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Validation of an immunoblot assay employing an objective reading system and used as a confirmatory test in equine infectious anaemia surveillance programs

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ABSTRACT

Equine infectious anaemia (EIA) is a blood borne disease that is listed among the notifiable diseases of the World Organisation for Animal Health (OIE). EIA is also regulated by the OIE for the international trading provisions and is generally subject to control programmes. Since 2011, Italy has been conducting a surveillance plan based on a three-tier diagnostic system, using a serological ELISA as screening test, an agar gel immunodiffusion test (AGIDT) as a confirmatory method, and an immunoblot (IB) as an alternative confirmatory assay for discordant results between the first two tests. As for the in-house competitive ELISA (c-ELISA) and the AGIDT, the Italian National Reference Laboratory for EIA (NRL) validated the IB according to the OIE guidelines, employing eight panels containing positive sera, including those from EIA virus (EIAV) proven infected horses, and negative horse, mule and donkey sera collected from different geographical areas. In addition, two international reference image panels were employed for the optimization and the validation of the digital image reading system adopted that allows an impartial measurement of the serum reactivity in the IB assay.

The immunological reactivity to EIAV antigens, p26, gp45 and gp90 adsorbed on the IB membrane, determines the serological status of the animal and for EIA, a p26 positive band together with at least one of the other antigen defines a subject as serologically positive for EIAV.

For validation, the parameters assessed were threshold values, analytical and diagnostic sensitivity and specificity, repeatability and reproducibility. These parameters were evaluated for each antigen as well as in combination, according to the diagnostic algorithm established above.

The validation data defined the IB as having a satisfactory sensitivity, specificity, repeatability and reproducibility for all antigens and species tested.

An instrumental recording of the results improves the confidence in using IB as a confirmatory test for EIAV, differently from the AGIDT that is read by an operator.

The advantages of using the IB are its higher sensitivity, to that of the AGIDT, which allows an earlier detection of infection that reduces the risk of transmission and therefore the incidence of the EIA, and its higher specificity to that of the ELISA which is based on the discrimination of subjects reacting only against the p26, the antigen used by all ELISAs available, which are not considered as infected by EIAV.

In particular, when this assay is used in outbreaks it can detect new cases earlier than the AGIDT, and therefore reduce the restriction period with an economic benefit for the animal owners and the public veterinary sanitary system.

Abbreviations: AGIDT, agar gel immunodiffusion test; AUC, area under curve; c-ELISA, competitive-enzyme linked immunosorbent assay; CI, confidence interval; CL, confidence level; CV, coefficient of variation; D_{se}, diagnostic sensitivity; D_{sp}, diagnostic specificity; ED_{se}, expected diagnostic sensitivity; ED_{sp}, expected diagnostic specificity; EIA, equine infectious anaemia; EIAV, equine infectious anaemia virus; FEK, fetal equine kidney; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HRP, rabbit anti-horse IgG peroxidase-conjugate; IB, immunoblot; ISO, international organization for standardization; JPEG, joint photographic experts group; LOD, limit of detection; Mab, monoclonal antibody; NCS, natural colour system®©; NRL, national reference laboratory for equine infectious anaemia; OIE, world organization for animal health; PBS, phosphate buffer saline; R^2, coefficient of determination; RGB, red, green, blue; ROC, receiver operating characteristic; SD, standard deviation; SE, standard error; TMB, 3,3′,5,5′-tetramethylbenzidine; U, expanded uncertainty

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1. Introduction

EIAV belongs to the *Retroviridae* family (Charman et al., 1976), subfamily *Orthoretrovirinae*, genus *Lentivirus* (Yaniv et al., 1986) transmitted by horse flies (*Tabanus* sp), deer fly (*Chrysops* sp), and stable fly (*Stomoxys calcitrans* sp), acting as mechanic vectors. The virus is also be transmitted during blood transfusions or iatrogenically, using blood contaminated needles, surgical instruments and horse trappings. The acute form of the disease that is typical of the horse in which it occurs occasionally is characterised by progressive anaemia, intermittent fever, emaciation and death, while the chronic and inapparent forms are the most frequent in horses, in mules and in donkeys (Cook et al., 2001; Autorino et al., 2016). The OIE lists EIA among the equine diseases that have "the potential for very serious and rapid spread", "particularly serious socio-economic consequences" and "major importance in the international trade of animals and animal products".[1] Even if its potential rapid spread has been recently reviewed (Bolfa et al., 2016), EIA remains particularly important for international trading purposes and for those countries conducting regional or national control programmes. For Italy a national surveillance and control plan with the serological testing of the target population was adopted in 2006, where a seropositive equid is either slaughtered or confined under specific biosecurity restrictions.

The OIE recommends the ELISA or the AGIDT for the evaluation of population freedom from infection, efficiency of eradication policies and demonstration of individual animal freedom from infection for EIA. As the sole use of AGIDT, as screening and confirmatory test, confers a low sensitivity to the surveillance system (Issel et al., 2013), since 2011, the NRL uses a three-tier diagnostic pathway. This diagnostic approach is based on the sensitivity and specificity of the tests employed, where highly sensitive ELISAs are used as screening tests (Nardini et al., 2017), the highly specific AGIDT is the confirmatory test for c-ELISA positive/equivocal samples, and the IB, characterised by both high sensitivity and specificity is the alternative confirmatory assay for ELISA/AGIDT discordant results.

The IB described in this study principally detects antibodies against the three main antigens of EIAV, a *gag* gene encoded protein (p26), and two *env* encoded glycoproteins (gp45, and gp90) (Rwambo et al., 1990a), through the development of a colorimetric reaction that can be read either visually or with the aid of digital instruments.

The advantage of method in improving the diagnosis of diseases, especially when used as a second tier assay, is described in several studies, e.g. neurocysticercosis, HCV, Lyme disease and HIV and is based on its higher specificity when compared to the ELISA (Gekeler et al., 2002; De Almeida Pondé, 2013; de Ory et al., 2018; Dickeson et al., 2016; Huang et al., 2018).

For EIA, the additional diagnostic value of this method, as an alternative confirmatory test, is consolidated from the evidence that ELISA-IB positive/AGIDT negative subjects are viremic (Issel et al., 2013; Scicluna et al., 2013) and therefore at risk of acting as reservoirs, and contributing to the persistence of the infection.

As the NRL works in a quality assurance system that is the ISO/IEC 17025:2017,[2] and since the IB was included in the EIA routine diagnostic three-tier pathway, the method was validated according to the OIE criteria, satisfying the requisites of this Organization for diagnostic tests of diseases that have a sanitary and economic impact.[3,4]

Validation of IB assays are reported for several diseases but are mainly focused on the evaluation of only some of the diagnostic parameters, such as sensitivity and specificity, while neglecting other aspects such as precision and analytic performance (Courouce et al., 1998; Frey et al., 2009; Schaller et al., 1999; Zaaijer et al., 1998). To the authors' knowledge few papers are available for EIAV IB validation, with one of these that also describes the method for the first time and another that reports the validation for an EIAV p26-based IB, according to the OIE guidelines in use at the time of its publication (Alvarez et al., 2007; Rossmanith and Horvath, 1989).

The present article describes the methodology and the results of an EIAV IB validation using eight different sera panels, containing reference and field positive and negative samples. Furthermore, a digital imaging based system was employed for an instrumental measurement and registration of the immunological reactivity of the three antigen bands, p26, gp45 and gp90.

In literature, two different digital imaging reading systems are described for measuring and recording the immunological reactivity of the IB (Gallego-Marín et al., 2006; Janes, 2016).

The present authors readapted the reading procedure proposed by Gallego-Marín et al., 2006, improving this by validating and optimizing the system using also two panels of grey scale colour images having an internationally recognised coding system.

In addition, the OIE validation procedure was chosen for its completeness in evaluating the diagnostic performance of the method and the results of this are presented and discussed. Particular focus is placed on the advantages of the use of a validated IB in the three-tier system, in terms of an improved efficiency of the EIA surveillance system due to early disease detection that reduces the spread of EIAV.

2. Materials and method

2.1. Methods employed in the validation

2.1.1. IB membrane preparation

The membranes employed in this study were produced by the Gluck Equine Research Centre (Lexington, Kentucky, USA) using the $EIAV_{Wyoming}$ strain (Malmquist et al., 1973; Rwambo et al., 1990b) propagated in FEK cells (Montelaro et al., 1982) and adsorbed on a nitrocellulose membrane using the Western blot technique (Rwambo et al., 1990a).

2.1.2. IB procedure

The optimization and standardization phases of the IB procedure were performed by the Gluck Equine Research Center (Lexington, Kentucky, USA) (Cook et al., 2001; Rwambo et al., 1990a). The method is run as follows: 4 mm wide strip of the sensitised nitrocellulose membrane is used for each serum sample that is tested at a dilution of 1/20 in a final volume of 3 ml of PBS pH 7.4 ± 0.2 containing 1% Blotting-Grade Blocker (Biorad, CA, USA). At the end of a 60' incubation at room temperature with gentle shaking, to ensure continuous contact between the serum sample and the IB membrane, the individual strips will be washed three times for 5' each time using PBS pH 7.4 ± 0.2 with 5% of NaCl and 0.05% Igepal® (Sigma Aldrich, Germany). A further 5' washing step is carried out using the previously described washing solution with the strips put all together. The strips are again incubated for 60' at room temperature, using gentle shaking in an HRP solution, appropriately diluted in PBS pH 7.4 ± 0.2 containing 1% Blotting-Grade Blocker. A cycle of 4 washings steps is then conducted as follows: a 10' washing with PBS pH 7.4 ± 0.2 with 5% of NaCl and 0.05% Igepal; a 10' washing with PBS pH 7.4 ± 0.2 with 5%

[1] OIE-World Organisation for Animal Health http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/0.01.1c_INTRODUCTION_HOW_TO_USE.pdf Accessed 14/03/2018.

[2] ISO/IEC 17025:2017 General requirements for the competence of testing and calibration laboratories. International Organization for Standardization.

[3] OIE-World Organisation for Animal Health http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.06_VALIDATION.pdf Accessed 14/03/2018.

[4] OIE-World Organisation for Animal Health Manual of Diagnostic Tests and Vaccinesfor Terrestrial Animals 2018 Section 3.6. Recommendations for validation of diagnostic tests http://www.oie.int/standard-setting/terrestrial-manual/access-online/ Accessed 14/03/2018.

of NaCl and two 5' washings with PBS pH 7.4 ± 0.2. In a further step, the strips are incubated and gently shaken for 4', at room temperature, in TMB Stabilized Substrate for HRP (Promega, WI, USA). On blocking colour development, by washing the strips using deionized water, these are immediately scanned to avoid fading of the colour.

The criteria employed to define the outcome of the sample reactivity as positive or negative for EIAV antibodies, is similar to that adopted for HIV (Mahajan et al., 2010): an equid is serologically positive for EIAV when the presence of antibodies is detected against p26 and for either gp45 or gp90, differently the equid is negative.

2.1.3. c-ELISA and AGIDT

The c-ELISA described in this study is the screening method used at the NRL and in several other official laboratories in Italy. The method procedure and its validation according to OIE criteria were already described by the authors (Nardini et al., 2016): briefly the c-ELISA uses a recombinant p26 antigen, expressed in *E. coli;* a Mab anti-p26 as catcher; a Mab anti-p26, different from the previous, conjugated with HRP and orthophenylenediamine as substrate.

The AGIDT employed is that reported by the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals,[5] using the same recombinant p26 antigen of the c-ELISA and an internal control set composed of a positive, a weak positive and negative sera, certified with the international reference sera provided by the USDA, an OIE Reference Centre for EIA.

2.2. Validation criteria

The OIE validation criteria considered for the IB are reported in Table 1[3].

2.3. Panels employed in the validation

Two reference grey scale colour panels and eight panels of sera were employed in the validation process. Characteristics of each panel are described in Table 2. The panels are numerically identified and will be referred to in this manner in the rest of the paper, accompanied by a brief description within brackets.

Panel 1 was composed of 19 A6 format colour panels that cover the NCS entire grey scale, excluding the white and the black (NCS Colour Centre Italia, Milan, Italy). Panel 2 was composed of 19 jpeg files, each represented by a rectangle filled with an RGB coded grey colour, corresponding to one of the NCS colour of Panel 1, prepared using the open source software LibreOffice Impress. More information is provided in the "Fitness for intended purpose(s), optimization and standardization" paragraph.

Panel 3 consisted of two USDA reference sera, one positive and one negative. Panel 4 contained 60 negative sera, with 30 horse sera collected in Iceland, an EIAV free country,[6] and another set of 30 horse sera from Italy. The latter group was defined as negative in the c-ELISA and AGIDT described above. Panel 5 and Panel 6 were employed, together with Panel 3, for the assessment of the analytical specificity.

Panel 5 included sera positive for other equine viruses and sera of other animal species positive for other lentiviruses. Panel 6 contained EIAV positive sera from distinct geographic areas defined as positive according to one of the following criteria: a) belonging to experimentally infected animals, b) positive in IB, c) positive to both AGIDT and ELISA or d) positive to PCR for EIAV and confirmed by sequencing (Dong et al., 2012).

[5] OIE-World Organisation for Animal Health http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.05.06_EIA.pdf Accessed 14/03/2018.

[6] World Animal Health Information Database (WAHIS) Interface http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail Accessed 14/03/2018.

Table 1
Parameters assessed, according to OIE guidelines[3], in the validation process of the immunoblot assay for the detection of antibodies against equine infectious anaemia virus.

Assay validation criteria
1. Fitness for intended purpose(s)
2. Optimization
3. Standardization
4. Robustness
5. Repeatability
6. Analytical sensitivity
7. Analytical specificity
8. Thresholds (cut-offs)
9. Diagnostic sensitivity
10. Diagnostic specificity
11. Reproducibility
12. Ruggedness

The minimum number of positive and negative samples (n) required for Panels 7, 8 and 9 used for the estimation of the diagnostic sensitivity (D_{se}) and diagnostic specificity (D_{sp}), was calculated with the following formulae:

$$n = t^2 * ED_{se} * (1\text{-}ED_{se})/ d^2 \quad (1)$$

$$n = t^2 * ED_{sp} * (1\text{-}ED_{sp})/d^2 \quad (2)$$

where *t* is the value of the *t*-distribution for the chosen CL, ED_{se} is the expected diagnostic sensitivity, ED_{sp} is the expected diagnostic specificity and *d* is the SE; all values are expressed as proportions. The values set for the assessment of the diagnostic parameters were: for mule and horse sera, ED_{se} and ED_{sp} = 0.99; CL = 0.99, SE = 0.05; while a CL equal to 0.95 was used for the estimation of the number of donkey samples required to evaluate the same diagnostic parameters. Panel 7, 8 and 9 respectively consisted of positive and negative horse, mule and donkey sera. Categorization criteria for positivity were those employed for Panel 6, while negativity was assessed either by IB or by AGIDT and c-ELISA.

Panel 10 was composed of 20 sera divided into 4 groups of 5 sera each, according to their reactivity level as defined by the digital image based reading system: negative (pixel value ≤ 30), weak positive (30 < pixel value ≤ 50); medium positive (51 < pixel value ≤ 70) strong positive (pixel value > 70). The classification of the reactivity of the sera composing this panel was set after the standardization and the choice of the threshold values for the different antigens (see Thresholds paragraph). This panel was employed for evaluating precision at different levels of reactivity of the sera that however, does not correlate with the clinical evolution of the disease or with its immune control (Hammond et al., 2000).

2.4. Fitness for intended purpose(s), optimization and standardization

As previously mentioned, the purposes listed by the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals for which the IB can be employed are individual animal freedom from infection and confirmation of clinical cases.

For this, the instrumental reading of the test represented an additional value of the IB, differently from what is possible with the AGIDT. The digital image based reading system of the IB results set up by the NRL is described as follows. The optical reading system has two components: the UN-SCAN-IT gel_{TM} Analysis Software (Silk Scientific, Inc., USA), further referred to as software, and the scanner that for this validation was a TASKalfa 5501i (Kyocera Corporation, Japan). Panels 1 and 2 were employed to set a reading procedure and assess the variability of the reading system arising from the use of the scanner and the

Table 2
Panel of samples (sera/reference colours) employed for the validation of the immunoblot assay for the detection of antibodies against equine infectious anaemia virus.

Panel 1: NCS®© Grey scale panel[a]	19
Panel 2: RGB colour panel [a]	19
Panel 3: Secondary reference sera [a]	1 – Negative 1 – Positive
Panel 4: Thresholds panel	30 Negative sera from Italy 30 Sera from EIAV-free country (Iceland)[b]
Panel 5: Sera positive for other equine viruses and for other lentiviruses [a]	Positive sera for – N° Visna Maedi virus – 5 Caprine arthritis encephalitis virus – 5 Equine Viral Arteritis – 5 Equine Herpes Virus-1 – 5 Equine Herpes Virus-4 – 5
Panel 6: Positive sera from foreign countries	Area Country – N°: Africa Nigeria[c] – 5 Asia China[d] – 5 Europe France[e] – 5 Germany[f] – 5 Hungary[a] – 1 North America Canada[g] – 5 USA[h] – 5 South America Argentina[i] – 3 Brazil[h] – 2
Panel 7: Horse sera [a]	27 – EIAV positive sera 27 – EIAV negative sera
Panel 8: Mule sera [a]	27 – EIAV positive sera 27 – EIAV negative sera
Panel 9: Donkey sera [a]	16 – EIAV positive sera 16 – EIAV negative sera
Panel 10: Precision panel[a]	5 – Negative sera 5 – Weak Positive sera 5 – Medium positive sera 5 – Strong positive sera

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[g] Dr. Fatima Belayat, Laboratoire d'hygiène vétérinaire et alimentaire, Agriculture Canada, St-Hyacinthe, Québec; Canada.
[h] Dr. Charles J. Issel, Gluck Equine Research Centre, University of Kentucky, Lexington, Kentucky.
[i] Dr. María Barrandeguy, Laboratorio de Virus Equinos Instituto de Virología, INTA Castelar, Argentina.

software. The E-paint website[7] was used to convert each colour of Panel 1 (NCS) to the RGB coding system (Supplementary material 1); the setting values for the use of the web page were the following: Choose Range = NCS1950; Choose colour = "each code of Panel 1". The RGB values were thus employed to set the filling colour of the rectangles composing Panel 2.

The reading of each colour of Panel 2 was repeated ten times with the software parameters set as follows: Gel analysis mode – segment analysis mode; Colour mode – grey scale intensity; Background correction – no background correction; Optical intensity calculation – linear; Scan direction – top to bottom; Digitizing speed – standard. An example of a reading is shown in Supplementary material 2. Maximum and mean pixel density were measured and used as reference values for each shade of grey in comparison with those obtained in the next step that employs the scanner.

Each Panel 2 colour was scanned ten times with the following scanner settings: Image type – JPEG, Resolution – 600*600 dpi; Clarity – maximum (+5); Density correction – 0. The resulting images were analysed as explained above for Panel 2. An example of a reading is shown in Supplementary material 3.

Pixel values were compared and scanner settings were adjusted to obtain similar outcomes for the two panels and to have a linearity range covering lower pixel values that confers a higher sensitivity to the system and the detection of weak positive reactions (see Results).

2.5. Thresholds

Threshold values were set according to the OIE validation procedure employing Panel 4 (Threshold panel) (Jacobson, 1998) based on the normalized maximum pixel value mean and SD calculated for the negative reaction for each antigen corresponding to the same height of the membrane where the positive control would have reacted. The pixel data were normalized by subtracting to the maximum pixel value, the mean pixel value of a blank zone, identified for each strip, as a zone outside the three antigen bands, but still presenting a background colour, thus allowing the comparison among strips having different backgrounds. The threshold values were set as the normalized maximum pixel value mean plus two SD.

The data obtained was further confirmed by performing both the ROC analysis, employing Panels 7, 8 and 9 (see Diagnostic performance paragraph), and the analysis of the reactivity of the positive serum of Panel 3 (secondary reference sera) diluted from 1/500 to 1/5500 with intervals of 1/500, and comparing their results to the estimated threshold value obtained for Panel 4. The threshold values obtained were employed to categorize the panels.

2.6. Analytical performance

2.6.1. Analytical specificity

Analytical specificity defines the capability of the assay to distinguish between the target analyte and other components present in the matrix and is based on three aspects, which are selectivity, exclusivity, and inclusivity.

Selectivity defines how the test can detect the target analyte in the presence of interfering substances, such as matrix components and degraded products. Five repetitions of Panel 3 (secondary reference sera) were tested with the defined procedure, as well as using deionized water in substitution of the test solutions. The results in terms of normalized maximum pixel value mean for each antigen were compared using the z-test with a CL of 95%.

Exclusivity is the ability of the assay to detect the target analyte, excluding all other cross-reactive biological substances, which could be present in the sample. The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals considers this characteristic as an essential feature for a confirmatory test, such as the IB. For this, even if no cross-reactive pathogens are reported for EIAV, Panel 5 composed of sera positive for the most common equine viral diseases (Slater, 2014) and for other Lentiviruses was analysed to confirm this.

Inclusivity is the ability of the assay to recognize various serotypes of the same biological entity. For the three EIAV antigens, a higher variability was reported for gp90 EIAV domain, then for p26 and gp45 (Chong et al., 1991; Craigo et al., 2009; Zheng et al., 2000). Panel 6, which is composed of positive sera collected from different areas of the world was analysed to verify the inclusivity of the assay.

2.6.2. Analytical sensitivity

As EIAV antibodies content cannot be precisely measured in a serum sample, LOD of the IB was indirectly determined by comparing it with

[7] E-paint website https://www.e-paint.co.uk/Lab_values.asp Accessed 14/03/2018.

that of the AGIDT and the c-ELISA, analysing the positive serum of Panel 3 (secondary reference sera) diluted 1/100, 1/500 and up to 1/5500 with 1/500 dilution intervals, using the three methods. The results were expressed as the $\log_{10}$ difference of the last dilution detected as positive by IB to that of each of the other two assays.

2.7. Diagnostic performance in horses, mules and donkeys

Panel 7 (horse sera), 8 (mule sera) and 9 (donkey sera) were analysed and the normalized maximum pixel value mean was used to perform a ROC analysis for each antigen and species and cross-breed, employing STATA SE v.12.0 software for Windows (StataCorp LP, Texas, USA). Diagnostic specificity and sensitivity for the chosen threshold value were automatically reported as outcome of the ROC analysis and the relative confidence intervals were calculated by the Clopper-Pearson exact method. Sensitivity and specificity were also calculated for the qualitative result (positive or negative).

2.8. Repeatability and reproducibility

Panel 10 was employed for the evaluation of both precision parameters. For repeatability, four sera, one for each level of reactivity (strong, medium, weak positive and negative), were simultaneously analysed in double replicates by two operators in two independent runs, one week apart. Intra and inter-run mean, SD and CV were calculated using the normalized maximum pixel value mean obtained for each reactivity level and antigen. Huber test (Huber, 1965) for detection of outliers was used first within a run, for the same serum, and then with data from the two runs. The s_r was calculated as indicated by the ISO 21748:2017,[8] considering the suitable data of the two runs with the following formula:

$$s_r = \sqrt{[\Sigma \text{ (variance * number of replicates per run)/total number of runs}]} \quad (3)$$

Reproducibility was assessed with all samples of Panel 10 tested in a single run by three different laboratories, including the NRL, of the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M. Aleandri". In panel 10, two sera, with the same level of reactivity and deriving from the same serum, were tested to evaluate the within-laboratory repeatability, as reported in the OIE guidelines[3]. Data were elaborated as for repeatability to obtain the S_R.

Expanded uncertainty (U) was calculated according to OIE guideline[4] as twice the value of the CV, the repeatability parameter that includes more sources of variability, for each level of reactivity. The estimated value was applied to the threshold levels to obtain the CI at 95%.

3. Results

3.1. Fitness for intended purpose (s), optimization and standardization

Results of mean pixel value, SD and CV for Panel 1 and 2 with a density value of 0 are reported in Supplementary material 4. For Panel 2, the SD and the CV resulted 0 and therefore omitted. With this density value, the first three colours of the panel were read as 0 (Fig. 1A) and the range of linearity (Fig. 1B) was from S1000 N to S9000 N ($R^2 = 0.997$). The purpose of the assay is to detect positivity at the earliest time possible, therefore, the authors opted for a higher sensitivity of the test by setting the density value of the scanner at +3 that can detect the weakest pixel values. This modification improved the detection of the assay relative to the lighter greys (Supplementary material 4) and the linearity range was obtained between S500 N and S6500 N (Fig. 1C and D, $R^2 = 0.998$). A plateau representing darker greys was detected above this range and considered acceptable, as these would still be correctly classified as positive.

3.2. Thresholds

In Table 3, normalized maximum pixel value mean, SD and CV for each antigen are reported separately for the Iceland negative sera and the Italian negative sera of panel 4 as well as considering all these sera together. The data from the comparison of the negative panel with the positive diluted serum is shown in Fig. 2, which established that the thresholds values were 40, 30 and 20 pixels, respectively for p26, gp45 and gp90. For practical purposes, the authors set a threshold value of 30 pixels for all the three antigens. The appropriateness of this value was also evaluated using data from the ROC analysis of the sera of all species (see Diagnostic performance in horses, mules and donkeys).

3.3. Analytical performance

3.3.1. Analytical specificity

3.3.1.1. Selectivity. The modified procedure, using deionized water in place of the test solutions, resulted in a flattened pixel value readings, compared to that of the standard procedure, that was significantly lower for all antigens (see Supplementary material 5).

3.3.1.2. Exclusivity. All the sera of Panel 5 (sera positive for other equine viruses and for other lentiviruses) were negative for the three antigens.

3.3.1.3. Inclusivity. IB detected all sera of Panel 6 (positive sera from different geographic areas) as positive.

3.3.2. Analytical sensitivity

The $\log_{10}$ difference between IB and AGIDT was 2.26, while that with the ELISA was 1.48. See Supplementary material 6 for the comparison of the results of IB, ELISA and AGIDT on successive dilutions of the positive serum of Panel 3.

3.4. Diagnostic performance in horses, mules and donkeys

Results of the ROC analysis for each antigen and species are reported in Table 4, in terms of AUC, best threshold value (automatically defined) and diagnostic sensitivity and specificity of the assay and relative CI. The diagnostic parameters for the chosen threshold of 30 pixels are shown in Table 5, together with the diagnostic parameters assessed for the qualitative outcome with all sera classified correctly. ROC curves for each antigen and species are presented in Supplementary material 7.

3.5. Repeatability and reproducibility

Values of s_r and s_R for each antigen and reactivity level are reported in Table 6. Details on normalized maximum pixel value mean, SD and CV are available in Supplementary material 8 and 9, respectively for repeatability and for reproducibility. The 95% CI for each reactivity level are shown in Fig. 3. The pixel value, above which a result based on a threshold value of 30 pixels is positive with a 95% CI, was around 40 for all the antigens. Images of IB membranes representing some of the stages of the validation before and after conversion to grey scale are reported in Fig. 4.

4. Discussion

Validation of immunoblot assays is rarely carried out both in human (Leung et al., 2001; Veijola et al., 2008) and in veterinary medicine

[8] ISO 21748:2017 Guidance for the use of repeatability, reproducibility and trueness estimates in measurement uncertainty evaluation. International Organization for Standardization.

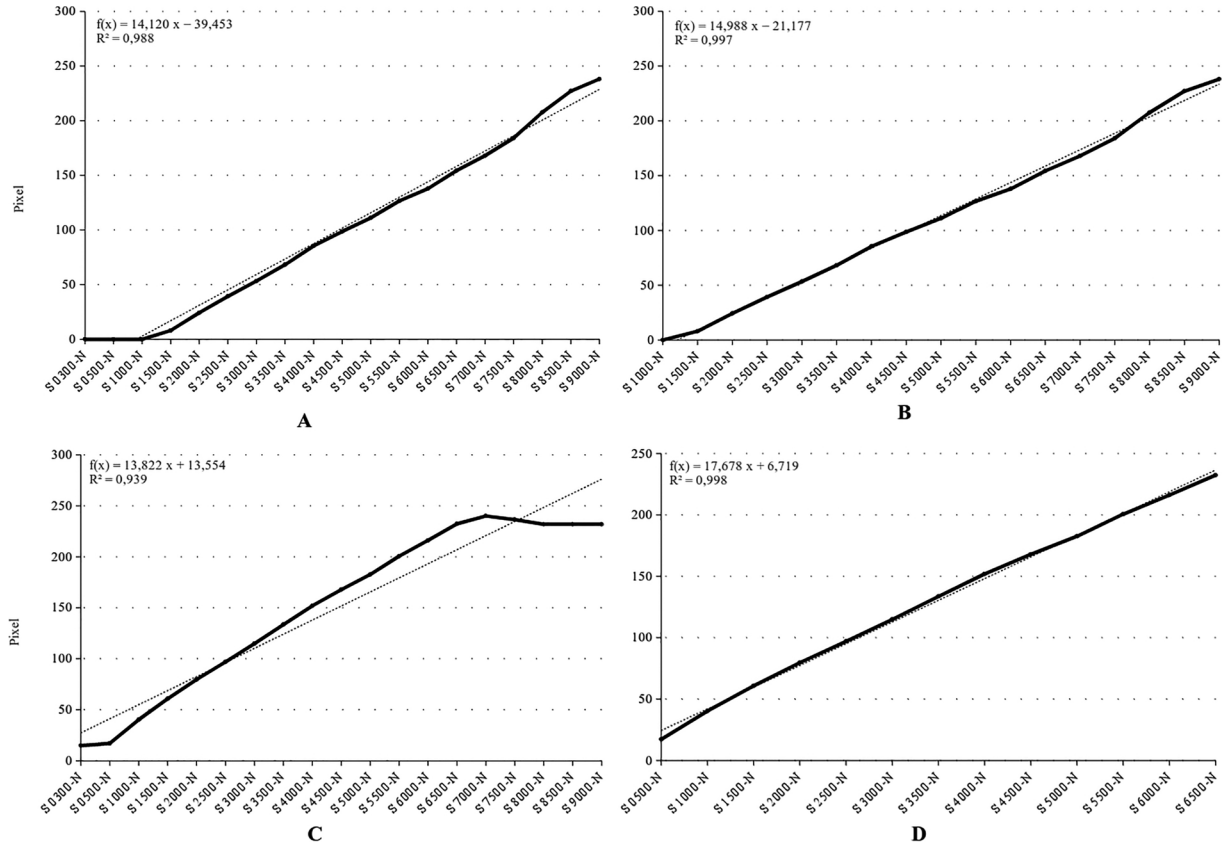

Fig. 1. Graphs of maximum pixel value mean of Panel 1 at different scanning settings.
Legend Fig. 1: Panel A: complete curve of reading with Density set at 0; Panel B: linearity curve of the reading with Density set at 0; Panel C complete curve of reading with Density set at +3; Panel D: linearity curve of reading with Density set at +3. On the X axis are the 19 NCS shades of greys ordered from the lighter (S0300-N) to the darkest (S9000-N); on Y axis are the means of pixel values of 10 readings by the UniScan Software. Black lines represent mean pixel values, dashed lines represent the linearity curve. At the top left corner, linearity equations and coefficient of determination (R^2) value are reported. R^2 values close to 1 indicate a good linearity of the data.

Table 3
UniScan Software reading results in terms of normalized maximum pixel value mean of IB membranes of Panel 4 (negative sera), reported for the three antigens (p26, gp45 and gp90) as overall and separated by country of provenance (Iceland and Italy).

Normalized maximum pixel value	p26	gp45	gp90
Panel 4			
Mean	15.59	9.33	15.74
SD	9.44	7.55	8.63
CV	67.03	66.03	65.03
Iceland negative sera			
Mean	9.67	5.03	14.94
SD	5.29	3.46	8.01
CV	54.68	68.89	53.65
Italian negative sera			
Mean	20.81	13.03	16.47
SD	9.25	8.17	9.21
CV	44.46	62.70	55.93

SD: standard deviation; CV: coefficient of variation.

(Schaller et al., 1999), and those available mostly investigate their diagnostic performance. The validation of laboratory assays, based on OIE guidelines[3], provides essential information on their suitability for the intended purpose and allows comparison between different methods (ELISA and AGIDT), or similar methods with different techniques (e.g. compare different ELISA set-ups (Nardini et al., 2017)).

A digital based reading system was chosen to overcome the subjective optical reading of a sample reactivity in IB, as is still the case for AGIDT that relies on the experience of the reader. This choice required further standardization and optimization stages, together with an evaluation of the variability of the digital reading system. As was expected, the software had no variability in reading the greys of Panel 2 that were created without scanning.

The results of Panel 1 had very low values of SD and CV (in terms of a hundredth of a pixel), indicating that the reading system does not contribute to the variability of the outcome which, on the other hand, is influenced by the repeatability and reproducibility of the IB procedure. The only step in the reading process that is still subjective is the choice of the blank zone for the normalization of data that is completely up to the operator. This issue will be discussed further on, together with the precision parameters.

The optimization phases requested changes of the scanning parameters to increase the sensitivity for the lighter greys. This feature is important in epidemiological frameworks, similar to the Italian one, where the overall prevalence is at a very low level (0.14% in Italy according to the NRL) and where the majority of animals with an AGIDT strong reactivity are already detected. A weak seropositive reaction could be due to a new infection, with a mounting antibody response, or to low levels of antibodies, as described in certain circumstances, such as during the chronic phase of the infection in animals defined as low responders (Issel et al., 2013). In both cases, the sera of such subjects are likely to present discordant results between ELISA and AGIDT, and only occasionally result positive by molecular methods due to the varying viremia levels through time that is typical of an EIA infection (Autorino et al., 2016; Ricotti et al., 2016).

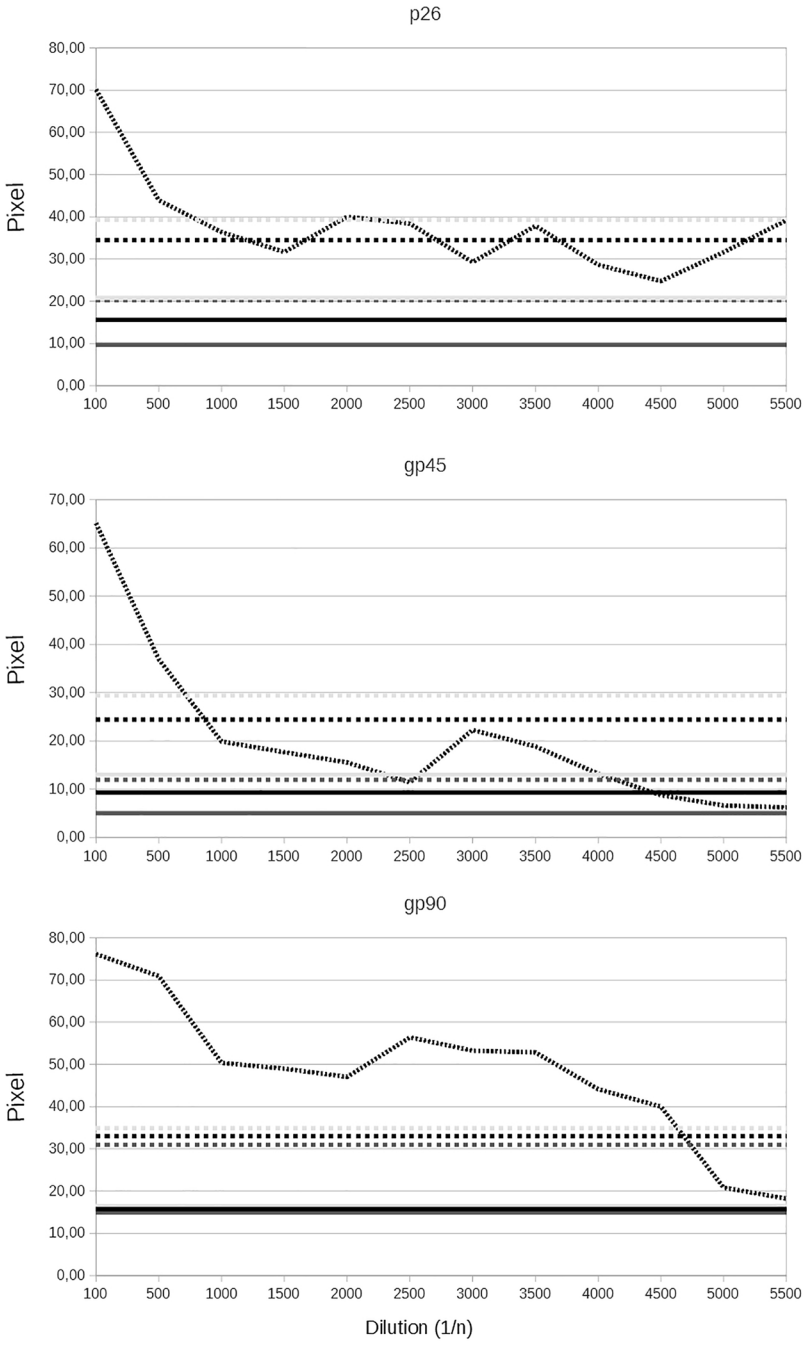

Fig. 2. Comparison of normalized maximum pixel value mean of negative sera and serial dilutions of a positive serum.
Legend Fig. 2: The graph at the top represents p26 antigen values, the middle one, gp45 antigen values and the bottom one, the gp90 antigen values. On X axis are the reciprocals of the dilutions of positive serum tested (from 1:500 to 1: 5500); on Y axis are the normalized maximun pixel value mean of readings by the UniScan Software. Dashed black line represents normalized maximun pixel value mean for the positive serum, complete black line represents the normalized maximun pixel value mean of Panel 4 considering all sera together; dotted black line is the normalized maximun pixel value mean of all sera of Panel 4 plus two standard deviation (SD); complete dark grey line represents the mean value of negative sera from Iceland; dotted dark grey line is the normalized maximun pixel value mean of Iceland sera plus two SD; complete light grey line represents the normalized maximun pixel value mean of Italian negative sera; dotted light grey line is the normalized maximun pixel value mean of Italian negative sera plus two SD.

The IB, the third and the last tier of the diagnostic pathway, should be capable of detecting even a weak antibody response. On the contrary, it is not necessary to discriminate between the intensity of stronger reactivity values, as once the reading curve reaches the plateau the high sensitivity of the system is not affected.

The colour conversion of the IB membrane from coloured to grey scale is carried out after scanning to ensure that this is independent from the scanner in case this is replaced.

The set-up of the threshold values was defined with three different methods, which provided similar results. For the threshold value of 30 pixels, the diagnostic performance of the IB is satisfactory in terms of sensitivity for all antigens and all the sera of the different species. This threshold pixel value was purposely chosen, first to optimize the reading and the interpretation of data and second to improve the sensitivity of the gp90 for horse sera that increases from 0.97 to 1.

Even if in some cases lower than the expected 0.99, the specificity for the three antigens together resulted high in the various conditions evaluated, ranging from 0.89 to 1 which could appear as a risk to classify some subjects as false positive, but this is the case only when considering the reactivity to the individual antigens.

In our case, since the result of a sample is based on the reactivity to more than one antigen, with p26 as compulsory, the probability that a negative horse results positive to p26 and to gp90 is 0.007, to p26 and to gp45 is 0.0021, and positive to all antigens is 0.00021 and therefore exceptional. In the three-tier system, samples tested in IB are previously analysed in ELISA, therefore, as the specificity of the c-ELISA is 0.808, the probability for a negative sample to result positive is 0.192 (Nardini et al., 2016). When combining the probability of a false negative result for both methods, the overall probability of a false positive result decreases to 0.00004. In fact, the diagnostic parameter measured with the qualitative outcome resulted perfect. These estimations will be verified by the results obtained from the use of the method in the routine. The

Table 4
Results of the ROC analysis performed on the immunoblot assay for the detection of antibodies against equine infectious anaemia virus employing horse, mule and donkey sera, displayed for the three antigens (p26, gp45 and gp90) present on the membrane. Threshold values were automatically determined by the software employed for the ROC analysis (STATA SE v.12.0).

Antigen	ROC	Horse	Mule	Donkey
p26	AUC (CI*)	1.00 (0.92–1.00)	1.00 (0.90–1.00)	1.00 (0.90–1.00)
	Threshold	35	21	32
	Se (CI*)	1.00 (0.86–1.00)	1.00 (0.82–1.00)	1.00 (0.79–1.00)
	Sp (CI*)	1.00 (0.84–1.00)	1.00 (0.82–1.00)	1.00 (0.79–1.00)
gp45	AUC (CI*)	0.98 (0.89–0.99)	0.96 (0.84–1.00)	0.97 (0.85–1.00)
	Threshold	25	18	11
	Se (CI*)	0.92 (0.73–0.99)	0.96 (0.76–1.00)	1.00 (0.79–1.00)
	Sp (CI*)	0.93 (0.73–1.00)	1.00 (0.82–1.00)	0.94 (0.70–1.00)
gp90	AUC (CI*)	0.99 (0.92–1.00)	0.96 (0.84–1.00)	1.00(0.90–1.00)
	Threshold	43	34	35
	Se (CI*)	0.97 (0.81–1)	0.96 (0.76–1,00)	1.00 (0.79–1.00)
	Sp (CI*)	1.00 (0.84–1.00)	0.96 (0.76–1,00)	1.00 (0.79–1.00)

ROC: receiver operating characteristic; AUC: area under curve; Se: sensitivity; Sp: specificity; CI: Confidence interval. * CI were calculated with a CL of 99% for Horse and Mule and 95% for Donkey. Thresholds are expressed as number of pixel.

Table 5
Diagnostic performance of the immunoblot assay for the detection of antibodies against equine infectious anaemia virus on horse, mule and donkey sera for the three antigens (p26, gp45 and gp90) with threshold between positive and negative set at a value of 30 pixel.

	Threshold	30		
Antigen		Horses	Mules	Donkeys
p26	Se (CI*)	1.00 (0.86–1.00)	1.00 (0.82–1.00)	1.00 (0.79–1.00)
	Sp (CI*)	0.93 (0.73–1.00)	1.00 (0.82–1.00)	1.00 (0.79–1.00)
gp45	Se (CI*)	0.86 (0.65–0.97)	0.63 (0.37–0.85)	0.75 (0.40–0.95)
	Sp (CI*)	0.97 (0.78–1.00)	1.00 (0.82–1.00)	1.00 (0.79–1.00)
gp90	Se (CI*)	1.00 (0.86–1.00)	0.96 (0.76–1.00)	1.00 (0.79–1.00)
	Sp (CI*)	0.90 (0.73–0.98)	0.89 (0.65–0.99)	1.00 (0.79–1.00)
Qualitative outcome	Se	1.00	1.00	1.00
	Sp	1.00	1.00	1.00

Se: sensitivity; Sp: specificity; CI: Confidence interval. * CI were calculated with a CL of 99% for Horse and Mule and 95% for Donkey.

Table 6
Repeatability (s_r) and reproducibility (s_R) standard deviations and expanded uncertainty (U) for each antigen and reactivity level expressed as number of pixels.

	Antigen	Negative	Weak positive	Medium positive	Strong positive
s_r	p26	6.76	12.49	7.48	12.36
	gp45	6.75	8.02	9.74	4.10
	gp90	4.38	9.91	7.13	12.69
s_R	p26	2.68	4.28	5.34	5.82
	gp45	3.80	3.94	3.64	2.72
	gp90	3.17	7.14	4.30	7.62
Expanded uncertainty (U) (95% CI)	p26	30 (22–38)	30 (21–39)	50 (40–60)	70 (59–81)
	gp45	30 (14–46)	30 (18–42)	50 (42–58)	70 (61–79)
	gp90	30 (18–42)	30 (21–39)	50 (43–59)	70 (54–86)

s_r : repeatability standard deviations ; s_R : reproducibility standard deviations; CI: Confidence interval. For the expanded uncertainty the value set as threshold between each reactivity level and the next are reported, together with the value of CI within round brackets.

same apparent specificity issue was also detected for the c-ELISA, but was not confirmed by its use in the routine, as the effective proportion of false positive reactions was estimated around 0.0019 (Nardini et al., 2016). A diagnostic evaluation, even if performed according to all the statistical requirements, could be influenced by the number of samples with unusual characteristics that during the routine use of the method may constitute a very small number of the total examined.

A lower sensitivity for the gp45, for all the species examined was obtained in the ROC analysis with a cut-off of 30 pixels that is due to the variable reactivity of the gp45 that is also generally weaker than for the other antigens. This data is also supported by the NRL results of the last seven years of surveillance activity and by other authors (Alvarez et al., 2007; Issel et al., 2013). As stated by Rwambo et al. (1990a), antibodies against gp45 are the last to appear during the initial stages of infection. Another hypothesis could be that their amount is the lowest when compared to those produced against p26 and gp90 and have a lower avidity.

The choice of a unique threshold value could cause an underestimation of the sensitivity to the gp45, but not of the assay as a whole, which is guaranteed by the interpretation criteria adopted for the assay. This criteria, which assigns the result on the basis of a serological reaction to more than one antigen (at least p26 and one *env* band), is analogous to that adopted for the HIV diagnosis (Mahajan et al., 2010; Zaaijer et al., 1998; Huang et al., 2018) and guarantees a high specificity for the diagnosis of EIA, avoiding the slaughtering of false positive subjects.

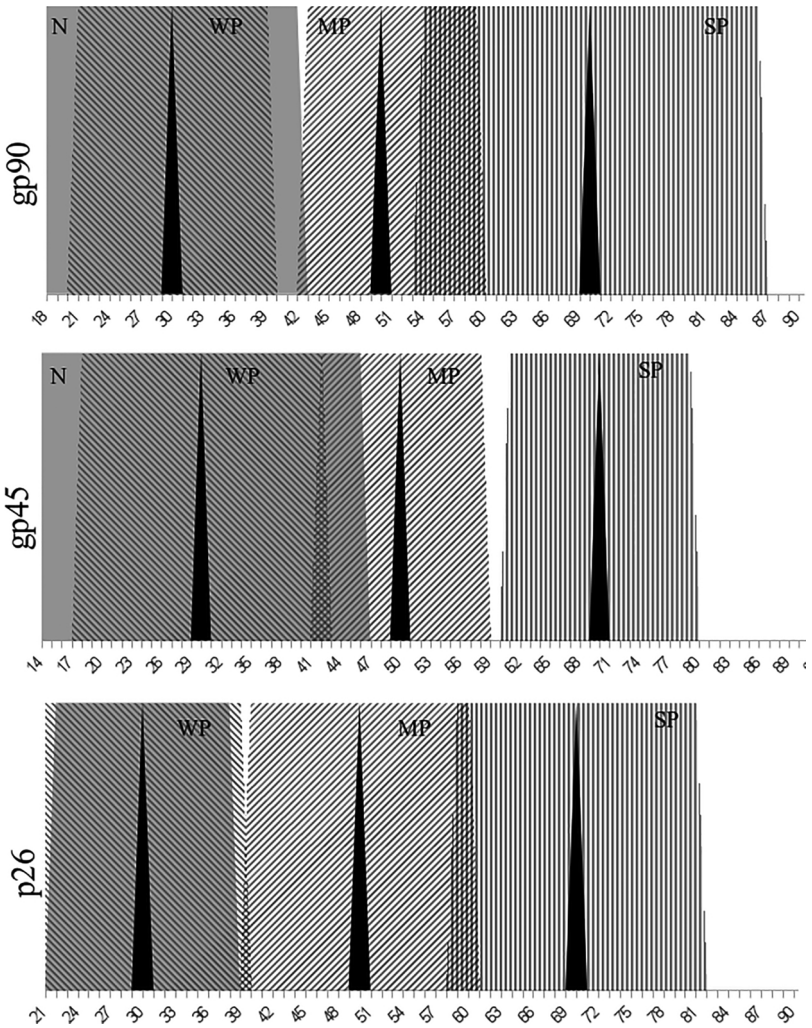

Fig. 3. 95% Confidence Interval (CI) for each antigen and reactivity level.
Legend Fig. 3: Black spikes: threshold values. Grey zone (N): negative (pixel value ≤ 30), Lines to the top left corner (WP): weak positive (30 < pixel value ≤ 50); Lines to the top right corner (MP): medium positive (51 < pixel value ≤ 70); Vertical lines (SP): strong positive (pixel value > 70).

In the previous version of the protocol (Cook et al., 2001), the positivity to each antigen was established by comparing it to that of the internal positive control for the same antigen. The positive control serum belongs to a horse with the weakest IB reaction that was proved to be still capable of transmitting EIAV, as confirmed by EIA transmission by horse inoculation (Issel and Adams, 1982). The rationale was that to represent a risk, a seropositive horse must be capable of transmitting the virus and therefore when adopting a conservative approach, weaker IB serological reactions were defined as negative. The normalized maximum pixel value mean of this serum were retrospectively calculated and resulted for respectively for the mean and median, 66 for p26, 53 and 57 for gp45 and 88 and 82 for gp90, classifying the sample as having a medium/strong positivity.

However, as pixel values of the negative sera of Panel 4 were never above those of the positive control, it was decided to assign a positive result to any sample with a reaction higher than the threshold level in consideration of the U of the method and not excluding the possibility that equids with a weaker reaction than the internal positive control could also transmit the infection.

Independently, the positive control is still required as an internal control for the evaluation of successful outcome of the run. The IB sensitivity and specificity level, defined by the threshold level which is to be adopted by the diagnostic laboratory could be more or less restrictive and is in function of the local epidemiological situation and of the objective of the surveillance plan as set by the national authorities.

The assay presented here resulted selective, exclusive and inclusive. In fact, the IB, employing the $EIAV_{Wyoming}$ strain, is capable of detecting serological reactions of subjects from different geographical areas, indicating that the epitopes of the three antigens of this EIAV are relatively conserved (Scicluna et al., 2013). The negative reactions of the sera positive to other equine and retro-viral infections confirmed the absence of cross-reactive antibodies against EIAV.

The analytical sensitivity of the IB resulted 100 times higher than that of AGIDT and 30 times higher respect to that of the c-ELISA. This data together with the result of the diagnostic sensitivity confirmed the suitability of IB as an alternative confirmatory test in case of ELISA positive-equivocal/AGIDT negative samples. This is also in view of the fact that in particular for the horse and mule sera panels (27 samples) used for the evaluation of the diagnostic sensitivity, these were made up of samples that were either experimentally infected or PCR sequenced positive and with around 80% of these that were AGIDT negative. Thus the IB presents, at the same time, a higher sensitivity than the AGIDT, correctly detecting as positive, ELISA positive/AGIDT negative samples. It also presents a higher specificity than the ELISA, discriminating aspecific results of samples obtained during the screening phase that were in part due to a reactivity in IB to only the p26 band, which is the antigen in common with the ELISAs used in Italy, and for this classified as negative. This reactivity is ascribed to interspecies determinants in

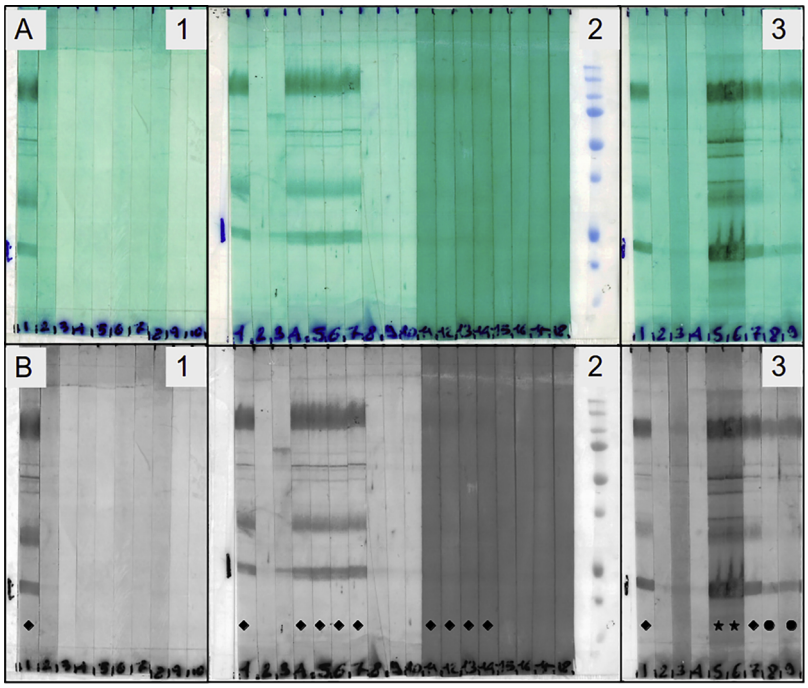

Fig. 4. Examples of immunoblot membranes before (Panel A) and after conversion in grey (Panel B).
Legend Fig. 4: Panel A1/B1: Threshold determination on negative samples from Iceland. Panel A2/B2: Selectivity assessment. Lines: 3-10: samples analysed with the standard procedure. Lines: 11-18: samples analysed employing deionized water instead of all solutions. Panel A3/B3: Repeatability panel. Black diamonds: Internal positive control, considered as Medium positive in repeatability panel. Black stars: Strong positive serum. Black circles: weak positive serum, consisting in internal positive control diluted 1/5 in negative horse serum. All remaining lines represent negative sera.

the major core antigens of retroviruses and lentiviruses (Issel et al., 2013).

Even if the present study compared the specificity of the IB to only the c-ELISA, a previous comparison study of the authors showed that its performance is comparable to that of the other five serological assays commercially available in Italy, one of which is also validated by the NRL (Nardini et al., 2017; Scicluna et al., 2018).

Relative to the evaluation of other parameters of the assay, CV and SD values are in general below the defined limits (Jacobson, 1998). In fact, most of the CVs evaluated were below 15% with some exceptions represented by the CVs of the negative and weak positive sera that exceptionally reached 50%, which was expected because the pixel values for these categories are very low and therefore small differences have a greater weight than for the other reactivity levels and for this considered as less significant[4] (Paré and Simard, 2004). The same consideration can be applied to the gp45 CVs.

Unexpectedly the s_r resulted higher than the s_R, although both were acceptable for all the antigens; this data requires confirmation by further evaluations. The variability expressed as 95% CI with the U computed according to OIE validation procedures[3] resulted in an overlapping of the reactivity of the negative and weak positive serum. As a consequence, the threshold values were set at 30 pixels and values between 30 and 40, should be evaluated in view of the individual and overall reactivity for the different antigens and a further serum sample of the individual could be requested to monitor the evolution of the antibody levels.

This validated IB could be employed and reproduced throughout the network of other NRLs. An impartial reading requested the use of a specific software, which could represent a limitation use of the method that should be taken into account.

The specific software was chosen for its ease of use, but others are available, e.g. Image J[9] that is an open source and for which some instructions to the western blot data analyses are available,[10,11] or other products such as the ChemiDoc System (Biorad®) or Adobe Photoshop (Adobe Systems, CA, USA), that are economically demanding. Laboratories that intend to adopt the method have other different options, which are or use the classical reading of the images by the operator, or employ a software for measuring pixel intensity. At present, the NRL can provide IB membranes of its own production and support in the validation of the method in other laboratories.

5. Conclusion

In the three-tier system, the main advantage of the IB as a confirmatory test, complementary to AGIDT, is the capability of the early detection and removal of cases, which increases the efficiency of the surveillance programme.

The European regulation, Directive 2009/156 CE does not consider the use of the IB as a confirmatory or complementary test to the AGIDT as is indicated by OIE.

For this, the Italian Regulation on EIA has defined as suspect of EIA, an equid that is AGIDT negative/IB positive. These animals, together with those living on the same premises, are subjected to restriction movement measures and serological monitoring that is carried out every six months.

On the bases of the results of different authors, the regulations of a country or a region should take into account that an AGIDT negative/IB positive animal is equivalent to an AGIDT positive animal in terms of risk of transmission of the disease.

The present study is the first report of a validation of an IB for detection of antibodies against three antigens of EIAV that is also based on an impartial reading of the result as well as its possible recording, thus conferring a greater reliability in its use as a confirmatory test than the AGIDT for its independence from the operator's personal evaluation of the reactivity of a sample.

As it is the only method of its kind that has been validated, its comparison can be only discussed with those of other EIA serological methods that confirmed its "fitness for purpose".

Finally, as its validation is compliant with international standards, as those of the OIE regulations, the NRL, as for the other methods in use for the serological diagnosis of EIA, will proceed with its submission for accreditation according to ISO/IEC 17025:2017[2].

[9] https://imagej.net/Image_Intensity_Processing.
[10] http://www.yorku.ca/yisheng/Internal/Protocols/ImageJ.pdf.
[11] http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.01.012.

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