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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