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Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Is a diagnostic system based exclusively on agar gel immunodiffusion adequate for controlling the spread of equine infectious anaemia?



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ARTICLE INFO

Article history:

Received 12 November 2012

Received in revised form 26 February 2013

Accepted 27 February 2013

Keywords:

Equine infectious anaemia

Serology

Mule

Diagnostic sensitivity

Three-tiered system

ABSTRACT

To improve the efficiency of the National equine infectious anaemia (EIA) surveillance program in Italy, a three-tiered diagnostic system has been adopted. This procedure involves initial screening by ELISA (Tier 1) with test-positive samples confirmed by the agar gel immunodiffusion test (AGIDT) (Tier 2) and, in the case of ELISA positive/AGIDT negative results, final determination by immunoblot (IB) (Tier 3). During this evaluation, 74,880 samples, principally collected from two Regions of Central Italy (Latium and Abruzzo) were examined, with 44 identified as negative in AGIDT but positive in both ELISA and IB. As the majority of these reactions occurred in mules, an observational study was conducted in this hybrid equid species to investigate if there is a correlation between plasma-associated viral loads and serological reactivity, to test the hypothesis that false-negative or very weak positive AGIDT results are associated with elite control of EIA virus (EIAV) replication accompanied by reduced transmission risks. The study animals consisted of 5 mules with positive AGIDT readings, along with another 5 giving negative or very weak positive results in this test. All mules were seropositive in Elisa and IB. Samples were collected routinely during an initial 56-day observation period, prior to dexamethasone treatment lasting 10 days, to determine the effect of immune suppression (IS) on clinical, humoral and virological responses. All mules were monitored for a further 28 days from day 0 of IS. None of the animals experienced relevant clinical responses before IS and there were no significant changes in antibody levels in ELISA, IB or AGIDT. However, plasma-associated viral-RNA (vRNA) loads, as determined using TaqMan[®] based RT-PCR, showed unexpectedly high sample to sample variation in all mules, demonstrating host-mediated control of viral replication is not constant over time. Furthermore, there was no apparent correlation between vRNA loads and antibody reactivity in serological tests. Analysis of PCR products established all mules were infected with viruses possessing nucleotide sequence similarity, varying from 77 to 96%, to previously identified European EIAV strains. Following IS, all mules showed increases in plasma-associated vRNA loads, suggesting control of EIAV replication is mediated by immune responses in this hybrid species. However, only three mules showed anamnestic humoral responses to rises in viral loads, as defined by at least a four-fold increase in ELISA titre, while two remained AGIDT-negative. This study demonstrates that viral loads in equids with consistent ELISA/IB positive-AGIDT negative to very weak positive test results

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(Group N) can be equivalent to those that produce clearly positive results in all three serologic tests (Group P). Therefore, such animals do not pose inherently lower risks for the transmission of EIAV. Consequently, the exclusive use of the AGIDT, as prescribed by the World Organization of Animal Health (OIE) for diagnosis of EIA prior to the international movement of horses, can report as negative some EIAV-infected equids. These results dramatically underscore the necessity of combining the specificity of AGIDT with tests with higher sensitivity, such as the ELISA and the power of the IB to enhance the accuracy of EIA diagnosis.

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1. Introduction

Equine infectious anaemia is a blood borne disease of equids that has nearly a worldwide distribution and is caused by a lentivirus (Equine Infectious Anaemia Virus—EIAV) of the *Retroviridae* family. Clinical signs linked with EIAV are highly variable, although infected equids often experience an acute febrile episode with concomitant thrombocytopenia followed by a chronic stage in which recurring disease episodes are associated with non-specific signs of fever, anaemia, oedema, thrombocytopenia and various wasting syndromes. In most cases, these febrile episodes eventually cease and, despite remaining persistently infected, the animal defined as inapparent carrier enters a prolonged phase with no overt clinical signs (inapparent carrier). The fact that disease signs are not specific and that most equids enter the inapparent carrier stage 12–24 months following exposure to EIAV, makes clinical diagnosis difficult to achieve (Leroux et al., 2004). Virological diagnosis of the infection is also problematical (Hines and Maury, 2001), because in vitro viral isolation is technically complicated, as is the use of diagnostic molecular methods mainly due to the genetic heterogeneity of the virus and the variation of the vRNA/proviral DNA loads during the infection (Leroux et al., 2004). For this, suspect cases and surveillance programmes for the disease are usually based on serological testing (Issel et al., 1999) employing methods recommended by the OIE (2012 a,b).

Since 2006, Italy implemented a National surveillance programme for the control of EIA, based on the annual serological control of all equids above the age of six months. To increase the effectiveness of the surveillance system, a three-tiered diagnostic system, similar to that already described by Issel et al. (1999), was applied to routine samples arriving at the Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana (IZSLT) diagnostic laboratory, as well as, to those sent for confirmatory purposes by the network of authorized laboratories to the Italian National reference centre for EIA (CRAIE), which is within the IZSLT. Using this serial testing diagnostic approach, samples were first examined by ELISA with sera reacting positive in this test confirmed by the AGIDT. Positive ELISA, but negative AGIDT samples were submitted to CRAIE for examination in IB. Equids are defined as serologically positive for EIAV when their sera, examined in the three-tiered system, react either in the AGIDT and/or with two or more of the major EIAV structural proteins (p26 and gp45 or gp90) in IB. When this diagnostic system was applied to 74,880 samples collected during 2009 and 2010, principally from the two Regions in Central Italy (Latium and Abruzzo) with the

highest EIA seroprevalence, 44 sera were identified as ELISA/IB positive-AGIDT negative when submitted to CRAIE for final determination. In a diagnostic system for EIA, based exclusively on AGIDT as both the screening and confirmatory test, such samples would have been misclassified as negative. Details of the results of the surveillance activity, conducted in Italy for EIA between 2006 and 2011, will be presented in a separate study. However, it is important to note that many numerous samples sent to the CRAIE have reactivity in AGIDT that is extremely close to the detection limit of this assay. Therefore, it is very likely that without the three-tiered system, diagnosis of EIA in these cases would have either been missed or delayed.

Analysis demonstrated the frequency of samples with the ELISA/IB positive-AGIDT negative or very weak positive reaction pattern was significantly higher in mules than in horses (Odds ratio = 103. 95% CI 76.22–139.2). This suggests that serological diagnosis may be more problematic in *E. caballus* × *E. asinus* and therefore it is important to understand the role this hybrid equid species plays in dissemination of EIAV. Unfortunately, information regarding EIA in mules is limited to a single publication describing the clinical and pathogenic evolution of the disease in naturally and experimentally infected animals (Spyrou et al., 2003).

For this, a longitudinal observational study was conducted on a group of 10 naturally infected mules, to investigate if there is a correlation between plasma-associated viral loads and serological reactivity, to test the hypothesis that false-negative or very weak positive AGIDT results are associated with elite control of EIAV, accompanied by reduced transmission risks. The study group consisted of 5 mules with positive AGIDT readings along with another 5 giving negative or very weak positive results in this test, defined also as score ≤ 1 , as reported by the scoring scheme in Fig. 1 A. All mules were seropositive in ELISA and IB. Following an initial 56-day observation period, the experimental subjects were IS with dexamethasone to determine if clinical, humoral and virological responses were similar to those previously reported in horses (Kono et al., 1976; Craigo et al., 2007).

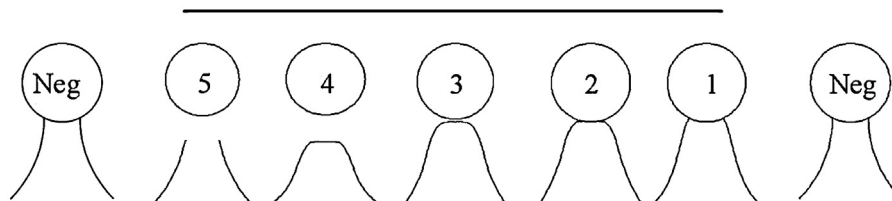
2. Materials and methods

2.1. Experimental animals and design

The study was carried out on ten mules, numbered 1–10 (see Table 1 for the general characteristics of the study group). These animals were acquired from five separate outbreaks, located in five different adjacent Provinces of Central Italy and included 5 animals positive in all

A. Quantitative scoring scheme for AGID test reactions.¹

Positive Reactions

B. AGIDT test scoring of the 10 mules²

Days Post-IS	Mule N°									
	3	6	7	8	5	1	9	2	4	10
-7	0	0	1	1	1	2	2	3	3	3
0	0	0	0	1	1	2	2	3	3	3
7	0	0	1	1	1	2	2	3	3	2
14	0	0	0	1	1	2	2	3	3	3
21	0	0	1	1	1	2	4	3	3	4
28	0	0	1	1	2	2	4	3	3	4

C. C-ELISA titres of the 10 mules²

Days Post-IS	Mule N°									
	6	3	7	8	5	1	2	9	10	4
-7	0	0	6	48	12	48	192	24	192	576
0	0	6	6	24	12	192	192	48	192	576
7	0	6	0	12	12	48	192	48	192	576
14	0	0	6	12	12	48	192	12	192	384
21	0	12	12	48	12	48	192	576	576	192
28	0	12	48	48	192	48	192	576	576	192

Fig. 1. Serological reactions of mules on AGID and C-ELISA tests. The results presented refer to the weekly blood samples collected between 7 days pre-IS and 28 days post-IS. The wells represented here is were the sample is distributed. This is placed between 2 wells, each containing an internal positive control serum.

¹The well containing the antigen is opposite the serum sample well. These reactions represent the spectrum of intensity of positive AGIDT reactions: positive samples are graded from 5 to 1. In the table and text, "Neg" is also expressed as "0" for numerical consistency. A score of "1" is a "very weak positive reaction" that is difficult to interpret by some readers and that might be reported as negative on routine tests. Samples with such reactions should be confirmed in IB. Scores from "1" to "5" should be reported as positive in AGIDT.

²Shading in darker grey and lighter grey was done to group the mules with lower, (referred in text as Group N) or higher (referred in text as Group P) serological C-ELISA/AGIDT reactivity respectively. Within each group, results are presented by rank (please see text for explanation). Although the ranking order within each group differs in AGIDT and ELISA, the groups are the same in AGIDT and ELISA, not surprising because both assay detect antibodies against the p26 antigen of EIAV.

serologic tests of the three-tiered system, to which belonged mules 1, 2, 4, 9 and 10 and 5 with negative or very weak positive reaction in the AGIDT, to which belonged mules 3, 5, 6, 7 and 8. Mule 6 was the only animal negative in both AGIDT and ELISA.

The animals were also verified for the presence of other frequently occurring infections in equids (Equine herpesvirus and Piropasms), to control if their presence effected the evolution of this study. On recruitment, defined as the day when the mules were collected from the holdings, all were positive for *Babesia caballi* or *Theileria equi* or for both in

commercial serological ELISAs (*Babesia equi/caballi* Antibody test Kit, cELISA-VMRD, Inc.[®]) carried out according to the instructions included in the kit. For this, treatment for sterilization was carried out a week before the beginning of the observation period, using imidocarb (Intervet[®]) according to the dosage indicated. During the experiment, the animals were also tested at weekly intervals for EHV-1 and -4 infections, using a Real-time PCR (RT-PCR) carried out as described by Damiani et al. (2005).

From day 0 of the observation period, the animals were housed in a covered enclosure with all animal handling and

Table 1

Characteristics of the study group regarding age, sex, serological reactivity observed on testing during surveillance and duration of immune suppression treatment in days of the mules used in the study.

Mule identification no.	Age	Sex	Serological result (ELISA/AGIDT/IB) ^a	Duration of IS treatment in days
1	12	Female	+/2/+	8
2	2	Female	+/3/+	10
3	30	Female	+/0/+	10
4	22	Female	+/3/+	10
5	9	Fe male	+/1/+	10
6	8	Female	±/0/+	10
7	11	Castrated male	+/1/+	10
8	17	Female	+/1/+	10
9	11	Female	+/2/+	10
10	7	Female	+/3/+	10

^a Serological results for ELISA and IB are reported qualitatively as positive (+) or negative (-). The AGIDT reactivity is reported as scores as described in Fig. 1A. The mules that had registered scores 0 or 1 are respectively mules 3 and 6 and mules 5, 7 and 8.

experimentation procedures performed under veterinary supervision, according to current European Regulations on Animal Experimentation. The study was performed adopting biosecurity measures for the risk management of EIAV transmission, especially iatrogenically. Furthermore, although the study period was not concomitant to the insect vector activity, the mules were regularly treated with an insect repellent, Tri-Tec 14 (Farnam1).

The mules were monitored daily for a total of 84 days for signs indicative of EIA (Leroux et al., 2004): alteration of the general state of the animal, fever, anaemia, oedema and jaundice. Rectal temperature was measured daily at the same hour. Blood samples, collected into vacuum tubes with EDTA or with no anticoagulant, were taken daily from the jugular vein for serological and virological analyses. Plasma and leukocytes were obtained from blood with anticoagulant as following; volumes of 5 ml of blood were processed on collection by centrifuging at 500 × g for 5 min to separate the plasma from the erythrocytes. The plasma was then transferred into a new tube, centrifuged at 1200 × g for 30 min and the cell-free resulting plasma was collected and stored. The remaining pellet of leukocytes was resuspended in 0.5 ml of transport medium consisting of 50% phosphate buffer solution containing Penicillin G 10,000 IU/ml, Streptomycin 0.01 g/ml and Amphotericin B 0.25 mg/ml and 50% glycerol. Nasal swabs for the verification of the presence of EHV-1 and -4, were also collected and immediately immersed in 0.5 ml of transport medium. Following an overnight incubation at 4 °C, the swabs were centrifuged for 20 min at 1000 × g and the supernatants were collected in new tubes. All biological samples were stored at -80 °C until analyzed.

2.2. Pharmacologically induced immune suppression

All the animals were injected intramuscularly with dexamethasone (Rapison[®]) (0.11 mg/kg bw/die) on day 57 from the beginning of the experiment, using the procedures described by Kono et al. (1976) and Tumas et al. (1994). The length of treatment for each animal was

dependent on the delayed-type hypersensitivity (DTH) ratio, estimated as described below, with dexamethasone administered until the ratio was less than or equal to 1. During treatment, the mules were monitored by complete blood count and for physical adverse reactions. Skin tests for DTH reactions, as described by Baus et al. (1996), were performed on the first day of dexamethasone treatment and repeated again on the day scheduled as end of treatment. For the skin test, two small areas on the neck were shaved and cleaned. The mules were inoculated intradermally respectively at each site with 50 µg of Phytohemagglutinin (PHA – Sigma[®]) in 1 ml of saline and with 1 ml of saline alone. Net increase in skin thickness was measured using constant tension callipers 24 h post-injection of antigen and control. The DTH ratios are calculated as the ratio of the skin thickness for the antigen (PHA) reaction and control (saline) reaction

2.3. Serological analyses

The serum samples collected on recruitment and during the study were examined in the three-tiered system as already described in the introduction.

The AGIDT and the competitive immunoassay (C-ELISA) cited below both use the recombinant p26 as antigen, produced by the IZSLT, as follows: the sequence of the reference strain Wyoming of EIAV was used for cloning and expression of the capsid protein p26 (Genbank accession number: M16575; K03334; M11337; M14855).

The p26 coding sequence was amplified from a 1547-bp BamHI-XbaI fragment, cloned into pGEM-3Zf (+/-) vector (Langemeier et al., 1996) kindly provided by Dr. F.R. Cook (Gluck Equine Research Centre, University of Kentucky, USA).

Primers used to amplify the p26 sequence are reported below. These were modified at their 5' ends, introducing restriction sites for subsequent cloning. Underlined sequences correspond to restriction sites for NcoI (p26U) and XbaI (p26L). The primers positions reported in brackets refer to the genome of the Wyoming strain (Genbank accession number: M16575): upper primer (p26U): A TCC ATG GTA GAT GGG GCT GGA AAC AG (Wyoming strain position number, 838–865, lower primer (p26L): AG TCT AGATTA AAG TGC TTT TGC CAA TAA CA, (Wyoming strain position number, 1553–1522).

Amplification mixture was as follows: 1 ng of pGEM-3Zf (+/-) 1547-bp BamHI-XbaI construct; 40 µM each dNTP, 0.2 µM each primers p26U and p26L, 3 U of Ultma DNA pol (Applied Biosystems, Foster City, CA, USA), DNA pol Ultma buffer 1X; 1.5 mM MgCl₂, and H₂O-DEPC added to reach a total volume of 100 µl. PCR was conducted using the following thermal profile: 80 °C for 5 min; 95 °C for 1 min; 25 cycles at: 94 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s; final step at 72 °C for 7 min. The PCR product was digested with restriction enzymes NcoI and XbaI and cloned into the expression vector pTHIO-HIS (Invitrogen, Life Technologies, Carlsbad, CA, USA), following manufacturer's instructions. Proteins expressed by the pTHIO-HIS vector were fused at their N-terminal portion to a modified version of the *E. coli* thioredoxin (TrxA, molecular weight of 12 kDa) under the control of the *trc* inducible promoter.

The resulting construct (pMTR1) was used to transform *E. coli* TOP10 strain (Invitrogen, Life Technologies, Carlsbad, CA, USA) and transformed colonies were grown on selective medium containing ampicillin, following standard procedures (Sambrook et al., 1989). The transformed colonies were screened for the presence of pMTR1 by restriction enzyme analysis of plasmidic DNA, purified with the commercial kit (Plasmid-Mini Kit, Qiagen, Hilden, Germany), according to manufacturer's instructions. The identity and sequence of the cloned insert were checked by sequencing analysis, using an ABI310 - Applied Biosystems DNA sequencer. The TOP 10 (pMTR1) clone was then inoculated in Luria Broth containing ampicillin and the expression of the fusion protein, cloned under the control of the *trc* promoter, was induced by the addition of 1 mM isopropyl- β -thiogalactopyranoside. Culture suspension was centrifuged for 10 min at $2000 \times g$ at 4°C and the pellet was resuspended in 20 mM Tris/HCl pH 8, 2.5 mM EDTA, 5 mM imidazole, as indicated by the expression system kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). After three sonication steps, 20 s each, the suspension was subjected to three freeze-and-thaw cycles at -80°C and $+37^\circ\text{C}$. Bacterial lysate was then centrifuged at $12,000 \times g$ for 10 min at 4°C . Both supernatant and pellet were controlled by immunoblotting for the presence of the p26 recombinant protein, using an international EIA positive reference serum acquired from the National Veterinary Services Laboratories, USDA. The expressed fusion protein was detected in the supernatant of the bacterial lysate and had the expected molecular weight of around 38 kDa.

A total of 800 field samples (400 positive and 400 negative) of equids collected during activity of the CRAIE was employed to calculate the respective diagnostic specificity (Dsp) and sensitivity (Dse) of the recombinant protein used in AGIDT and C-ELISA, as prescribed by Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2012 a,b) using as reference method the AGIDT employing the cell culture derived antigen from the Wyoming strain.

The Dse and Dsp for the AGIDT was 100%, while for the C-ELISA, the Dse was 100% and the Dsp was 80.3%. In the case of the latter method, the lower Dsp value was not considered critical since it was being used as a screening test and therefore all positive samples had to be confirmed by other the tests, AGIDT and/or IB.

Following is a brief description of the methods used in the three-tiered system. Sera were examined using the AGIDT described by the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2009). The samples were tested undiluted and results were expressed as a score from 0 to 5, on the basis of the position of the precipitation reaction (Fig. 1A) as described by Issel et al. (1999).

The C-ELISA was carried out as already reported in detail by Scicluna et al. (2008) and Issel et al. (2013) with the following modification: each serum sample was two fold serially diluted starting from two separate initial dilutions of 1/4 and 1/6, up to 1/1024 and 1/1536 respectively, to obtain narrower in-between dilution intervals. The results are interpreted using the following

equation: Percentage Inhibition (PI) = $100 - (\text{mean optical density of sample} / \text{mean optical density of negative control} \times 100)$. The sample is considered negative if the PI is less than 30 per cent, positive if greater than 50 per cent, and equivocal if between 31 and 49%. The C-ELISA results were expressed as the reciprocal of the last dilution still positive.

The membranes employed for the IB were supplied by Dr. C.J. Issel (Gluck Center, KY, USA) and details of their preparation and the IB procedure used are as described by Issel and Cook (1993) and Issel et al. (1999), with the following modifications: individual strips of the membrane were cut and used for each sample and the sera were tested at 1/20 and in a final volume of 450 μl . Rabbit anti-horse IgG conjugate (Sigma[®]) was used at the appropriate working dilution and at the same previously reported final volume. Samples are defined as positive when they present an equal or more intensive staining as seen for that of the internal positive control, relative to the band of the major core protein p26, as well as, for at least one of the two envelope proteins of EIAV (gp90 and gp45). The same samples were also examined at a higher dilution of 1/80, in an attempt to detect temporal variation in the reactivity for the three major IB proteins, as subtle differences become obscured at the 1/20 dilution.

2.4. Virological analyses

2.4.1. Estimation of plasma associated RNA viral loads of EIAV using a quantitative RT-PCR (qRT-PCR)

The plasma samples collected throughout the observation period were analyzed for the estimation of plasma associated vRNA loads, using a qRT-PCR as described below, targeting a region of the exon 1 of the *tat* gene of EIAV.

Total RNA was extracted from 140 μl of plasma, using the QIAamp[®] Viral RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer instructions, employing the automatic extractor QIAcube[®]. The RNA was extracted in double replicate from each sample and its concentration was measured using a spectrophotometer. The extracted RNA was either used immediately or stored at -80°C until further analysis. Reverse transcription was carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and 200 ng of the extracted RNA, contained in a volume of 30 μl , with the addition of the following reagents: 6 μl of 10X random hexamer primers, 6 μl 10X RT-Buffer, 2.4 μl dNTP mix 100 mM, 3 μl of 5 U Multi Scribe Reverse Transcriptase and 12.6 μl of H_2O -DEPC obtaining a total volume of the reaction mixture equal to 60 μl . Synthesis of the cDNA was carried out using AB Veriti 96 well Thermal Cycler (Applied Biosystems, Carlsbad, USA) with the following thermal profile: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min.

The design of oligonucleotide primers and probe for the qRT-PCR was carried out by Dr. F.R. Cook (Gluck Center, KY, USA), based on the conserved regions of the exon 1 of the *tat* gene of the viral genome, predicted by alignment of EIAV sequences published in GenBank (Accession Numbers: AB008197, AF327877, AF033820, JX480631,

JX480632, JX480633, JX480634), amplifying a region of 119 bp. Position and sequences of primers and probe are as following and are those referred to the EIAVuk strain – GenBank no. AF016316:

MkIII Forward: 5'-GGC GCC CGA ACA GGG ACC-3' (EIAVuk position number, 310–327).

MkIII Reverse: 5'-TGG CCA GGA ACA CCT CCA GAA GAC-3' (EIAVuk position number, 405–428).

Probe EIAV is a Locked Nucleic Acid (LNA) Fluorescent: 5'-FAM-T[+G]A ACC T[+G]G [+C]TG ATC G[+T]A G[+G]A-3'BHQ 1 (EIAV uk position number, 353–373).

For the qRT-PCR, TaqMan[®] Universal PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA) was used with the following composition: 12.5 µl of TaqMan[®] 2X Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 900 nM of forward primer, 900 nM of reverse primer, 300 nM of probe, 5 µl of cDNA and H₂O-DEPC to reach a total volume of 25 µl.

The qRT-PCR was carried out using AB 7900HT Fast Real-Time PCR System with the following thermal profiles: 50 °C for 2 min, 95 °C for 10 min, and 50 cycles of 95 °C for 15 s, 52 °C for 30 s and 60 °C for 1 min. In each run of the qRT-PCR, a quantified internal control, developed as described further on, was included for the estimation of the viral RNA copies/ml of the plasma sample.

All data were analyzed using the ABI 7900HT Sequence Detection Systems software package ver. 2.4 (Applied Biosystems, Foster City, CA, USA).

2.4.2. Construction of the internal standard for qRT-PCR

The RT-PCR product relative to the primer pair EIAV MkIII, obtained from the plasma of mule 4, was purified using the QIAquick[®] PCR Purification kit (Qiagen, Hilden, Germany) and was cloned in pCRII-TOPO vector-TOPO TA Cloning[®] Dual Promoter kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). A ligation reaction was used to transform the competent cells ONE SHOT TOP 10 (Invitrogen, Life Technologies, Carlsbad, CA, USA), following the instructions supplied by the manufacturer. The presence of the cloned PCR products was verified by sequencing of the recombinant plasmids using both set of primers EIAV MkIII and primers internal to the pCRII-TOPO vector. Plasmid DNA used for the in vitro transcription was purified by the Nucleo Spin Plasmid kit (Macherey-Nagel, Düren, Germany), linearized with EcoRV by restriction enzyme cleavage, and its concentration was measured by spectrophotometry (Bio Photometer, Eppendorf, Hamburg, Germany). The in vitro transcription was performed with the MEGA Script[®] T7/Sp6 kit (Ambion, Austin, Texas, USA) according to the manufacturer's instructions, using 1 µg of the linearized DNA plasmid as template. The in vitro transcribed RNA was treated with 2 U of DNase I (Ambion, Austin, Texas, USA) for 6 h at 37 °C. Following inactivation, the RNA was purified by adding ammonium acetate and ethanol and incubating the mixture for 30 min at –20 °C. The resulting RNA pellet was resuspended in H₂O-DEPC and conserved at –80 °C until further use. Before freezing, the number of RNA molecules was calculated on the basis

of the RNA concentration measured using a spectrophotometer.

The LOD of the assay was established using the in vitro transcribed RNA, generated with the EIAV MkIII primers. Aliquots of log₁₀ dilutions of the RNA were prepared to cover a range of dilutions between 3.77 × 10⁹ to 1 copy/µl and stored at –80 °C in volumes of 5 µl and used only once. The LOD test was carried out on three independent occasions and the Ct (threshold cycle) values were used for the construction of a standard curve. Threshold limit was set in the exponential phase of the reactions and Ct-values greater than 45 were considered as negative.

The standard curve was obtained from the linear regression line through the data points on a plot of Ct versus the logarithm of the concentration of the internal standard. The amount of RNA in the unknown samples was determined by interpolation on the standard curve of the Ct value obtained for each sample and corrected to RNA copies/ml of plasma. The Ct value of each sample was the mean obtained from the analyses of the sample extracted and examined in double replicate.

2.4.3. RT-PCR for EHV-1 and -4

The RT-PCR for EHV-1 and -4 is briefly described as follows (Damiani et al., 2005): total RNA was extracted from 140 µl of the supernatant of the nasal swab obtained as described above, using the QIAamp[®] Viral RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer instructions, employing the automatic extractor QIAcube[®] and its concentration was measured using a spectrophotometer.

The design of the primers and probes was based on the sequences contained in the conserved regions of the *glycoprotein E* gene. The sequence and the position of the primers and probes is as following: EHV Forward primer – 5'-CCACGGCTAGGAGAGCACTT-3', EHV Reverse primer – 5'-CKRGTCCTCAAGCTCCCAACTCT-3', EHV-1 probe 5'-FAM-TCGGGCACAAAAT-MGB-3', EHV-4 probe 5'-VIC-ATC-CATCTGAGGCAACAA-MGB-3'.

The position on the EHV-1 V592 (GenBank AY464052) of the forward primer is from 135,198 to 135,217, for the reverse primer from 135,275 to 135,295 and for the probe is 135,229 to 135,241. The position on the EHV-4 (NS80567-Genbank AF030027) of the forward primer is from 133,697 to 133,716, for the reverse primer from 133,774 to 133,794 and for the probe 133,752 to 133,769.

For the Real Time-PCR, TaqMan[®] Universal PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA) was used with the following composition: 12.5 µl of TaqMan[®] 2X Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 900 nM of forward primer, 300 nM of reverse primer, 120 nM of the EHV-1 probe, 240 nM of the EHV-4 probe, 5 µl of cDNA and H₂O-DEPC to reach a total volume of 25 µl.

The amplification was carried out using AB 7900HT Fast Real-Time PCR System with the following thermal profiles: 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 60 °C for 1 min.

All data were analyzed using the ABI 7900HT Sequence Detection Systems software package ver. 2.4 (Applied Biosystems, Foster City, CA, USA).

2.4.4. Sequencing of the products obtained in the nested-PCR for EIAV

Preliminary sequencing was carried out on a region of 313 bp of the *gag* gene of the proviral DNA extracted from the leukocytes obtained from blood samples collected on recruitment, using the diagnostic nested-PCR described by Cappelli et al. (2011), to which minor modifications were applied. Genomic DNA was extracted from the leukocytes using QIAamp[®] DNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer instructions. The primers used for the nested-PCR were those reported by Cappelli et al. (2011), based on consensus sequences obtained from the alignment of Asian, North American and European viral isolates. Both reaction mixtures were prepared using Platinum[®] Pfx DNA Polymerase kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). The master mix for PCR 1 and 2 was composed of 5 μ l of 10X Buffer, 0.5 mM of dNTPs, 2 mM MgSO₄, 0.025 U/ μ l of Platinum[®] Pfx DNA Polymerase (Invitrogen, Life Technologies, Carlsbad, CA, USA), and H₂O-DEPC for a total final volume of 50 μ l. For the 1st round of PCR, 0.2 μ M of the forward primer (EIAV DNA ITA F 5'-GAATGGAGCAAAGCGCTCA-3'), 0.2 μ M of the reverse primer (EIAV DNA ITA R 5'-CTGCCAGGCACCACATCTA-3') and 5 μ l of cDNA were used while for the 2nd round, the primers used were 0.2 μ M of the forward primer (EIAV DNA ITA NSTD F 5'-TGTGGGCGCTAAGTTTGGTG-3'), 0.2 μ M of the reverse primer (EIAV DNA ITA NSTD R 5'-TTTCTGTTCCAGCCC-CATC-3') and 5 μ l of the PCR1 product. Amplification was carried out using Gene Amp[®] PCR System 9700 (Applied Biosystems, Foster City, CA, USA) and the thermal profiles used for the first and second reaction were as following: 94 °C for 10 min; 35 cycles for 94 °C for 15 s (PCR1)/20 s (PCR2), 52 °C for 30 s, 72 °C for 40 s (PCR1)/30 s (PCR2) and final extension at 72 °C for 10 min. The length of the amplicons was respectively 547 bp for PCR1 and 313 bp for PCR2.

Subsequently, the nested-PCR products were purified using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and sequenced using the internal PCR primers of nested-PCR and the Big Dye Terminator Cycle Sequencing Ready Reaction kit, version 3.1 (Applied Biosystems, Foster City, CA, USA) in an automated sequencer (ABI PRISM[®] 310 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were compared to those published in GenBank using Nucleotide Blast (BLASTn) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

3. Results

3.1. Clinical profiles and verification of pharmacologically induced IS

There were no reports of disease in any of the mules before entry into this trial and none experienced important clinical signs commonly associated with EIA during the

initial 56-day observation period. This is consistent with the supposition that all animals had attained inapparent carrier status, along with active control of viral replication. Dexamethasone administration was continued until all mules were clinically immune suppressed, as defined by a DTH ratio equal to or less than 1. With the exception of mule 1, that required a 10-day treatment, this DTH ratio was obtained, following 8 daily doses of dexamethasone in the rest of the animals. Side effects commonly associated with pharmacological immune suppression, such as increased digital pulse, were not detected. Following IS, six mules, had transient febrile episodes (≥ 39 °C) that were considered to be consistent with EIA (Leroux et al., 2004). The IS induced recrudescence of EHV-1 was excluded as a potential cause of these febrile episodes as nucleic acids associated with this virus were only detected in a single nasal swab from mule 6, collected on day 12 post-IS. Despite the presence of EHV-1, this individual did not develop any overt signs of disease. A more complete description of the clinical signs induced by IS in these naturally infected mules study will be presented in a separate manuscript.

3.2. Serological responses

Results of the serological analyses in the AGIDT and the C-ELISA are reported respectively in Fig. 1B and C. The data shown are limited to one week before and four weeks after the first dose of dexamethasone, since previous to this period, the serological results were similar to those reported on day 7 pre-IS. The serological results were ranked on the basis of the total sum of the AGIDT scores and of the ELISA reciprocal titres obtained for each mule, relative to the reported period. The ranked results designated two levels of serological reactivity, one higher, defined as Group P including mules 1, 2, 4, 9 and 10 and one lower, defined as Group N, including mules 3, 5, 6, 7 and 8, with the same animals present, in the 2 groups for the AGIDT, as well as, the C-ELISA (Fig. 1 B and C), indicating a correlation of the reactivity detected by the two methods. It is also important to note that the animals that in Table 1 are described as having a negative to very weak positive reaction in AGIDT are the same as those included in Group N.

IS had only a minimal impact on humoral responses. In the case of Group N subjects, mules 3 and 6 remained negative in AGIDT, while mule 7 alternated between negative and a score of 1 and mule 8 retained a score of 1 throughout the entire 28-day post-IS observation period. Only in the case of mule 5 was there any evidence of an anamnestic antibody response with the AGIDT score increasing from 1 to 2, 28-days post-IS (Fig. 1B). Similar results were observed in Group P animals where apparent increases in AGIDT reactivity were only observed in mules 9 and 10 (Fig. 1B). In general terms, the more sensitive ELISA mirrored these results. Three mules of the Group N animals had no significant increases in ELISA titre pre- and post-IS, including mule 6 that remained negative throughout the entire experiment (Fig. 1 C). On the other hand, mule 5 had a 16-fold increase in ELISA titre confirming the AGIDT result while a more modest, 4–8 fold, increase was

observed in mule 7 (Fig. 1C). Within the Group P animals, ELISA titres in mules 1, 2 and 4 remained relatively constant pre- and post-IS, while mule 9 had a 12–24 fold increase and a 3-fold increase was seen in mule 10. Results that are again consistent with AGIDT (Fig. 1C). On the other hand, serum samples from both Group N and Group P mules reacted strongly in IB, recognizing p26, gp45 and gp90 at a serum dilution of 1/20 (data not shown; see Issel et al., 2013). At this serum dilution no differences in IB reactivity over time could be detected in samples collected from any of the mules either pre- or post-IS. However, when the same serum samples were tested in IB at a dilution of 1/80, an increase in reactivity was noted against gp45 in mules 6, 7 and 8 post-IS. Interestingly these animals are all in Group N and they had relatively low levels of reactivity in C-ELISA/AGIDT tests and, from the 3, only mule 7 seroconverted in the C-ELISA that employs the p26 antigen.

3.3. Estimation of plasma associated RNA viral loads using qRT-PCR

The standard curve obtained using \log_{10} serial dilutions of an in vitro transcribed EIAV derived RNA is linear (regression coefficient value of 0.9932) with a range between 10^9 (Ct = 4,86) and 0,4 molecules/50 μ l (Ct = 40,02). Standard curves, produced with the same in vitro transcribed EIAV derived RNA, were used to determine (absolute quantification) vRNA loads in all samples collected pre and post-IS.

Temporal evolutions of the plasma vRNA loads for the total observation period are reported in Fig. 2 for Group N

and Fig. 3 for Group P, while Table 2 reports the summary of qRT-PCR assay results of plasma samples collected from mules before and after IS. Plasma associated vRNA was detectable in one or more samples collected prior to IS from 6 of the naturally infected mules in the study, including two (mules 3 and 5) with no or very weak positive reactivity in AGIDT (Figs. 2 and 3). Furthermore, vRNA detection in mules 3 and 5 was not restricted to an isolated time-point, and reached or even exceeded the copy numbers observed pre-IS of some of the animals (mules 1, 2 and 10) of Group P (Figs. 2 and 3), producing strong reactions in AGIDT. Another unexpected finding was that in some animals (Group P: mules 1, 2, 4 and 10; Group N: mules 3, 5 Fig. 2 and 3) there was extensive pre-IS temporal variation in plasma-associated vRNA loads, that in the case of mule 5 fluctuated by as much as $\log_{10}^{5.48}$ during the initial 56-day observation period.

As predicted, dexamethasone treatment induced significant increases in amounts of plasma-associated vRNA in all 10 mules, although the size of these increases differed by $\log_{10}^{4.48}$ between animals and the timing of peak vRNA titres varied between days 9 and 19 post-IS (Table 2), except for mule 1 whose vRNA peak was detected at -7 pre-IS. However, these differences between individuals in post-IS plasma-associated vRNA responses had no obvious correlation with serological reactivity in AGIDT. In addition, there was no apparent association between peak vRNA titres and post-IS anamnestic responses in the other serological assay, the C-ELISA. For example, ELISA reactivity in mule 9 increased 12–24 fold at 21 days post-IS while its maximum vRNA titre did not exceed $\log_{10}^{3.0}$ (Figs. 1C

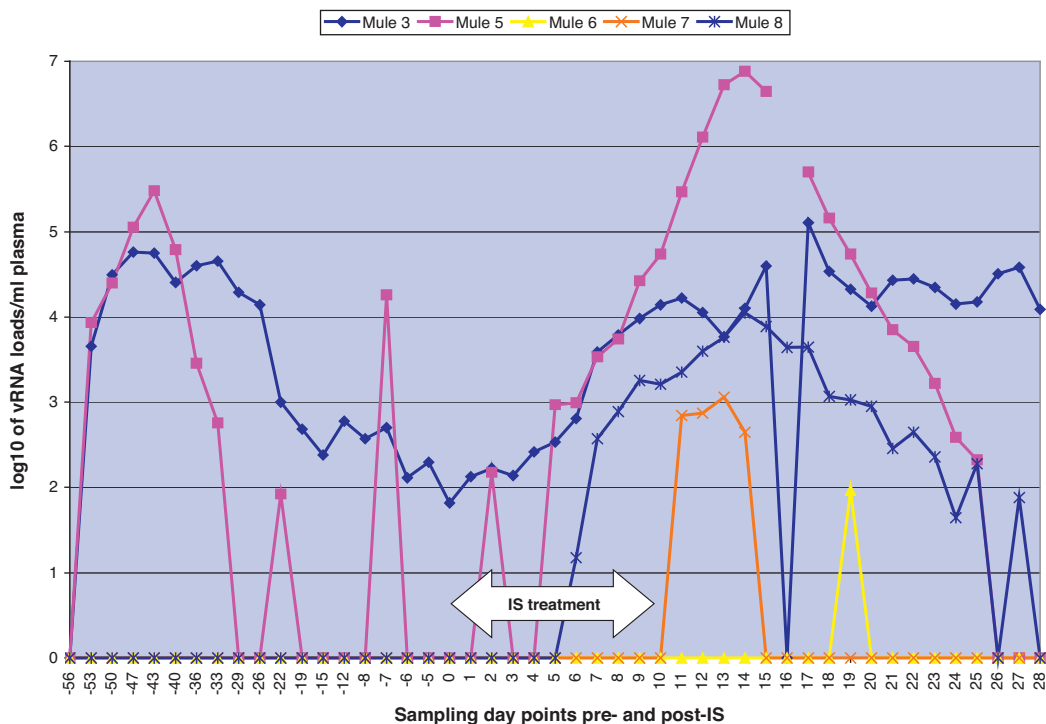


Fig. 2. Temporal evolution of the plasma vRNA loads of Group N. The vRNA loads are reported as \log_{10} /ml of plasma for each animal in this group (Mules 3, 5, 6, 7 and 8) and refer to sampling day points relative to the pre- and post-IS period.

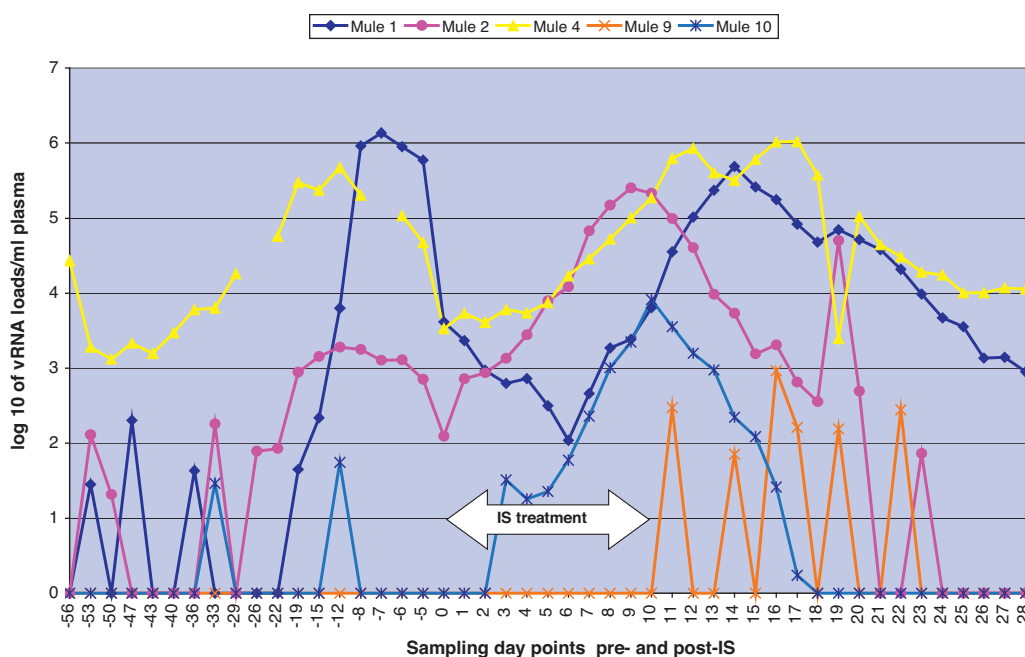


Fig. 3. Temporal evolution of the plasma vRNA loads of Group P. The vRNA loads are reported as \log_{10}/ml of plasma for each animal in this group (Mules 1, 2, 4, 9 and 10) and refer to sampling day points relative to the pre- and post-IS period.

Table 2

Summary of qRT-PCR assay results of plasma samples collected from mules before and after immune suppression^a

Mule number	Number of samples tested	Number positive	Per cent positive	Maximum titre ^b	Time of maximum titre ^c
Group N					
3	47	45	96	5.1	+17
5	46	30	65	6.8	+14
6	47	1	2	2.0	+19
7	47	4	9	3.0	+13
8	47	21	45	4.0	+14
Group P					
1	47	39	83	6.1	-7
2	47	34	72	5.4	+9
4	45	45	100	6.0	+16 = 17
9	47	6	13	3.0	+16
10	47	17	36	3.9	+10

^a Mules were gathered and received dexamethasone at 0.11 mg/kg for 8 days, except mule 1 who was treated for 10 days. Each mule was tested by qRT-PCR nineteen times prior to IS, except for mule 4 which was tested for 17 times; each was also sampled daily for 28 days after initiation of IS except for mule 5 that was tested for 27 days.

^b Titre is estimated from Ct data and expressed as \log_{10} copies/ml.

^c Time of maximum titre is expressed as days and as “-” if sample was collected prior to immunosuppression and “+” if collected after the first dexamethasone treatment.

and 3). On the other hand, serological responses following IS in mule 1 remained unchanged while its maximum plasma-associated vRNA titre was over a \log_{10}^3 higher than in mule 9 (Fig. 1C and Table 2).

3.4. Sequences of the nested-PCR products

Sequencing of nested-PCR products confirmed that all the mules were infected by EIAV, thereby validating the serological responses observed during this study. Nucleotide BLAST searches demonstrated each of the mules in this study were infected with EIAV isolates similar to those previously reported as European strains

by Quinlivan et al. (2007) and Cappelli et al. (2011) with a similarity varying from 77 to 96%. In detail, sequences of six animals (mules 1, 2, 6, 7, 9 and 10) had a similar identity to EIAVRom-4 (GU060662.1), two (mules 4 and 8) to EIAVita-1 (EU240733.1) and 2 (mules 3 and 5) to EIAVita-90 (HQ888862.1). The sequencing results presented here are to be considered as preliminary, especially because similarity of some isolates with those already known is as low as 77%. Since the region sequenced is short, 313 bp, the sequencing of a longer segment of the *gag* gene and/or other regions of the EIAV genome are necessary to better define the identity of the strains detected in this study.

4. Discussion

The primary goal of this study was to expand the sparse database on equids reported as equivocal or negative in AGIDT but positive on other tests for anti-EIAV antibodies. Previous studies involving a horse with this pattern of reactivity suggested that it had extremely low viraemia titres because EIAV was only transmitted to recipient animals in one of six horse inoculation attempts using 5 to 300 ml of heparinized whole blood or washed mononuclear cell preparations from 300–500 ml of whole blood (Issel and Adams, 1982). As antibody production is dependent on exposure to a critical antigenic mass, it might be postulated that low serum antibody titres against EIAV and hence no or only weak reactivity in AGIDT will occur in equids that maintain unusually low tissue-associated viral loads. In addition, there is evidence from experimental infections, involving viruses derived from infectious molecular clones, that some equids are capable of exerting superior control (elite controllers) over EIAV replication (Cook et al., 2003). Although a low antigenic mass model may account for false-negative AGIDT results in some equids, this study suggests it may not be the only mechanism involved. For example, all serum samples obtained from mule 3 failed to react in AGIDT whereas those from mule 5 only produced a test score of 1. However, pre-IS plasma-associated vRNA levels in these two animals were as high if not higher than any of the Group P animals. Consequently, based on the likely assumption of a direct correlation between vRNA and viral antigen levels it can be concluded that low EIAV-specific serum-antibody titres in mules 3 and 5 is not based on the superior or elite control of EIAV replication.

Another significant finding from this study is that plasma-associated vRNA loads do not remain constant over time in all mules. Indeed in one subject there were differences of $\log_{10}^{5.48}$ in vRNA amounts during the 56-day initial observation period. These results suggest that inapparent carrier status in some animals involves highly dynamic relationships between viral replication and host-mediated mechanisms of control. It remains to be determined if such large fluctuations in plasma-associated vRNA are dependent on individual host and/or virus strain characteristics or if they are related to other factors such as the length of time post-infection. In the present study, no statistical analysis of the above results was possible since the number of subjects in the study group was limited and therefore the data derived was not sufficient for such elaborations. However, this study demonstrates that equids with low to null serological responses in AGIDT and no apparent clinical signs of the disease cannot be considered at zero or even minimal risk for the transmission of EIAV. In fact, as this study demonstrates, risk in terms of tissue-associated viral loads may change significantly in some equids even on a daily basis.

Unfortunately, detection of animals with low titres of virus-specific serum antibodies represents a major challenge when diagnosis of EIA is based solely on the use of the AGIDT, especially in cases where this level of reactivity is constant over time.

From the data obtained in this study and that of Issel et al. (2013), the exclusive use of the AGIDT will result in animals being falsely reported as negative, thus allowing the free movement of EIAV-infected equids with an increase in the risk for transmission of the disease. Interpretation of results in AGIDT can be highly subjective with many weak positive samples misinterpreted by inexperienced laboratory personnel (Issel et al., 2013). However, the equivocal reactions presented by some of the mules enrolled in this study cannot be attributed to misreading AGIDT test results because the reactions persisted through time and the same samples were read and confirmed by different highly-trained operators in separate laboratories using kits from different manufacturers (Issel et al., 2013). Furthermore, the length of time from initial diagnosis to completion of this study, that for these animals was at least 240 days, excludes the possibility that recent exposure could account for low levels of EIAV-specific serum antibodies in these animals. Therefore, it seems that some equids simply fail to produce sufficient antibodies for detection in AGIDT. Although the mechanism(s) associated with induction of poor humoral responses against EIAV requires further investigation, such animals are problematic, in that they are not detectable in the single tier diagnostic system, reliant solely on AGIDT that is currently employed by most countries. Furthermore, even in a two-tiered system (initial screening in ELISA followed by verification in AGIDT) animals such as mule 3 could easily be declared “negative” after confirmatory AGIDT testing. On the basis of this study and the considerable experience acquired during the Italian surveillance programme, a three-tiered diagnostic pathway, with final confirmation by IB in designated reference laboratories, appears to offer the most accurate and effective diagnostic algorithm for EIA, in terms of current technology.

Use of PCR-based techniques for detection of EIAV nucleic acids is complicated by a high level of genetic variation, that is a hallmark of lentiviral biology and the low copy numbers encountered in some inapparent carrier animals. The fact that both PCR-based techniques employed in this study were capable of amplifying viral sequences from all 10 mules suggests conservation of their primer and probe (in the case of the Taqman[®] assay) binding sequences, at least among European isolates of EIAV. However, viral sequences were not amplified from all animals at every sample time-point. In fact, viral sequences were only detected in some mules post-IS. Consequently, while these PCR-based assays may prove to be valuable research tools or useful for detecting EIAV in cases of recent exposure when viral replication occurs at high levels, they are not suited for routine diagnostic use, since, as clearly shown in this study, EIAV is known to fluctuate widely in quantity and through time.

The international community has attempted to harmonize requirements for testing for animal diseases and to standardize reagents to assure a level of agreement. In the case of EIA, these standards revolve around what is currently accepted as the gold-standard serological test for EIA: the AGIDT. This is understandable, as the AGIDT is the only serological test that research has proven a high

level of statistical agreement between virus presence (as measured in horse inoculation tests) and specific antibody presence. Other serological tests, approved by national and international bodies, have been designed to produce results aligned to those of the AGIDT. The results presented here and earlier (Issel et al., 2013), from field and experimental cases, have demonstrated a high degree of agreement between specific EIAV genetic material presence and antibodies in equids that are reported as negative in AGIDT but positive on ELISA and IB tests. As these appear to be more numerous than previously expected, and approaching 20% in some estimates, the international community, in our opinion, needs to expand studies on EIA diagnostics to determine a more effective standard.

Each commercial producer of test kits for EIA has established their kit sensitivity using only AGIDT comparisons. Likewise, a number of countries develop their own reagents because of economics, and some use smaller formats for AGIDT that may be more difficult to interpret, especially with samples with low levels of anti-p26 antibodies. Additionally, there appears to be no strict quantitative standard for antigen content in AGIDT kits, making it virtually impossible to standardize interpretation of results on samples with low levels of anti-p26 antibodies in this subjective test. Likewise, as all the ELISA test kits for EIA have been set to manufacturers standards, one should expect different levels of sensitivity on samples with lower levels of anti-p26 antibodies. This study clearly demonstrates the value and need for additional diagnostic tools and better standardization for surveillance of EIA. Historically in the US, the majority of errors reported were those where samples with lower levels of anti-p26 antibody were tested by AGID. Error in interpretation of EIA test results can be minimized by using an ELISA-first strategy.

At the current time, the IB appears to be the test of choice for detection of EIAV-infected equids. This is thought to be because immune responses to the envelope proteins gp90 and gp45 can be discriminated in the IB, even though the envelope proteins are present in virus particles in very low quantities relative to the p26 antigen. Unfortunately, commercial kits to detect these are not yet available. The IB test utilizes gradient purified EIAV that has been produced from a laboratory adapted Wyoming strain of EIAV and it is surprising that it has proven effective in detecting antibodies against EIAV strains from all regions where it has been investigated (Europe, Asia, and the Americas). It appears that the envelope proteins of this strain contain sufficient broadly reactive epitopes in a denatured state on immunoblot membranes to provide such utility.

When one considers the diagnosis of EIA in toto, neither clinical signs, serological data or virus detection alone can be relied upon for 100% accuracy. Data presented here suggest that the IB test using the Wyoming cell-adapted strain of EIAV is the most effective current test in detecting antibodies against EIAV. When combined with the strengths of the ELISA tests for EIA (for higher sensitivity) and the AGIDT (for specificity), the IB test can help establish a practical and effective diagnostic algorithm in a three-tiered laboratory system. When the IB test is applied more widely, it is logical to assume and experience dictates that some equids will be found with lower level of

antibody against EIAV than found in the serum used in this study as the reference weak positive serum.

5. Conclusions

Despite the scientific data confirming the lower sensitivity of the AGIDT (Issel et al., 2013), substitution with alternate, more sensitive, methods for the serological diagnosis for EIA has till now received resistance by regulatory organisms. In fact legal issues could emerge not only between different countries, but also between different official bodies of the same country when contending a discordant serological result for EIA, diagnosed with different methods that are not all officially recommended.

Taking into account the increasing evidence of the limitations of AGIDT in diagnosing all EIAV-infected animals, it is essential to reconsider the present OIE diagnostic recommendations in prescribing this test for international trade, by combining the specificity of the AGIDT, with the higher sensitivity of the ELISA/IB tests.

Such modification would act as an incentive for more countries to adopt a three-tiered diagnostic system, thus increasing the accuracy of surveillance programmes that are based on the identification and removal of EIA serologically positive animals. The examination in series, using the different serological tests, will be especially relevant not only in the final phases of the eradication of the infection when the probability of recent infections is higher, but also for the diagnosis of equids with a constant weak positive to null AGIDT reactivity. A further advantage of this diagnostic approach, observed during the Italian surveillance programme, was the precocity it demonstrated when used in existing outbreaks for the early identification of incident cases.

In conclusion, to reach the highest possible degree of accuracy for the diagnosis of EIA, the scientific data till now available strongly supports the three-tiered serological system with the appropriate integration of molecular based diagnostic methods. This is necessary to cover the widest spectrum of possible virological and humoral reactions the EIAV infected animals may present.

Acknowledgments

We would like to thank Dr. Raniero Lorenzetti and Dr. Maurizio Zini of the Biotechnology Department of the IZSLT for having produced and supplied the recombinant p26 described in this paper.

This study was conducted within the research project “Diagnostic, pathogenetic and epidemiological evaluations aimed at acquiring elements for the risk analysis of the diffusion of Equine Infectious Anemia – IZS 02/10 RC” funded by the Italian Ministry of Health.

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