DEVELOPMENT OF A CELL CULTURE ATTENUATED VACCINE AGAINST LUMPY SKIN DISEASE: CULTURAL BEHAVIOUR AND POTENCY OF THE CANDIDATE VACCINE VIRUS

BY

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ABSTRACT

The candidate vaccine virus was found to spread by contiguity in bovine kidney cell (BKC) culture. The cytopathic effects (CPE) in this culture correlated well with the infectivity detected. The titres of the released virus never exceeded that of the cell associated virus though the difference between the two titres decreased steadily with the progress of sheet destruction. Therefore, to obtain high titre materials, it seems necessary to harvest both the infected cells and the supernatant fluid early after the CPE involve the whole of the sheet cover (after 24 hours). Three cycles of repeated freezing and thawing are suggested for harvesting of the infected cells as it does not affect virus infectivity.

Cell culture infectivity and cattle immunizing titres also correlated well. The cattle protective $dose_{50}$ was determined as equivalent to $5TCID_{50}$ and 1000 times as many was proposed for experimental field dosage.

ملخص

أوضحت نتائج التجارب لدراسة تكاثر فيروس اللقاح في المزارع الخلوية لكلي العجـــول إنـــه لإنتاج لقاح يحتوي على كمية عالية من الفيروس، لابد من حصد الخلايا المصابة إضافة إلى السائل الخلوي.

كمـــا وجد أن الجرعة الواقية (50%) في الأبقار تساوي خمسة أضعاف الجرعة المعديـــة (50%) في المزارع الخلوية وعليه فقد اقترح استخدام ما يعادل 1000 جرعة واقية (50%) كجرعة تجريبية بالحقل.

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INTRODUCTION

It was established by Abd El Raouf *et al*, 2003 that the 66th passage of "Massalamia" lumpy skin disease virus (LSDV) in bovine kidney cell (BKC) culture, while capable of protecting Zebu cattle against virulent challenge of LSDV is safer and grows more vigorously in cultured kidney cells than the 54th passage of the virus. Vigorous growth in the medium of propagation and consequential production of high titre material for the purpose of vaccination is not extravagant. Indeed one of the advantages that promoted the use of cell culture for vaccine production is that, infected cultured cells could yield large amount of virus (Piercy, 1966). Nevertheless, increased adaptation to cultural growth and decreased cattle pathogennicity might often be accompanied by a declined in-*vivo* viral multiplication, with a corresponding loss of immunogenicity. The 66th passage of "Massalamia" LSDV is no exception; it has shown less ability than the 54th passage in eliciting neutralizing antibody response.

The objectives of this work is firstly, to study the mechanisms of infection and release of the candidate vaccine virus in the medium of propagation, a lore which is basic for production of high titre preparations. Secondly to determine the immunising titres of the culture-virus preparations in order to evaluate the potency of the candidate vaccine virus and estimate a field dose. Finally to assess thoroughly the post-vaccinal localized reactions before field trials.

MATERIALS AND METHODS

Virus: The "Massalamia" strain of LSDV modified by 66th passages in BKC culture and cloned by limiting dilution technique, was used for all experiments. The freeze-dried ampoules of the virus-labeled LSDV-BKC/P_{66} clone "B' were reconstituted each in one ml of sterile deionized distilled water before use.

Culture and Culture Media: This was already described by (Abd El Raouf *et al*, 2003).

Titration of Virus Infectivity: Titration of the virus infectivity was carried out in tissue culture tubes (Abd El Raouf *et al*, 2003) and in microtitre plates. Micro-titre titrations of the virus infectivity were conducted in 96 well flat bottomed microtitre tissue culture plates

(Flow Laboratories). Virus dilutions were prepared in a diluent consisting of 94% Glasgow Modified Eagle's Medium (GMEM) that contained 0.2% $NaHCO_3$ (W/V), 1% Hepes buffer (1M., Flow Laboratories) and 5% tryptose phosphate broth. One hundred microlitres (μl) volume of each virus dilution was pipetted with a microtitre pipette into each of 5 wells of the microtitre plate. Each well then received 100 μl of secondary BKC suspension, prepared in media similar to that used for preparation of virus dilution, to which 7% of bovine serum was added. The plate was shaken lightly, sealed with an adhesive tape and incubated at 37°C with a source of humidity and 5% CO_2 atmosphere. Examination for cytopathic effects (CPE) was carried out on the 3rd, 5th, 7th and 9th day post-seeding. Titres were expressed as tissue culture infective $dose_{50}$ ($TCID_{50}$) calculated following the method of Karber (1931).

Growth Curve: A method similar to that described by Plowright and Witcomb (1959) was adopted. Small falcon tissue culture flasks (surface area 25cm²) were seeded simultaneously with equal amounts of primary BKC suspension and incubated at 37°C. When complete monolayers were established, the outgrowth medium was discarded and the cell sheet in each flask was then washed twice with 10 ml of prewarmed phosphate buffered saline ((PBS). Each culture flask then received one ml of the virus preparation and incubated at 37°C ± 0.5 for two hours. Control cultures were inoculated with one ml of PBS instead of virus and were treated in the same way as infected cultures. One uninoculated control culture flask was immediately removed and the cell sheet dispersed with trypsin-versine solution. The viable cells were counted by means of Neubauer haemocytometer to determine roughly the multiplicity of infection. Following absorption, the inoculum was decanted and stored at-30°C for titration. The cultures received three further washes with 10 ml of prewarmed PBS, in an effort to remove any unabsorbed virus. Cultures were then refed with outgrowth media, incubated at 37°C and examined daily by light microscopy to assess the progress of CPE and sheet destruction.

One virus infected flask culture was harvested at 0 hour following absorption i.e. prior to demonstrable formation of new

virus. The cell sheet was washed five times with 10 ml of PBS, refed with 10 ml of outgrowth medium and frozen at-30°C. At each 24 hrs interval after the end of absorption time, one infected culture was harvested. The medium was decanted, clarified at 2000 r.p.m for 10 minutes and the supernatant was stored at -30°C. This fraction represented "cell free" virus (CFV). The cell sheet was given a single wash to remove detatched cells and then frozen after addition of 10 ml of fresh outgrowth medium. Immediately prior to titration, frozen flasks were subjected to three cycles of repeated freezing at -30°C and thawing at 37°C then clarified as mentioned before. The supernatant represented "cell associated" virus (CAV). All virus samples were titrated simultaneously using secondary BKC of the same batch of cell in which the growth curve experiment was conducted.

Production of Plaques on Monolayer Culture of BKC Without Agar Overlay:
Tube cultures of secondary BKC were inoculated with 0.2 m1 of 1/10 dilution of the virus and allowed one hour at 37°C for absorption. The inoculum was discarded and the cell sheets were washed three times with prewarmed PBS. A group of infected cultures was refed with outgrowth medium containing 10% normal bovine serum whereas another group was refed with outgrowth media containing 10% LSDV immune bovine serum. Medium change was carried out daily for both groups using outgrowth medium with normal and immune sera respectively.

Control cultures, inoculated with PBS, were divided also into two groups and treated in the same way as infected cultures.

All cultures were observed daily under an inverted light microscope for development of plaques, LSD virus CPE or any other abnormality.

Cultures with discrete plaques were washed three times with prewarmed PBS, subjected to three cycles of repeated freezing and thawing then clarified by light centrifugation. The cell associated virus was assayed for infectivity by titration and for specificity by neutralization test using reference LSDV antiserum.

Determination of The Protective Dose $_{50}$ of The Candidate Vaccine Virus:
Eighteen calves of a local Zebu breed between 9 to 12 months of age, with no detectable serum neutralizing antibodies as tested by microserum neutralization test (MSNT) were shaved at the right side of the neck.

Ten fold serial dilutions of LSDV-BKC/P $_{66}$ were prepared in PBS starting with dilution $10^{-0.5}$ and ending with dilution $10^{-5.5}$. One ml of each of the last four dilutions ($10^{-2.5}$,$10^{-3.5}$,$10^{-4.5}$,$10^{-5.5}$) was inoculated subcutaneously into each of four experimental animals. Two experimental animals were left as uninoculated contact controls. Concurrent titration of the inoculated virus was carried out in tissue culture tubes.

Animals were observed clinically for 24 days. Body temperatures were recorded daily at a fixed time and post-vaccinal localized swellings were measured by means of calipers.

On the 24th day post-vaccination, all vaccinated animals and the two uninoculated controls were challenged with LSDV-BKC/P_2 using intradermal titration method (Davies and Mbugwa, 1985). Withstanding challenge was judged by absence of skin reaction to the lowest dilution of challenge virus inoculated, which was 10^{-1}. Using the method of Karber (1931) the 50% protective dose was determined.

RESULTS

Effect of Repeated Cycles of Freezing and Thawing on Virus Infectivity:
Since repeated cycles of freezing and thawing were adopted for release of the intracellular virus in the growth curve experiment, it was essential to study their effect on virus infectivity. For this purpose clarified virus harvest was subjected to three cycles of freezing at -30°C (2hrs each) and thawing at 37°C. Samples were taken before the start and after each cycle, kept at 4°C and then titrated simultaneously. The three cycles of freezing and thawing did not affect the virus infectivity. The virus titre before freezing was $10^{4.9}$ $TCID_{50}$ and $10^{5.3}$ $TCID_{50}$ /ml after the first cycle and $10^{5.1}$ $TCID_{50}$/m1 after the 2nd and 3rd cycles.

Growth Curve: Each flask culture was inoculated with $10^{5.7}$ $TCID_{50}$ at an apparent multiplicity of infection of 0.3. After two hours absorption at 37°C, the infectivity of the inoculum was $10^{5.1}$ $TCID_{50}$ (loss of about 75% of the input virus), while that of a sample of the

inoculum kept at 37°C for two hours (without absorption) was $10^{5.5}$ $TCID_{50}$.

Cytopathic effects typical to "Neethling" type virus were observed by the light microscope as early as 48 hours post-inoculation in all infected cultures. From the 4th day onward no islets of normal cells could be seen.

The results of titration of the CFV and the CAV are given in Table (I) and Fig.(1). Production of new virus was demonstrable at 24 hours post-inoculation both as CAV and CFV (released virus). The CAV increased rapidly up to the first 48 hours then at a slower rate to reach a peak on the 5th day post-inoculation; 24 hours after the CPE involved the whole of the sheet cover. After that it showed a steady decline in proportion to the destruction of the cell sheet. The CFV exhibited, to some extent, a steady rise from the first day up to the end of the experiment. The titre of the CFV never exceeded that of the CAV, only on the 7th day post-inoculation, when about 65% of the sheet cover was detatched, was it equal to that of the CAV. The difference between the CAV and CFV was more than one log unit on the 1st three days of the experiment, when the CPE involved 50% or less of the sheet cover. On the last four days, when the CPE involved the whole of the sheet cover, the difference was less than one log unit and it decreased steadily with the progress of sheet destruction.

Table (I): The Growth of the Candidate Vaccine in Primary BKC Culture

Time P.I. (hours)	C.F.V* (released virus)	C.A.V*	Difference #	C.F.V./CAV ratio	%of CFV to the whole infectivity detected
0	-	3.13	-	-	-
24 (1day)	2.63	3.63	1.00	1:10	9
48 (2days)	4.13	5.63	1.50	1:31	3
72 (3days)	4.63	5.88	1.25	1:17	5.5
96 (4days)	5.50	6.25	0.75	1:5	16.6
120 (5days)	5.88	6.38	0.50	1:3	25
144 (6 days)	5.63	6.00	0.37	1:2	33
168 7(days)	5.63	5.63	0.00	1:1	50

* = Log_{10} $TCID_{50}$, # = Difference between CFV and CAV in log units, O hour = After 2 hrs adsorption.

Prduction of Plaques on Monolayer Culture of BKC Without Agar Overlay:

Monolayer cultures inoculated with the vaccine virus and refed with outgrowth media containing normal serum showed regular diffuse CPE of "Neethling" type virus. On the other hand, infected cultures refed with outgrowth media containing LSD-immune serum showed focal CPE on the 4th day post-inoculation that proceeded to discrete plaques. After the 8th day post-inoculation, the focal CPE in these cultures started to diffuse. Control cultures inoculated with PBS and refed with outgrowth media containing normal or LSD-immune sera did not show any form of CPE.

Virus harvest from cultures showing discrete plaques produced, when inoculated in BKC cultures, the regular CPE of "Neethling" type virus. The identity of the virus was confirmed by virus-serum neutralization test using a reference LSDV hyperimmune serum (PIRBRIGHT). The reference hyperimmune serum neutralized comp-

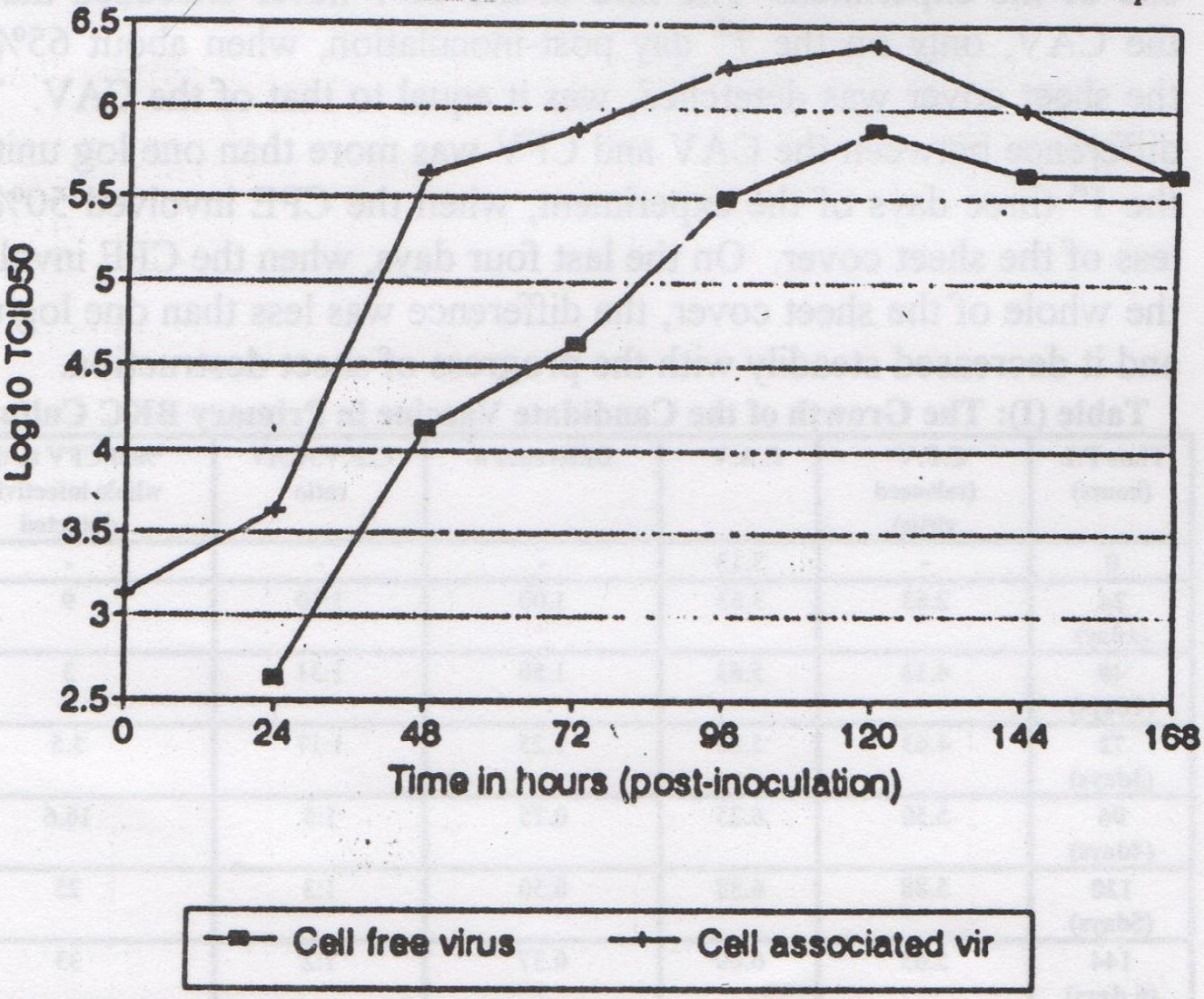

Figure (1) : Growth Curve of The Candidate Vaccine in Primary BKC Culture

letely the 10^{-2} dilution of the virus (the lowest dilution used), whereas the virus/normal serum mixture produced a titre of $10^{5.3}$ $TCID_{50}$/m1.

Cattle Protective $Dose_{50}$ of The Candidate Vaccine Virus: According to the result of the concurrent titration in BKC culture, the titre of the vaccine virus used for animal inoculation was $10^{6.2}$ $TCID_{50}$. It followed that the employed dilutions ($10^{-2.5}$, $10^{-3.5}$, $10^{-4.5}$, and $10^{-5.5}$) were containing 5000, 500, 50, and 5 $TCID_{50}$/m1 (volume of inoculum) respectively. Table(II) shows that all vaccinated animals were protected against the intradermal challenge with the exception of two animals out of four vaccinated with $5TCID_{50}$. The reactions to challenge that appeared in these two animals were milder than those recorded in the two in-contact controls with regard to the titre of the reacting challenge virus and the nature of the reaction. From these results, it could be directly interpreted that one cattle PD_{50} was equivalent to $5TCID_{50}$.

Table (II): Determination of PD_{50} of LSDV-BKC/P_{66} For Cattle (Post-Vaccinal and Post-Challenge Reactions)

		Post-vaccinal reaction			Post-challenge reaction			
Animal No.	Vaccine dose ($TCID_{50}$)	Generalized	Maximum size of localized reaction	Date of appearance	Generalized	Localized (titre of challenge virus**)	Nature of the reaction	No. of animals protected per group
2382	5000	Nil	6.3x6.8cm	8th day P.1	Nil	N.D	-	
2662	"	"	+	" " "	"	Nil	-	
3079	"	"	+	7th " P.1	"	"	-	3/3
3081	"	"	-			"	-	
2672	500	Nil	6.8x7cm	7th day P.1	Nil	Nil	-	4/4
2682	"	"	-	-	"	"	-	
2683	"	"	++	8th day P.1	"	"	-	
3122	"	"	+		"	"	-	
2663	50	Nil	-	-	Nil	Nil	-	4/4
2678	"	"	++	12th day P.1	"	"	-	
3038	"	"	4X7cm	11th day P.1	"	"	-	
3120	"	"	-	-	"	"	-	
2670	5	Nil	+	17th day P.1	Nil	Nil	-	2/4
2681	"	"	-	-	"	10^{22}	*	
2687	"	"	-	-	"	Nil	-	
2690	"	"	-	-	"	$10^{2.7}$	*	
2388	Uninocul-	-	-	-	Nil	$10^{2.7}$	#	
3072	ated Contact Controls	-	-	-	"	$10^{3.7}$	#	0/2

**Cattle skin infective dose $_{50}$ /m1, *2-3 fold increase in skin thickness appeared by the 7th day post-challenge, # 2-3 cm local swelling with formation of sit-fast at dilution 10^{-1} and 10^{-2} inoculation sites, +Hardly detectable subcutaneous nodule, ++ Easily detectable subcutaneous nodule.

Post-Vaccinal Reaction to Varying Doses of The Candidate Vaccine Virus: Following inoculation of doses as low as $5TCID_{50}$, and as high as 5000 $TCID_{50}$ no generalized reaction was detected in any animal, whereas localized reactions at the inoculation sites were detected in nine animals out of sixteen inoculated. They were similar to those noticed before (Abd El Raouf *et al* 2003). Moreover, it was also evident that the size of the localized reaction differs with individual animals rather than with groups receiving different doses of the virus (Table II).

DISCUSSION

The growth curve of the vaccine virus in BKC culture indicated that the infectivity detected in culture correlated with the CPE observed and that the rate of virus production and accumulation in cells was greater than the rate of release. Therefore, it is essential to harvest both the infected cells and the supernatant fluid. The optimum time for harvesting the infected culture is after the CPE involves the whole of the sheet cover and before advanced destruction of the cell sheet takes place. Three cycles of repeated freezing and thawing did not affect the virus titre and may be used for harvesting of the infected cells.

Plowright and Witcomb (1959) studied the growth of a low passage "Neethling" type virus in BKC culture. The peak of released virus was reached on the 5th day post-inoculation and by the 7th day post-inoculation half to two thirds of the cell sheet was cleared, yet the released virus did not exceed in quantity 6% of the total infectivity of the culture even at the latest stage. In the growth curve of this candidate vaccine similarly the peak of the released virus was reached on the 5th day post-inoculation and two days later two thirds of the cell sheet were cleared but the released virus reached 25 to 50% of the total infectivity detected. This may imply that the atten-uated virus is released more rapidly from the cells.

The formation of plaques by the vaccine virus in BKC culture in the presence of LSDV-antiserum indicated that it can spread in this type of culture from an infected cell to neighbouring cells by a mechanism which bypassed the extra-cellular fluid. Plowright and

Witcomb (1959) expected this mode of virus transmission to play a role in dissemination of infection in BKC culture. According to Tyrell (1963) this cell-to-cell transfer is expected whenever little release of virus occurs. Direct cell-to-cell passage has also been verified for vaccinia virus in chick embryo monolayer culture (Postlethwaite 1960) and in HeLa cells (Nishmi and Keller, 1962). The latter authors argued that as vaccinia virus has shown, in differ-ent type of tissues and monolayer cultures as in suspension cultures, a tendency to remain cell bound even at the late stages of infection, it would seem that this criterion is inherent in the nature of the virus rather than the host cell. Though, there is no proof that this mode of virus transmission might operate in-*vivo* in case of natural infection, but had that been the case the importance of cell-mediated immunity (CMI) would be much greater than anticipated. Spread by contiguity has been suggested to play a part in in-*vivo* viral dissemination in small pox (Downie, 1951), herpes simplex (Hoggan and Roizman, 1959) and measles (Rapp *etal* 1960). Protection in all these infections is predominantly a function of CMI (Bloom and Ragger-Zisman 1975).

The PD_{50} of the candidate vaccine virus was determined as equivalent to $5TCID_{50}$. Nevertheless, this figure may be taken with reservation because of the difference in susceptibility of cattle to LSDV. Against this reservation, no discrepancy was noticed in the result of the experiment conducted to determine the PD_{50}. In addition, one animal, which had reacted locally to $5TCID_{50}$ of the vaccine virus, was completely resistant to challenge. If this post-vaccinal reaction taken as an indication of high susceptibility, then resistance to challenge was due to no other reason than protection by the low dose of the vaccine virus. Also, animals that reacted to challenge after vaccination with $5TCID_{50}$ manifested a degree of protection as judged by the lower titre of the reacting challenge virus and the milder nature of the post-challenge reaction compared to that observed in the control animals. These results and those presented in the preceding article of this series (Abd El Raouf *et al* 2003) showed beyond doubt that even very small doses of the candidate vaccine can confer short term resistance to challenge. However, long term resista-nce remains to be studied, though immunity against generalized pox infection is

known to be prolonged. On the other hand, it is remark-able that the determined PD_{50} of this candidate vaccine is comparable to those of other cell culture propagated vaccines. The PD_{50} of the "Kedong" strain of sheep pox used for immunization against LSD (Capstick and Coackley 1961) and of strain 0240 of Kenyan sheep and goat pox virus used for vaccination against sheep pox (Kitching *et al* 1987) are equivalent to 8.9 and one $TCID_{50}$ respectively.

The value of the PD_{50} together with the nature or size of risk that animals encounter in the field, both, determine the minimal requirement of virus titre that the field dose should meet to ensure adequate immunity. Of the latter factor there is little information. Nevertheless, 5000 $TCID_{50}$ or 1000 PD_{50} per/dose is chosen for experimental field dosage. This figure meets the requirement of $10^{3.5}$ $TCID_{50}$ per dose recommended by the Office Internationale d'Epizootie (O.I.E 1992) for LSD cell culture vaccines. It also seems appropriate with other practical considerations such as virus titres that could be achieved constantly in production and the stability of the final product. Growth curve experiment has showed that titres above 10^6 $TCID_{50}$/m1 are achievable. Lypholised preparaions of the vaccine virus of $10^{6.2}$ $TCID_{50}$/m1 were scored during this work. The stability of the final product needs yet to be studied but there is no reason so far to suppose other than what expected of pox viruses.

Fifty six percent of the animals vaccinated with varying doses showed post-vaccinal localized reactions. The similarity of the size of these reactions between groups receiving different doses of the vaccine suggest, in a further indication of safety, that there is little relation between the dose of the vaccine virus and size of localized reactions. The cutaneous reactions did not show necrosis or sloughing which preclude the chance of virus shedding. They are not associated with fever, yet the possibility of biting insects transmitting the virus horizontally in the field, although slight, does exist. Experimental serial transmission of the vaccine virus needs yet to be studied. Weiss (1968) reported that the South African vaccine against LSD produced granulomatous lumps in about 50% of the vaccinated animals. Localized reactions following pox virus vaccination are common and are generally looked upon as an effortless way for assessment of "vaccine

take". Nevertheless, final judgement of safety and efficacy of a vaccine is possible only after extensive field trials.

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