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PREPARATION OF AN INACTIVATED LUMPY SKIN DISEASE VIRUS VACCINE

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SUMMARY

Lumpy skin disease (LSD) local strain (Ismalia 88) was adapted in MA104 cell line and propagated for seven serial passages to obtain a titer or $10^{8.7}$ /ml TCID50. To determine the optimum time for harvestation of the adapted virus, sequential follow up of the growth behavior of LSD on MA 104 was carried out by studying the growth curve of the virus in the cells at 2,12,16,24,48,72,96 and 120 hours post inoculation. The virus was inactivated using different concentration of binary ethyleneimine (BEI) including 0.5, 1.2 and 5%. The inactivated virus was used to prepare the suitable inactivated LSD local vaccine. Virus preparations with titers of 10^6, 10^7, and 108 TCID50/ml were adjuvated with 2 types of adjuvants (alhydragel at a final concentration of 30% and maracol oil). The vaccine different preparations were characterized using different techniques including the adsorption affinity assay, drop test, emulsion viscosity and stability as well as keeping quality. In addition, the vaccine preparations were tested for the sterility and safety. Thirty five calves divided into 7 groups were inoculated with the different vaccine preparations to evaluate the vaccine potency. Sera were collected weekly post inoculation for 4 weeks and at 2nd and 3rd and 4th months post inoculation. The sera were tested by virus neutralization test and ELISA to evaluate the immune response against the inoculated LSD vaccine preparations. The results of the in vivo experiment revealed that the double oil emulsion vaccine preparation elicit-

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ed the highest antibody response against LSD at 8 weeks post vaccination whereas the alhydragel vaccine preparations yielded the highest antibody response at 4 weeks post vaccination.

INTRODUCTION

Lumpy skin disease (LSD) is a serious viral disease of cattle caused by a capripoxvirus (known as Neethling virus). LSDV of cattle and the very closely related viruses of sheep and goat pox are currently sub-classified as members of genus Capripox (Woods, 1988). Although, Capripox viruses (field and vaccine isolates) of cattle, sheep and goat posses a high degree of nucleotide sequence homology (Black et., 1986) and the pathological lesions of capripoxvirus infection in sheep and cattle are similar (Burdin, 1959), most of the capripoxvirus strains do not have an absolute host specificity (Capstick, 1959; kitching and Taylor, 1985;yeruham et al.,1994). Cross protection between the three capripoxviruses occurs in vivo (Yeruham, 1994; Capstick and Coackley, 1961; Davies and Mbugwa, 1985).

LSD was reported for the first time in Egypt in 1988 through cattle imported from Somalia (Fayed et al., 1988; Anonymous, 1988). In 1989 the disease was spread rapidly and caused several outbreaks in different governorates (Suez, El-Tal El-Kabir in Ismalia governorate [highest incidence of the disease] and El- sharkia). The disease was diagnosed clinically and pathologically (Ali et al., 1990) and the virus was isolated form samples obtained from the first outbreak (Ismalia governorate) which was investigated by Ali et al., (1990). Several local isolates of LSD were identified later during an outbreak of the disease in Nag-Hamadi, New Valley, Mousha El- Badary, Menia, Assuitt and Upper Egypt (El-Allawy et al., 1992).

The control strategy of LSD was based on the use of the live attenuated strains of capripoxviruses (Capstick and Coackley,1961; Carn, 1993). In Egypt, the application of sheep pox virus (Romanian strain) was used to control LSD outbreaks (Michael et al., 1996). Several published studies have convincingly suggested the need of development of an attenuated cell culture vaccine from LSD to control the disease (Nawathe et al., 1982; Asagba, 1984; Sewell and Bracklesby, 1990). Recently the Egyptian local isolate of LSD was adapted in MDBK cell line and was used in the production of LSD vaccine in Egypt and proved its safety and efficiency (Aboul soud, 1996; Daoud et al., 1998 [b]). The live attenuated vaccines depend on the passage levels in cell culture which could affect both immunogincty and virulence. Besides, live attenuated vaccines can cause the disease in immunosupressed individual by reverting to a more virulent phenotype (Singh and OfHagan, 1999). Moreover, the used live modified LSD virus vaccines residual their pathogenicity and produce a large local reaction at the site of

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the inoculation which some stockowners find unacceptable (OIE, 1996). However, inactivated vaccines are considered the base and the favorite to apply policy to eradicate the disease in several countries.

In the present study, we report the preparation of an inactivated LSD vaccine using the local cell culture adapted strain. In addition to in vivo comparison between two types of adjuvants used in vaccine preparations as well as the evaluation of the immune response produced after vaccination with the prepared LSDV vaccine.

MATERIALS AND METHODS

1-Propagation of lumpy skin disease virus on MA104 cell line:

Local isolate of LSD (Ismalia 88 strain) adapted on MDBK cell line [$10^{4.7}$ TCID50 /ml] was used in the study. The virus was propagated on MA104 cell line for seven successive passages.

2-Titeration of LSDV on MA 104 cell line:

Virus infectivety assay was carried out as previously described (Frey and Liess, 1971) suing 96 well microtiter tissue culture plate. The virus infectivety titer was determined according to method of Reed and Meunch (1938).

3-Growth curve of LSDV on MA104 cell line:

Monolayers of MA104 were prepared in Cell culture staining chamber and incubated over night in CO2 incubator. The cells were inoculated with the virus and the culture supernatants were collected at zero , 2,12,16,24,48,72, 96 and 120 hour post inoculation. Virus titer for each supernatant was determined by virus infectivety assay as described above.

4-Virus inactivation:

Binary ethyleneimine (BEI) was used for virus inactivation. BEI was prepared by cyclization of 0.1 M of 2-bromo-ethylamine hydrobromide in 0.2N NaoH in a water bath (37°C) for 1-2 hours. Different concentrations of BEI 0.5 1 and 5% were mixed with LSDV. Samples were taken after zero,1,3, 6,9,12,18,24,30 and 36 hours and assayed for virus infectivety to determine the concentration and time at which complete inactivation was obtained. Virus inactivation was stopped by addition of cold sodium thiosulphate at a final concentration of 2%. To evaluate the BEI inactivation virus preparations were inoculated in cell culture and examined daily for the presence of CPE , in mean time four calves were injected with the inactivated virus and observed daily to detect any clinical symptoms.

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5-Different vaccine Preparations:

5-1-Preparation of 30% alhydragel LSD vaccine:

Inactivated virus preparations with a titer of 106, 107 and 108 were adjuvated with alhydragel (2% strength and 1.3% ALO dry matter, Superfos, Copenhagen, Denmark) at a final concentration of 30% as previously described (Roshdy, 1992) and used in vivo experiment.

5-2- Preparation of double oil emulsion LSD vaccine:

Different preparation of the inactivated LSDV with a titer of 106,107 and 108 were concentrated 10x of its original volume using polyethylene glycol 6000 as described (Roshdy, 1996). The concentrated virus preparations were then emulsified with double oil phase which consist of 9 parts Marcol oil 52 {Esso chemical company} and 1 part Arlacel. Double oil emulsion vaccine preparations were prepared as one part of antigen and one part of oil phases emulsified by ultra sonic emulsifier and 2% V/V Tween 80 in normal saline and re-emulsified (Barteling et al., 1990).

6- Characterization of the LSD vaccine preparations:

6-1- Characterization of alhydragel prepared LSD vaccine:

Alhydragel prepared vaccine was evaluated by using adsorption affinity to congo- red (Roshdy, 1996).

6-2- Characterization of double oil emulsion prepared LSD vaccine:

The oil emulsion LSD vaccine preparations were evaluated for the emulsification process using [a] the drop test as previously described (Roshdy, 1996) [b] emulsion viscosity (Cunliffe and Graves, 1963) [c] emulsion stability (Cunliffe and Graves, 1963) and [d] keeping quality (Roshdy.1996).

7- Vaccine sterility and safety:

The vaccine preparations were cultured on thiglycolate broth, Sabaroud nutrient agar, blood agar and phenol dextrose media to test the sterility of the vaccine. Safety test was conducted by S/C inoculation of 10 ml of each vaccine preparations in a susceptible cattle . Postvaccinal observations for one week to record the presence of fever, local or general lesions had been applied.

8- In vivo experimental studies on the prepared vaccine:

8-1- Animals:

Thirty five calves 6-8 months age were seronegative from LSD antibodies (by SNT and ELISA) were classified into seven groups five each.

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The animals were vaccinated S/C with one ml / dose form the prepared LSD vaccine preparations as follow: group 1,2 and 3 were received the al-hydralgel vaccine preparations including the preparations with a virus titer of 10^8,10^7 and 106 respectively where as group 4, 5 and 6 received the double oil emulsion vaccine preparations including the preparations with a virus titer of 108,107 and 10^6 respectively and group 7 was left as control. All animals were kept in an insect proof isolated area and observed for one week with daily record of body temp. The immune response of the animals to LSD Vaccine was measured using the SNT and ELISA (House et al.,1990).

9-Statistical analysis:

The obtained results were statistically analyzed using the methods of Snedecor (1956) and Cocharn and Cox (1960).

RESULTS

LSDV (Ismalia strain) propagated on MA104 cell for 7 successive passages showed an increase in virus titer with a log difference form $10^{4.7}$ to $10^{8.7}$ TCID50/ml (Table 1). The CPE induced by the virus was characterized by cell rounding and shrinking resulting in empty patches in the cell monolayer start to appear 3 days post inoculation and ended by cellular degeneration like those of tissue sections. The sequential microscopical changes developed by Ismalia strain of LSDV were similar in each passage in MA104 cells.

The virus infectivity rate was recorded in a growth curve study where a slow drop in virus titer has been observed 2hrs post inoculation in cell culture followed by a gradual increase at 12hrs and reached its peak at 120h as it became $10^{8.7}$ TCID50/ml (Table 2). There was a vigorous increase in the titer between 48 and 72 hrs post inoculation. Indeed, It was clearly demonstrated that the time elapsed by the virus in the cells till completed its maximum titer was ranged between 48 to 120 hrs. To determine the ability of BEI to completely inactivate the propagated LSDV, different concentrations of BEI were used at different intervals of incubation. As shown in table (3), BEI was able to inactivate completely the virus after at least 18 hrs of incubation at a concentration of 1%. By increasing the BEI concentration to 2% and 5%, the time of incubation needed to induce its effect was reduced to 9 and 3 hrs, respectively. W hen inoculating the calves with the inactivated virus preparations no clinical symptoms have been observed confirming the process of inactivation of each virus preparation used in the study (the selected concentration was 2% of BEI). Vaccine preparations were prepared using different virus titers (10 log 6, 7 and 8) and two types of adjuvants including 30% alhydragel containing 1.3% aluminum oxide that showed a good

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adsorption affinity when tested using adsorption affinity to Congo red (Table 4) and oil adjuvant. The oil adjuvated LSD vaccine preparations examined for a period 5 months for its stability, viscosity and sterility. The vaccine preparations remained stable and quite emulsified at 4°C, room temperature and 37°C beside its sterility along the 5 months period of examination. Moreover, when inoculating in cell culture or 10x dose S/C in 4 calves, the inactivated vaccine preparations found to be safe with no CPE in MA104 cells and/or clinical symptoms including fever, local or general lesions or death have been recorded. The immune response of the prepared vaccine was determined by inoculating the vaccine preparations in calves and measuring the level of antibody produced against LSDV using the virus neutralization test (SNT) and ELISA. The study was conducted in high-containment Facility (insect proof isolated area) and all animals used were seronegative to LSDV before being vaccinated. Serum samples were taken weekly for one month then at 8, 12 and 16 weeks post vaccination and assessed by SNT and ELISA. The vaccinated animals did not reveal or manifest any thermal reactivity after vaccination with the different vaccine preparations along the period of observation (one month) as there was no change in body temperature recorded daily after vaccination. In the first three groups of vaccinated calves, groups 1, 2 and 3 (the alhydrgel LSD vaccine), All vaccinated animals developed antibodies with detectable neutralization titer which could be determined by the SNT. The highest neutralization index detected by the three different preparations was observed at 4 weeks post inoculation. The vaccine preparation containing the virus titer of 10^7 TCID50/ml showed a superior response than the others (106 and 10^8 TCID50/ml) which developed lower neutralizing index along the interval weeks in the study. Although the alhydragel preparation containing 10^6 virus titer revealed a high neutralization index in the first 4 weeks post vaccination than the preparation containing 10^8 TCID50ml, its decline phase of antibodies was sharper in the period between 8 and 12 weeks post vaccination (Figure 1). The results of SNT demonstrated clearly that the vaccine preparation containing the virus titer 10^7 revealed high induction of neutralizing antibodies compared to the other preparations even in the decline phase of the antibody titers. Examining the same sera by ELISA , It was also observed that all the animals had antibodies to LSD detected by ELISA. As expected, the vaccine preparation containing virus with titer of 10^7 TCID50/ml being the higher in the amount of antibodies (optical densities) than the other preparations (Figure 2). Noting that the maximum antibodies titer expressed as neutralization index induced by the alhydragel vaccine preparations was 1.2 (figure 1). On the other hand, the maximum OD obtained in the sera from vaccinated

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calves in groups 1,2 and 3 was 0.39 (figure 2). Groups 4, 5 and 6 were received the different oil adjuvated LSD vaccine preparations. T esting the sera collected from these groups at interval times in the study by SNT and ELISA revealed potent immune responses than those received the alhydragel vaccine preparations. Figures 3 and 4 demonstrate the neutralization index and the mean OD obtained by SNT and ELISA, respectively. With weekly monitoring, the titers reached its peak at 8 weeks post vaccination with a higher neutralization index approximately double of the one obtained by the alhydragel preparations and even decrease slowly till the end of the experiment where it was also considered protective levels (NI 1.5) of neutralizing antibodies with the preparations contained 10^7 TCID50/ml. The ELISA results of the tested sera from the vaccinated calves with oil adjuvated vaccine preparations revealed detectable amount of antibodies reached its maximum level at 8 weeks post vaccination and slowly decreased with considerable higher level than those animals vaccinated with the alhydragel vaccine preparation. Indeed, calves vaccinated with the prepared vaccine preparations developed detectable neutralizing antibodies as early as 7 days post vaccination and lasted for at least 4 months in case of the oil adjuvated vaccine preparations with a protective level of neutralizing antibodies and exhibiting no detectable illness and a normal temperature of 38°C. The five unvaccinated calves lacked detectable antibodies and had a normal temperature during the course of the experiment.

Table (1): Virus infectivity titer of LSD (Ismalia strain) propagated on MA104 cell line .

No. of Passage on MA104 cell line	virus infectivity titer
Original titer	$10^{4.7}$ $TCID_{50}$/ml
1	$10^{5.2}$ $TCID_{50}$/ml
2	$10^{5.8}$ $TCID_{50}$/ml
3	$10^{6.4}$ $TCID_{50}$/ml
4	$10^{7.2}$ $TCID_{50}$/ml
5	$10^{7.8}$ $TCID_{50}$/ml
6	$10^{8.2}$ $TCID_{50}$/ml
7	$10^{8.7}$ $TCID_{50}$/ml

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Table (2): One step growth curve of LSDV propagated on MA104 cell line .

No. of Passage on MA104 cell line	virus infectivity titer
0	10^3
2	10^2
12	$10^{2.3}$
16	$10^{2.8}$
24	10^3
48	$10^{3.5}$
72	10^7
96	10^8
120	$10^{8.7}$

Table (3): The effect of BEI inactivation process on LSDV expressed in TCI50/ml.

0.5%	BEI concentration			
	0.5%	1%	2%	5%
0	$10^{8.7}$ $TCID_{50}$/ml	$10^{8.7}$ $TCID_{50}$/ml	$10^{8.7}$ $TCID_{50}$/ml	$10^{8.7}$ $TCID_{50}$/ml
1	$10^{8.4}$ $TCID_{50}$/ml	$10^{7.2}$ $TCID_{50}$/ml	$10^{6.1}$ $TCID_{50}$/ml	$10^{2.5}$ $TCID_{50}$/ml
3	10^8 $TCID_{50}$/ml	$10^{5.5}$ $TCID_{50}$/ml	$10^{4.3}$ $TCID_{50}$/ml	Zero
6	$10^{7.7}$ $TCID_{50}$/ml	$10^{4.3}$ $TCID_{50}$/ml	$10^{1.8}$ $TCID_{50}$/ml	Zero
9	$10^{7.4}$ $TCID_{50}$/ml	$10^{2.6}$ $TCID_{50}$/ml	Zero	Zero
12	10^7 $TCID_{50}$/ml	10^2 $TCID_{50}$/ml	Zero	Zero
18	10^5 $TCID_{50}$/ml	Zero	Zero	Zero
24	$10^{3.9}$ $TCID_{50}$/ml	Zero	Zero	Zero
30	$10^{1.7}$ $TCID_{50}$/ml	Zero	Zero	Zero
36	Zero	Zero	Zero	Zero

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Table (4): Adsorption affinity of Ahydragel to Congo red

Concentration of gel	Congo red solution (1 ml. Contains 0.77mg)			
	69.3 mg	84.7mg	100mg	115mg
30%	-a	--	--	--

a. (-) Supernatant is colorless or faint pink color.

Fig. (1): Mean neutralization index of vaccinated cattle with Alhydragel LSD vaccines as measured by SNT.

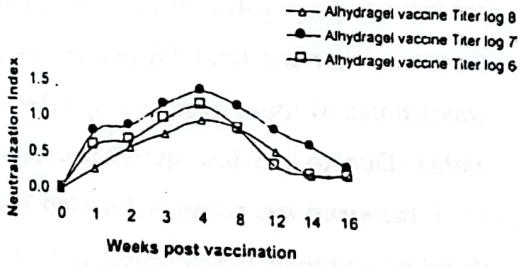


Fig. (2): Mean optical deinsty of vaccinated cattle with Alhydragel LSD vaccines as measured by ELISA.

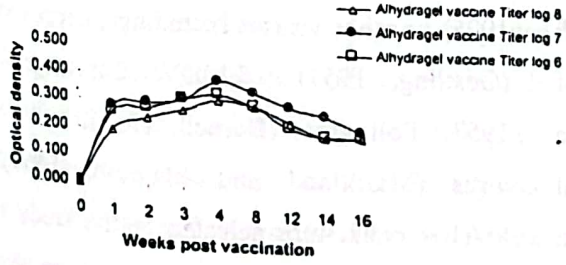


Fig. (3): Mean neutralization index of vaccinated cattle with DOE LSD vaccines as measured by SNT.

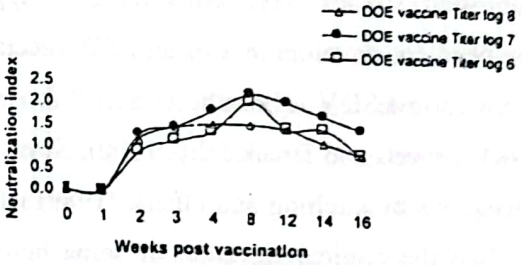
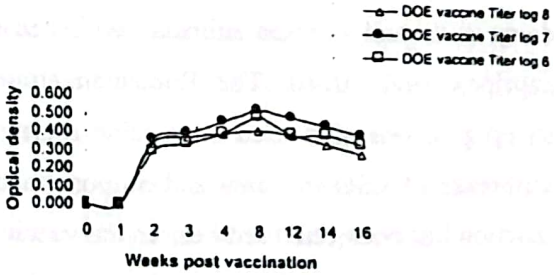


Fig. (4): Mean optical deinsty of vaccinated cattle with DOE vaccines as measured by ELISA.



DISCUSSION

Initially, the control strategy of LSD was based on the use of the live attenuated strains of capripoxviruses (Capstick and Conackley, 1961, Carn, 1993). Several researchers have used the sheep pox virus vaccines and the results have shown that these vaccines were sufficiently related to the LSD to induce cross protection (Davis, 1976; and Capstick,1959). Although, The application of the sheep pox vaccine against LSD during outbreaks was successful to control LSD infection, Several published studies have convincingly suggested the need for development of an LSD vaccine derived form LSDV '(Nawathe et al.,1982; Asagba, 1984; Sewell and Bracklesby, 1990). Several observations by kitching and others, (1989) who described the clinical response of some heifers to experimental vaccination with strain 0240/KSGP including enlarged regional lymph node, pyrexia, secondary papules. Moreover, recently Yeruham and others (1994) reported the incidence of generalized reactions with LSD-like lesion in lactating dairy cattle and stressed animals vaccinated with capripox strain 0240. The Romanian strain of sheep pox was also used as vaccine during the outbreaks of LSD in Israel and no post vaccinal reaction has been observed besides the vaccinated animals did not serve as potential source of infection by horizontal transmission (Yeruham and others , 1994). Micheal et al., (1996) have also used the Romainian strain of sheep pox vaccines and there is an evidence of some cases of LSD still reported which may be probably due to strains of capripoxvirus may not be specific to either sheep or cattle which suggesting that there are variants of a single species (Kitching and others 1989) or may be due to improper vaccine delivery and application. In the present study, we describe the preparation of an inactivated LSDV vaccine. The development of such vaccine from a locally isolated LSDV is of need specially after the appearance of the disease again in upper Egypt (El-Allaway et al.,1992). LSDV (Ismalia strain) was propagated on MA104 cell line to increase its titer and the CPE induced by the virus was similar to those observed by others (Yousif, 1995). Due to the low initial titer of the virus used, the virus was considerable slow in inducing its effect on the cells and subsequently in increasing its titer in appropriate time as indicated that the best time of harvestaion of virus with high titer is obtained after 120 hrs post inoculation. This observation is previously reported by others who propagated LSDV (Ibrahim, 1993 and Yousif, 1995) or other viruses including , herpes simplex (Gostling, 1957) , FMDV (Cartwright et al, 1957), Poliovirus (Darnell, 1958), and vaccinia virus (Mailltland and Magrath, 1957). The MA104 cells were selected in the study for the propagation of the virus because of the yield of a good virus titer when compared with the MDBK

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cells no change in antigenicity (Yousif, 1995). BEI has been used to inactivate LSD propagated in MDBK without alteration on its immunogenic properties (Bahnemann, 1975, Blackburn, 1991 and Shahin, 1998). It is clearly demonstrated that BEI used in the study has the ability to completely inactivate the propagated LSDV. In addition, there was no live virus effect in cell culture or in calves when tested for virus residue after inactivation. Inspection of the composite results of vaccine preparation and in vivo experimental trial indicates a number of findings, 1) Testing of the alhydragel adjuvated LSD vaccine preparations revealed a good adsorption with Congo red with a concentration of 30% as previously described (Roshdy, 1992 and 1996); 2) Marked stability, viscosity, and sterility of the oil adjuvated vaccine preparations over a period of 5 months; 3) The good safety profile of the vaccine preparations clearly demonstrated in MA104 cells or 10x doses in calves with no record of CPE or post vaccinal reactions; 4) The vaccine preparations containing the virus with a log 10^7 revealed a better immune response compared to the other ; 5) the oil adjuvated vaccines induced potent immune response peaked at 2 months and lasted with a considerable level of antibodies until 4 months and being superior than those induced by the vaccines adjuvated with alhydragel. The results of the in vivo experiment clearly demonstrated that the prepared vaccines have the ability of developing a protective levels of neutralizing antibodies with no adverse reactions post vaccination. The inactivated LSD vaccine presented here has several advantages over the live attenuated vaccines. First, its ability to induce a protective level of neutralizing antibodies against LSD compared to those obtained by sheep pox and LSD live attenuated vaccines (Micheal et al., 1996 and Daoud et al., 1998 [b]); secondly, the concentration of BEI used in the study to inactivate the virus insured that the antigenicity of the virus was not affected and the virus titer steadily decreased in direct relation with time of incubation like others (Bahnemann, 1975 and 1991; Shahin, 1998); thirdly, live attenuated vaccines can cause the disease in immunosupressed individuals or stressed animals (Kitching and others, 1989; Yeruham and others, 1994) or can revert to a more virulent phenotype (Sing abd OíHagan, 1999). In addition, in some countries it was reported that the used live modified LSDV vaccines residual their pathogenicity and produce a large local reaction at the site of inoculation which come stockowner find unacceptable (OIE, 1996). Indeed, due to the safety and the immunogoncity of inactivated vaccines they are considered the favorite vaccine to apply policy to eradicate the disease. The selection of an effective and potent adjuvant which will serves as antigen reservoir preventing rapid elimination and promoting sustained release of the antigen will be of added interest. Besides, the combination of more than one adjuvant will be a new trend in vaccine pro-

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duction as the immunomodulators influence both the magnitude of immune response which might reveal Th1/Th2 balance and hence the isotope of the produced antibody and DTH (Cox and Coulter, 1997). Moreover, there are some adjuvants which are capable of cytolytic delivery (stimulate CTL response) and targeting the antigen which increase the efficiency of delivary of antigen to APCs (Singh and OfHagan,1999).The use of such adjuvants or combination of more than one of them require more detail on the nature of the viral proteins. Indeed, in the present study, we propose the inactivated LSD vaccine preparation and application for long term control and eradication of the LSD. In addition, the trail of using a new generation of adjuvatns and combination between then will be our area of interest in future studies.

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