Preliminary studies on preparation and evaluation of a local isolate tissue culture propagated pigeon pox vaccine

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Abstract

The current study was conducted to use the propagated Egyptian strain of pigeon pox Qaluobeia 2017 virus on primary chicken embryo fibroblast (CEF) in preparation of tissue culture (VERO) specific pigeon pox virus vaccine (PPV) for pigeons. The titer of this virus was $10^{5.5}$ $TCID_{50}$ /ml at the 20th passage, and induced an acceptable reaction when it was tested for pathogenicity in pigeons. After addition of an equal volume of Lactalbumin sucrose stabilizer the virus fluid was lyophilized showing titer of $10^{5.0}$ $TCID_{50}$/ml in VERO cells. Keeping quality control tests revealed that such vaccine was free from the foreign contaminants, safe and potent. The humoral antibody level in the serum of vaccinated pigeons was measured by serum neutralization test (SNT) which proved that the induced pigeon pox antibodies had 1.3 neuralizing index (NI) from the 2nd week and reached its peak (2.8 NI) at 4th week post vaccination. Pigeons withstand the inoculation with the virulent PPV after 3 weeks from vaccination with no symptoms of PPV disease on contrast to control non- vaccinated pigeons with protection percent 95%.

This study recorded the production of a safe and potent PPVV from local PPV strain (Qaluobeia -2017) of less cost than that prepared on specific pathogenic free Embroynated chicken egg (SPF-ECE)

1. Introduction

Pigeon pox is adisease caused by pigeon pox virus (PPV) that is classified within *family Poxviridae subfamily Chordopoxvirinae* and *genus Avipoxvirus* (**Andraw, 2012**). It produces economic losses within the infected pigeons. The disease causes cutaneous diphtheritic or systemic changes which create problems due to the lesions induced around the mouth or eyes with ulcerations in the oral cavity making an affected bird reluctant to drink or eat causing dehydration or starvation ending by death **(Tripathy and Reed ,1997 and Hemanth et al ., 2014).**

Pigeon pox is a serious virus disease in pigeon causing mortalities especially in young pigeon characterized by the development of discrete proliferative nodular skin lesions (cutaneous form) or

fibrino-necrotic lesion in the mucous membrane of the upper respiratory tract (diphtheritic form) **(Sumaya 2005).**

A chicken embryo cell culture system derived from a specific pathogen free embryo is defined and shown to be highly susceptible to the pigeon pox virus (PPV). The high susceptibility of the system and the growth characteristics of the virus suggest that the host tissue specificity for PPV persists after the cells have differentiated in cell cultures. The cell system consists of cell strains derived from primary cell cultures. PPV was adapted to the cell system, and an experimental batch of vaccine was prepared from the 5th passage and tested. The cell culture vaccine has higher biological properties than a conventional vaccine prepared on the chlorioallantoic membrane of embryonated eggs **(Ael-Zein et al., 1974).**

In Egypt, pigeon pox vaccine was prepared by propagation of pigeon pox virus (Hungarian strain) on the chorioallantoic membrane (CAM) of 9-11 day -old specific pathogen free (SPF) embryonated chicken eggs **(Helmy et al., 1967).**

Pigeon pox virus infection is worldwide in its distribution **(Smits et al., 2005).**
Pigeon pox virus can be used as a vaccine against fowl and pigeon poxvirus infection and also against other avian poxviruses **(Simon, C. W. and Morten, T. 2011).**

The present study aim to prepare and evaluate local pigeon pox vaccine from isolated and identified local pigeon pox virus in 2017 on Vero cell line.

2.Material and Methods

2.1. Viruses

An Egyptian strain of pigeon pox virus Qaluobeia 2017 isolated identified antigenically and genetically and adapted in CEF primary culture. **(Aboul Soud et al., 2018)** was supplied by Veterinary Serum and Vaccine Research Institute.

Virulent pigeon pox virus was kindly supplied by pox vaccine department, Veterinary Serum and Vaccine Research Institutes, Abbasia, Cairo. It had a titer of $10^6 EID_{50}$ / ml and used for challenge of experimentally vaccinated pigeons.

2.2. Susceptible pigeons:

One hundred thirty of susceptible pigeons of 45 day - old were used in this study, for monitoring of the pathogenicity of the cell culture adapted virus and for vaccine evaluation. These pigeons were divided as follows: Thirty pigeons were used to test the pathogenicity of the cell culture propagated

virus, sixty pigeons were used for testing the safety of the prepared vaccine and Forty pigeons were used for detection of the potency and duration of immunity.

2.3.Tissue cultures

2.3.1. Chicken Embryo Fibroblast cell culture (CEF)

Chicken embryo fibroblast cell cultures were prepared according to the method described by **Olfat (2006)** using 10 days-old–SPF chicken embrynted. It was used for primary propagation of the PPV virus.

2.3.2. Vero cell culture

Vero cell line were kindly supplied from Pox Research Department VSVRI and used for propagation of the virus, virus titration and SNT

2.4. Earle's Minimum Essential Medium (MEM).

It was obtained from Sigma Chemical Company, USA and used as growth medium containing 10 % Newborn calf serum or as maintance medium containing 2% Newborn calf serum to use in CEF preparation, subculture and virus propagation on cell culture.

2.5. Stabilizer:

Lactalbumin sucrose stabilizer (5% lactalbumin hydrolysate and 2.5 % sucrose) was prepared according to the **OIE (2012).**

2.6. Pigeon pox hyper-immune sera:

Hyper-immune serum against PPV was obtained from Pox Research Department VSVRI, and used in Virus Neutralization Test as appositive control

2.7. Serum samples:

Serum samples were collected from all birds weekly before and after vaccination and challenge for detection of antibody levels by serum neutralization test.

2.8. Pigeon Pox virus propagation in Chicken Embryo Fibroblast cell culture.

PPV propagated and titrated on CEF .The virus isolate was inoculated in to CEF for 7 successive passages. CEF tissue culture cells prepared from 12 days old embryonated SPF eggs. The embryos were aseptically collected and minced. The minced tissues were repeatedly washed with Hanks balanced salt solution (BSS) until the supernatant fluid was completely cleared of blood cells and cellular debris. The washed tissues were resuspended in 0.25% trypsin solution. Ten ml. of the trypsin

solution was added for each embryo. The tissues were then agitated at room temperature using a trypsinizing flask and a magnetic stirring device. The agitation was continued until a fairly homogenous suspension with relatively few large articles was obtained. This process took about one 20 minutes. The trypsinized tissue suspension was filtered through two or four layers of sterile gauze and centrifuged using sterile Eppendorf caped 50ml tubes at speed of 800 r.p.m. for a ten minute period. The supernatant fluid was discarded. The cells were suspended and centrifuged again. The supernatant fluid was discarded. The packed cells were resuspended in 1:400 dilution with the growth medium, and were agitated through the dispensing process. The growth medium used for cell propagation consisted of Hanks salt solution containing 0.5% lactalbumin hydrolysate and new borne calf serum. Antibiotic like Penicillin at the rate of 50 units and 0.50 microgram of dihydrostreptomycin per ml. of fluid were also optionally added to the medium. The growth medium containing the resuspended cells was added to each culture flask (prescription bottle of modern oval type) and incubated at 37oC.The monolayers fibroblast cells were generally well developed after about twenty-four hours. After two days incubation, the growth medium was discarded and replaced with maintenance medium. Inoculation of the CEF tissue cell with pigeon pox virus .The inoculated cell cultures were incubated at 37 oC. About three to five days of incubation following virus inoculation, cytopathogenic effect (CPE) was noticeable by microscopic observation. At this time both cells and fluids were harvested and the resulting virus suspension was used for serial passages in CEF tissue cultures. After a relatively 7 passages of the pigeon pox viruses in CEF it was adapted to VERO.

2.9. Propagation and titration of Pigeon pox virus in Vero cell line

Adapted virus on CEF was propagated and titrated on Vero cell line as the method described by **Mishra and Mallick (1994)** and **Olfat, et al.,(2005)**.

2.10. Testing the pathogenicity of the cell culture adapted Egyptian pigeon pox virus in susceptible pigeons:

The cell culture adapted virus was inoculated in 20 susceptible pigeons by feather follicle route and 10 susceptible pigeons were used as control non- inoculated according to **Nakano et al. (1972)**. The inoculated pigeons were observed for 15 days for the appearance of local and general post inoculation reaction.

2.11.Preparation and lyophilization of tissue culture pigeon pox vaccine

The virus fluid was added to lactalbumin sucrose stabilizer at a ratio of (l: l) according to **OIE (2012)**. This mixture was distributed in sterile neutral glass vials (2ml/ vial) and submitted to freeze drying process (Lyophilization process).

2.12. Evaluation of the cell culture pigeon pox vaccine (Quality control):

2.12. 1. Sterility:

It was carried out according to **OIE (2012)**, where random samples of the lyophilized vaccine were inoculated separately into tubes of nutrient agar, Sabouraud agar and thioglycolate medium and mycoplasma medium Also, the lyophilized vaccine was examined for any extraneous viruses.

2.12.2. Safety:

40 susceptible pigeons were inoculated with 10^3TCID$_{50}$ /dose of the prepared vaccine according to **OIE (2012)**; and another ten pigeons were inoculated with 10 times such dose.

five pigeons were kept as a contact control and another five ones were kept as an isolated control group.

2.12.3. Potency and duration of immunity:

Fourty susceptible pigeons were vaccinated by inoculation of the prepared vaccine using feather follicle route according to **Branson and Kip (1995)** in addition to 20 pigeons left as non-inoculated control pigeons. Challenge test was applied by inoculation of the virulent pigeon pox virus by feather follicle route in vaccinated and susceptible control pigeons at l month post vaccination, all birds were subjected to daily observation of gross lesions and collection of serum samples.

2.12.4 Serological assay using Serum neutralization test (SNT) :

It was carried out according to **Carol and Marinescu (1971)**.

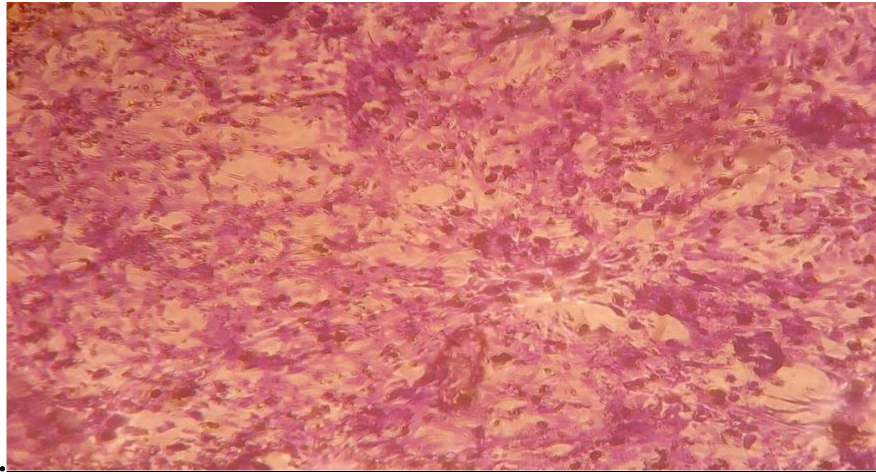
3. Results

3.1. Propagation of Pigeon Pox virus on CEF.

PPV propagate on ECF showed CPE detected at the 3rd passage characterized by cells rounding, aggregation and cell detachment, (photo 1).

The virus titer was 3, 3.5, 4, 4.5 log10 TCID50/ml for the 4th, 5th, 6th and 7th passage respectively.

Photo (1): CPE of propagated PPV on CEF showing rounding of cells, cell detachment, intracytoplasmic inclusion bodies and giant cell Formation



3.2.Propagation and titration of isolated PPV on Vero cell line

Propagation of isolated PPV on Vero cell line revealed that the virus CPE appeared clearly at the 3rd passage with titer 3.0 log10 $TCID_{50}$/ml then reaches 5.5 log10 $TCID_{50}$/ml at the 17th passage as shown in table (1).

Table (1): Titration and harvestation time of the propagated virus on Vero cell line

Virus passage No.	Harvestation time/day	Virus titer log_{10} $TCID_{50}$/ml
1	6	2
2	6	2.5
3	6	3.0
4	5	3.5
5	5	3.5
6	5	3.5
7	5	4.0
8	5	4.0
9	5	4.5
10	5	4.5
11	4	4.5
12	4	4.5
13	4	5.0
14	4	5.0
15	4	5.2
16	4	5.2
17	4	5.5
18	4	5.5
19	4	5.5
20	4	5.5

3.3. Pathogenicity test of propagated virus

The result of pathogenicity test carried out on susceptible pigeons presented in table (2) showing that the safe virus passage was number twenty.

Table (2) pathogenicity test of propagated PPV on Vero cell line.

Virus passage	No. of inoculated birds	No. of birds showing generalized lesion post vaccination	No. of birds showing localized lesion post vaccination	Pigeon with acceptable post vaccination take
4	4	1	3	0
8	4	1	3	0
12	4	1	3	0
16	4	1	3	0
20	4	0	0	4

3.4.Pigeon pox virus vaccine titer after lyophilization

The prepared virus fluid was lyophilized after addition of lactalbumin sucrose stabilizer (l: l) to obtain the end product. This lyophilized vaccine was retitrated in VERO cells showing a titer of $10^{5.0}$ $TCID_{50}$ /ml.

3.5.Quality control of the prepared PPV vaccine

3.5.1. Sterility test:

Bacterial culture of PPV vaccine proved to be free from any bacterial and fungal contamination and extraneous viruses.

3.5.2. Safety test:

Inoculation of PPV vaccine in susceptible pigeons with 10 times of the recommended dose proved that the produced vaccine was safe to be used in pigeons. Where the vaccinated birds did not show any undesirable symptoms refer to PP.

3.5.3. Challenge test:

Vaccination of Susceptible pigeons with Prepared vaccine by feather follicle method and challenged after 3weeks with virulent PPV revealed that 2 birds showed post challenging pox lesions and the other 38 birds remained healthy without local or generalized post challenging lesion ,This meaning that the protection percent of vaccinated pigeons were 95%,

3.5.4. Serum Neutralization test .

Serum neutralization test was carried on the serum samples collected from vaccinated pigeons and challenged one. The results were expressed as neutralization index (NI) which subsequently calculated as VT minus SVT (virus titer minus serum virus titer) and the NI ≥ (1.5) consider as positive results. The result which presented in Table (3) revealed that, the level of neutralizing antibodies in all vaccinated Pigeons serum samples is above 1.5 NI.

Table (3): Results of the Neutrization Index of sera collected from vaccinated pigeons with tissue culture PPVV and challenged pigeons.

Weeks post vaccination	PPV NI. In Vaccinated pigeons	Control non vaccinated pigeons
0	0.40	0.04
1	0.40	0.06
2	1.35	0.05
3	2.20	0.08
4	2.8	0.09
5	2.8	0.06
6	*2.7*	*0.04*
7	2.6	0.05
8	2.2	0.06
9	2.1	0.06
10	2	0.07
11	1.9	0.09
12	1.7	0.08
14	1.6	0.05
16	1.6	0.06
18	1.5	0.07
20	1.5	0.05

4. Discussion

Vaccination plays a key role in the modern poultry industry and without it the productivity would not have progressed so successfully and as rapidly as it has over the last few decades **(Frank et 1a. 2001).** As vaccination is the only mean for controlling pigeon pox disease **(Tripathy and Reed, 2003),** pigeon pox was controlled in Egypt by egg-adapted pigeon pox vaccine (Hungarian strain) **(Helmy et al., 1967).**

Through the present study, the PPV tissue culture vaccine was prepared from local pigeon pox isolate (Qaluobeia 2017 strain). where it was propagated on chicken embryo fibroblast for 7passeges.

The cytopathic effect (CPE) of the propagated virus on CEF appeared after the 3rd passage as cell rounding and cell aggregation (photo no.1).

Adaption of PPV in CEF revealed that CPE appeared clearly at the 3rd passage after 6 day post inoculation with titer 2.0 log_{10} $TCID_{50}$/ml then reaches complete CPE at the 4th passage as the virus titer reached 3 log_{10} $TCID_{50}$/ml and reached 4.5 log_{10} $TCID_{50}$ at The 7th passage In agreement to the obtained data by **Olfat et al., (2005)** as gradual appearance of CPE was observed by the 3rd passage.

Adaptation and Propagation of PPV on VERO cells revealed that CPE appeared clearly at the 3rd passage with titer 3.0 log10 TCID50/ml then reaches 5.5 log10 TCID50/ml at the 17th passage,Confirming to the obtained data by **Olfat et al., (2005).**

Adapted Qaluobeia Strain on VERO cells of a titer $10^{5.5}$ $TCID_{50}$ ml, was tested for detecting its pathogenicity in susceptible pigeons. The inoculated pigeons showed slight thickening of skin and takes at the site of inoculation, no local or general symptoms appeared. These results agreed with **Buller and Palumbo (1991)** and **Tripathy and Reed (1997).**

The prepared virus fluid was lyophilized after addition of lactalbumin sucrose stabilizer (l: l) to obtain the end product. This lyophilized vaccine was reiterated in VERO cells showing a titer of $10^{5.0}$ $TCID_{50}$ /ml with a slight decrease in the titer after lyophilization which seemed to be non significant in the vaccine efficacy. Evaluation of the prepared vaccine proved that the vaccine was sterile and (free from any bacterial, fungal and mycoplasma contaminants and also free from extraneous viruses) .Also, it was completely safe when inoculated either by the protective dose or with 10 times this dose showing no adverse effects attributable to the vaccine in agreement with the recommendation of **(OIE, 2012)**. Challenge is considered the master test to measure the immunizing capacity of the vaccine against pox infection, so the prepared vaccine was tested by challenging the immunity of the vaccinated pigeons with the virulent pigeon pox virus. The results indicated that vaccinated pigeons were able to overcome the virus infection with protection percent reach 95%. These results agree with those of **Frank et al.(2001)** As mentioned by **(Soad et al., 2007)**, the antibody response to vaccination is use(as an indirect measure of the immunogenicity of the vaccine and accordingly the immune status of vaccinated pigeons was estimated by serum neutralization test (SNT). In Table (3) , the observed results showed that the neutralizing antibodies reached their maximum neutralizing index (NI.) 2.8 at 4weeks post vaccination and NI and reached (1.5) at 20weekes in a parallel with those of **Michael et al. (1986)**.

From the obtained results, the present study proved that the use of cell line (VERO) for production of pigeon pox vaccine has several advantages over the use of SPF chicken embryos which are expensive and required special holding facilities with intensive labors. Whereas cell line is not subjected to these factors and can be made available easily and continuously by Simple procedures. Thus reaching the production of PPVvaccine on tissue culture is considered to be more economic and safe as the incidence of contamination is relatively restricted, in addition to the easiness in production.

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الملخص العربي

دراسات أوليه لتحضير وتقييم لقاح جدرى الحمام النسيجى المحضر من عترة محليه.

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المعمل المركزى للرقابه على المستحضرات الحيويه البيطريه**

تهدف هذة الدراسه لتحضير لقاح جديد لفيروس جدرى الحمام على خلايا الزرع النسيجى بدلا من اللقاح المحضر على بيض اجنه الدجاج لتفادى المشاكل المرتبطه بتحضير اللقاح على البيض من تكلفه عاليه وظهور ملوثات بكتريه وفطريه وفيروسيه.

فى هذا البحث تم استعمال عترة فيروس جدرى الحمام المعزوله محليا بقسم بحوث الجدرى من حمام مصاب بالمرض من محافظه القليوبيه عام 2017 وقد تم تقييم خصائص الفيروس المعزول مسبقا بعد تمريرة على خلايا اجنه بيض الدجاج بعد التمريرة السابعه وكانت قوته العياريه $4.5\log_{10}TCID_{50}/ml$

وقد تم استكمال تمرير واستضعاف الفيروس السابق على خلايا الزرع النسيجى من نوع خلايا كلى القرد الافريقى الاخضر(فيرو)لعدد عشرون تمريرة وتم قياس القوة العياريه وكانت تساوى5.5log10TCID50/ml

تم تقييم اللقاح المحضر باجراء اختبارات النقاوة حيث ثبت خلوة من اى ملوثات بكتريه وفطريه اوفيروسيات اخرى .

كذلك تم تقييم امان اللقاح باجراء اختبار الامان بعشرة اضعاف الجرعه الحقليه واختبار التحدى باستخدام باستخدام فيروس جدرى الحمام الضارى على عدد من الطيورواظهرت النتائج عدم ظهور اى اعراض جانبيه بعد الحقن باللقاح.

وقد اظهر اختبار التحدى بالفيروس الضارى قدرة اللقاح على التحدى لفيروس المرض الضارى بنسبه 95% بعد 21يوم من التحصين تم قياس مستوى المناعه على عينات مصل باختبار المصل المتعادل واظهرت النتائج ان مستوى الاجسام المناعيه وصل لاعلى معدل له عند الاسبوع الرابع بعد التحصين بمعدل . 2.8NI

وتخلص هذة الدراسه الى ان لقاح جدرى الحمام النسيجى المحضر محليا يمكن استعماله مستقبلا فى تحصين الحمام بدلا من اللقاح الحالى نظرا لكفاءته وانخفاض تكلفته الاقتصاديه.