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Tissue culture-based preparation and validation of the turkey pox virus vaccine

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ABSTRACT

Turkey Pox Virus (TKPV) is one of avipox virus affecting poultry breeding causing many economic losses due to general skin lesion on the non-feathered area of the body (cutaneous form) and may be fatal in case of diphtheritic form. Turkey breeding in Egypt is getting a lot of attention lately so the disease of turkey get more concern, the most important disease that causes loses is (TKPV). The most effective way to control the disease is vaccination the birds with suitable vaccine protecting against the circulating virus therefor this study for production of TKPV vaccine. TKPV was isolated by inoculation of eleven day old embryonated chicken egg on chorioallantoic membrane (CAM) then the egg adapted TKPV was propagated on chicken embryo fibroblast (CEF) till adaptation at the 15th passage when titer was reaching to log $10^{5.5}$ TCID 50/ ml . The adapted virus was transmitted to Vero cell line to produce the qualified vaccine. All quality control measures approved that the vaccine is ready to be used to control the TKPV disease in Egypt. C4L like gene expression was employed to evaluate TKPV virulence via Quantitative RT-PCR to ensure TKPV attenuation that begin from the 15th passage till complete attenuation at the 20th passage. Once all procedures are finished, the lyophilized live attenuated TKPV vaccine is prepared for use in control the disease in Egypt.

Introduction

Turkey pox virus (TKPV) is one of Avipox viruses which are characterized by the development of separate nodular proliferative skin lesions on the non-feathered area of the body (cutaneous form) and fibrino-necrotic and proliferative lesions in the mucous membrane of the upper respiratory tract, mouth and esophagus (diphtheritic form) (Esposito *et al*., 1991). The economic importance of pox infection in turkey is attributed to retardation of weight, blindness, decreased egg production and impaired fertility (Tripathy and Reed, 1997).

The course of the disease in turkey flocks may be 2-3 weeks and severe outbreaks often last 6, 7 or even 8 weeks with high mortalities reaching 50% of the infected birds (Winterfield *et al*., 1985).

Fowl pox vaccine is used to vaccinate turkey against pox infection by the wing-web method, but the virus may spread and infect the head region causing generalization of the pox disease (Nakhla *et al*., 2010). So, this study aimed to the preparation of specific turkey pox vaccine from the local Egyptian isolate, which is a homologous vaccine producing higher protection percent than the heterologous one and avoid post vaccinal reaction caused by Fowl pox vaccine. Also, Quantitative RT-PCR was used to evaluate Turkey pox virus virulence and complete attenuation of the virus by evaluating the expression of C4L like gene which responsible for virulence in TKPV.

The aim of vaccination is to give the birds a mild attack of pox so that after recovery they will be immune to virulent infection. Protection is effective 2 to 3 weeks after vaccination. Both cell mediated and humoral immunity develops following vaccination or natural exposure and provides protection pigeon pox live virus vaccines are available and routinely used for vaccination of chickens and pigeons (Landolt and Kocan, 1976).

Materials and methods

Propagation and titration of the virus on Embryonated chicken eggs

Specific pathogen-free (SPF) eggs were obtained from the SPF Production Farm, Koum Osheim, El-Fayoum, Egypt. The eggs were kept in the incubator at 37°C with a humidity of 40-60%. They were used for titration of egg adapted vaccines, according to CFR (2012).

Propagation and titration of the selected TKPV isolate on African Green Monkey Kidney Cells (Vero)

TKPV was isolated on ECE and propagated and adapted on the ECE using Chorioallantoic membrane route (CAM) for inoculation (Haydar *et al*., 2017). ECG adapted TKPV was inoculated into chicken embryo fibroblast (CEF) obtained from Veterinary Serum and Vaccine Research Institute (VSVRI) used as primary cells for preparation of the virus to be adapted on Vero cell line according to Kafafy *et al*. (2018). Firstly the virus collected after five days without appearance of cytopathic effect (CPE) and it is called (blind passage). Then the CPE appeared after five days and after ten passages the CPE appear after 4-5 days. TKPV was transmitted to grow on Vero cell line according to OIE (2018) and examined daily for cytopathic effect (CPE). Thq2e titre was calculated according to Reed and Muench (1938).

Study of the growth and cytopathic changes of three virus samples

Multiplication, cytopathic changes, harvesting time of cell free and cell associated virus were studied and performed according to El-Bagoury *et al*. (2021).

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Growth Kinetics (GK) of TKPV isolate in Vero cells

Using different multiplicity of infection (MOI) of TKPV isolate (virus dilutions from 10^{-1} to 10^{-6}). This technique was performed according to Trabelsi *et al*. (2012).

Vaccine preparation

Commercial, live-attenuated vaccines against TKPV aren't available, this study used live attenuated FPVV according to Bailey et. al. (2002). The vaccine preparation was done in the form of live attenuated tissue culture vaccine (Radwan and Mikhael, 2020).

Experimental Design

Fifty susceptible turkey birds of 45 days old were used in this study for vaccine evaluation. The birds were housed in separate negative pressure filtered isolators and provided with autoclaved commercial water and feed. These turkey birds were divided as follows:
Group 1 (G1): Thirty birds were used to test the vaccine for detection of the potency and duration of immunity, plus ten as contact control.
Group 2 (G2): Ten birds were kept unvaccinated as controls.

Quality control of the TKPV vaccines

Sterility

It was carried out according to OIE (2018), 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 by ECE inoculation and PCR.

Safety

A quantity of the vaccine virus equivalent was administered as ten doses to each of ten susceptible birds for each group via wing web. The birds were observed daily for 21 days with recording any abnormalities (take, pock or death).

Potency and duration of immunity

Infectivity of the live TKPV vaccine by titration in Vero cell line

Adapted tissue culture vaccines titrated on Vero cell line as the method described (Nakhla *et al*., 2005) and $TCID_{50}$ was estimated according to the method described by Reed and Muench (1938).

Efficacy

Twenty susceptible birds were vaccinated by injection of the recommended vaccinal dose of each tested vaccines using wing web route according to Branson and Kip (1995), in addition to five birds were left as non-vaccinated contact control birds. The birds were observed daily for ten days after vaccination and record the post-vaccinal reaction formation in vaccinated birds.

The Challenge test was applied by the inoculation of the virulent Turkey pox virus by the wing web route in vaccinated and susceptible control birds at 21 days post-vaccination. All birds were subjected to a daily observation of gross lesions for ten days, and the deaths and the numbers of surviving birds that show clinical signs of disease were recorded.

C4L like gene expression was employed to evaluate TKPV virulence via Quantitative RT-PCR

Gene expression was employed to evaluate Turkey poxvirus virulence via Quantitative RT-PCR. The primer was designed by picking up on GenBank according to Ember *et al*. (2012).

Procedures of Quantitative RT-PCR

Purification of total RNA

Total RNA purification from blood samples according to manufacturer protocol.

C-DNA synthesis

1 µg of total RNA was reverse-transcribed into single-stranded complementary DNA by using QuantiTects Reverse Transcription Kit (Qiagen, USA) using a random primer hexamer in a two-step RT-PCR reaction.

Q-PCR reaction preparation

Real-time PCR was performed to evaluate mRNAs of Interleukin- IL-1β, Interleukin-6 and Insulin-like growth factors 1 (IGF-1) genes using by Rotor-Gene Q (Qiagen, USA) with β-actin as house-keeping gene. C-DNA amplicons were amplified via Maximas SYBR Green/Fluorescein qPCR Master Mix through specific primers which prepared according to manufacturer protocol; Forward primer 1 TAGCGGAAAAAGGAGGCGAA, Reverse primer 1 CTGTGCCACGTCCATAAATCA

Thermo Scientific Maxima SYBR Green/Fluoresce in qPCR Master Mix (2X) is a ready-to-use solution optimized for quantitative real-time PCR and two-step real-time RT-PCR. It contains SYBR® Green I dye and is supplemented with fluorescein passive reference dye. Only template and primers need to be added. The SYBR Green I intercalating dye allows for DNA detection and analysis without using sequence-specific probes. dUTP is included in the mix for optional carryover contamination control using uracil-DNA glycosylase (UDG).

Thermal cycling conditions was designed according to Lee *et al*. (2019) as follow: 10 min at 95°C, followed by 45 cycles of 95°C for 10s, 60°C for 15s and 72°C for 15s. The conditions of the melting curve analysis were 72–95°C, increased by 1°C per second s -1. 1 µg of total RNA was reverse-transcribed into single-stranded complementary DNA by using QuantiTects Reverse Transcription Kit (Qiagen, USA) using a random primer hexamer in a two-step RT-PCR reaction in which any genomic DNA (gDNA) contamination was eliminated using gDNA without buffer.

Rotor-Gene Q collected data automatically and analyzed the value of threshold Cycle (Ct) which normalized to an average Ct value of the house-keeping genes (ΔCt) and the relative expression of each representative was calculated as $2^{-\Delta Ct}$.

Relative expression

Using $2^{-\Delta\Delta ct}$, relative expression of target gene were estimated as follow:

Control group was applied as calibrator. On the other hand, other dietaries groups were represented as tested groups for both of target and reference genes.

Threshold cycler numbers (Ct) of target gene were normalized to reference genes, for tested and control groups according to following equations:

Δ Ct (tested) = Ct (target in the tested groups) – Ct (ref. in test group)
Δ Ct (calibrator) = Ct (target in control) – Ct (ref. in control)
Δ Ct of tested genes were normalized to the Δ Ct of the calibrator as follow: ΔΔ Ct = Δ Ct (test) - Δ Ct (calibrator)
Relative gene expression fold change was estimated as follow:
Fold changes = (2-ΔΔct)

Results

Propagation and titration of the selected TKPV isolates on ECE and African Green Monkey Kidney Cells (Vero)

The inoculated ECE showed small diffused pock lesions in CAM having diameter of about 2-3 mm were observed (Fig. 1). The inoculated chicken embryo fibroblast (CEF) as primary cells at the beginning the passaging occurred by blind passages for 4-5 passages then show cytopathic effect (CPE) as granulation of cells followed by rounding and aggregation forming giant cells. The control cells were rounded in monolayer in intact sheet as shown in Fig. 2.

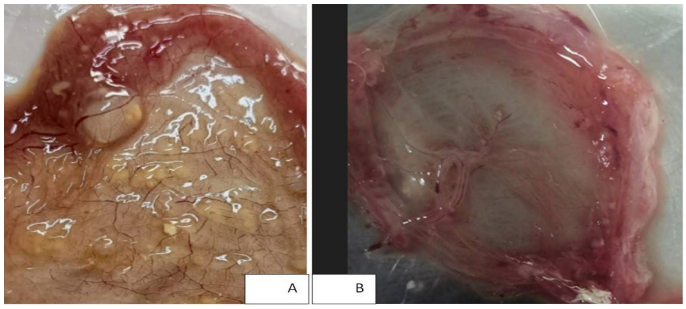

Fig. 1. A: Small diffused pock lesions in CAM having diameter of about 2-3 mm. B: Normal CAM without any changes.

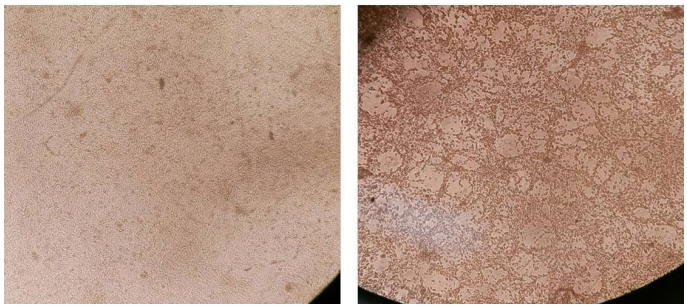

Fig. 2. A: normal CFP rounded cells in confluent monolayer sheet. B: CPE appear as aggregation of the cells with free area forming giant cells.

Inoculated Vero cell show granulation of cells followed by cell rounding and aggregated separately; this occurred after 3 days post inoculation (DPI) and then completely detached in some samples after 5 days. The control Vero cell culture spindle shape cells in monolayer confluent sheet. Results are shown in Fig. 3.

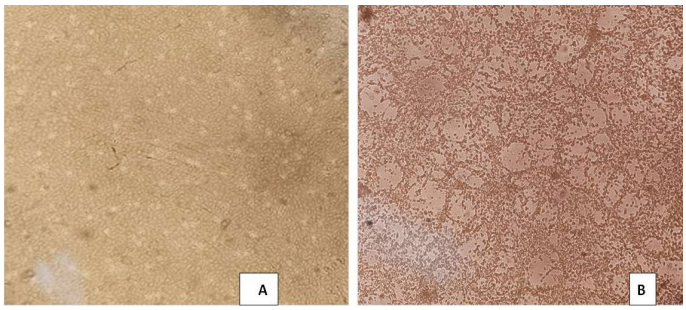

Fig. 3. A. Normal Vero cell culture spindle shape cells in monolayer confluent sheet. B. Vero cell show granulation of cells followed by cell rounding and aggregated separately.

Passages and titres of virus isolate in Vero cells

The titres of the virus increase by passaging on Vero cell line, the result showed in Table 1.

Table 1. The effect of passaging on TKPV titre.

Virus Passages	Virus Titre (Log_{10} $TCID_{50}$/ml)
3rd	3.2
6th	3.6
8th	4.1
10th	4.3
12th	4.9
15th	5.2

TCID50/ml: Tissue Culture Infective Dose 50/ ml.

Growth curve of cell free and cell associated viruses of a TKPV isolate (CFV & CAV)

Results of infectivity titration of CFV & CAV at various hours' intervals PI (2, 12, 24, 36, 48, 72, 96 and 120 h PI) are presented in Table 2.

Which indicated that the titre of CFV decreased from $log10^{5.2}$ $TCID_{50}$/ml to $log10^{4.9}$; $log10^{3.5}$ and $log10^{2.5}$ $TCID_{50}$/ml at 2, 12 and 24 h PI respectively. The titres of CAV were $log10^{3.5}$, $log10^{3.0}$ and $log10^{3.5}$ $TCID_{50}$/ml at the same hours. The significant infectivity virus titres of CAV were $log10^{5.2}$ $TCID_{50}$/ml at 96 h PI. At 120 h PI, the CFV titres increased and reached $log10^{5.2}$ $TCID_{50}$/ml while CAV were $log10^{4.5}$ $TCID_{50}$/ml.

Table 2. Results of infectivity titration of CFV &CAV at various hours' intervals Post inoculation (PI).

Hours post Inoculation (HPI)	Titres of CFV & CAV (log_{10} $TCID_{50}$/ml)	
	CFV	CAV
0	5.2*	5.2*
2	4.9	3.5
12	3.5	3
24	2.5	3.5
36	2.5	3.5
48	2.5	4.1
72	4	4.5
96	4.9	5.2***
120	5.2**	4.5

CFV: Cell Free Virus; CAV: Cell Associated Virus; *: Titre of the original virus (whole culture virus); **: maximum titre of CFV; ***: maximum titre of CAV.

Efficacy and Quality control of the TKPV vaccine

Efficacy

Takes were detected at the site of vaccination 4th DPV and increased to the maximum at the 7th DPV to reach 90% for TKPV vaccine (27 birds from 30 birds show Postvaccinal reaction) as shown in Fig 4.

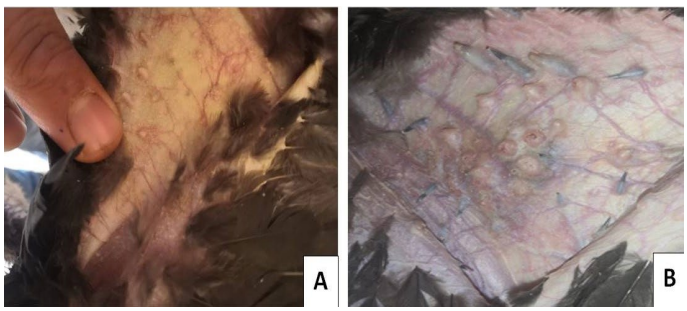

Fig. 4. A: Takes appearance at the site of Vaccination 4 days post vaccination. B: Takes appearance at the site of Vaccination at 7 days post vaccination.

Sterility test

Vaccine was proved to be free from any bacterial, fungal, or extrane-

Table 3. Effect of passage on gene expression.

Samples	Gene tested experiment (TE)	Gene tested control (TC)	Housekeeping Gene experiment (HE)	Housekeeping Gene Control (HC)	ΔCt values for the Experiment (ΔCTE)	ΔCt values for the control (ΔCTC)	Delta Ct Value (ΔΔCt)	$2^{-\Delta\Delta Ct}$ (Expression fold change) -fold
Original	31.9	22	30.7	20	1.2	2	-0.8	1.7
5th passage	31.5	22	30.1	20	1.4	2	-0.6	1.5
10th passage	31.6	22	30	20	1.6	2	-0.4	1.3
15th passage	32.3	22	30.2	20	2.1	2	0.1	0.99
20th passage	32.7	22	30.1	20	2.6	2	0.6	0.65

ous virus's contamination.

C4L like gene expression via Quantitative RT-PCR

C4L like gene is responsible for virulence of pox viruses

The result of gene expression is shown in Table 3. The result ensures attenuation of the gene beginning from the 15th passage till complete attenuation at the 20th passage.

Discussion

Vaccination is the most effective method to control virus diseases in turkey. TKPV is the most dangerous disease in turkey especially after the spread of turkey breeding. Fowl pox vaccine is used to vaccinate turkey against pox infection by the wing-web method, but the virus may spread and infect the head region causing generalization of the pox disease (Nakhla *et al*., 2010). The first vaccine against avian pox virus adapted on tissue culture (Vero cell line) was pigeon pox virus vaccine, production of a safe and potent PPVV from local PPV strain of less cost than that prepared on specific pathogenic free embroynated chicken egg (SPF-ECE) (Kafafy *et al*., 2018)

Isolation of the TKPV on ECE and the pock lesion appear on CAM after 4-5 days of inoculation as shown in Fig. 1. A chicken embryo cell culture system derived from a specific pathogen free embryo was shown to be highly susceptible to the TKPV, where CPE appear as granulation of cells followed by rounding and aggregation forming giant cells. The control cells were rounded in monolayer in intact sheet as shown in Fig. 2. The most suitable tissue culture for the production of TKPV is Vero cell line to produce the qualified vaccine. Inoculated Vero cell show granulation of cells followed by cell rounding and aggregated separately; this occurred after 3 days post inoculation (DPI) and then completely detached in some samples after 5 days. The control Vero cell culture spindle shape cells in monolayer confluent sheet. Results are shown in Fig .3. The tissue culture adapted vaccines were titrated on Vero cells showing a titer of $10^{5.2}$ $TCID_{50}$/dose.

The titres of the virus increase by passaging on Vero cell line, the result showed in Table. 1, reaching $10^{5.2}$. The titre of CFV decreased from $log10^{5.2}$ $TCID_{50}$/ml to $log10^{4.9}$; $log10^{3.5}$ and $log10^{2.5}$ $TCID_{50}$/ml at 2, 12 and 24 h PI respectively. The titres of CAV were $log10^{3.5}$, $log10^{3.0}$ and $log10^{3.5}$ $TCID_{50}$/ml at the same hours. The significant infectivity virus titres of CAV were $log10^{5.2}$ $TCID_{50}$/ml at 96 h PI. At 120 h PI, the CFV titres increased and reached $log10^{5.2}$ $TCID_{50}$/ml while CAV were $log10^{4.5}$ $TCID_{50}$/ml

Evaluations of the vaccines proved that they were sterile, free from any bacterial, fungal and mycoplasma contaminants and free from extraneous viruses, the vaccine is in agreement with the recommendation of OIE (2018). The birds inoculated with the vaccine showed slight thickening of skin and scales at the site of inoculation (Takes formation) in 90% of inoculated birds), no local or general symptoms appeared. Takes were detected at the site of vaccination on the 4th DPV and increased to the maximum on the 7th DPV to reach 90% as shown in Fig. 4. These results agreed with Radwan and Mikhael (2020).

The role and contribution of the C4L-like gene to the pathogenicity of viruses have been evaluated. Bioinformatics analysis showed that C4L is conserved in six orthopox virus species and shares 43% amino acid identity with VACV protein C16, a known virulence factor (Ember *et al*., 2012). C4L like gene expression was employed to evaluate TKPV virulence via Quantitative RT-PCR. The result ensures attenuation of the gene beginning from the 15th passage till complete attenuation at the 20th passage.

Conclusion

As a consequence of the study's acquired data, it can be concluded that vaccination against TKPV using a local isolate protect effectively against the illness and aids in the control of the virus in Egypt as all results lead to effective immune response.

Conflict of interest

The authors declare that they have no conflict of interest.

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