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Entry-competent-replication-abortive African horse sickness virus strains elicit robust immunity in ponies against all serotypes

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ABSTRACT

African horse sickness virus (AHSV) is an *Orbivirus* within the *Reoviridae* family, spread by *Culicoides* species of midges, which infects equids with high mortality, particularly in horses and has a considerable impact on the equine industry. In order to control the disease, we previously described Entry Competent Replication Abortive (ECRA) virus strains for each of the nine distinct AHSV serotypes and demonstrated their potential as vaccines, first in type I interferon receptor (IFNAR-/-) knockout mice, and then in ponies. In this report we have investigated whether or not a combination ECRA vaccine comprising nine vaccine strains as two different cocktails is as efficient in ponies and the duration of the immunity triggered by ECRA vaccines. In one study, a group of ponies were vaccinated with a cocktail of 4 vaccine strains, followed by a vaccination of the remaining 5 vaccine strains, mimicking the current live attenuated vaccine regimen. In the second study, ponies were vaccinated with a single ECRA-AHSV strain and monitored for 6 months. The first group of ponies developed neutralising antibody responses against all 9 serotypes, indicating that no cross-serotype interference occurred, while the second group developed robust neutralising antibody responses against the single serotype that were sustained at the same level throughout a 6-month study. The results support our previous data and further validate ECRA vaccines as a safe and efficacious replacement of current live vaccines.

1. Introduction

African horse sickness (AHS) is an arthropod transmitted viral disease, most commonly infecting horses, donkeys, and other equids. Infection can manifest in a number of distinct forms, the most lethal of which is the pulmonary form with a fatality rate of 95% in susceptible horses [1]. Early symptoms of this include mild depression, sweating, and coughing, and progress to pulmonary oedema, and respiratory distress in the terminal stages. A cardiac form is characterised by high fever, conjunctivitis, abdominal pain, shortness of breath, and subcutaneous oedema, and has a fatality rate of between 50 and 70%. The most common form in susceptible animals is a mixed form, which presents with signs of both the pulmonary and cardiac forms. In contrast to horses, donkeys generally experience a mild subclinical form, which is generally non-lethal. Zebras rarely experience symptoms, and are considered the natural reservoir of the virus, with other species believed to be incidental hosts [2].

Currently African horse sickness virus (AHSV) is endemic only in sub-Saharan Africa. However, sporadic outbreaks have been reported in North Africa, Pakistan, India, Spain, and Portugal, and more recently in Thailand [3] and Malaysia [4]. Such outbreaks have a severe economic impact on the local horse industry due to both loss of livestock, and introduction of controls on animal movement. More importantly, due to the changing distribution of *Culicoides* midges, the key vector of AHSV, there is a very real possibility that AHS will also spread to regions previously considered at low risk. This has already occurred for the related bluetongue virus (BTV), which has recently spread into Northern Europe [5,6]. Current vaccination strategies rely on the use of a multivalent live attenuated vaccine, however this is considered unsafe due to the potential for reversion and reassortment with wild-type strains, and has not been licenced in Europe [7,8]. It is therefore essential to develop safer, more effective vaccine strategies [9].

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AHSV is a non-enveloped, structurally complex capsid virus with a genome of 10 dsRNA segments. The virus particle is made up of 7 structural proteins organised in concentric layers forming a double capsid. The outer capsid consists of highly variable VP2 and a less variable VP5, whilst the inner capsid, or core, consists of two layers of protein, a surface layer of VP7 and an inner layer VP3. The inner core surrounds the segmented RNA genome, and the replication complex consisting of minor enzymatic proteins VP1, VP4, and VP6 [10].

Nine distinct AHSV serotypes (AHSV1 to AHSV9) have been described based on the outer capsid protein VP2, the determinant of serotype that mediates virus entry into the host cells. Consequently, several vaccine candidates have been generated in recent years targeting VP2; however, as yet none have been licenced [11–14].

Towards the aim of developing a safe and effective AHSV vaccine, we first generated replication abortive virus strains deficient in minor protein VP6, essential for virus replication [15]. These VP6 deficient virus strains remained able to enter AHSV-susceptible cells or inoculated animals, however are unable to replicate fully due to their inability to synthesise progeny double-stranded RNA. These Entry-Competent-Replication-Abortive (ECRA) strains undergo a single round of protein synthesis and then replication stops. Propagation of ECRA vaccine strains is achieved in a complementary cell line expressing a functional VP6 protein in *trans*. Limiting the vaccine strains to a single round of protein expression effectively induces innate immunity but eliminates the risk of viraemia and thus minimises the possibility of re-assortment with wild type strains [15–18]. In previous studies, ECRA strains of AHSV were capable of inducing a protective immune response when type I interferon receptor (IFNAR-/-) mice were immunized with either an ECRA-AHSV1 or ECRA-AHSV4 vaccine strain and then challenged with the homologous strain of virulent virus. No vaccinated mice developed clinical symptoms upon challenge and AHSV RNA replication was shown to be minimal. Further studies were undertaken in ponies, a natural host for the virus, which were vaccinated with either a monovalent AHSV4 vaccine or a cocktail containing ECRA-AHSV 1/4/6/8, in a prime and boost regimen. Serology data demonstrated a strong immune response in all vaccinated animals and the generation of high titre neutralising antibodies against all included serotypes. During subsequent challenge studies with the virulent AHSV4 strain, no significant clinical symptoms were observed in any of the vaccinated ponies, confirming the ECRA viruses as being capable of generating a protective immune response consistent with the presence of neutralising antibody [18]. Together these data demonstrate that ECRA-AHSV strains serve as safe and effective vaccine candidates. However, both studies were of relatively short duration despite the fact that equines are typically kept for extended periods. The longevity of the immune response elicited by such vaccines therefore is an important yet currently undefined parameter. Similarly, the extension of the proof-of-principle already reported to include a vaccine capable of protecting against all 9 serotypes without interference or strain dominance in the observed immune response to each different serotype is an important dimension if the ECRA vaccines are to be widely used. Here we report on both these aspects of AHSV ECRA vaccines.

In one study, ponies were immunised with a multivalent ECRA-AHSV vaccine against all 9 AHSV serotypes to determine the feasibility of a multivalent ECRA-AHSV approach. In a second study, ponies were inoculated with a monovalent ECRA-AHSV vaccine and the immune responses generated by vaccination were monitored for a period of six months. Both seroconversion and serotype specific neutralisation assays were performed to assess the presence of an antibody response over the period of the studies. Additionally, limited cytokine assays were performed post vaccination to detect common markers of T-helper type 1 (Th1), T-helper type 2 (Th2), and T-regulator (Treg) responses. Our data extend the previous studies and show that ECRA vaccines meet many of the criteria for use in the field to control AHS disease.

2. Materials and method

2.1. Cells and viruses

BSR Cells (a subclone of BHK-21 cells) were maintained in Dulbecco modified Eagle Medium (DMEM; Sigma) supplemented with 5% foetal bovine serum (FBS; Sigma). The stable cell line BSR-VP6 was grown in DMEM + 5% FBS supplemented with 7.5 μg/ml puromycin (Sigma). Mammalian cell lines were cultured at 37 °C in a 5% CO_2 humidified atmosphere.

Wild type AHSV serotypes 1 to 9 were kindly provided by S. Zientara (ANSES, France). All AHSV serotypes were passaged once in BSR cells, titrated, and used for subsequent experiments.

2.2. Plasmids

The plasmids for the AHSV reverse genetics system have been described previously. Protein expression plasmids were generated by inserting the coding regions of the desired viral proteins into the pCAG-PM vector. T7 plasmids carrying the full-length segments of AHSV were generated in pUC19.

The AHSV1 S9 mutants (S9 multistop) containing 11 stop codons through the s9 gene was created previously by gene assembly [18].

2.3. Recovery of entry competent replication abortive AHSV variants

VP6 deficient AHSV strains were generated as previously described [18,19]. Briefly, BSR cells stably expressing the AHSV1 VP6 protein were transfected with pCAG plasmids expressing 4 AHSV1 proteins, VP1, VP3, VP4, and NS2, and subsequently transfection with RNA transcripts of AHSV segments 1 to 8, 10, and the AHSV1 S9 multistop mutant. ECRA AHSV mutants of each serotype were produced by substitution of appropriate segments as described previously [19]. Recovered virus was plaque-purified and titred on BSR-VP6 cells.

2.4. Preparation of vaccine cocktails

Early passage ECRA AHSV stocks (1–2 passages in BSR-VP6 cells) were amplified in BSR-VP6 cells at a MOI of 0.1 in antibiotic free media, harvested when complete cytopathic effect was visible, and titrated on BSR-VP6 cells. Cocktails were produced by combining 10^5–10^6 pfu of each ECRA-AHSV serotype, and supplementing with 10% trehalose. Stocks were then frozen in liquid nitrogen and stored at −80 °C until vaccination. Mock vaccine doses were prepared from an equivalent number of BSR-VP6 cells lysed by sonication.

2.5. Multivalent ECRA-AHSV vaccination of ponies

To test the multivalent ECRA-AHSV vaccine protocol 6 ponies were purchased from a riding stable in Val-d'Oise, France. 5 of the 6 were injected subcutaneously all 9 serotypes of ECRA-AHSV in two sequential injections. Cocktail 1 (ECRA-AHSV 1/4/7/9) was injected on day 0, and Cocktail 2 (ECRA-AHSV 2/3/5/6/8) was injected on day 21, with each strain present at $5x10^6$ pfu except ECRA-AHSV2 which was slightly lower, $4x10^5$ due to poor growth of ECRA-AHSV2. The inoculation titre of each individual strain was

adjusted such that the total titre was equivalent to both our previous studies [19] and current LAV strategies.

Serum and whole blood samples were collected from each animal weekly between day 0 and day 83. In addition, a single pony was mock-vaccinated with lysate from uninfected cells. This study was performed in strict accordance with the French guidelines and recommendations on animal experimentation and welfare (ComEth Anses/ENVA/UPEC, Ethics APAFiS number: 2,019,032,008,313,852 (#19849)).

2.6. Long term ECRA-AHSV vaccination of ponies

To determine the duration of ECRA-AHSV immunity 7 ponies were purchased from a stud farm in Normandy, France. 6 of these were injected subcutaneously with 10^7 Plaque Forming Units of ECRA-AHSV 4 twice, once on day 0 and again on day 21, in line with our previous studies [19]. The remaining pony was mock vaccinated with lysate of an equivalent number of uninfected cells. Animals were inspected daily for the development of clinical signs, and serum and whole blood samples were collected weekly from each animal between days 0 and 173. This study was performed in strict accordance with the French guidelines and recommendations on animal experimentation and welfare (ComEth Anses/ENVA/UPEC, Ethics APAFiS number: 2017061311362547).

2.7. Clinical monitoring

Each group of ponies was monitored on a daily basis, up to 83 days after vaccination, for the development to AHSV clinical signs as described previously [19].

2.8. Viraemia

Blood samples were taken every 7–14 days for a minimum of 85 days post vaccination. These were processed and analysed by real-time AHSV RT-PCR as described PCR to detect viral replication [19].

2.9. Serology of ECRA AHSV vaccine trials

Serum samples were analysed using a commercially available competitive ELISA AHSV4 VP7 Antibody Test kit (ELISA Ingezim AHSV Compaq) according to manufacturer's instructions. To detect neutralising antibody responses, virus neutralisation assays were performed against virulent AHSV as described previously [19]. Long term immunity was determined at a 90% neutralisation threshold and multivalent immunity at 50%.

2.10. Cytokine analysis

Cytokine levels were measured in the blood of vaccinated ponies without prior antigenic stimulation at days 0 (prime), 10–14, 21 (boost) and 31 post-vaccination by Luminex assay. Briefly, plasma was prepared within 30 min of blood collection and stored at −20 °C until analysis. Cytokines were measured using the MILLIPLEX MAP Equine Cytokine/Chemokine Magnetic Bead Panel (Merck) used as recommended by the manufacturer for the detection of IL2, 4, 6, 10, 17, TNFα and IFNγ.

3. Results

3.1. Generation of ECRA AHSV cocktails

As in our previous work all of our VP6 deficient ECRA-AHSV vaccine strains were generated by reverse genetics in a AHSV1 VP6 complementary BSR cell line [18,19]. Each serotype was generated separately by sequential transfection of 5 viral protein expression vectors (VP1, VP3, VP4, NS1, NS2), followed by transfection of 10 capped T7 RNA transcripts of the AHSV genomic RNA segments, of which the S9 had been replaced with S9 multistop. AHSV1 was used as the basis for all strains of ECRA AHSV, with various serotype specific segments substituted, as described previously [18] and shown in Table 1.

Multivalent cocktail vaccines were then produced based upon phylogenetic analysis of VP2, with phylogenetically distant serotypes combined [20]. The vaccine cocktail 1 contained ECRA AHSV 1, 4, 7, and 9, as these four serotypes are most distant from each other whilst Cocktail 2 contained the remaining ECRA AHSV strains 2, 3, 5, 6, and 8. Each cocktail was formulated such that serotypes in each cocktail shared a maximum of 51% sequence homology within VP2, in an attempt to minimise intra-cocktail cross reactivity.

3.2. Assessment of the immune response to multivalent ECRA-AHSV innoculation

To assess the ability of our complete ECRA-AHSV vaccination to generate a protective antibody response, five ponies (Blue, Orange, Green, Purple, and Red) were injected sequentially with the two cocktail vaccines 21 days apart, as shown in Fig. 1a, alongside a Control animal (Black) vaccinated with lysate from uninfected cells. Vaccination with Cocktail 1 of four vaccine strains (ECRA-AHSV 1/4/7/9) was performed on day 1, whilst the second immunisation with Cocktail 2 of five vaccine strains (ECRA-AHSV 2/3/5/6/8) was performed on day 21, in line with our previous ECRA-AHSV protocols [19], and live attenuated vaccine strategies.

Following inoculation, animals showed a marginal (<1°C) increase in temperature which lasted no more than 1 day and a mild cutaneous reaction at the site of inoculation which lasted maximally for a few days. No other adverse clinical events were noted throughout the full monitoring period of 83 days. These reactions were consistent with previous observations of the ECRA vaccines [18,19] and are consistent with innate immune responses triggered by the initial inoculums. Importantly, no symptoms typical of AHSV were noted in any animal at any time following vaccination.

Sera were collected from all ponies approximately every 7 days (Fig. 1a) for the duration of the study to monitor antibody responses to ECRA-AHSV in each animal using a standard AHSV group-specific VP7 antigen ELISA Test (Fig. 1b). All animals showed a low antibody response after inoculation with cocktail 1, which increase upon inoculation with cocktail 2. Of the five ponies, four (Orange, Green, Purple, and Red) had high levels of antibody pro-

Table 1
RNA segment substitutions required for recovery of ECRA-AHSV strains for all 9 serotypes of AHSV. ECRA-AHSV1 serves as the parental strain for all ECRA-AHSV viruses, with serotype specific substitutions in the structural proteins VP2, VP3, VP5, and VP7, as required. Table modified from *Lulla et al (2016)* [18].

	AHSV1 segment exchanged for serotype specific segment		
	VP2 + VP5	**VP7**	**VP3**
ECRA-AHSV1*			
ECRA-AHSV2	+	+	+
ECRA-AHSV3	+	+	
ECRA-AHSV4	+	+	
ECRA-AHSV5	+	+	+
ECRA-AHSV6	+	+	+
ECRA-AHSV7	+	+	+
ECRA-AHSV8	+		
ECRA-AHSV9	+		

*ECRA-AHSV1 serves as the parental strain for all ECRA-AHSV strains.

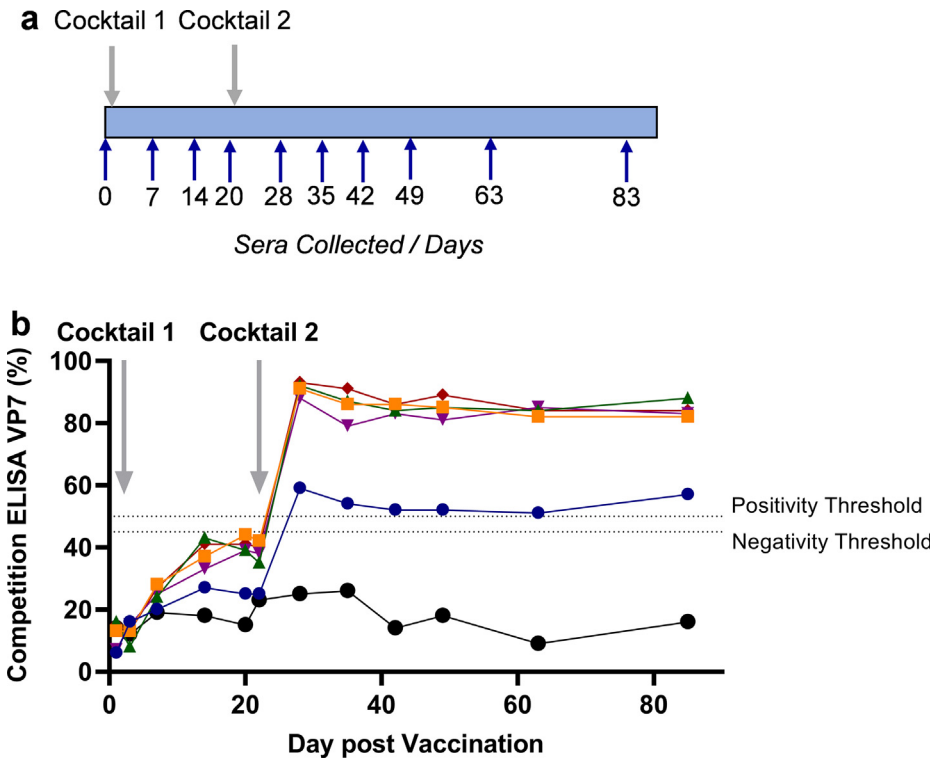

Fig. 1. a. Time course of the multivalent AHSV Trial. **b.** Seroconversion of animals after multivalent ECRA-AHSV vaccination. Animals were vaccinated with 2 different ECRA-AHSV cocktails 21 days apart, with ECRA-AHSV1/4/7/9 on day 0, and ECRA-AHSV2/3/5/6/8 on day 21. The induction of the immune response was monitored in vaccinated animals by VP7 group specific competitive ELISA.

duction (85–90%) and one (Blue) had slightly lower (~60%), with all maintaining these levels for the duration of experiment. The control animal showed no evidence of any VP7 antibody response.

To investigate whether the vaccinated ponies developed AHSV serotype specific neutralising antibody responses, we used neutralisation assays to determine the titre of neutralising antibody against virulent strains of all 9 serotypes (Fig. 2). Consistent with our previous data, sera collected prior to the second vaccination dose with the Cocktail 2, demonstrated minor evidence of serum neutralisation against the serotypes of the Cocktail 1, the first vaccination dose. Nevertheless, all animals showed some evidence of neutralising antibody compared to the control at day 20. Two ponies Red and Purple, however, showed evidence of a stronger response. Purple achieved an anti-AHSV4 titre of 16, whilst Red generated neutralising antibodies to AHSV4 and AHSV9 at titres 32 and 16 respectively. By day 42, 21 days after inoculation with Cocktail 2, all animals had developed detectable antibody responses to all 9 AHSV serotypes, although the levels of neutralising antibodies produced against each serotype did vary between animals. Overall ECRA-AHSV2 elicited the weakest neutralising antibody response, with Purple pony achieving only a titre above 8. This is consistent with the lower titre of virus included in the vaccine cocktail and was not, therefore, unexpected. Importantly, little difference was observed in the titres of antibodies against the other serotypes in Cocktail 2 compared to those in Cocktail 1. This is important given that in our previous study peak antibody production was achieved at 35 days, 14 days after a booster of the same serotypes was given [19]. Note that all vaccinated ponies demonstrated enhanced response to AHSV serotypes present in Cocktail 1, after immunisation with Cocktail 2, although the extent of boosting varies between ponies and serotypes. Each of the 5 vaccinated ponies showed enhanced neutralisation of AHSV1, with titres rising from < 8 at day 20 to 32 at day 42 for Blue, Green, Purple, and Red, and from 8 to 64 for Orange. Additionally, all vaccinated ponies apart from Red demonstrate enhancement in the neutralising antibody responses to certain serotypes present in Cocktail 1 (Blue to AHSV7 and AHSV9; Orange to AHSV7; Green to AHSV4 and Purple, AHSV4 and AHSV7). Interestingly, several ponies also achieved comparable enhanced titres against Cocktail 2 serotypes that were not included in the Cocktail 1 (Blue, to AHSV6; Orange, to AHSV3 and AHSV6; Green, to AHSV3 and AHSV8; Purple, to AHSV 8; and Red, to AHSV6 and AHSV8). Together these data suggest a cross serotype priming/boosting-like activity.

One important caveat to this study is that each cocktail contained different compositions of serotypes, as such, each animal was only vaccinated against each serotype once, however our data demonstrate that multivalent vaccination with our ECRA-AHSV strains remains a feasible possibility. The detection of neutralising antibodies against all 9 serotypes in all vaccinated animals, albeit to varying levels, suggests that there is minimal cross-serotype interference between ECRA-AHSV strains, and that a vaccination protocol containing all 9 ECRA vaccine strains should feasibly elicit protective immunity to all AHSV serotypes. Importantly all animals achieved specific antibody titres of at least 8, against all 9 serotypes, equivalent to the lowest titre shown to be protective against homologous serotype challenge in our previous study [19].

3.3. Long term assessment of the protective response to ECRA-AHSV vaccination

Previously we have shown that vaccination of ponies with a monovalent ECRA-AHSV, results in a protective immune response against virulent virus challenge. To determine the duration of this protection 6 ponies (Cyan, Pink, Lilac, Jade, Gold, and Brown) were vaccinated with ECRA-AHSV4, alongside a mock control (Grey), in a

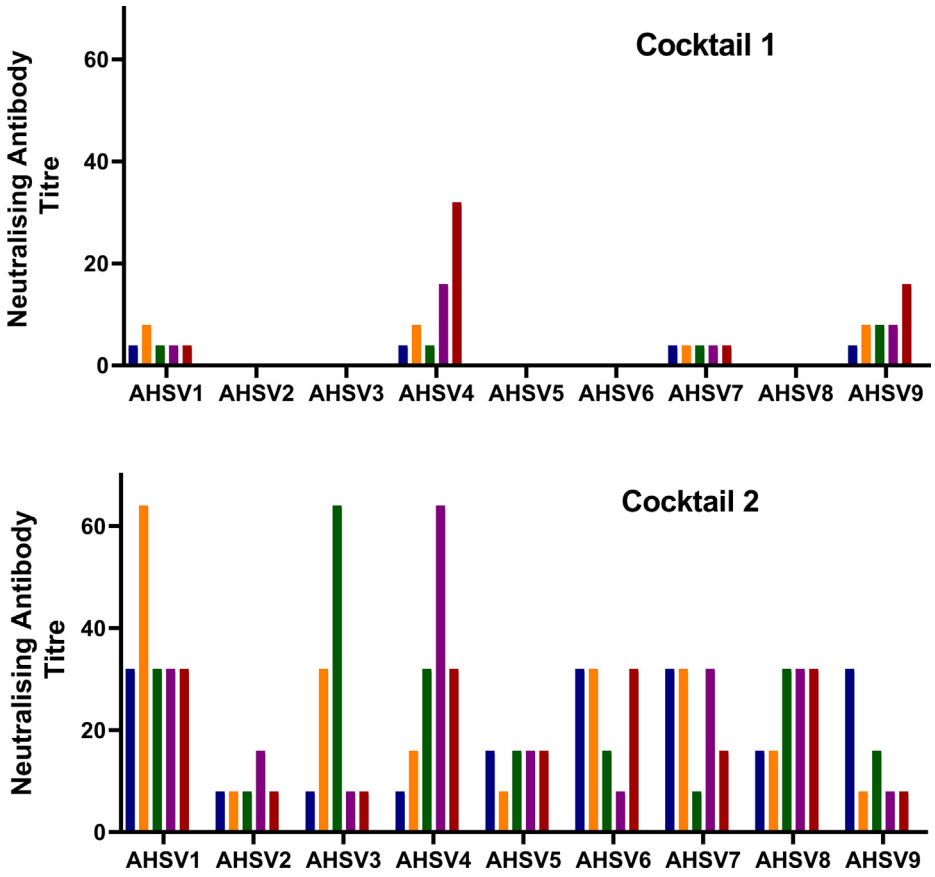

Fig. 2. Serum neutralisation activity in ponies after multivalent ECRA-AHSV vaccination. Neutralizing antibody titres of vaccinated animal sera were determined by serum neutralisation assay 21 days after receipt of Cocktail 1, immediately prior to Cocktail 2, and at 42 days. Neutralising antibody was never detected in the serum of the control animal indicated in Black.

prime boost strategy described previously [19]. Serum and blood samples were collected regularly for 173 days, and serological assays were performed to monitor the immune response.

The development of an immune response was initially monitored by ELISA, detecting the group specific VP7 antigen (Fig. 3). All vaccinated ponies had developed a high titre of antibodies against VP7 (between 73% and 94% in a competitive ELISA), by day 28, demonstrating that all animals were seroconverted. In 5 of the 6 vaccinated ponies (Cyan, Pink, Lilac, Gold, and Brown) the antibody titre remained high for the duration of the study (up to day 173 post vaccination). One vaccinated pony (Jade) did show inconsistent antibody titres (between 48 and 73%) during the experiment but VP7 antibody remained detectable throughout the study. No anti-VP7 antibody was detected in any of the sera collected from the control animal.

Serotype specific immunity was determined by serum neutralisation assay (Fig. 4). On day 21 vaccinated animals showed only very low levels of neutralising antibodies, consistent with the very

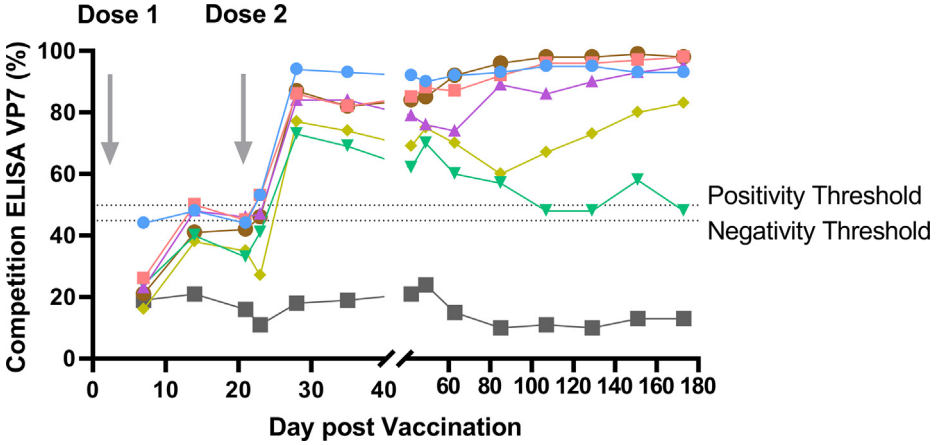

Fig. 3. Seroconversion of animals after ECRA-AHSV4 Vaccination. The induction of the immune response was monitored in vaccinated animals by VP7 group specific competitive ELISA.

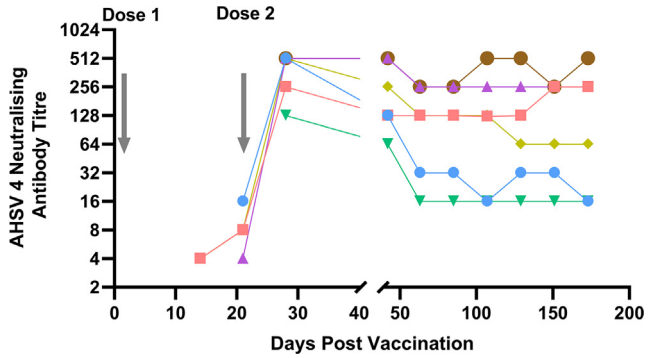

Fig 4. Long term serum neutralisation activity of sera collected from ECRA-AHSV4 vaccinated ponies. Sera collected throughout the study were examined for serotype specific neutralisation by serum neutralisation assay against AHSV serotype 4. Titres are expressed as the reciprocal of the highest dilution that maintained 90% neutralisation compared to the control animal in Grey.

limited replication possible following the first inoculation. By day 28 however, 1 week after the booster immunisation, all vaccinated animals had developed AHSV4 specific neutralising antibody titres above 64. Peak antibody titres were visible in all vaccinated ponies between days 28 and 42. After 28 days animals Cyan and Jade demonstrated a considerable drop in neutralising antibody, from 512 and 256 respectively, to 16 by the end of the study. Gold demonstrated a less dramatic drop from 512 at day 28 to 64 on day 173. In contrast the antibody titres of animals Pink, Lilac, and Brown remained consistently high (above 128) for the duration of the study. Importantly neutralising antibody titres remained above 8 in all vaccinated animals for the duration of the study demonstrating that vaccination with our ECRA AHSV strains is capable of eliciting a sustained protective neutralising immune response.

3.4. Cellular innate immunity in vaccinated animals

Usually inactivated vaccines induce a predominantly antibody - T-helper type 2 (Th2) mediated response (Th2-driven) but fail to activate T-helper type 1 (Th1) driven cellular immune responses which are the key for the elimination of intracellular infections [21]. This is in contrast to live-attenuated and vector vaccines which elicit generally balanced Th1-Th2 immune responses. With the aim to characterize the innate immune responses after ECRA-AHSV4 vaccination and to analyze if ECRA-AHSV4 vaccines could trigger Th1 and or Th2-mediated immune responses, cytokine levels were analysed by quantitative Luminex (Fig. 5). Our data showed that ECRA-AHSV4 prime-boost vaccination could indeed induce Th1 cytokine levels significantly in three ponies: Gold and Brown, IFNγ protein at 10 and 21 days post vaccination (dpv) and Lilac, TNFα protein at 21 and 31 dpv; Gold, at 10 dpv and Th2 cytokines in two animals: Lilac, IL10-protein and Brown, IL4-protein from 10 to 31dpv; while Th17, a cytokine induced during T regulatory cell (Treg) activation, was secreted in ponies Jade and Gold at days 10 and 31 post vaccination. Our results demonstrate that ECRA-AHSV4 vaccines are capable of inducing both Th1 and Th2 immune responses. Further, the induction of Treg cytokine profiles in two ponies was associated with lower antibody responses in these animals.

We also assessed the Th1 and Th2 responses of animals vaccinated with multivalent ECRA-AHSV vaccines of two different cocktails, thus each animal has been vaccinated only once with each vaccine strain. Only few animals demonstrated induction of low level of Th1-immune responses and only one animal, Blue, showed increased TNFα secretion on day 31 post vaccinations (data not shown). However, none developed Th2 or Treg-mediated responses. Note that the vaccine strains are different for prime and boost doses. Altogether, these data are consistent with both inactivated and live virus vaccine immune responses [21].

4. Discussion

With the growing risk of AHSV spreading beyond its traditional boundaries, there is a clear and present need for a safe and effective AHSV vaccine. Current vaccination strategies still rely on a live attenuated vaccine (LAV) although issues with both safety and efficacy have been identified [9]. First and foremost is the potential for re-assortment between vaccine strains and infecting virus, allowing reversion of the attenuated virus to virulence [7,8]. Whole sequence analysis of AHSV isolates from outbreaks in Gambia and South Africa provides clear evidence that vaccine derived strains have gained enhanced virulence through such re-assortment and have caused AHSV outbreaks. In addition, the lack of complete serotype coverage within the live attenuated vaccine has allowed the emergence of those strains not included. LAV AHSV vaccines are given in two doses, the first containing Serotype 1, 3, and 4, and the second serotypes 2, 6, 7, and 8, and, omitting serotypes 5 or 9 completely for reasons of safety and efficacy. However, their exclusion from the vaccine has led to their spread, with outbreaks of both in the areas where LAV is used [9]. Furthermore, the protection afforded by the LAV is not complete as vaccinated horses are still able to experience a subclinical infection [22].

The use of inactivated AHSV has also been investigated as a potential vaccination strategy [23]; however, the results from these studies suggest that such a strategy is not yet feasible. In the absence of a robust adjuvant, protection is variable, with only a fraction of vaccinated animals developing a full protective immune response. In addition, even in those animals which respond positively to vaccination, immunity is slow to develop, limiting the feasibility of using inactivated AHSV as an emergency vaccine. Additionally, long term immunity is dependent on subsequent booster vaccinations, every 6–12 months [24]. With a robust adjuvant inactivated AHSV may serve as a viable vaccine candidate, however the current drawbacks mean that a live attenuated vaccine remains the preferred approach. Another key concern with inactivated vaccines, and also LAVs, is that their production requires the handling and processing of live infectious virus.

Previously we demonstrated that ECRA AHSV vaccine strains, severely attenuated by not being able to complete the replication cycle, are nevertheless capable of eliciting a strong immune response, protecting inoculated animals against subsequent exposure [18,19]. All vaccinated animals seroconverted and developed neutralising antibodies but the study was limited to a subset of serotypes used for the vaccination. In the current study we have shown that inoculation with ECRA AHSV strains of all serotypes in two sequential cocktails elicits an immune response against all 9 AHSV serotypes, including neutralising antibodies. Importantly, our study included AHSV serotype 5 which is excluded from the LAV due to cross serotype interference, and subsequent failure to elicit a protective immune response [9]. Our previous data provided proof-of-principle for a multivalent vaccine with minimal risk of cross serotype interference, and the data presented here shows that this is indeed the case for all AHSV serotypes.

Of note in this study, vaccination was performed using different virus cocktails. In our previous studies, boosting was performed with at least one serotype present in the priming vaccination, as designed to achieve maximal levels of seroconversion and neutralising antibody response [19]. However, it was possible that the observed boosting effect was restricted to serotype specific antibody. In the current study however, a more general cross-

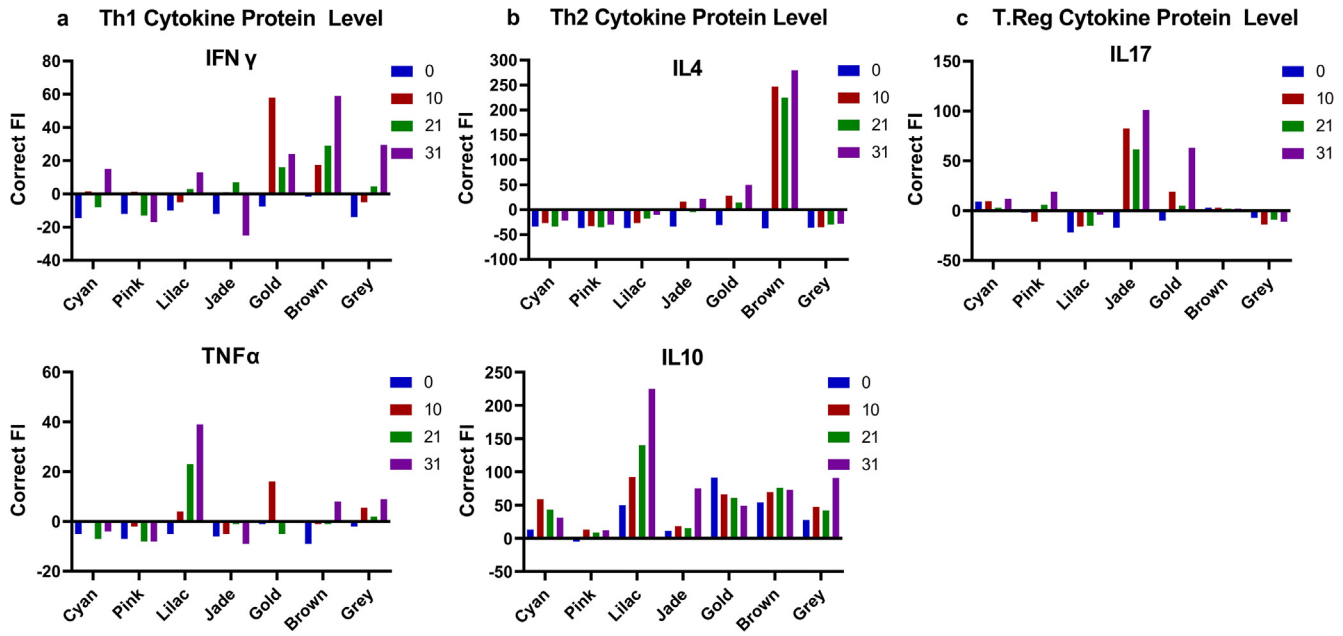

Fig. 5. Cytokine Analysis of ponies vaccinated with ECRA AHSV4. Sera collected throughout the study were examined for cytokine expression by Luminex Assay, to monitor (a) Th1, (b) Th2, and (c) T.Reg. response.

serotype boosting effect is apparent. Importantly, neutralising antibody titres were raised as part of a general increase in anti-AHSV titre. Maximum antibody titre against all serotypes, regardless of cocktail, was achieved after the second vaccination, in particular, neutralising antibody against Cocktail 1 serotypes were almost undetectable prior to the Cocktail 2 vaccination, but rose against all 9 serotypes to reach almost comparable levels. This cross-serotype enhancement is likely due to conserved sequences within the VP2 and VP5 proteins of the various serotypes. Alternatively, it could be a result of the shared structural elements within the ECRA-AHSV strains, all of which have been produced by serotype specific substitutions within the parental ECRA-AHSV1 strain [18].

Alongside the multivalent vaccine, we also examined the duration of ECRA-AHSV induced immunity against one selected serotype, AHSV 4. Vaccinated ponies were found to remain seroconverted for the duration of the study, approximately 6 months, with high levels of neutralising antibody detectable throughout. Given the lack of complete protection by the LAV, and the need for 8 vaccinations over 6 years [9], an immunisation schedule including fewer administrations would be a considerable improvement.

As noted, a common drawback of inactivated vaccines is the failure to trigger a complete immune response. Inactivated vaccines typically elicit a strong Th2 response, with minimal Th1 activity. This results in the generation of neutralising antibodies, but often fails to stimulate strong cellular immunity [21,25]. Whilst non-replicative, the ECRA-AHSV virus strains reported here retain the ability to enter cells and undergo a single round of protein expression, with replication blocked at the assembly stage. This means that infected cells develop the hallmarks of infections, allowing the detection of the ECRA viruses, triggering of a cytokine cascade, and induction of a T-cell mediated response. During the ECRA-AHSV4 trial, four of the six ponies demonstrated increased levels of Th1 cytokines post vaccination, with 3 showing an additional strong induction of Th2 cytokines. Together with the generation of high titres of neutralising antibody, these data lend support to the hypothesis that ECRA-AHSV viruses are capable of inducing a balanced immune response, featuring both antibody and cellular activities.

Ponies vaccinated with the multivalent vaccines demonstrated a reduced cytokine response, both Th1 and Th2, when compared to the monovalent trial. This is likely a result of each animal receiving only a single dose of each serotype overall. Antibody titres were also lower, suggesting that more complex cocktails and or further boosts may be necessary to achieve peak immunity. Ideally, future protection trials would address this issue although these are necessarily complicated as a result of the biosecurity required.

Given the continuing spread of *Culicoides* midges throughout Europe, and accompanied emergence of the related Bluetongue virus, there is a very real possibility that AHSV will soon follow and spread into previously low risk areas. Together with the issues with the current live attenuated vaccine strategies, there is an urgent need for a safe and effective vaccine against AHSV. By permitting cell entry and viral protein expression, ECRA-AHSV vaccine strains have been generated that stimulate immune responses, akin to a live attenuated vaccine, without the requirement for adjuvants. However, unlike the live vaccine, our ECRA vaccine strains do not replicate in non-complementary cells, and thus have safety profiles more commonly associated with killed vaccines, specifically the lack of reversion. Despite this, replication in the complementing cell line is similar to the live virus, such that manufacturing should be equivalent to both LAVs and inactivated vaccines. Additionally, production of our ECRA strains does not require the handling of live virus.

The data presented here, together with our previous studies, suggest that ECRA-AHSV strains can serve as safe effective vaccines against all serotypes of AHSV.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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