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Diagnostic DIVA tests accompanying the Disabled Infectious Single Animal (DISA) vaccine platform for African horse sickness

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ABSTRACT

African Horse Sickness Virus (AHSV) (*Orbivirus* genus, *Reoviridae* family) causes high mortality in naïve domestic horses with enormous economic and socio-emotional impact. There are nine AHSV serotypes showing limited cross neutralization. AHSV is transmitted by competent species of *Culicoides* biting midges. AHS is a serious threat beyond the African continent as endemic *Culicoides* species in moderate climates transmit the closely related prototype bluetongue virus. There is a desperate need for safe and efficacious vaccines, while DIVA (Differentiating Infected from Vaccinated) vaccines would accelerate control of AHS. Previously, we have shown that highly virulent AHSV with an in-frame deletion of 77 amino acids (aa) in NS3/NS3a is completely safe, does not cause viremia and shows protective capacity. This deletion mutant is a promising DISA (Disabled Infectious Single Animal) vaccine platform, since exchange of serotype specific virus proteins has been shown for all nine serotypes. Here, we show that a prototype NS3 competitive ELISA is DIVA compliant to AHS DISA vaccine platforms. Epitope mapping of NS3/NS3a shows that more research is needed to evaluate this prototype serological DIVA assay regarding sensitivity and specificity, in particular for AHSVs expressing antigenically different NS3/NS3a proteins. Further, an experimental panAHSV PCR test targeting genome segment 10 is developed that detects reference AHSV strains, whereas AHS DISA vaccine platforms were not detected. This DIVA PCR test completely guarantees genetic DIVA based on *in silico* and *in vitro* validation, although test validation regarding diagnostic sensitivity and specificity has not been performed yet. In conclusion, the prototype NS3 cELISA and the PCR test described here enable serological and genetic DIVA accompanying AHS DISA vaccine platforms.

1. Introduction

African horse sickness virus (AHSV) is the causative agent of the OIE listed disease African Horse Sickness [1]. AHSV (genus *Orbivirus, Reoviridae* family) consists of nine serotypes [2–4]. Orbiviruses of which bluetongue virus (BTV) is the prototype are non-enveloped multi-protein layered RNA viruses with ten double stranded genome segments S1-10 encoding seven structural proteins VP1-7 and at least four non-structural proteins NS1-4 [2,5,6].

Mortality by AHSV infection is over 90% in fully susceptible domestic horses, whereas zebras and African donkeys rarely show clinical signs [7]. AHS is still endemic to sub-Saharan Africa causing animal losses, and reduction of draft power, transportation and trade [8]. AHSV and BTV are transmitted by biting *Culicoides* midges [9–11]. The habitat of competent midges is expanding and *Culicoides* species in moderate climates have become competent BTV vectors [12–16], assuming that historically AHS-free countries are at risk [17,18]. Overall, AHS outbreaks lead to huge economic losses to the equestrian industry and enormous socio-emotional impact on owners of horses.

Vaccination is the most effective control of insect-borne diseases. Conventionally live-attenuated AHS vaccines (LAVs) are used in Africa but have not been licensed in Europe because of safety issues. LAVs do not enable Differentiation Infected from VAccinated individuals (DIVA). Experimental AHS vaccines like subunit and vectored vaccines are mainly based on the VP2 immune response and are DIVA compatible with the recommended ELISA detecting VP7 antibodies (Abs), however, none of these have reached the market yet [19–24].

More recently, promising virus-based vaccine platforms applicable for all nine serotypes have been developed. Examples are Disabled Infectious Single Cell/Cycle (DISC) vaccine or Entry

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Competent Replication-Abortive vaccine lacking VP6 expression [25,26], AHSV deficient for NS4 protein [27], and Disabled Infectious Single Animal (DISA) vaccine deficient for NS3/NS3a protein [28–30]. Regarding efficacy, except for the respective missing protein, these virus-based vaccines induce full immune responses including cytotoxic T-cell responses to highly conserved viral proteins potentially contributing to (cross) protection [31]. Importantly, in contrast to VP6 and NS4, NS3/NS3a protein is immunogenic in horses and a promising viral antigen for serological DIVA [32]. Similar virus-based vaccines have been developed for BTV, including the DISA vaccine platform ([33] and references therein). Indeed, the BT DISA vaccine platform is DIVA compatible with accompanying panBTV NS3 based ELISAs (serological DIVA) [34–36]. In addition, OIE recommended panBTV PCR tests targeting S10 are DIVA compatible (genetic DIVA) with the BT DISA vaccine platform [37–39].

Genome segment 10 (S10[NS3/NS3a]) of AHSV has been subdivided into three phylogenetic lineages [40,41]. Accordingly, reference strains AHSV1-9 represent three S10 groups consisting of AHSV serotype 1 and 2 (group α), 3 and 7 (group β), and of AHSV serotype 4, 5, 6, 8 and 9 (group γ). However, S10 subgrouping and the AHSV serotype is not correlated *per se* because of reassortment events [42].

Complete safety and protective capacity of AHS DISA vaccine platforms has been previously shown [30]. Here we demonstrate DIVA compatibility with AHS DISA vaccine platforms of (1) a prototype NS3 competitive ELISA (serological DIVA), and (2) a S10 based panAHSV PCR test (genetic DIVA) as developed in this study.

2. Materials and methods

2.1. Cell lines and viruses

BSR cells (a clone of baby hamster kidney cells [43] were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 5% foetal bovine serum (FBS), and antibiotics (100 IU/ml Penicillin, 100 μg/ml Streptomycin and 2.5 μg/ml Amphotericin B).

Except for AHSV reference strains for all nine serotypes (supplied by The Pirbright Institute, UK), viruses in this study have been generated using reverse genetics [28–30]. 'Synthetic' reassortants of LAV strain AHSV4LP (accession numbers: KM820849–KM820858) representing phylogenetic S10 groups α and β contain S10 originating from reference strains AHSV2 and 3 (accession numbers: KF860005 and KM886363) [29]. S10 mutant of AHSV4LP 'AUG total' [29] contain three STOP codons, 10 silent mutations in the conserved region from nucleotide 150 to 163, and 13 in-frame AUG -> GCC mutations (Figs. 1 and 2). The latter was previously designated DISA4 [30]. 'Synthetic' virulent AHSV5 FR (accession numbers: KM886344–KM886353 [44]) and DISA5 – AHSV5 with an in-frame deletion of 77 amino acid (aa) codons (77aa deletion) in S10 – have been described previously [30]. The derivative of AHSV5 lacking LD motif PPYNFASAP in NS3/NS3a (9aa deletion) was kindly provided by C.A. Potgieter, Deltamune, South Africa.

2.2. Mutants of genome segment 10

Plasmids containing cDNA flanked by the T7 promoter and restriction enzyme sites suitable for run-off transcription were synthesized by Genscript Corporation Piscataway, NJ, USA. An overview of S10 mutants is presented in Fig. 1. S10 sequences were confirmed after full segment amplification using primers F-full-S10 and R-full-S10 (Table 1) and the One-step RT-PCR kit (Qiagen) followed by conventional sequencing with appropriate primers [28]. Details of the 5′-terminal part of S10 and putatively expressed NS3/NS3a protein are presented in Fig. 2A and B, respectively.

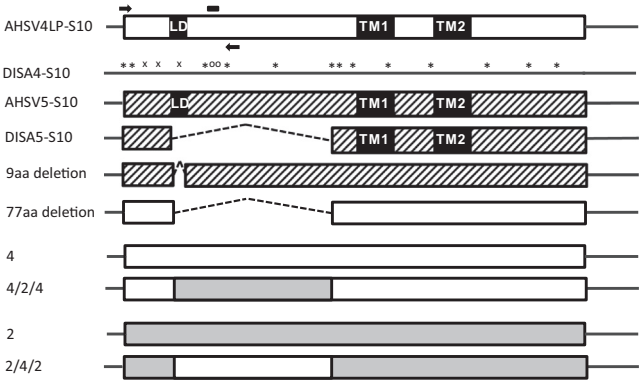

Fig. 1. Schematic overview of mutations in genome segment 10. Locations of primers and probe are indicated by arrows and a box, respectively. Introduced AUG -> GCC, multiple silent mutations and introduced STOP codons are indicated by *, oo and x, respectively. S10 RNA sequences are indicated by lines, deletions by dashed lines, and putatively translated NS3 related ORFs are represented by boxes. Filled, open and striped boxes indicate S10 of AHSV2, 4 and 5, respectively. Fig. 2 shows details of the 5′- terminal part of S10[NS3/NS3a].

2.3. Immunoperoxidase monolayer assay (IPMA)

Protein expression was determined by immunoperoxidase monolayer assay (IPMA) of infected or transfected BSR monolayers according to standard procedures [45]. Transiently expressed NS3/NS3a protein was studied in BSR monolayers infected with recombinant fowlpox virus expressing DNA dependent T7 RNA polymerase [46] followed by transfection with plasmid containing S10 under control of the T7 promoter as previously described [47]. Briefly, immunostaining was performed with monoclonal Ab (MAb) directed to VP5 (VP5 MAb), to NS3 (NS3 MAb) or 500x diluted α-NS3 rabbit serum followed by conjugated α-mouse serum or conjugated α-rabbit serum, respectively. MAb 10AE12 is directed to VP5, and MAbs 8F9, 1B4, 1E7, 4D3 and 6E6 are directed to NS3 of AHSV4LP (Ingenasa, Spain). Mono-specific polyvalent α-NS3 rabbit serum (α-NS3 rabbit serum) was raised against bacterially expressed AHSV 4LP NS3 protein (group γ) with several mutations in putative transmembrane regions to increase bacterial protein expression (will be published elsewhere). Conjugated rabbit α-mouse serum and conjugated goat α-rabbit serum were commercially purchased (DAKO, Amstelveen, the Netherlands). Generally, IPMA with VP5 MAb was performed to monitor virus infection. IPMA with NS3 MAbs was performed to map epitopes, and immunostaining with α-NS3 rabbit serum confirmed expression of NS3/NS3a protein.

2.4. Genetic DIVA

EDTA blood samples were analysed with panAHSV PCR-tests based on S4, S5 and S10. Conserved sequences of primers and probes were identified by the Basic Local Alignment Search Tool for nucleotide sequences (BLASTN). Briefly, total nucleic acid was extracted from 100 μl EDTA blood using a MagNA Pure Compact Nucleic Acid Isolation kit I on a MagNA Pure 96 according to the methods described by the manufacturer (Roche), and eluted in 100 μl water. Real time PCR tests were performed according to the all-in-one method as described [48]. Primers and probes were synthesized by Eurogentec and Tib Molbiol, respectively, and are listed in Table 1. Typically, real-time RT-PCR assays (20 μl final volume) consisted of 800 nM primers each, 300 nM of probe, 2 mM MnO_2, 7.5 μl RT-PCR mix (Roche LC480 RNA Master HybProbes) and 5 μl of purified RNA. RT-PCR was performed in the LightCycler 480 (Roche): 98 °C for 20 s (dsRNA denaturation), 55 °C for 20 s and 61 °C for 10 min (reverse transcription), 95 °C for 30 s, and

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