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Evaluation of Protective Efficacy of Inactivated Thermostable Vaccine Against Nairobi Sheep Disease Virus

Ngari P. Muriuki[1,2*]
A. K Nyamache[1],
[1]Department of Biochemistry, Microbiology & Biotechnology, Kenyatta University, P.O. Box 43844-00100, Nairobi, Kenya.

Ithinji G. D[2],
Ngari P. Muriuki[1,2*]
[2]Kenya Agricultural and Livestock Research Organization (KALRO), Veterinary Science Research Institute (VSRI)-Muguga, P.O Box 32-00902, Kikuyu, Kenya.

Leonard Ateya[3],
Yatinder S. Binepal[3],
[3]Kenya Agricultural and Livestock Research Organization (KALRO), Biotechnology Research Institute (BioRI)-Kabete, P.O. Box 57811-00100, Nairobi, Kenya.

Caroline Wasonga[4],
[4]Department of Biochemistry, University of Nairobi, P. O. Box 30197 - 00100. Nairobi, Kenya.

Joel Lutomiah[5],
[5]Arbovirus/Viral Hemorrhagic Fever Laboratory, Center for Virus Research, Kenya Medical Research Institute, P.O. Box 54840-00200, Nairobi, Kenya.

Muthamia M. Kiraithe[6†]
[6]Department of Research & Development, Kenya Veterinary Vaccine Production Institute (KEVEVAPI), P.O. Box 53260-00200, Nairobi, Kenya.

Correspondence Author:- Ngari P. Muriuki[1,2*]

[†]Equally Contributing Author:- Muthamia M. Kiraithe[6†]

Abstract:- Sheep and goats (n=4/group) were inoculated with thermos-stabilized inactivated Nairobi sheep disease virus vaccine. Four unvaccinated animals for each group were kept as control. Vaccinated groups were given a booster vaccine dose 21 days later. Immune response was monitored by neutralizing antibody titers were determined by micro-plaque reduction neutralization test and confirmed by immunofluorescence assay. Two doses of the inactivated vaccine stimulated a strong immune response in the vaccinated animals. The vaccinated and mock group were challenged with virulent 1473 strain of the Nairobi sheep disease virus. All animals developed fever and viremia with varying degrees between sheep and goats post challenge. Mock vaccinated sheep developed high viremia levels relative to the vaccinated group and developed severe disease. In contrast, mock vaccinated goats showed a slight temperature compared to vaccinated goats. After challenge, two control sheep died from the disease whereas the vaccinated sheep survived. Vaccinated sheep suffered mild to moderate clinical reactions with pyrexia. Formalin inactivated vaccine fully protected the animals against the lethal 1473 challenge virus.

I. INRODUCTION

The reemerging pathway of Nairobi sheep disease virus in Africa and Asia (Krasteva et al., 2020a) is a major public health concern for Europe, Australia and Americas about the introduction of this disease in this regions. Vaccination remains the most robust measure to prevent the disease and prevent introduction to the new regions in the event of an outbreak of the disease.(Shi & Dong, 2021). Deployment of the vaccine focusing on spatial temporal space of endemic areas will slow or stop the disease spread to new regions. Currently there no efficient and economically affordable thermostable vaccine for Nairobi sheep disease. Methanol precipitated vaccine and live attenuated vaccine immune response to challenge virus was inadequate to protect the animals (Davies, Otieno, et al., 1977). The available vaccine limits their use due to their inefficiencies similar challenges posed by most vaccines under development. Recent studies have by (Hartlaub et al., 2021) however, showed formalin inactivated orthonairovirus can elicit immune response. Thermo-tolerant Nairobi sheep was developed from purified I34 strain to reduce the challenges associated with live attenuated vaccines and reduce delivery cost associated with the cold chain. Live attenuated vaccine could lead to establishment of vaccine derived virus by acquiring virulence in the host animal. Circulation of this virus could reduce the effectiveness of the vaccine program. Residual infectivity and pathogenicity

ruminants of a live attenuated vaccine cannot be excluded when dealing with naïve genetically selected small as it could result to a disease (Cosseddu et al., 2016). The wider implications will limit vaccine use for effective disease control and could lead to trade and movement restrictions of animals

From previous work of this research, I34 strain was profiled as the Nairobi sheep disease virus strain producing superior immune response that could cross protect against heterologous strains. Formalin inactivated Nairobi sheep disease virus vaccine was developed from the I34 strain grown in cell culture, concentrated and purified, inactivated, thermos-stabilized and adjuvanted to boost immune response. Protective efficacy of the formalin inactivated vaccine was evaluated by experimental infection of sheep and goats with a virulent 1473 strain and results reported in this study.

II. MATERIALS AND METHODS

A. Ethical Statement.

Animal vaccine trial studies were approved by the Animal care and Use committee of Veterinary Science Research Institute (VSRI)-Muguga of Kenya Agricultural and Livestock Research organization (KALRO) with approval code No: **KALRO-VSRI/IACUC018/27082019**

B. Virus and Cells.

Nairobi sheep disease virus strains I34 and 1473 bio-banked in Biotechnology Research Institute (Bio-RI) Kabete was used for all in-vitro and in-vivo experiment. Strain I34 was used as the vaccine seed for the formalin inactivated vaccine. Lethal 1473 strain was used as the challenge virus for the experimental animals' post vaccination. Vaccine seed and challenge virus was revived respectively from 4th and 3rd passage of lyophilized cell culture virus stock isolated by Davies and colleagues(Davies, Mungai, et al., 1977). Virus were grown in BHK21 cell line cultivated in GMEM medium supplemented with 5% fetal bovine sera. Inactivated vaccine was prepared according to the procedure described by (Möller et al., 2015; Pavel et al., 2020) from NSDV virus purified through a sucrose gradient.

Serological analysis of immune response to the inactivated virus antigen was evaluated on day 0, 7, 14, 21-, 28-, 35- & 42-days post vaccination and 0, 7 &14 days post challenge. Blood was collected from the from experimental animal in serum separating tubes (SST), kept at +4 ^{0}C before separating by centrifugation at 3000 rpm for 15 minutes. Serum was aliquoted and heat inactivated at 56 ^{0}C for 30 minutes and stored at -20 ^{0}C. Serum neutralizing antibody titers was determined in a serum neutralization test (SNT) and micro plaque reduction neutralization test against the Nairobi sheep antigen according to test protocol described (Chowdhury et al., 2015; Hartlaub et al., 2021) with slight modification

C. Virus Titration.

- *Plaque Assay*

Viral stock was prepared from the infected BHK 21 cell supernatant stored at -80 0C. supernatants were titrated in a 96 well plate for micro plaque units (PFU) as described by (Zhao et al., 2021) with slight modification. BHK 21 cells were grown in 96 well plate after which 10-fold dilution of Nairobi sheep disease virus and incubated for 1hour. Cells were maintained for 5-6 days with 1% carboxy methyl cellulose in GMEM BHK21 medium supplemented with 2 % fetal bovine serum. Infected cells were then fixed with chilled 10% formaldehyde containing 0.6% crystal violet for 30 minutes. The fixative and dye were then aspirated and plaques visualization was done using a fluoresce microscope.

- *Fluorescence Focus Assay*

Viral stock was prepared from the infected BHK 21 cell supernatant stored at -80 ^{0}C. supernatants were titrated in chamber slides by focus forming units (FFU) assay as described by (Dzagurova et al., 2020) with slight modification. BHK 21 cells were grown in 8 well chamber slides after which 10-fold dilution of Nairobi sheep disease virus and incubated for 1hour. Cells were maintained over night with GMEM BHK21 medium supplemented with 5 % fetal bovine serum. Infected cells were then fixed with chilled 80% ethanol for 15 minutes. The fixative was then washed and cells were permeabilized with 0.2% tween 20 in PBS overnight. Sheep sera containing primary antibody (1:50) specific to virus was added after blocking with 5% skimmed milk for 1hour. After washing with 0.2% Tween 20 in PBS the wells were incubated with secondary FITC conjugated anti-sheep IgG antibody (1:2000) and incubated for 45 minutes. Visualization was done using a fluoresce microscope.

D. Antibody Neutralization Test.

- *Plaque Reduction Neutralization Test (PRNT50)*

Plaque reduction neutralization test (PRNT50) was performed in a 96 well plate (micro plaque reduction neutralization assay) and 24 well plate respectively. BHK 21 cells were grown in GMEM supplemented with 10 % fetal bovine serum. Test sera was inactivated at 56 0C and diluted serially from 1:4 in virus diluted in GMEM supplemented with 5% fetal bovine sera. Serum virus incubated at 4 0C overnight before transferring to confluent monolayer of BHK 21 cells in triplicates for each sample. Virus was allowed to adsorb for one hour and the supernatants were washed and monolayer overlaid with GMEM containing 1% carboxy-methyl cellulose for 5 days. Cells were then fixed with 10% formaldehyde containing 0.6 % crystal violet in phosphate buffered saline for 30 minutes. Plaque reduction was visualized under a microscope. Neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution showing 50% reduction in the number of PFU relative to the control wells. Each serum dilution was replicated thrice and the average reading considered neutralizing titer for a particular end point.

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E. Virological Analysis

Animals were sampled for plasma daily for 7 days post challenge and viremia. Blood was collected from the jugular vein puncture in EDTA tubes for seven days. Plasma was transported in a cool box and separated at +4 0C before being stored at -80 0C. Plasma was analyzed to determine the viremia levels for the vaccinated group relative to that of the mock vaccinated. Virus titers were determined by plotting a standard curve of viremia post challenge in a 10 fold serial dilution on a confluent BHK21 monolayer as described by (Wichgers Schreur et al., 2021). Cells were maintained with Glasgow Minimum Essential Medium (GMEM). With 2% fetal bovine serum. Cytopathic Effect (CPE) was read daily for a maximum of seven days.

III. RESULTS

Efficacy of formalin inactivated thermostable Nairobi sheep disease virus vaccine in sheep and goats in compliance to KALRO IACUC for evaluation of veterinary vaccine. NSDV vaccine was developed using I34 strain passaged 3 times in BHK21 cell line. The virus was scaled up the same cells, concentrated and purified through sucrose gradient. Formaldehyde at a concentration of 0.01% was used to inactivate the virus.

Eight Red Maasai sheep and eight goats (n=4/group) were vaccinated with approximately 200ug of formalin inactivated NSDV vaccine via the intramuscular route (IM). Mock group was inoculated with sterile 0.01M phosphate buffered saline. Fourteen days post vaccination all the animals were given a booster dose in similar matrices. All animals were challenged via the intravenous route with 5mls of 10^5 TCID50 of the NSDV 1473 challenge virus. All the vaccinated animals did not present any clinical signs or related outward event following vaccination. Temperature reaction curve showed increased rectal temperature with onset on day 2 and 3 **Fig 1 (a).** Sheep were observed to present slightly higher temperature as compared to goats. Vaccinated sheep elevated temperatures normalized by day 5 compared to the mock vaccinated sheep. Similarly, goats recorded a slight temperature reaction compared to sheep. High temperatures in the mock vaccinated sheep were associated with high viremia determined by virus isolation on BHK21 cell lines. **Fig 1(b).** Viremia was detectable throughout the period of pyrexia determined by virus isolation of BHK 21 cell line. Plasma serial diluted in tenfold of was transferred to confluent monolayer cells and virus titer determined accordingly by day five. Two ewes in the mock vaccinated acutely group died on days 9 and 12 post challenge. No mortalities occurred in goats following inoculation of the animals with 1473 virulent strains. A slight temperature increase was observed in goats with unvaccinated recording higher temperatures than vaccinated. In contrast, Onset of clinical signs of fever with peak temperature of 41 ^{0}C was observed in mock vaccinated sheep. Vaccinated ovine also recorded slightly lower temperature compared to mock vaccinated sheep. Onset of diarrhea 3-8 days post challenge, was observed in the mock vaccinated animals. Clinical score was recorded every two days showed, mock vaccinated sheep had a higher clinical index than the vaccinated sheep. There was no significance between the mock vaccinated goats and vaccinated goats' clinical index

All vaccinated animals developed neutralizing titer (log) after vaccination with a booster dose eliciting a higher neutralizing titer. A spike in neutralizing titers was observed in the animals' post challenge. The titers in the control animals were similar for the goats and sheep

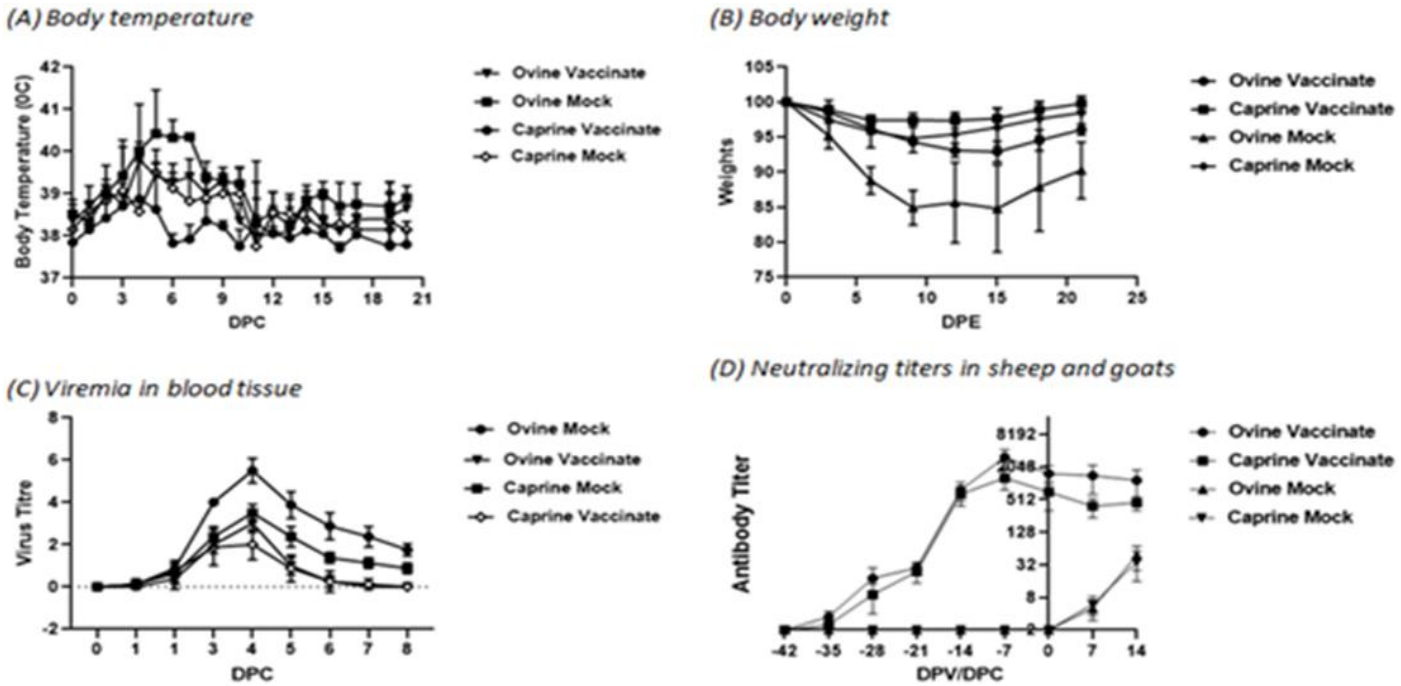

Fig 1 (A) Rectal temperature (B) body weight loss. (B) Viremia in blood and (D) Neutralizing antibody titer for the mock vaccinated and vaccinated animals; Sheep (***, p=0.0002; ****, p<0.0001; ****, p<0.0001; ****, p<0.000) and goats (****, p<0.0001; *, p<0.0225; ****, p<0.0001; ****)

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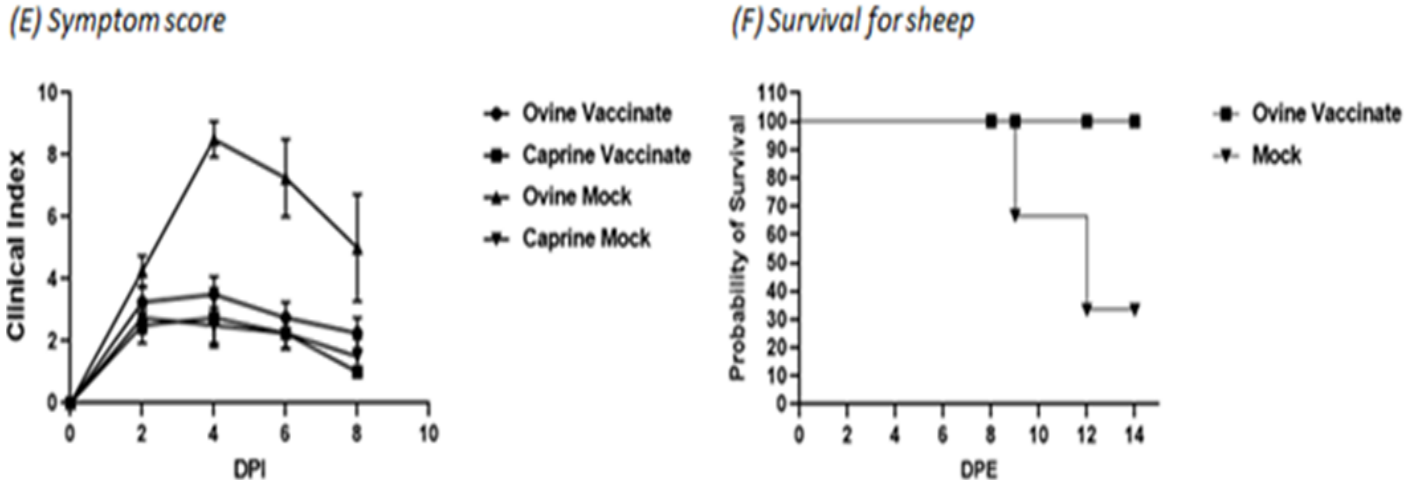

Fig 2 (a) symptom curve showing differences in clinical index; Ovine vaccinates vs Ovine mock (****, P<0.0001); Caprine vaccinate vs Caprine mock (ns, P=0.9591); Ovine mock vs caprine mock (****, P=0.0001); Ovine vaccinated vs caprine vaccinated (*, P=0.0103). (b) Ovine survival curve for mock vaccinated and vaccinated group.

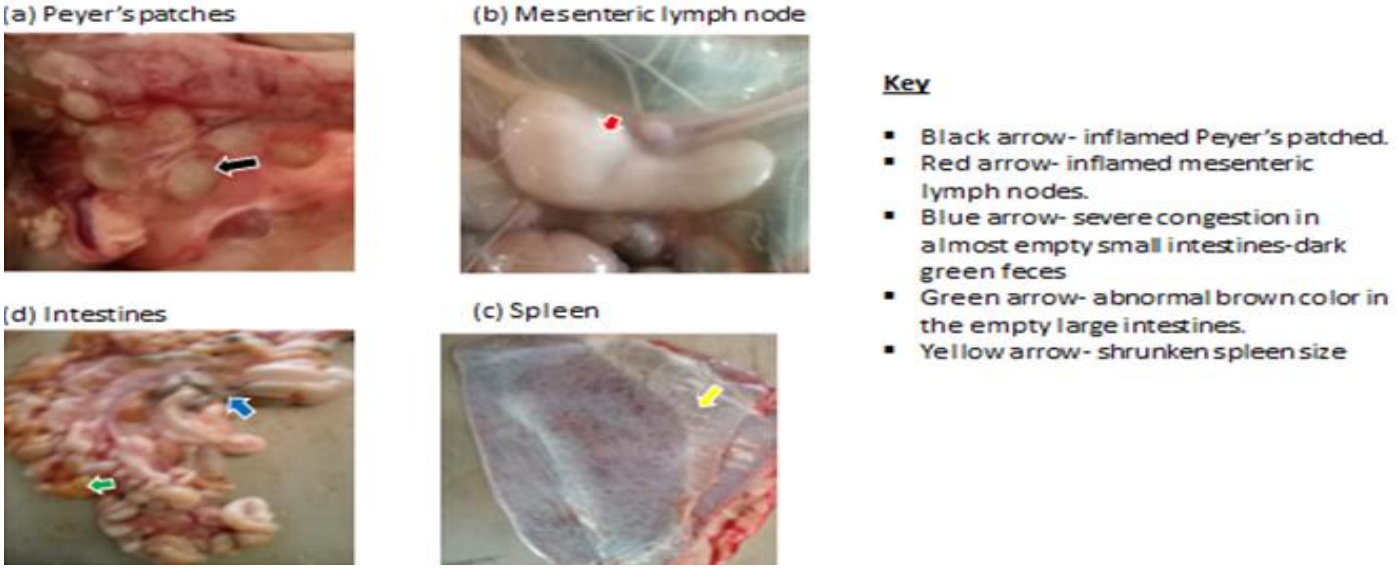

Fig 3 Post mortem micrograph of mock vaccinated sheep (a) inflammation of Peyer's patches (b) inflammation of mesenteric lymph nodes (c) Severe gastroenteritis associated with congestion in the gut (d) Atrophy of the spleen

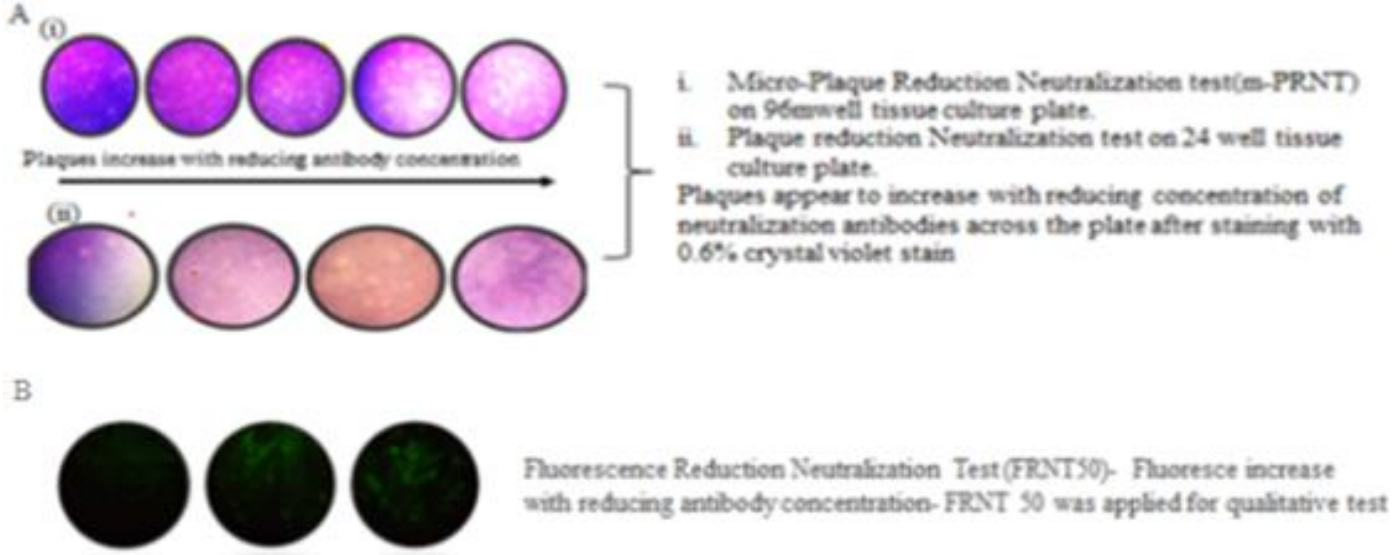

Fig 4 Micrograph showing (A) Plaque reduction Neutralization Test (PRNT)- Plaques increasing with reducing serum neutralizing titers. (B) Fluorescence Reduction Neutralizing test (FRNT50)- Fluorescence increase with reducing serum neutralizing titers

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From post mortem results, the animals died of severe gastroenteritis presented by lesions in the gut. Inflammation of the lymph nodes was observed in mesenteric lymph nodes the payer's patches. All the mock vaccinated significantly lost more weight compared to their respective vaccinated groups**.** Higher weight loss was observed in mock vaccinated sheep than mock vaccinated goats relative to their respective vaccinated groups. The spleen of one of the mock ovine that died was observed reduced in size suggesting damage of the peripheral immune possibly caused reduced appetite of the sheep by 3 days post infection. Throughout the observation period of 28 days neither did the mock nor do vaccinated goats showed any clinical signs except a slight elevation in rectal temperature. Moreover, viremia in goats determined from blood was observed to be very low compared to sheep.

IV. DISCUSSION

Nairobi sheep disease is an important reemerging disease of small ruminants in Africa and Asia (Krasteva et al., 2020b). Climate change influence emerging pathway in areas of circulation in Africa and Asia and has become endemic in new regions of China (Marczinke & Nichol, 2002; Walker et al., 2015). Establishment of the disease vector in new regions of USA, New Zealand, and China (Li et al., 2020; Sheng et al., 2019; Tufts et al., 2020) present major concerns over the spread of this disease to new regions. Incursion of Nairobi sheep disease to new regions would have considerable negative economic impacts globally. The diseases could cause direct losses from death, reduce production in affected areas and impose trade restrictions between NSD affected and non-affected areas (Adams et al., 2021). Vaccination is the most effective methods of reducing direct and indirect disease burden attributed to mortalities and morbidity by the virus circulation. Currently, Available vaccines in the market against Nairobi sheep disease virus have proved to be ineffective in preventing the disease. Earlier studies by (Hartlaub et al., 2020) have shown that formalin inactivated Nairobi sheep disease virus can produce neutralizing antibodies against the virus. Use of inactivated vaccine eliminate the potential for vaccine induced disease when the virus regains its virulence in the host animal (Cosseddu et al., 2016). Whole inactivated virus vaccine presents a safer alternative to live attenuated. This study evaluated the protective efficacy of an inactivated Nairobi sheep disease virus against Nairobi sheep disease.

Cell culture based vaccine was prepared using a method previously described by (Elveborg et al., 2022) and adjuvanted with Alum to enhance immune response against co-inoculated NSDV antigen. Two doses of the vaccine were given 21 days apart as the efficacy of inactivated vaccine is highly dependent on dosage (Pavel et al., 2020). Neutralizing antibodies in the vaccinated animals were detectable as early as during the first week with the second dose leading to significantly higher antibody titers after day 21 post vaccination. Vaccination provided full protection in sheep and goats against the NSDV 1473 lethal challenge strain.

However, the significant increase in temperature between the mock vaccinated and the vaccinated animals indicate the pathogenicity of the NSDV challenge. A sharp increase in body temperature between the ovine control and ovine vaccinated animals was observed from 2- 9 after challenge depicting the increasing viremia. All mock vaccinated sheep developed severe symptoms and two animals succumbed to the disease. Vaccinated sheep developed mild symptoms with one sheep showing moderate disease presenting with blood-stained feces. Viremia was detectable in both groups during pyrexia. All vaccinated sheep recovered by day 8 post infection, in contrast, a slight rectal temperature change was observed in caprine mock with caprine vaccinated temperature remaining within the normal range. Temperature reaction marked the onset of the disease in the animals. Peak viremia was reached on day 4 post challenge with average $\log_{10}$ 100TCID50 3, virus titer of 5.5 for the ovine mock, 3,5 for caprine mock, 3.0 for ovine vaccinate and 2.0 for caprine vaccinate. Gradual decrease for viremia was observed for the groups with ovine vaccinate and caprine vaccinate having undetectable virus by day 7 post challenge. Mock vaccinated groups had detectible viremia levels with an average of $\log_{10}$ TCID50 1.75 and 0.875 for ovine mock and caprine mock vaccinated respectively. On day 9 and 12 post challenge two mock vaccinated sheep died from severe hemorrhagic gastroenteritis.

Infectious challenge in mock vaccinated groups elicited low neutralizing titers detectable in first detected in sheep by day 7 post challenge. Neutralizing titers in goats were detectable in goats by day 14. One log decrease was observed in the neutralizing titer far the vaccinated animal followed by a subsequent increase in neutralizing antibody titers by day 14 attributed to humoral response induced by the infectious challenge

V. CONCLUSION

This study evaluated the immunogenicity and protective efficacy of formalin inactivated Nairobi sheep disease vaccine in experimental conditions. Results obtained from this research demonstrated that this vaccine is safe and effective against challenge with NSDV 1473 challenge virus. Inactivated vaccine protect sheep from developing severe symptoms of the disease. Goat is not a good model for Nairobi sheep disease. Further research is required to optimize the dosage and evaluation in larger field studies is warranted.

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