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Safety and immunogenicity of a live attenuated Rift Valley Fever recombinant arMP-12ΔNSm21/384 vaccine candidate for sheep, goats and calves

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ABSTRACT

Rift Valley fever (RVF) causes serious health and economic losses to the livestock industry as well as a significant cause of human disease. The prevention of RVF in Africa is a global priority, however, available vaccines have only been partially effective. Therefore, the objective of this study was to evaluate the safety and immunogenicity of a live, attenuated recombinant RVFV arMP-12ΔNSm21/384 nucleotide deletion vaccine candidate in domestic ruminants. Evaluation involved testing to determine the infectivity titer of the vaccine virus in Vero cells for industrial scale up vaccine production. Safety experiments were conducted to determine the potential of the vaccine virus to revert to virulence by serial passages in sheep, the possibility of virus spread from vaccinated sheep and calves to unvaccinated animals, and the potential health effects of administering overdoses of the vaccine to sheep, goats and calves. The immunogenicity of 3 doses of 10^4, 10^5 and 10^6 Tissue Culture Infectious $Doses_{50\%}$ ($TCID_{50}$) of the vaccine was assessed in 3 groups of 10 sheep and 3 groups of 10 goats, and doses of 10^5, 10^6 and 10^7 $TCID_{50}$ was evaluated in 3 groups of 10 calves subcutaneous vaccintation. The results showed that the infectivity titer of the vaccine virus was $10^{8.4}$ $TCID_{50}$/ml, that the vaccine did not spread from vaccinated to un-vaccinated animals, there was no evidence of reversion to virulence in sheep and the vaccine overdoses did not cause any adverse effects. The immunogenicity among sheep, goats and calves indicated that doses of 10^4–10^6 $TCID_{50}$ elicited detectable antibody by day 7 post-vaccination (PV) with antibody titers ranging from 0.6 log to 2.1 log on day 14 PV with sustained titers through day 28 PV. Overall, these findings indicated that the RVFV arMP-12ΔNSm21/384 vaccine is a promising candidate for the prevention of RVF among domestic ruminants.

1. Introduction

Rift Valley fever (RVF) is a mosquito-borne disease of ruminants and human caused by Rift Valley fever virus (RVFV) of the order Bunyavirales, family Phenuiviridae, genus Phlebovirus [1,2]. The disease poses a veterinary and public health as well as an economic threat in affected areas [3]. The RVFV genome contains a large (L), medium (M) and small (S) RNA segment of negative sense or ambisense polarity. The L segment encodes for the viral RNA-dependent RNA polymerase while the M segment encodes for the external glycoproteins (Gn and Gc) and the non-structural protein (NSm) that affects viral pathogenicity. The S segment is ambisense and codes for the nucleoprotein (N) and the non-structural proteins (NSs), a major virulence factor [4]. The geographic distribution of the RVFV enzootic region has expanded since the first recorded outbreaks during 1930–1931 in Kenya [5,6]. Major RVF outbreaks were reported during 1977 and 2003 in Egypt, East Africa between 1997 and 2008, South Africa, 1950–2011 and West Africa between 1987 and 2016 as well as outside mainland Africa (Madagascar and Comoros islands) and the Arabian Peninsula (Saudi Arabia and Yemen) between 1979 and 2010 [7–12].

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The development of effective prevention measures for RVF is a global priority because of the devastating impact to human and animal health in Africa and neighbouring countries as well as the threat of the spread and potential impact of RVFV beyond the enzootic region [4]. Several live-attenuated vaccines have been developed for livestock with the RVFV Smithburn and Clone 13 being the more commonly used vaccines. The Smithburn vaccine has been reported to be abortigenic and teratogenic, and Clone 13 is temperature sensitive with a potential teratogenic effect among pregnant sheep based on experimental studies [13–16]. Also, these two vaccines are not designed to elicit an immune response that can be used to distinguish naturally infected from vaccinated animals (DIVA). The live-attenuated RVF MP-12 candidate vaccine was developed by mutagenizing a pathogenic wild-type RVFV strain (ZH548) isolated from a RVF patient in Egypt [17]. This candidate was shown to be safe and efficacious in ruminants and nonhuman primates and human volunteers [18–24]. However, one report claimed that the RVF MP-12 caused abortions in sheep and fetal malformation, but this alleged observation has not been confirmed [25]. The RVF MP-12 parent vaccine virus was used to develop a recombinant RVFV arMP12-NSm-deletion (del) (21/384) and RVFV MP-12NSs-del (16/198) vaccine candidates with nucleotides deleted from the non-structural M and S viral RNA segments [26,27]. The primary reason for the deletion of the non-structural genes was to provide the potential for developing a vaccine with a biomarker that would be DIVA compatible. The deletion vaccine candidates were prepared by reverse genetics technology using the RVFV MP-12 consensus RNA sequence bit having nucleotides 21-384 and 16-198 deleted in the non-structural regions of the genome M and S segments, respectively [26–28]. Experimental studies in sheep, including pregnant animals and in calves demonstrated that these recombinant vaccines were safe, efficacious and non teratogenic [29–31]. However, the neutralizing antibody response of sheep to arMP-12NSs16/198 was poor in comparison to the RVFV arMP-12ΔNSm21/384 candidate [30]. Therefore, the latter candidate was selected for further studies as a more immunogenic vaccine candidate in target domestic ruminant animal species, including sheep, goats and cattle.

Although the original RVFV arMP-12ΔNSm21/384 vaccine candidate was found to be safe and efficacious in sheep and calves, the recording of the data during the development and the laboratory facilities in the United States was not in compliance with Good Laboratory Practices (GLP) [26,27]. Since the ultimate goal is to develop and license an improved veterinary vaccine for the prevention of RVF in livestock, the primary aim of this study was to employed reverse genetic technology to regenerate the RVF arMP-12ΔNSm21/384 candidate vaccine virus under Good Manufacturing Practices (GMP). As additional aims, the replication characteristics of this vaccine virus was determined in vertebrate cells as an assessment for industrial scale up capacity to mass produce the vaccine candidate, and the safety and immunogenicity of a lyophilized formulation of the vaccine was evaluated in calves, sheep and goats.

2. Material and methods

2.1. Good manufacturing practices

All procedures and data recording and storage involved in this study were performed in accordance with GMP at the Multichemical industry (MCI) Sante Animale biopharmaceutical facility located in Mohammedia, Morocco. Also, the MCI quality management system received triple certification Quality-Safety-Environment (QSE) according to ISO9001, OHSAS 18001 and ISO 14001. As an animal health company, MCI specializes in the production and marketing of veterinary vaccines and medicines.

2.2. Rescue of the RVF MP-12NSm-del virus

The recovery of RVF arMP-12ΔNSm21/384 virus from the parent recombinant arRVF MP-12 vaccine candidate was performed by a reverse genetic technique as described previously [26,27]. Briefly, the infectious recombinant RVF arMP-12ΔNSm21/384 virus was generated by transfection of BHK/T7-9 cells with 6 plasmids synthesized by Genescript (pProT7-avS (+), pProT7-avM (+) NSmdel21/384, pProT7-avL (+), pT7-IRES-vN, pT7-IRES-vL, pCAGGS-vM) using a stably express T7 RNA Polymerase. The virus was propagated by one passage on confluent monolayers of Vero cells to confirm the identity of the virus using PCR and nucleotide sequencing. A stock preparation of the virus was titrated on the same cells using both a plaque assay and tissue culture infectious dose$_{50}$/ml (TCID$_{50}$/ml) methods in Vero cells [28,32].

2.3. RVF arMP-12ΔNSm21/384 virus sequencing

The identity of the rescued RVF arMP-12ΔNSm21/384 virus was confirmed by qualitative real time polymerase chain reaction assay (QPCR) that targeted the L and M viral RNA segments of the virus [33], and then sequenced in Genewiz laboratories (GENEWIZ Global Headquarters; USA), using Next Generation Sequencing technology (NGS) Illumina method 1x50bp SR, HiSeq2500, High Output, per lane (V4 chemistry). Extraction of the viral RNA was performed as recommended by QIAamp Viral RNA Mini Kit (QIAGEN). The cDNA was prepared using a random primers kit Ovation RNA-Seq System V2 (8rx) kit that provided a rapid, simple and sensitive whole transcriptome RNA based on Ribo-SPIA technology (NuGEN's).

2.4. Viral replication kinetics

Cell cultures, including Vero and Baby Hamster Kidney 21 (BHK-21) cells were propagated in Dulbecco's Modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS) in 6 wells microplates at 37 °C and 5% CO_2. When the culture monolayers were 90% confluent, 0.1 ml of the RVF arMP-12ΔNSm21/384 virus was inoculated onto each culture at a concentration of 0.1, 0.01 and 0.001 multiplicity of infection (MOI) to determine the infectivity titers. The virus was adsorbed on cell cultures for 60 minutes (min) and then the monolayers were washed with phosphate buffered saline (PBS) and 10 ml of DMEM supplemented with 1% FBS were added to each culture. Cultures with the virus inoculum were incubated at 37 °C and 5% CO_2. Every 24 hours (h), culture supernatant was sampled to determine the infectivity titers of the extra-cellular virus, and cultures were frozen and thawed before titration in the above cells to determine the infectivity titers of the total intracellular and extra-cellular virus. Infectivity titers based on the detection of cytopathic effect (CPE) were calculated by the Reed-Muench method and expressed as TCID$_{50}$/ml throughout this report [34].

2.5. Vaccine production process optimization and industrial feasibility

The pre-master RVF arMP-12ΔNSm21/384 vaccine candidate virus seed stock was produced by infecting Vero cells propagated in roller bottles and then the virus yield was mixed at a concentration of 40% with a stabilizer consisting of 4% peptone, 8% sucrose and 2% glutamate. The vaccine was freeze-dried in a lyophilizer according to a cycle of 46 h and then tested for sterility, identity and adventitious agent [35,36].

The optimization of RVF arMP-12ΔNSm21/384 vaccine virus yield for industrial production feasibility was determined by comparing the replication of the virus in Vero E6 cells propagated in roller bottles (4250 cm^2) versus cell factory (6300 cm^2) bottles. Briefly, confluent monolayers of Vero cells were inoculated with the virus at a MOI of 0.01, and after 48 h incubation of the cells and inoculum at 37 °C, the supernatant was harvested and used to determine the infectivity titers based on the detection of CPE in Vero cells. The antibody titers were calculated by the Reed-Muench method and infectivity endpoints expressed as $TCID_{50}$/ml [34].

2.6. Purity testing

The pre-master RVF arMP-12ΔNSm21/384 vaccine virus seed stock was tested for adventitious agents (viruses and bacteria) by inoculating aliquots of un-neutralized and neutralized virus onto confluent monolayers of Vero, Madin Darby bovine kidney (MDBK) and lamb testis cells. The infectivity of the virus was neutralized by incubating equal volumes of the virus suspension and pathogen free RVF monospecific anti-serum for one hour at 37 °C. Cells were observed microscopically once daily for 7 days for cytopathic effect and for hemadsorption of red blood cells following the initial inoculation of cells and 3 subsequent blind passages. Confirmation of the identity of the virus was determined by performing neutralization of infectivity test in Vero cells using pathogen free RVF monospecific anti-serum and by performing PCR assay for RVF viral RNA by two qPCR that targeted the L and M viral RNA segments of the virus.

2.7. Animals

All animals were purchased from local vendors, including sheep of Sardi species (6–9 months old), goats of Beni Arous species (6–9 months old) and calves of Brune de l'Atlas species (6–12 months old) that were housed in the MCI Animal Biosafety Level 3 (ABSL 3) animal facility. All animal experiments involving the evaluation of the RVF arMP-12ΔNSm21/384 vaccine were carried out in accordance with international guidelines for the care and handling of experimental animals as described in a protocol approved by "The internal ethic committee for animal experiment, MCI Santé Animale". Animals were manually restrained and aliquots of 10 ml of blood were obtained from the jugular vein of each animal using a 20 gauge needle attached to a 10 ml syringe. Serum expressed after overnight at 4 °C for each blood sample was used to determine the antibody response of the vaccinated animals using an enzyme-linked immuno-adsorbent assay (ELISA) and virus neutralization test (VNT) as described below. ELISA testing was only performed on selected sera samples. The animals were then divided into 2 groups, including the ones to be vaccinated and those to serve as controls as described below. Each group of animals was managed and utilized in accordance with a protocol approved by the MCI Institutional Animal Care and Use Committee.

2.8. Vaccine spread

An experiment was performed to determine if the RVF arMP-12ΔNSm21/384 vaccine virus spread from vaccinated to unvaccinated animals. Ten sheep and 10 calves were each vaccinated SC with a dose of 10^5 and 10^6 $TCID_{50}$ of the vaccine virus, respectively. Vaccinated animals were kept in the same pens with 10 unvaccinated sheep or 10 unvaccinated calves. Blood samples were obtained as described above on days 0, 7, 14, 21 and 28 PV from both the control and vaccinated animals. Samples from cohabitant animals on days 0 and at day 28 PV were tested for IgG antibody by an ELISA and then all samples were tested for antibody at 1:3 serial dilutions by VNT.

2.9. Vaccine virus reversion to virulence

The potential for the RVF arMP-12ΔNSm21/384 vaccine virus to revert to virulence was tested by performing 5 serial passages of the virus in sheep. A group of 5 animals were inoculated SC with a dose of 10^5 $TCID_{50}$ and after 4 days of observations for elevated temperature and other signs of illness, animals were euthanized with sodium pentobarbital (120 mg/kg intravenously) and samples of spleens were mixed with PBS and re-injected into a second group of 5 naïve animals. Three additional passages were conducted similarly. The last passage was conducted on a group of 10 sheep that were observed for 21 days before euthanasia. At each passage, the spleen mixture was analyzed for RVF viral RNA by PCR and 3 blind passages were made on Vero cells in an attempt to detect viral CPE. Spleen mixtures of each passage in animals were tested for RVF viral RNA by two qPCR assays that targeted the L and M viral RNA segments of the virus [33,37]. RVF viral RNA extracted from the RVFV arMP-12ΔNSm21/384 vaccine virus were included in each test run as controls to verify that the qPCR assay performed properly.

2.10. Vaccine immunogenicity and overdose safety

The projected doses of RVFV arMP-12ΔNSm21/384 based on the infectivity titer of the virus for use to evaluate the immune response were 10^4 and 10^5 $TCID_{50}$ for goats and sheep, and 10^5 and 10^6 $TCID_{50}$ for calves subcutaneous vaccination (SC). The safety of an overdose of the RVF MP-12NSm-del vaccine virus was evaluated by vaccinating 10 sheep and 10 goats each SC with a dose 10^6 $TCID_{50}$, and 10 calves SC with 10^7 $TCID_{50}$. Five sheep, 5 goats and 5 calves were used as unvaccinated control. Blood samples as described above were collected at days 0, 7, 14, 21 and 28 post vaccination (PV) and at days 0, 7 and 14 for the animals that received the overdose, and all samples were tested for RVFV antibody by the VNT as described below. All animals were observed and examined daily for abnormal local and systemic reactions, including inflammation at the injection site, nasal discharge, hyper-salivation, anorexia, asthenia or diarrhea. Also, rectal temperature was recorded for each animal two days prior to vaccination and at the time of vaccination and on day 14 PV.

2.11. Virus neutralization

The immune response of animals to the RVF arMP-12ΔNSm21/384 vaccine virus was determine by testing sera samples collected from the animal PV using the VNT method as described in the OIE Terrestrial Manual [38]. The test was based on serial 1:3 dilutions of heat inactivated sera, mixed with a constant dose of RVF arMP-12ΔNSm21/384 virus (100 $TCID_{50}$), and then incubated for one hour, and inoculated onto Vero cells to be observed for CPE on day 5 of the incubation period. A mixture of equal volumes of the virus dose and DMEM was incubated for one h and then tested on Vero cells to verify the dose of virus used in the VNT. The neutralizing antibody titer was calculated in accordance with Reed and Muench method [32].

2.12. ELISA

Sera samples from sheep, goats and calves were tested at a 1:100 dilution for RVFV antibody by an ELISA using a commercial kit ID Screen® Rift Valley Fever Competition Multi-species (ID.Vet Innovative Diagnostics). Briefly, sera samples were added to 96 well plates, coated with a recombinant RVF nucleoprotein (NP)

and incubated for 1 h at 37 °C. After washing the wells of the plates with PBS, 100 μl of an anti-NP peroxidase conjugate was added to fix the remaining free NP epitopes. After 30 min of incubation at room temperature, 100 μl of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution were added to each well. The reaction was stopped after 15 min at room temperature by the addition of 100 μl of 0.16 M sulfuric acid, and then the reactivity results were read at 450 nm. Antibody positive and negative cut-off values were calculated as recommended by manufacturer with the sera samples being negative, if a percentage of competition was S/N > 50%, doubtful if a percentage of competition was 40% < S/N ≤ 50% and positive if a percentage of competition was S/N ≤ 40%.

2.13. Statistical analyses

The differences between RVFV antibody titers obtained with different doses of the RVFV arMP-12ΔNSm21/384 vaccine was tested for significant using a one-way analysis of variance (ANOVA) followed by a student *t*-test. Values of p = ≤0.05 were considered significant.

3. Results

3.1. Rescue of the RVF arMP-12ΔNSm21/384 virus

BHK/T7-9 cells transfected with the 6 arRVF MP-12 virus plasmids synthesized by Genescript (pProT7-avS (+), pProT7-avM (+) NSmdel21/384, pProT7-avL(+), pT7-IRES-vN, pT7-IRES-vL, pCAGGS-vM) showed CPE on day 5 post inoculation (P.I). The infectivity titer of the virus in Vero cells was $10^{3.4}$ $TCID_{50}$/ml and $10^{2.9}$ PFU/ml. The virus was then inoculated onto a confluent monolayer of Vero cells. Sixteen individual RVF arMP-12ΔNSm21/384 virus clones were isolated and of the two clones that showed the highest infectivity titers, one clone was selected to develop a RVFV vaccine candidate. The titers of the selected virus clone after one passage on Vero cells was $10^{7.0}$ $TCID_{50}$/ml and $10^{7.3}$ PFU/ml.

3.2. RVF arMP-12ΔNSm21/384 virus clone sequencing

The reads alignments of the RVFV arMP-12ΔNSm21/384 clone sequences were mapped to L, M and S reference sequences (accession numbers DQ375403 DQ380206, DQ380151) using CLC Genomics Server program (GENEWIZ company). The analysis of the FASTAQ read alignments of the RVFV arMP-12ΔNSm21/384 clone confirmed that the L and S segments were identical to the RVFV arRVF MP-12 at 99.96% and 99.94.3%, respectively. The M RNA segment was compared to the sequence of the original RVFV arMP-12ΔNSm21/384. The results showed that the sequence included cDNA fragments that matched with RVFV arMP-12ΔNSm21/384 sequences at 100%, but not with parental RVFV MP-12 and that all positions in the reference sequence were covered by the read values, thus confirming the presence of corresponding M-segment sequence in the generated virus.

3.3. Viral replication kinetics

The kinetics of the RVFV arMP-12ΔNSm21/384 virus replication in Vero and BHK − 21 cells are shown in Tables 1 and 2. The maximum infectivity titer on BHK −21 cells was $10^{8.0}$ $TCID_{50}$/ml and was first observed on day 2 P.I. at an MOI of 0.1 of total virus. On Vero cells, the maximum titer of the virus was $10^{8.4}$ $TCID_{50}$/ml on day 2 P.I. at a MOI of 0.1. The titer of RVFV arMP-12ΔNSm21/384 virus decreased progressively up to day 4 P.I. when complete lysis was observed for all of the cells. A difference of 1.2–1.7 logs was observed between the titer of the extracellular and the total virus.

3.4. Vaccine production process optimization and industrial feasibility

The RVFV arMP-12ΔNSm21/384 vaccine virus was inoculated at a MOI of 0.01 onto Vero cells propagated in factory and roller bottles. The highest titers obtained were $10^{7.4}$ and $10^{8.4}$ $TCID_{50}$/ml for extracellular and total virus, respectively on day 2 P.I. in cells propagated in factory bottles. The titer of virus in cells propagated in roller bottles was $10^{7.2}$ $TCID_{50}$/ml for extracellular virus and $10^{8.2}$ $TCID_{50}$/ml for total virus.

3.5. Lyophilized vaccine virus

The lyophilized RVF arMP-12ΔNSm21/384 vaccine virus retained its identity and residual moisture, and had a final titer of $10^{6.8}$ $TCID_{50}$ per vial. It was free from any adventitious agents; no cytopathic or hemadsorbant effects after 3 passages was observed, and no extraneous agents was detected by PCR analysis at the end the incubation period. The vaccine was stable at 24 °C for 12 days, at 37 °C less than 3 days and at 4 °C more than 12 months (Daouam S., 2018, unpublished data).

3.6. Animal testing

The safety of an overdose of the RVFV arMP-12ΔNSm21/384 vaccine virus was evaluated by vaccinating 10 sheep and 10 goats each with a dose 10^6 $TCID_{50,}$ and 10 calves each with 10^7 $TCID_{50.}$ Five sheep, 5 goats and 5 calves were used as unvaccinated controls. All animals, including the controls remained in good health based on the absence of any rise in body temperature, and no clinical signs or local inflammation at the injection site during the 14 day PV observation period.

The possibility that the RVFV arMP-12ΔNSm21/384 vaccine virus could spread among animals was addressed by housing 10 sheep and 10 calves vaccinated subcutaneously (SC) with a dose of 10^5 and 10^6 $TCID_{50}$ of the vaccine, respectively, in the same pen with 10 unvaccinated sheep and 10 unvaccinated calves. Assay of blood samples obtained on day 28 PV indicated that the vaccinated animals developed RVFV neutralizing antibody and that all of the samples from unvaccinated animals were negative for RVFV antibody, thus indicating that virus did not spread from the vaccinated to the unvaccinated animals.

The potential reversion of the RVFV arMP-12ΔNSm21/384 vaccine virus to virulence was tested by 5 serial passages of the virus in sheep. The body temperature of all vaccinated animals remained within normal limits and none showed clinical signs or any local inflammation. Viral RNA was not detected by PCR and virus was not detected by assays for CPE in Vero cells of homogenates of the spleen that were obtained post-mortem from sheep after each of the 5 serial passages, including 3 blind passages after each serial passages in Vero cells, thus demonstrating that the virus did not revert to virulence.

The immunogenicity of the RVFV arMP-12ΔNSm21/384 vaccine was evaluated by vaccinating 20 sheep, 20 goats and 20 calves, SC with 2 different doses (n = 10 animals/dose) of the vaccine for each species, including 10^4 and10^5 $TCID_{50}$ for sheep and goats and 10^5 and 10^6 $TCID_{50}$ for cattle. The antibody response to the overdoses of the vaccine are also included to compare the 3 administered doses of the vaccine for each species (Fig. 1). The antibody titers elicited by the 3 doses did not differ significantly (p = >0.05) for sheep and goats. Also, the overall antibody response profile was somewhat similar in sheep and goats with antibody being detectable on day 7 PV followed by an increase with peak titers on day 14 PV, and then titers decreased or remain about the same through

Table 1
Kinetics of RVF arMP-12ΔNSm21/384 virus replication of extracellular and total virus in Vero cells.

Hours post inoculation	Extracellular virus			Total virus		
	0.1*	0.01	0.001	0.1	0.01	0.001
24	6.4**	5.8	4.8	7.5	7.9	6
48	7.0	7.0	5.9	8.4	8.2	7.3
60	6.9	7.4	6.9	7.7	8.0	7.9
72	6.5	7.5	7.5	7.4	7.8	7.5
96	6.3	6.7	6.9	7.5	7.5	7.2

* MOI.
** Infectivity titer (log $TCID_{50}$/ml).

Table 2
Kinetic of RVF arMP-12ΔNSm21/384 vaccine virus replication of extracellular and total virus in BHK- 21 cells.

Hours post inoculation	Extracellular virus			Total virus		
	0.1*	0.01	0.001	0.1	0.01	0.001
24	6.0**	4.4	3.3	7.7	6.3	4.8
48	6.5	6.3	5.3	8.0	8.0	7.2
60	6.2	6	5.7	8.0	7.7	8
72	6.7	6	6.4	7.2	7.7	7.4
96	6.4	5.7	6	6.9	6.5	7.0

* MOI.
** Infectivity titer (log $TCID_{50}$/ml).

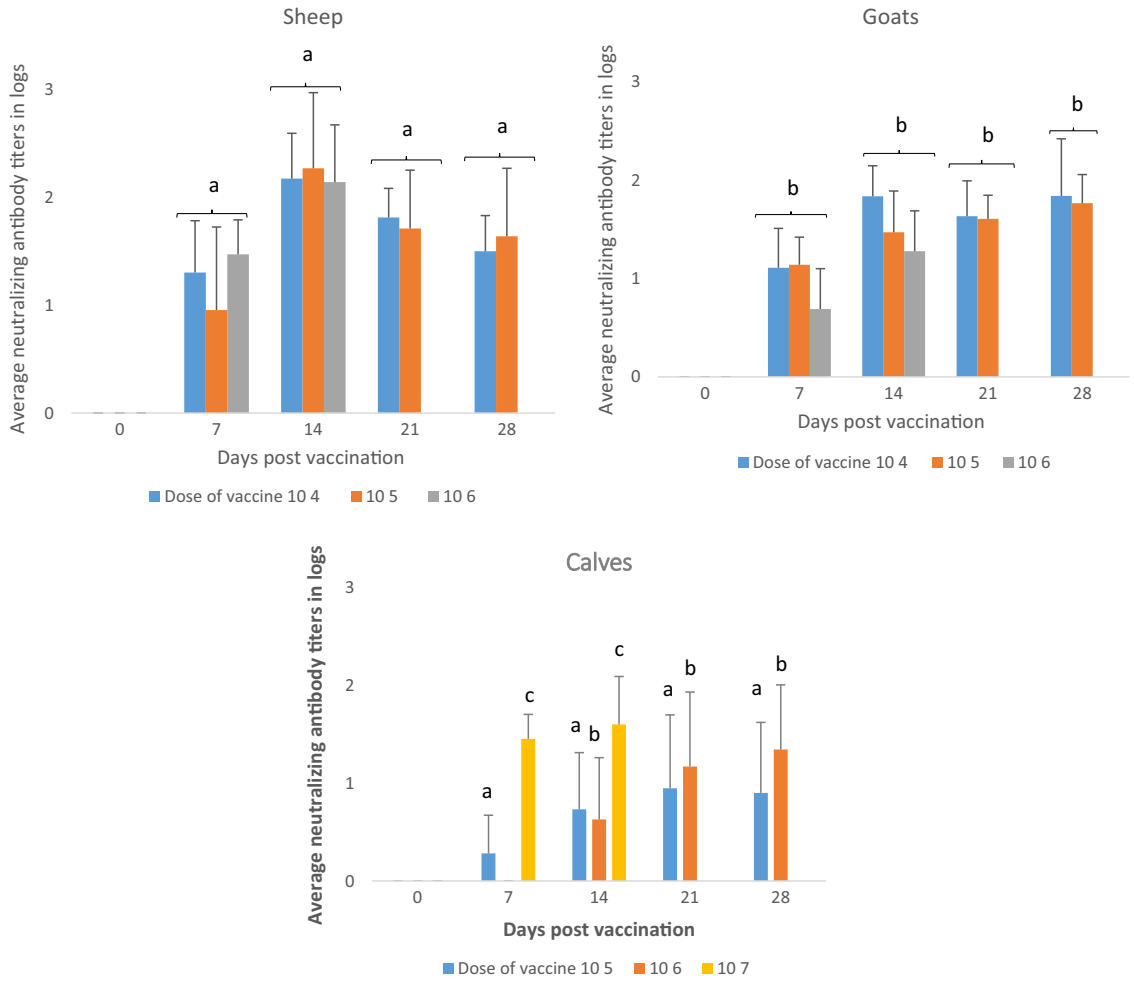

Fig. 1. Average antibody titers of vaccinated sheep goats and calves with different doses of RVF arMP-12ΔNSm21/384 vaccine expressed in log $_{10}$ values. Blood samples were not taken from the animals that received an overdose of the vaccine after day 14 PV, including 10^6 $TCID_{50}$ for sheep and goats, and 10^7 $TCID_{50}$ for cattle. The antibody titers elicited by the 3 doses of the RVFV arMP-12ΔNSm21/384 vaccine did not differ significantly ($p \geq 0.05$) for sheep (a) and goats (b). Antibody titers increased significantly ($P \leq 0.05$) for calves (a – c) with escalating doses of the vaccine ranging from 10^5 to 10^7 $TCID_{50}$.

Table 3
Summary of ELISA RVFV IgG antibody detected in sheep vaccinated subcutaneously with 10^4 $TCID_{50}$ of RVFV arMP-12ΔNSm21/384 vaccine candidate.

	Day 7		Day 14		Day 21		Day 28	
Animal #	OD*	Antibody	OD	Antibody	OD	Antibody	OD	Antibody
218	0.59	Positive	0.24	Positive	0.30	Positive	0.21	Positive
212	0.76	Doubtful	0.43	Positive	0.35	Positive	0.27	Positive
146	0.99	Negative	0.38	Positive	0.29	Positive	0.26	Positive
141	0.52	Positive	0.23	Positive	0.27	Positive	0.19	Positive
134	0.39	Positive	0.25	Positive	0.22	Positive	0.23	Positive
154	0.60	Positive	0.41	Positive	0.26	Positive	0.19	Positive
133	0.68	Doubtful	0.25	Positive	0.30	Positive	0.30	Positive
225	0.56	Positive	0.29	Positive	0.26	Positive	0.26	Positive
222	0.77	Doubtful	1.90	Negative	0.38	Positive	0.41	Positive
204	0.99	Negative	0.26	Positive	0.20	Positive	0.12	Positive

* – Optical density values.

Table 4
Summary of ELISA RVFV IgG antibody detected on day 7 post-vaccination in goats vaccinated subcutaneously with 10^4 $TCID_{50}$ of RVFV arMP-12ΔNSm21/384 vaccine candidate.

Dose 10^4TCID_{50}		
Animal #	OD*	Antibody
406	0.22	Positive
401	0.76	Positive
403	0.18	Positive
404	0.15	Positive
402	0.16	Positive
416	0.51	Positive
412	0.15	Positive
407	0.36	Positive
418	0.15	Positive
414	0.22	Positive

* Optical density values.

day 28 PV. Sheep developed detectable antibody by day 7 PV, including 90% of the animals that received a dose of 10^4, 60% that received a dose of 10^5 and 100% of the animals that received a dose of 10^6 $TCID_{50}$. Antibody titers peaked on day 14 PV when all animals had developed RVFV antibody. In goats vaccinated with doses of 10^4, 10^5 and 10^6 $TCID_{50}$, RVFV antibody was detected on day 7 PV, including 90% of the animals that received the 10^4 dose, 100% that received the 10^5 dose and 70% of the animals that received the 10^6 $TCID_{50}$ dose (Fig. 1). The maximum antibody titers were observed on day 14 PV.

Among calves vaccinated with 10^5 $TCID_{50}$ of the RVFV arMP-12ΔNSm21/384, 30% had developed antibody by day 7 PV and 60% had antibody by day 14 PV (Fig. 1). At a dose of 10^6 $TCID_{50}$, 70% of the animals had antibody by day 14 PV. In animals vaccinated with a dose of 10^7 $TCID_{50}$/ml, antibody was detected in all animals on day 7 PV. In contrast to sheep and goats, the antibody response in calves increased significantly ($p = <0.05$) with increasing doses of the vaccine and the dose of vaccine required to elicit antibody in most of the calves was higher (10^7 $TCID_{50}$). In unvaccinated control sheep, goats and calves, RVFV antibody was not detected during 28 day PV period in any of the animals.

Only a selected sub-sample of the sera from vaccinated animals were tested by ELISA as an additional assay for verifying the immune response. All sera samples obtained on day 28 from unvaccinated sheep and calves that were housed in pens with the same species of RVFV arMP-12ΔNSm21/384 vaccinated animals to evaluate for shedding of the vaccine virus were negative for RVFV IgG antibody (data not shown). The detection of RVFV ELISA arMP-12ΔNSm21/384 IgG antibody on selected days PV and selected doses of the vaccine for sheep, goats and calves, respectfully are presented in Tables 3, 4 and 5. Among 10 sheep, antibody was detected on day 7 PV in 50% that received 10^4 $TCID_{50}$ of the vaccine SC, and rates increased to 90% on day 14 and to 100% on days 21 and 28 PV. Antibody was detected on day 7 PV in 100% of 10 goats vaccinated SC with 10^4 $TCID_{50}$ of the vaccine. The immune response of calves, including 10 vaccinated SC with 10^5 and 10 calves vaccinated SC with 10^6 $TCID_{50}$ was 70% and 90%, respectively on day 28 PV. Overall, the immune response based on the detection of IgG antibody in each of the animals species were comparable to the results obtained by the VNT.

4. Discussion

Although the need for an improved RVF livestock vaccine has been recognized for many years, efforts have been slow and difficult to develop such a vaccine. The reasons can in part be attributed to the limited market in Africa, and the questionable views that

Table 5
Summary of ELISA RVFV IgG antibody detected on day 28 post-vaccination in calves vaccinated subcutaneously with 10^5 and 10^6 $TCID_{50}$ of RVFV arMP-12ΔNSm21/384 vaccine candidate.

Dose 10^5 $TCID_{50}$			Dose 10^6 $TCID_{50}$		
Animal #	OD*	Antibody	Animal Number	OD	Antibody
62	1.42	Negative	54	0.12	Positive
29	0.09	Positive	45	1.23	Negative
20	0.09	Positive	56	0.34	Positive
85	0.08	Positive	749	0.42	Positive
55	0.34	Positive	65	0.38	Positive
92	0.38	Positive	57	0.06	Positive
39	0.06	Positive	18	0.18	Positive
745	0.32	Positive	50	0.13	Positive
32	0.97	Negative	63	0.10	Positive
67	0.90	Negative	38	0.31	Positive

* Optical density values.

utilization of a vaccine is not feasible because RVF epizootic/epidemics are unpredictable and can be absent for more than 10 years. Therefore, the development of a safe and effective vaccine that can respond to any unexpected outbreak becomes a priority. As supported by the results of this study and previous observations, the RVF arMP-12ΔNSm21/384 vaccine was found to be suitable for the latter purpose in that only one dose is likely to elicit rapid protective immunity for sheep and calves, and the results of this study indicated that the vaccine induced a similar response in goats [29–31]. Although the duration of antibody response was not determined beyond 28 days PV, studies involving the parent RVFV MP-12 vaccine showed that human volunteers retained neutralizing antibody of 1:20 or greater 5 years after a single dose of the vaccine [24]. While the formulation of the parent RVFV MP-12 vaccine differs from that of the RVFV arMP-12ΔNSm21/384, the immune response to the 2 vaccines in goats was comparable in this study and in a previous study conducted in sheep [30].

In this study, we successfully rescued a clone of RVFV arMP-12ΔNSm21/384 by reverse genetic, and then prepared and tested a freeze-dried formulation of the vaccine. Nucleotide sequencing confirmed that the identity of the generated clone was identical to the original RVFV arMP-12ΔNSm21/384. The replication kinetic of the vaccine virus in Vero and BHK-21 cells at 3 different MOIs showed that the highest virus yield of $10^{8.4}$ $TCID_{50}$/ml was obtained in Vero cells infected at a MOI of 0.1 when total virus was harvested on day 2 PI. Similarly, for the RVFV CL 13 vaccine virus, the peak virus yield was reported to occur after 2 or 3 days P.I., depending on the MOI. Viral replication was associated with cells rounding up, detaching from the plate and lysis after 5–6 days [42,45].

Our results of safety and immunogenicity testing of the RVFV arMP-12ΔNSm21/384 in sheep, goats and cattle showed that the vaccine elicited antibody responses with no adverse effects, even with an overdose of the vaccine. However, one of the most important safety criteria for live vaccines is the absence of reversion to virulence and spread to unvaccinated animals. Our results demonstrated that the RVFV arMP-12ΔNSm21/384 vaccine virus did not spread from vaccinated to un-vaccinated animals. Further studies regarding the possibility of shedding and/or spread of the vaccine virus are needed, including experiments designed to evaluate viral shedding in excreta such as nasal and ocular swabs or testing the potential spread to highly susceptible species i.e. younger or immunocompromised animals. The results of our preliminary experiments did not reveal any evidence of reversion to virulence after 5 serial passages of the vaccine virus in RVFV immunologically naïve sheep. Indeed, live attenuated vaccines such as RVFV Clone-13 that possess a single gene deletion, can potentially revert to virulence by gene reassortment or combination with field strains [39]. This is virtually impossible for RVFV arMP-12ΔNSm21/384 virus because it was derived from the attenuated parent RVF MP-12 vaccine that has multiple attenuated mutations in all 3 viral RNA segments [40]. In addition, the parent MP-12 virus is stable genetically in cells based on limited viral replication cycles [41,42], and has also been shown to be safe following vaccination of 62 human volunteers [23,24]. Another potential advantage of the RVFV arMP-12ΔNSm21/384 vaccine is the deletion of the non-structural genes of the viral RNA M segment that could provide a negative bio-marker to differentiate infected from vaccinated animals (DIVA) [43].

The safety of the RVFV arMP-12ΔNSm21/384 vaccine in pregnant animals was not tested in this study. However, previous reports indicated that the parent RVF MP-12 vaccine was immunogenic, non abortigenic and protective of sheep and cattle and their fetuses against experimental challenge with virulent RVFV [18,20]. In contrast, one study suggested that RVF MP-12 caused abortions and fetal malformation in ewes when vaccinated at an early stage of pregnancy [25], whereas, the results of limited studies indicated that the RVFV arMP-12ΔNSm21/384 was safe when administered to pregnant sheep [31].

In this study, we did not perform any challenge studies of animals vaccinated with the arMP-12ΔNSm21/384 vaccine to assess antibody titers as a correlate of protective efficacy because appropriate biosafety facilities were not available. However, the development of neutralizing antibody in all sheep, cattle and goats vaccinated with arMP-12ΔNSm21/384 in this study is likely to correlate with protection. While a more conclusive assessment of the potential protective role of antibody for these animals will require challenge studies, the results of a previous study showed that antibody titers of less than 1:100 were protective for sheep vaccinated with this vaccine and challenged with virulent RVFV [31]. In addition, RVFV MP-12 antibody titers of 1:20 were considered protective for *Rhesus macaques* and titer of 1:10 to 1:20 protected hamsters against a lethal challenges of virulent RVFV [22,44]. Our antibody titers for sheep were within this range, but the titers cannot be interpreted as correlates of protection for cattle and goats. However, neutralizing antibody have been shown to be the most commonly used correlates of RVFV protective immunity among both human and animals [4,15,20,22,24,31,44]. The immunological response in each of the animal species was based on the initial screening of selected animals for IgG antibody by an ELISA and the subsequent use of the VNT for assaying all animals for neutralizing antibody titers. Overall, the results indicated that the most all animals that were antibody positive by the ELISA were positive by the VNT. The antibody titers in sheep and goats after vaccination with 10^4 $TCID_{50}$ suggested that the results were compatible with the industrial scale up vaccine production system. These results were supported by a dose escalation study of RVFV arMP-12ΔNSm21/384 in ewes showed that 10^3 PFU/ml stimulated an antibody response comparable to doses of up to 10^5 PFU/ml of this virus [18]. In addition, studies performed with CL13 showed that in sheep and goats, a dose of 10^3 $TCID_{50}$ conferred a long-lasting and protective antibody response [45].

Among the calves, the antibody response was delayed, dose dependent and the titers were lower as compared titers for sheep and goats. This antibody response was comparable to the observations reported for cattle vaccinated with the CL13 vaccine [45]. Furthermore, our findings indicated that calves should be vaccinated with a dose of at least 10^5 $TCID_{50,}$ and was consistent with a previous study that showed cattle vaccinated with as low as 10^5 PFU/ml of RVFV MP-12 vaccine were protected against challenge by virulent RVFV [20]. These findings are comparable to those for cattle vaccinated with different doses of the CL13 candidate vaccine (10^5, 10^6, 10^7 and 10^8 $TCID_{50}$) [45]. All animals seroconverted with detectable neutralizing antibody as early as day 7 PV and lasting for 116 days or longer PV.

While most sheep, goats and calves developed antibody in response to the different doses of arMP-12ΔNSm21/384 in this study, the antibody titers were more comparable to those reported for domestic ruminants following vaccination with CL 13T, but lower than titers reported for sheep and cattle vaccinated with the RVFV arMP-12ΔNSm21/384 vaccine in the United States and Canada [29–31,45]. However, the use of different methods and different species of animals precluded a valid comparison of the immune response of domestic ruminants to RVFV vaccines.

5. Conclusions

Overall, the findings of this study implied that the RVF arMP-12ΔNSm21/384 is a promising vaccine candidate for the prevention of RVF among domestic ruminants in Africa.

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Conflict of interest

All authors declared no conflict of interest.

Authorship

All authors attest that they meet the ICMJE criteria for authorship. The following authors, Z. Boumart, S. Daouam, Z. Bamouh, M. Jazouli and M.EL Harrak contributed substantially to the conception and design of the study, the collection and analysis and interpretation of data and the preparation of the manuscript, and B. Dungu, and K. Omari Tadlaoui contributed substantially to the conception or design of the work; and D.M. Watts and G.E. Bettinger contributed to the preparation of the manuscript and interpretation of the data for the work, and all authors participated in the revision, and agreed to be accountable for all aspects of the work and approved the final version this manuscript. All authors reviewed the journal policies detailed in the guide for authors.

Appendix A. Supplementary material

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