EQUINE INFECTIOUS ANEMIA: SHOULD THE AGAR IMMUNODIFFUSION TEST STILL BE USED FOR SCREENING AND AS UNIQUE CONFIRMATORY TEST?

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Abstract

Following the introduction in 2007, of an extraordinary surveillance programme, imposing the serological control of the Italian equine population for infectious anemia (EIA), our laboratory adopted as screening test, a previously validated in-house p26 CElisa. Samples with positive or doubtful results, were subsequently confirmed by the agar immunodiffusion (AGID), the prescribed confirmatory test. The Western blot (WB) assay, recommended by World Organisation Animal Health (WOAH) as complementary test, was applied for the first time in the EIA control programme, for routine samples with equivocal results occurring in our laboratory (positive/doubtful CElisa-negative AGID). Over a 17-month period, 253 sera resulted reactive out of the 32 448 horses bled in the province of Rome. Among the former, 83 were confirmed positive in AGID. For those available, i.e. 91 samples, further analysis in WB was carried out. Ten of the CElisa pos/AGIDneg horses were confirmed positive, because reactive with both core protein p26 band and at least one of the 2 surface glycolproteins bands, gp45 and gp90, considered this as specific response pattern to an EIA infection. These preliminary results demonstrate the greater sensitivity of the in-house CElisa compared to AGID, important characteristic in a screening test. For such equivocal results, the in series use of Elisa and AGID and/or WB, improves the overall specificity of EIA diagnosis, essential in the eradication of this low prevalent infection.

Introduction

EIA is a viral equid disease transmitted by insect vectors and, not less important, by iatrogenic means. In 2007, an extraordinary surveillance programme for the control of EIA was introduced in Italy, imposing the serological control of all horses, above the age of 3 months. The surveillance was implemented due to a series of important outbreaks which had occurred over a short period, in spring of 2006. In view of the high number of samples which were to be tested, our laboratory adopted the use of a previously validated in-house p26 CElisa as a screening test, in substitution of the more time consuming and laborious AGID. The method was validated in an inter-laboratory test, involving 11 National Official Laboratories, testing a panel of positive and negative sera supplied by the "National Veterinary Services Laboratories – United States Department of Agriculture, obtaining values of 99% and 96% respectively for relative diagnostic sensitivity and specificity(1). As prescribed by the WOAH, all Elisa positive and doubtful sera were subsequently confirmed in AGID. Due to a number of equivocal results on replicate samples, i.e. positive and doubtful in CElisa and negative in AGID, the WB assay, indicated as complementary test by, was applied for the first time in the EIA control programme, in Italy.

Using in this study as reference method, the WB, the preliminary results presented here indicate a greater sensitivity for the in-house Celisa, when compared to the AGID, essential property in a screening test which even if it compromises its specificity. The CElisa used in series together with AGID and /or WB, improves the overall sensitivity as well as the specificity of EIA diagnosis, indispensable for the control and eradication of this low prevalent infection.

Materials e methods

The in–house C-Elisa was developed in collaboration with the Istituto Zooprofilattico Sperimentale delle Regioni Lombardia ed Emilia Romagna. The method is as here briefly described: 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 sensitisation, on inert microplates, serum samples are diluted 1/3 in PBS pH 7,2 – 7,4, containing yeast extract (0.05%) and mouse serum (1%) together with the following internal controls: a antigen control, a positive and negative control and a blank reaction control. The recombinant p26 antigen is added to all samples and controls, prepared in double replicates. At the end of a 75' incubation at 37°C, the samples and controls are transferred onto the previously washed sensitised plate. Terminated the distribution of the samples, the horseradish conjugated tracer Mab is added, so as to then proceed with another incubation under the same conditions as before. The reaction is developed by the addition of OPD substrate and stopped after 10', using 1M sulphuric acid. The samples reactivity is read at 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 X 100).

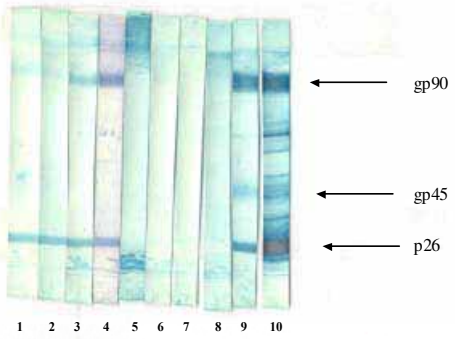
The sample is considered negative if the PI is $\leq$ 30%, positive if $\geq$ to 50%, doubtful if PI is between 30 and 50%.

The AGID is conducted as described by the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals OIE 2004 (2), using again as antigen the recombinant p26 produced by our Institute (2). After 24–48 hours, the plates are examined for the precipitation reaction, typical of a positive serological reaction.

The WB is conducted as described by C. Issel *et. al.* (3), diluting 1/20 the samples as well as the weak positive control, and 1/50 the strong positive control. The reagents, as well as the internal controls, were supplied by the same authors. A sample is positive for EIA virus (EIAV) when reactive with both core protein p26 band and at least one of the 2 surface glycoproteins bands, gp45 and gp90, considered this as specific response pattern to an EIA infection (Figure 1).

EIAV Immunoblot - Lanes N°1 to 4 – pos samples N° 5 to 8 – neg samples N° 9, 10 –weak and positive controls

Figure 1



The samples tested were from horses present in the province of Rome tested within the EIA surveillance programme during 2007 and the 1st semester of 2008 and their number is reported in Table 1. The Elisa positive or doubtful samples were confirmed in AGID, while the WB was conducted for all available equivocal samples , the number of these samples is reported in Table 1 and 2.

Results

The number of samples tested in the CElisa are reported in Table 1. In 2007, 53 of the 162 CElisa positive samples tested were confirmed in AGID on the total of the 18 159 samples controlled, while in 2008, 36 of the 91 were confirmed of the 14 289 samples controlled. Both years confirm the EIA infection as sporadic.

Table 1

Year of sampling	2007	2008
N° of samples tested in elisa	18 159	14 289
	N° samples (% on total)	
CElisa pos/AGID pos	51 (70%)	36 (57.1%)
CElisa pos/AGID neg	22 (30%)	27 (42.9%)
total	73	63
CElisa doubt. / AGID pos	2 (2.2%)	0 (0%)
CElisa doubt. / AGID neg	87 (97.8%)	28 (100%)
total	89	28

Of the total samples testing positive in Elisa i.e. corresponding to 253 horses, over the total period reported, a total of 89 of these subjects were confirmed (35%) as EIA positive using the AGID as confirmatory test.

When 91 of the discordant results were also tested in WB the, 10 further horses were found positive (11%) as highlighted in Table 2, increasing the positivity of EIA positive horses from 35% to 39%.

Table 2

Reactivity of Horses examined in WB	2007	2008	Total
CElisa pos / AGID pos / WB pos	23	2	25
CElisa pos / AGID neg / WB Pos	**2**	**5**	**7**
CElisa pos / AGID neg / WB neg	4	16	20
CElisa doubt. / AGID neg / WB neg	22	13	35
CElisa doubt. / AGID pos / WB pos	1	0	1
CElisa doubt. **/ AGID neg / WB pos**	**0**	**3**	**3**
Total	52	39	91

Even more interesting, is the case of 4 horses, which were tested at subsequent times, starting with an CElisa pos /AGID neg / WB neg reactivity which, on further sampling, were all confirmed as positive in WB, while 2 also became positive in AGID.

Discussion

Subsequent to exposure to EIA, the virus replicates in the monocytes/macrophages cell lineage and although viremia is as early as 5-7 days post-infection, it is infrequently persistent and therefore in case of negativity cannot be used for the definitive diagnosis of this infection. On the other hand, once a horse is infected with EIAV, it is assumed that it will become positive for antibodies to the virus around 20 – 30 days post-infection, in a serologic test and will remain infected and test positive for the rest of its life. This is the keystone upon which current control programmes for EIA are based. The principal proteins to which the horse responds are mainly 3, the core protein p26, the most abundant viral protein and the two glycoproteins (gp) 45 and 90, the most immunoresponsive (4).

However, the majority of the EIA serological methods, both AGID and Elisa, are based on the reactivity to the group, genetically conserved, immunereactive, core protein, p26 .

While the AGID test is the official confirmatory test, it is still also widely used as screening test. In view of the preliminary results here presented as well the results from the comparison of AGID with Elisa reported in other papers, we must reconsider the use of this test in such a context.

Reasons for such discordant results can be, on one hand, the higher analytical sensitivity of the Elisa in the presence of low levels of p26 antibodies or a minor specificity of this test in the presence of cross-reactive antibodies directed against interspecies antigens. Another factor conditioning the higher sensitivity of the Elisa could be due to the less subjective reading of the enzyme test.

In view of the data which is presented here, in accordance with what has been reported by other authors (3, 5, 6 and 7) the following considerations should be made:

- should the AGID test still be used as a screening test?
- in case of equivocal results with Elisa, should the AGID be the only confirmatory test or should it always be followed in case of a negative result by the WB?
- should an animal with a discordant result between an Elisa and the AGID and or WB be recontrolled again at least after the period considered as maximum for an antibody response, i.e. 90 days later?
- could the molecular diagnostic methods come in aid to solve equivocal results?

Independently from which test to use and in which context, these should be constantly controlled to ensure maximum diagnostic accuracy by the use of control panels, as also the laboratory technical efficiency, should be assessed through the periodic participation to proficiency trails.

In the case of EIA, severe sanitary restrictions are imposed, principally represented by the permanent confinement of the infected horse and after the removal of the former, by a 90 day standstill of the in-contact horses within which period they must remain negative. All this represents great potential economic losses. In consideration of this, the accurate diagnosis of EIA should be the drive for the development of serological methods based on scientific proof data, as essential tool in the aid of an improved control of this economically important equine infection.

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