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Short communication

Development and evaluation of a new lateral flow assay for simultaneous detection of antibodies against African Horse Sickness and Equine Infectious Anemia viruses

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A B S T R A C T

African horse sickness (AHS) and equine infectious anemia (EIA) are both notifiable equid specific diseases that may present similar clinical signs. Considering the increased global movement of horses and equine products over the past decades, together with the socio-economic impact of previous AHS and EIA outbreaks, there is a clear demand for an early discrimination and a strict control of their transmission between enzootic and AHS/EIA-free regions. Currently, the individual control and prevention of AHS or EIA relies on a series of measures, including the restriction of animal movements, vector control, and the use of several laboratory techniques for viral identification, amongst others. Despite being widely employed in surveillance programmes and in the control of animal movements, the available serological assays can only detect AHS- or EIA-specific antibodies individually. In this work, a duplex lateral flow assay (LFA) for simultaneous detection and differentiation of specific antibodies against AHS virus (AHSV) and EIA virus (EIAV) was developed and evaluated with experimental and field serum samples. The duplex LFA was based on the AHSV-VP7 outer core protein and the EIAV-P26 major core protein.

The results indicated that the duplex LFA presented a good analytical performance, detecting simultaneously and specifically antibodies against AHSV and EIAV. The initial diagnostic evaluation revealed a good agreement with results from the AHS and EIA tests prescribed by the OIE, and it highlighted the usefulness of the new AHSV/EIAV duplex LFA for an on-field and point-of-care first diagnosis.

African horse sickness (AHS) is an infectious, non-contagious, vector-borne disease of equids, characterized by alterations in the respiratory and circulatory functions (OIE, 2012). This disease is highly lethal for horses, being less severe for mules, donkeys and zebra (Boinas et al., 2009; Lord et al., 2002; Mellor and Hamblin, 2004; Wilson et al., 2009). Although AHS is enzootic in sub-Saharan Africa, outbreaks have occurred in the Middle East, Morocco and Iberian Peninsula, causing a considerable economic impact to the equestrian industry (Coetzer and Erasmus, 1994; Howell, 1960; Lhafi et al., 1992; Mellor, 1993; Mellor and Hamblin, 2004; Portas et al., 1999; Rodriguez et al., 1992; Sanchez-Vizcaino, 2004). AHS is caused by a double-stranded RNA virus (AHSV) that belongs to the *Orbivirus* genus of the *Reoviridae* family. Currently, there are nine antigenically distinct serotypes of AHSV recognized worldwide. The virus capsid contains seven structural proteins, including the VP7 outer core protein that is a group-specific antigen recognized by all the nine serotypes (Chuma et al., 1992). Taking into account that this protein is highly conserved among all AHSV serotypes, and that horses develop antibodies against the infecting serotype of AHSV in an early phase (within 8–12 days post-infection (Maclachlan and Guthrie, 2010)), the VP7 protein is often the target protein used in

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AHS diagnostic assays (Chuma et al., 1992; Maree and Paweska, 2005; OIE, 2012). In fact, two of the prescribed tests for international trade by the World Organization for Animal Health (OIE) are aimed at detecting specific antibodies against AHSV, using soluble VP7 protein (indirect and competitive blocking enzyme-linked immunosorbent assays (ELISA)). At the moment, there is no effective treatment for AHS, and its control relies mainly on vaccination based on live attenuated AHSV South African strains (OIE, 2012; OIE 2015; Coetzer and Erasmus, 1994), together with the restriction and regulation of horse movements between endemic, epidemic and disease-free areas. The adoption of measures that prevent horses being bitten by infected vectors is also an important component for disease control (OIE, 2015). Despite allowing horses to survive in endemic areas, the available vaccination strategy is not sufficiently safe and efficient in non-endemic countries, and it does not differentiate infected from vaccinated animals. Alternative vaccination strategies have been developed in the past (Alberca et al., 2014; Castillo-Olivares et al., 2011; Chiam et al., 2009; Guthrie et al., 2009; Roy and Sutton, 1998), but unfortunately none of these vaccines are commercially available. Here, surveillance programmes are fundamental to determine the AHSV status of the country, and rapid and effective diagnostic techniques are envisaged for an early AHS detection (de Vos et al., 2012; OIE, 2015; Sanchez-Matamoros et al., 2015).

Equine infectious anemia (EIA) is a persistent viral infection of equids with a world-wide prevalence that has a significant economic impact in the equine industry (Cook et al., 2013). Its etiological agent is the equine infectious anemia virus (EIAV), which is an enveloped single-stranded RNA virus classified in the Lentivirus genus of the Retroviridae family (Cook et al., 2013; Matheka et al., 1976; Weiland et al., 1977). At the moment, there is no vaccine or treatment for EIA. Inactivated and attenuated live EIAV vaccines were used in the past, but, in order to avoid the interference of vaccine antibodies with diagnostic tests, the strategy for EIA control changed to quarantine (OIE, 2014). EIA is mainly diagnosed by serological tests since infected equids carry the EIAV for life. The presence of antibodies against the major core protein of EIAV (P26) in the agar gel immunodiffusion test (AGID) is the established serological indicator of the viral infection, and it is the prescribed test for international trade by the OIE (Alvarez et al., 2015, 2010; Cook et al., 2013; OIE, 2014; Scicluna et al., 2013). Other techniques, such as ELISA and immunoblotting are used for screening purposes (Issel et al., 2013; Nardini et al., 2016).

The increased global movement of horses and equine products over the past decades together with the socio-economic impact of the previous AHS and EIA outbreaks, and considering that both diseases are listed by the OIE and may present similar clinical signs, it is of utmost importance to rapidly discriminate both diseases at an early phase. This will allow to control their transmission between enzootic and AHS/EIA-free regions, and to implement effective surveillance procedures that determine the AHSV/EIAV status of the countries of interest (OIE, 2012, 2014). The available serological techniques used for independent detection and control of AHS and EIA diseases often require specific laboratory facilities, skilled technicians and time-consuming protocols, which delay the diagnosis in an outbreak scenario. New approaches are now emerging to advance the high-throughput and cost-effective detection of AHSV-specific antibodies, such as Luminex assay (Craigo et al., 2012; Sanchez-Matamoros et al., 2015; Wang et al., 2010). However, there is still a clear scope for improvement in the simultaneous and differential diagnosis of AHS and EIA diseases using rapid and point-of-care technologies.

In this work, a rapid, one-step, duplex LFA based on the recombinant VP7 and RP26 proteins was developed for simultaneous detection of AHSV-specific and EIAV-specific antibodies in serum samples.

The VP7 and P26 recombinant proteins were produced in the baculovirus expression system (BES) and in *Escherichia coli*, respectively. Three different coloured carboxyl-modified latex microspheres of 300 nm (PolymerLabs) were used in the AHSV/EIAV duplex LFA: red particles covalently conjugated with recombinant VP7 protein, blue particles covalently conjugated with recombinant P26 protein, and green particles covalently conjugated with the LFA control detector reagent (BSA-Biotin protein complex). The mixture of latex microspheres was dispensed onto the conjugate pad (Operon) by using the Matrix 1600 dispenser (Kinematic Automation, Inc.). Recombinant P26 and VP7 proteins were also applied onto the nitrocellulose (NC) membranes (Millipore) at 0.2 mg/mL and 0.125 mg/mL, resulting in test line T1 and T2 capture reagents, respectively. The anti-biotin IgG monoclonal antibody was used as the control line capture reagent. Capture reagents were dispensed using the Matrix 1600 platform (Kinematic Automation, Inc.).

Apart from the NC membrane and the conjugate pad, the duplex LFA strips comprised an absorbent pad (Ahlstrom), and a special sample pad (Cytosep 1662), which was here included considering a possible application for blood samples. The duplex LFA kit was composed by test strips assembled individually into a plastic housing, and by droppers containing the dilution buffer (Tris-HCl 250 mM at pH 7.5, NaCl 150 mM buffer, with casein and sodium azide as blocking and preservative agents, respectively).

Initially, both AHSV and EIAV LFA were developed individually, optimising the test conditions for each LFA; and then, the optimal individual test conditions were applied to the duplex LFA and further assessed jointly. Several VP7 and P26 protein concentrations were studied for the latex conjugation and capture reagents on the membrane, together with different dilution buffers and sample pads. The optimal protein concentrations of AHSV capture reagents and protein-latex conjugated microparticles differed from those of EIAV, in which the P26 recombinant protein was dispensed and conjugated at higher concentrations than the VP7 recombinant protein.

In this study, only serum samples were analysed by the ASHV/EIAV duplex LFA, conducting the following protocol: 10 μL of sample are applied onto the round window of the cassette, followed by 3–4 drops (120 μL) of dilution buffer. The mixture migrates through the conjugate pad and the nitrocellulose membrane by capillarity. In the presence of antibodies towards AHSV or EIAV, these will bind first to the VP7 or P26 protein-conjugated microparticles, forming an immune complex. As the duplex LFA was developed using a Double Recognition (DR) format (Venteo et al., 2012), which relies on the ability of an antibody to recognize two epitopes at the same time, this immune complex will then react with the immobilized VP7 and P26 proteins on the membrane, making the test line visible (T1/T2). After ten minutes, the visual observation of different coloured lines illustrated the presence or absence of AHSV's and/or EIAV's antibodies, as shown in Fig. 1. If red and green lines are visible, the sample is positive for AHSV; if blue and green lines are visible, the sample is positive for EIAV; and finally, if only a green line is visible, the sample is negative for both AHSV and EIAV. In any case, the green control line must always appear; otherwise the test has to be considered invalid.

The analytical sensitivity of the AHSV/EIAV duplex LFA was analysed with two-fold serial dilutions of individual AHSV and EIAV reference positive serum and a mixture of both positive sera as well (Table 1). These positive serum samples were kindly provided by the European reference laboratory for AHSV (Laboratorio Central de Veterinaria (LCV), Algete, Spain) and from the Equine Virus Laboratory of CICVyA-INTA (Centro de Investigaciones en Ciencias Veterinarias y Agronómicas – Instituto Nacional de Tecnología Agropecuaria, Buenos Aires, Argentina). The AHSV positive reference serum was obtained from a pool of sera of horses vaccinated with the inactivated serotype AHSV-4, and characterized as

Table 1
Summary of serum samples used in the study.

Serum specificity	No. of samples	Description	Laboratory of origin
AHSV reference	1 (pool)	Experimental: horses vaccinated with inactivated AHSV-4	Laboratorio Central Veterinaria (LCV), Algete, Spain (EU AHSV reference laboratory)
EIAV reference	1 (pool)	Serum from two naturally infected horses	CICVyA-INTA, Equine Virus Laboratory, Argentina
AHSV serotypes	9	Experimental guinea pig sera	Veterinary Research Institute, Onderstepoort, South Africa
AHSV serotypes	9	Experimental goat sera	US Department of Agriculture, Plum Island, USA
AHSV serotypes	9	Experimental horse sera	Pirbright Institute, UK
BTV serotypes	24	Experimental sheep sera	Pirbright Institute, UK
EHDV serotypes	8	Experimental cattle sera	Pirbright Institute, UK; National Veterinary Services Laboratories, USA
AHSV	66	Field positive (Ivory Coast 2012, Cameroon 2013)	VISAVET-UCM, Spain; ANSES, France
AHSV	95	Field negative (Ivory Coast, Cameroon, Canada, Argentina, Spain)	VISAVET-UCM, Spain; ANSES, France; INGENASA, Spain
EIAV	39	Field positive (Argentina endemic regions)	CICVyA-INTA, Equine Virus Laboratory, Argentina
EIAV	63	Field negative (Canada, Argentina, Spain)	CICVyA-INTA, Equine Virus Laboratory, Argentina; INGENASA, Spain

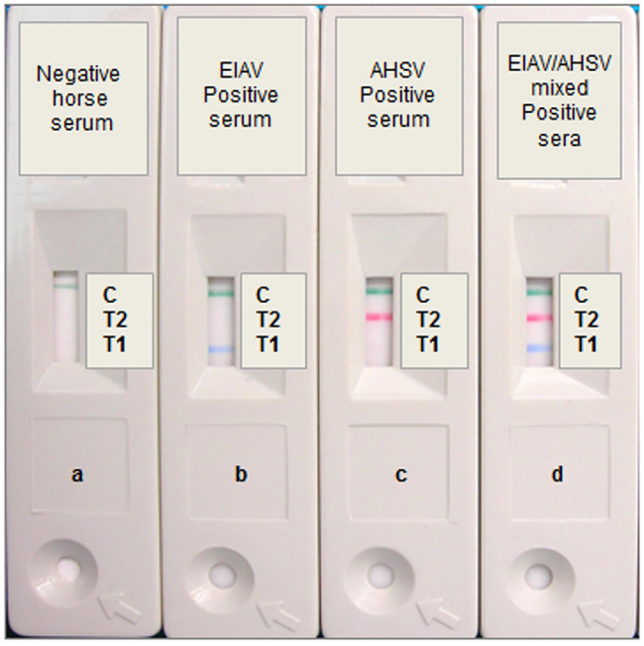

Fig. 1. Visual observation and interpretation of the AHSV/EIAV duplex LFA. Green visible line: the test is valid (a). If only the green line is observed, the sample is negative for AHSV and EIAV. Green and blue visible lines: the sample is positive for EIAV (b). Green and red visible lines: the sample is positive for AHSV (c). Green, red and blue visible lines: the sample is positive for both AHSV and EIAV (d). C–Control line, T2–VP7 (AHSV) test line, T1–P26 (EIAV) test line. This experiment was conducted with AHSV and EIAV reference positive sera. The double positive sample was obtained from the mixture of both reference positive sera.

positive down to the 1/64 dilution by the OIE's prescribed ELISA (INgezim AHSV Compac Plus, INGENASA) (OIE, 2012). The EIAV positive serum came from a pool of sera from the terminal blood collection of two naturally infected horses, and it was characterized by AGID following the OIE's instructions (OIE, 2014), and by ELISA (INgezim Anemia DR, INGENASA), in which it was positive down to the 1/256 dilution (data not shown). Sera was diluted in running buffer, and each dilution was treated as an independent sample. As shown in Fig. 2A, the duplex LFA detected both AHSV and EIAV positive serum samples down to the 1/32 dilution. The same limit of detection was obtained when analysing the mixture of both sera or each AHSV and EIAV positive serum samples individually. The 1/32 dilution of the AHSV positive reference serum corresponded to a blocking percentage of 96%, determined by the AHSV blocking ELISA (INgezim AHSV Compac Plus, INGENASA), and the 1/32 dilution of the EIAV positive serum corresponded to a S/P value (OD of sample/OD of positive control) of 1.8, evaluated by the EIA DR ELISA (INgezim Anemia DR, INGENASA) (data not shown). Although the sensitivity of the blocking and DR ELISAs appear to be higher than the Duplex LFA (positive at 1/64 and 1/256 dilution for AHSV and EIA respectively), this test presents other advantages to take into consideration such as multiplex detection, easy and rapid to perform, that allows its use at field.

The AHS's analytical sensitivity of the duplex LFA was further assessed by using the specific monoclonal antibody 4AH9 directed against the VP7-AHSV protein (Ranz et al., 1992). The estimation of the limit AHS-antibody concentration that the duplex LFA could detect, was useful for the quality control of the different batches of the assay. All the produced batches detected the 4AH9 mAb down to 3.125 μg/mL (0.375 μg per test).

The AHSV/EIAV duplex LFA was able to detect the 9 different serotypes of AHSV (AHSV1-AHSV9), as observed in Fig. 2B. The reactivity against the 9 serotypes were tested with sera from experimentally infected guinea pigs, goats and horses, from AHSV at the Veterinary Research Institute, Onderstepoort (South Africa) and at the US Department of Agriculture ARS-AD & Research Division (Plum Island Animal Disease Lab., USA) and Pirbright Institute (UK), respectively.

Regarding the analytical specificity, 24 serum positive samples from sheep infected with Bluetongue virus (BTV) serotypes 1–24 (provided from Pirbright Institute, UK), and 8 sera from cattle infected with Haemorrhagic disease virus (EHDV) serotypes 1–8 (provided from Pirbright Institute, UK and/or National Veterinary Services Laboratories, USA) were analysed. No cross-reactivity was observed except for the BTV-17 serotype, which presented a weak signal with the test line of AHSV (data not shown). These results showed a good analytical sensitivity and specificity of the test, and no cross-reactivity was observed between the two test lines when analyzing AHSV-, EIAV-, BTV-, or EHDV-positive reference sera.

A panel of 161 AHSV field sera and 102 EIAV field sera were analysed to estimate the diagnostic sensitivity and specificity of the AHSV/EIAV duplex LFA. All serum samples were previously evaluated by the corresponding reference methods (ELISA for the AHSV sera, and AGID for the EIAV sera), which classified them as positive or negative samples.

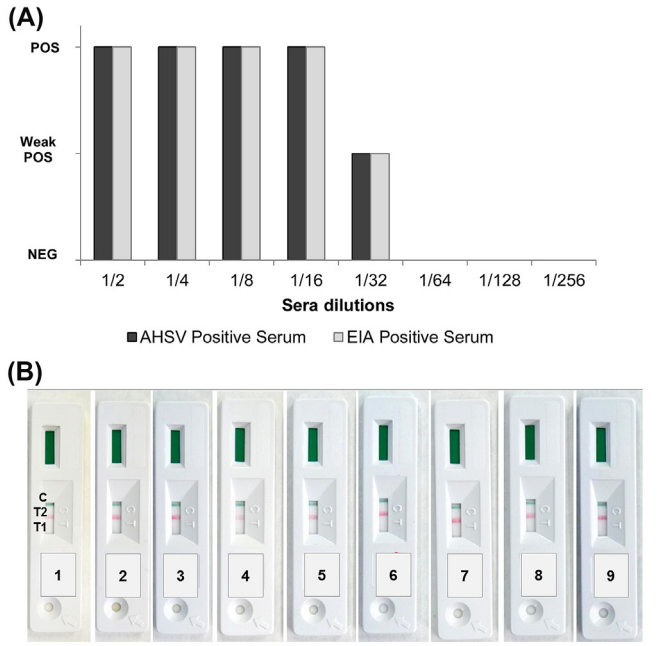

Fig. 2. Analytical sensitivity of the AHSV/EIAV duplex LFA. (A) Serial dilutions of each AHSV and EIAV reference positive sera and their corresponding result of the immunochromatography: POS–Positive result, Weak POS–Weak positive result, NEG–Negative result. (B) Detection of the 9 different AHSV serotypes by the AHSV/EIA duplex LFA: C–control line (green), T2–VP7 (AHSV) test line (red), T1–P26 (EIAV) test line (not visible, blue).

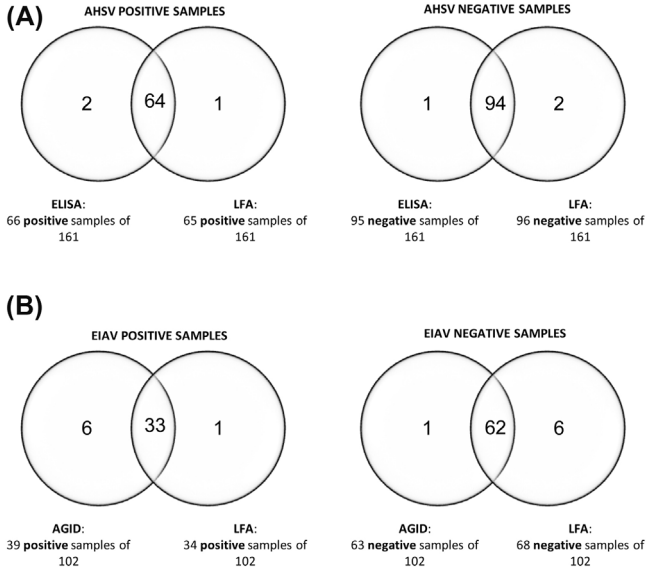

Fig. 3. Diagnostic sensitivity and specificity of the AHSV/EIAV duplex LFA. (A) Venn diagram's analysis of AHSV positive and negative field sera: comparison of the results between the duplex LFA and ELISA. (B) Venn diagram's analysis of EIAV positive and negative field sera: comparison of the results between the duplex LFA and AGID.

Sixty six of the AHSV field samples used in this study were classified by ELISA as positive (14 sera from vaccinated or infected horses, and 52 sera from suspicious horses from endemic regions (Ivory Coast, 2012 and Cameroon, 2013), provided by ANSES (European Union Reference Laboratory for equine diseases), and 95 sera were considered negative (14 from Cameroon and Ivory Coast endemic regions, and 81 from AHSV/EIAV-free areas of Canada, Argentina and Spain). Regarding the EIAV field samples, 39 sera were classified by AGID as positive (from naturally infected animals in Argentina endemic regions), and 63 sera were considered negative (13 sera from Argentina endemic regions, 50 from AHSV/EIAV-free areas of Canada, Argentina and Spain).

Diagnostic sensitivity and specificity of the LFA were estimated in a Microsoft Excel spreadsheet, considering the number of true positives (TP), the number of true negatives (TN), the number of false positives (FP), and the number of false negatives (FN). Sensitivity was calculated as 100× TP/(TP + FN), and specificity was calculated as 100 x TN/(TN + FP).

Concordance between LFA and ELISA (for AHSV) or AGID (for EIAV) methods was estimated, with 95% confidence intervals, in the MedCalc software (version 10.1.7).

Fig. 3A presents the comparison between the AHSV field sera's detection by the duplex LFA and by ELISA. The AHSV/EIAV duplex LFA recognized 64 of the 66 positive serum samples, and reported 94 of 95 negative serum samples. From this analysis, the duplex LFA showed a diagnostic sensitivity of 97% and a diagnostic specificity of 99% for the AHS detection.

Regarding the EIAV field serum samples (Fig. 3B), the duplex LFA detected 33 of 39 positive sera, and 62 sera were read as negative from a total of 63 negative samples. The duplex LFA showed a diagnostic sensitivity of 85% and a diagnostic specificity of 98% for the EIA detection.

None of the 161 AHSV serum samples gave a positive reactivity with the EIAV test line, and none of the 102 EIAV serum samples cross-reacted with the AHSV test line. Moreover, results from the AHSV/EIAV duplex LFA presented a good agreement with those from the AHSV ELISA (Kappa = 0.96, 95% CI: 0.95–0.97) and from the EIAV AGID (Kappa = 0.85, 95% CI: 0.79–0.90).

Although the duplex LFA presented a diagnostic performance similar to the OIE's prescribed tests for international trade, it correlated better with the AHSV ELISA than the EIAV AGID. Some AGID-positive samples were not detected by the duplex LFA, and this result suggests that further optimisations of the P26 protein's concentration immobilized on the nitrocellulose membrane and/or conjugated to the latex beads should be conducted in order to overcome false negative results. In the specific case of EIA, false negatives are critical because of the increased risk of disease's transmission by the free movement of infected equids (Alvarez et al., 2010; Cook et al., 2013; Issel et al., 2013; Scicluna et al., 2013). Nevertheless, more samples should be tested in order to fully characterize and compare both serological methods.

In the absence of a safe and effective vaccination strategy, rapid and reliable diagnostic techniques are essential to establish suitable and effective sanitary control measures and to impede AHS and EIA's transmission. Surveillance programmes and the restriction of animal movements between enzootic and AHS/EIA-free regions are also fundamental control measures, in which, serological tests have an important role on the determination of the disease-free status of either a specific country/area or animal (Issel et al., 2013; OIE, 2012, 2014; Sanchez-Matamoros et al., 2015; Scicluna et al., 2013). Despite being widely-used, the serological tests recommended by the regulatory organisations diagnose individually the ASH or EIA, and they may also present technical difficulties that delay the diseases' identification and discrimination in remote areas. The duplex LFA developed in this work is a rapid, low-cost and novel alternative to detect simultaneously AHSV-specific and EIAV-specific antibodies and to differentially diagnose both AHS and EIA diseases, which can present similar clinical signs. Although in this preliminary evaluation the new pen side presented slightly lower sensitivity than the traditionally-used ELISA or AGID, it offers the advantage of a rapid positive/negative result (in ten minutes) without requiring special skills or expensive equipment. Moreover, by being tailor-made for serum or blood samples, the duplex LFA supports an on-field and point-of-care diagnosis in small field labs.

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