Volume 9, Issue 6, June – 2024 International Journal of Innovative Science and Research Technology
ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUN1674

Evaluation of Infectivity and Immunogenicity of Sugar Stabilized Nairobi Sheep Disease Vaccine

Ngari P. Muriuki[1, 2 *], A. K Nyamache[1], Leonard O. Ateya[3], Yatinder S. Binepal[3],
Caroline Wasonga[4], Muthamia M. Kiraithe[5], Ithinji G. D[2†]

1. Department of Biochemistry, Microbiology & Biotechnology, Kenyatta University, P.O. Box 43844-00100, Nairobi, Kenya.
2. Kenya Agricultural and Livestock Research Organization (KALRO), Veterinary Science Research Institute (VSRI)-Muguga, P.O Box 32-00902, Kikuyu, Kenya.
3. Kenya Agricultural and Livestock Research Organization (KALRO), Biotechnology Research Institute (BioRI)-Kabete, P.O. Box 57811-00100, Nairobi, Kenya.
4. Department of Biochemistry, University of Nairobi, P. O. Box 30197 - 00100. Nairobi, Kenya.
5. Department of Research& Development, Kenya Veterinary Vaccine Production Institute (KEVEVAPI), P.O. Box 53260-00200, Nairobi, Kenya.

*Correspondence; Ngari P. Muriuki
† Equally contributing Author

Abstract:- This work presents a cost-effective approach that can tremendously reduce the financial and logistical burden for vaccine delivery. Strict requirement of a cold chain account about 80% of the total cost of vaccination program. This research paper detail the use of inexpensive FDA approved biocompatible sugars for stabilizing inactivated Nairobi sheep disease virus vaccine. We evaluated sugar additives that improve the storage and maintain the antigenic properties of inactivated vaccine. Low concentrations of trehalose and sucrose maintain the residual infectivity of the virus. Nairobi sheep disease virus vaccine was lyophilized in trehalose and sucrose and subjected to accelerated stability test at temperatures; 25 ^{0}C, 37 ^{0}C and 40 ^{0}C. Results of this study showed the sugar stabilizers can retain residual infectivity for 14 days at 40^{0}C. Stabilization effect of trehalose and sucrose preserve the efficacy of inactivated vaccine stored at 40 ^{0}C for despite loss of infectivity.

I. INTRODUCTION

Thermal labile vaccine requires constant storage under a cold chain from production and dispensation in order to maintain vaccine efficacy (Matthias et al., 2007). These requirements pose a significant economic and logistic challenges for vaccination programs. Global climate change significantly increases vaccine delivery challenges compounded with increased spread of infectious disease.(Asad & Carpenter, 2018; Ebi & Nealon, 2016; Kurane, 2010; Wu et al., 2016). Versatile thermal stabilization technologies and platforms would significantly increase the access to vaccine and greatly decrease the vaccine delivery cost especially in underdeveloped countries. Developing countries are the worst hit by the burden of infectious disease being the most affected by high morbidity and mortality. Despite vaccinations being the most effective method in the control of infectious disease both for human and animal diseases, developing countries lack the costly cold chain infrastructure needed for vaccination programs. Unreliable power supply disrupts the network of refrigerated storage and distribution the cold chain facilities. (Azimi et al., 2017; Zhang et al., 2021). Most vaccines currently available require a strict requirement of a cold chain in refrigeration to maintain their stability and viability ensuring that they remain infectious and effective. Lack of a thermostable vaccine whose use is not limited to strict requirements of refrigeration has been identified as the main challenge affecting global health due to high cost of vaccine delivery and reduced efficacy of the vaccination programs, (Walgate, 2003). Thermal stable vaccines offer a simple, cost-effective platform for achieving the goal of universal access to vaccines. Lyophilizing vaccines in trehalose and sucrose offers an inexpensive and FDA approved method, significantly extending the vaccine shelf-life. Disaccharides offers their cost-effective thermal stabilization option due to their ability to form a solid in which antigen (protein, DNA or RNA) can be immobilized with zero chemistry enabling them to survive desiccation. (Sakurai et al., 2008). Lyophilized vaccines and biopharmaceutical products are cryopreserved in stabilizers and excipients formulations from non-reducing sugars (Iturriaga et al., 2009). Sugars such as sucrose and trehalose can be utilized as stabilizing agents to enhance the stability and viability to reduce limitations associated with cold chain requirements especially in developing countries. Sugar stabilization reduce the logistical and financial burden on vaccine delivery particularly in developing countries hence increase the vaccine accessibility globally. Trehalose and sucrose offer a cost-effective FDA approved stabilizer formulations which are able to maintain the stability and viability without refrigeration.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUN1674

II. MATERIALS AND METHODS

➢ *Cells and Virus Propagation*

BHK cells were propagated in Glasgow minimum essential medium (GMEM) supplemented with 10% fetal calf serum (FCS Sigma Aldrich). BHK cells were inoculated with Nairobi Sheep Disease Virus and cells maintained with GMEM containing 5% FCS. Infected cells were incubated at 37 ^{0}C and routinely monitored for CPE and the virus was after 5 days post infection at 80% CPE and stored in -80 ^{0}C.

➢ *NSDV Antigen Preparation*

BHK 21 cells clone K21 were propagated in T175 in GMEM BHK21 medium supplemented with 10% FCS for cell growth. Confluent monolayer cells were inoculated with 2mls of NSDV infected culture 10^8 TCID50. The virus was allowed to adsorb for 1 hour at 37 ^{0}C in a humidified CO_2 incubator after which the monolayer was washed with plain 0.01M phosphate buffered saline after. Cells were maintained with GMEM BHK21 medium supplemented with 2% FCS and monitored daily to observe for infected cells. Cells were incubated at 37 ^{0}C in a humidified CO_2 incubator until cytopathic effect (CPE) advanced in 80-90% of cells in 3-5 days. Infected cultures were harvested by scrapping of the infected cells using cell scrapers and the infected viral cells pooled together. The culture was then centrifuged at 3000 rpm for 30 minutes and the pellet retained. Cell pellets were suspended in 0.01M Phosphate Buffer Saline (PBS) + 0.5% BSA in the ratio 50:1 of the original infected fluid culture. The virus culture was ultra-sonicated on ice in ice six times, 30 sec each with 30 sec between each period. Cell lysate was ultra-centrifuged at 42,000 rpm for 30 minutes and aliquots of 0.5mls of the supernatant (antigen) were made and stored at -80. Viral protein was quantified using a nano drop with 0.01M phosphate buffered saline as the standard.

➢ *Virus Inactivation and Stabilization of NSD*

Virus was inactivated with 0.01% formaldehyde using method described by two different stabilizers were used; 10% Trehalose and 10% sucrose in 5% lactalbumin hydrolysate (LAH) prepared in Hank's balanced salt solution (Gibco HBSS, pH 7.2 (Mariner, 2017). Stabilizers were added to the viral harvest in 1:1 ration to give final concentration of 2.5% of LAH and 5% Trehalose in LAH+ Trehalose (LT) and 5% sucrose in in LAH+ Sucrose (LS).

➢ *Viral Accelerated Stability Test*

Residual stability of the lyophilized virus for trehalose and sucrose stabilizer formulations was conducted at 25 ^{0}C, 37 ^{0}C and 40 ^{0}C. Exposure of the lyophilized vaccine in vials to the test parameters was done in triplicates with periodic monitoring of temperatures. Tests viral stability tests conducted using protocol described by (Allison et al., 1981) for measles vaccine subjecting all the batches of trehalose and sucrose each at 25 ^{0}C, 37 ^{0}C and 40 ^{0}C with stabilized viral seed as control to determine the residual infectivity.

Batch 1 of trehalose and sucrose was subjected at room temperature (RTP) at 25 ^{0}C by placing the vaccine on the bench. Batches 2 and 3 of trehalose and sucrose were put in two incubators set at 37 ^{0}C and 40 ^{0}C respectively. Sampling points for all batches were days 0, 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21. The viral seed controls for each batch was titrated and residual viral infectivity determined.

➢ *Quantification of Residual Viral Infectivity (TCID50)*

Residual viral infectivity was quantified by titrating each batch at each time point and comparing the residual infectivity by estimating the tissue culture infectious dose TCID50. Cells were grown in a 96 well plate to 80% confluence and three replicates were infected with 100ul tenfold diluted stabilized viral sample. The plate was incubated in a humidified CO_2 incubator at 37 °C for 6 days and CPE monitored daily on an inverted microscope. The residual viral infectivity titers were determined by Spearman Kerber formulae and subsequent decay curve regression analysis to determine the accelerated stability of the virus at different temperature in trehalose and sucrose. Stability of the virus in different stabilizer formulations was determined and compared by t tests, estimating the differences in viral titers at the same parameters for each stabilizer formulations.

➢ *Immunogenicity and efficacy in BALB/c mice*

Purified NSDV antigen was titrated in BALB/c mice to determine the effective dose. Twelve mice (n=4/group) were immunized intraperitoneally (IP) each group with 5ug, 10ug and 20ug respectively. The mice were bled in weekly intervals, seven days apart up to day 35 post immunization. Neutralizing antibody titers were determined by micro-plaque reduction neutralization assay.

Six weeks old mice ware immunized intraperitoneally with inactivated NSDV in three matrices; Vaccine dried in trehalose incubated at 40 ^{0}C for 14 days; Inactivated NSDVv vaccine dried in sucrose stored at 40 ^{0}C for 14 days: and Inactivated NSDv vaccine frozen in -80 ^{0}Cto determine its immunogenicity. Two matrices of the lyophilized vaccine were composed of inactivated NSDv+ 5% Trehalose and Inactivated NSDv+ 5% stored at 40^{0}C for fourteen days prior to be used in this study. A total of 16 mice (n=4/group) were immunized with each matrix per group and a control group immunized with 100uL inactivated vaccine stored at -80 ^{0}C. A booster dose was given 14 days after the first immunization with the same formulation of the first immunization. At day 28 post immunization, the mice were challenged with 100ul of 10^6TCID50 of the 1473 challenge virus. The mice were bled at day 0, 14, 28 and day 35 via the tail snip method and the blood was +4 ^{0}C over-night before spinning down serum at 3000rpm for 30 minutes. During challenge the mice were monitored for 7days for temperature, weight change and clinical scores made.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUN1674

III. RESULTS

Residual infectivity for the two matrices trehalose and sucrose exhibited a biphasic degradation composed of sharp initial loss of titer, followed by gradual loss in titer throughout the exposure period. Sugar stabilizer composed of trehalose vaccine matrix had a high initial titer 6.45 $\log_{10}$ TCID50/ml and sucrose score a low initial titer of 5.57 $\log_{10}$ TCID50/ml. Stabilizer formulation composed of 5% Trehalose + 2.5% Lactalbumin hydrolysate was determined observed to be a superior stabilizer for room temperature, 37^0C and 40^0C. There was total loss in residual infectivity by day 9 for both trehalose and sucrose.

Table 1: Residual Infectivity of Nairobi Sheep Disease Virus Stabilized in Trehalose and Sucrose Sugar Formulations in Lactalbumin Hydrolysate

DPE	LAH +Trehalose (25^0C)	LAH + Trehalose (37^0C)	LAH + Trehalose (40^0C)	LAH + Sucrose (25^0C)	LAH + Sucrose (37^0C)	LAH + Sucrose (40^0C)
0	6.45	6.45	6.45	5.57	5.57	5.57
1	6.05	5.98	4.8	5.54	4.51	4.51
3	6	5.8	3.9	5.51	4.14	3.69
5	5.95	5.5	3.08	5.49	4.02	3.07
7	5.95	5.48	2.15	5.47	3.97	2.12
9	5.94	5.4	1.33	5.47	3.86	1.24
11	5.93	5.37	n. d	5.42	3.74	n. d
13	5.8	5.27	n. d	5.3	3.59	n. d
15	5.36	5.18	n. d	5.29	3.5	n. d
17	5.36	5.14	n. d	5.22	3.43	n. d
19	5.29	5.09	n. d	5.11	3.33	n. d
21	5.29	4.91	n. d	4.81	3.2	n. d

**DPE-Days Post Exposure. *LAH- Lactalbumin hydrolysate. *n. d- not detected.*

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUN1674

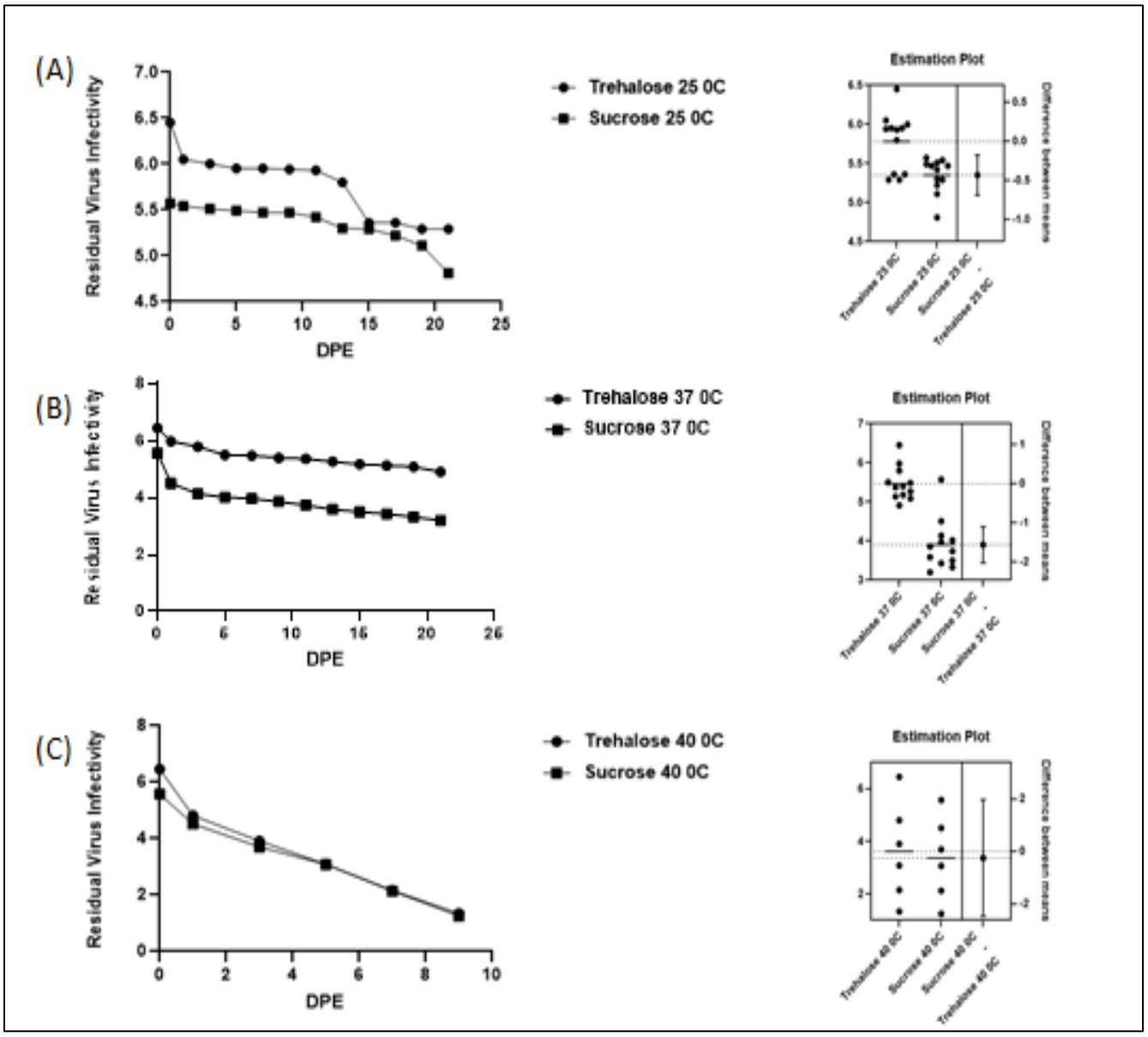

Fig 1. Residual infectivity of NSDV stabilized in trehalose and sucrose and estimation plot showing mean differences between the two stabilizers and levels of significance determined by ANOVA at p=0.05 and Tukey's multiple comparison at P=0.05: {(A) Residual infectivity at 25^0C and mean difference at 25^0C (ns, P=0.8081). (B) Residual infectivity at 37^0C. and mean difference at 37^0C (***, P=0.0004) (C) Residual infectivity at 40^0C; and mean difference at 40^0C (ns, P=0.9952). Residual infectivity for trehalose stabilized vaccine matrices at 25^0C compared at 37^0C and 40^0C (ns, P=0.9392; ****, P<0.0001) and Sucrose stabilized matrices at 25^0C compared at 37^0C and 40^0C (**, P=0.0013; ***, P=0.0002)

Stability of the virus at 25 ^{0}C for trehalose stabilized vaccine is as good as that of sucrose with mean difference differing insignificantly **(Figure 1(a)).** However, residual stability for trehalose stabilized vaccine exposed at 37 ^{0}C, observed to be higher than that of sucrose **(Figure 1(b))**. The sharp gradual decrease at 40 ^{0}C in residual infectivity observed for trehalose and sucrose was similar for both matrices limiting application for long term use in extreme temperatures **(Figure 1(c))**.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUN1674

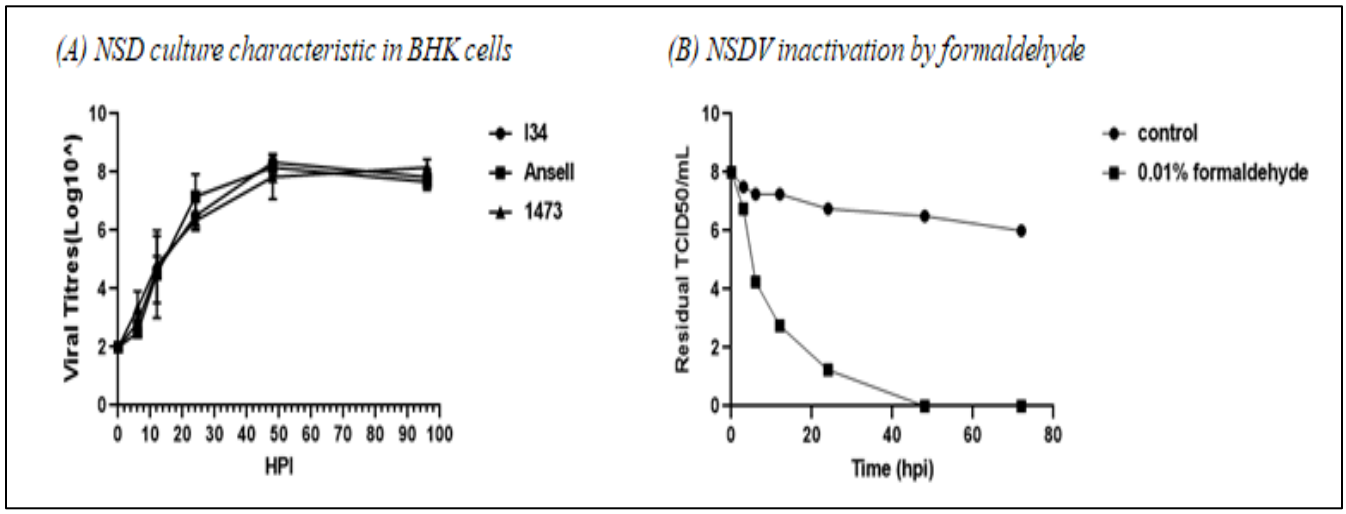

Fig 2 (A) Nairobi Sheep Disease Virus Culture Characteristic in BHK Cells, (B Residual Infectivity on Formalin Inactivation.

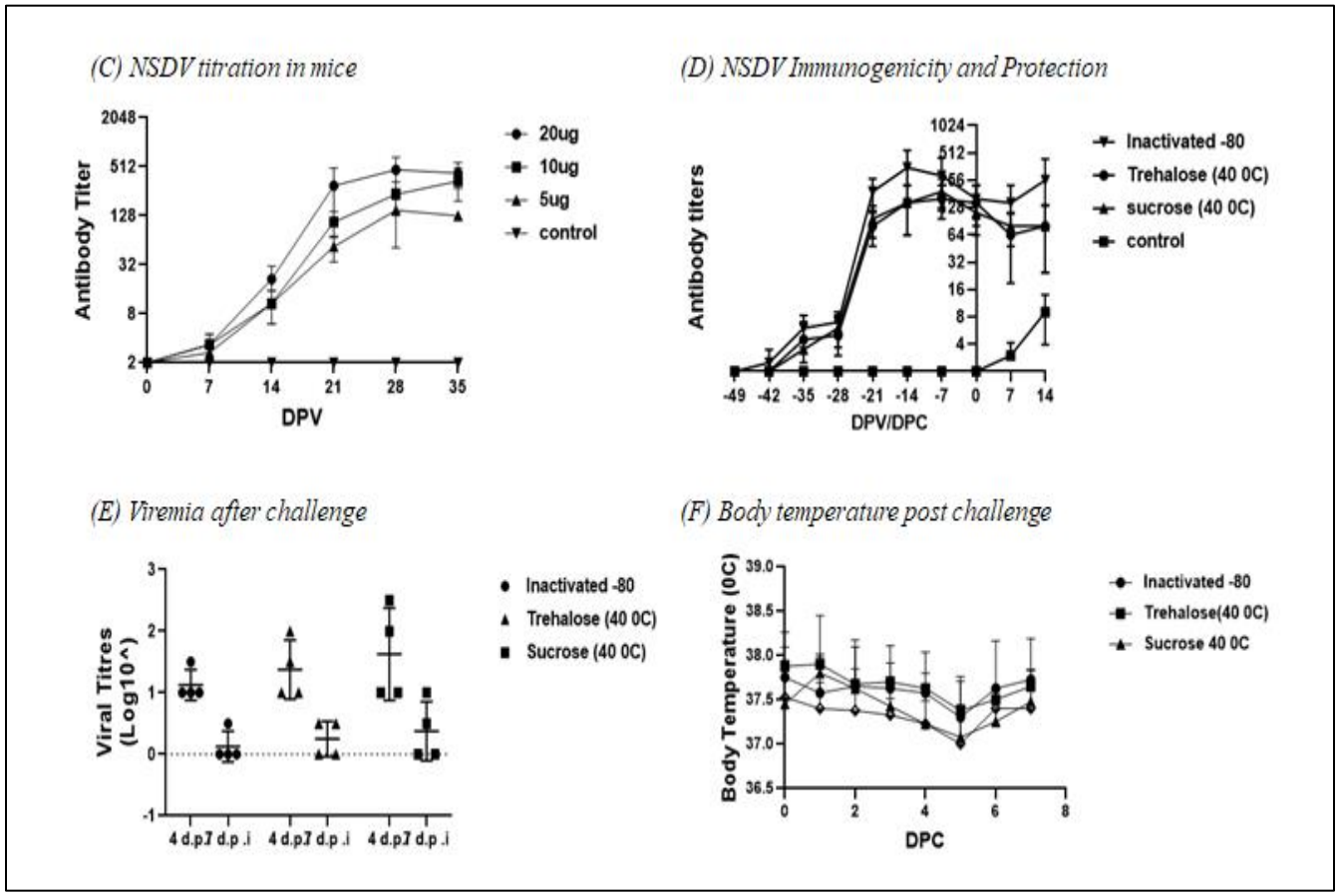

*Fig 3:. (C) Titration of inactivated NSD vaccine in BALB/c mice with 20ug, 10ug, 5ug compared to PBS control group by Two-way ANOVA and Tukey's multiple comparison at P=0.05 (****, P<0.0001; ***, P=0.0003; ns, P=0.1440). Dose comparison for 20ug, against 10ug, 5ug and control was significant (**, P=0.0071; ****, P=0.0001; ****, P=0.0001) (D) Antibody response in mock vaccinated mice (simulating total loss in immunogenicity), mice vaccination with sugar stabilized vaccine exposed at 40^0C compared to control mice vaccinated with inactivated vaccine stored at -80^0C (****, P<0.0001; **, P=0.0077; *, P=0.0370) (E) Viremia in vaccinated BALB/c mice. (F) Body temperature post challnge*

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUN1674

Nairobi sheep disease virus was observed to be fully inactivated by 0.01% formaldehyde at +4 ^{0}C. Viral culture was observed to be stable at +4 ^{0}C and exhibited a gradual decay as shown in ***Figure 2*** Sugar-stabilized vaccine lost all its residual infectivity when stored at 40 ^{0}C by day 9 post exposure. Despite loss in infectivity, the viral antigenic properties were preserved and there was no observable loss in efficacy when tested in mice. Both trehalose and sucrose preserved the antigenic properties. This result needs to be further optimized at wide range of temperatures in long-term stability test.

IV. DISCUSSION

The main challenge in preservation of vaccines is possible loss of potency due to a compromised cold chain integrity during storage and during transportation. Parameters to consider to ensure maintenance of vaccine quality is the lyophilizing agent formulation, virus type and temperature. These factors must be carefully regulated to ensure viability of the vaccine is preserved (Pastorino et al., 2015). Stringent requirement of a cold chain for vaccine storage account for up to 80% of the total cost of vaccination strategy and is susceptible to failure and loss of vaccines (Portnoy et al., 2015). Use of simple sugars such as trehalose and sucrose offer a cost-effective and simple sugar membrane technology to thermos-stabilize viral vaccines. Stabilization of vaccine in the fibrous matrix ensures prolonged storage and maintenance of viability when exposed to extreme temperatures (Caremans et al., 2020.; Leung et al., 2019).

This study evaluated the residual infectivity of Nairobi sheep disease virus thermos-stabilized in trehalose and sucrose. Low freezing temperature of -80 ^{0}C was considered as the optimal storage temperature. Freeze dried NSDV vaccine was exposed in three matrices for 21 days simulating the extreme environmental temperatures; matrice 1 at RTP 25 0C, matrice 2 at 37 ℃ and matrice 3 at 40 ^{0}C. Residual infectivity of the virus was then determined to evaluate the sugar's ability to maintain the vaccine infectivity. Exposure of the lyophilized vaccine formulations for trehalose and sucrose at temperature of 25 ^{0}C for 21 days showed a gradual decrease in residual infectivity. Residual plot of viral titers plotted against the exposure period showed an initial sharp decrease of viral titers within period within the first 2-3 days followed by gradual decrease in residual viral infectivity throughout the exposure period.

Trehalose stabilizer formulation showed a gradual decrease in residual infectivity at 25 ^{0}C. Residual titers curve showed an initial sharp drop within the first three days with a gradual loss in residual infectivity throughout the period of 21 days. Similar biphasic degradation curve of residual titers against the exposure period was observed with sucrose stabilizer formulation. Biphasic loss in residual infectivity at 37 ^{0}C was observed with a rapid initial loss compares to room temperature followed by a gradual linear decay. At 40 ^{0}C, rapid linear decay in infectivity titers at each time point was observed with undetectable infectivity by day six for both stabilizer matrices as earlier reported by (Mariner et al., 2017). This indicates that the vaccine is stable for use in harsh environment without strict requirement of a cold chain.

Table 2: Average residual infectivity of sugar stabilized Nairobi sheep disease virus

Stabilizer	Initial titer	Sample Size	Regression Equation	P value	Initial Titer Range	Shelf-Life Range
Trehalose 25 ^{0}C	6.272	12	Y = -0.04868*X + 6.272	<0.0001	6.107 to 6.436	102.9 to 174.7
Trehalose 37 ^{0}C	6.029	12	Y = -0.05599*X + 6.029	<0.0001	5.826 to 6.231	85.20 to 149.2
Trehalose 40 ^{0}C	5.778	6	Y = -0.5184*X + 5.778	0.0009	4.930 to 6.627	9.270 to 14.53
Sucrose 25 ^{0}C	5.636	12	Y = -0.05664*X + 5.636	<0.0001	5.524 to 5.747	76.35 to 144.95
Sucrose 37 ^{0}C	4.708	12	Y = -0.07960*X + 4.708	0.0002	4.331 to 5.084	45.21 to 90.59
Sucrose 40 ^{0}C	5.232	6	Y = -0.4477*X + 5.232	0.0001	4.778 to 5.686	10.34 to 13.61

Nairobi sheep disease virus stabilized in both trehalose and sucrose rapidly lost its infectivity within the first five days. Despite loss in infectivity, trehalose and sucrose stabilized vaccines maintained their efficacy. The sugars preserved the antigenic proteins of the inactivated vaccine. Regression curve determined that the residual infectivity could be maintained for a maximum of 14 days. Sugar stabilization for NSDV need to be further optimized to determine the long-term stability under extreme temperatures.

BALB/c mice vaccinated with trehalose and sucrose stabilized vaccine stored at 40 ^{0}C for 14 days generated neutralizing antibodies against the NSD virus. Mice group vaccinated with inactivated vaccine stored at -80 ^{0}C used as a control group. Mock vaccinated group was also included to simulate total loss in efficacy. The mean difference in neutralizing antibody titers between trehalose, sucrose and the mock group was found to significantly different using two-way ANOVA at P<0.0001. Using Dunnett's multiple comparison, all the groups differed significantly at p=0.05, trehalose* (p=0.037), sucrose** (p=0.0077) and mock**** (p<0.0001).

Both trehalose and sucrose stabilized inactivated vaccine conferred protection with correlated visible signs including piloerection in day 2 and 3 prior to recovering. The viral titers for the all the vaccinated groups correlated closely at day 3 with only one mouse in the sucrose matrix having detectable viral titer of 10^1 at day five. All the mice had resolved their infection at day five. Results obtained from trehalose

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUN1674

stabilized and sucrose stabilized vaccine data showed no significant statistical difference at p value of 0.05 to the mice control group with inactivated vaccine stored at -80 ^{0}C.

V. CONCLUSION

Nairobi sheep disease virus can be inactivated effectively using formalin concentration 0.01% at +4 ^{0}C within 48 hours. This study deduced that trehalose and sucrose are effective cryoprotectant for viral vaccine. Trehalose is a superior cryoprotectant than sucrose as determined by the end point residual infectivity. Trehalose and sucrose preserve viral protein antigenic properties.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Kenyatta University, Kenya Agricultural and Livestock Research organization for facilities to carry out this work.

- *Data Availability*

All data and material that support the findings of this study are included in this manuscript.

- *Funding*

This study was fully funded by Kenya Climate Smart Agriculture Project (KCSAP) through the Ministry of Agriculture, Livestock, Fisheries State Department for Crop Development and Agricultural Research grant number KCSAP/COMP.2/243.

- *Conflict of Interest*

The authors declare no conflict of interest.

REFERENCES

[1]. Allison, L. M. C., Mann, G. F., Perkins, F. T., & Zuckerman, A. J. (1981). An accelerated stability test procedure for lyophilized measles vaccines. Journal of Biological Standardization, 9(2), 185–194. https://doi.org/10.1016/S0092-1157(81)80022-4

[2]. Asad, H., & Carpenter, D. O. (2018). Effects of climate change on the spread of zika virus: a public health threat. Reviews on Environmental Health, 33(1), 31–42. https://doi.org/10.1515/REVEH-2017-0042

[3]. Azimi, T., Franzel, L., & Probst, N. (2017). Seizing market shaping opportunities for vaccine cold chain equipment. Vaccine, 35(17), 2260–2264. https://doi.org/10.1016/J.VACCINE.2016.12.073

[4]. Caremans, J., Valgaeren, H., Huizing, M., & Rompaey, V. Van. (2020). Virucidal activity of formaldehyde solutions used for preservation of allograft tympano-ossicular systems. Royal Belgian Society for Ear, Nose, Throat, Head and Neck Surgery. 16(4), 202-208. https://doi.org/10.5152/B-ENT.2021.20119

[5]. Ebi, K. L., & Nealon, J. (2016). Dengue in a changing climate. Environmental Research, 151, 115–123. https://doi.org/10.1016/J.ENVRES.2016.07.026

[6]. Iturriaga, G., Suárez, R., & Nova-Franco, B. (2009). Trehalose Metabolism: From Osmoprotection to Signaling. International Journal of Molecular Sciences, 10(9), 3793-3810. https://doi.org/10.3390/IJMS10093793

[7]. Kurane, I. (2010). The effect of global warming on infectious diseases. Osong Public Health and Research Perspectives, 1(1), 4–9. https://doi.org/10.1016/J.PHRP.2010.12.004

[8]. Leung, V., Mapletoft, J., Zhang, A., Lee, A., Vahedi, F., Chew, M., Szewczyk, A., Jahanshahi-Anbuhi, S., Ang, J., Cowbrough, B., Miller, M. S., Ashkar, A., & Filipe, C. D. M. (2019). Thermal Stabilization of Viral Vaccines in Low-Cost Sugar Films. Scientific Reports, 9(1), 1–11. https://doi.org/10.1038/s41598-019-44020-w

[9]. Mariner, J. C., Gachanja, J., Tindih, S. H., & Toye, P. (2017). A thermostable presentation of the live, attenuated peste des petits ruminant's vaccine in use in Africa and Asia. Vaccine, 35(30), 3773–3779. https://doi.org/10.1016/j.vaccine.2017.05.040

[10]. Matthias, D. M., Robertson, J., Garrison, M. M., Newland, S., & Nelson, C. (2007). Freezing temperatures in the vaccine cold chain: A systematic literature review. Vaccine, 25(20), 3980–3986. https://doi.org/10.1016/j.vaccine.2007.02.052

[11]. Pastorino, B., Baronti, C., Gould, E. A., Charrel, R. N., & De Lamballerie, X. (2015). Effect of chemical stabilizers on the thermostability and infectivity of a representative panel of freeze-dried viruses. PLoS ONE, 10(4), 1–12. https://doi.org/10.1371/journal.pone.0118963

[12]. Portnoy, A., Ozawa, S., Grewal, S., Norman, B. A., Rajgopal, J., Gorham, K. M., Haidari, L. A., Brown, S. T., & Lee, B. Y. (2015). Costs of vaccine programs across 94 low- and middle-income countries. Vaccine, 33(S1), A99–A108. https://doi.org/10.1016/j.vaccine.2014.12.037

[13]. Sakurai, M., Furuki, T., Akao, K. I., Tanaka, D., Nakahara, Y., Kikawada, T., Watanabe, M., & Okuda, T. (2008). Vitrification is essential for anhydrobiosis in an African chironomid, Polypedilum vanderplanki. Proceedings of the National Academy of Sciences of the United States of America, 105(13), 5093–5098. https://doi.org/10.1073/PNAS.0706197105

[14]. Walgate, R. (2003). Gates Foundation picks 14 grand challenges for global disease research. Bulletin of the World Health Organization, 81(12), 915–916.

[15]. Wu, X., Lu, Y., Zhou, S., Chen, L., & Xu, B. (2016). Impact of climate change on human infectious diseases: Empirical evidence and human adaptation. Environment International, 86, 14–23. https://doi.org/10.1016/J.ENVINT.2015.09.007

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/IJISRT24JUN1674

[16]. Zhang, W., Deng, H., Liu, Y., Chen, S., Liu, Y., & Zhao, Y. (2021). Antiviral Effectivity of Favipiravir Against Peste Des Petits Ruminants Virus Is Mediated by the JAK/STAT and PI3K/AKT Pathways. Frontiers in Veterinary Science, 8(722840), 1-12. https://doi.org/10.3389/fvets.2021.722840