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Current Opinion in Virology

Reverse genetics approaches: a novel strategy for African horse sickness virus vaccine design

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African horse sickness (AHS) is a devastating disease caused by African horse sickness virus (AHSV) and transmitted by arthropods between its equine hosts. AHSV is endemic in sub-Saharan Africa, where polyvalent live attenuated vaccine is in use even though it is associated with safety risks. This review article summarizes and compares new strategies to generate safe and effective AHSV vaccines based on protein, virus like particles, viral vectors and reverse genetics technology. Manipulating the AHSV genome to generate synthetic viruses by means of reverse genetic systems has led to the generation of potential safe vaccine candidates that are under investigation.

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Current Opinion in Virology 2020, **44**:49–56

This review comes from a themed issue on **Preventive and therapeutic vaccines**

Edited by **Luis Martinez-Sobrido** and **Aitor Nogales**

For a complete overview see the Issue and the Editorial

Available online 10th July 2020

https://doi.org/10.1016/j.coviro.2020.06.003

Introduction

African horse sickness virus (AHSV) causes lethal disease in horses and is transmitted by hematophagous biting midges of the genus *Culicoides* [1,2]. AHSV infects mainly equids, causing high mortality rates up to 90% in horses, while mules and donkeys are less susceptible [3]. The virus belongs to genus *Orbivirus*, family *Reoviridae*, and nine serotypes (AHSV-1 to AHSV-9) have been identified upon the specificity of their reactions with neutralizing antibodies (NAbs) [4,5]. AHSV virion is a non-enveloped isometric particle composed of three concentric protein layers surrounding 10 lineal double-stranded RNA genome segments [6]. Together with the seven structural viral proteins (VP), the genome encodes other five non-structural (NS) proteins [6–8].

Despite endemicity of AHSV is constrained to Sub-Saharan Africa, the virus has caused devastating losses in indigenous horses outside of its current endemic zone during epidemics in Middle East, India, Pakistan, North Africa and Europe caused by multiple serotypes [9]. Recently (February the 24th, 2020), new outbreaks of AHSV-1 with an unknown origin have been documented in racehorses in Thailand, with 191 confirmed cases and 175 deaths, and a 91.62% of fatality rate, being the first AHSV outbreak described in this country (https://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=33768). The increasing global trade and the climate changes may facilitate the spread of vector-borne diseases, as shown by recent outbreaks of Bluetongue and Smallenberg viruses and demonstrating the rising viral transmission by *Culicoides* in non-endemic areas [10,11]. This suggests that AHSV can also emerge outside of Africa, causing huge direct and economic losses in horse industry as occurred in the past [12]. This scenario requires the development of an effective and safe vaccine capable to protect equids against all AHSV serotypes.

New approaches in vaccine generation against AHSV

Currently, the control of AHSV in endemic African countries relies on a polyvalent live attenuated vaccine (LAV) administering seven serotypes in two doses; AHSV-5 and AHSV-9 are not included in the vaccine since cross-protection with serotypes 8 and 6 respectively has been documented [3,13]. Of concern, LAVs are associated with reversion to virulence, vector's transmission, absence of DIVA (Differentiating Infected from Vaccinated Animals) capacity, teratogenicity, and gene reassortment that lead to the establishment of new genetic variants [3,14–18]. To address the need for safe and more effective vaccines, several candidates have been evaluated including subunit vaccines, virus like particles (VLPs), avian reovirus muNS protein microspheres (MS), recombinant poxviruses and reverse genetic approaches [19–27,28••,29••,30–36,37•] (Table 1).

The VP2 capsid protein is the most variable AHSV antigen and determines virus serotype [38]. As VP2 is

Table 1

Main approaches to develop vaccine candidates against AHSV

Vaccine candidate	Article	Level of protection or immune responses in animal models or host
Subunit VP2, alone or in combination with VP5 and VP7 (AHSV-4 or 5)	Roy *et al.*; Martinez-Torrecuadrada *et al.*; Scanlen *et al.*; Aksular *et al.* [23,26,31,69]	Protection in mice and horses against homologous challenge
Multiserotype cocktail of VP2 (serotypes 2, 4, 5, 6, 9)	Kanai *et al.* [32]	Low cross-neutralizing antibody response for genetically related AHSV-8
Plant-produced single or quimeric VLPs	Dennis *et al.*; Kutkowska *et al.* [34,36]	NAbs levels induced in horses similar to those obtained with AHSV LAVs
ALVAC canarypox-VP2/VP5 (AHSV-4)	Guthrie *et al.* [30]	Horses were protected against homologous challenge upon immunization with adjuvant
MVA-VP2 (AHSV-4)	Castillo-Olivares *et al.*; Calvo-Pinilla [20,41]	No viremia or clinical signs after challenge with homologous serotype in mice
MVA-VP2 (AHSV-9)	Alberca *et al.* [22]	Full protection against lethal challenge with homologous AHSV serotype
Cocktail of MVA-VP2	Manning *et al.* [27]	Simultaneous vaccination with MVA-VP2 of two serotypes (4 and 9) triggered NAbs against a third serotype (AHSV-6)
DNA/MVA or MVA/MVA-VP2/NS1 (AHSV-4)	De la Poza [24]	Reduced viremia upon infection with heterologous serotype (AHSV-9) in mice
muNS/MVA-NS1 (AHSV-4)	Marín-López *et al.* [37•]	No viremia or clinical signs after challenge with heterologous AHSV-9 in mice
RG ECRA-AHSV-1 with Seg 2 of AHSV-4	Lulla *et al.* [58••]	Survival in absence of body weight loss after AHSV-4 challenge in mice
Multiserotype cocktail ECRA-AHSV-1/4/6/8	Lulla *et al.* [29••]	Partial protection of ponies against AHSV-4 challenge
RG DISA AHSV-5	Van Rijn [28••]	DISA AHSV-5 partially protected ponies after homologous challenge

the main target for virus neutralizing antibodies (NAbs) [38,39] that are related with protection [40–42], several potential vaccines under investigation rely in the induction of VP2 NAbs; however these do not offer full cross-protection among serotypes. Subunit vaccines based on VP2 produced by baculovirus expression system have been analyzed either singly or in combination with VP5 and VP7 inducing protective immunity against homologous AHSV-4 [23,26,31]. A multiserotype cocktail of subunit VP2 vaccine (serotypes 2, 4, 5, 6, 9) was tested in guinea pigs eliciting a low cross-neutralizing antibody response for genetically related AHSV-8 [32]. In addition, recombinant baculovirus expression systems that allowed the assembly of VLPs have been reported [33–36]. Currently, transient expression in plants is being used for a relatively easy production of VLPs. A plant-produced AHSV-5 VLP vaccine was shown to induce comparable NAbs levels to those obtained with AHSV LAV against serotype 5 [33,36]. Sera from horses immunized with AHSV-5 VLPs also elicited similar antibody titres towards AHSV-8. In further studies, plant-produced triple chimeric AHSV-1/AHSV-3/AHSV-6 VLPs, composed of a combination of capsid proteins, induced moderate NAbs titres against AHSV-6 in horses [34].

Otherwise, promising poxvirus vaccines have targeted protective humoral and cellular immune responses against AHSV. ALVAC canarypox expressing AHSV-4 VP2 and VP5, formulated with adjuvant protected horses against the homologous AHSV serotype [30]. Another poxvirus, modified Vaccinia virus Ankara (MVA) expressing AHSV-4 VP2 [21] elicited protective immunity against homologous AHSV in mice upon heterologous regimen (DNA prime/MVA boost) [24] or alone (one or two doses of MVA) [20,23]. In horses, prime/boost with MVA expressing VP2 from serotype 9 provided sterilizing protection against a lethal dose of AHSV-9 without any adjuvant in the vaccine composition [22]. Interestingly, simultaneous vaccination with MVA-VP2 of serotypes 4 and 9 triggered NAbs against serotype 6 [43•]. After four months, vaccination with MVA-VP2 (AHSV-5) of previously immunized horses induced an anamnestic response towards AHSV-5, 4, 6 and 9 as well as the cross-reactive AHSV-8.

As antigenic variability of AHSV is the main hurdle of cross-protective immunity, several studies have been focused on NS1 protein with a highly conserved amino acid sequence among all serotypes (97.26–99.82% sequence identity). Importantly, CD8 T-cell epitopes have been identified in NS1 in mice and they are conserved among AHSV serotypes [44]. As cross-reactive T-cell responses are critical for multiserotype protection, vaccines based on NS1 have been analyzed. Immunization with DNA/MVA expressing AHSV-4 NS1 or two doses of MVA-NS1 reduced viremia in mice after challenge with a heterologous serotype, AHSV-9 [24]. In a more recent work, NS1 from AHSV-4 was incorporated

into avian reovirus muNS protein microspheres (MS-NS1) and combined to MVA-NS1 to test protective immunity in mice. This combinatorial immunization afforded sterilizing protection after infection with heterologous serotype 9 and it would be a promising universal vaccine against AHSV [37•].

Reverse genetics AHSV systems

Since the first reverse genetics (RG) system was designed to generate synthetic poliovirus from cDNA [45], this experimental approach has gathered an increasingly interest over the years among the virology community. RG techniques have become one of the most powerful tools to decipher key viral aspects such as structure, pathogenicity and immunogenicity, working as an alternative for vaccine development platforms in parallel.

RG systems exist for all major groups of animal RNA viruses. For picornavirus, coronavirus, flavivirus or arterivirus, positive-strand RNA virus RG systems are mainly focused on delivery of either transcribed genomic RNA into the cell cytoplasm or cDNA under the control of a viral transcription promoter such as T7 or CMV [46]. Negative-stranded and double-stranded RNA viruses, like paramyxovirus, orthomyxovirus, rotavirus or reovirus, usually require additional helper constructs to introduce the RdRP and other proteins essential for genomic replication [46].

Regarding the family *Reoviridae*, several plasmid-based or RNA transcript-based RG systems have been depicted [47,48•,49,50•,51–55]. The most significant reoviruses causing diseases in ruminants and equids are BTV and AHSV, respectively. Their ten-segmented dsRNA genomes have turned out to be a challenging factor for RG systems development, although RG strategies for BTV have been successfully implemented [47,54]. In the case of AHSV, a few RG approaches have been developed so far.

The capability of isolating AHSV core proteins, which makes generation of core transcripts possible, was exploited to establish a double-transfection RG system [56•]. In consequence, reassortant AHSV viruses can be obtained in as much as the serotype specificity of the rescued viruses relies on the RNA transcripts used in the second transfection event. Moreover, not only core transcripts can be utilized but plasmids including T7-derived segments also enable recovery of AHSV virions [56•].

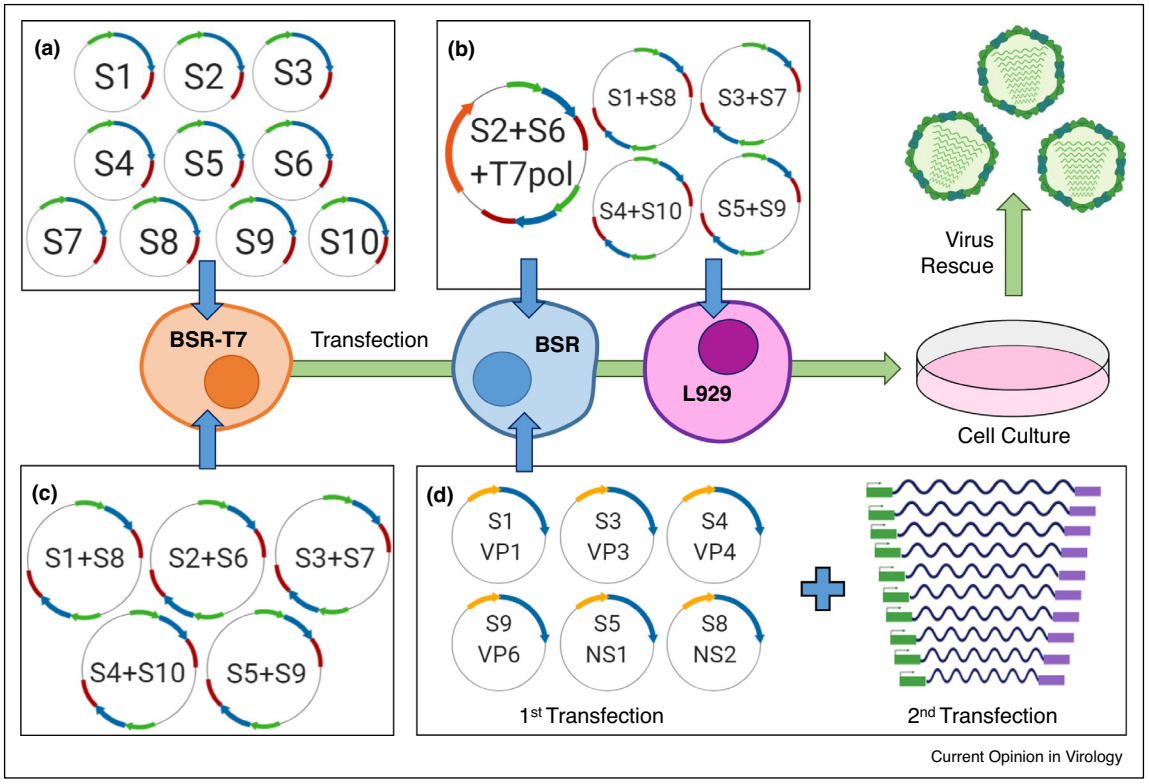
Four whole plasmid-based RG systems have been proposed and evaluated with considerable success. Transfection of BSR-T7 cell line with plasmids containing a full-length cDNA copy of single AHSV-4 genome segments under control of the T7 RNA polymerase promoter and enclosed at 3′ end with the hepatitis delta virus (HDV) ribozyme led to rescue of competent AHSV-4 (Figure 1a). Nonetheless, optimized virus rescue can be attained by transfection of double expression plasmids either in BSR-T7 cell line (Figure 1b) or in BSR (alternatively L929 cells) (Figure 1c) as long as plasmids expressing T7 polymerase are used [48•]. Finally, as previously described for BTV [57], a RG system strategy based on the combination of six expression plasmids encoding VP1, VP3, VP4, VP6, NS1 and NS2, along with transfection of a complete set of T7 transcripts (Figure 1d) allows to rescue reassortant or mutated viruses [50•]. However, viral titers ($<10^6$ PFU/mL) achieved by using this RG system are limited for molecular manipulations. An almost identical double transfection approach was applied to produce replicative-incompetent AHSV particles by means of transfection of a multistop segment 9 (encoding VP6 and NS4) and an additional expression plasmid encoding for VP7, combined with the utilization of a complementing BSR-VP6 cell line [58••]. Selection of AHSV-1 expression plasmids besides diverse combinations of capped T7 RNA transcripts yielded higher viral titers for AHSV-1 and reassortant-defective AHSV variants [58••]. Oftentimes, a similar RG strategy has been successfully applied for the study of the role and localization of different AHSV proteins, showing that NS3 protein is dispensable for attenuated virus recovery [28••,59,60] or evidencing the interaction of NS1, NS2 and NS4 with host cell nuclear components [61].

Reverse genetics systems for AHSV vaccine development

The use of RG technology has constituted the base for the development of new generation modified live attenuated vaccines (MLAVs), through targeted modifications and directed attenuation. Several MLAVs have been generated and tested as vaccine candidates [62–64]. Two different approaches have been addressed in order to develop vaccine candidates against AHSV, following similar strategies previously designed to generate BTV MLVAs [65,66]: Entry Competent Replication-Abortive (ECRA) viruses (formerly known as Disabled Infectious Single Cycle (DISC) vaccines) and Disabled Infectious Single Animal (DISA) vaccine strains (Figure 2).

ECRA viruses are deficient in VP6 and cannot complete the whole replication cycle due to the lack of function of VP6 as part of the transcriptase and packaging complex. However, they still initiate the replication cycle and synthesize a single round of viral mRNAs following entry and express viral proteins in normal cells. In contrast, for vaccine production, the *in trans* expression of VP6 in a helper cell line is required, to allow viral growth [66]. For AHSV, ECRA-AHSV viruses have been generated for all the nine serotypes by introducing multiple stop codons in the coding region of segment S9, then disrupting the expression of VP6 and also NS4 protein (encoded in the same segment) [58••]. Previous works reported that NS4 is not essential for BTV replication *in vitro* but antagonizes Interferon-I expression *in vivo* [67,68] so, likely, the absence of NS4 would positively affect the immune

Figure 1

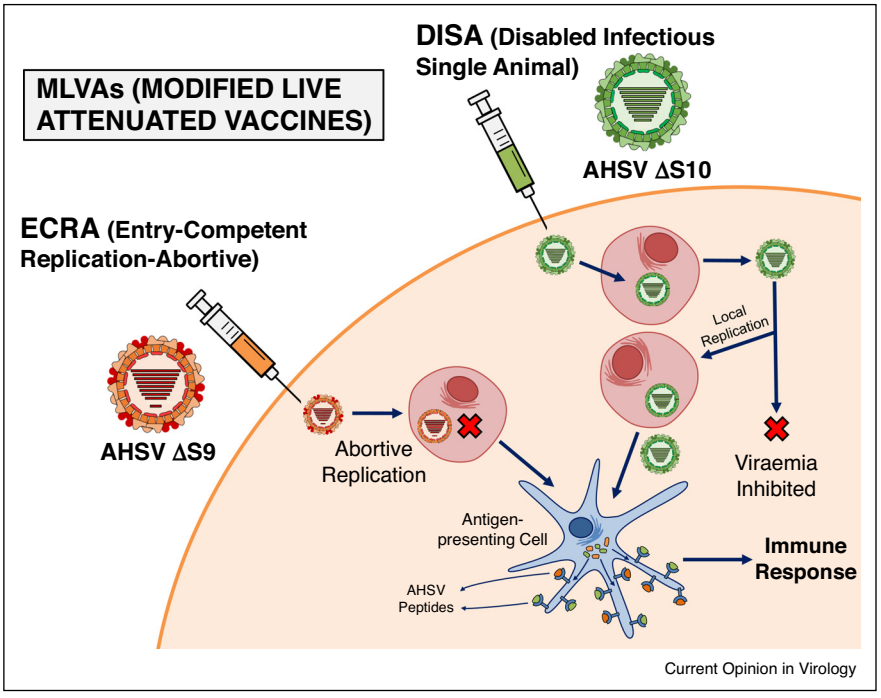


Representation of different developed plasmid-based RG strategies for AHSV.
The T7 RNA polymerase promoter is represented in green, the ORF of AHSV segments are colored blue and the hepatitis delta virus (HDV) ribozyme is emphasized in red. The CAG promoter is showed in yellow. **(a)** Ten plasmids containing the AHSV cDNA segments are co-transfected into BSR cells that express constitutively the T7 polymerase (BSR-T7). The viral positive-sense mRNAs with native 5′ and 3′ ends are produced due to cytoplasmic transcription of transfected cloned cDNAs. **(b)** Only five plasmids encoding the viral genome via transcription cassettes containing two AHSV cDNA segments are co-transfected into BSR-T7 cells in an identical procedure to that for the previous ten-plasmids RG system. **(c)** T7 polymerase is encoded in the plasmid that includes the AHSV segments 2 and 6 (S2-S6) under control of a CMV promoter. Co-transfection of this set of 5 plasmids is conducted in cells that do not express constitutively the T7 polymerase (BSR or L929). **(d)** A double transfection procedure is performed using BSR cells. First, transfection of six expression plasmids containing AHSV cDNA segments 1, 3, 4, 5, 8 and 9 is performed. A second transfection event of a whole set of ten T7 transcripts representing all AHSV dsRNA segments results in virus rescue. Expression plasmids enhance virus recovery events as it optimizes the formation of the primary replication complex. In all previous cases, once the virus is recovered, BSR cells are used for viral amplification and isolation is conducted by plaque assay.

response by DISC vaccination for AHSV (although it has to be determined). Based on stability and level of viral replication, AHSV-1 was selected as a backbone for use in an RG system. Protective efficacy studies using ECRA-AHSV-1 variants (AHSV-1 and 4, exchanging Seg 2 of AHSV-4 in AHSV-1 backbone) were performed in the IFNAR(−/−) mouse model [58••]. Safety of these candidates was tested after immunization (10^7PFU), showing no clinical signs and viremia. After a prime-boost immunization (10^7 PFU) and challenge with AHSV-1 or AHSV-4, ECRA-AHSV immunized animals led to a significant reduction of AHSV RNA levels in mouse organs and blood for both AHSV-1 or 4, and complete survival in absence of body weight loss was observed in immunized mice challenged with AHSV-4. In subsequent studies, two different vaccine regimes, a monovalent (ECRA-AHSV-4) vaccine and a multivalent cocktail vaccine of 4 different AHSV serotypes (ECRA-AHSV-1/4/6/8) (10^7 PFU each) were assessed in ponies followed by AHSV-4 challenge [29••]. Specific VP7 and neutralizing antibodies were detected in immunized animals before challenge. After infection, partial clinical protection based on survival, clinical signs and viremia levels was observed in immunized animals when compared with non-immunized ones, which presented higher levels of viremia and developed the typical clinical signs of AHS disease as hyperthermia, respiratory distress, edema of the eyelids or pulmonary edema among others.

Another strategy based on RG has been generated for vaccine design against AHSV, the Disabled Infectious Single Animal (DISA) vaccine strains. These viruses lack the functional gene of the non-structural protein NS3/NS3a, then interrupting viral egress, inhibiting the

Figure 2



Schematic representation of the modified live attenuated vaccines (MLVAs) based on RG systems against AHSV.
ECRA (Entry-competent Replication-Abortive) vaccines, formerly known as DISC (Disabled Infectious Single Cycle) vaccines, are deficient in VP6 and NS4, both encoded in segment 9. As a consequence, the replication cycle cannot be completed, although expression of viral proteins leads to an immune response. DISA (Disabled Infectious Single Animal) vaccines are based on attenuated viruses lacking the non-structural protein NS3/NS3a, encoded by segment 10. Therefore, viral egress is interrupted, inhibiting viraemia and allowing only local replication. The delayed egress of new viral particle results into a more prolonged antigen exposure and induces a potent immune response.

presence on virus in blood and allowing only local replication in infected cells; reducing the risk of propagation or transmission by midge vectors during feeding [59]. RG-generated DISA-AHSV-4, with a total deletion of NS3/3a, was used for horse immunization ($n = 2$) following a prime/boost regimen (4×10^4TCID50) [28••]. No adverse reactions were detected in vaccinated animals. Seroconversion was observed, showing the peak of VP7 antibodies after boost (35 dpv). After challenge with AHSV-4, a horse developed severe clinical signs and high fever and viremia, and finally was euthanized. The second horse developed mild edema of the supraorbital fossae, slightly elevated body temperature and viremia, becoming negative at 28 dpi and survived. In the same study, DISA-AHSV-5, with an in-frame deletion of amino acid codon 25–101 in the S10 (77aa deletion in NS3/3a), was used to test safety ($2 \times 10^{7.7}$TCID50) and efficacy (2×10^5 TCID50) in ponies. After confirming the absence of side effects, clinical signs and viremia, and the presence of AHSV VP7 specific antibodies, immunized ponies were challenged with AHSV-5. Three out of four immunized animals survived to the infection and showed a delay in viremia, with lower titers compared to control ponies. Thus, DISA vaccine partially protected against AHS although did not induce measurable NAbs titers. The better results obtained in the latter experiment compared to that performed in horses might be due to the differences in vaccine's doses and strains, virulence between strains used for the challenge or susceptibility to AHSV between horses and ponies.

Conclusion

Several research groups have developed promising vaccine candidates against AHSV. These approaches show improvements compared to marketed vaccines such as safety and allow a DIVA strategy. AHSV vaccines based on poxvirus recombinant vectors, such as MVA and canarypox [22,30], have displayed high levels of protection with absent of clinical signs and viremia in immunized horses. Although further optimization of reverse genetics vaccines is needed to abolish viremia completely in vaccinated animals, reverse genetics technology to create ECRA and DISA AHSV vaccines looks promising. Further research will be necessary to determine the optimal dose requirement and to perform a deep characterization of immune responses elicited for these vaccines. As the activation of cytotoxic CD8 T cells and other subsets of immune cells have been shown to have a key role in the virus clearance, cell-mediated immunity by AHSV RG vaccines need to be elucidated in the future. In

any case, having reverse genetics systems that allow the rapid development of safe and effective vaccines against the different serotypes of the virus by single S2[VP2] exchange, makes these vaccine platforms promising AHSV vaccine candidates for all current AHSV serotypes.

Conflict of interest statement

Nothing declared.

Acknowledgements

This work was supported by the Spanish Ministry of Science (AGL2017-82570-R) and EU Horizon 2020 Program (NO.727393-PALE-Blu). Sergio Utrilla-Trigo was a recipient of a predoctoral fellowship from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Centro de Investigación en Sanidad Animal (program FPI-SGIT-2018).

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