VALIDATION OF A COMPETITIVE ELISA FOR THE DETECTION OF ANTIBODIES ANTI-p26 OF EQUINE INFECTIOUS ANEMIA VIRUS IN EQUINE SERA

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Introduction
Equine infection anemia (EIA) is a viral infection of equidae, caused by a virus (EIAV) of the Lentivirus genus, Retroviridae family. Hematophagous insects belonging to the Tabanidae, Hippoboscidae and Muscinae families are involved as passive vectors in viral transmission. The infection is usually persistent, and can occur as an asymptomatic form or with recurrent febrile episodes. Since 2006, Italy has adopted a National Surveillance Plan (NSP) consisting in a serological screening of all animals except for meat horses. According to the Italian Regulations, the official confirmatory test is the Agar Gel Immunodiffusion (AGID) test which detects antibodies anti-p26, a highly conserved capsidic protein of the virus. Since this technique has a low detection limit, development of new and more sensitive tests has become necessary. WHOA Manual (2) indicates ELISA, immunoblotting and PCR as confirmatory tests for the serological diagnosis of EIA. The ELISA, for its characteristics, is the most suitable for screening purposes. The aim of this paper is to present the results of validation of a competitive ELISA (c-ELISA) for the detection of antibodies anti-p26 of EIAV in equine sera.

Materials and methods
The procedure of the c-ELISA, object of this validation, is briefly described as following. A 96-well microplate is pre-adsorbed overnight at 4°C with a monoclonal antibody (Mab) anti-p26. Serum samples and a recombinant antigen p26, produced in E. coli, are mixed on a separate plate and incubated at 37°C for 75 minutes. At the end of the incubation, the serum-antigen mix is transferred onto the pre-absorbed plate and a second Mab conjugated with horseradish peroxidase is added. After another incubation at 37°C for 75 minutes, orthophenyl-diamine substrate is added and the plate is incubated at room temperature for 15 minutes in the dark. The reaction is stopped by the addition of sulphuric acid and the optical density (OD) is read with a 492nm filter. Sera are categorized as positive, negative or equivocal according to the percentage inhibition (PI), calculated as the ratio between the sample and the internal negative control.

Validation of the c-ELISA was performed according to WHOA Manual guidelines (3) and the aim of this test was for screening purposes. For its validation the following parameters were evaluated:

Analytical specificity was estimated at three different levels:
1. Selectivity, defined as the capability to detect the target analyte in the presence of other interferences, was evaluated by changing the composition of the wash solution and processing positive and negative International Reference Serum (IRS).
2. Exclusivity, considered as the capacity to discriminate target analyte from other crossreactive analytes, was evaluated processing ten sera positive for each of the following virus: Feline Immunodeficiency Virus, Feline Leukaemia Virus and Visna Maedi Virus. Each sample was repeated ten times.
3. Inclusivity which is how a test can differentiate between different serovars. p26 protein is highly conserved in this virus and (4) thus evaluation was not necessary.

Analytical sensitivity is represented by limit of detectability (LOD) and in this validation, the LOD of ELISA was compared with the AGID LOD, analysing progressive dilutions of a positive IRS in both methods.

Repeatability was evaluated estimating the standard deviation (S) of the OD values of 30 repetitions of a negative IRS; this value was compared with another set of 30 values, processed by the same person during the same session.

Reproducibility was estimated taking into account both qualitative and quantitative characteristics of ELISA test.

Results
For statistical analysis XL-Stat 2011 ® was used.

1. ELISAs performed changing the washing solution did not correctly recognise positive and negative sera.
2. All sera positive for other Lentivirus were classified as negative by the ELISA.
3. LOD of ELISA test is at least 1 Log10 more sensitive than AGID.
4. Comparison between two data sets for evaluation of repeatability did not show significant differences with test F (p=0.42) and S, resulted to be equal to 0.184.
5. Statistical K value was equal to 0.967.
6. Comparison between seven data sets for evaluation of reproducibility did not show significant differences with test F (p=0.092) and S, resulted to be equal to 0.129.
7. Dse and Dsp resulted respectively 100 and 80.3%.
8. Positive and negative predictive values resulted respectively 94.8% and 100%.
9. Efficacy of ELISA which is the percentage of samples correctly identified (positive and negative together) compared with the GS resulted equal to 95.7%
10. Test Bias which is the ratio between apparent and real prevalence resulted 1.05.

Discussion and conclusions
The results of the parameters evaluated for the validation of the c-ELISA are all satisfactory. In particular, the lower Elisa LOD would allow not only the detection of "poor responders" when using the AGID only, but also the possibility of detecting earlier, new cases. Although the Dsp is 80%, this value is still acceptable for a screening test compared to the advantages that it presents to the AGID, i.e the possibility of its standardization, the objectivity of the results reading, the lower amount of reagents used and the higher processing of samples per unit time. The use of this diagnostic test is an added value, together with other measures, in a control programme for AIE.

References
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