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Research Article

Effect of Different Stabilizers on the Stability of the Lyophilized Bovine Ephemeral Fever Virus

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Abstract

Purpose: This work aims to select the best stabilizer for lyophilization [freeze-drying] of bovine ephemeral fever virus [BEFV] that provide better physical appearance and high stability in different storage temperatures.

Materials and Methods: Each of the four portions of live BEF virus suspension was stabilized in the ratio of 1:1 [v/v] with one of four different stabilizers 10% lactose with 2% peptone; 10% skimmed; 0.5% gelatin with 2% sorbitol and 5% lacto albumin hydro lysate with 2.5% sucrose. All these mixtures were freeze dried [lyophilized] where it was noticed that skimmed milk showed better physical appearance than other stabilizers. Each one of the 4 dried mixtures were divided into 4 portions, kept at0, 4, 37 and -20° C respectively. Samples were collected for virus titration monthly from virus samples kept under 0; 4 and -20 ° C, and every day from samples kept at 37oC.

Results: After lyophilization the loss of virus titer was 0.5log10TCID50/mL using lactose and peptone, gelatin with sorbitol and lacto albumin hydro lysate and sucrose while the loss of virus titer was 0.25log10TCID50/mL using skimmed milk. The best storage temperature for all stabilized BEF virus preparation is -20oC showing any reductions in virus titer/ month intervals followed by 0oC. There is no difference on vaccinated calves' immune response to the different stabilized BEFV.

Conclusion: Skimmed milk provides a better physical appearance and thermo stability than other used stabilizers.

Keywords: *Stabilizers; Bovine ephemeral fever virus; Gelatin; Sorbitol; Lacto albumin hydro lysate*

Introduction

Bovine ephemeral fever virus [BEFV] is an acute febrile, arthropod-born viral disease of cattle and water buffalo clinically characterized by sudden onset of fever, stiffness, lameness and spontaneous recovery within three days with high morbidity and mortality rates. The disease causes significant economic losses due to drop in production in dairy herds and reduction in condition of prime animals or disruption of stock movement and markets [1].

BEF is endemic in tropical and subtropical regions of Africa, Asia, Australia and the Middle East [2] and caused by a virus belongs to the Ephemero virus of the Rhabdoviridae family [3]. In Egypt, the disease was first described since 1924 and several subsequent outbreaks of BEFV had been occurred in summer of 2000, 2001, 2004, 2010 and 2012 [4,5].

Control of insect vectors and vaccination of susceptible hosts are two arms of BEF control [6].There are known potent safe BEF vaccines include attenuated and inactivated cell culture vaccines which provide vaccinated animals with adequate levels of specific antibodies [7-13]. Nowadays the used vaccine in Egypt is a live virus inactivated on the time of vaccination using a specific diluent containing saponin [13].

It is known that live vaccines are based on attenuated strains of the virus that retains limited ability to reproduce in the animal body, but is capable of inducing immune response. In order to ensure high immunogenicity, a live vaccine should have high reproductive activity [high titer] [14]. To maintain a high infectious titer, vaccine strains are subjected to lyophilization. Prior to lyophilization, the biological product is dissolved in a protective medium. This technological stage stabilizes the properties of the bio preparation and considerably increases shelf life of the product.

Our aim was to select the optimal protective medium for lyophilization of live BEF virus. Such media [stabilizers] include lactose and peptone; 10% skimmed milk; gelatin and sorbitol and 5% lacto albumin hydro lysate and 2.5% sucrose.

Materials And Methods

Ethical approval

Care and use of laboratory animals in this study were approved by the Medical and Veterinary Research Ethics Committee at the National Research Centre in Egypt (No., 20/053).

BEF virus

BHK-21 cell culture adapted strain of BEF virus of a titer $10^{7.5}$ $TCID_{50}$/ml [15] were supplied by the Veterinary Serum and Vaccine Research Institute [VSVRI], Abbasia, Cairo and used for preparation of lyophilized virus with 4 different stabilizers and in serum neutralization test to follow up the levels of induced antibodies in vaccinated calves for six months.

Cell culture

Baby hamster kidney cell line [BHK-21] established by [16] and supplied by VSVRI were used preparation of BEF virus suspension and in serum neutralization.

Virus propagation in tissue culture

Confluent BHK cell lines in roller flasks were inoculated with BEF virus using MOI 1:1 and incubated at 37° C and examined daily for CPE. When complete CPE was obtained, such flasks were subjected to two cycles of freezing and thawing and the harvest was aseptically centrifuged for 10 minutes at 3000 rpm in a cooling centrifuge and tested for sterility and titration.

Virus titration

BHK-21 cell lines were used for titration of the prepared BEF virus suspension using serial tenfold dilutions of the virus through application of the microtiter technique according to [17] and the virus titer was expressed as $\log_{10}$ $TCID_{50}$/ml of the original inoculums using the formula of [18]. Virus titration was carried out before and after lyophilization.

Preparation of BEF virus suspension for the lyophilization [Freeze-drying]

The titrated BEF virus suspension was divided into 4 portions to be stabilized using the follow stabilizers:

- Stabilizer [1] consisted of 10% lactose and 2% peptone according to [19].
- Stabilizer [2] consisted of 10% skimmed milk added to the virus suspension in according to [20].
- Stabilizer [3] consisted of 0.5% gelatin and 2% sorbitol according to [21].
- Stabilizer [4] consisted of 5% lacto albumin hydro lysate and 2.5% sucrose according to [22].

Each portion of BEF virus suspensions was stabilized with one of the mentioned stabilizer added as 1:1 [v/v] then dispensed in neutral sterile vials [2.5ml/vial] and subjected to freeze-drying [lyophilization] process.

Lyophilization [Freeze-drying] process

The lyophilizing technique was carried out on Tofflonlyophilizer apparatus. A total of 2.5 ml freeze-drying system was filled into a sterilized glass vials covered with a semi permeable rubber stopper. The glass vial's inner diameter was 1.9 cm. The vials were placed on the shelf of the freeze-dryer, which had been pre cooled to - 60 °C, so that quick freezing could be realized [23]. After cooling for 2h, the primary drying began. The shelf temperature was set at -32°C, and the vacuum was controlled under 10 Pa [24]. The primary drying process lasted for 16h. Then the shelf was wormed up to 20 °C at a rate of 0.2 °C/min and held for 6h. After freeze-drying, the vials were sealed and kept at room temperature for 2 h [25] then kept at - 20°C till subjected for evaluation of the effect of lyophilization process.

Stability of the lyophilized BEF virus preparations

Samples from each lyophilized BEF virus preparations were kept at 4 ° C, 20 ° Cand 37° C and subjected for virus titration on month intervals up to 6 months while those kept at 37 ° C were titrated on 24 hours intervals up to 7days post preparation.

Evaluation of the immunogenicity of the lyophilized BEF virus

Fifteen native breed calves of about 6 - 8 months of age and free from BEF antibodies as screened by serum neutralization test were divided into 5 groups [3calves/group] as follow:

- Group [1] vaccinated with the vaccine preparation [1].
- Group [2] vaccinated with the vaccine preparation [2].
- Group [3] vaccinated with the vaccine preparation [3].
- Group [4] vaccinated with the vaccine preparation [4].
- Group [5] was kept without vaccination as test control.

Each virus vial was dissolved in 20ml of the specific diluent containing saponin and supplied by VSVRI leaving for 10 minutes to allow virus inactivation the each animal received 2ml inoculated S/C in the neck side followed by a second dose 2 weeks later according to [13].

Serum neutralization test [SNT]

SNT was carried out using the microtiter technique according to [26] on serum samples obtained from vaccinated calves of week intervals 4 times post vaccination then on month intervals up to 6 months post vaccination. The end point of neutralizing antibody titers was expressed as the reciprocal of the final dilution of serum inhibiting the CPE according to [27]

Results

Through the present work BEF virus was subjected to freeze dried process [lyophilization] using four different stabilizers including 10% lactose with 2% peptone; 10% skimmed milk; 0.5% gelatin with 2% sorbitol and 5% lacto albumin hydro lysate with 2.5% sucrose.

Figure 1 of lyophilized BEFV demonstrated that virus prepared with skimmed milk stabilizer reviled better physical properties [more compact solid disk] than other preparations.

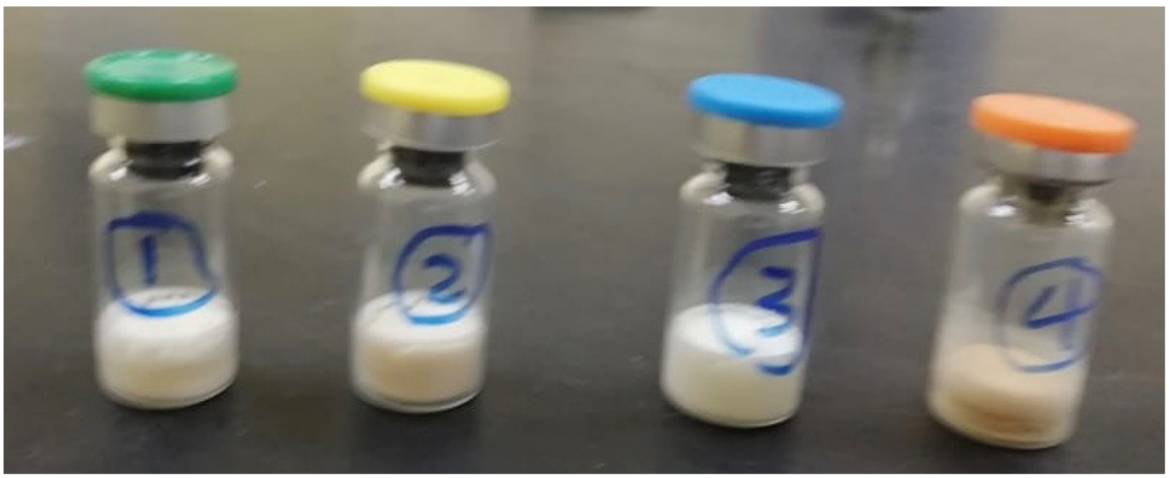

Figure 1: *Lyophilized Bovine Ephemeral Fever (BEF) living virus using different stabilizers.*
[1] BEFV stabilized with10% lactose and 2% peptone.
[2] BEFV stabilized with 10% skimmed milk.
[3] BEFV stabilized with 0.5% gelatin and 2% sorbitol.
[4] BEFV stabilized with 5% lacto albumin hydro lysate and 2.5% sucrose.

Virus titration before and after lyophilization (Table-1) revealed that the loss of virus titer was $0.5\log_{10}TCID_{50}$/mL using lactose and peptone, gelatin with sorbitol and lacto albumin hydro lysate and sucrose while the loss of virus titer was $0.25\log_{10}TCID_{50}$/mL using skimmed milk.

Titrated Stabilized virus	Virus titer [$\log_{10}TCID_{50}$/mL]		
	Before lyophilization	After Lyophilization	Loss during lyophilization
Stabilized virus-1	↑ 7 ↓	6.5	0.5
Stabilized virus-2		6.75	0.25
Stabilized virus-3		6.5	0.5
Stabilized virus-4		6.5	0.5

Table 1: *Titer of different lyophilized Bovine Ephemeral Fever (BEF) virus preparations.*
[1] BEFV stabilized with lactose and peptone.
[2] BEFV stabilized with 10% skimmed milk.
[3] BEFV stabilized with gelatin and sorbitol.
[4] BEFV stabilized with 5% lacto albumin hydro lysate and 2.5% sucrose.

Regarding the stability of the prepared lyophilized BEF virus preparations, table 2 showed that the best storage temperature for all stabilized BEF virus preparation is -20°C showing no reductions in virus titer/month intervals followed by 0°C which reduce the virus titer by 0.25$log_{10}TCID_{50}$/ml/month. At 4°C the lowest reduction of virus titer [0.25$log_{10}TCID_{50}$/ml/month] was that recorded with skimmed milk stabilizer while it was 0.5$log_{10}TCID_{50}$/ml/month for other stabilizers.

Storage temperature	Loss virus titer [$log_{10}TCID_{50}$/ mL during storage			
	BEFV [1]	BEFV [2]	BEFV [3]	BEFV [4]
4°C	0.5/Month	0.25/Month	0.5/Month	0.5/Month
37°C	1/Day	0.5/Day	0.75/Day	0.75/Day
0°C	0.25/Month	0/Month	0.25/Month	0.25/Month
-20°C	0/Month	0/Mont	0/Month	0/Month

Table 2: *Stability of different lyophilized Bovine Ephemeral Fever (BEF) virus preparation at different temperatures.*

[1] BEFV stabilized with 10% lactose and 2% peptone.

[2] BEFV stabilized with 10% skimmed milk.

[3] BEFV stabilized with 0.5% gelatin and 2% sorbitol.

[4] BEFV stabilized with 5% lacto albumin hydro lysate and 2.5% sucrose.

Regarding calves vaccination with the lyophilized BEFV preparations after their dissolving in the specific solvent containing saponin, the results of SNT (Table-3) showed that all animals receiving two doses of such preparations exhibited a detectable level of specific BEF antibodies by the first week post the first dose of vaccination with a mean titer 8 increased gradually after the second dose till reach a peak titer of 128by the first month post the second vaccination and still unchanged with this level up to 4 months post last vaccination [6months post the first vaccination; the period of experimental work].

Used vaccine	Mean BEF serum neutralizing antibody titer [a]								
	Zero time	1WPV[b]	2WPV	1WPB[c]	2WPB	1MPB[d]	2MPB	3MPB	4MPB
Stabilized virus-1	0	4	8	16	64	128	128	128	128
Stabilized virus-2	0	8	16	32	64	128	128	128	128
Stabilized virus-3	0	4	8	32	64	128	128	128	128
Stabilized virus-4	0	8	16	32	64	128	128	128	128
No vaccination	0	0	0	0	0	0	0	0	0

Table 3: *Mean Bovine Ephemeral Fever (BEF) serum neutralizing antibody titer in vaccinated calves.*

[1] BEFV stabilized with lactose and peptone.

[2] BEFV stabilized with 10% skimmed milk.

[3] BEFV stabilized with gelatin and sorbitol.

[4] BEFV stabilized with 5% lacto albumin hydro lysate and 2.5% sucrose.

aAntibody titer= the reciprocal of the final serum dilution which neutralized 100 TCID50 of BEF virus.

bWPV= week post vaccination.

cWPB= week post booster.

dMPB = month post booster.

Discussion

Stability of living viral vaccines is an essential parameter for the determination of product changes in maintenance period, efficacy and maintenance of biological properties of the vaccines. Vaccine stability is a vital factor to keep vaccine potency and efficiency, as its potency

decays over time and during temperature changes. The choice of stabilizers for viral vaccine formulation could be conceders a corner stone in production of living virus vaccines [28].

The obtained more compact disk of lyophilized BEF vaccine using skimmed milk than those obtained by other used stabilizers came in agreement with the findings of [29-32]when they prepared an attenuated rift valley fever virus vaccine, Brucella melitensis Rev 1 vaccine and PPR vaccine using skimmed milk in comparison with other stabilizers they mentioned that skimmed milk made more compact mass [cake] of the vaccine in the respective vials providing an effective preservative for the virus during freeze drying process.

Virus titration showing that the less loss in lyophilized BEF virus ($0.25\log_{10}TCID_{50}$/mL) was recorded with skimmed milk while such loss was $0.5\log_{10}TCID_{50}$/ mL with other used coming in agreement with those of [33] who mentioned that during the Lyophilization process, the water of product is frozen and then subjected to a high vacuum [freeze-drying]. These factors consider as stress factors and lead to damage and decrease in the viability of viruses. Similar results were obtained by [30-32] who also found that prepared FP fowl pox] and PPR [peste des petits ruminants] vaccines with variable concentrations of skimmed milk after lyophilization showed the same decrease in virus titer [0.25 $\log_{10}EID_{50}$] but decrease was [0.5 $\log_{10}EID_{50}$] by using Lactalbumen sucrose stabilizer as shown in table 2 which indicate the role of skimmed milk maintaining of virus titer in agreement with [33].

To successfully carry out international immunization programs, confirming the stability of vaccines is crucial. The thermal stability of vaccines is of concern to the vaccine manufacture, health authorities, research government institutions, and philanthropic organizations attempting to raise the distribution of vaccines in countries with bad infrastructure, unreliable transportation and substandard storage facilities for the preservation of vaccines requiring refrigeration.

World Health Organization [WHO] sets guidelines for the stability evaluation of vaccines [38] and recommends conducting regular and accelerated stability studies. These guidelines aim to provide a framework for shelf life and storage conditions, monitor vaccine stability in the post licensure period and support manufacturing changes by demonstrating consistency of vaccine batches. Such guidelines can be used for the selection of optimal stabilizing conditions, shelf life estimation, temperature excursion modeling, and investigation of changes to manufacturing process that may potentially alter vaccine stability [39]. Many different stability indicating assays can be used, including viral titer, immunochemical assays, liquid chromatography and gel electrophoresis [40,41]. An often used parameter is shelf life, this refers to the time period during which a drug product remains capable of acceptable performance.

Depending on the present results of lyophilized BEF virus stability, it could be suggested that all BEF stabilized virus preparation through the present study could be stored at 4°C for 12 months; at 37°C for not more than 3-4 days to avoid the reduction in virus titer to the non-protective titer which should not be less than 6 $\log_{10}TCID_{50}$/ml on the time of inactivation within the time of vaccination as recommended by [13] and at 0 or -20°C for more than 12 months reaching 24 months [10]**.** These findings agree with those of [19], [29,33] [30-32] using the same stabilizers with rabies virus, RVF; Duck hepatitis virus; Brucella; Fowl Pox and Pigeon pox viruses. In addition it is well known that due to their thermo sensitivity, most vaccines must be kept refrigerated from production to use [34,35].

In addition skimmed milk stabilizer is more efficient than the used lacto albumen sucrose stabilizer as 10% skimmed milk reduced the losses in virus titer during the process of lyophilization and during different thermos-stability tests than the used lacto albumen sucrose stabilizer in addition to the more better physical appearance of the final lyophilized product and more decrease in cost in comparison with the other used stabilizer [36]**;** other stabilizers may be preferable than skimmed milk where they do not contain animal proteins where the use of substances that contain animal proteins is now not recommended due to the risk of transmission of prion diseases and more stringent requirements to the composition of the preparations are imposed [37].

Following up the titers of induced BEF neutralizing antibodies in vaccinated calves using the different lyophilized BEF virus preparation (using specific solvent containing saponin according to 13), similar results o were obtained by [9,11,42-44], that concluded that

vaccination of cattle with 2 doses of the inactivated BEF vaccine induce high levels of immunity against BEF virus infection. In addition [13] stated that the use of live BEF vaccine which was inactivated on the time of administration induced high levels of specific BEF neutralizing antibodies where the diluent of such vaccine contains saponin which act as virus in activator and immune stimulant. However it is clear that no one of the used stabilizers affect the immune response of vaccinated calves where all of them exhibited similar levels of BEF antibody titers. There are no available data discussed the effect of different stabilizers on the immune response of vaccinated animals.

In conclusion and according to the present obtained results, it could be concluded that skimmed milk stabilizer is more efficient than the used lacto albumen sucrose stabilizer where it reduced the losses in BEF virus titer during the process of lyophilization and during different storage temperatures in addition to the better physical appearance of the final lyophilized product.

Bibliography

1. George St TD. "Studies on the pathogenesis of bovine ephemeral fever in Sentinal cattle. I-Virology and serology". *Veterinary Microbiology* 10.6 (1985): 493-504.
2. Yeruham I., *et al.* "Bovine ephemeral fever in beef cattle herds in the Jordan Valley". *Israel* 152.3 (2003): 86-88.
3. Wunner WH., *et al.* "In virus taxonomy, 6th report of the Intern". *International Committee on Taxonomy of Viruses* (1995): 288-293.
4. Kasem S., *et al.* "Isolation and Characterization of Bovine Ephemeral Fever Virus in Delta Provinces, Egypt 2012". *Global Veterinarian* 13.6 (2014): 972-976.
5. Albehwar AM., *et al.* "Molecular characterization of recent isolates of BEF virus in Egypt". *Global Journal of Medical Research: (G) Veterinary Science and Veterinary Medicine* 18.2 (2018): 6-14.
6. St George TD. "Effective treatment of bovine ephemeral fever". *Australian Veterinary Journal* 75 (1997): 221-222.
7. Inaba Y., *et al.* "Formalin-inactivated, aluminum phosphate gel-adsorbed vaccine of bovine ephemeral fever virus". *Arch Gesamte Virusforsch* 42 (1973): 42-53.
8. Tzipori S and Spradbrow PB. "A cell culture vaccine against bovine ephemeral fever". *Australian Veterinary Journal* 54.7 (1978): 323-328.
9. Vanselow BA., *et al.* "Field trials of ephemeral fever vaccines". *Veterinary Microbiology* 46.1-3 (1995): 117-130.
10. Daoud AM., *et al.* "Preparation of attenuated bovine ephemeral fever vaccine". *Beni-Suef Veterinary Medical Journal* 2 (2001a): 627-634.
11. Daoud AM., *et al.* "Preparation of inactivated bovine ephemeral fever vaccine (BEF) in Egypt". *Beni-Suef Veterinary Medical Journal* 2 (2001b): 619-626.
12. Sahar MM Saber. "Epidemiological studies on ephemeral fever in cattle, Ph. D. Vet. Sci. Thesis (Infectious diseases)". *Faculty of Veterinary Medicine, Cairo University* (2004).
13. Albehwar AM., *et al.* "Comparative evaluation of the potency of traditionally inactivated bovine ephemeral fever vaccine and that one inactivated on the time of vaccination. 14th Sci.Cong.2010". *Faculty of Veterinary Medicine, University Egypt* (2010): 51-61.
14. WHO. "Expert committee on biological standardization. Geneva, 19 to 23 October 2009. WHO recommendations to assure the quality, safety, and efficacy of influenza vaccines (human, live attenuated) for intranasal administration (2009).
15. Azab AMH., *et al.* "Susceptibility of different cell cultures to bovine ephemeral fever virus. 6th Vet Med. Zag. Conf (2002): 41-55.

16. Mackpherson LA and Stocker MGP. "Polyoma transformation of hamster cell colonies; an investigation of genetic factors affecting cell competence". *Virology* 16 (1962): 147.

17. Rossiter PB., *et al.* "Micro neutralization system for use with different strains of pest des petit ruminant's virus and rinderpest virus". *Tropical Animal Health and Production* 17.2 (1985): 75-81.

18. Reed LJ and Muench H. "A simple method for estimating fifty percent (50%) ends points". *The American Journal of Tropical Medicine and Hygiene* 27 (1938): 493-497.

19. WHO. "Laboratory techniques in rabies, Fifth edition 1 (1996): 261- 268.

20. OIE (Office International Des Epizootic). Recommended Diagnostic Technique and Requirements for biological products 1(2012): 507-514.

21. Kang MS., *et al.* "Development of a stabilizer for lyophilzation of an attenuated duck viral hepatitis vaccine". *Poultry* (2010): 1167-1170.

22. Thachamvally Riyesh., *et al.* "Evaluation of Efficacy of Stabilizers on the Thermo stability of Live Attenuated Thermo-adapted Peste des petits ruminants Vaccines". *Virologica Sinica* 26.5 (2011): 324-337.

23. Wang J and Zhang H. "The influence of one-step −80 °C cryopreservation on the osteogenesis differentiation ability of human bone marrow-derived mesenchymal stem cells". *Guangdong Medical Journal* 28.3 (2007): 365-367.

24. Zhou XL., *et al.* "Freeze-drying of human platelets: influence of saccharide, freezing rate and cell concentration". *Cryo Letters* 28.3 (2007): 187-196.

25. Shao-Zhi Z., *et al.* "Preliminary study on the freeze-drying of human bone marrow-derived mesenchymal stem cells". *Journal of Zheijang University SCIENCE B - Biomedicine and Biotechnology* 11.11 (2010): PMC2970899.

26. Ferreira ME. "Prubadamicroneutralizationproesludaes de anticurpos de la fiebreaftosablincentro, Panoamericana de ubrea Akrosa 211 (1976): 17-24.

27. Singh KV., *et al.* "Colostral transfer of rinderpest neutralizing antibodies to offspring of vaccinated dams". *Canadian Journal of Comparative Medicine and Veterinary Science* 31.11 (1967): 295-298.

28. Cardoso FMC., *et al.* "REVIEW: Viral vaccine stabilizers: status and trends". *Acta Virologica* 61.3 (2016): 231-239.

29. Salama LS., *et al.* "Trails for using skimmed milk as a stabilizer for attenuating Rift Valley Fever virus vaccine". *Egyptian Journal of Agricultural Research* 4.82 (2004): 1905-1915.

30. Ghazy A., *et al.* "The use of different stabilizers for improving integrity of the locally prepared lyophilized Brucella melitensis Rev 1Vaccine". *Journal of Veterinary Medical Research* 25.1 (2017): 59-67.

31. Latif MZ., *et al.* "Effect of Stabilizers on Infectivity Titer of Freeze Dried Peste Des Petits Ruminants Virus Vaccine". *Pakastan Veterinary Journal* 38.2 (2018): 169-173.

32. Ayatollah I Bassiouny., *et al.* "Efficacy of skimmed milk as stabilizer in comparison with lacto albumen sucrose in production of fowl and pigeon pox vaccines". *International Journal of Research and Development (IJRD)* 2.4 (2019).

33. Alexander DSN., *et al.* "Optimization of a freeze-drying cycle of a viral vaccine based on changes in temperature, time and geometry of the vials". *Journal of Applied Pharmaceutical Science* 5.2 (2015): 022-029.

34. Brandau DT., *et al.* "Thermal stability of vaccines". *Journal of Pharmaceutical Sciences* 92 (2003): 218-231.

35. Chen D and Kristensen D. "Opportunities and challenges of developing thermostable vaccines". *Expert Review of Vaccines* 8.5 (2009): 547-557.

36. Sally M AbdElsalam., *et al.* "Extraction of RB51 rough lipopolysaccharide antigen for evaluation of locally prepared RB51 vaccine". *Bioscience Research* 15.3 (2018): 2253-2259.

37. Emea. "Questions and answers on bovine spongiform encephalopathies and vaccine (2001).

38. WHO/BS/06.2049. Guidelines on Stability Evaluation of Vaccines (2006).

39. Schofield TL. "Vaccine stability study design and analysis to support product licensure". *Biologicals* 37.6 (2009): 387-396.

40. Zhang J., *et al.* "Stabilization of vaccines and antibiotics in silk and eliminating the cold chain". *Proceedings of the National Academy of Sciences of the United States of America* 109.30 (2012): 11981-11986.

41. Hasija M., *et al.* "Forced degradation studies: an essential tool for the formulation development of vaccines". *Vaccine Development and Therapy* 3 (2013): 11-33.

42. Uren MF., *et al.* "Effective vaccination of cattle using the virion G protein of bovine ephemeral fever virus as an antigen". *Vaccine* 12.9 (1994): 845-852.

43. Safaa M Khalid. "Some studies on bovine ephemeral fever in cattle. M.V. Sci. Thesis (Infectious Diseases), Fac. Vet. Med. Zagazig Univ., Benha Branch, Moshtohor (2004).

44. Daoud AM., *et al.* "Some studies on bovine ephemeral fever in buffaloes". 4th Inter. Sci. Conf. Mansoura (2005): 687-701.

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