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Role of stabilizers in preparation of a potent sheep pox vaccine

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SUMMARY

Production of live attenuated sheep pox vaccine sustained the elevated temperatures during freeze-drying, transportation storage and vaccination in unequipped tropical and subtropical zones of the world, is highly recommended. For this reason, eight stabilizer formulas were individually used for preparation of eight sheep pox vaccines, which were lyophilized and then titrated and accordingly four vaccine formulas were eventually selected that should be tested for thermoprotectivity to select the best stabilized vaccine. These selected vaccines were tested for sterility; potency (vaccination and challenge) and safety in susceptible sheep. The collected blood sera were subject to serological examination for estimating the antibody response by ELISA.

The results proved transcendence of sheep pox vaccines stabilized with 10% trehalose alone or in combination with 5% lactalbumin hydrolyste in the thermoprotectivey, thereby improvement vaccination efficacy.

INTRODUCTION

Sheep pox is an important infectious disease, caused by Variola Ovina virus. According to Hutyra and March (1983), the earliest record of sheep pox dates back to the second century A.D. It still persists as a problem of sheep industry in various parts of the world. In endemic areas, the disease causes considerable economic losses to the farmers mainly in form of mortality. Mortality rate in lambs is as high as 90% and more than 30% in adults; it causes abortion, mastitis and leaves considerable skin defects (Mahmood et al., 1988). Annual mass vaccination is the method of choice in checking the spread of the disease (Carn, 1993).

Different stabilizers were used for production of live vaccines, such as peptone or lactalbumin hydrolysate or a mixture of the

latter with sucrose (Chifney et al., 1973); Skimmed milk (Ramisse et al., 1978), lactalbumin hydrolysate and sucrose (Rizkallah, 1994 and OIE, 2004); or lactose peptone tris buffer (Ozawa et al., 1965 and Rizkallah, 1994).

Trehalose is a multifunctional carbohydrate that can improve storage stability of biological materials. Liu et al., (2005) conducted that trehalose could be extremely beneficial for stabilizing the protein structure of the virus for restoring the potency of the virus after dehydration.

Accordingly the objective of the present study is to provide an improved stabilizer for lyophilized live sheep pox vaccine having storage stability without great reduction in titre.

MATERIAL AND METHODS

Material

1. Viruses:-

1.1. Kenyan sheep pox virus:

The reference Kenyan sheep pox virus strain was kindly supplied from foreign animal disease diagnostic laboratory (FADDL), Plum Island, USA, and was used for production of sheep pox vaccine.

1.2. Virulent sheep pox virus:

Egyptian strain of sheep pox virus was obtained from the Pox Department, Veterinary Serum and Vaccine Research Institute (VSVRI) Abbassia, Cairo, the virus had been previously isolated from local outbreak (Sabban, 1960) and was used for challenge test.

2. Animals:-

A total of 43 susceptible lambs of 4 - 6 months age apparently healthy. These lambs were not vaccinated against sheep pox disease; they were previously screened for sheep pox antibodies in their sera by using enzyme linked immunosorbent assay (ELISA). These lambs were used in titration, vaccination and challenge tests for sheep pox tissue culture Vaccines as well as for studying the safety test and for serological test (ELISA).

3. Cell culture:-

African green monkey Kidney cell line (Vero):-

It was kindly supplied by Dr. J. House, FADDL, Plum Island, USA. These cells were used for production of sheep pox vaccines and their titration.

4. Tissue culture media:-

It was Minimum Essential Medium (MEM) with Earle's salts and L-glutamine without Sodium bicarbonate. It was supplied by Sigma Chemical Company U.S.A.

5. Biological reagents:-

5.1. Bovine Serum:

Virus and mycoplasma screened newborn calf serum (of less than 14 days old) produced by Gibco Laboratories, New

Zealand lot 6834217D sterile A. It was used for media supplementation as 10% for growth media and 2% for maintenance media.

5.2. Anti-sheep pox serum:

It was kindly supplied from VSVRI, Pox Department. In which it was prepared according to Kalpana et al.,(1995) and Pandey and Singh (1972).

5.3. Antigens:

Partially purified tissue culture soluble viral antigen (TCSA):-

It was prepared according to El-Bana (1978). It was used as a positive control antigen in ELISA.

6. Buffers and solutions:-

6.1. Bicarbonate solution:

7.5% sterile sodium bicarbonate solution was used for adjusting the pH of media.

6.2. Antibiotic stock solution:

It was prepared according to United State Environmental Protection Agency (USEPA, Manual of Methods for Virology, 1984).

6.3. Trypsin solution:

It was purchased from DIFCO Laboratories, Detroit, Michigan, USA and prepared according to Lennetta (1964).

6.4. Phosphate buffer saline (PBS): pH 7.4

It was prepared according to the formula of Oxoid Manual (1982). The buffer was used as a washing and diluting solution.

6.5. Buffers used in ELISA:

They were prepared according to USEPA, Manual of methods for Virology (1984).

7. Stabilizers:-

7.1. Sucrose:

It was obtained from Sigma Chemical Company. It was used as 2.5, 5 and 10% concentrations in preparation of lactalbumin sucrose stabilizers.

7.2. Lactalbumin hydrolysate:

It was obtained from DIFCO laboratories, Detroit, Michigan, USA. It was used as 5% concentration in preparation of different stabilizers.

7.3. Trehalose:

Trehalose (α-D- glucopyranoside dehydrate) was obtained from SERVA Electrophoresis Company. It was used as 2.5, 5 and 10% concentrations in preparation of lactalbumin trehalose or as 5% and 10% alone.

Methods

1. Propagation of sheep pox virus "Kenyan strain":

Sheep pox virus "Kenyan strain" was propagated in African green monkey kidney cell (VERO cells) according to Singh and Rai (1991) and Rizkallah (1994).

2. Evaluation of adapted sheep pox virus: Titration of Kenyan sheep pox virus:-

The harvested sheep pox virus (SPV) was titrated in Vero cell culture according to House (1990).

3. Production of sheep pox vaccines:

Eight formulas of sheep pox vaccines were prepared by the following steps:

3.1. Preparation of virus fluid:-

Five milliliters of the prepared solution of the adapted Kenyan sheep pox virus were inoculated in Roux bottles containing confluent growth of Vero cell cultures. The supernatant fluid constitutes the virus fluid.

3.2. Preparation of stabilizers:-

Eight formulas of stabilizers were prepared as follows:

I. The 1st three formulas (1, 2 and 3) composed of lactalbumin hydrolysate "LAH" (5%) and sucrose "S" (2.5%; 5% and 10%) respectively.

II. The 2nd three formulas (4, 5 and 6) composed of LAH (5%) and Trehalose Dihydrate (2.5%; 5% and 10%) respectively.

III. -The 7th formula composed of Trehalose Dihydrate (5%).

-The 8th formula composed of Trehalose Dihydrate (10%).

N.B. All the formulas were sterilized by autoclaving according to Kitching et al.,(1986).

3.3. Preparation of different sheep pox vaccines:-

According to OIE Manual volume I (2004), the different formulas of sheep pox vaccine were prepared by mixing of each stabilizer solution with the virus fluid of attenuated live sheep pox virus at the ratio 1:1 (v:v). 2 ml of each vaccine (mixture) was distributed into 10 ml sterile neutral glass vials, stoppered, then submitted to lyophilization, capsulated and stored at -20°C.

4. Titration of sheep pox vaccines before and after lyophilization:

The different vaccine formulas were titrated directly before and after lyophilization according to Tiwari and Negi, (1995). (The preparated sheep pox vaccine should have a minimum $\log_{10}$ 4.5 $TCID_{50}$ per ml after freeze drying, equivalent to a field dose of $\log_{10}$ 2.5 $TCID_{50}$, OIE, (2004)).

5. Evaluation of the selected sheep pox vaccines:

5.1. Sterility test:-

Sterility test was carried out according to OIE Manual volume I, (2004).

5.2. Safety test:-

Each of the four selected potent sheep pox vaccine formulas were tested for its safety according to OIE Manual volume 1, (2004). 16 lambs divided into 4 groups, each group is formed of 4 lambs.

In each group two lambs were inoculated intradermally (I/D) with 100-fold of the filed dose, of one vaccine formula, two lambs left as contact non-inoculated control.

The animals were observed daily for one month for any local or systemic lesions.

5.3. Vaccination and challenge:

As described by Sabban, (1960) and Rizkallah, (1994), twenty four apparently healthy susceptible lambs were divided into 4

groups, each of 6 lambs, beside another 3 lambs were isolated in a separate pen.

N.B. according to the results of titration of the eight formulas before and after lyophilization, four formulas that recorded the least titer losses were selected.

5.4. Serological assays:

Serum was collected from sheep just before and after vaccination in a weekly interval for one month and again just before challenge and for 3 weeks post challenge. Samples were stored at -20°C until examined by ELISA.

RESULTS

The results of propagation of sheep pox virus "Kenyan strain" in Vero cell culture:

By titration of the daily harvested virus, it was found that the highest titer was $10^{6.8}$ $TCID_{50}$/ml at the 5th day post inoculation where nearly 90% CPE was present.

Titration of different formulae of sheep pox vaccines before and after lyophilization:

Titration was applied directly before and after lyophilization according to Tiwari and Negi, (1995).

The titer of sheep pox virus vaccine was expressed by $TCID_{50}$ and calculated according to the method of Reed and Muench, (1938), for selection of the higher prophylactically effective titers.

Table (1) Show the titer of the 8 prepared vaccine formulas:

Table (1): Effect of lyophilization on different sheep pox vaccine formulas:

SP Vaccine formula	Stabilizer component %	Titration before lyophilization log_{10} ($TCID_{50}$)*	Titration after lyophilization log_{10} ($TCID_{50}$)*	Losses in titres log_{10} ($TCID_{50}$)
1st	5LA / 2.5S	$10^{6.7}$	$10^{5.9}$	0.80
2nd	5 LA / 5 S	$10^{6.6}$	$10^{5.35}$	1.25
3rd	5 LA / 10S	$10^{6.5}$	$10^{5.5}$	1.00
4th	5LA / 2.5T	$10^{6.6}$	$10^{6.1}$	0.50
5th	5 LA / 5 T	$10^{6.6}$	$10^{5.4}$	1.20
6th	5 LA / 10T	$10^{6.6}$	$10^{6.2}$	0.40
7th	5 T	$10^{6.5}$	$10^{5.55}$	0.95
8th	10 T	$10^{6.8}$	$10^{6.5}$	0.30

* = The average of the titer of 3 experimentally prepared lots.
LA = Lactalbumin hydrolysate. S = Sucrose.
T = Trehalose Dihydrate. $TCID_{50}$ = Tissue culture infective dose fifty.

Selection of the lyophilized vaccines having higher titer:

After titration of the lyophilized vaccines and according to the results (table 1), four vaccines were selected, that were 1st, 4th, 6th and 8th vaccine formulas for comparative studies and detection of the best one. These formulas called F_1, F_2, F_3 and F_4, respectively.

Sterility test:-

This test was carried out according to OIE Manual volume I, (2004).

Vaccination and challenge:-

1. Vaccination of lambs:

Four lambs in each group were vaccinated with the field dose (10^{-2}) of a separate sheep pox vaccine formula; two were kept as non-vaccinated contact controls.

2. Challenge test:-

The virulent virus used was the local Egyptian sheep pox strain isolated in Egypt by Sabban,(1960), this virulent strain was used to challenge the immunity of all sheep vaccinated with any formula of vaccine produced, beside the lambs tested for safety in addition to the contact and isolated control sheep. All lambs injected intradermally in the inner side of the right thigh. Body temperature of all animals and any clinical signs were recorded for 3 weeks post-inoculation.

Results of safety test:-

Indicated that none of the animals inoculated for safety test developed pyrexia, only moderate nodule at the inoculation site four to seven days after inoculation, but these nodules were no longer detectable by day 10 post injection. While the contact non-inoculated susceptible control lambs showed no general or local reaction. After challenge the injected lambs developed a hypersensitivity reaction at the site of challenge within 48 hours. On the other hand the contact and isolated control animals developed the classical form of pox infection by day 5-7 after infection.

Serological assays: (Solid phase ELISA)

According to House et al., (1990), the ELISA test was applied on sheep sera.

It was noticed that antibodies appeared from the 1st week and increased till reaching maximum titers by 21 and 28 days post vaccination.

After challenge, all vaccinated and control lambs showed antibodies from the 7th days till 21st days post challenge (time of testing). The results differ according to vaccine formula. From the results it was noticed from the comparison between the F_1 stabilizer and F_2, That trehalose is superior to sucrose in inducing the protectivity after vaccination and challenge in contrast nearly no significant difference between F_3 and F_4 in which 10% Trehalose was used in combination with lactalbumin hydrolysate or alone. On the other hand, a significant difference was recorded in the protectivity of F_3 and F_4 in comparison with F_1 or F_2

stabilizers in which it was increased by increasing the $^{00}/_{0}$ (wt. /vol.) of trehalose.

It was also noticed in the four vaccines that antibody titer after challenging of the vaccinated group overcome that recorded after challenging of the control non vaccinated groups. From the obtained results, it noticed that the antibody titer of the contact non vaccinated group persist allover the time post vaccination (one month) without any significant increase, that was in agreement with the results of challenge test which mean that the sheep pox vaccines virus not transmitted from the vaccinated animals to the susceptible contact Lambs by aerosol or contact methods.

Table (2): Results of ELISA reading (optical density = OD) of sera collected from sheep tested for protection and safety of F_1 of sheep pox vaccine:

Tested lambs	Animal number	28 Days post vaccination				21 Days post challenge			
		F_1	F_2	F_3	F_4	F_1	F_2	F_3	F_4
Vaccinated	1	1.87	2.18	2.35	3.00	2.00	2.00	2.39	2.80
	2	2.30	2.00	2.74	2.90	1.90	1.80	2.85	3.00
	3	2.50	2.20	3.10	3.20	2.20	2.20	3.00	3.20
	4	2.00	2.30	2.86	3.10	2.10	2.40	2.73	3.00
Contact control	1	0.31	0.38	0.49	0.25	1.90	1.95	2.00	1.90
	2	0.30	0.40	0.57	0.35	2.00	1.80	1.90	2.20
Safety	1	2.00	2.30	3.35	2.70	2.10	2.50	3.20	3.22
	2	2.50	1.95	3.22	3.35	2.50	2.10	3.54	3.58
Contact control	1	0.33	0.37	0.41	0.30	1.70	1.74	1.85	2.00
	2	0.40	0.30	0.40	0.35	1.80	1.95	2.20	1.80

F_1 = the 1st formula of prepared sheep pox vaccine, that stabilized with 5% LA and 2.5% S (wt. /vol.).
F_2 = the 2nd formula of prepared sheep pox vaccine, that stabilized with 5% LA and 2.5 % T (wt. /vol.).
F_3 = the 3rd formula of prepared sheep pox vaccine, that stabilized with 5% LA and 10% T (wt. /vol.).
F_4 = the 4th formula of prepared sheep pox vaccine, that stabilized with 10% T (wt. /vol.).
N.B. B.V. = before vaccination or inoculation (0.29).
N.B.$_2$: Sample positive ≥ one consider protective (Williams, 1987).
N.B.$_3$: Isolate control sheep gave the same results of the contact control.
Average of post-challenge ELISA reading of:
F_1: -Vaccinated animals = 2.08 -Contact control = 1.68
F_2: -Vaccinated animals = 2.14 -Contact control = 1.61
F_3: -Vaccinated animals = 2.72 -Contact control = 1.50
F_4: -Vaccinated animals = 2.99 -Contact control = 1

DISCUSSION

An object of the present study is to provide an improved stabilizer to have a prolonged store stability with diminished reduction in titre, for this reason eight stabilizers were individually used for preparation of different sheep pox vaccines in which each was mixed with an equal volume of the sheep pox virus for preparation of eight sheep pox vaccine formulas, the stabilizers were composed mainly from 5% lactalbumin hydrolysate (wt/vol) mixed with different percentages of sucrose or trehalose (2.5, 5 or 10% wt/vol) or composed of trehalose alone (5 or 10% wt/vol).

The mean loss of sheep pox virus titre following freeze-drying is given in table (1). The results proved a significant difference in the losses of the different tested vaccines ranged from log10 0.3 TCID50 in case of 10% Trehalose stabilizer to log10 1.25 TCID50 of 5%LA: 5%Sucrose, accordingly four stabilizers were eventually selected as the stabilizers used in preparing of sheep pox vaccine as follows:

- The 1st vaccine formula (F1) that contains a stabilizer composed of 5% Lactalbumin hydrolysate: 2.5% Sucrose.
- The 4th vaccine formula (F2) that contains a stabilizer composed of 5% Lactalbumin hydrolysate: 2.5% Trehalose.
- The 6th vaccine formula (F3) that contains a stabilizer composed of 5% Lactalbumin hydrolysate: 10% Trehalose.
- The 8th vaccine formula (F4) that contains a stabilizer composed of 10% Trehalose.

These four formulas beside its protectivity during lyophilization they have the higher titers.

In comparison between the two used carbohydrates, sucrose and Trehalose that present with the same ratio in F1 (control vaccine that contain 5% lactalbumin: 2.5% sucrose) and F2 (that contain 5% lactalbumin: 2.5% trehalose instead of sucrose), the mean titre losses after lyophilization was 0.8 log10 TCID50 in sucrose condition while it was 0.5 log10 TCID50 in case of Trehalose. Also the titer of F1 was $10^{5.9}$ and that of F2 was $10^{6.1}$ (table 1) which was corresponded to Sampedro et al., (1998), who reported that during freeze-drying and rehydration, the protective efficiency of carbohydrates was as follows: Trehalose > maltose > sucrose. Joi and Rojiv, (2003) stated that Trehalose found to be very effective in the stabilization of labile proteins during lyophilization and exposure to high temperature in solution. This essentially due to the difference in their

cosolvent molecular structure and their solution physico-chemical properties.

The prepared vaccines were tested for sterility to detect any possible contaminant and the results proved that they were negative to any contaminating agents as bacteria, moulds and fungi when inoculated on specific media.

For detection of the evermore effective and safer vaccine from the selected four formulas, vaccination and challenge test in susceptible sheep was applied, The post vaccinal reaction were similar to those previously reported by Pierre et al., (1979) and Rizkallah, (1994). Slight reactions and slight rise in temperature could be explained that the vaccine stimulated the immune system in the susceptible lambs to produce antibodies against SPV. According to Ramyar and Hessami ,(1970), challenge test was considered as the direct method used for measuring the immunizing capacity of the vaccine. We found that the mild post challenge reaction appeared on the previously vaccinated sheep, this is due to the circulating antibodies derived through vaccination, which limits spread virus in animals, but dose not prevent replication of the virus at the site of infection. The vaccinated lambs with tissue culture sheep pox virus vaccine resisted the infection with the virulent virus; which was in agreement with (Carn,1993; Solyom et al., 1980; Das and Mallick, 1984; Wang and Jiang, 1985; Ergin et al., 1988 and Rizkallah, 1994).

On the contrary; both contact and isolated control sheep showed severe reaction produced by the virulent SPV at the site of inoculation and around this place, with increase in the body temperature, depression and loss of appetite for several days beside generalization of pox infection in three control animals indicating that there had been no aerosol or contact transmission of vaccines virus under the experimental conditions. These clinical features of sheep pox have been outlined by many workers as Blood and Henderson, (1974); Afshar et al., (1986) and Rizkallah, (1994).

By challenging all the sheep (inoculated; contact and isolated controls); the injected vaccinated sheep resisted the challenge while both contact and isolated non inoculated control sheep showed severe local and systemic reactions, which meant that they had no immunity and proved that there was no spread of the vaccinal virus to the contact unvaccinated control sheep.

After application of vaccination and challenge test we selected a serological enzyme linked immunosorbent assay (ELISA) for estimating the antibody response to the

different vaccines in which Williams, (1987), showed that ELISA proved to have great potentiality as a quantitative serological tool in the detection of antibodies against several viral infections and its sensitivity and specificity are superior to other serological tests.

The antibody response to sheep pox vaccine was measured by S/P ratio which is considered protective when it was above 1, (House et al., 1990).

The results were presented in table No. (2). It was noticed that antibodies reaching maximum titers by 21 and 28 days post vaccination.

The results differed according to vaccine formula. From the obtained results after challange it was noticed that a significant difference was recorded in the protectivity of F3 and F4 in comparison with F1 or F2 stabilizers in which it was increased by increasing the (wt. /vol.) of Trehalose. The results were in agreement with Pitaksuteepong, (2005), who revealed that Trehalose is one of the substances used with a specific antigen to enhance the immune response.

ELISA reading explained the difference effect between sucrose and trehalose (F1 and F2) and revealed the immunoprotectivity of trehalose in F3 and F4 in comparison with F2. This may reflect on the efficiency of the vaccine in the field and on the duration of immunity.

It was also noticed in the four vaccines that antibody reading after challenging of the vaccinated groups were in average 2.08, 2.14, 2.72 and 2.99 respectivelly that overcome that recorded after challenging of the control non vaccinated groups which were 1.68, 1.61, 1.5 and 1.0 (Table 2). That was in compatible with Carn, (1993). From the obtained results, it has been noticed that the antibody titre of the contact non vaccinated group persist negative (less than 1) allover the time post vaccination (one month) without any significant increase, and this was in agreement with the results of challenge test, meaning that the sheep pox vaccines virus not transmitted from the vaccinated animals to the susceptible contact one. Which are in conformity with Aboul-Soud, (1995); Olfat, (2000) and Magda et al., (2003), who proved by biochemical assays and by ELISA the difference between vaccinated and or infected and non vaccinated animals with sheep pox, goat pox or lumpy skin disease.

CONCLUSION

We concluded that sheep pox vaccines stabilized with 10% trehalose alone or in

combination with 5% lactalbumin hydrolyste have longer store stability, more thermoprotectivity and less titer reduction as well as stronger humeral and cell mediated immunity which were measured by ELISA and challenge tests respectively. The use of trehalose as sheep pox vaccine as stabilizer improve vaccination efficacy which reflect positively on farmers and national income.

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دور المثبتات في تحضير لقاح لجدري الاغنام

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إنتاج لقاح حي مستضعف لجدري الأغنام يتحمل درجات الحراره خلال عمليات التجفيد و النقل والتخزين والتحصين في المناطق القاريه وشبة القاريه الغير مجهزه في العالم هو أحد المطالب الهامه لذا استخدمت في هذه الدراسة ثمانية نماذج من المثبتات (stabilizers) لتحضير ثماني لقاحات لجدري الأغنام و قد تم إختبارها عياريا بعد التجفيد – واختيرت الأربعه لقاحات التي سجلت أعلا عياريه – وهي اللقاح الضابط (المستخدم حقليا) والمحتوي علي المثبت المركب من خليط من لاكت البيومن هيدروليسيت ٥% + سكروز ٢,٥% - بالأضافه إلي لقاحين حل فيهما التريهالوز محل السكروز في اللقاح الضابط بنسبة ٢,٥% ، ١٠% علي التوالي واللقاح الرابع المستخدم فيه التريهالوز بمفرده بنسبة١٠% لأختبار ثباتها الحراري والمناعي وإختبار اللقاح الاكفأ (الأفضل) حيث اختبرت نقاوتها ومعامل الأمانSafety (في الأغنام المحقونه بمائه ضعف الجرعه الحقليه) - كما تم تحصين اربع مجموعات من الأغنام بالجرعة الحقليه مع وجود اغنام ضوابط غير محصنه وقابلة للعدوي مخالطة ،معزوله – ثم أجرى إختبار التحدي وجمعت عينات سيرم الدم اسبوعيا قبل وبعد التحصين و التحدي واجري عليها إختبار الأليزا لأستبيان الإستجابه المناعيه السيرولوجية – وثبت من النتائج تفوق اللقاحين المستخدم فيهما التريهالوز بنسبة١٠% سواء علي انفراد أو مخلوط مع ٥% لاكت البيومن هيدروليسيت في إطالة فترة كفاءة اللقاح – مما يساعد علي زيادة قدرته وسلامة التحصين والإقلال من معدلات الأصابه بالمرض مما يعود بالفائده علي الفلاح والدخل القومي.