Stability of a formalin-inactivated Rift Valley fever vaccine: Evaluation of a vaccination campaign for cattle in Mozambique

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ABSTRACT

In Africa and the Arabian Peninsula, outbreaks of Rift Valley fever (RVF) are characterized by abortions in gestating animals and high mortality rates among domestic ruminants. An immunization program using a formalin-inactivated vaccine was initiated in Mozambique in 2002 to control RVF in cattle. In this intervention, the vaccine must be transported for more than a week within the country before it can be administered to the animals, and it is practically impossible to maintain low storage temperatures during that time. Here, we evaluated the influence of transportation conditions on the efficacy of the vaccine. Sixty-three previously unvaccinated and RVF virus seronegative cattle were divided into four groups, which were given vaccine that had been stored for 1 week at 4 °C ($n = 9$, group A), at 25 °C ($n = 8$, group B), or alternating between 4 and 25 °C ($n = 8$, group C), or under the temperature conditions ordinarily occurring during transportation within Mozambique ($n = 38$, group D). The antibody responses induced were monitored for 6–9 months and in some animals up to 21 months. Two immunizations (3 weeks apart) with the formalin-inactivated vaccine induced a long-lasting neutralizing antibody response that was still detectable up to 21 months later. The antibody titers in the animals did not differ significantly between the temperature-assigned vaccine groups A, B, and C, whereas they were significantly higher in group D. These results show that the formalin-inactivated RVF virus vaccine is stable, and, importantly, it is not adversely affected by the variation in temperature that ordinarily occurs during transport within Mozambique.

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1. Introduction

The first report of Rift Valley fever (RVF) among sheep in Kenya was published in 1930 [1], and since then the disease has become widespread and endemic in Sub-Saharan Africa and Egypt, and later also in the Arabian Peninsula. The causative agent is the RVF virus (RVFV), which belongs to the genus *Phlebovirus* of the family Bunyaviridae. The tripartite RNA genome (S, M, and L segments) is of negative or ambisense (S segment) polarity and encodes four structural proteins [2]. The highly immunogenic nucleocapsid (N) protein, which is encoded by the S segment, is increasingly being used as the antigen of choice in serological assays [3,4]. The membrane-associated Gn and Gc glycoproteins are encoded by the M segment, and constitute the targets for virus-neutralizing antibodies [5].

RVFV can infect a broad range of animal hosts, and outbreaks of this disease often occur after heavy rains and flooding have generated favorable breeding conditions for the mosquito vectors [6,7]. Viral infections in humans are usually manifested as mild, self-limiting febrile illness, although in some cases there are more severe symptoms, such as encephalitis, loss of vision, and hemorrhagic fever [8]. In animals infected with RVFV, spontaneous abortions are frequently observed among gestating domestic ruminants, and the mortality rate can reach 100% among newborn and young individuals. RVF can be subclinical in older animals, but clinical manifestations are also often severe in adults, with mortality rates ranging from 10% to 70%, depending on the age, species, and breed of the infected host [9]. Epizootics of RVF have resulted in rigorous restrictions on the trade of animals and animal products,

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which in turn have had a considerable socio-economic impact, not only on livestock keepers and food producers, but on the non-agricultural sector as well [10].

Vaccination of livestock is the main measure used to control RVF in endemic areas. Many promising vaccine candidates are under evaluation [11–13], although animals are currently being immunized with the live-attenuated Smithburn strain [14] or formalin-inactivated RVFV vaccines [15]. Unfortunately, the Smithburn strain has retained teratogenic characteristics, as noted in studies of pregnant cows and goats [16,17]. Compared to the live-attenuated strain, the formalin-inactivated vaccines are safer for use in gestating animals, but they are also less immunogenic, and annual boosters are required to maintain protective immunity [18].

Outbreaks of RVF are reported regularly in several of the Sub-Saharan countries [19,20], although the situation regarding this disease in Mozambique is unclear. Strong evidence of RVFV transmission in Mozambique was obtained as early as the 1960s [21], and this was confirmed in the 1980s, when virus-specific antibodies were detected in 2% of pregnant women in the country [22]. Furthermore, surveys of cattle in Zambezia Province (the central region of Mozambique) revealed high seropositivity, which led to introduction of an immunization program in 2002 using a formalin-inactivated vaccine [23].

In the present study, we investigated whether different storage and transport conditions affect the ability of formalin-inactivated RVFV vaccine to induce immune responses in cattle. For that purpose, a commercial vaccine was stored under four different conditions: at 4 °C, at 25 °C, alternating between 4 °C and 25 °C, or according to the standard procedures stipulated by the Mozambican Directorate of National Veterinary Services (see Section 2.3). The antibody responses induced by the vaccine were monitored by ELISA and plaque reduction neutralization tests (PRNTs) for 6–9 months, and in some animals up to 21 months.

2. Materials and methods

2.1. Cells and viruses

Vero cells (ATCC no. CCL-81) were grown in Medium 199 (Gibco, Life Technologies Corporation) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 5% heat-inactivated fetal bovine serum (FBS). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. The RVFV strain ZH548 [24], was propagated and titrated in Vero cells before being used in immunofluorescence assays (IFAs) and PRNTs.

2.2. Animals

The experimental procedures using animals were approved by the Mozambican Board of Agriculture (ethical permission no. 555/DNSV/2008, Maputo). Thirty mixed breed cattle were raised in a free-range feeding system in the Namaacha District, Maputo Province (Fig. 1), with the main diet based on grass and hay to which mineral salts and food supplements were added during the dry season (March–August). These animals were divided into three groups (designated A, B, and C) initially with 10 animals in each. An additional 41 head of mixed-breed cattle (designated group D) were raised in the Alto Molocue District, Zambezia Province (Fig. 1), under conditions similar to those described for groups A–C, but without food supplements during the dry season. Two more animals were kept as negative controls in the Namaacha District, Maputo Province. None of the cattle had previously been vaccinated against RVF. Characteristics of the animals are given in Table 1.

Fig. 1. Map of Mozambique and neighboring countries. The map shows the capital city of Maputo (italics) and the provinces referred to in the article (bold capital letters), as well as the city of Quelimane and the approximate locations of the Namaacha and Alto Molocue Districts.

2.3. Vaccination and sample collection

In Maputo Province, vials containing formalin-inactivated RVFV vaccine and aluminum hydroxide gel adjuvant (Onderstepoort Biological Products, batch no. 398) were stored for 1 week at 4 °C (according to the manufacturer's instructions), at 25 °C, or alternating between 4 and 25 °C (at 12-h intervals); thereafter, the vaccine was administered to the cattle in groups A, B, and C, respectively. For group D in Zambezia Province, vaccine in vials was transported as currently stipulated by the Directorate of National Veterinary Services before it was administered to the cattle. More precisely, the vials were sent by air from Maputo to Quelimane and thereafter by car to the Alto Molocue District (Fig. 1), and the vaccine was subsequently given to the animals. The delivery took approximately 1 week, and the vials were stored on ice or at 4 °C when possible.

The animals were inoculated subcutaneously (16 G ½ in. needle) lateral on the neck with 2 mL of vaccine on day 0 and boosted on day 21, according to the manufacturer's instructions. Blood samples were collected by venipuncture of the coccygeal vein on the day of primary vaccination and thereafter for 287–630 days in groups A–C and for 147 days in group D. Samples collected on days 45 and 48 were analyzed together and are referred to below as having been taken on day 45. The blood samples were stored at 4 °C and later centrifuged at $1500 \times g$ to obtain sera, which were stored at −20 °C pending analysis.

2.4. Immunofluorescence assay

Bovine serum samples diluted 1:40 in PBS were incubated with monolayers of acetone-fixed RVFV-infected Vero cells for 30 min at

Table 1
Characteristics of the cattle.

Group	Province	Animal[a]	Age (yr)	Sex	Weight[b] (kg)	0	10	20	30	45	87	117	147	207	267	630
A (4 °C)	Maputo	A1	5	F	300	•	•	•	•	•	•	•	•	•	•	x
		A2	4	F	260	•	•	•	•	•	•	•	•	•	•	•
		A3	3	F	250	•	•	•	•	•	•	•	•	•	•	•
		A5	8	F	400	•	•	•	•	•	•	•	•	•	+	+
		A6	8	F	380	•	•	•	•	•	•	•	•	•	•	•
		A7	8	F	380	•	•	•	•	•	†					
		A8	8	F	360	•	•	•	•	•	•	•	•	x		
		A9	8	F	360	•	•	•	•	•	•	•	•	•	•	•
		A10	8	F	300	•	•	•	•	•	•	•	•	•	•	•
B (25 °C)	Maputo	B2	5	F	320	•	•	•	•	•	•	•	•	x		
		B4	4	F	300	•	•	•	•	•	•	•	x			
		B5	5	M	340	•	•	•	•	•	•	•	•	•	•	•
		B6	6	F	320	•	•	•	•	•	•	•	•	†		
		B7	6	F	300	•	•	•	•	•	•	+	•	•	•	•
		B8	8	F	350	•	•	•	•	•	•	•	•	•	•	•
		B9	8	F	250	•	•	•	•	•	•	•	•	•	•	x
		B10	8	F	300	•	•	•	•	•	•	•	•	•	•	•
C (4/25 °C)	Maputo	C1	8	M	280	•	•	•	•	•	•	•	•	•	•	•
		C2	4	F	260	•	•	•	•	•	•	•	•	•	•	•
		C3	6	F	340	•	•	•	•	•	•	•	•	•	•	•
		C5	5	F	350	•	•	•	•	•	•	•	•	•	•	•
		C6	5	F	300	•	•	•	•	•	•	•	•	•	•	•
		C7	8	F	260	•	•	•	•	•	•	•	•	x		
		C8	8	F	250	•	•	•	•	•	•	•	•	•	•	x
		C10	8	F	400	•	•	•	•	•	•	•	•	•	•	•
D (customary)[d]	Zambezia	D1–D5	4–8	n/a	n/a	•	•	•	•	•	•	•	•			
		D7	4–8	n/a	n/a	•	•	•	•	•	•	•	+			
		D8–D29	4–8	n/a	n/a	•	•	•	•	•	•	•	•			
		D30	4–8	n/a	n/a	•	•	•	•	•	•	•	+			
		D31	4–8	n/a	n/a	•	•	•	•	•	•	•	•			
		D33–D39	4–8	n/a	n/a	•	•	•	•	•	•	•	•			
		D41	4–8	n/a	n/a	•	•	•	•	•	•	•	•			
	Maputo	ctrl1	6	F	300	•	•	•	•	•	•	•	•	•	•	•
		ctrl2	5	F	300	•	•	•	•	•	•	•	•	•	•	•

[a] Animals A4, B1, B3, C4, C9, D6, D32, and D40 were excluded due to pre-exposure to Rift Valley fever virus.

[b] Weight was estimated using a tape measure.

[c] Symbols indicate samples that could not be collected (+) or were not available because an animal died (†) or was stolen or sold (x).

[d] Vaccine was transported according to the customary procedures of the Mozambican Directorate of National Veterinary Services (see Section 2.3). F, female; M, male; n/a, not available; ctrl, control.

37 °C. Next, the cells were washed 3 × 10 min in 0.9% NaCl solution, and then fluorescein-conjugated anti-bovine IgG antibody (Cappel, MP Biomedicals Inc.) was added to a final dilution of 1:50. Thereafter, the samples were incubated for 30 min at 37 °C and then washed as above and mounted. The presence of anti-RVFV antibodies was determined by fluorescence microscopy, and all serum samples were analyzed in duplicate.

2.5. ELISA

Full-length RVFV N protein (GenBank ID: AF134534) [25] was produced from a prokaryotic expression vector pET14b (Novagen), and the recombinant N protein antigen was purified from *Escherichia coli* cell lysates under native conditions using Ni-NTA agarose (Qiagen), as described elsewhere [26]. Indirect ELISA was performed as reported by other authors [27] with these minor changes: the plates were coated with 3 μg/mL recombinant N protein, and the antigen-antibody complexes were visualized by use of peroxidase-conjugated anti-bovine IgG antibody (Jackson ImmunoResearch Laboratories) diluted 1:5000 and TMB substrate (3,3′,5,5′-tetramethylbenzidine, Sigma Aldrich). The reaction was stopped with 50 μL of 1 M H_2SO_4, and the absorbance was measured at 450 nm. The ELISA cut-off value was calculated using the results for 25 negative bovine sera and the Student t-distribution test with 99% confidence intervals, as described in detail in the literature [28]. Negative and positive bovine serum control samples were included in each plate. All samples were analyzed in duplicate, and the titers were determined as the reciprocal of the highest serum dilution that resulted in a reading above the cut-off value.

2.6. Plaque reduction neutralization test

Heat-inactivated serum samples were serially diluted twofold in Minimal Essential Medium (MEM, Invitrogen) supplemented with 3% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, and then incubated with 60 PFU of RVFV for 30 min at 37 °C. Adsorption of the serum-virus mixture to Vero cells was allowed to proceed for 1.5 h at 37 °C, and then the cells were washed, overlaid with MEM medium containing 0.75% carboxy methyl cellulose (Sigma Aldrich), and incubated for 4 days at 37 °C under 5% CO_2. The cells were subsequently fixed in 4% formaldehyde, and plaques were visualized by counterstaining with crystal violet. The neutralization titers were determined as the reciprocal of the highest serum dilution that reduced virus infectivity by 80%. A titer of 20 was considered positive after evaluating the assay using 25 negative bovine sera. All procedures using viable RVFV were carried out in a biosafety level 3 containment laboratory.
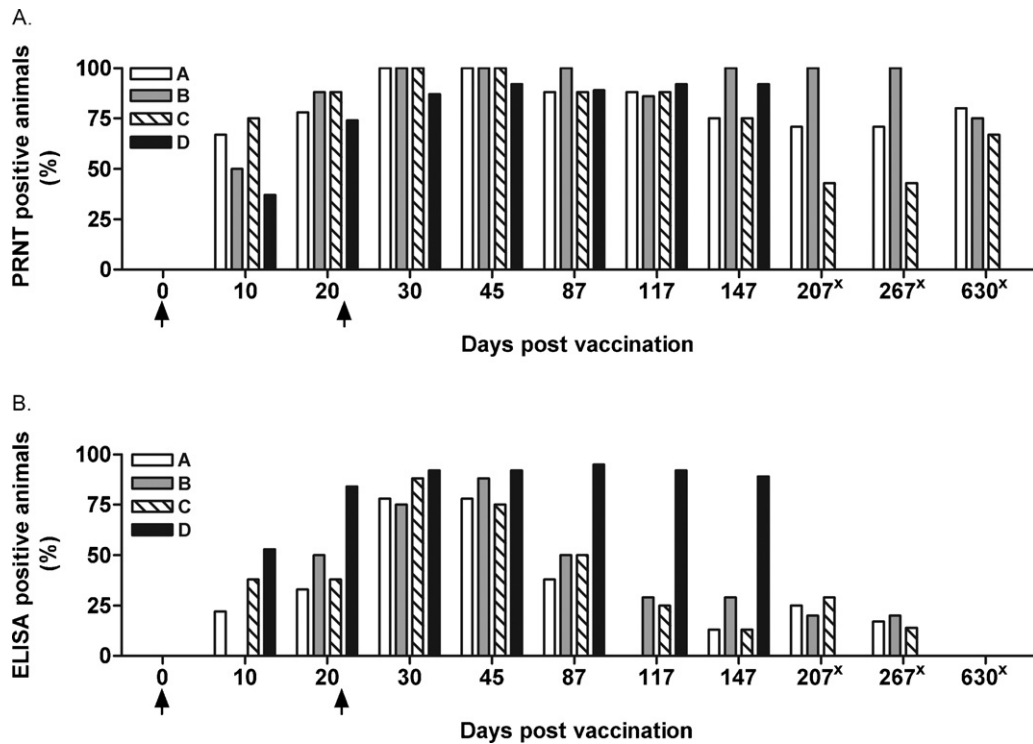
Fig. 2. Percentage of seropositive cattle in each of the four vaccine groups. Formalin-inactivated RVFV vaccine was stored for 1 week at 4 °C, at 25 °C, alternating between 4 °C and 25 °C, or in vials transported in the manner customary in Mozambique and then administered twice (3-week interval, day 0 and day 21) to animals in groups designated A, B, C, and D, respectively. The percentage of seropositive animals was determined on the indicated days by plaque reduction neutralization tests (A) and indirect ELISA against N protein (B). Arrows along the *x*-axis indicate immunization days. No data were available for the animals in group D on the days marked with a superscript 'x'.

2.7. Statistical methods

Statistical correlations were analyzed using Spearman's rank test. The Kruskal–Wallis test was applied to achieve the initial comparisons of the antibody responses exhibited by the four vaccination groups, and, if systematic variations were found, the Mann–Whitney *U* test was carried out to compare the vaccine-induced titers of the animals in the individual groups. Statistical analyses were performed with Statistica™ 10 (StatSoft Inc.), and graphs were visualized using GraphPad™ Prism 4 (GraphPad Software Inc.).

3. Results and discussion

The sporadic reports of outbreaks of RVF in neighboring countries illustrate that there is a continuous risk to Mozambique, because it is one of the most frequently flooded countries in the southern part of Africa [29]. Notably, Mozambique has been spared from RVF in humans or livestock, with the exception of the outbreaks that occurred in 1969 and 1994, which caused abortions and deaths in cattle and water buffalo [9,21,30]. Nevertheless, surveillance of cattle in Zambezia Province (Fig. 1) in 1996 and 2001 revealed seroprevalence of 37% and 53%, respectively [23]. Accordingly, in 2002, a vaccination program was initiated in districts in that province to prevent RVFV infections in cattle. Inasmuch as there was considered to be a high risk of RVF after the flooding that had affected the southern part of the country in 2000 and the central region in 2001, the vaccination program was extended in late 2002 to include the provinces of Gaza and Manica (Fig. 1) [31]. However, after 2004, vaccination of livestock was once again limited to only a few districts in Zambezia Province.

The antibody status of the cattle to be used in our study was initially unknown, and thus, to start with, we assessed RVFV seronegativity by IFA, and the results were later confirmed by PRNT (data not shown). A seropositivity rate of 7% was found among the 41 animals kept in Zambezia Province (group D), which was a relatively low level compared to the sero-surveillance data previously recorded in that province [23]. On the other hand, 17% of the 30 animals in Maputo Province (groups A, B, and C) were positive for anti-RVFV antibodies, which was a surprisingly high rate. It was in this region that the last small outbreaks of RVF in cattle were reported in 1969 [21]. These results indicate that RVFV is circulating in Maputo Province, but it is either not causing manifest clinical disease or not being diagnosed.

Cattle that were found to have elevated RVFV antibody titers before the primary immunization were not included in the study (Table 1). Furthermore, none of the negative control animals showed seroconversion over the experimental period, analyzed as the presence of antibodies against the N protein or by virus neutralization tests (data not shown).

The ability of the bovine antisera to neutralize RVFV *in vitro* was analyzed by PRNT. Our data show that the majority of the animals in all four groups (78%, 88%, 88%, and 74% in A, B, C, and D, respectively) seroconverted rapidly in response to the primary vaccination (Fig. 2A). After the second immunization, all animals in groups A, B, and C had elevated PRNT titers (≥80) (Figs. 2A and 3A). However, four of the animals in group D (*n* = 38) failed to seroconvert 9 days after receiving the booster dose; three of those four seroconverted by day 45, whereas the fourth animal did not develop detectable anti-N protein or neutralizing antibodies over the entire experimental period. In general, neutralizing antibody titers showed small peaks on days 30–45 and thereafter declined relatively rapidly in groups A–C but not in group D (Fig. 3A). Interestingly, the antibody titers of the cattle in group D peaked on day 45 and remained high until day 147 (Fig. 3A). Furthermore, two-thirds of the animals in groups A, B, and C still had detectable levels of neutralizing antibodies 267 days after vaccination (Fig. 2A). It can be mentioned that comparable
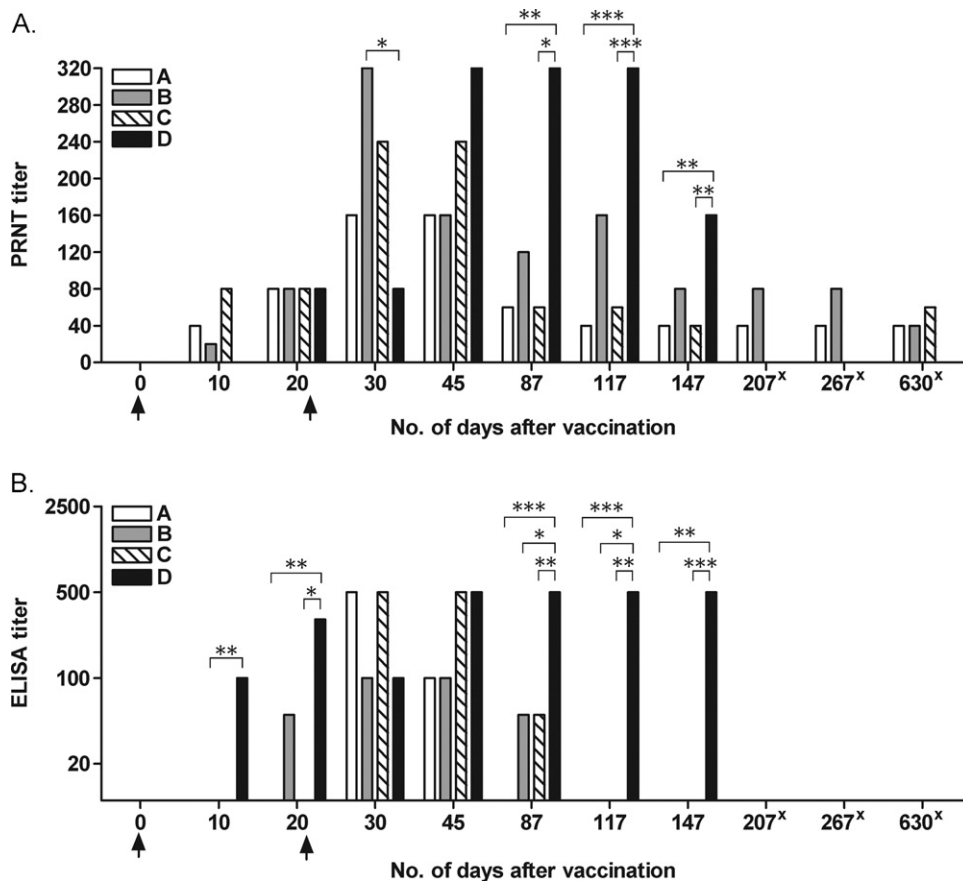
Fig. 3. Bar charts showing the median antibody titers in the vaccinated cattle. Four groups of cattle were immunized with formalin-inactivated RVFV vaccine that had been stored at 4 °C (group A), 25 °C (group B), or alternating between 4 °C and 25 °C (group C), or had been transported within Mozambique according to the practice stipulated in that country. Two doses were administered (primary vaccination on day 0 and booster on day 21), and the vaccine-induced neutralizing antibody responses (A) and anti-N antibody responses (B) were determined by a plaque reduction neutralization test and indirect ELISA, respectively. Serum samples were collected from the animals on the indicated days. No data were available for group D on the days marked with a superscript "x". Arrows along the x-axis indicate immunization days. Brackets and asterisks indicate values found to be significantly different by the Mann–Whitney U test (*$p < 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

long-lasting titers of neutralizing antibodies had previously been observed by other researchers in a study using a comparable formalin-inactivated RVFV vaccine [32]. We also noted that 73% of the animals in our investigation still had neutralizing antibody titers in the range of 40–320 (median 40) (Figs. 2A and 3A) 630 days after the initial vaccination. The extent and duration of the protective immunity induced by formalin-inactivated RVFV vaccines are considered to be "short termed", and consequently annual booster immunizations are given to cattle to maintain immunity. However, our observations indicate that the neutralizing antibody responses induced by the formalin-inactivated RVFV vaccines may be more robust and long-lasting than expected.

Indirect ELISA based on recombinant N protein has the potential for use in routine diagnostics and epidemiological studies of RVF [33]. Therefore, we decided to monitor the levels of anti-N-protein antibody in serum samples of the vaccinated cattle. Anti-N antibody responses were detected in about one-third of the animals after the primary vaccination, and nearly 80% of the animals were seropositive nine days after the booster immunization, regardless of treatment group (Fig. 2B). In contrast to the neutralizing antibody responses, circulating anti-N antibodies could only be detected for a short period of time. On day 147 after the primary immunization (126 days after booster), the majority (74%) of the animals in groups A–C were negative for antibodies against the recombinant N protein (Fig. 2B), whereas such seronegativity was observed in only 14% of the animals in group D at that time (Fig. 2B). By the end of the study period, all but four of the investigated animals

had exhibited anti-N antibody responses after the two immunizations.

We also noted that, after receiving the immunizations, the animals that had low vaccination anti-N protein titers (≤ 100) showed neutralizing antibody titers similar to those seen in the animals that had stronger vaccination anti-N antibody responses. The ELISA used in this study measures antibodies directed toward the N protein, while the PRNT mainly measures neutralizing antibodies directed toward the glycoproteins. Thus the level of anti-N antibodies may not be relevant as an indicator of vaccination status. It is possible that the lack of a relationship between anti-N and neutralizing antibody titers (group A, Spearman's rank test) can be explained by differences in assay sensitivity, however our analyses indicate that serology based on the response to N protein may not be the method of choice for evaluating the efficacy of immunization with formalin-inactivated RVFV vaccines.

Formalin-inactivated vaccines are in general more stable than attenuated vaccines [34]. Efforts have been made to increase the stability of formalin-inactivated RVFV vaccines even further by lyophilization [35], but the preparations used in our study were in fluid form, and, to our knowledge, no evaluations have been performed to determine the efficacy of that type of formalin-inactivated RVFV formula after storage or transportation at ambient temperatures.

In our investigation, analysis of the impact of the different vaccine storage conditions on neutralizing antibody titers measured after immunization (Fig. 3A) revealed no significant

differences between the four experimental groups on days 10, 20, and 45, or between groups A, B, and C on days 207, 267 and 630 (Kruskal–Wallis test). However, the neutralizing antibody responses differed significantly between the four groups on days 30, 87, 117, and 147 after the first vaccination ($p = 0.023$, 0.004, 0.000, and 0.001, respectively; Kruskal–Wallis test). Accordingly, the individual groups of animals were subjected to further analysis using the Mann–Whitney U test. On day 30, neutralizing antibody titers were higher in group B than in group D, which might be explained by the earlier antibody peak (Fig. 3A). However, from day 45 and onward, the results were reversed, that is, the titers in group D were elevated (albeit only slightly) compared to those in group B (Fig. 3A). Furthermore, somewhat surprisingly, the antibody titers observed on days 87, 117, and 147 were higher in group D than in groups A and C (Fig. 3A).

A similar pattern was observed concerning the levels of anti-N protein antibody (Fig. 3B), which differed between the groups on days 10, 20, 87, 117, and 147 ($p = 0.035$, 0.006, 0.001, 0.000, and 0.000, respectively; Kruskal–Wallis). Comparisons of the individual groups regarding the results for the indicated sampling days revealed that the titers measured in group D were higher than those in groups A and C, and also higher than those in group B on some of the days (Mann–Whitney U test) (Fig. 3B). As for the neutralizing antibody titers, the anti-N protein antibody responses did not differ significantly between groups A, B, and C on days 207, 267, and 630 after vaccination. Several unrelated factors, such as the breed and the nutritional status of the animals may influence vaccine-induced immune responses [36], which might explain the higher antibody titers we observed in the animals in group D. Moreover, in general, the vaccine from vials stored at the recommended temperature (4 °C) induced equivalent or slightly lower antibody levels compared to the vaccine stored at ambient temperatures (alternating between 4 and 25 °C) or at 25 °C. These effects were noted in cattle belonging to the same herd (groups A, B and C), which suggests that storage for 1 week under adverse conditions does not impair the ability of the vaccine to induce the humoral antibody responses.

In summary, of the animals included in this study, only one failed to acquire a detectable antibody response after two vaccinations. Furthermore, the antibody responses were still detectable up to 21 months after the immunizations. Based on the data presented here, we conclude that the storage and transport conditions used in Mozambique do not have an adverse effect on the antibody responses induced by the formalin-inactivated RVFV vaccine.

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