Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Reverse genetics approaches: a novel strategy for African horse sickness virus vaccine design

Eva Calvo-Pinilla¹, Alejandro Marín-López², Sergio Utrilla-Trigo¹, Luis Jiménez-Cabello¹ and Javier Ortego¹

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.

Addresses
¹ Centro de Investigación en Sanidad Animal (CISA), Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Valdeolmos, Madrid, Spain
² Section of Infectious Diseases, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT, USA

Corresponding author: Ortego, Javier (ortejo@inia.es)

Current Opinion in Virology 2020, 44:49–56
This review comes from a themed issue on Preventive and therapeutic vaccines
Edited by Luis Martínez-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
1879-6257/© 2020 Elsevier B.V. All rights reserved.

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 linear 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 (Differentially 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
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 NAb; 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 NAb 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 NAb against a third serotype (AHSV-6) [22]. Full protection against lethal challenge in horses against homologous AHSV serotype.

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

### Table 1

| 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.; Martínez-Torrecuadrada et al.; Scanlen et al.; Aksular et al. [23,26,31,69] | Protection in mice and horses against homologous challenge                                           |
| Multiseroype cocktail of VP2 (serotypes 2, 4, 5, 6, 9) | Kanai et al. [32]                          | Low cross-neutralizing antibody response for genetically related AHSV-8 NAb levels induced in horses similar to those obtained with AHSV LAVs |
| Plant-produced single or quimeric VLPs                | Dennis et al.; Kutkowska et al. [34,36]     | Horses were protected against homologous challenge upon immunization with adjuvant                  |
| ALVAC canarypox-VP2/VP5 (AHSV-4)                      | Guthrie et al. [30]                        | No viremia or clinical signs after challenge with homologous serotype in mice                      |
| MVA-VP2 (AHSV-4)                                      | Castillo-Olivares et al.; Calvo-Pinilla [20,41] | Full protection against lethal challenge with homologous AHSV serotype                              |
| MVA-VP2 (AHSV-9)                                      | Alberca et al. [22]                        | Simultaneous vaccination with MVA-VP2 of two serotypes (4 and 9) triggered NAb against a third serotype (AHSV-6) |
| Cocktail of MVA-VP2                                   | Manning et al. [27]                        | Reduced viremia upon infection with heterologous serotype (AHSV-9) in mice                         |
| DNA/MVA or MVA/MVA-VP2/NS1 (AHSV-4)                   | De la Pozo [24]                           | No viremia or clinical signs after challenge with heterologous AHSV-9 in mice                       |
| mNS/MVA-NS1 (AHSV-4)                                  | Marín-López et al. [37]                    | Survival in absence of body weight loss after AHSV-4 challenge in mice                             |
| RG ECRA-AHSV-1 with Seg 2 of AHSV-4                   | Lulla et al. [58**]                        | Partial protection of ponies against AHSV-4 challenge in mice                                       |
| Multiseroype cocktail ECRA-AHSV-1/4/6/8               | Lulla et al. [29**]                        | DISA AHS-5 partially protected ponies after homologous challenge                                  |
| RG DISA AHSV-5                                         | Van Rijn [28**]                           | DISA AHS-5 partially protected ponies after homologous challenge                                  |
into avian reovirus muNS protein microspheres (MSNS1) 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 arbovirus, 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 (<10⁶ 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 MLAVs [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 response.
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. Safety of these candidates was tested after immunization (10^7 PFU), 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. 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
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 × 10^4 TCID50) [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 × 10^7.5 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 Umilla-Trigo was a recipient of a predoctoral fellowship from the Instituto Nacional de Investigacion y Tecnologia Agraria y Alimentaria, Centro de Investigacion en Sanidad Animal (program FPI-SGIT-2018).

**References and recommended reading**
Papers of particular interest, published within the period of review, have been highlighted as

- of special interest
- of outstanding interest

1. Du Toit R: The transmission of bluetongue and horse sickness by Culicoides. *Onderstepoort J Vet Sci Anim Ind* 1944, 19:7-16.
2. Carpenter S, Mellor PS, Fall AG, Garros C, Venter GJ: African horse sickness virus: history, transmission, and current status. *Annu Rev Entomol* 2017, 62:343-358.
3. Mellor PS, Hamblin C: African horse sickness. *Vet Res* 2004, 35:445-466.
4. Howell PG: The isolation and identification of further antigenic types of African horsesickness virus. *Onderstepoort J Vet Res* 1962, 29:139-149.
5. McIntosh BM: Immunological types of horse sickness and their significance in immunization. *Onderstepoort J Vet Res* 1958, 27:465-538.
6. Roy P, Mertens PP, Casal I: African horse sickness virus structure. *Comp Immunol Microbiol Infect Dis* 1994, 17:243-273.
7. Zwart L, Potgieter CA, Clift SJ, van Staden V: Characterising non-structural protein NS4 of African horse sickness virus. PLoS One 2015, 10:e0124281.
8. Quan M, van Vuuren M, Howell PG, Groenewald D, Guthrie AJ: Molecular epidemiology of the African horse sickness virus S10 gene. *J Gen Virol* 2008, 89:1159-1168.
9. Aklilu N, Batten C, Gelaye E, Jenberie S, Ayelet G, Wilson A, Belay A, Asea Y, Oora C, Maan S et al.: African horse sickness outbreaks caused by multiple virus types in Ethiopia. *Transbound Emerg Dis* 2014, 61:185-193.
10. Gibbens N: Schmallenberg virus: a novel viral disease in northern Europe. *Vet Rec* 2012, 170:58.
11. Purse BV, Brown HE, Harrop L, Mertens PP, Rogers DJ: Invasion of bluetongue and other orbiviruses infections into Europe: the role of biological and climatic processes. *Rev Sci Tech* 2008, 27:427-442.
12. Portas M, Boinas FS, Oliveira ESJ, Rawlings P: African horse sickness in Portugal: a successful eradication programme. *Epidemiol Infect* 1999, 123:337-346.
13. Coetzter JAW, Guthrie AJ: African horse sickness. In *Infectious Diseases of Livestock*, edn 2. Edited by Coetzter JAW, Tustin RC. Oxford University Press; 2004:1231-1246.
14. Weyer CT, Quan M, Joone C, Lourens CW, MacLachlan NJ, Guthrie AJ: African horse sickness in naturally infected, immunised horses. *Equine Vet J* 2012, 45:117-119.
15. Weyer CT, Grewar JD, Burger P, Rossouw E, Lourens C, Joone C, le Grange M, Coetzee P, Venter E, Martin DP et al.: African horse sickness caused by genome reassortment and reversion to virulence of live, attenuated vaccine viruses. *South Africa, 2004-2014. Emerg Infect Dis* 2016, 22:2087-2096.
16. Dungu B, Potgieter C, Von Teichman B, Smit T: Vaccination in the control of bluetongue in endemic regions: the South African experience. *Dev Biol (Basel)* 2004, 119:463-472.
17. Von Teichman BF, Dungu B, Smit TK: In vivo cross-protection to African horse sickness serotypes 5 and 9 after vaccination with serotypes 8 and 6. *Vaccine* 2010, 28:6505-6517.
18. Von Teichman BF, Smit TK: Evaluation of the pathogenicity of African horsesickness (AHS) isolates in vaccinated animals. *Vaccine* 2008, 26:5014-5021.
19. El Garch H, Crafford JE, Amouyal P, Durand PY, Edlund Toulemonde C, Lematre L, Cozette V, Guthrie A, Minke JM: An African horse sickness virus serotype 4 recombinant canarypox virus vaccine elicits specific cell-mediated immune responses in horses. *Vet Immunol Immunopathol* 2012, 149:76-85.
20. Castillo-Oliveres J, Calvo-Pinilla E, Casanova I, Bachanek-Bankowska K, Chiaram R, Maan S, Nieto JM, Ortego J, Mertens PP: A modified vaccinia Ankara virus (MVA) vaccine expressing African horse sickness virus (AHSV) VP2 protects against AHSV challenge in an IFNAR-/- mouse model. *PLoS One* 2011, 6:e16503.
21. Chiaram R, Sharp E, Maan S, Raso S, Mertens P, Blacklaws B, Davis-Poynter N, Wood J, Castillo-Oliveres J: Induction of antibody responses to African horse sickness virus (AHSV) in ponies after vaccination with recombinant modified vaccinia Ankara (MVA). *PLoS One* 2009, 4:e9907.
22. Alberca B, Bachanek-Bankowska K, Cabana M, Calvo-Pinilla E, Viaplana E, Frost L, Gubbins S, Urniza A, Mertens P, Castillo-Oliveres J: Vaccination of horses with a recombinant modified vaccinia Ankara virus (MVA) expressing African horse sickness (AHS) virus major capsid protein VP2 provides complete clinical protection against challenge. *Vaccine* 2014, 32:3670-3674.
23. Akcular M, Calvo-Pinilla E, Marin-Lopez A, Ortego J, Chambers AC, King LA, Castillo-Oliveres J: A single dose of African horse sickness virus (AHSV) VP2 based vaccines provides complete clinical protection in a mouse model. *Vaccine* 2018, 36:7000-7010.
24. de la Pozza F, Calvo-Pinilla E, Lopez-Gil E, Marin-Lopez A, Mateos F, Castillo-Oliveres J, Lorenzo G, Ortego J: NS1 is a key protein in the vaccine composition to protect IFNAR(-/-) mice against infection with multiple serotypes of African horse sickness virus. *PLoS One* 2013, 8:e70197.
25. Roy P: Multiple gene expression in baculovirus system. Third generation vaccines for bluetongue disease and African horsesickness disease. *Ann N Y Acad Sci* 1996, 791:318-332.
26. Roy P, Bishop DH, Howard S, Atchison H, Erasmus B: Recombinant baculovirus-synthesized African horsesickness virus (AHSV) outer-capsid protein VP2 provides protection against virulent AHSV challenge. *J Gen Virol* 1996, 77:2053-2057.
27. Manning NM, Bachanek-Bankowska K, Mertens PPC, Castillo-Oliveres J: Vaccination with recombinant Modified Vaccinia Ankara (MVA) viruses expressing single African horse sickness virus VP2 antigens induced cross-reactive virus neutralising antibodies (VNAbs) in horses when administered in combination. *Vaccine* 2017, 35:6024-6029.
28. van Rijn PA, Maris-Veldhuis MA, Potgieter CA, van Gennip RGP: African horse sickness virus (AHSV) with a deletion of 77 amino acids in NS3/NS3a protein is not virulent and a safe promising AHS Disabled Infectious Single Animal (DISA) vaccine platform. *Vaccine* 2018, 36:1925-1933.

In this study, Disabled Infectious Single Animal (DISA) vaccine strains lacking the functional gene of the non-structural protein NS3/NS3a, were used for horse and pony immunizations. These strains were based on AHSV-4 or AHSV-S (DISA-AHSV-S/5) and conferred different levels of protection in the animals.

29. Lulla V, Losada A, Lecollinet S, Kerviel A, Lilin T, Saillau C, Beck C, Zientara S, Roy P: Protective efficacy of multivalent replication-
abrotive vaccine strains in horses against African horse sickness virus challenge. Vaccine 2017, 35:4262-4269.

Following the previous study, two vaccine regimes based on a mono-valent (ECRA-AHSV-4) vaccine and a multivalent cocktail vaccine of 4 different AHSV serotypes (ECRA-AHSV-1/4/6/8) were assessed in ponies followed by AHSV-4 challenge. Both regimes conferred total protection against the challenge.

30. Guthrie AJ, Quan M, Lourens CW, Audonnet JC, Minke JM, Yao J, He L, Nordgren R, Gardner IA, Maclachlan NJ: Protective immunization of horses with a recombinant canarypox virus vectored vaccine co-expressing genes encoding the outer capsid proteins of African horse sickness virus. Vaccine 2009, 27:4434-4438.

31. Martinez-Torrecaudrada JL, Diaz-Laviada M, Roy P, Sanchez C, Vela G, Sanchez-Vizcaino JM, Casal JF: Full protection against African horse sickness (AHS) in horses induced by baculovirus-derived AHS virus serotype 4 VP2, VP5 and VP7. J Gen Virol 1996, 77:1211-1221.

32. Kanai Y, van Rijin PA, Maris-Veldhuis M, Kaname Y, Athmaram TN, Roy P: Immunogenicity of recombinant VP2 proteins of all nine serotypes of African horse sickness virus. Vaccine 2014, 32:4932-4937.

33. Dennis SJ, O’Kennedy MM, Rutkowski D, Tseko T, Lourens CW, Hitteroth II, Meyers AE, Rybicki EP: Safety and immunogenicity of plant-produced African horse sickness virus-like particles in horses. Vet Res 2018, 49:105.

34. Rutkowski DA, Mokoena NB, Tseko TL, Dibakwane VS, O’Kennedy MM: Plant-produced chimeric virus-like particles - a new generation vaccine against African horse sickness. BMC Vet Res 2018, 15:432.

35. Maree S, Maree FF, Putterill JF, de Beer TAP, Huismans H, Theron J: Synthesis of empty African horse sickness virus particles. Virus Res 2016, 213:184-194.

36. Dennis SJ, Meyers AE, Guthrie AJ, Hitteroth II, Rybicki EP: Immunogenicity of plant-produced African horse sickness virus-like particles: implications for a novel vaccine. Plant Biotechnol J 2016, 18:442-450.

37. Marin-Lopez A, Barreiro-Pineiro N, Utrilla-Trigo S, Barrales D, Benavente J, Nogales A, Martinez-Costas J, Ortego J, Calvo-Pinilla E: Cross-protective immune responses against African horse sickness virus after vaccination with protein NS1 delivered by avian reovirus mNS1 microspheres and modified vaccinia virus Ankara. Vaccine 2020, 38:882-889.

The authors tested an immunization approach comprises NS1 of AHSV-4 incorporated into avian reovirus mNS1 microsphere (MS-NS1) and/or expressed using recombinant modified vaccinia virus Ankara vector (MVA-NS1). The results indicated that immunization based on MS-NS1 and MVA-NS1 afforded complete protection against the infection with homologous AHSV-4. Moreover, priming with MS-NS1 and boost vaccination with MVA-NS1 triggered NS1 specific cytotoxic CD8+ T cells and prevent AHSV disease in IFNAR (−/−) mice after challenge with heterologous serotype AHSV-9. The induction of cross-protective immune responses is highly important since AHS can be caused by nine different serotypes.

38. Burrage TG, Trevejo R, Stone-Marshat M, Laegreid WW: Neutralizing epitopes of African horse sickness virus serotype 4 are located on VP2. Virology 1993, 190:799-803.

39. Bentley L, Fehrsen J, Jordan F, Huismans H, du Plessis DH: Identification of antigenic regions on VP2 of African horse sickness virus serotype 3 by using phage-displayed epitope libraries. J Gen Virol 2000, 81:993-1000.

40. Calvo-Pinilla E, de la Poza F, Gubbins S, Mertens PP, Ortego J, Castillo-Olivares J: Antiserum from mice vaccinated with modified vaccinia Ankara virus expressing African horse sickness virus (AHSV) VP2 protects when it is administered 46h before, or 48h after challenge. Antiviral Res 2015, 116:27-33.

41. Calvo-Pinilla E, de la Poza F, Gubbins S, Mertens PP, Ortego J, Castillo-Olivares J: Vaccination of mice with a modified Vaccinia Ankara (MVA) virus expressing the African horse sickness virus (AHSV) capsid protein VP2 induces virus neutralising antibodies that confer protection against AHSV upon passive immunisation. Virus Res 2014, 180:23-30.

42. Calvo-Pinilla E, Gubbins S, Mertens P, Ortego J, Castillo-Olivares J: The immunogenicity of recombinant vaccines based on modified Vaccinia Ankara (MVA) viruses expressing African horse sickness virus VP2 antigens depends on the levels of expressed VP2 protein delivered to the host. Antiviral Res 2018, 154:132-139.

43. Manning NM, Bachanek-Bankowska K, Mertens PPC, Castillo-Olivares J: Vaccination with recombinant Modified Vaccinia Ankara (MVA) viruses expressing single African horse sickness virus VP2 antigens induced cross-reactive virus neutralising antibodies (VNAbs) in horses when administered in a single combination. Vaccine 2017, 35:6024-6029.

In this study, the administration of two different MVA expressing VP2 from serotypes 4 and 9 induced neutralising antibodies against the homologous AHSV serotypes. A booster with MVA-VP2 of AHSV-5, given four months later to ponies resulted in the induction of NAbs against serotypes 4, 5, 6, 8 and 9. The anamnestic antibody response following the MVA-VP2-AHSV-5 boost suggests that it is possible some shared epitopes exist between different serotypes.

44. de la Poza F, Marin-Lopez A, Castillo-Olivares J, Calvo-Pinilla E, Ortego J: Identification of CDB T cell epitopes in VP2 and NS1 proteins of African horse sickness virus in IFNAR(−/−) mice. Virus Res 2015, 210:149-153.

45. Racanelli VR, Baltimore D: Cloned poliovirus complementary DNA is infectious in mammalian cells. Science 1981, 214:916-919.

46. Stobart CC, Moore ML: RNA virus reverse genetics and vaccine design. Viruses 2014, 6:2531-2550.

47. Boyce M, Selma GC, Roy P: Development of reverse genetics systems for bluetongue virus: recovery of infectious virus from synthetic RNA transcripts. J Virol 2008, 82:8339-8348.

48. Conradie AM, Stassen L, Huismans H, Potgieter CA, Theron J: Establishment of different plasmid only-based reverse genetics systems for the recovery of African horse sickness virus. Virology 2016, 499:144-155.

In this work, three plasmid only based reverse genetics systems for AHSV recovery were developed. Initially, ten plasmids containing AHSV-4 segments 1-10 were transfected in cells expressing the T7 polymerase, allowing for recovery of AHSV-4 from cDNA plasmids only. A more efficient viral recovery was achieved by reducing the number of plasmids used from 10 to 5. In addition, cloning of the T7 polymerase into one of these five plasmids enabled AHSV rescue in cell lines that have not been engineered to express T7 RNA polymerase. The authors showed that recovery of specific reassortant viruses can be attained by this method.

49. Kanai Y, Komoto S, Kagawagi T, Nouda R, Nagasawa N, Onishi M, Matsuura Y, Taniguchi K, Kobayashi T: Entirely plasmid-based reverse genetics system for rotaviruses. Proc Natl Acad Sci USA 2017, 114:2349-2354.

50. Kaname Y, Celma CCP, Kanai Y, Roy P: Recovery of African horse sickness virus from synthetic RNA. J Gen Virol 2013, 94:2259-2265.

The authors report the recovery of AHSV from a complete set of RNA transcripts synthesized in vitro from cDNA clones. In addition, they demonstrated the generation of mutant and reassortant AHSV genomes, their recovery, stable passage, and characterization.

51. Kagawagi T, Kanai Y, Tani H, Shimojima M, Sajio M, Matsuura Y, Kobayashi T: Reverse genetics for fusogenic bat-borne orthoreovirus associated with acute respiratory tract infections in humans: role of outer capsid protein sigmaC in viral replication and pathogenesis. PLoS Pathog 2016, 12: e1005455.

52. Kobayashi T, Oorns LS, Ikizler M, Chappell JD, Dermody TS: An improved reverse genetics system for mammalian orthoreoviruses. Virology 2010, 398:194-200.

53. Matsuo E, Saeki K, Roy P, Kawano J: Development of reverse genetics for Ibaraki virus to produce viable VP6-tagged IBV. FEBS Open Bio 2015, 5:442-453.

54. Pretorius JM, Huismans H, Theron J: Establishment of an entirely plasmid-based reverse genetics system for bluetongue virus. Virology 2015, 486:71-77.

55. Yang T, Zhang J, Xu Q, Sun E, Li J, Lv S, Feng Y, Zhang Q, Wang H et al.: Development of a reverse genetics system for...
epizootic hemorrhagic disease virus and evaluation of novel strains containing duplicative gene rearrangements. J Gen Virol 2015, 96:2714-2720.

56. Matsuo E, Celma CC, Roy P: A reverse genetics system of African horse sickness virus reveals existence of primary replication. FEBS Lett 2010, 584:3386-3391.

57. Matsuo E, Roy P: Bluetongue virus VP6 acts early in the replication cycle and can form the basis of chimeric virus formation. J Virol 2009, 83:8842-8848.

58. Lulla V, Lulla A, Wernike K, Aebischer A, Beer M, Roy P: Assembly of replication-incompetent African horse sickness virus particles: rational design of vaccines for all serotypes. J Virol 2016, 90:7405-7414.

59. van de Water SG, van Gennip RG, Potgieter CA, Wright IM, van Rijn PA: VP2 exchange and NS3/NS3a deletion in African Horse Sickness Virus (AHSV) in development of disabled infectious single animal vaccine candidates for AHSV. J Virol 2015, 89:3764-3772.

60. van Gennip RG, van de Water SG, Potgieter CA, van Rijn PA: Structural protein VP2 of African horse sickness virus is not essential for virus replication in vitro. J Virol 2017, 91.

61. Boughan S, Potgieter AC, van Staden V: African horse sickness virus NS4 is a nucleocytoplasmic protein that localizes to PML nuclear bodies. J Gen Virol 2020, 101:366-384.

62. Cai Y, Iwasaki M, Motooka D, Liu DX, Yu S, Cooper K, Hart R, Adams R, Burdette T, Postnikova EN et al.: A lassa virus live-attenuated vaccine candidate based on rearrangement of the intergenic region. mBio 2020, 11.

63. Nogales A, Baker SF, Ortiz-Riano E, Dewhurst S, Topham DJ, Martinez-Sobrido L: Influenza A virus attenuation by codon deoptimization of the NS gene for vaccine development. J Virol 2014, 88:10525-10540.

64. Jimenez-Guardano JM, Regla-Nava JA, Nieto-Torres JL, DeDiego ML, Castano-Rodriguez C, Fernandez-Delgado R, Perlman S, Enjuanes L: Identification of the mechanisms causing reversion to virulence in an attenuated SARS-CoV for the design of a genetically stable vaccine. PLoS Pathog 2015, 11:e1005215.

65. Feenstra F, van Gennip RGP, Maris-Veldhuis M, Verheij E, van Rijn PA: Bluetongue virus without NS3/NS3a expression is not virulent and protects against virulent bluetongue virus challenge. J Gen Virol 2014, 95:2019-2029.

66. Matsuo E, Celma CC, Boyce M, Vianrouge C, Sailleau C, Dubois E, Breden E, Thiery R, Zientara S, Roy P: Generation of replication-defective virus-based vaccines that confer full protection in sheep against virulent bluetongue virus challenge. J Virol 2011, 85:10213-10221.

67. Ratini M, Caporale M, Golder M, Franzoni G, Allan K, Nunes SF, Armezzani A, Bayoumy A, Rixon F, Shaw A et al.: Identification and characterization of a novel non-structural protein of bluetongue virus. PLoS Pathog 2011, 7:e1002477.

68. Ratini M, Shaw AE, Barry G, Gu Q, Di Gialleandro L, Janowicz A, Varela M, Randall RE, Caporale M, Palmarini M: Bluetongue virus NS4 protein is an interferon antagonist and a determinant of virus virulence. J Virol 2016, 90:5427-5439.

69. Scanlen M, Paweska JT, Verschoor JA, van Dijk AA: The protective efficacy of a recombinant VP2-based African horse sickness subunit vaccine candidate is determined by adjuvant. Vaccine 2002, 20:1079-1088.