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Efficacy of three candidate Rift Valley fever vaccines in sheep

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ABSTRACT

Rift Valley fever virus (RVFV) is a mosquito-transmitted Bunyavirus that causes high morbidity and mortality among ruminants and humans. The virus is endemic to the African continent and the Arabian Peninsula and continues to spread into new areas. The explosive nature of RVF outbreaks requires that vaccines provide swift protection after a single vaccination. We recently developed several candidate vaccines and here report their efficacy in lambs within three weeks after a single vaccination. The first vaccine comprises the purified ectodomain of the Gn structural glycoprotein formulated in a water-in-oil adjuvant. The second vaccine is based on a Newcastle disease virus-based vector that produces both RVFV structural glycoproteins Gn and Gc. The third vaccine comprises a recently developed nonspreading RVFV. The latter two vaccines were administered without adjuvant. The inactivated whole virus-based vaccine produced by Onderstepoort Biological Products was used as a positive control. Five out of six mock-vaccinated lambs developed high viremia and fever and one lamb succumbed to the challenge infection. A single vaccination with each vaccine resulted in a neutralizing antibody response within three weeks after vaccination and protected lambs from viremia, pyrexia and mortality.

1. Introduction

RVFV is a zoonotic mosquito-borne Bunyavirus of the Phlebovirus genus. The RVFV genome comprises three single-stranded RNA segments [1]. The large (L) segment encodes the viral polymerase. The medium (M) segment encodes two surface glycoproteins Gn and Gc [2]. This segment also encodes a non-structural protein named NSm, which was shown to have an anti-apoptotic function, and a 78-kDa protein of which the function is not known [3]. The small (S) segment encodes the nucleocapsid protein and a non-structural protein named NSs. The latter is recognized as the major virulence factor of the virus by counteracting host innate immune responses [4–7].

The first recorded outbreak of RVF occurred on the shores of Lake Naivasha in Kenya in 1930, where 3500 lambs and 1200 ewes died of acute liver necrosis within a period of seven weeks. This outbreak and follow up studies revealed that the virus affects cattle, goats as well as humans [8,9]. RVFV has since been responsible for large outbreaks across the African continent and the Arabian Peninsula [10–15], resulting in many casualties among both domesticated livestock and humans. RVF epidemics are generally preceded by explosions of mosquito vector populations after periods of exceptionally heavy rainfall [16]. In semi-arid areas where the virus is endemic, outbreaks tend to occur every 25–35 years, whereas in savannah grasslands outbreaks occur more often, on average every 5–15 years. This cyclical nature of RVFV epidemics is still poorly understood. The largely unpredictable and explosive nature of RVFV outbreaks requires that vaccines are available that induce swift immunity after a single vaccination. These vaccines should not only be available to countries where RVFV is currently endemic but also to countries outside these areas since potential mosquito vectors are globally prevalent [17–19].

The first vaccine that was developed to control RVFV in livestock was produced by attenuation of a field isolate by serial passage in mouse brain [20]. This so-called Smithburn vaccine is still commercially available and can be used to protect adult animals from disease. Due to residual virulence, use of the Smithburn vaccine in gestating and young animals, which are the most susceptible to disease, is however not recommended. The commercial vaccine based on inactivated whole virus, which was included in the current study, is expensive to produce and requires a booster and annual revaccination for optimal protection. Considering these shortcomings, efforts were made to develop safer live-attenuated vaccines. This work resulted in the development of the MP-12 vaccine [21], the Clone 13 vaccine [7] and, more recently, in a recombinant

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virus that lacks both the NSs and NSm coding regions [22]. Several efficacy and safety trials in natural target species were recently reported [23–27] and the Clone 13 vaccine was recently registered and commercialized in South Africa.

The vaccines based on live-attenuated RVFV that are now available hold great promise for the future control of RVFV in endemic areas. Mass deployment of these vaccines outside these areas could however suffer from safety concerns. Subunit vaccines, DNA-based vaccines or vector vaccines can provide alternatives of optimal safety. The efforts made in the past decades to develop novel RVFV vaccines were recently reported in several comprehensive reviews [28–30].

Four different candidate vaccines were recently developed in our laboratory. The first is a recombinant avian paramyxovirus that expresses the RVFV structural glycoprotein Gn [31] or both Gn and Gc [32]. The LaSota strain of the Newcastle disease virus (NDV) that was selected for this application is used across the world as a vaccine for the control of NDV and is proven highly safe, even in the natural target species. Mammals are not natural reservoirs of NDV, which further adds to the safety of this approach and minimizes the chance of vaccination failure due to pre-existing immunity in the field. Another advantage of this vaccine is its efficient and low-tech production in embryonated eggs. We previously demonstrated that the NDV-based RVFV vaccine (here referred to as NDV-GnGc) provides protection in mice and that a single vaccination in sheep results in a neutralizing antibody response [32].

In another approach, more focused on application in humans, we developed two subunit vaccines. The first is based on the ectodomain of the Gn structural glycoprotein, the second on virus-like particles (VLPs) resulting from the co-expression of the Gn and Gc proteins in *Drosophila* cells. Both vaccines were shown to provide complete protection against a lethal RVFV challenge dose in mice [33]. With the aim to further improve the efficacy of VLPs, we recently developed RVFV replicon particles, here referred to as nonspreading RVFV (NSR). NSR particles are capable of genome replication but incapable of autonomous spread. A single intramuscular vaccination with the NSR vaccine without added adjuvant provided solid protection in the mouse model [34].

We now report the efficacy of our candidate vaccines in sheep. Lambs were vaccinated once and challenged with a highly virulent RVFV isolate within three weeks after vaccination. Antibody responses were analyzed by virus-neutralization tests and a commercial ELISA and viremia was monitored by quantitative real-time PCR and virus isolation. We show that a single vaccination with these novel vaccines induces a neutralizing antibody response and protects against viremia, pyrexia and mortality.

2. Materials and methods

2.1. Preparation of the challenge virus

The RVFV virus that was used for challenge was previously rescued from cDNA [34]. The recombinant 35/74 (rec35/74) virus was derived from the sequence of strain 35/74 [35] and was titrated on baby hamster kidney (BHK) cells as tissue culture infective dose 50 ($TCID_{50}$) using the Spearman–Kärber algorithm [36,37] as previously described [34]. The virus was handled under biosafety level-3 laboratory conditions in class-III biosafety cabinets.

2.2. Preparation of the vaccines

The ectodomain of the Gn protein was produced using the *Drosophila* expression system (Invitrogen, Carlsbad, CA, USA) essentially as previously described [33], although an improved purification procedure was used. Briefly, the sequence encoding the ectodomain of the Gn protein was fused to a sequence encoding a combined FLAG-tag/enterokinase (EK) cleavage site and three Strep-tags separated by glycine linkers to allow easy detection and purification of the monomeric protein. The gene was introduced in pMT/BiP/V5-HisA (Invitrogen), which was used to express the protein in *Drosophila* Schneider 2 (S2) cells. The protein was purified from the culture medium using Strep-Tactin Sepharose according to the manufacturer's recommendations (IBA, Göttingen, Germany) and concentrated using an Amicon® Ultra-4 concentrator with a molecular mass cut-off of 30 kDa (Millipore, Billerica, MA, USA). The protein, named GneS3, was formulated in Stimune water-in-oil adjuvant (Prionics, Lelystad, The Netherlands) to a final concentration of 20 μg/ml.

Production of the NDFL-GnGc, here referred to as NDV-GnGc [32] and the NSR vaccine [34] were previously reported. The administered doses and routes of vaccination are depicted in Table 1.

Table 1
Route and dose of vaccines.

Vaccine	Route	Dose[a]	Adjuvant
OBP vaccine	Subcutaneous	Prescribed	Aluminum hydroxide gel
NDV-GnGc	Intramuscular	2×10^7 $TCID_{50}$	None
NSR	Intramuscular	10^7 $TCID_{50}$	None
GneS3	Subcutaneous	20 μg	Stimune water-in-oil

[a] All vaccines were administered in a volume of 1 ml.

2.3. Vaccination and challenge

Thirty conventional European breed lambs were purchased from a commercial sheep farm in The Netherlands and divided over five groups. Lambs were vaccinated once at the age of six weeks (day 0), as depicted in Table 1. Mock-vaccinated lambs were inoculated with PBS. On day 19 (days post challenge [DPC] 0), all lambs were challenged via the intraperitoneal route with 10^5 $TCID_{50}$ of RVFV rec35/74. EDTA blood samples were collected daily starting from day 19 (DPC 0) until day 26 (DPC 7) and again on days 28, 30, 33, 35, 37 and 40 (DPC 9, 11, 14, 15, 17 and 20). Serum samples were collected on days −7, 0, 7, 14, and daily from day 19 (day of challenge) to 26 (DPC 7) and finally on days 33 (DPC 14) and 40 (DPC 20). Body weights were determined weekly, on DPI −7, −1, 6, 13, 18, 25, 32, 39. Rectal body temperatures were determined on days −2 to 4 and, starting from day 17 (DPC −2), daily until the end of the experiment.

This experiment was conducted in accordance with the act on Experimental Animals of The Netherlands and approved by the Ethical Review Committee of the CVI-WUR.

2.4. Quantitative real-time PCR

Viral RNA was isolated from plasma samples using the QuickGene DNA tissue kit S (DT-S, Fuji Photo Film Europe GmbH, Dusseldorf, Germany) with the following modifications. Proteinase K solution (EDT, DT-S kit, 30 μl) and 3 μl polyadenylic acid A (polyA 5 μg/μl, Sigma, St. Louis, MO, USA) were added to 250 μl lysis buffer (LDT, DT-S kit). Of this mixture, 250 μl was added to 300 μl plasma. The mixture was heated at 72 °C for 10 min in a heating block and stored at −20 °C until further analysis. RNA isolation was subsequently performed using the QG-Mini80 Workflow (Fuji Film). The lysate was mixed with 350 μl 99% ethanol before loaded on the column. After three wash steps with 750 μl wash buffer (WDT, DT-S kit) the RNA was eluted with 50 μl elution buffer (CDT, DT-S kit). The material was stored at −70 °C until further analysis.

RNA samples (5 μl) were used for quantitative Taqman reverse-transcriptase real-time PCR (qPCR). The LightCycler RNA

Amplification Kit HybProbe (Roche, Almere, The Netherlands) was used and primers, probes and cycling conditions were used as previously described [38].

2.5. Virus isolation

Virus isolation from blood was performed on plasma samples. The plasma was mixed with an equal volume of culture medium (CO_2-independent medium [GIBCO™, Carlsbad, CA, USA] supplemented with 100 U/ml penicillin [GIBCO] and 100 μg/ml streptomycin [GIBCO], 2 mM L-glutamine [GIBCO] and 5% fetal calf serum [FCS]). To prevent clotting of the plasma following contact with divalent cations present in the culture medium, 10 ml medium was put in a Vacutainer® heparin tube (BD, Franklin Lakes, NJ, USA) and after mixing combined with 30 ml of culture medium. The amount of heparin present in the resulting 40 ml of medium was sufficient to prevent clotting during virus isolation assays. The plasma samples were diluted by 2-fold dilution steps and added to 96-wells plates containing 40 000 BHK cells/well. The plates were incubated for 1 h at RT, after which the culture medium was replaced. After five days, the plates were scored for cytopathic effect.

2.6. Serology

The ID Screen competition ELISA was used for the detection of RVFV-specific antibodies, according to the instructions of the manufacturer (ID-VET Montpellier, France). This ELISA makes use of plates coated with recombinant RVFV nucleocapsid (N) protein. Specifically, the ELISA detects competition of N protein-specific antibodies present in sera with a peroxidase-conjugated diagnostic antibody. Peroxidase activity is detected by conversion of 3,3′,5,5′-tetramethylbenzidine.

Neutralizing antibodies were detected by virus neutralization test (VNT). Briefly, approximately 200 $TCID_{50}$ of virus was incubated with two-fold serial dilutions of sera for 2.5 h at room temperature, after which BHK cells were added. After a 4–5 days incubation at 37 °C, the cultures were scored for cytopathic effect. Fifty percent end point titers were calculated using the Spearman–Kärber method as described previously [32].

2.7. Clinical chemistry

Clinical chemistry was performed with serum collected on the day of challenge (study day 19, DPC 0) and subsequently on days 20–25 (DPC 1–6), and on days 32 (DPC 14) and 38 (DPC 21). Enzyme analysis was performed using the Spotchem EZ SP-4430 analyser (Menarini Diagnostics, Valkenswaard, The Netherlands) using strips capable of detecting alkaline phosphatase (ALP), alanine transaminase (ALT), creatinine, total protein (TP) and blood urea nitrogen (BUN).

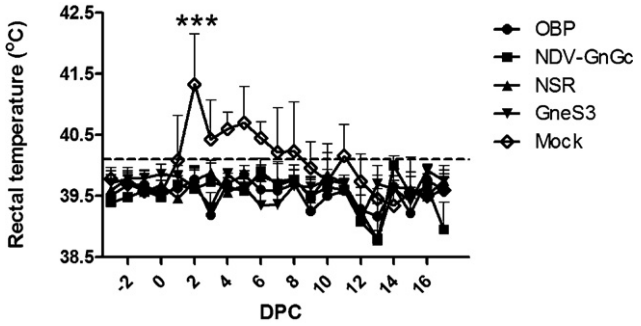

Fig. 1. Rectal temperatures of vaccinated and mock-vaccinated (mock) lambs before and after challenge with RVFV. Rectal body temperatures (°C) were determined daily. Fever was defined as a body temperature above 40.1 °C (interrupted line). Rectal body temperatures of vaccinated lambs are depicted as averages ($n = 6$) with SD. Rectal body temperatures of mock-vaccinated lambs determined after DPC 8 are depicted as averages of five measurements since one lamb from this group died on this day. A one-way ANOVA with Bonferroni's post test was used to determine the statistical significance of peak rectal temperatures between groups, which is denoted by asterisks (***$p < 0.0001$).

2.8. Statistical analysis

Statistical analyses were performed with the one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Statistical differences with p-values <0.05 were considered significant.

3. Results

3.1. Vaccination and challenge

After acclimatization for one week, all lambs were vaccinated as depicted in Table 1. On different time points after vaccination, the injection sites were inspected for possible adverse reactions. These inspections revealed mild to moderate swelling in four and five out of six lambs vaccinated with the OBP and GneS3 vaccine,

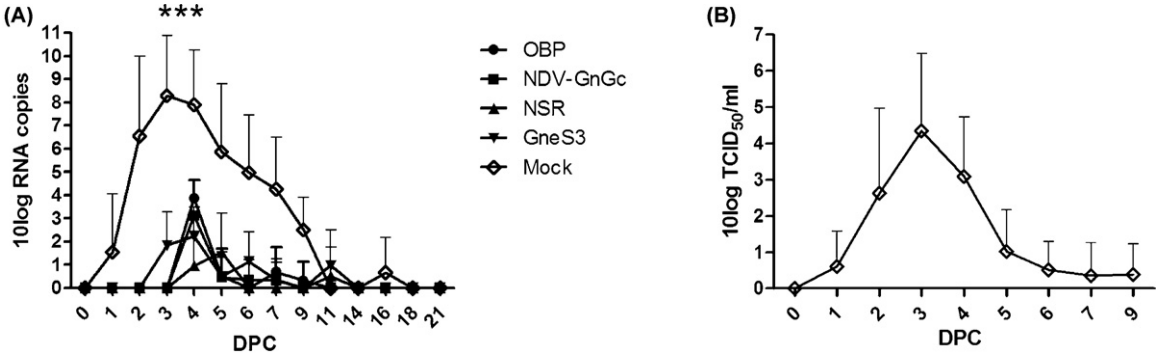

Fig. 2. Monitoring of viremia in vaccinated and mock-vaccinated lambs. (A) Detection of viral RNA by qPCR in plasma samples of vaccinated and mock-vaccinated lambs obtained at different days post challenge (DPC) with RVFV. A repeated measures one-way ANOVA with Bonferroni's post test was used to determine the statistical significance of differences in viral RNA levels (***$p < 0.0001$). Results obtained from vaccinated lambs are depicted as averages ($n = 6$) with SD. The results obtained from mock-vaccinated lambs obtained after DPC 8 represent averages of 5 determinations, since one of the lambs died on this day. (B) Virus titers detected in plasma samples obtained from mock-vaccinated lambs at selected DPC. Virus isolations using plasma samples of vaccinated lambs were negative and are not depicted.

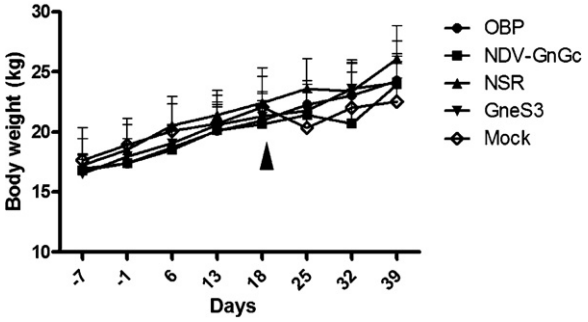

Fig. 3. Body weights of lambs vaccinated once with the indicated vaccines. The moment of challenge infection is indicated by the arrowhead. Body weights of vaccinated lambs are depicted as averages ($n = 6$) with SD. Body weights of mock-vaccinated lambs determined on days 32 and 39 are averages of 5 determinations since one lamb of this group died on day 27.

respectively. No adverse reactions at injection sites were observed in lambs vaccinated with NDV-GnGc or NSR (data not shown).

After challenge the rectal temperatures in the control group peaked at 2 DPC (Fig. 1). Peak rectal temperatures and the total days of fever (rectal body temperature $\geq 40.1\,^{\circ}C$) were both significantly lower (one-way ANOVA, $p < 0.0001$) in all vaccinated groups compared to the mock-vaccinated group.

Viral RNA in plasma samples was detected by qPCR. High levels of viral RNA were detected in all but one of the mock-vaccinated animals, peaking on DPC 3 (Fig. 2A). Mean viral RNA levels, determined between DPC 0–21, were significantly lower in all vaccinated groups (repeated measures one-way ANOVA, $p < 0.0001$) compared to the mock-vaccinated group.

Virus was isolated from plasma samples obtained from five of six mock-vaccinated lambs. In accordance to PCR results, the viral load peaked on day DPC 3 (Fig. 2B). Because viral RNA levels in plasma samples of vaccinated lambs were very low as determined

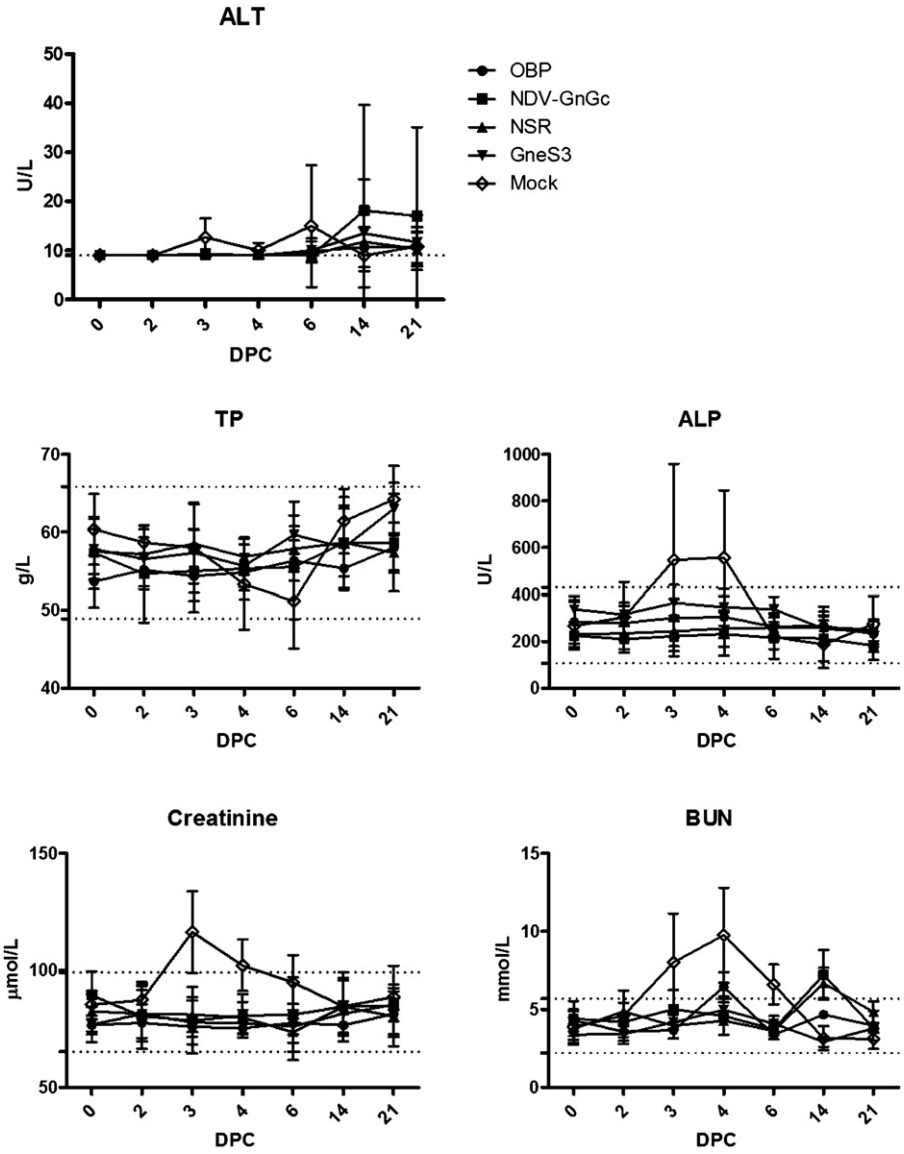

Fig. 4. Biochemical analysis of serum samples obtained from lambs vaccinated with the indicated vaccines or mock-vaccinated lambs at different time points post challenge (DPC) with virulent RVFV. Alkaline phosphatase (ALP), alanine transaminase (ALT), creatinine, total protein (TP) and blood urea nitrogen (BUN) concentrations measured in serum of vaccinated lambs are depicted as averages ($n = 6$) with SD. The results obtained with sera from mock-vaccinated lambs on DPC 14 and 21 are averages of 5 determinations, since one of the lambs died on DPC 8. Repeated measures one-way ANOVA with Bonferroni's post test was used to determine the statistical significance of differences in biochemical values. The results of these analyses are described in Section 3.

by qPCR, only selected samples with the highest PCR signals were used for virus isolation. These samples were either obtained on DPC 4 or 5 (Fig. 2A) and were tested in fourfold. No virus was isolated from these samples.

The body weights of the lambs were determined weekly. The lambs in the mock-vaccinated control group all displayed weight loss in the first two weeks after challenge (between days 18 and 25). Some weight loss, at a later time point (between days 25 and 32), was noted in five lambs vaccinated with NDV-GnGc and two lambs vaccinated with the NSR vaccine (Fig. 3). Repeated measures ANOVA demonstrated that body weights determined between DPC −1 and 39 of mock-vaccinated lambs differed significantly from those of lambs vaccinated with the NSR vaccine ($p < 0.05$).

To investigate the occurrence of liver and renal damage, biochemical analysis was performed on serum samples using the Spotchem EZ dry chemistry analyzer. Statistical significance was tested by repeated measures one-way ANOVA with Bonferroni's correction. Hepatic dysfunction was assessed by sequential measurements of serum alkaline phosphatase (ALP), alanine transaminase (ALT) and total protein concentrations (TP) (Fig. 4). Total protein concentration is assumed to represent mostly albumin levels. In mock-vaccinated lambs, ALP and ALT levels were clearly increased when compared to vaccinated lambs and TP levels were clearly decreased. Statistical significance ($p < 0.05$) was however only achieved when comparing ALP levels in plasma obtained from mock-vaccinated lambs (mock) and NDV-GnGc-vaccinated lambs.

The concentrations of blood urea nitrogen (BUN) and creatinine were measured to assess renal function. BUN levels in serum obtained from mock-vaccinated lambs were clearly on average higher than levels detected in the serum of vaccinated lambs (Fig. 4). Statistical significance was however not achieved.

Creatinine levels in serum from mock-vaccinated lambs were significantly higher when compared to levels detected in serum from vaccinated lambs (OBP vaccine, $p < 0.0005$; GneS3, $p < 0.005$; NSR, $p < 0.005$; NDV-GnGc, $p < 0.05$).

The liver of the one mock-vaccinated lamb that succumbed to the infection obtained at necropsy on DPC 8 was successfully used for virus isolation. No virus was isolated from any of the livers obtained from surviving lambs at the end of the experiment.

3.2. Antibody responses

Sera obtained on the day of vaccination (DPC −19) and at the day of challenge (DPC 0), which corresponds to 19 days post vaccination, were analyzed for the presence of neutralizing antibodies by VNTs as previously described [31]. Five out of six lambs in the group vaccinated with the OBP vaccine developed detectable levels of neutralizing antibodies (Fig. 5). Neutralizing antibodies were also detected in two lambs vaccinated with the NDV-GnGc vaccine and four lambs vaccinated with GneS3. All six lambs vaccinated with the NSR vaccine developed neutralizing antibodies and the titers in this group were also the highest (Fig. 5). No neutralizing antibodies were detected in mock-vaccinated lambs before challenge, but after challenge these sera contained the highest levels of neutralizing antibodies. The one mock-vaccinated lamb that did not display viremia also did not develop detectable levels of neutralizing antibodies. After challenge, neutralizing antibodies were detected in three lambs vaccinated with the OBP vaccine, five lambs vaccinated with NDV-GnGc or GneS3-vaccines and in all lambs vaccinated with the NSR vaccine.

All serum samples were also analyzed by the commercial ID Screen® Rift Valley Fever Competition ELISA (ID-VET, Montpellier, France), which detects antibodies against the N protein of RVFV. All sera obtained from control lambs that developed viremia were scored positive in the ELISA (Fig. 6). Sera obtained from lambs vaccinated with the OBP vaccine were all scored negative. In the groups vaccinated with GneS3, NDV-GnGc or NSR, one lamb in each group was scored positive after challenge.

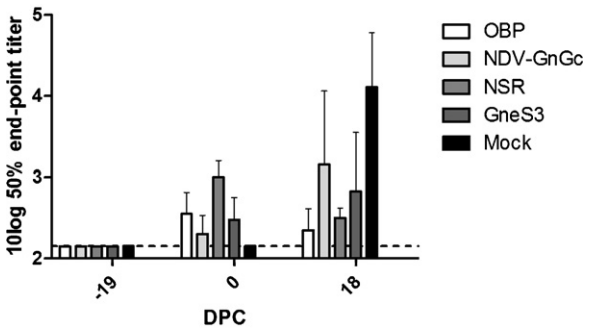

Fig. 5. Virus neutralization test with sera obtained from lambs vaccinated with the indicated vaccines or mock-vaccinated lambs obtained before challenge and at 18 days post challenge (DPC). Detection of neutralizing antibodies in sera obtained before vaccination (DPC −19), 19 days after the first vaccination (DPC 0) with the indicated vaccines and 18 days after challenge (DPC 18). Results obtained with plasma of vaccinated lambs are depicted as averages ($n = 6$) with SD. The results obtained with plasma from mock-vaccinated lambs on DPC 18 are averages of 5 determinations, since one of the lambs died on DPC 8. The detection limit of the assay is depicted by the interrupted line.

4. Discussion

We here report the efficacies of three novel RVFV candidate vaccines and a commercially available inactivated vaccine in sheep. The sheep in this study were vaccinated once at the age of six weeks and challenged 19 days later. Based on previous experimental work and field observations, RVFV infection in sheep of this age was expected to result in high viremia and fever, but only low mortality [39]. In addition, it was anticipated that clinical signs in sheep that do not succumb to the infection remain unapparent. Considering this, the primary aim of our study was not to prevent morbidity and mortality, but instead to investigate if our vaccines can significantly reduce viremia. Reduction of viremia by vaccination is likely to decrease the chance of RVFV transmission by mosquito vectors and thereby reduce the number of livestock and human casualties during epidemics.

Analysis of plasma samples by qPCR and virus isolation demonstrated that all but one of the mock-vaccinated lambs developed high viremia. The reason why one of the lambs did not develop high viremia remains unclear. In mock-vaccinated lambs, RNA levels approaching 10^{10} copies/ml were detected in plasma samples and virus isolations on these samples revealed titers of up to 10^6 $TCID_{50}$/ml. This result demonstrates that monitoring viremia by the M segment-based qPCR is much more sensitive than virus isolation. RNA levels in the blood were strongly reduced by vaccination with any of the vaccines and we were unable to isolate virus from plasma samples of vaccinated lambs. The results obtained from PCR analysis of plasma samples suggest that our vaccines do not provide sterile immunity within three weeks after vaccination but that vaccination does reduce viremia to levels undetectable by virus isolation. Recent studies suggested that vaccines based on live-attenuated RVFV can provide sterile immunity in sheep after a single vaccination. In contrast to the current work, titers of challenge virus in the blood of unvaccinated animals were not reported in these studies [23,25], making it difficult to appropriately qualify this suggestion.

Clinical disease was monitored by body weight measurements and biochemical blood analysis. Biochemical blood analysis revealed signs of liver and kidney damage in mock-vaccinated

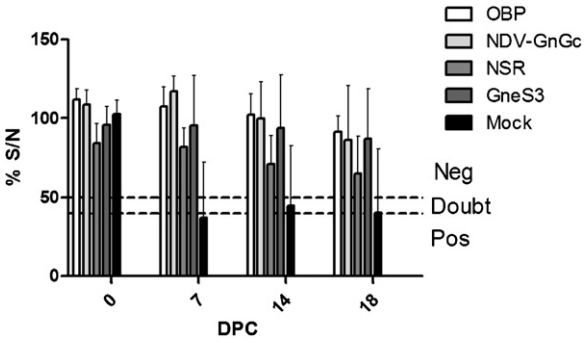

Fig. 6. Detection of N protein-specific antibodies in sera obtained from lambs vaccinated with the indicated vaccines or mock-vaccinated lambs at different days post challenge (DPC). S/N percentages less than or equal to 40% are considered positive, values between 40 and 50% are considered doubtful and values greater than 50% are considered negative. Results obtained with sera of vaccinated lambs are depicted as averages ($n = 6$) with SD. The results obtained with sera from mock-vaccinated lambs on DPC 14 and 18 are averages of 5 determinations, since one of the lambs died on DPC 8.

lambs in the first week after challenge and this correlated with a clear drop in body weight. Five lambs vaccinated with the NDV-GnGc vaccine and two lambs vaccinated with the NSR vaccine also revealed some weight loss in the second week after challenge. Although some variations in biochemical parameters were found in vaccinated animals, this did not correlate with weight loss and no viral RNA nor live virus was detected in the blood at these time points. The relevance of these findings therefore remains unclear.

Neutralizing antibodies are currently the only established correlate of protection against RVFV. A single vaccination with any of the vaccines evaluated in the current work resulted in a neutralizing antibody response within three weeks after vaccination. Interestingly, challenge infection of lambs vaccinated with the NDV-GnGc or GneS3 vaccine resulted in a clear boost of the neutralizing antibody response, whereas challenge infection of lambs vaccinated with either the OBP or the NSR vaccine did not boost this response (Fig. 5). A boost in neutralizing antibodies is likely to result from replication of the challenge virus in the vaccinated animal, leading to the suggestion that vaccination with the OBP vaccine or the NSR vaccine resulted in the strongest immunity. Analysis of sera for antibodies against the N protein also provides insight into replication of the challenge virus in the vaccinated animals. Only one lamb in each group vaccinated with the candidate vaccines seroconverted for antibodies against N, again demonstrating that vaccination strongly reduced replication of the virus.

It was interesting to observe that neutralizing antibody levels in some animals declined somewhat within three weeks after vaccination (Fig. 5). Similar findings were recently recorded during studies with the Clone 13 vaccine [24]. The consequences of this finding remain speculative, particularly since we found that lambs even lacking detectable levels of neutralizing antibodies at the moment of challenge were protected from viremia and disease. Considering this, it will be valuable to elucidate the correlates of protection provided by the different candidate vaccines in future studies.

In conclusion, we here demonstrate efficacy of our vaccine candidates in the major natural target species after a single vaccination. Further evaluation of the vaccines will include studies on minimum protective dose, onset and duration of immunity as well as safety and efficacy trials in gestating animals.

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