Vaccine 38 (2020) 7108–7117

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Safety and efficacy of inactivated African horse sickness (AHS) vaccine formulated with different adjuvants

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ARTICLE INFO

Article history:
Received 30 June 2020
Received in revised form 24 August 2020
Accepted 29 August 2020
Available online 10 September 2020

Keywords:
African Horse Sickness
Inactivated vaccine
Adjuvant

ABSTRACT

African horse sickness virus (AHSV) is a virus species in the genus *Orbivirus* of the family *Reoviridae* causing African Horse Sickness (AHS) in equids with a mortality of about 95% in naïve horses. AHS causes serious losses in developing countries where horses play a central role in draft power and transportation. There are nine AHSV serotypes inducing no or low cross-neutralizing antibodies. AHSV is spread by biting *Culicoides* midges. AHS is endemic in sub-Saharan Africa, and a serious threat outside Africa, since *Culicoides* species in moderate climate conditions are spreading the closely related bluetongue virus. AHS outbreaks will be devastating for the equestrian industry in developed countries. Live-attenuated vaccines (LAVs) are licensed, marketed and in use in Africa. Their application is controversial with regard to safety issues. LAVs are not allowed in AHS-free countries. We here studied inactivated AHSV with different adjuvants in guinea pigs and horses. Subcutaneous and intramuscular vaccination were studied in horses. Local reactions were observed after prime and boost vaccination. In general, neutralizing antibodies (nAbs) titres were very low after prime vaccination, whereas boost vaccination resulted in high nAb titres for some adjuvants. Vaccinated horses were selected based on local reactions and nAb titres to study efficacy. Unfortunately, not all vaccinated horses survived virulent AHSV infection. Further, most survivors temporarily developed clinical signs and viremia. Further, the current prototype inactivated AHS vaccine is not suitable as emergency vaccine, because onset of protection is slow and requires boost vaccinations. On the other hand, inactivated AHS vaccine is completely safe with respect to virus spread, and incorporation of the DIVA principle based on NS3/NS3a serology and exploring a vaccine production platform for other serotypes is feasible. A superior adjuvant increasing the protective response without causing local reactions will be required to develop payable and acceptable inactivated AHS vaccines.

1. Introduction

African Horse Sickness (AHS) is an OIE listed disease of equids causing mortality up to 95% for naïve horses, while zebras and African donkeys rarely show clinical signs [1,2]. The causative agent, African horse sickness virus (AHSV), genus *Orbivirus*, family of *Reoviridae*, causes different forms of disease ranging from mild fever, subacute and acute infections [3–5]. AHS outbreaks lead to huge economic losses by horse deaths, reduction of draft power, and blockade on transportation and trade [6]. AHSV is transmitted by biting *Culicoides* midges [7–9]. AHS is endemic in sub-Saharan Africa but this area could expand by climate change [10,11]. AHS epidemics has been periodically reported outside Africa, such as in India, Pakistan [12–14], Thailand [15], and on the Iberian Island [16,17] which expanded to Morocco. [18]. AHS outbreaks are devastating and result in large economic losses in the equestrian industry, and an enormous socio-emotional impact on owners of pet horses. Findings for closely related prototype orbivirus bluetongue virus (BTV) could imply that AHS-free countries in a moderate climate are also at risk [19–21,22,23].

Vaccination against insect-borne diseases such as AHS is the most effective control measure. Multivalent cocktails of live-attenuated vaccines (LAVs) for AHSV serotypes are commercially available and applied in Africa [24,5]. The last European AHS

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https://doi.org/10.1016/j.vaccine.2020.08.072
0264-410X/

outbreak was initially controlled with a multivalent cocktail of LAVs, and later on with monovalent LAV for serotype 4 (AHSV4LP) [16]. The major concern of LAVs is safety with regard to residual virulence, reverse to virulence, reassortment, and subsequently leading to virus spread by midges, reproduction losses and virulent AHSV variants [18,25]. In addition, infected equids cannot be distinguished from vaccinated horses according to the DIVA principle (DIVA = Differentiation of Infected from VAccinated individuals) [26]. These disadvantages hamper disease control and safe movement and trade of LAV vaccinated horses.

Orbiviruses, like AHSV, of which bluetongue virus (BTV) is the prototype orbivirus, are non-enveloped multi-layered RNA virus with ten double stranded genome segments Seg1-10 encoding seven structural proteins VP1-7 and at least four non-structural proteins NS1-4 [27–29]. Nine AHSV serotypes have been recognized inducing no or low cross neutralizing antibodes mainly determined by the serotype specific immunodominant VP2 protein encoded by Seg-2[VP2] [30]. Promising AHS vaccine candidates have been developed, such as subunit-, VLP-, and viral vector vaccines [31–33]. Recovery of AHSV by reverse genetics has been published [34,35], and have resulted in attenuated replicating AHS vaccine platforms lacking VP6, NS4, or NS3/NS3a [36–38]. None of these new generation vaccine candidates have been marketed yet [24]. Purified inactivated AHS vaccine for serotype 4 has been developed, and could serve as DIVA vaccine based on the absence of antibodies (Abs) against AHSV non-structural proteins [39,40]. Several inactivated AHS vaccines have been studied, but none of these are officially licensed [41–45]. In contrast, inactivated vaccine for several serotypes of the closely related BTV are successfully applied in European countries, for review [46]. In the light of preparedness and prophylactic vaccination, safe AHS vaccines for all serotypes should be available, or should be instantly available to rapidly induce protection. Preferably, these should be DIVA vaccines to enable serological monitoring of the vaccinated equine population for wild type AHSV infection in order to quickly eradicate and regain the AHS-free status.

Reverse genetics for AHSV4LP has been used to exchange outer shell proteins of all nine serotypes, and to delete dispensable NS3/NS3a protein [34,35]. These AHSV NS3/NS3a knockout variants could be considered for antigen production for inactivated AHS vaccines for all serotypes with DIVA potential based on NS3/NS3a serology [40,47]. As a first step, we here studied inactivated AHSV4LP formulated with different adjuvants to study safety and efficacy in guinea pigs and horses.

2. Materials and methods

2.1. Cell lines and viruses

Vero (green monkey) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 3% foetal bovine serum, antibiotics (100 IU/ml penicillin, 100 μg/ml Streptomycin and 2.5 ug/ml Amphotericin B).

AHSV strain HS32/62–10S-10BHK-3LP-7Vero (AHSV4LP) is the live-attenuated vaccine (LAV) for serotype 4 generated>50 years ago [2]. The ancestor virulent AHSV4 (AHSV HS 32/62) was originally isolated from a spleen and passed once in suckling mice and four times on BHK21 cells (AHSV HS 32/62/1s/4bhk). This virus was used to infect suckling mice to generate challenge inoculum.

2.2. Production of inactivated AHS vaccine

AHSV4LP was produced on monolayers of Vero cells (60% confluence) by infection with a multiplicity of infection of 0.1. Starting from 48 h post infection (hpi), culture medium was harvested every two hours and replaced by an equal volume of fresh culture medium from 48 to 56, 72–80, and 96–100 hpi. Each harvest was virus titrated by endpoint dilution. Harvests containing sufficient high virus titres were pooled. Pooled harvests contained $10^{7.5}$ $TCID_{50}$/ml AHSV4LP, and were inactivated by binary ethylenimine (BEI) at 37 °C [48,49]. To ensure complete inactivation by monitoring, a parallel virus batch was inactivated for 24 h, seeded with infectious AHSV4LP, and inactivation was continued for another 24 h. During the inactivation process, small aliquots of the parallel batch were taken and virus titrated to monitor the inactivation. After 48 h of inactivation, the original virus batch of AHSV antigen containing an equivalent of $10^{7.5}$ $TCID_{50}$/ml AHSV4LP was released from the veterinary biosafety unit of Wageningen Bioveterinary Research (WBVR), neutralized by adding sodium thiosulphate and the pH was checked. The absence of infectivity was confirmed by virus titration and confirmed after shipment to Deltamune, Lyttelton, South Africa before formulation of vaccine batches.

Adjuvants Montanide™ 206 VG and ISA 35 are oil-based adjuvants. 206 VG has been used in previous GP trails in combination with baculovirus expressed VP2 proteins [30], but is not recommended for use in horses [50]. Montanide™ IMS 3012 and PetGel A (PetGel) are water-based adjuvants. PetGel is in particular recommended for pet animals, including horses [50,51]. Immune stimulating complex ISCOM G3 (G3) is a water-based adjuvant and has been previously used in horses [52–54].

2.3. Diagnostic assays

Sera were tested with the commercially available competitive ELISA for group specific VP7 Abs (VP7 cELISA) (Ingezim AHSV Compac Plus, INGENASA, Madrid, Spain), and for NS3 Abs with the experimental NS3 competitive ELISA (NS3 cELISA) (Ingenasa, Madrid, Spain) [37,47]. Results of both cELISAs were expressed as blocking percentage (100-value). According to the supplier of the extensively validated VP7 cELISA, >50% blocking is positive, 45–50% blocking is doubtful, and < 45% blocking is considered negative. The experimental NS3 cELISA has not been validated yet. Previous results evidenced a threshold of 30–40% blocking [47].

Serum samples of indicated days were tested with the serum neutralization test (SNT) to determine neutralizing antibody (nAb) titres against AHSV serotype 4 [37,55]. SNTs were performed in duplicate and nAb titres were expressed by the highest serum dilution showing 100% neutralization.

EDTA blood samples were tested with the panAHSV real-time RT-PCR test as described [47].

2.4. Animal trials

2.4.1. Immunization of guinea pigs

The guinea pig (GP) experiment was performed under the guidelines of the European Community and was approved by the Committee on the Ethics of Animal Experiments of Central Veterinary Institute (currently, Wageningen Bioveterinary Research, WBVR)(permit 2012.027). Six groups of six GPs were subcutaneously (sc) immunized twice with an interval of three weeks with 4 × 100 ul per GP per immunization at four spots on the back on day 0 and day 21. Short before immunization, inactivated AHSV antigen was formulated with adjuvant according to the respective suppliers. Group 1 was immunized with a 50% emulsion of AHSV antigen in 206 VG (206 VG 50/50). PetGel was used in two final concentrations: group 2; 5% PetGel, and group 3; 2.5% PetGel. Group 4 was immunized with a 75% emulsion of AHSV antigen in ISA 35 (ISA35 25/75). IMS 3012 was used in two final concentrations of 50% or 30% (Group 5; IMS 3012 50/50 and Group 6; IMS 3012 30/70). Serum samples were collected by orbital punction on days 0, 21 and 42, and tested for AHSV directed Abs.

2.4.2. Vaccination and challenge experiments in horses

Horse trials were performed under the guidelines of the South African Department of Agriculture Forestry and Fisheries (DAFF) (12/11/1/1/9). The animal trials were approved by the Committee on the Ethics of Animal Experiments of Deltamune Ltd (permits CR/12/78, CR/12/78 repeat 1, and CR/13/126). Suitable horses (boerperd or boerperd-cross) of 1–4 years old on a South African premise were screened for AHSV Abs. The private game reserve keeps a herd of > 300 free ranging horses. The reserve is located on an altitude of > 1.500 m and is historically free of AHS. As a result, the horses were kept under natural, standard farm conditions throughout the trial up to AHSV challenge. Horses grazed on natural pastures and water was provided ad libitum.

Healthy horses free of AHSV and AHSV Abs were selected to enter the horse trial and were randomly grouped. Formulated inactivated AHS vaccine was administered in the mid third of the neck; prime vaccination at the left side, and boost vaccination at the right side. Vaccinated horses remained on the premise, and were monitored on indicated days for local inflammatory reactions. Local reactions were blindly quantified by direct measurement across the widest and tallest aspects, while the height of lesions was subjectively estimated. The consistency and sensitivity of the lesion was attained by direct palpation. Serum samples were collected on indicated days. After clotting, sera were cooled and transported to the laboratory for further processing.

2.4.2.1. Trial 1. Four groups (A-D) of four horses were formed. Vaccines were freshly formulated consisting of undiluted inactivated AHSV antigen (equivalent of ± $10^{7.5}$ $TCID_{50}$/ml) and 25% ISA 35 (total volume: 1.4 ml), 5% PetGel (total volume: 1.1 ml), IMS 3012 50:50 (total volume: 1.4 ml), or IMS 3012 30:70 (total volume: 2 ml). One horse served as negative control for serology tests. Horses were subcutaneously (sc) vaccinated with indicated vaccine batches, and received a boost vaccination at 27 days post prime vaccination (27 dpv/0 dpb). The experiment was finalized at 61 dpv/34 dpb. Abs against VP7 and NS3/NS3a were determined by cELISAs, and nAb titres against serotype 4 were determined by SNT for sera of the indicated days.

2.4.2.2. Trial 2. Four groups of five horses were formed. As described for trial 1, vaccines were freshly formulated. Each vaccination consists of 2 ml of freshly formulated vaccine containing undiluted inactivated AHSV antigen (equivalent of ± $10^{7.5}$ $TCID_{50}$/ml) and 10% PetGel or G3. Horses were two times vaccinated subcutaneously (sc) or intramuscularly (im) with an interval of four weeks on 0 dpv and 27 dpv. Boost vaccinations of horses was intended with the same vaccine by the same route. Unfortunately, two horses received G3 formulated vaccine after prime vaccination with PetGel formulated vaccine and vice versa for one horse, and two different vaccination route were used for one horse. Based on the highest nAbs titres on three weeks post boost vaccination (49 dpv) and the fewest and less severe local reactions after vaccinations, eight horses were selected for challenge with virulent AHSV4.

Vaccinated horses, together with naïve horse 927, free of AHSV and AHSV Abs, were transported to BSL-II insect-proof animal facilities of Deltamune, Lyttelton, South Africa to conduct infection with virulent AHSV4. Horses were individually housed in horse permanent, brick stables (9 m^2 per horse) allowing visual and auditory contact with other horses. Wood-shavings were provided as bedding material and manure and soiled bedding were removed daily. Horses were fed 1–1.5 kg commercial horse-pellets per horse per day, and grass hay (in hay net) and water were provided ad libitum. After acclimatisation, horses were intravenously (iv) infected at the jugular vein with 1 ml of prepared mouse-brain suspension, equivalent to one mouse bra in and typically contain $1x10^6$ $TCID_{50}$ virulent AHSV4 on day 125 dpv/0 dpc. Horse 927 served as challenge control and one horse was considered sufficient to meet criteria of animal welfare regarding the 3 R logic: Refinement, Replacement, Reduction.

Body temperature was measured twice daily for the first two weeks and daily thereafter. Horses were frequently examined for clinical signs depending on the general health status. According to the ethical rules of animal welfare, diseased horses were euthanized in case of severe clinical signs of AHS. EDTA blood samples were taken daily for PCR testing, and serum samples were collected on indicated days post challenge. This part of the study was finalized two weeks after challenge (139 dpv). Surviving horses were less frequently monitored for a longer period, and were released from the insect proof premise after repeated negative PCR testing.

3. Results

3.1. Production of AHSV antigen

AHSV4LP was produced on monolayers of Vero cells. Culture medium was harvested every two hours starting from 48 h post infection (hpi). Each two-hours harvest contained 10^{7-8} $TCID_{50}$/ml AHSV up to 80 hpi. Then, virus production dropped below 10^7 $TCID_{50}$/ml and monolayers showed increased cell damage (not shown). Four harvests per daytime of two successive days were pooled. This antigen batch contained approximately $10^{7.5}$ $TCID_{50}$/ml AHSV4LP. Inactivation was monitored for samples taken from a parallel virus batch. After 24 h, inactivation was completed and the parallel batch was seeded with infectious AHSV. Again, after 24 h of inactivation no infectivity was detected in this seeded parallel virus batch. Completion of the inactivation of the original virus batch was confirmed by infection of fresh monolayers of Vero cells at WBVR, and was re-confirmed after shipment at Deltamune (not shown).

3.2. Immunogenicity of inactivated AHSV formulated with different adjuvants in guinea pigs

Three weeks after prime immunization (21dpv), guinea pigs (GPs) remained negative for VP7 Abs (Table 1). Three weeks after boost immunization (42 dpv/21 dpb), one GP in group 1 (206 VG (50/50)), three GPs in group 2 (5% PetGel), one GP in group 3 (2.5% PetGel) and group 4 (ISA 35 (25/75)), and two GPs in group 6 (IM3012 (30/70)) were positive for VP7 Abs (>50% blocking). Three GPs, one in groups 3, 5, and 6, were interpreted as doubtful (D)(45–50% blocking). The other 25 GPs in total remained seronegative for VP7 Abs at 42 dpv/21 dpb.

nAb titres against AHSV4LP were determined on days 0, 21 and 42 (Table 1). At day 21, 4 out of 36 GPs showed a very low nAb titre of 2–6. At 42 dpv/21 dpb, groups 4 (ISA 35 (25/75)) and 6 (IMS 3012 (30/70)) had nAb titres varying from 2 to 64, whereas 1–3 GPs in groups 1, 2, 3 and 5 showed very low nAb titres of 2–12 but most GPs were negative for nAbs. These results were very disappointing as GPs have been used previously as alternative animal model in order to study Ab responses prior to test vaccine candidates in horses. Still, based on these results, ISA 35 (25/75) and IMS 3012 (30/70) were the most promising adjuvants to formulate AHS vaccine.

3.3. Horse trial 1

Four formulations of inactivated AHSV4LP were studied in four groups of four horses for Ab responses and local reactions (Table 2 and supplemented data Table S1). Subcutaneous (sc) prime vaccination with adjuvant ISA 35 resulted in soft swellings in all horses on the next day (1 dpv) (Fig. 1a). Swellings in three horses became

Table 1
Serological results of immunization of guinea pigs. Guinea pigs (GPs) were randomized in six groups of six GPs. GPs were subcutaneously (sc) immunized twice with an interval of three weeks with 4 × 100 ul per GP per immunization at four spots on the back on day 0 and day 21. Inactivated AHSV (equivalent of ± $10^{7.5}$ $TCID_{50}$/ml) was formulated with indicated adjuvants on days 0 and 21: **group 1**: 206 VG (50/50), **group 2**: PetGel 5%, **group3**: PetGel 2.5%, **group 4**: ISA 35 (25/75), **group 5**: IMS 3012 (50/50), **group 6**: IMS 3012 (30/70). Sera on days 0 (0 dpv), 21 (21 dpv/0 dpb) and 42 (42 dpv/21 dpb) were analysed for Abs. Results of the VP7 cELISA (ELISA) were expressed as blocking percentage (100-value). Blocking percentage of < 45% is negative (-); 45–50% is doubtful (D); and > 50% blocking is positive (+). nAb titres for serotype 4 were determined by the serum neutralization test (SNT). nAb titres were expressed by the highest serum dilution showing 50% neutralization.

		ELISA			SNT		
		0	21	42	0	21	42
group 1	1	–	–	–	–	–	–
206 VG (50/50)	2	–	–	–	–	–	–
	3	–	–	–	–	–	–
	4	–	–	–	–	–	–
	5	–	–	+	–	–	6
	6	–	–	–	–	–	2
group 2	7	–	–	–	–	–	–
PetGel (5%)	8	–	–	–	–	–	–
	9	–	–	+	–	–	–
	10	–	–	–	–	–	–
	11	–	–	+	–	–	12
	12	–	–	+	–	–	–
group 3	13	–	–	–	–	–	–
PetGel (2.5%)	14	–	–	D	–	–	6
	15	–	–	+	–	–	8
	16	–	–	–	–	–	–
	17	–	–	–	–	–	–
	18	–	–	–	–	2	6
group 4	19	–	–	–	–	–	8
ISA 35 (25/75)	20	–	–	–	–	–	6
	21	–	–	–	–	–	24
	22	–	–	–	–	–	8
	23	–	–	–	–	–	32
	24	–	–	+	–	–	32
group 5	25	–	–	–	–	–	8
IMS 3012 (50/50)	26	–	–	D	–	–	–
	27	–	–	–	–	–	–
	28	–	–	–	–	–	2
	29	–	–	–	–	–	–
	30	–	–	–	–	–	–
group 6	31	–	–	+	–	4	24
IMS 3012 (30/70)	32	–	–	–	–	3	32
	33	–	–	–	–	6	64
	34	–	–	D	–	–	24
	35	–	–	+	–	–	6
	36	–	–	–	–	–	2

Table 2
Serological results of immunization of horses. Four groups (A-D) of four horses were formed. Vaccines were freshly formulated consisting of undiluted inactivated AHSV antigen (equivalent of + $10^{7.5}$ $TCID_{50}$/ml) and 25% ISA 35 (1.4 ml), 5% PetGel (1.1 ml), IMS 3012 50:50 (1.4 ml), or IMS 3012 30:70 (2 ml). Total volumes of the vaccine dose are in brackets. Sera collected prior to the start of the experiment (−91), on 27 post prime immunization (dpv) and 61 dpv (34 days post boost immunization (dpb) were tested for Abs. Results of the VP7 cELISA are expressed as blocking percentage (100-value). Neutralizing Ab titres (nAb titres) for serotype 4 were determined by the serum neutralization test. The respective serum nAb titres are expressed as the highest dilution of serum showing 100% neutralization. ID: identification number, control: non-vaccinated control horse.

		days post vaccination (post booster)				
		VP7 cELISA			nAb titre	
adjuvant	ID	−91	27(0)	61(34)	27(0)	61(34)
ISA 35 (25%)	785	9	42	75	8	64
	774	6	65	90	<2	16
	771	8	58	79	<2	64
	804	11	43	93	<2	64
Pet Gel (5%)	783	11	nd	nd	nd	nd
	726	10	80	98	32	256
	793	7	76	99	2	≥256
	873	11	81	99	16	128
IMS 3012 (30:70)	849	13	52	75	2	4
	777	12	28	75	<2	8
	760	11	31	85	<2	2
	769	11	30	69	<2	4
IMS 3012 (50:50)	765	9	26	nd	<2	2
	789	10	34	76	2	8
	837	11	32	76	<2	2
	952	11	56	95	<2	8
control	851	nd	13	12	<2	<2

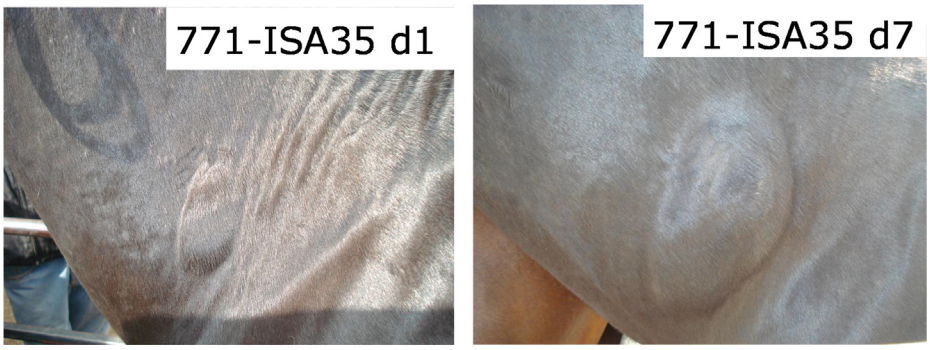

Fig. 1a. Local reactions. Pictures were taken after the first sc vaccination on 1 dpv (d1) and 7 dpv (d7) corresponding to Day 1 and Day 7 in Table S1.

very flat but persisted for five weeks (34 dpv). The adjuvant 5% PetGel induced soft inflammatory local reactions that became firm short after immunization in two out of three horses, and remained visible for a longer time for one horse (Table S1). Unfortunately, one horse of the PetGel group, horse 783, had to be removed from the study due to unrelated injury (snake bite in one leg). Adjuvant IMS 3012 induced local reactions for one day for both 50:50 and 30:70 formulations, and these remained visible for one week for two out of eight horses, and up to five weeks for one horse. Eventually, local reactions disappeared in all horses of both IM 3012 groups (day 61) (Table S1).

Boost vaccination caused local reactions again (Table S1). Group ISA 35 and PetGel showed swellings that slightly tended to be more severe than after prime sc vaccination, but were vanished after 61 days (Fig. 1b), except for one horse of group PetGel (Table 2). Local reactions for IM 3012 groups also tended more severe after boost vaccination but disappeared eventually at dpv 61/34 dpb. In general, local reactions for IM 3012 groups were fewer and less severe than for groups ISA 35 and PetGel (Table S1).

VP7 Abs were detected at 27 dpv, but not all horses of all groups were conclusively positive (>50%), except for group PetGel (Table 2). Two or three horses of the other groups were interpreted negative for VP7 Abs (<45%). The mean % blocking of groups ISA 35 and PetGel was higher than for IMS 3012 groups at 27 dpv. After boost vaccination, all groups were conclusively positive at 61 dpv/34 dpb. Group PetGel showed the maximum of ± 100%, whereas horses of the other groups varied between 69 and 95%. The unvaccinated control horse 851 remained negative (±13% blocking).

nAb titres against serotype 4 were determined on 27 dpv and 61 dpv/34dpb). On 27 dpv, nAbs were hardly detected, except for group PetGel showing nAb titres of 2–32 (Table 2). On 61 dpv/34 dpb, group ISA 35 and group PetGel showed nAb titres of 16–64 and 128–256, respectively, whereas both IMS 3012 groups showed very low nAb titres of 2–8. The unvaccinated control horse 851 remained negative for nAbs (<2). Clearly, inactivated AHSV4 formulated with 5% PetGel induced the highest nAb titre, whereas IMS 3012 formulated vaccines did not induce promising nAb titres with regard to protection against AHS. In conclusion, 5% PetGel is the most promising formulation, although improvement by minimizing adverse effects and increase of the mean nAb titres is needed to guarantee safety and protection.

3.4. Horse trial 2

3.4.1. Local reactions and Ab responses after vaccination

To find a completely safe inactivated AHS vaccine, two new vaccine formulations were studied by two vaccination routes. One formulation was based on previously tested 5% PetGel, but we increased the percentage to 10% in order to enhance the immune response. The other studied adjuvant immune stimulating complex ISCOM G3 (G3) was selected, since it has been shown safe in horses in combination with other antigens [52–54]. Groups of five horses were sc or intramuscularly (im) vaccinated with undiluted inactivated AHSV4 formulated in 10% PetGel or G3. After im prime vaccination, no local reactions nor any discomfort was observed, except for horse C3C of the im, G3 group (Table S2). In contrast, sc vaccination resulted in swellings on day 1 (1 dpv) in both groups, except for horse C68. These swellings in the sc, G3 group disappeared shortly. Swellings after sc vaccination with 10% PetGel formulated vaccine were still recorded for all five horses on day 7 (7 dpv), and for two horses these were still visible on day 61.

Horses were boost vaccinated at 27 dpv/0 dpb at the right side of the neck. Like for prime vaccination, boost vaccination resulted in more severe local reaction for sc vaccination than im vaccination (Table S2). Sc boost vaccination resulted in local reactions for one day for G3 and for about one week for 10% PetGel. One of these horses, horse 84A, had still a visible reaction on day 34 (61 dpv). G3 formulated vaccine induced no local reactions after im boost vaccination, whereas 10% PetGel showed tender swellings in two

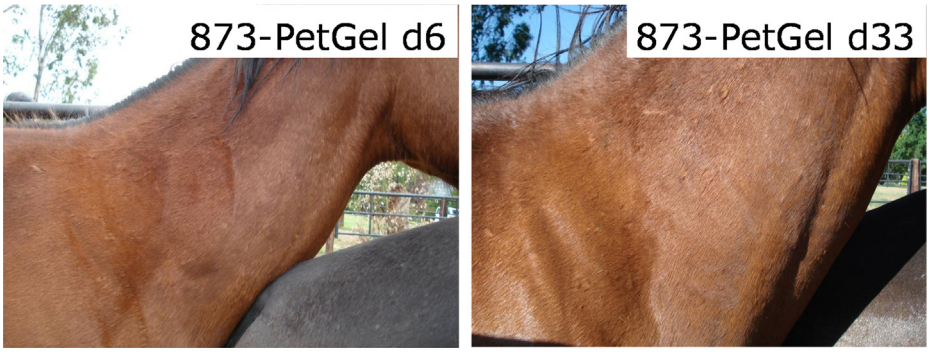

Fig. 1b. Local reactions. Pictures were taken after sc boost vaccination on 6 dpb (d6) and 33 dpb (d33) corresponding to Day 34 and Day 61 in Table S1.

out of five horses. These were still present after 7 days (day 34/7 dpb) but eventually disappeared after five weeks (day 61/34 dpb). In contrast, local reactions caused by G3 formulated vaccine were very minor and disappeared shortly. Note, horses B64 and 71D sc prime vaccinated with 10% PetGel were accidentally sc boost vaccinated with G3 formulated vaccine. Further, horses C64 and 84A accidently received 10% PetGel formulated vaccine by the sc route, whereas these were im or sc prime vaccinated with G3, respectively (Table S2).

In conclusion, im vaccination resulted in fewer and less severe local reactions than sc vaccination. Further, adjuvant G3 formulated vaccine induced fewer and less severe reactions than 10% PetGel formulated vaccine. Finally, boost vaccination tended to induce more severe local reactions, irrespective of heterologously used adjuvant.

VP7 Abs were detected in all groups (Table 3). After sc or im prime vaccination with 10% PetGel formulated vaccine, Mean VP7 cELISA results were positive (±60%) at 27 dpv, although horses 125 and F38 (sc, PetGel) were interpreted negative, 39 and 26%, respectively. All horses of both G3 groups induced VP7 Abs at 27 dpv but were interpreted negative (<45%), except for horse 969 (51%). Obviously, irrespective of the application route, prime vaccination with G3 formulated vaccine resulted in a weaker VP7 response than with PetGel formulated vaccine. After boost vaccination, all horses of all groups were conclusively positive by VP7 cELISA at 49 dpv/22 dpb (Table 3). Even more, all groups showed a mean % blocking close to the maximum (91–99%), except for group sc, G3 with a mean blocking of 88%.

NS3/NS3a directed Abs were determined with the experimental NS3 cELISA. Results showed obvious variation, including up to 41 and 42% for horses 71D and B29 at 0 dpv, respectively (Table 3). The cut-off value is not determined yet by lack of validation data but is likely 30–40% [47]. In general, horses remained negative for NS3 Abs after vaccinations based on this provisional threshold value, except horse A09 showing 57% blocking at 49 dpv/22 dpb.

nAb titres for serotype 4 were determined for sera of 0 dpv, 27 dpv/0 dpb and 49 dpv/22 dpb (Table 3). nAbs titres were not detected at 0 dpv confirming that horses were negative for AHSV Abs at the start of the experiment. At 27 dpv, nAb titres of 4 to 32 were determined for three out of five horses of each PetGel group, while two horses of each group remained below the threshold of 4. Except for horse 005 (nAb titre of 4), all horses of both G3 groups did not show nAbs at 27 dpv. Three weeks post boost vaccination (49 dpv/22 dpb), all horses of all four groups showed nAb titres (Table 3). Note, two out of three horses with nAb titres of $\geq$ 512 were accidentally boost vaccinated with heterologous adjuvant. In conclusion, PetGel groups showed promising nAb titres of 64 to $\geq$ 512. Further, Group im, G3 had slightly lower nAbs titres between 4 to $\geq$ 512, whereas group sc, G3 showed nAb titres of 8 to 64.

3.4.2. Challenge with virulent AHSV4

A limited number of vaccinated horses was selected to study protection (underlined horse numbers in Table 3). Selection criteria were a combination of high nAb titres and minor local reactions, since companies and horse owners will be reluctant to further develop and use such vaccines, respectively. Sc vaccinated horses were not selected because of severe local reactions (sc, PetGel) or low nAb titres (sc, G3). Horses C64, 84A, 71D and B64, accidentally vaccinated with different adjuvants (Table S2), were excluded from the challenge study despite of high nAb titres (Table 3). All five horses of group im, PetGel and horses A09, C3C, and 005 of group im, G3 were selected. These horses showed no or very mild local reactions and had nAb titres varying from 32 to $\geq$ 512 at 49 dpv/22dpb promising protection against virulent AHSV4.

The challenge control horse 927 had fever (>40 °C) started on 2 dpc (Fig. 2A), and clinical signs such as discomfort started two days later at 4 dpc. Viremia by PCR testing started on one day after challenge (1 dpc) and rapidly increased to Cp < 20 at 6 dpc (Fig. 2B). Oedema of the supra-orbital fossae was obvious one day later at 5 dpc (not shown). The next day (6 dpc), control horse 927 was euthanized due to severe clinical signs; dyspnoea, dehydration as observed by > 50% haemoconcentration (not shown), and > 2 °C

Table 3
Serology results after vaccination. Four groups of five horses were formed. Each vaccination consists of 2 ml of freshly formulated vaccine containing undiluted inactivated AHSV antigen (equivalent of ± $10^{7.5}$ $TCID_{50}$/ml) and 10% PetGel or G3. Horses were two times vaccinated subcutaneously (sc) or intramuscularly (im) with an interval of four weeks on 0 dpv and 27 dpv. Sera collected on day 0, 27 and 49 were tested for Abs. Results of the VP7 cELISA and the NS3 cELISA are expressed as blocking percentage (100-value). Neutralizing Ab titres (nAb titres) against serotype 4 were determined by the serum neutralization test (SNT). nAb titres are expressed as the highest serum dilution resulting in 100% neutralization. Accidental heterologous boost vaccinations are indicated in bold. Chip: identification number, 1st: prime vaccination, 2nd boost vaccination, im: intramuscular, sc: subcutaneous, neg: <2. Horses with underlined chip numbers were selected for AHSV challenge, see Figs. 2 and 3.

		vaccination		VP7 cELISA (%)			NS3 cELISA (%)			SNT (dilution)		
Chip	Sex	1st	2nd	0	27	49	0	27	49	0	27	49
658	F	im, PetGel	im, PetGel	24	61	101	14	−1	22	neg	8	≥ 512
24C	F	im, PetGel	im, PetGel	22	55	100	8	9	6	neg	<4	256
A03	F	im, PetGel	im, PetGel	16	50	93	13	0	10	neg	<4	128
D6C	F	im, PetGel	im, PetGel	24	59	97	32	7	20	neg	4	128
80A	F	im, PetGel	im, PetGel	10	83	103	10	33	23	neg	8	64
B64	M	sc, PetGel	**sc, G3**	20	85	101	3	−8	−8	neg	32	≥ 512
C72	F	sc, PetGel	sc, PetGel	20	65	100	20	4	15	neg	4	256
71D	M	sc, PetGel	**sc, G3**	21	87	90	41	1	26	2	8	256
125	F	sc, PetGel	sc, PetGel	13	39	96	−1	−13	2	neg	<4	256
F38	F	sc, PetGel	sc, PetGel	12	26	84	11	−12	−2	neg	<4	64
C64	F	im, G3	**sc, PetGel**	15	21	90	−10	−8	−1	neg	<4	≥ 512
A09	F	im, G3	im, G3	13	31	100	10	27	57	neg	<4	256
C3C	F	im, G3	im, G3	18	37	94	7	7	27	neg	<4	64
005	F	im, G3	im, G3	15	33	86	25	−7	18	2	4	32
B34	F	im, G3	im, G3	23	37	83	18	1	−1	neg	<4	4
B29	F	sc, G3	sc, G3	18	33	85	42	2	7	neg	<4	64
84A	F	sc, G3	**sc, PetGel**	20	41	97	25	−1	3	neg	<4	64
C1C	M	sc, G3	sc, G3	18	32	89	7	8	8	neg	<4	16
C68	F	sc, G3	sc, G3	17	24	85	28	−9	−33	neg	<4	16
969	F	sc, G3	sc, G3	12	51	86	4	−3	−10	neg	<4	8

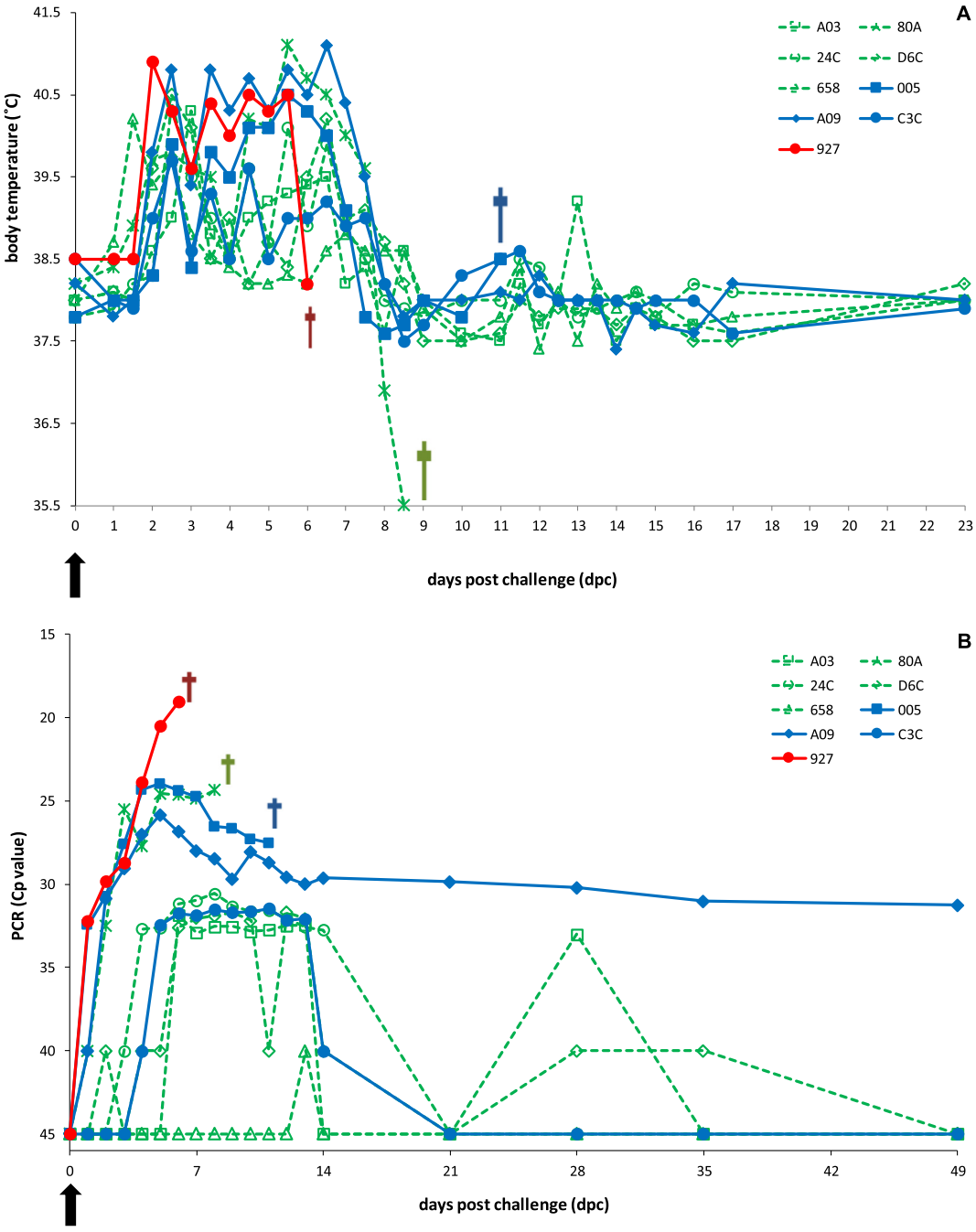

Fig. 2. Body temperature and viremia after challenge with virulent AHSV4. Selected horses (underlined in Table 3) were intravenously challenged on day 0 (dpc) (filled arrow) corresponding with 125 dpv and 98 dpb, see Fig. 3. Inactivated AHSV was formulated with PetGel (green open symbols) or with G3 (blue filled symbols). A cross indicates death or euthanizing for ethical reasons in the time course of the experiment. **A**: The body temperature was frequently measured twice daily in the morning and the afternoon on indicated days after challenge. **B:** Viremia was determined by semi-quantitative PCR-testing and expressed as Cp value Negative results are set on Cp = 45. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

drop of the rectal temperature to 38.2 °C (Fig. 2A). Post mortem examination confirmed lethal African Horse Sickness (AHS).

On the day of challenge, 0 dpc/125 dpv/98 dpb, VP7 seronversion was only slightly decreased compared to that on 49 dpv/22dpb (Fig. 3A). All surviving horses boosted for VP7 Abs after AHSV4 challenge to the maximum of 100% blocking and seroconverted for NS3 Abs (Fig. 3A and 3B). Vaccinated horse 80A (im PetGel) developed viremia up to Cp = 25 (Fig. 2B). Clinical signs related to AHS started on 2 dpc, and included fever (Fig. 2A), swelling of the supra-orbital fossae, severe thrombocytopenia, discomfort and inappetence (not shown). In conjunction with a severe sudden drop in rectal temperature (<37 °C), slightly increased haemoconcentration, severe discomfort, depression, and respiratory dyspnoea was observed on 8 dpc. Horse 80A was euthanized for humane reasons at the end of 8 dpc. Horses 24C, A03 and D6C of group im, PetGel survived, but developed elevated body temperature or fever (>40 °C) was limited for one or two days (Fig. 2A). Clinical signs related to AHS were absent or very mild (not shown). Viremia remained low (Cp > 30) (Fig. 2B), and turned negative at 49 dpc or earlier. Horse 658 of group im PetGel developed fever at the end of 1 dpc followed by elevated body temperature the next day (2 dpc). Clinical signs were very mild.

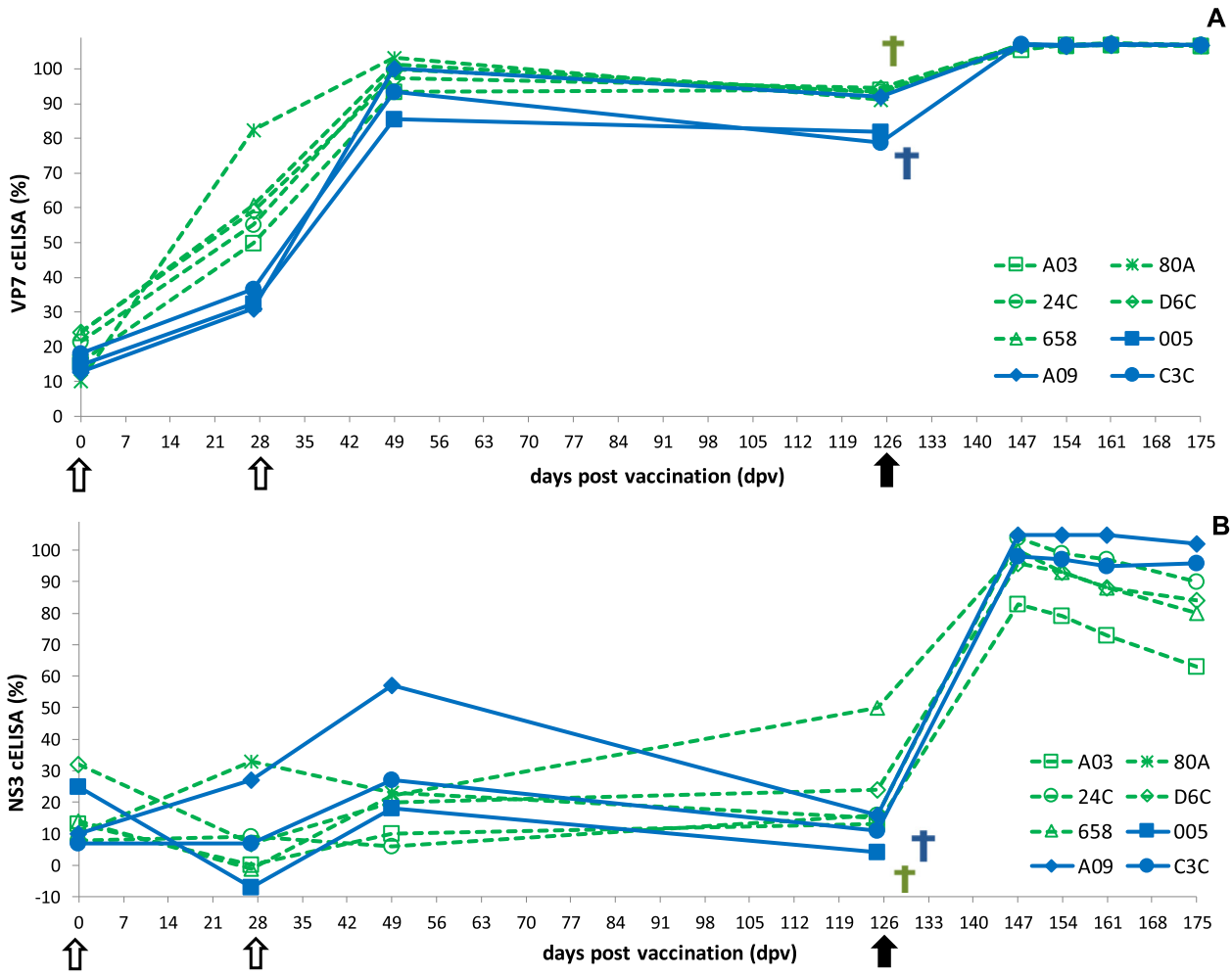

Fig. 3. Results of the VP7 cELISA and the NS3 cELISA after vaccination followed by AHSV4 challenge. Selected horses (underlined in Table 3) were intramuscularly vaccinated on day 0 (0 dpv) and 27 dpv (open arrows) with inactivated AHS vaccine formulated with PetGel (green open symbols) or with G3 (blue filled symbols). Horses were intravenously challenged with virulent AHSV4 on day 125 dpv 0 (filled arrow). A cross indicates death or euthanizing for ethical reasons in the time course of the experiment. **A**: VP7 Abs were determined with the VP7 cELISA and expressed as blocking percentage (100-value). **B**: NS3 Abs were determined with the experimental NS3 cELISA and expressed as blocking percentage (100-value).

Horses 005 and A09 of group im G3 developed clinical signs after AHSV4 challenge, including fever from 2 to 8 dpc (Fig. 2A), in-appetence, and increased respiratory effort. Horse 005 also developed oesophageal paralysis, evident on 9 dpc, and due to increasing unease, depression, and poor prognosis horse 005 was euthanized two days later on 11 dpc. Horse A09 fully recovered but remained PCR positive up to seven weeks post challenge (Fig. 2B). The peak of viremia of horse A09 was close to that of non-survivors 005 and 80A. Horse C3C (im G3) suffered from a mild thrombocytopenia between 5 and 13 dpc, but developed no fever nor other clinical signs related to AHS. Slightly elevated body temperature was measured on several days post challenge, in particular at the end of each day (Fig. 2A). Viremia of horse C3C persisted for two weeks but was limited to Cp > 30 (Fig. 2B). Remarkably, surviving from AHSV challenge was roughly correlated to nAb titres > 2 months earlier (49 dpv/22/dpb (Table 3). Horses 80A and 005 did not survive with nAb titres of 64 and 32, respectively, whereas six surviving horses had nAb titres of 64 to $\geq$ 512 at two months prior to challenge with lethal AHSV4.

4. Discussion

In order to develop inactivated AHS vaccines, inactivated AHSV4LP formulated with different adjuvants was studied as prototype vaccine on efficacy and safety with regard to local reactions. Cell culture adapted AHSV4LP was produced on monolayers of Vero cells by serial refreshing of culture medium, and harvests of culture medium were pooled and used as AHSV antigen. Multiple culture medium harvests of one cell culture prior to cytopathogenic effect (CPE) each contained > 10^7 $TCID_{50}$/ml AHSV4LP. Pooled harvests are suggested to be free of cell components and free of non-structural AHSV proteins. Costly down processing steps, such as purification and concentration of produced AHSV antigen [42,45], were not needed.

The Ab responses by formulated inactivated AHSV strongly depended on the amount of antigen, adjuvant and vaccination route (Table 1-3). ISA 206VG formulated inactivated AHS vaccine induced much lower nAb titres in GPs than large amounts of baculovirus expressed VP2 formulated with the same adjuvant [30]. Group 6 (IMS 3012 30:70) contained more inactivated AHSV than group 5 (IMS 3012 50:50) and induced indeed higher Ab responses (Table 1). Similarly, a correlation between the amount of antigen and Ab response has been shown [44]. Using the here described harvest method, the amount of AHSV4LP antigen is an equivalent of ±$10^{7.5}$ $TCID_{50}$/ml, which corresponds to that of other inactivated AHS vaccine candidates [42,45], and commercially available inactivated vaccines for Bluetongue [56].

Adjuvant greatly affects the efficacy of dead vaccines. Different formulations were first tested in GPs. However, adjuvants ISA 35, IMS 3012 and PetGel induced a low VP7 Ab response in GPs compared to that induced in horses (Tables 1 and 2). Adjuvant ISA 27VG also induced higher nAb titres in horses by im vaccination than in GPs by immunization in the pre-scapular region [44]. Apparently, the GP model is not predictive for Ab responses in horses (Table 1 and 2). Therefore, we did not evaluate results of the GP trial in detail, but selected the best formulations to be used in horses. Subcutaneous (sc) vaccination with IMS 3012 formulated vaccine induced low nAb titres in horses (Table 2). Local reactions by ISA 35 in horses after sc vaccination were similar as for 5% PetGel formulated vaccine (Table S1). nAb titres raised by 5% PetGel were however much higher (Table 2). Therefore PetGel was selected as preferred adjuvant to be further studied in horses.

Studies on adverse effects and inflammatory reactions after vaccination with inactivated AHS vaccine have been scarcely or not reported [43], although this safety aspect is extremely important for the equestrian industry and for owners of pet horses. Sc vaccination induced temporary or long-term local reactions in some vaccinated horses (Tables S1 and S2). Ronchi et al observed temporary mild inflammatory reactions after sc injection of ISA 27VG and Montanide™ gel without AHSV antigen [45]. We compared intramuscular (im) vaccination with sc vaccination of formulated inactivated AHS vaccine for two different formulations with PetGel and MoreinX G3 (G3) as adjuvants (Table S2). Clearly, local reactions by im vaccinations were less than by sc vaccinations but some local reactions were still observed for both formulations (Table S2). By coincidence, we mixed up prime-boost vaccination for four horses and these were vaccinated with heterologous adjuvants (10% PetGel and G3, and vice versa) and by different routes. These horses were not selected for challenge because of local reactions after sc boost vaccination (Table S2). Im vaccination with 10% PetGel or G3 formulated vaccine induced promising high nAb titres (Table 3).

Except for horse B34 (im G3 group) with a nAb titre of 4, horses im vaccinated twice with 10%PetGel or G3 formulated vaccine were selected to study protection against virulent AHSV4 (Table 3). Horses 80A and 005 had the lowest nAb titres of 64 and 32, respectively, and did not survived AHSV challenge. Horses, 658, C3C, 24C, A03, D6C, and A09 with nAb titres of $\geq$ 64 survived AHSV challenge, although they developed viremia and fever for one or more days (Fig. 2). Thus, the threshold of the nAb titre for protection is approximately 64 (horses C3C and 80A). Apparently, the nAb titre on 49 dpv/22 dpb, two months before AHSV challenge, was indicative for protection against lethal AHSV later on. In conclusion, six out of nine horses, that were im vaccinated twice with 10% PetGel or G3 formulated AHS vaccine, were protected against lethality but not against disease and not against viremia (Fig. 2). Clearly, the efficacy of this prototype inactivated AHS vaccine needs improvement. Others achieved full protection short after vaccinations with concentrated AHSV antigen in combination with other adjuvants [39,44], or also observed incomplete protection after several annually repeated vaccinations [43].

Culture medium harvests containing AHSV4LP prior to CPE will minimize the amount of non-structural NS3/NS3a protein in antigen batches. This implies serological DIVA (Differentiating Infected from Vaccinated) based on NS3 serology as shown for purified inactivated AHS vaccine [40]. Previously, a non-validated NS3 cELISA was successfully used a DIVA test accompanying DISA vaccine lacking 77 amino acids in NS3/NS3a[47]. Here, horse sera on 0 dpv showed blocking up to 42% (Table 3), indicating that the threshold value of the NS3 cELISA is about 45% blocking. Horses A09 and horse 658 showed 57% at 49 dpv, and 50% at 125 dpv, respectively (Fig. 3B). Seropositivity for NS3/NS3a Abs after vaccinations could be caused by traces of NS3/NS3a protein. NS3/NS3a protein of AHSV is not essential for virus replication *in vitro* [34,57]. AHSV lacking NS3/NS3a enables DIVA by NS3-based serology [37,47]. Furthermore, exchange of serotype specific outer shell proteins has been explored [34,35,58]. Taken together, these AHSV variants lacking NS3/NS3a expression could be used for the production of antigens for inactivated DIVA vaccines for different AHSV serotypes.

Ideally, vaccination is completely safe, fully protective, cheap, and distinguishable (DIVA). Above all, vaccine should be licensed and accepted by users to guarantee its application. The ultimate vaccine profile is balancing between safety, efficacy and costs. However, this 'balance' can vary for users and countries because of the local situation with respect to AHS and the local equestrian industry. Owners of pet horses and high performance horses will be very reluctant to use vaccine with even a small chance on inflammatory reactions. Still, inactivated AHS vaccine is completely safe with regard to reverse to virulence, uncontrolled spread and reassortment. In contrast, many experimental AHS vaccines, such as viral vector vaccines and AHSV-based vaccine candidates, are completely safe with regard to local reactions [24]. Likely, boost vaccinations will be required for full and long-lasting protection, in particular taking the lifespan of horses and nine AHSV serotypes into account. DIVA vaccination will support safe trade and movement of horses, will be helpful for disease control, and will shorten the time to regain the AHS-free status. DIVA compatibility of inactivated AHS vaccine is in particular feasible with AHSV antigen free of NS3/NS3a protein, like for the non-transmittable replicating AHS DISA vaccine platform [37,47]. Of course, other experimental vaccine approaches not using VP7 are DIVA compatible with the extensively validated and commercially available VP7 cELISA.

Summarizing, advantages of inactivated AHS vaccine compared to LAVs are safety with regard to vaccine spread and DIVA compatibility. Safety with regard to inflammatory reactions after vaccination are not in favour compared to experimental replicating AHS vaccines and applied LAVs. Improvement of the efficacy by subsequent boost vaccinations or higher antigen load are not preferred and will be more expensive, in particular taking broad protection against all nine serotypes into account. Therefore, safety and efficacy should be improved by a much better adjuvant resulting in a faster and stronger protective response without causing inflammatory effects. Further research on adjuvant for use in horses is needed to develop cost-competitive inactivated AHS DIVA vaccines that are completely safe, efficacious and acceptable for horse owners.

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.

Acknowledgments

Montanide™ adjuvants were kindly provided by Seppic Montanide™, France. MoreinX G3 was kindly provided by MoreinX AB, Sweden. The authors thank dr. Paloma Rueda, Ingenasa, Madrid, Spain for supplying the prototype NS3 cELISA. We are grateful to the animal caretakers of Deltamune, and the horse reserve. We thank René van Gennip for fruitful discussions and critical reading of the manuscript. This research was funded by the Dutch Ministry of Agriculture, Nature and Food Quality (CVI-projects 1691063000, 1630047700 and 1600025-01).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2020.08.072.

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