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EFFICACY OF SKIMMED MILK AS STABILIZER IN COMPARISON WITH LACTO ALBUMEN SUCROSE IN PRODUCTION OF FOWL AND PIGEON POX VACCINES

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ABSTRACT

Aim: *This work is a trail to decrease cost of vaccine production and improve the physical properties of the produced fowl pox (FPV) and pigeon pox (PPV) vaccines.*

Materials and methods: *Two batches of live attenuated FPV and PPV were prepared. These batches were divided into 2 portion where the first portion was mixed with an equal volume of lacto albumin (5%) and sucrose (2.5%) stabilizer, while the second portion was mixed with an equal volume of skimmed milk (10 %) stabilizer. All these mixtures was subjected to freeze drying process where it was found that skimmed milk has a better physical properties than lactalbumen sucrose stabilizer, each one of the 4 dried mixtures (1- FPV with lacto albumin sucrose 2- FPV with skimmed milk 3- PPV with lacto albumin sucrose 4- PPV with skimmed milk) were subdivided into 3 portions, kept under 4, 37 and -20 °c respectively. Samples were collected monthly from lyophilized vaccines kept under 4 and -20 °c, while the portion which kept at 37°c the sample collected every day.*

Results: *The titer of FPV and PPV samples kept at 4 °c and -20 °c decreased by 1.5 and 0.25log_{10} respectively after 6 months in case of using skimmed milk but in case of using lacto albumin sucrose the titer decreased by 2 and 0.50log_{10} after 6 months, the virus titer of samples collected daily at 37 °c decreased by 2.25 log_{10} in case of using skimmed milk and 2.75 log_{10} in case of using lacto albumin sucrose respectively. The keeping quality test applied on the prepared vaccines showed that all of them were safe and potent.*

Conclusion: *This study showed that skimmed milk is cheaper to FPV and PPV vaccine and provides a better in physical properties and thermo stability than lacto albumin sucrose stabilizer*

KEYWORDS: *Stabilizer; Fowl Pox; Pigeon Pox .*

1. INTRODUCTION

Fowl pox virus (FPV) and Pigeon pox virus (PPV) are belonging the family *poxviridae*, subfamily *Chorodopoxvirinae* and genus *Avipoxvirus* [1]. These viruses have two forms, the cutaneous form or dry form. This form is characterized by formation of proliferative lesions on the unfeather skin such as wattle and comb. The other form is called wet pox or diphtheritic form, in this form; raised white solid nodules grow on the mucous membranes. The nodules increase in size and converted to yellowish diphtheritic membrane. These lesions are present on the mucous membrane of the trachea, larynx, esophagus and mouth [2, 3].The infection with avian pox is mainly with a low mortality rate in chickens but may be reaching to 65-100 % in outbreak in pigeon [4].

Vaccine shelf life can be determined throw the rate of viability and in consequences loses of its potency at a recommended storage temperature. So that the stability of vaccines has a major influence on the success and effectiveness of vaccination programs worldwide and may be responsible for vaccine miscarriage with the results of deterioration of whole vaccination programs [5].The production of live attenuated vaccine is mainly obtainable in lyophilized (freeze drying) form and can be produced at wide scale with low cost. When skimmed milk is used as main stabilizer in production of different avian vaccines that's will gave use lyophilized disc vaccine was obtained with much better physical appearance [6].

Different concentrations of skimmed milk were used as stabilizer in preparation of Rift Valley Fever Vaccine (RVF) where the best concentration from skimmed milk was 10%. It was noticed that skimmed milk maintained thermo stability of the live attenuated vaccine as the virus titer was $10^{7.6}$ TCID50/ml reaching $10^{4.6}$ TCID50/ml after 12 months at 4°c, while the titer of RVF vaccine reached $10^{6.3}$ TCID50/ml at -20°c after 12 months. It also made the physiochemical properties of the final product better [7].

When the skimmed milk was used as stabilizer for production of Pest des Petits Ruminants live attenuated vaccine the virus titer of vaccine decreased by $10^{0.3}$TCID50/ml after 6 months in - 4 °C, So skimmed milk is considered as one of the most suitable stabilizers used for manufacture of attenuated Pest des Petits Ruminants live attenuated vaccine [8].

Ghazi et al [9] mentioned that, the using of different types of stabilizers in preparation of brucella vaccine batches such as skimmed milk, sucrose, sodium glutamate, gelatin, casein, sucrose and sodium glutamate lead to collapse (shrinkage) of lyophilized disc in a number of vials in prepared batches but these batches met the specification recommended by OIE (2012) for 12 months post-production in vaccine batches with skimmed milk stabilizer.

Also Latif et al [10] tested different stabilizers for PPR vaccine as the stabilizers having carbohydrates and hydrolyzed proteins like Lactalbumen hydro lysate sucrose-(LS), Lactalbumen hydro lysate sorbitol-(LSbG), Tris Trehalose-(TT), and Goat skimmed milk-(GSM) were evaluated to protect the infectivity titer of the virus and effective to make compact mass of PPR virus vaccine.

The present study was planned to evaluate the use of skimmed milk as stabilizer for both FPV and PPV vaccines production instead of using Lactalbumen sucrose stabilizer, in a trail to improve the physical property and stability.

2. MATERIAL AND METHODS

2.1. Fowl and Pigeon pox vaccines

Vaccinal (Baudate, egg adapted strain), and Vaccinal (Hungarian, Egg adapted strain) pigeon pox were supplied by Pox Research Department VSVRI for were used for preparation of experimental vaccine batches according to OIE [11].

2.2.Specific pathogenic free embroynated chicken eggs

SPF embroynated chicken eggs (ECE) kindly supplied from Qum Oshem farm and used for propagation and titration of FPV and PPV and neutralization test according to Namaa [12].

2.3. Experimental chickens

One hundred chickens 2 weeks old were divided into 4 groups 25 chicken for each group.

Group no (1) vaccinated with field dose (10^3log10EID50/ml) of sucrose lactalbumen FPV.

Group no (2) inoculated with 10x skimmed milk concentration used in vaccine production as allergy test for the skimmed milk.

Group no (3) vaccinated with field dose (10^3log10ECID50/ml) of skimmed milk FPV.

Group no (4) non vaccinated control chickens.

2.4. Experimental pigeon

One hundred pigeons 2 weeks old were divided into 4 group's 25 pigeons for each group and vaccinated with PPV as the same manner as described above.

2.5. Serum samples

Blood samples were collected from all birds before and after vaccination throw wing vein and left for coagulation and serum collection to measure the protective level of pox antibodies by VNT.

2.6. Stabilizer

Different concentration of skimmed milk (10%, 15% and 20%) were added to the infective fluid of attenuated viruses with an equal volume according to Shahid and Usman [6] in addition to using lacto albumin Sucrose stabilizer according to OIE [11].

2.7. Preparation of experimental PP and FP vaccines

Different batches of FP and PP vaccines were prepared according to OIE [11] using different concentrations of skimmed milk added to equal amount of harvested virus fluid in addition to 2

batches of PP and FP vaccine by using equal amount of lactalbumen Sucrose Stabilizer, all prepared vaccines were lyophilized by freeze drying lyophilization.

2.8. Pigeon pox and fowl pox prepared vaccines titration

The previously prepared vaccines were titrated after lyophilization according to Namaa [12] to select the best concentration of skimmed milk to be used for PP and FP vaccines preparation.

2.9. Challenge test

Challenge test was carried out according to OIE [11].

2.10. Evalution of the prepared vaccines

2.10.1. Sterility test

The prepared vaccines were tested for their sterility for bacterial and fungal contamination according to OIE [11].

2.10.2. Safety test

The reconstituted vaccines were inoculated in 5pigeons and 5 chickens by 10x field dose according to OIE [11].

2.10.3. Potency test

Virus neutralization test (VNT) was applied on serum samples collected from vaccinated chickens and pigeons with FPV and PV vaccines according to OIE [11].

2.10.4. Thermo stability test

Samples of the different prepared vaccines with different concentrations of the stabilizer were kept at various temperatures (table 1) and virus titration test was applied according to Soleimani [13].

Table (1): Thermo stability test for prepared vaccines

Temperature	Interval time for titration
37 C	Every 12 hour for 72hour
-4C	Every month for 6 months
-20C	Every month for 6 months

3. RESULTS

3.1. Safty test of skimmed milk

Inoculated pigeons and fowl with 10 X concentration of skimmed milk used in vaccine production showing no adverse reaction or side effect up to 2 weeks post inoculation.

3.2. Titration of prepared FP and PP vaccines before and after lyophilization with different skimmed milk concentrations

The titer of prepared FP and PP vaccines with different concentrations of skimmed milk after lyophilization showed the same reduction in virus titer (0.25 $\log_{10}EID_{50}$) in all prepared vaccines while it was (0.5 $\log_{10}EID_{50}$) by using lactalbumen sucrose stabilizer as shown in Table (2) so its suitable to use the less concentration (10%) skimmed milk stabilizer as its gives same results with more suitable economic value.

Table (2) titration of FP and PP vaccines before and after lyophilization

Tested vaccine formula	Virus titre ($\log_{10}EID_{50}$ /ml)			
	Before Lyophilization		After Lyophilization	
	FPV	PPV	FPV	PPV
Vaccine fluid without stabilizer	6.50	6.25	-	-
Vaccine fluid with 10%skimmed milk	6.50	6.25	6.25	6.0
Vaccine fluid with 15% skimmed milk	6.50	6.25	6.25	6.0
Vaccine fluid with 20% skimmed milk	6.50	6.25	6.25	6.0
Vaccine fluid with 2.5% sucrose and 5% lactalbumen	6.50	6.25	6.00	5.75

3.3. Physical properties of the prepared vaccines

The results in Photos (1 and 2) of lyophilized FP and PP vaccines prepared with skimmed milk stabilizer reviled more better physical properties (more compact solid disk) than those prepared with Lactalbumen sucrose stabilizer as shown in photos (3 and 4).

Photo (1)
FP vaccine prepared with skimmed milk stabilizer

Photo (2)
PP vaccine prepared with skimmed milk stabilizer

Photo (3)
FP vaccine prepared with Lactalbumen sucrose stabilizer

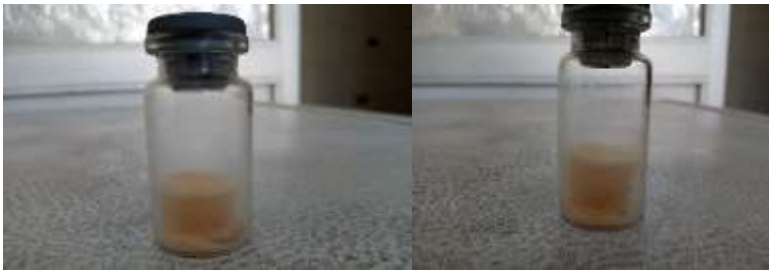

Photo (4)
PP vaccine prepared with Lactalbumen sucrose stabilizer

3.4. Thermo stability tests of the prepared vaccines

3.4.1. Thermo stability of the prepared lyophilized FP and PP vaccines at 37°c

The prepared FP and PP vaccines with 10% skimmed milk showing less reduction in virus titer (2.25 log 10EID50 /ml) lower than FP and PP vaccines prepared by using lactalbumen sucrose stabilizer (2.75 log 10EID50 /ml) kept at 37 °C for 1week as shown in table (3).

Table (3): thermo stability of the prepared FP and PP vaccines kept at 37 °c

Titre	Titre log_{10} EID_{50}/ml				log_{10} reduction/ml			
Vaccine type	FPV	FPV	PPV	PPV	FPV	FPV	PPV	PPV
Stabilizer used	SKM	LAC	SKM	LAC	SKM	LAC	SKM	LAC
Before lyophilization	6.5	6.5	6.25	6.25	0.0	0.0	0.0	0.0
0 time after lyophilization	6.25	6.00	6.00	5.75	0.25	0.50	0.25	0.50
1st day	6.25	6.00	6.00	5.75	0.25	0.50	0.25	0.50
2nd day	6.00	5.75	5.50	5.50	0.50	0.75	0.75	0.75
3rd day	5.50	5.50	5.25	5.25	1.00	1.00	1.00	1.00
4th day	5.50	5.00	5.00	5.00	1.00	1.50	1.25	1.25
5th day	5.00	4.50	5.00	4.50	1.50	2.00	1.25	1.75
6th day	5.00	4.00	4.50	4.00	1.50	2.50	1.75	2.25
7th day	4.25	3.75	4.00	3.50	2.25	2.75	2.25	2.75

SKM: Skimmed milk stabilizer
LAC: Lactalbumen sucrose stabilizer

3.4.2. Thermo stability of the prepared lyophilized FP and PP vaccines at 4°C and 0 °C

The prepared FP and PP vaccines with 10% skimmed milk showed less reduction in virus titer (1.5) and (0.25) at 4°C and -20°C respectively lower than FP and PP vaccines prepared by using lactalbumen sucrose stabilizer (2.0) and(0.5)at 4 °C and -20°C for 6 months as shown in Tables (4) and (5).

Table (4): thermo stability of the prepared FP and PP vaccines at 4°C.

Time	Titre log_{10} EID_{50}				log_{10} reduction			
Vaccine type	FPV	FPV	PPV	PPV	FPV	FPV	PPV	PPV
Stabilizer used	SKM	LAC	SKM	LAC	SKM	LAC	SKM	LAC
Before lyophilization	6.50	6.50	6.25	6.25	0.0	0.0	0.0	0.0
0 time after lyophilization	6.25	6.00	6.00	5.75	0.25	0.5	0.25	0.5
1st month	6.25	6.00	6.00	5.75	0.25	0.5	0.25	0.5
2nd month	6.25	6.00	6.00	5.75	0.25	0.5	0.25	0.5
3rd month	6.25	6.00	6.00	5.75	0.25	0.5	0.25	0.5
4th month	6.25	5.5	6.00	5.25	0.25	1.0	0.25	1.0
5th month	5.75	5.0	5.50	4.75	0.75	1.5	0.75	1.5
6th month	5.00	4.5	4.75	4.25	1.5	2.0	1.5	2.0

SKM: Skimmed milk stabilizer
LAC: Lactalbumen sucrose stabilizer

Table (5): thermo stability of the prepared FP and PP vaccines at -20 °C.

Time	Titre log_{10} EID_{50}				log_{10} reduction			
Vaccine type	FPV	FPV	PPV	PPV	FPV	FPV	PPV	PPV
Stabilizer used	SKM	Lac	SKM	LAC	SKM	LAC	SKM	LAC
Before lyophilization	6.5	6.5	6.25	6.25	0.0	0.0	0.0	0.0
0 time After lyophilization	6.25	6.00	6.0	5.75	0.25	0.50	0.25	0.50
1st month	6.25	6.00	6.0	5.75	0.25	0.50	0.25	0.50
2nd month	6.25	6.00	6.0	5.75	0.25	0.50	0.25	0.50
3rd month	6.25	6.00	6.0	5.75	0.25	0.50	0.25	0.50
4th month	6.25	6.00	6.0	5.75	0.25	0.50	0.25	0.50
5th month	6.25	6.00	6.0	5.75	0.25	0.50	0.25	0.50
6th month	6.25	6.00	6.0	5.75	0.25	0.05	0.25	0.05

SKM: Skimmed milk stabilizer
LAC: Lactalbumen sucrose stabilizer

3.5. Evaluation of the prepared vaccines

3.5.1. Sterility test

Bacterial and fungal cultures of prepared vaccines proved to be free from any bacterial and fungal contamination.

3.5.2. Safety test

Inoculation of prepared FPV and PP vaccines in chickens and pigeons with 10 times of the recommended dose proved that the produced vaccine were safe to be used. Where the vaccinated birds did not show any undesirable symptoms refer to FP and PP.

3.5.3. Challenge test

Challenge test applied against the prepared FPV and PPV vaccines in vaccinated chickens and pigeons using wing web route were presented in table (6) reveling protection percent in vaccinated chickens with both FPV vaccines were 96% while the protection percent in vaccinated pigeons with both PPV vaccines were 92%.

Table (6): Results of challenge test in vaccinated and control birds by virulent pox virus

Challenge Time	bird group	No. of challenged bird/group	No. of birds showing lesion post challenge			Protection percent (%)
			*5DPC	7 DPC	10 DPC	
4 **WPV	1	25	0	1	1	96% (24/25)
	2	25	0	1	1	96% (24/25)
	3	25	0	1	2	92% (23/25)
	4	25	0	1	2	92% (23/25)
	Control chicken	20	5	15	20	0%
	Control Pigeon	20	7	13	20	0%

*DPC= days post challenge
**WPV = week post vaccination
Group (1) vaccinated chicks with FPV vaccine with Skimmed milk (10%)
Group (2) vaccinated chicks with FPV Lacto albumin sucrose.
Group (3) vaccinated pigeons with PPV vaccine with skimmed milk (10%)
Group (4) vaccinated pigeons with PPV vaccine with Lacto albumin sucrose.

3.5.4. Potency test of the vaccines in chickens and pigeons (Virus notarization test –Alpha procedure).
The results were presented in table (7) showed that, chickens and pigeons vaccinated with both FPV and PPV vaccines showing protective notarizing index(NI) after 2 weeks from, while negative results shown with the serum of the control chickens and pigeons [NI> 1.5 considered as positive result according to OIE (2012)

Table (7): Results of the VNT for chickens and pigeons vaccinated with FP and PP vaccines

Type of vaccine	NI of fowl vaccinated with		NI of control chickens	NI of pigeon vaccinated with		NI of control pigeon
*WPV	FPV with SKM	FPV with LAC	----	PP with SKM	PP with LAC	------
0	0.4	0.3	0.3	0.3	0.3	0.2
1WPV	0.9	1.1	0.2	1.1	1.3	0.3
2 WPV	1..6	1.5	0.3	1.8	1.7	0.3
3 WPV	2.3	2.3	0.3	2.4	2.4	0.3
4 WPV	2.1	2.0	0.3	2.3	2.2	0.2
5 WPV	1.9	1.7	0.3	2.0	1.8	0.3

*WPV = week post vaccination

4. DISCUSSION

Usually viral vaccine producers hope to increase their production with a maximum possibility of cost reduction. One of the methods that help in such purpose is to use alternative cheap and effective stabilizer like skimmed milk.

The safety of skimmed milk was tested in pigeons and chickens by inoculation of 10x concentration used in vaccine preparation and the result showed its safety in all birds where no adverse reaction or side effect was detected that was explained by Khokha and Werb [14] and Latif et al [10]. Who explained that the skimmed milk contains proteins (lactalbumen, lactglobulins, casein, and lactoferrin), lactose and minerals The albumin and globulins of skimmed milk may be recognized as self-antigen in caprine so, does not induce any untoward reaction in vaccinated birds where source of milk casein is not from liver, but is milk acini. In addition to that milk casein is in contrast to other non-hydrolyzed casein which can make anaphylactic shock in animals.

The titer of prepared FP and PP vaccines with different concentrations of skimmed milk after lyophilization showed the same reduction in virus titer (0.25 $\log_{10}EID_{50}$) in all vaccines prepared using different concentrations of skinned milk but it 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 it agrees with Alexander et al [15] 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. To prevent such damages, stabilizers are added to vaccine suspensions before freeze-drying.

The physical appearance of the lyophilized disks present in Photos (1 and 2) for FP and PP vaccines prepared with skimmed milk stabilizer showed the more better physical properties (more compact solid disk) than those prepared with lactalbumen sucrose stabilizer as shown in photos (3 and 4) similar results obtained by Salama et al [7],Ghazy et al [9] and Latif et al [10] 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.

The vaccines are combination of components (biological and non-biological) that are sensitive to environmental factors and changes in non-biological ingredients of vaccines by different factors. So, biological changes may be occurred especially in live vaccine as decrease in virus titer which consequently affects the vaccine potency as stated by Boris et al [16] and Razieh et al [17]. So, to determine product changes in maintenance period and ensure safety and efficacy of vaccines, stability study of biological products is needed. Stability is the ability of a vaccine to retain its chemical, physical, microbiological and biological properties within specified limits throughout its shelf life [18,19-

14].The reduction in the titer of prepared FP and PP vaccine with 10% skimmed milk stabilizer was (2.25 $\log_{10}$ EID_{50}) while it was (2.75 $\log_{10}$ EID_{50}) in the prepared FP and PP vaccine with lactalbumen sucrose stabilizer kept at 37 °C for 1week, the reduction of the titer of prepared FP and PP vaccine with 10% skimmed milk stabilizer was (1.5 $\log_{10}$ EID_{50}) while it was (2 $\log_{10}$ EID_{50}) in prepared FP and PP vaccine with lactalbumen sucrose stabilizer kept at 4 °C for 6 months. The reduction in the titer of prepared FP and PP vaccine with 10% skimmed milk stabilizer was (0.25 $\log_{10}$ EID_{50}) less than the prepared FP and PP vaccine with lactalbumen sucrose stabilizer (0.5 $\log_{10}$ EID_{50}) kept at -20 °C for 6 months.as shown in Tables (3), (4) and (5).These results indicate the efficacy of skimmed milk to maintain viability of lyophilized vaccines under lyophilization and different environmental conditions. Similar results obtained by [10] who concluded that the skimmed milk is the most suitable stabilizer in comparison with Lactalbumen hydro lysate sucrose-LS, Lactalbumen hydro lysate sorbitol(LSbG), Tris Trehalose(TT), Tris sucrose(TS) in maintaining infectivity titer of the vaccine virus, also Salama et al [7] mentioned that 10% skimmed milk is the best concentration stabilizer of choice for attenuated RVF vaccine and the prepared vaccine being suitable for use when stored at 37 °C for 48 hour and 6 months at 4 °C and can be stored at -20 °C without loss in its titer till 9 months also Shahid and Usman [6] reported that freeze dried Newcastle disease virus vaccine with skimmed milk maintained NDV stability with low reduction in mean infectivity.

All the prepared vaccines match to OIE [11] for sterility as shown to be free from bacteria and fungi and the NI of VNT showed protective level of antibodies after 2 weeks from vaccination in all vaccinated bird as shown in Table (7). The beak of antibody titer was detected in the 3rd week in all vaccinated bird groups similar results were optioned by Omar et al [20].The Protection percent of vaccinated chickens and pigeons. It with FPV and PP vaccines with skimmed milk and lactalbumen sucrose stabilizers were 96%, 92%, respectively on contrast it was 0% in control non vaccinated chicken and pigeons in these results are the same as obtained by Namaa [12] who examined the protection rate for FPV vaccine prepared by using lactalbumen sucrose.

5. CONCLUSION

The present study proved that skimmed milk stabilizer is more efficient than the used lactalbumen sucrose stabilizer as 10 % skimmed milk reduced the losses in virus titer during the process of lyophilization and during different thermo stability tests than the used lactalbumen 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.

Recommendations

This study recommended the use of skimmed milk stabilizer instead of using lacto albumen sucrose during production of FPV and PP vaccines in order to obtain more stable vaccines with higher virus **titer under different storage temperature with reduction of vaccine costs.**

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