow established guidelines. The initial dilution of serum to 1 : 6 for use in this test makes direct comparison impossible with kits from the USA which use undiluted serum. In the IT C-ELISA test, however, results of sera from experimentally infected horses were highly correlated with the results from the US ELISA test kits. Following immunosuppression, serologic evidence of active virus replication was suggested using the IT C-ELISA test in three of the five mules, AGIDT interpretations changed from negative/ equivocal to positive in two of those three, and there were qualitative changes in recognition of the gp90 and gp45 antigens in immunoblot in the third.

AGID and ELISA test kits for EIA manufactured in the USA are designed to test undiluted serum. When these data are considered together with the Italian C-ELISA data from the mules (where samples had endpoint titres of up to 1 : 24 and significant recognition of EIAV major proteins in immunoblots were interpreted as Negative by AGIDT), it is clear that AGID testing is well below the desired threshold for detection of all EIAV-infected equids. It would seem to be appropriate for the international standards groups responsible for establishing the set point for reference standards for EIA testing to revisit them in light of these data.

Experimental infections

The most compelling data emanate from our experimental studies using strains of EIAV that have been engineered to contain functional or real deletion of the accessory gene product S2. This deletion renders the virus less able to replicate *in vivo*, but with no apparent effect on replication in cell cultures. The effect of this lower replication is a corresponding increase in the percentage of intentionally infected horses that develop antibodies against EIAV p26 protein that are below the detection level for positive in AGID and/or ELISA tests. As all these horses had evidence of active virus infection as measured by PCR assays, in some cases exceeding 10^5 copies per millilitre, the serology reported as 'negative' clearly represents true false negative results. In these cases, the ELISA test results were often reactive, but below the cut-off line for positive.

As a consequence of these experiences, the authors recommend that samples with reactivity in a single ELISA format be tested further in multiple ELISA test formats and, if reactive in several, tested further in immunoblot tests. As the data clearly demonstrate, if only AGID testing were done, these horses would escape routine detection and would be able to move freely as EIA test-negative, EIAV-infected equids.

EIAV genetic signal detection – PCR strengths and limitations

Data presented here document the presence of EIAV genetic material in plasma samples or leucocytes from horses and two mules with AGIDT reactions of ≤ 1 . To date, current thought suggests that equids with such reactions are either infected with weakened strains of EIAV that replicate poorly (eg, the D9 deletion), or that the individual host is more genetically resistant to the particular strains of EIAV presented to them. The net result in both cases is lower viral burdens and lower immune stimulation, which may or may not persist at static levels through time. In fact, data presented here and elsewhere (Craig and others 2007a) indicate the utility of immunosuppression with dexamethasone for documenting EIAV genetic sequences in the blood of some seropositive equids, which may be temporally associated with significant changes in antibody levels against EIAV proteins (fourfold or greater change in antibody level).

In our previous vaccine trials using the $\Delta S2$ and D9 strains, immunosuppression has been used as an adjunct to evaluate the protection

afforded by immunisation of horses after challenge with pathogenic strains of EIAV. Some of the horses with no prior evidence of the challenge virus (apparent sterile protection against challenge) develop clinical signs of EIA with higher levels of EIAV sequences after a 10-day treatment course with dexamethasone. Thus, tissue reservoirs of EIAV, and possible presence of latent virus, cannot be ruled out in these cases without immunosuppression, and conclusions based on absence of virus from routine virus isolation or PCR must be stated with caution. In other words, this provides additional evidence supporting our continued reliance on serologic evidence of infection with EIAV because of the nature of the infection and difficulty in detecting virus in circulating blood of all infected equids at all times.

Development of PCR-based assays for routine diagnosis of EIAV infections faces two major hurdles. The first is the significant genetic variation that occurs both between geographically distinct isolates and during the course of an infection, while the second, as indicated above, is that plasma-associated viral RNA loads are maintained at exceptionally low levels in some infected individuals. It is only comparatively recently that studies have revealed the extent of genetic diversity between EIAV isolates. Until this information became available, nucleotide sequence data were limited mainly to closely related North American EIAV strains. Therefore, it is perhaps not surprising that PCR assays based exclusively on these initial sequences failed (Langemeier and others 1996, Cook and others 2002) to detect EIAV in the field (Quinlivan and others 2007, Cappelli and others 2011). Despite these difficulties, PCR has been used successfully to detect EIAV sequences in naturally infected horses. A nested PCR assay developed by Nagarajan and Simard (2001) was used to amplify a region of EIAV proviral DNA containing *gag* gene sequences in 88 of 112 horses from stables where EIA had been previously diagnosed. By contrast, only 81 of these were positive by AGID and, so, based on these results, this nested PCR assay is currently recommended by the OIE for the detection of EIAV. However, the PCR primers developed by Nagarajan and Simard (2001) did not detect equivalent viral *gag* sequences from the 2006 Irish and Italian EIAV outbreaks (Quinlivan and others 2007, Cappelli and others 2011) even in samples collected from clinical cases where tissue and blood-associated viral loads are expected to reach maximal levels (Harrold and others 2000). As a result, new PCR-based methods were developed to detect these European viral strains (Quinlivan and others 2007, Cappelli and others 2011) of which one described by Cappelli and others (2011) was demonstrated to possess cross-reactivity by the amplification of sequences from over 30 Italian and Romanian EIAV isolates not related to viruses directly responsible for the 2006 outbreaks (Cappelli and others 2011). Furthermore, this assay was shown to possess greater sensitivity than AGID. Perhaps more importantly, Cappelli and others (2011) found that although plasma-associated viral RNA was undetectable except in clinical EIA cases, proviral DNA could be amplified by PCR in all AGID-positive equids when blood mononuclear cell DNA was used as a template similar to that first described by Nagarajan and Simard (2001). Therefore, these authors may have addressed the second problem facing PCR development for EIAV, namely the low plasma-associated viral loads that typify inapparently infected EIAV carrier animals (Harrold and others 2000). However, experiments outlined here indicate that while the nested PCR primers described by Cappelli and others (2011) are capable of detecting EIAV proviral sequences from all five experimental mules, the results vary dependent on the time-point. In other words, samples collected at certain periods from some equids may produce false negative results when tested using PCR. At present, the reason for the discrepancy between these results and those of Cappelli and others (2011) is not known, although potential explanations include differences between nucleic extraction procedures or assay reagents, the possibility of mismatches between primer and viral target sequences limiting detection efficiency and/or the fact that in some equids, proviral DNA levels in blood monocytic cells may temporarily fall below the detection limit. The full details of the mule study will be presented elsewhere (G L Autorino, personal communication).

Despite its limitations, detection of genetic material, rather than actual isolation of an infectious agent, is increasingly being regarded as evidence of active infection in routine diagnostic applications. In fact,

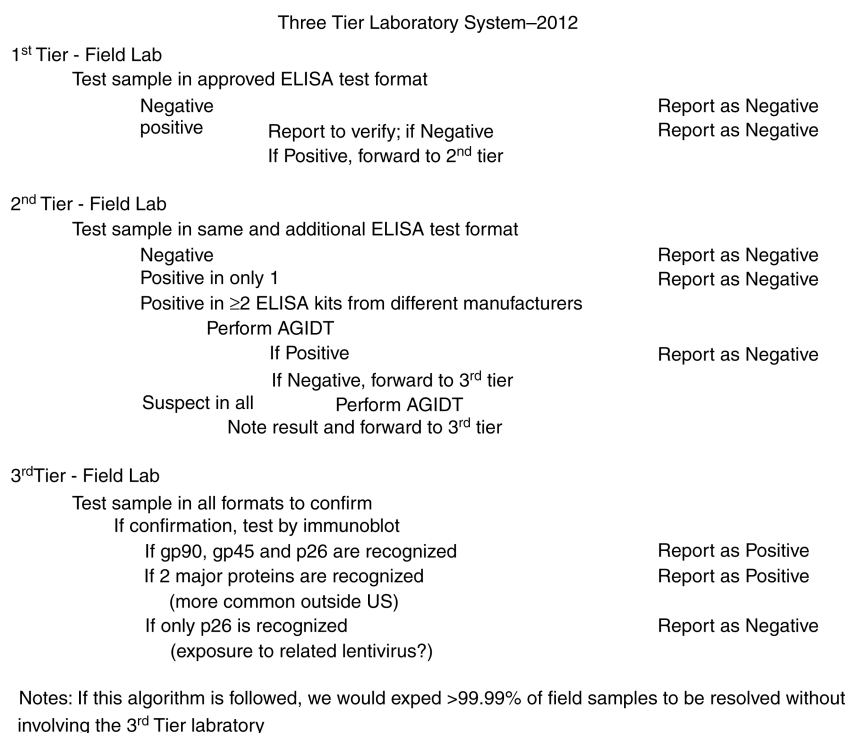


FIG 3: Proposed decision tree for serodiagnosis of EIA using the three-tiered laboratory system

in the case of EIAV where virus isolation in equine monocyte-derived macrophages present considerable technical difficulties (Hines and Maury 2001), PCR-based assays are the best available methodology for confirming presence of the virus, especially in cases of suspected recent exposure. Furthermore, real-time PCR-based tests can produce fully quantitative information about tissue-associated viral burdens. An additional advantage of using PCR-based methods against retroviruses is that two targets, viral RNA and proviral DNA, are available. Although further work must be done, data presented in this study support the conclusion of Cappelli and others (2011), that in clinically inapparent EIAV equids, proviral DNA is more easily detectable than viral RNA by PCR-based techniques. (Cappelli and others 2011) However, as the horse genome is populated with large numbers of retroviral-like sequences, it is imperative that all PCR products generated from cellular DNA samples be sequenced to confirm their identity.

Additional hesitations, concerns and recommendations

Caution must be exercised when using more sensitive serological techniques, especially those with lower accuracy (recognised higher rates of non-specific reactions). We contend that with testing for EIA, it is better to have resolvable laboratory errors with the more sensitive ELISA procedures than to rely on a less sensitive procedure that would result in continued free movement of EIAV-infected equids.

These considerations in the case of EIA prompt us to recommend immunoblot analysis of samples with positive (and suspect) results in more than one ELISA test kit. In very rare cases, such equids show specific reactivity only to the p26 antigen, in some cases giving sufficient reactivity to achieve a positive interpretation (≤ 1) in the AGIDT. In fact, the Italian experience related to a large population also reports that this value is low and estimated to be less than 0.04 per cent. Because of these cases, we strongly recommend requiring immunoblot confirmation on all AGIDT-positive samples with intensity of one or less. One possible explanation for these reactions is based on the presence of a cross-reactive group and even interspecies determinants in the major core proteins of retroviruses and lentiviruses. For example, serum from EIAV-infected horses recognised the major core protein of the human immunodeficiency virus (Montagnier and others 1984), and similar cross-reactions between other recognised and yet to be discovered retroviruses may also be possible. As these types of reactions are so rare, they may only be of academic interest. However, our experience suggests that such exposures may be more common than recognised, as a relatively high rate of

low-level reactivity against the p26 antigen is observed in equids tested by immunoblot.

Taken together, these data indicate that a number of EIAV-infected equids with antibodies against all three major proteins of EIAV can escape detection if only AGID testing is performed. The rate of such equids is not known, but based on these studies and our collective experiences, it is estimated at up to 20 per cent of the EIAV-infected population. If our control programmes are truly designed to detect EIAV-infected equids with serologic tests, current regulatory efforts should be modified to include the strengths of both the ELISA tests (for negatives) and the AGIDT (for positives) if we are to use the investment made by the horse industry wisely.

We maintain that reliance on serology is our best approach to control of EIA, because monitors for virus, or virus genetic material, may not be accurate measures of virus presence because of recognised dramatic variations in viral loads and/or viral genetic diversity. Our control programmes for EIA are based on the assumption that all equids infected with EIAV pose the same potential threat, a scientifically defensible position. We do not have the ability to accurately monitor virus replication in all infected equids, and we realise that stress can change virus presence in circulation by 100,000-fold within a short time. Thus, in our opinion the continued reliance on serology for diagnosis of EIA is warranted.

We have summarised our recommendations for optimal serological testing for EIA (Fig 3) based on technology available today, and deployed in a three-tiered approach.

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