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INFLUENCE OF PROPAGATION OF SHEEP POX VACCINAL STRAIN IN PRIMARY CULTURE OF OVINE EMBRYONIC HEART CELLS ON THE EFFICACY OF THE VACCINAL SEED VIRUS

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SUMMARY

Multiple propagation of seed viruses in cell cultures, used in manufacturing of vaccines, could cause relative weakness of the virus pathogenicity and immunogenicity after successive passages. This study was conducted to reamplify the pathogenicity and immunogenicity of sheep pox vaccinal seed virus throughout its passage on primary cell culture of ovine foetus heart muscle cell (OFHMC). The primary propagation was applied for 7 passages recording a titre of $10^{6.2}$ $TCID_{50}$/ml. The virus was transferred to Vero cell lines where its titre reached $10^{7.8}$ $TCID_{50}$/ml by the 11^{th} passage; while it recorded $10^{5.5}$ SID_{50}/ml by its titration in susceptible lambs, with producing remarkable lesions in the inoculation sites. An experimental batch of lyophilized sheep pox vaccine from the adapted virus fluid, was prepared. Sterility, safety and potency tests were successfully applied on the trial batch. The results were confirmed by monitoring the antibody levels in experimentally vaccinated lambs through the application of serum neutralization test and ELISA.

INTRODACTION

Small ruminants play an important role in the agricultural economy (**Carn, 1993**).Sheep pox is an important viral disease affecting sheep, characterized by fever, generalized papules, vesicles and pastules on the skin; while papules are present in the internal organs. It is caused by a capripox virus which is endemic in Africa; the Middle East and India (**OIE, 2004**). In the endemic situation, claims of 10% morbidity and low mortality leads to under estimation of production losses (**Kitching, 1988**).Vaccination is the only mean for controlling the disease (**Jadhav et al., 1989**).

Attenuated Kenyan sheep pox virus strains, propagate on Vero cell line succeeded to produce a safe, sterile and potent sheep pox vaccine (**Rizkallah, 1994)**, in Egypt, where hundred million doses are used for controlling capripox diseases (sheep pox and lumpy skin diseases) since

1994 (**General Organization of Veterinary Services "GOVS").**As capripox virus is more susceptible to grow on both primary or secondary cultures of lamb testis or kidney, or on cell lines as Vero cell line (**OIE, 2004**). The present study tried to propagate the Kenyan sheep pox virus in primary cultures of OFHMC, in order to enhance the viral replication for reaching a higher titer in comparison with the already obtained one.

MATERIALS AND METHODS

1. Animal

1.1. Ewes

Four, sheep pox seronegative late pregnant Baladi ewes were slaughtered for foetus delivering. Primary cultures of foetal heart cells were prepared from the delivered foetus.

1.2. Lambs

Eight sheep pox seronegative lambs were used to assay the pathogenicity and immunogenicity of the vaccine seed virus of Kenyan strain propagated on primary cultures of OFHMC, as well as Vero cell line.

2. Viruses

2.1. Vaccinal sheep pox virus

Kenyan strain of sheep pox virus was obtained from the Pox Vaccines Department, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo. The virus titre was 5.3 Log_{10} $TCID_{50}$/ml. It was used for propagation in OFHMC.

2.2. Virulent sheep pox virus

Egyptian strain of sheep pox virus (**Sabban, 1960)** was obtained from the Pox Vaccines Research Department, Veterinary Serum and Vaccine Research Institute , Abbasia, Cairo. It was used in challenge test.

3. Cell cultures

3.1. Ovine foetal heart muscle cells (OFHMC)

These cells were grown and maintained according to the methods described by **Davies and Otema (1978)**.The cells were used for propagation of sheep pox seed virus.

3.2. Vero cell line

African Green Monkey Kidney cell (Vero cell) was obtained from FADDL, Plum Island, USA. Vero cells were propagated and maintained in Pox Vaccines Research Department, VSVRI, according to the method described by **Ozawa and Hazrati (1964)**. The cells were used for production and titration of sheep pox vaccine.

4. Media and Serum

Minimal essential medium (MEM) based on Earl's balanced salt solution with 10% newborn calf serum was used as growth media and with 2-3% as maintenance media.

5. Lactalbumin– sucrose stabilizer

It consists of sucrose 2.5% and lactalbumin hydrolysate 5% (**Rizkallah, 1994)**.

6. Preparation of primary OFHMC

Heart was obtained from a foetus delivered from the pregnant ewe under aseptic condition and primary ovine foetus heart cells cultures were prepared as described by **Hoskins (1967)**. These were initiated from trypsinized heart cells suspension. The cells were grown in monolayer cell sheets and maintained according to the method described by **Davies and Otema (1978).**

7. Propagation of sheep pox virus on OFHMC

The propagation of sheep pox virus technique in cell cultures was applied according to **Rizkallah (1994)**. This technique was applied until complete adaptation of sheep pox virus on OFHMC. Sterility and titration of the heart cells adapted sheep pox virus were conducted according to **OIE (2008).**

8. Adaptation of sheep pox virus on Vero cell line

Serial passages of sheep pox virus were applied on Vero cell lines by the method described by **Prakash et al. (1994**). The adapted sheep pox virus on foetal heart muscle cells, which recorded the highest titre and cytopathic effect was inoculated into confluent sheet of Vero cells, till reaching clear cytopathic changes at constant time.

9. Titration of adapted sheep pox virus

9.1. Titration in-Vitro

Adapted sheep pox virus was titrated in Vero cell cultures according to **Tiwari and Negi (1995)**.

9.2. Titration in-Vivo

Adapted sheep pox virus was titrated in the skin of the flank of two susceptible lambs, as described by **OIE (2004)**. The virus was diluted from 10^{-2} to 10^{-8}, 0.2 ml (from each dilution) was inoculated intradermally at 3 sites in each lamb. The inoculated animals were observed for 7 to 15 days where daily clinical examination and recording of the body temperature were carried out. The virus titre was calculated according to **Reed and Muench (1938)**.

10. Preparation of a lyophilized sheep pox vaccine trial batch

The final product of sheep pox vaccine was prepared by addition of lactalbumin sucrose stabilizer to the adapted sheep pox virus fluid which adjusted to contain $10^{5.3}$ $TCID_{50}$/ml in a ratio 1:1; the mixture was lyophilized to obtain the final product.

11. Evaluation of the experimental batch of sheep pox vaccine

11.1. Sterility test according to **OIE Manual (2008)**.

11.2. Safety and potency test

It was applied according to **Sabban (1960) and OIE (2008)**. Six susceptible lambs were divided into 2 equal groups. Two lambs of the first group were inoculated with the field dose and two lambs from the second group were inoculated with 20 times the field dose. One lamb from each group was kept as a non-inoculated control. Challenge test was applied on inoculated and control lambs according to **Rizkallah (1994).** Serum samples were collected during the period of vaccination and challenge.

12. Seroconversion

12.1. Serum neutralization test (SNT)

It was applied according to **OIE Manual (2008)** for serum samples.

12.2. ELISA

ELISA was applied according to the method described by **House et al. (1990)**.

RESULTS

1. Propagation of sheep pox virus on foetal heart cells

The obtained results revealed by the initial passage, infected cultures of ovine foetus heart cells showed characteristic cytopathic effect in the form of bulging and aggregation of rounded cells with cytoplasmic extensions (Photo 2). Between the 3rd and 5th passage, cytopathic effect of the virus was evident as early as the 3rd day post inoculation (DPI) and completed by the 5th DPI at the 6th and 7th passages, where detachment of the infected cells, and the cell sheet showed plaque formation. The infectivity titres of the virus in ovine foetus heart cell cultures were recorded in (Table 1). Sterility tests showed that the virus fluid was free from bacteria, fungi and advantageous viruses other than sheep pox.

Table 1. The infectivity titre of sheep pox virus in primary cultures of OFHMC

Passage No.	Harvestation Time	Infectivity titre * log_{10} $TCID_{50}$/ml
1	7 DPI **	4.7
2	7 DPI	4.8
3	6 DPI	5.1
4	6 DPI	5.5
5	6 DPI	6.2
6	5 DPI	6.7
7	5 DPI	6.7

*Tissue Culture Infective Dose 50 **DPI: Days Post Inoculation

Photo (1): Normal cells (20x)

Photo 2: Cytopathic effect in ovine fœtus heart cells infected with sheep pox virus (40x)

2. Adaptation of sheep pox virus on Vero cell lines

The propagated sheep pox virus on foetal heart cells was transferred to confluent sheets of Vero cell lines. Serial passages were applied until clear and specific cytopathic effect obtained. Infected cells appeared as discrete foci of rounded–highly refractile cells and the stained monolayer cultures showed intracytoplasmic inclusions. Many of the cells show finely granular intracytoplasmic acidophilic matrices with basophilic inner bodies, often in the shape of a broken arc or continuous hoop towards the periphery of the cell.

3. Titration of adapted sheep pox virus

3.1. In-vitro

Titration of the adapted sheep pox virus serially passaged on Vero cell lines was applied in 96 wells tissue culture plates. The results were recorded in Table (2).

Table 2. Titration of the adapted sheep pox virus on Vero cell lines

Passage No.	Titre in Log_{10}
1	5.3
3	6.0
5	6.7
7	7.6
9	7.8

11	7.8

3.2. Titration in-vivo

By the 3rd day post inoculation, reddish nodular swellings appeared at the site of injection with formation of different forms of sheep pox lesion finally at 5th to 6th day; scab formation appeared. The titration is calculated by the formula of **Reed and Muench (1938)** and recorded $10^{5.5}$ SID_{50}/ml.

4. Evaluation of the adapted sheep pox vaccine experimental batch

4.1. Sterility

Results of the tested samples proved their freedom from aerobic and anaerobic bacteria and fungi when inoculated on specific media.

4.2. Safety and potency

During the 1st month the contact non-vaccinated susceptible control lambs showed no general or local reaction while the inoculated lambs with either field dose or 20 times field dose (group 1 and 2), showed slight rise in temperature with clear and variable nodular swelling at the site of injection (normal post vaccinal reaction). After challenge, the vaccinated lambs with field dose did not show any abnormal local or systemic reactions; while the non-vaccinated lambs showed severe local reaction in the site of inoculation, which consisted of highly reddish nodular swelling which continued to form all the pox lesion stages (papule, vesicle, pustule and scab). These lesions become generalized on all the unwooled parts of the animal body, especially around the eyes, nostrils and mouth. The signs were associated with severe increase in the body temperature which reached 41ºC, depression, loss of appetite and increased nasal discharge.

5. Seroconversion

5.1. Serum neutralization test: The results were presented in Table (3).

Table (3): Results of sheep pox neutralizing indices of sera of lamb vaccinated with adapted sheep pox virus

Tested lamb		Animal No.	Before vaccination	Days Post Vaccination				Days Post Challenge		
				7	14	21	28	7	14	21
1st group G1*	Vaccinated	1	0.1	0.8	1.4	2.2	2.0	1.9	2.0	2.0
		2	0.3	1.0	1.6	2.2	2.2	2.1	2.2	2.0
	Control	1	0.2	0.4	0.6	0.5	0.4	1.2	1.8	2.0
2nd group G2**	Inoculated	1	0.1	1.0	1.7	2.2	2.0	2.0	2.1	2.0
		2	0.0	0.9	1.5	2.0	1.9	1.8	2.1	2.0
	Control	1	0.3	0.3	0.3	0.4	0.3	1.1	2.0	2.1

N.B. Neutralizing index (NI) > 1.5 considered as the protective mean against sheep pox virus (**Cottral, 1978**).
1st group: Received one field dose 2nd group:Received 20 times field dose

5.2. ELISA

The results were presented in Table (4)
Table (4): S/P ratio of sera collected from lambs vaccinated with adapted sheep pox virus

Tested lamb		Animal No.	Before vaccination	Days Post Vaccination				Days Post Challenge		
				7	14	21	28	7	14	21
1st group G1*	Vaccinated	1	0.07	0.74	1.80	1.90	1.34	1.67	1.80	1.74
		2	0.14	0.91	1.66	1.97	1.69	2.13	2.45	1.59
	Control	1	0.14	0.07	1.10	0.09	0.09	1.25	1.58	1.68
2nd group G2**	Inoculated	1	0.11	0.80	1.46	2.05	1.77	2.25	2.25	1.84
		2	0.06	0.90	1.70	2.00	1.87	2.11	2.30	2.00
	Control	1	0.09	0.13	0.11	0.12	0.10	1.23	2.00	1.75

S/P: Sample positive ratio
N.B. Sample positive > 1.0 considered protective (**Williams, 1987**).
1st group: Received one field dose 2nd group Received 20 times field dose

DISCUSSION

Pox is one of the most serious contagious viral diseases causing high mortality and great economic losses among susceptible animals and birds (**Sabban, 1955, 1957 and 1960; Le Goff et al., 2009**). Large quantities of sheep pox vaccine were produced annually to cover the vaccination programs of sheep population in Egypt (**Rizkallah, 1994**).The major problem facing the vaccine production on large scale is the continuous efficacy of the vaccinal working seed virus used in this mass production, which could decrease due to its successive passages for long times.

In this study, re-amplification of the pathogenicity and immunogenicity of the vaccinal seed of Kenyan sheep pox virus, was applied by passaging the virus in primary cultures of ovine foetal heart cells (**Hoskins, 1967 ; Davies and Otema, 1978**).Sheep pox virus was primarily passaged on ovine foetal heart cells (Photos 1 and 2) as shown in Table (1) until reaching specific CPE of sheep pox virus within 5 days post inoculation at the 6^{th} and 7^{th} passage, with a titre of $10^{6.2}$ $TCID_{50}$/ml. This adapted virus was transferred on Vero cell lines on which the vaccine mass production was applied where complete adaptation occurs (**Rizkallah, 1994**).

As this seed virus would be used for inoculation on Vero cell lines, the adapted virus was tested by titration in both vitro and vivo. In-vitro, by titration on Vero cells, Table (2) recorded that the highest titre was obtained between the 9^{th} and 11^{th} passages reaching $10^{7.8}$ $TCID_{50}$/ml. There is an absolute correlation between the intensity of local skin lesion induced by sheep pox virus vaccine (post Vaccinal reaction) and its efficacy (**Kitching et al., 1986**). Thus, the pathogenicity and immunogenicity of the adapted seed virus was detected by its titration in susceptible lambs; showing a titre of $10^{5.5}$ SID_{50}/ml. It was remarked that the lesions, resulting from inoculation of different virus dilutions, were more detectable in size than that of the original seed virus; which proved that the seed virus was activated by its primary passage on ovine foetal heart cell as recorded by **Pierre et al. (1979**).

Quality control tests revealed that the prepared experimental sheep pox vaccine was free from any aerobic, anaerobic bacteria, moulds and fungi. As well, safety and potency tests were applied; it was observed that the vaccine was safe either by using the field dose or its multiplications (20 times) is in agreement with **Chandra et al. (1990) and OIE (2008)**.By challenging the vaccinated animals with virulent sheep pox virus, the vaccinated lambs showed complete protection while the control ones produced local and generalized lesions of sheep pox infection. Similar findings were described by **Jassim and Keshavamurthy (1982**).

The former results were confirmed by monitoring the antibody levels in vaccinated and challenged lambs through application of serum neutralization test and ELISA. Tables (3 and 4) screened that the antibody levels is in agreement with those obtained by **Cottral (1978) and Williams (1987).** So, it could be concluded that activation of the seed sheep pox virus was successding by its propagation in primary OFHMC, providing a potent seed virus able to be used for production of sheep pox vaccine having sufficient pathogenicity and immunogenicity, with desirable intensity of post vaccinal local reaction, which will give change to a quantitative and qualitative improvement of the production of sheep pox vaccine, helping in the control of lumpy skin disease in cattle and sheep pox in sheep.

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تاثير تمرير عترة لقاح فيروس جدرى الأغنام فى خلايا قلب جنين الضأن على كفاءة بذرة اللقاح

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الملخص العربى

التمريرات المتكررة لبذور الفيروسات فى خلايا الزرع النسيجى، والتى تستخدم فى تحضير اللقاحات، يمكن أن تسبب ضعف جزئى فى القوة المناعية والمرضية للفيروس بعد تمريرات متتابعة. قامت هذه الدراسة على تعظيم القوة المرضية والمناعية لبذرة فيروس جدرى الأغنام المستخدم فى إنتاج اللقاح عن طريق تمريره فى خلايا الزرع النسيجى الأولية المحضرة من خلايا قلب جنين الضأن. كان عدد التمريرات الأولية سبعة حتى تم تسجيل القوة العيارية بقيمة $10^{6.2}$ $TCID_{50}/ml$. وبعد ذلك تم نقل الفيروس لتمريره على خطوط خلايا الزرع النسيجى المستديمة (فيرو) ووصلت القوة العيارية على هذه الخلايا الى $10^{7.8}$ $TCID_{50}/ml$ بعد إحدى عشر تمريرة، بينما وصلت القوة العيارية الى $10^{5.5}$ SID_{50}/ml بعد معايرتها فى الحملان مع ظهور أثار واضحة مكان الحقن. تم تجفيد دفعة تجريبية من اللقاح المحضر من فيروس جدرى الأغنام المؤقلم. وتم إجراء اختبارات النقاوة والأمان والفاعلية عليها وكانت النتائج ناجحة وتم تاكيد هذه النتائج بمتابعة مستوى الأجسام المناعية فى الحملان المحصنة معملياً باختبارى التعادل المصلى والاليزا.