Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Shelf Life of Freeze-dried Foot and Mouth Disease Virus Stock Seeds

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Abstract

Foot-and-mouth disease viral strains SAT2/EGY/2012, A Iran 05, and FMDV O PanAsia-2 could remain contagious after freeze-drying with minimum losses in infectivity, with or without additives to virus suspensions. Over 12 months of storage at different temperatures, the infectivity titers of freeze-dried viruses were evaluated on a regular basis. Additive solutions were required to prevent virus degradation throughout freeze-drying and minimize infectivity loss during storage at 4°C and -20°C. Six additives were added as stabilizers forming 6 mixtures: (1)Culture medium without additives, (2)A combination of 5% lactalbumin hydrolysate and 10% w/v sucrose, (3)10% skim milk, (4)A mixture of 10% sucrose and 1% gelatine, (5)A mixture of 4% peptone and 1% gelatine and (6)5% Inositol to prepare virus suspensions for the lyophilization process. Evaluation of obtained lyophilized viruses stored at different temp findings suggested that freeze-dried foot-and-mouth disease virus can be shipped and stored for a brief period which can save storage and transportation expenses using a stabilizer formed of 10% w/v sucrose and 5% Lactalbumin hydrolysate or 10% skimmed milk storage at -20, 0, 4°C.

Keywords: Foot and Mouth disease virus, Freez-dried, Stabilizer.

Introduction

Animals with cloven hooves, such as domestic and wild bovids, are susceptible to foot-and-mouth disease (FMD) or hoof-and-mouth disease (HMD), an infectious and occasionally lethal viral disease [1,2]. A picornavirus, member of the genus Aphthovirus, is the causal virus. There are seven main serotypes of the foot-and-mouth disease virus: O, A, C, SAT-1, SAT-2, SAT-3, and Asia-1. Regional variation is evident in these serotypes, with the O, A, SAT2 serotypes being the most prevalent [3]. The virus produces blisters within the mouth and around the hoof that may burst and render the affected animal. The fever is high and lasts for two to six days. For animals with cloven hooves, FMD is a highly contagious vesicular illness caused by the FMD virus (FMDV) [4].

It is desirable for future reference that the biological properties of animal viruses namely, their infectivity and antigenicity remain intact for extended periods. But according to [5], FMDV is extremely thermolabile, and antigens break down into 12s sub-particles at moderate temperatures [6]. Low-temperature storage is therefore necessary to preserve FMDV's integrity.

It is better to keep purified 146s antigens above liquid nitrogen. High storage expenses within the laboratory and airfreight rates for shipments to laboratories abroad are caused by such temperature conditions. The presence of dry ice, or solid carbon dioxide, in packaging that are meant to keep antigens frozen during transportation presents additional handling challenges. A freeze-dried product could get around these issues. Benefits include easier antigen transportation, less mass needed for storage, and elimination of the need for the low temperatures needed to maintain antigenicity and infectivity [7].

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(Received 27 June 2024, accepted 24 July 2024)
DOI: 10.21608/EJVS.2024.299908.2200

Freeze-drying, or lyophilization, is now a commonly used method for virus sample preservation. As mentioned, lyophilized viruses have several significant benefits, including improved stability over extended periods of storage and the capacity to be delivered at room temperature without the need for special handling. Lyophilization is widely used in pharmaceutical companies and culture collections to preserve live virus stocks for use in production, research, and diagnostics. Because viral systems can differ greatly in terms of excipient selection, freezing/drying rates, and post-drying stability, optimizing the lyophilization method for wide applicability is a constant challenge. Understanding how various virus properties affect ideal lyophilization settings is important because it may have a big impact on how important virus samples are stored, transported, and made available. [8,9]

A key element in successful virus lyophilization (freeze-drying) is the choice of stabilizer. The particular virus family being maintained can have a significant impact on a stabilizer's effectiveness. It has been shown that the best defence against enveloped viruses is a culture medium enriched with gelatin and sucrose, which preserves the envelope shape and virus infectivity. Nonenveloped viruses, on the other hand, seem to be less stabilizer-dependent, with even the base culture medium providing adequate defence. It's interesting to note that, aside from picornaviruses, which are thought to be among the most labile viral kinds under these drying pressures, the freezing rate had very little effect on virus stability after lyophilization. This implies that, in comparison to stabilizer selection, freezing rate may be a less important feature for many virus types. Furthermore, it was found that the freezing rate and stabilizer had the most impacts right after the lyophilization process. The infectivity tended to converge throughout the storage period under all of the settings that were assessed [10].

It was demonstrated that, when freeze-dried Schwarz strain measles virus was stored at -70°C or freeze-dried and stored at -20°C, the sorbitol-gelatin stabilizer offered more satisfactory stability (r= +0.18), and their potency was monitored over 21 months virus suspensions in comparison to the glutamic acid-lactose. It was discovered that the sorbitol-gelatin combination worked well as a stabilizer when it came to freezing-dried Schwarz strain measles viral suspensions, which could be utilized as working lots or reference preparations [11].

It is well known that to facilitate freeze-drying process and maintain the biological activity of viral suspensions, some kind of additive solutions must be added. Many additives, such as peptone, Tris-buffer, calcium lactobionate, sodium glutamate, human serum albumin, inositol, dextran, non-fat skim milk, glucose, lactalbumin hydrolysate, and sucrose, have been studied for their effects on the freeze-drying of numerous animal viruses. To determine if different additive solutions were suitable as preservatives, the impact of these solutions on the infectivity of the FMDV was studied in the lyophilization process and the subsequent storage at various temperatures [7].

Freeze-drying, or lyophilization, can offer several benefits for preserving biological products like viruses, vaccines, and antiviral serums. This process can reduce the storage volume, raise the tolerable temperature range, and improve the overall shelf-life compared to the liquid state.

The present work was designed to determine the effect of lyophilization on the stability of Egyptian FMD virus strain seeds aiming to maintain their antigenicity and potency as seeds required for the production of highly potent vaccine.

Material and Methods

FMD viruses

Egyptian isolates of the FMD virus strains O PanAsia-2, A Iran 05, and SAT2/EGY/2012 of calves origin isolated, typed and subtyped at the Veterinary Serum and Vaccine Research Institute (VSVRI), Abasia, Cairo, and confirmed by the World Reference Laboratories, Pirbright, United Kingdom were subjected to the present experimental work.

Cell culture

Baby hamster kidney cell line (BHK21 clone13) kindly provided by The Animal Research Institute, Pirbright, UK was propagated at Foot and Mouth Disease Research Department (FMDRD) using minimum Essential Medium (MEM supplied by Gibco "G80 Gibco Limited, P.O. Box 35 Parisley, Scotland U.K.") with Eagle's salts supplemented with 10% newborn calf serum. The propagation and maintenance of the cell line was carried out by [12].

Virus inoculation in tissue culture

At a multiplicity of infection (MOI) of 1:1, each employed virus strain was introduced into confluent BHK cell lines in roller flasks and incubated at 37˚ C. These flasks were frozen and thawed twice until complete CPE was achieved. After that, the harvest was titrated and its sterility was evaluated by aseptically centrifuging it for ten minutes at 3000 rpm in a cooled centrifuge.

Virus Titrations in tissue culture:

BHK cell line was used for the infectiousness titrations of SAT2/EGY/2012, A Iran 05, and FMDV O PanAsia-2 using the microtiter technique as described by [13] in tissue culture 96-well plates. Control non-infected cells were included with incubation for two days at 37°C, and subjected to microscopic examination for detection of FMDV cytopathic changes (CPE) in comparison to the

control non-infected cells. Ultimately, by [14] description, the viral titer was given as $\log_{10}$ $TCID_{50}$.

Used Stabilizers

Six different stabilizers were used to lyophilize FMD virus strains in the following formulae:

1) Culture medium without additives
2) A mixture of 10% w/v sucrose (HiMedia, Mumbai, India) and 5% Lactalbumin hydrolysate according to [15]
3) 10% skim milk (Oxoid, Basingstoke, England) [16]
4) A mixture of 10% sucrose and 1% gelatine [10]
5) A mixture of 4% peptone and 1% gelatine [7]
6) 5% Inositol.

Separately, 50 mls. of distilled water were used to dissolve the gelatine and sucrose. Then, the gelatine was autoclaved for 150 minutes at 127°C, the pH was corrected to 7.3, and the mixture was autoclaved for 20 minutes at 121°C. After dissolving skim milk and sodium glutamate in 100 mls. of distilled water, the stabilizers' pH was brought to 7.3 and they were autoclaved for 20 minutes at 121°C.

Each stabilizer was mixed with each of the used FMD virus strains as 1:1 (V/V) then dispensed as 2.5ml / glass vial and subjected to a lyophilization process on Teflon lyophilize apparatus and covered with a semipermeable rubber stopper. The glass vial's inner diameter was 1.9 cm.

Freeze-drying process

Teflon lyophilization equipment was used to perform the lyophilizing process. Glass vials that had been sanitized and sealed with a semipermeable rubber stopper held a total of 2.5 ml of the freeze-drying system. The inner diameter of the glass vial measured 1.9 cm. In order to achieve rapid freezing, the vials were set on the shelf of the freeze-dryer, which had been precooled to −60 °C [17]. Following two hours of cooling, the initial drying started. According to [18], the vacuum was maintained at 10 Pa and the shelf temperature was fixed at -32°C. After a 16-hour primary drying process, the shelf was wormed up to 20°C at a rate of 0.2°C/min and held for 6 hours. Following freeze-drying, the vials were sealed and stored at room temperature for two hours before being kept at -20°C to assess the impact of the lyophilization process [19].

Stability of the lyophilized FMD virus preparations

Samples from each lyophilized FMD virus preparations were kept at 0, 4 and -20°C and subjected to virus titration at intervals of 1 week then 1, 3, 6- and 12-months post lyophilization.

Discussion

According to the role of FMD that affects the animal wealth dramatically, it is an essential job to preserve the seed viruses with good infectivity titers for a suitable shelf life for vaccine production. So, the present work was planned to determine the lyophilization effect on the infectivity stability of three Egyptian serotypes of FMDV as a preservation method. To establish this purpose three different stabilizers were used and the lyophilization process was followed by virus infectivity titration carried out on lyophilized virus samples kept at 0, -20 and 4°C for 1 week, 1,3,6 and 12 months post lyophilization.

Our present results as shown in Tables (1,2&3) depending on virus titration carried out through this study revealed that infectivity was lost during the process of freeze-drying and storage when no additives were used (media alone). The infectivity titer loss was also found to be more severe at high temperatures, where in the case of FMDV serotype (O), the lyophilization process reduced the titer by 1.4 $\log_{10}$ and lost 3.8 $\log_{10}$ after storage at 0°C, 2 $\log_{10}$ after storage at -20°C, and 4 $\log_{10}$ after 12-month at 4°C. Contrary to a 2-4 $\log_{10}$ drop in the absence of additives. The infectivity levels with the other stabilizers were maintained with values nearly identical to those before freeze-drying where it remained constant at -20°C for a full year following the freeze-drying process. The infectivity loss values were 0.2–0.3, 0.6–0.7, 0.5–0.6 and 0.7–0.8 using 10% skim milk, 5% lactalbumin, and 10% sucrose, 10% sucrose and 1% gelatine, using 4% peptone and 1% gelatine and using 5% inositol respectively. Thus, it appeared that the optimal lyophilization additives for FMDV serotype (O) are either 10% skimmed milk or 10% sucrose plus 5% lactalbumin.

The lyophilization of FMDV serotype (A) resulted in a titer decline of 3.3–4.2 $\log_{10}$ with culture medium without any additives. In contrast, the other addition solutions managed to maintain infectivity levels that were quite near to their pre-freeze-drying levels. Infectivity remained constant at -20°C for a full year following the freeze-drying process. Using 10% sucrose and 5% lactalbumin reduced infectivity loss by 0.2–0.3, using 10% skimmed milk reduced infectivity loss by 0.1–0.2, using 10% sucrose and 1% gelatine reduced infectivity loss by 0.2–0.6, using 4% peptone and 1% gelatine reduced infectivity loss by 0.5–0.7, and using 5% inositol decreased infectivity loss (0.2–0.4). Thus, the optimal lyophilization additives for FMDV serotype (A) are either 10% skimmed milk or 10% sucrose + 5% lactalbumin.

Lyophilized FMDV serotype (SAT2) stored at 0, -20 °C loss 3.9 $\log_{10}$, but at 4°C its loss 4.9 $\log_{10}$ after 12 months of storage. Unlike a 3.9–4.9 $\log_{10}$ drop without any additives, the other addition solutions were able to retain infectivity levels close to what they were before freeze-drying. After freeze-drying, infectivity held steady at -20°C for 12 months, where the virus infectivity losses were (0.2-0.5), (0.2-0.4), (0.5-0.7), (0.6-0.9), and (0.3-0.5) by using (10% sucrose and 5% lactalbumin), (10% skimmed milk), (10% sucrose and 1% gelatine), (4% peptone and 1%

gelatine) and (5% inositol) respectively. So, it is clear that the best additives with FMDV serotypes for lyophilization purposes are (5% lactalbumin, and 10% sucrose), (10% skimmed milk) and (5% inositol).

Researchers looked at how long foot-and-mouth disease viral strains could remain contagious after lyophilization, with or without additions to virus suspensions. To prevent virus degradation throughout the freeze-drying process and minimize any infectivity loss resulting from product storage at 4°C and -20°C, certain additive solutions were required. The virus's ability to be kept at an increased temperature of 37°C was extended by additive solutions made of 10% sucrose and 5% lactalbumin hydrolysate, 10% skimmed milk, 4% peptone and 1% gelatin, and 5% dextran, 1% sodium glutamate, and 5% sucrose. These findings suggest that freeze-dried FMDV antigens can be shipped and stored for a limited period without refrigeration, which can save storage and transportation expenses [7].

In this respect, it was shown that the stabilizing activity of culture medium without additives had never been thoroughly investigated before. It was found that the FMDV strains O/BFS 1860 and A22 IRQ 24/64 lost infectivity on freeze-drying and storage without additives [5] and [20] found that without any additions, a stabilizing effect could happen because the culture media contains calf serum and sucrose. Gelatine with sucrose and skim milk with sodium glutamate were expected to have some favourable protective activity [9,21]. Although earlier research had suggested that specific additives might have some favourable protective effects, the culture medium's stabilizing role on its own had not been fully investigated. However, the mixture may naturally stabilize due to the sucrose and serum content of the medium. [22].

The efficacy of freeze-drying FMDV antigens has been demonstrated in varied degrees in previous studies, some have found that the virus is resistant to infectivity loss whereas [23] found that type A22 virus in tissue culture supernatant was resistant to loss of infectivity on freeze-drying and storage while others have reported significant drops in infectivity in the absence of protective chemicals [24].

It was reported that many additive solutions improved stability during freeze-drying and storage. Regarding the present used stabilizers, it was concluded that the most successful stabilizing additive was discovered to be a combination of gelatin and sucrose, which successfully shielded all tested enveloped viruses. Additional studies have also demonstrated the effectiveness of this combination in maintaining the stability of enveloped viruses. The inclusion of gelatin-sucrose did not provide as much protection for the non-enveloped picornaviruses, nevertheless, sorbitol and a reduced gelatin content were shown to stabilize a picornavirus vaccine [9]. The culture medium, which included sucrose and serum, was sufficient to stabilize both the enveloped and non-enveloped coronaviruses. This supports earlier discoveries on the foot-and-mouth disease virus, or picornavirus. Picornaviruses were the most vulnerable to lyophilization-induced inactivation, with 2-4.2 log titer declines, yet, this family benefited from faster freezing in liquid nitrogen. During storage, adenoviruses were shown to demonstrate an unexpected jump in titer, this was most likely brought on by the viral particles dissolving [10]. Parallel to our results [25] found that 5 FMDV strains were resistant to freeze-drying in 5% lactalbumin hydrolysate and 10% sucrose, with infectivity declining 0.5-2.5 log over 1 year at -20°C and 4°C where sucrose may retain water content through hydrogen bonding or replacing lost water and Lactalbumin provide a protective coating. [26] found type O_1 BFS 1860 survived poorly on freeze-drying, but some additives (calcium lactobionate, inositol, sodium glutamate, glucose) provided moderate protection.

The less virus loss obtained using skimmed milk came in agreement with [27,28,29 and 30] who found that prepared FP and PPR vaccines with different concentrations of skimmed milk after lyophilization showed the same reduction in virus titer (0.25 log10EID50) but reduction was (0.5 log10EID50) by using Lactalbumin sucrose stabilizer indicating the role of skimmed milk maintaining of virus titer. Although skimmed milk induces less loss infectivity titers, it is not recommended for use in animal vaccines where it contains animal proteins that now are not recommended due to the risk of transmission of prion diseases [31].

Conclusion

According to our present investigation, we could say FMDV seeds could be preserved safely in a lyophilized form for a not short time when lyophilized with a suitable stabilizer composed of lactalbumin and sucrose and kept at a suitable temperature as 0 to -20 °C.

Acknowledgments

Authors are thankful to Prof. Dr. Mohamed Saad Director of Veterinary Serum and Vaccine Research Institute and All member of FMD department specially Prof. Dr. Sonia Ahmed Rizk, Head of the department. Also, thanks to Prof. Dr. Mohamed Hassan Khoudier for reviewing this work.

Funding statement

This work was funded by Veterinary Serum and Vaccine Research Institute

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

TABLE 1. Mean infectivity titer of FMDV serotype (O PanAsia-2)

Used stabilizer	Virus titer ($log_{10}TCID_{50}$/ml)						
	Before lyophilization	Post lyophilization	Storge temp. (^0C)	Periods of storage after lyophilization			
				1 W*	1 M**	3 M	6 M	12 M
Medium	8.2	6.8	0	6.5	5.5	4.2	3	3
			- 20	5.5	5.3	5.1	4.8	4.8
			4	6	5.4	3.7	2.8	2.8
10% sucrose 5% lactalbumin	8.2	8	0	7.9	7.9	7.9	7.7	7.7
			- 20	7.9	7.9	7.9	7.8	7.8
			4	8	7.9	7.8	7.7	7.7
10% skimmed milk	8.2	7.9	0	7.9	7.8	7.8	7.7	7.6
			- 20	7.9	7.9	7.9	7.8	7.7
			4	7.9	7.9	7.8	7.7	7.6
10% sucrose 1% gelatine	8.2	7.6	0	7.7	7.4	7.2	7	7
			- 20°C	7.7	7.4	7.2	6.9	6.9
			4 °C	7.6	7.5	7.3	7	7
4% Peptone 1% gelatine	8.2	7.7	0	7.7	7.7	7.5	7.1	7.1
			- 20°C	7.7	7.7	7.5	7.3	7.2
			4 °C	7.7	7.5	7.3	7	7.1
5% Inositol	8.2	8	0	8	7.5	7.3	7.2	7.2
			- 20°C	8	7.6	7.3	7.3	7.3
			4 °C	7.9	7.5	7.2	7.2	7.2

TABLE 2. Mean infectivity titer of FMDV serotype (A)

Used stabilizer	Virus titer ($log_{10}TCID_{50}$/ml)						
	Before lyophilization	Post lyophilization	Storge temp. (^0C)	Periods of storage after lyophilization			
				1 W*	1 M**	3 M	6 M	12 M
Medium	7.5	6.4	0	4.5	4	3.4	3	3
			- 20	5	4.5	3.6	3.2	3.1
			4	4	3.8	3	2.2	2.2
10% sucrose 5% lactalbumin	7.5	7.3	0	7.2	7.2	7.1	7	7
			- 20	7.3	7.2	7.1	7.1	7.1
			4	7.2	7.1	7.1	7	7
10% skimmed milk	7.5	7.2	0	7.2	7.2	7.2	7	7
			- 20	7.2	7.2	7.2	7.1	7.1
			4	7.1	7.1	7	7	7
10% sucrose 1% gelatine	7.5	7.1	0	7	6.9	6.8	6.8	6.6
			- 20°C	7	7	6.9	6.9	6.9
			4 °C	6.9	6.8	6.6	6.5	6.5
4% Peptone 1% gelatine	7.5	7.0	0	6.9	6.7	6.6	6.6	6.5
			- 20°C	6.9	6.8	6.8	6.7	6.5
			4 °C	6.8	6.6	6.4	6.3	6.3
5% Inositol	7.5	7.2	0	7.2	7.2	7.1	7	6.9
			- 20°C	7.2	7.2	7.1	7.1	7
			4 °C	7.1	7	6.9	6.8	6.8

TABLE 3. Mean infectivity titer of FMDV serotype (SAT2/EGY/2012)

Used stabilizer	Virus titer ($log10TCID_{50}$/ml)						
	Before lyophilization	Post lyophilization	Storge temp. (^0C)	Periods of storage after lyophilization			
				1 W*	1 M**	3 M	6 M	12 M
Medium	7.0	5.9	0	3	3	2.4	2	2
			- 20	3.3	3	2.5	2	2
			4	3	2.8	2	1	1
10% sucrose 5% lactalbumin	7.0	7.0	0	7	6.9	6.9	6.8	6.8
			- 20	7	6.9	6.9	6.9	6.8
			4	7	6.7	6.6	6.5	6.5
10% skimmed milk	7.0	7.0	0	7	6.9	6.8	6.8	6.7
			- 20	7	7	6.9	6.9	6.8
			4	6.9	6.8	6.8	6.7	6.6
10% sucrose 1% gelatine	7.0	6.9	0	6.8	6.8	6.7	6.6	6.3
			- 20°C	6.9	6.9	6.7	6.6	6.4
			4 °C	6.8	6.7	6.6	6.5	6.2
4% Peptone 1% gelatine	7.0	6.9	0	6.8	6.7	6.5	6.4	6.2
			- 20°C	6.8	6.8	6.6	6.5	6.3
			4 °C	6.8	6.7	6.5	6.4	6
5% Inositol	7.0	7.0	0	6.8	6.8	6.7	6.6	6.6
			- 20°C	6.9	6.9	6.8	6.8	6.7
			4 °C	6.8	6.7	6.7	6.6	6.5

*W= week **M= months

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الملخص

يمكن أن تظل السلالات الفيروسية لمرض الحمى القلاعية SAT2/EGY/2012 وA إيران 05 و2-FMDV O PanAsia معدية بعد التجفيف بالتجميد مع الحد الأدنى من نزول عياريتها، مع أو بدون إضافات إلى معلقات الفيروس. على مدار 12 شهرًا من التخزين في درجات حرارة مختلفة، تم تقييم عياريه الفيروسات المجفده على أساس منتظم. من أجل منع نزول العياريه للفيروس طوال عملية التجميد والتجفيد ولتقليل فقدان العياريه أثناء التخزين عند 4 درجات مئوية و20 درجة مئوية، كانت هناك حاجة إلى اضافه مثبتات. تمت إضافة ستة إضافات كمثبتات مكونة 6 مخاليط: 1-وسط زراعي بدون إضافات؛ 2- مزيج من 5% لاكتالبومين هيدروليزات و 10% وزن/حجم سكروز؛ 3-10% حليب خالي الدسم، 4- خليط من 10% سكروز و1% جيلاتين؛ 5-خليط من 4% ببتون و1% جيلاتين و6-5% اينوزيتول لتحضير المعلقات الفيروسية لعملية التجفيد. يشير تقييم الفيروسات المجفده بالتجميد التي تم الحصول عليها والمخزنة في درجات حرارة مختلفة إلى أنه يمكن شحن فيروس مرض الحمى القلاعية المجفد بالتجميد وتخزينه لفترة وجيزة من الوقت مما يمكن أن يوفر نفقات التخزين والنقل باستخدام مثبت يتكون من 10٪ وزن / حجم السكروز و 5% لاكتالبومين هيدروليزات أو 10% حليب منزوع الدسم تخزينه عند -20، 0، 4 درجة مئوية

الكلمات الدالة: فيروسات مرض الحمي القلاعيه – التجفيد – المثبتات.