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Comparative Evaluation on The Efficacy of Embryonated Chicken Egg Adapted and Tissue Culture Pigeon Pox Vaccines Against The Local Virulent Strain

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ABSTRACT

A total of 150 pigeons of 45 days old was used and divided into three groups; the first one was vaccinated with tissue culture adapted pigeon pox vaccine (TCAPPV), and the second was vaccinated with egg adapted pigeon pox vaccine (EAPPV) and the third as a non-vaccinated control group. Birds were observed for ten days post-vaccination (DPV) for the presence of takes. Cellular immunity was detected by lymphocyte proliferation assay on the whole blood for 21 DPV, and serum samples were collected weekly. The level of induced antibodies was detected by the neutralization test for six months post-vaccination. Twenty pigeons of each group were challenged by virulent pp virus at 28th DPV Takes were recognized at the site of vaccination at 4thDPV and increased to the maximum at7th DPV to reach 90% for TCAPPV and 98% for EAPPV. The peak of the cellular immunity by lymphocyte proliferation assay was at the 12thDPV when TCAPPV recorded 1.534 and EAPPV 2.037. Protection was 90% for TCAPPV and 100% for EAPPV. The peak of neutralizing index (NI) at 35thD.P.V for both vaccinated groups; It was 2.75 for TCAPPV and 3.25 for EAPPV. Both vaccines are still potent to the end of examination at the 6thmonth when NI was 1.5 for TCAPPV and 2.0 for EAPPV. This result shows that both eggs adapted PP and tissue culture PP vaccines are efficient in the protection of pigeons in Egypt despite the egg adapted vaccine is more preferable.

Keywords: cellular immunity, EAPPV, egg adapted, Pigeon pox, TCAPPV.

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INTRODUCTION

Embryonated chicken eggs are still and considered as one of the primary substrates for the production of different vaccines. They can support the replication of a wide range of viruses. This includes attenuated vaccines, i.e., defective viruses that have impaired potential to replicate in mammalian cells and can be used as a vaccine. Embryonated chicken eggs used for vaccine production must be certified to be free from a defined set of viral and bacterial contamination (Specific Pathogen Free–SPF). The VERO cell line (originated from African green monkey kidney cell) is allowed as a cell substrate for vaccine manufacturing based on the proven safety profile and lack of transformed phenotype for a defined number of passages.

The cell line has been used extensively for vaccine manufacture. Also, with adaptation to a primate-derived cell substrate, receptor binding sites on the virus are likely to change, resulting in a modified antigen pattern and, thus, a general effect on immunogenicity. This genetic adaptation may reverse attenuation for strains that have been developed via passaging in avian cells or create new strains replicating more efficiently in the cell compared to their wild type isolates. Such viruses may also obtain a higher pathogenic potential. For the above reasons, vaccine manufactures are reluctant (averse-opposed) to switch to mammalian cell lines, and a need for immortal avian cell lines has developed **(PCT, 2005).** However, this finding does not apply to all viruses relevant to vaccine development, in particular avian viruses. While some of these viruses replicate well on mammalian cell lines, virus growth is often weak. For other viruses, replication is more reduced and limited

to particular, specially adapted strain. **Soad (1986),** reported that cell line tissues weren't suitable for the growth of the avipox virus.

Pigeon pox disease is caused by pigeon pox virus (PPV) that is classified within the Poxviridae family subfamilies Chordopoxvirinae genus Avipoxvirus (A.P.V.) (**Andraw, 2012)**. It is endemic in Egypt, and it is one of the more critical poultry diseases that causes considerable economic losses **(Abdallah and Hassanin, 2013).** Pigeon pox is a serious viral disease in pigeon causing mortalities especially in young pigeon characterized by the development of discrete proliferative nodular skin lesions (cutaneous form) around the mouth or eyes and/or ulcerations in the oral cavity and fibrino-necrotic lesion in the mucous membrane of the upper respiratory tract (diphtheritic form) **(Sumaya, 2005)** which making an affected bird cannot drink or eat causing dehydration or starvation ending by death **(Tripathy and Reed ,1997 ; Hemanth *et al*., 2014).** Pigeon pox virus produced mild infection in chicken and turkey but was more pathogenic for pigeons **(Bossinger *et al.*, 1982)**. For these reasons, PP Vaccine is used not only for the vaccination of pigeons but also against pox infection in chickens and turkeys **(Gottstein *et al.*, 2004 ; Wang *et al.*, 2006)**.

In Egypt, pigeon pox vaccine was prepared by the 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).** The control against virus infection is realized by vaccination using attenuated vaccine either propagated on SPF embryonated chicken egg (SPF-ECE) or tissue culture **(Dasgupta *et al.*, 2007)**. In Egypt, **Namaa (1998)** prepared the fowlpox virus (FPV) vaccine from the whole CAM of the infected ECE, while **Soad (1986)** developed FPVvaccine on chicken embryo rough cells (CER) as a tissue culture adapted fowl pox vaccine.

Because SPF-ECEs are expensive, hardly handled during work, the need for economic, sensitive, and easily maintained cell cultures use for massive production of avipox vaccine was necessary **(Dasgupta *et al.*, 2007)**. **Joshi and Namital (2011)** agreed with **Sainova *et al.*, (2005) and Weli *et al.*, (2005)**, who succeeded in propagating APV in mammalian cell cultures and challenge the hypothesis that A.P.V. cannot undergo a full replication cycle in mammalian cells.

The present study aimed to compare the efficacy of embryonated chicken egg adapted and tissue culture adapted pigeon pox vaccines against the local virulent strain; and also in the evaluation of the quality of these vaccines in the protection of pigeon against pox infection.

MATERIALS AND METHODS

Material

1.Vaccines

1.1. Pigeon Pox Virus egg adapted vaccine

Commercial pigeon pox lives attenuated egg adapted vaccine batches produced by Veterinary Serum and Vaccine Research Institute (VSVRI) Abbasia Cairo was used.

1.2. Pigeon Pox Virus tissue culture adapted vaccine

Commercial pigeon pox lives tissue culture adapted attenuated vaccine batches produced by Veterinary Serum and Vaccine Research Institute (VSVRI) Abbasia, Cairo was used.

2. Viruses

2.1. Pigeon Pox Virus (PPV)

Pigeon pox virus was supplied by Reference Strain Bank (RSB), Central Lab for Evaluation of Veterinary Biologic (CLEVB), Abbasia, Cairo, Egypt.

2.2. Virulent PPV

A local isolate of PPV virulent Pigeon pox virus was supplied by Reference Strain Bank (RSB), Central Lab for Evaluation of Veterinary Biologic (CLEVB), Abbasia Cairo. It had a titer of 10^6EID_{50}/ml and used for challenge immunity of experimentally vaccinated pigeons.

3. 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).**

4. Tissue cultures for titration

4.1. **Chicken Embryo Fibroblast cell culture (CEF)** Obtained from Central Lab for Evaluation of Veterinary Biologic CLEVB **according to Olfat (2006)**.

4.2. Vero cell culture

African green monkey kidney (Vero) cells from the central laboratory for evaluation of veterinary biologics (CLEVB) and maintained, according to **Soad (1986).**

5. Earle's Minimum Essential Medium (MEM)

It was obtained from Sigma Chemical Company, USA, and used as a growth medium containing 10% Newborn calf serum or as a maintenance medium containing 2% newborn calf serum.

6. Susceptible pigeon

One hundred and thirty of susceptible squabs 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 pigeons were divided as follows:

G1: Fifty squabs were used to test the cell culture vaccine for detection of the potency and duration of immunity, plus ten as contact control.

G2: Fifty squabs were used to test the egg adapted vaccine for detection of the potency and duration of immunity, plus ten as contact control.

G3: Ten squabs were kept unvaccinated as controls.

7. Samples

7.1. Whole blood

Samples were collected from squabs at 0,1,3,5,7,10,12,16,19 and 21 DPV for estimation of cellular immunity.

7.2. Serum samples

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

8. Kits

XTT Cell Viability Assay Kit: The kit was used in the lymphocyte proliferation assay.

Methods

1. Evaluation of pigeon pox vaccines (Quality control)

1.1. Sterility

It was carried out according to **OIE (2018)**, where random samples of the lyophilized vaccine were inoculated separately into tubes of nutrient and blood agar, Sabouraud agar and thioglycolate medium and mycoplasma medium. Also, the lyophilized vaccine was examined for any extraneous viruses by ECE inoculation and PCR.

1.2. Safety

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

1.3. Potency and duration of immunity

1.3.1. Titration of Pigeon pox virus vaccines

Infectivity of the live PP virus vaccine by titration in embryonated chicken eggs:

Embryonated chicken egg adapted vaccine was titrated on the embryonated chicken egg as the method described **CFR (2012)** and EID_{50} was estimated according to the method described by **Reed and Muench (1938).**

-Infectivity of the live PP virus vaccine by titration in Vero cell line

Adapted tissue culture vaccines titrated on Vero cell line as the method described by **Mishra and Mallick (1994)** and **Olfat *et al.*, (2005)** and $TCID_{50}$ was estimated according to the method described by **Reed and Muench (1938).**

-Infectivity of the live PP virus vaccine by titration in CEF cell line

Adapted tissue culture vaccines titrated on CEF cell line as the method described by **Olfat (2006)** and $TCID_{50}$ was estimated according to the method described by **Reed and Muench (1938).**

1.3.2. Efficacy

Fifty susceptible squabs were vaccinated by injection of the recommended vaccinal dose of each tested vaccines using feather follicle route in the thigh according to **Branson and Kip (1995),** in addition to 10 pigeons were left as non-vaccinated contact control pigeons. 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 pigeon pox virus by the feather follicle route in vaccinated and susceptible control pigeons 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.

1.4. Evaluation of the cell-mediated response: Assay of lymphocyte proliferation

Whole blood was collected 1, 3, 5, 7, 10, 12, 16, 19 and 21 days post-vaccination for estimation of the cellular immunity. It was applied according to the method adopted by **Charles *et al.* (1978) and Lucy (1984).**

1.5. Serological assay using Serum Neutralization Test (SNT)

Serum neutralization test (SNT) was done according to the method described by (**Carol and Marinescu 1971)**, for the detection of antibody levels after vaccination and the challenge of different squabs group and the results were calculated according to the formula of **Reed and Muench (1938).**

2.Quality control of Pigeon pox vaccines

2.1. Sterility test

Pigeon pox vaccine (PPV) vaccines were proved to be free from any bacterial, fungal, or extraneous viruses contamination.

2.2. Safety test

Inoculation of PPV vaccines in susceptible squabs with ten filed doses did not show any notable clinical signs of PP or unfavorable reactions or deaths; so it was proved that the tested vaccines were safe to be used in pigeons.

2.3. Titration of Pigeon pox virus vaccines

a. Infectivity of the egg adapted live PP virus vaccine by titration in embryonated chicken eggs

The vaccine titer was 3.6 $\log_{10}$ EID_{50}/dose.

b. Infectivity of the tissue culture attenuated PP virus vaccine by titration in Vero and CEF cell line

The vaccine titer was 3.0 $\log_{10}$ $TCID_{50}$/dose on Vero cell, and it was 3.3 $\log_{10}$ $TCID_{50}$/dose on CEF.

2.4. Potency and duration of immunity

2.4.1. Efficacy

Takes were detected at the site of vaccination 4th DPV and increased to the maximum at 7th DPV to reach 90% for tissue culture adapted vaccine and 98% for egg adapted vaccine as shown in table (1).

2.4.2. Evaluation of the cell-mediated response

a- Assay of lymphocyte blastogenesis:

Lymphocyte proliferation test was carried out on the whole blood collected from vaccinated pigeons. The results were expressed as optical density (OD). They showed that the peak of the cellular immunity by lymphocyte proliferation assay was at 12 DPV when the tissue culture adapted vaccine recorded 1.534 and the egg adapted vaccines recorded 2.037as shown in table (2).

b- Challenge test

Vaccination of the susceptible pigeon with under-examined vaccines by feather follicle method and challenged with virulent PPV revealed that the protection percent was 90% for tissue culture adapted vaccine and 100% for egg adapted vaccine as shown in table (3).

2.4.3. Serum Neutralization test

A serum neutralization test was carried out on the serum samples collected from vaccinated pigeons. The results were expressed as neutralizing index, and the peak of NI was 35 DPV for both treated groups; it was recorded 2.75 for tissue culture adapted vaccine and 3.25 for the egg adapted vaccine. As well as both vaccines still potent to the end of examination at six months when the neutralizing index (NI) was 1.5 for tissue culture adapted vaccine and 2.0 for egg adapted vaccine as shown in table (4).

The neutralizing index (NI) of birds vaccinated with any of the two vaccines appeared from the second-week post-vaccination.

RESULTS

Table 1: Post vaccinal reaction (takes) of pigeons vaccinated with TCPP, EAPP vaccines and control non-vaccinated

Squabs Group	No. of tested birds	No. of Tested birds		Takes %
		+ ve	- ve	
G1	50	45	5	90
G2	50	49	1	98
G3	30	0	30	0

G1= Cell culture adapted vaccine vaccinated group
G2= Egg adapted vaccine treated group.
G3= Control group contact and isolated control.
NB: The vaccine is potent if at least 90% of the vaccinated birds show vaccine takes.

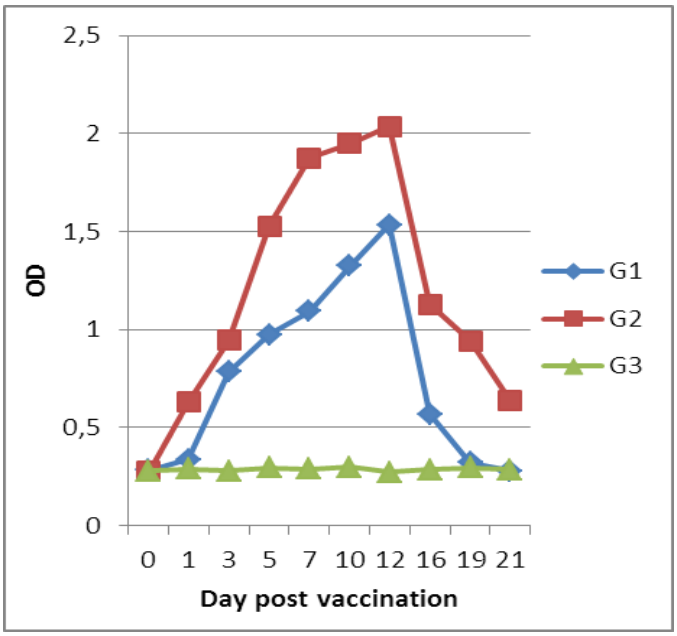

FIG 1: Cell-mediated immune response of pigeons vaccinated with TCPP, EAPP vaccines and control non vaccinated (expressed as optical density)

Table 2: Cell-mediated immune response of pigeons vaccinated with TCPP, EAPP vaccines and control non vaccinated (expressed as optical density).

Vaccinated Groups	Days post-vaccination									
	0	1	3	5	7	10	12	16	19	21
G1	0.281	0.335	0.783	0.972	1.091	1.325	1.534	0.569	0.321	0.278
G2	0.276	0.629	0.946	1.528	1.874	1.950	2.037	1.128	0.942	0.637
G3	0.277	0.288	0.279	0.294	0.287	0.297	0.272	0.285	0.293	0.285

G1= Cell culture adapted vaccine vaccinated group. G2= Egg adapted vaccine treated group.
G3= Control group contact and isolated control.

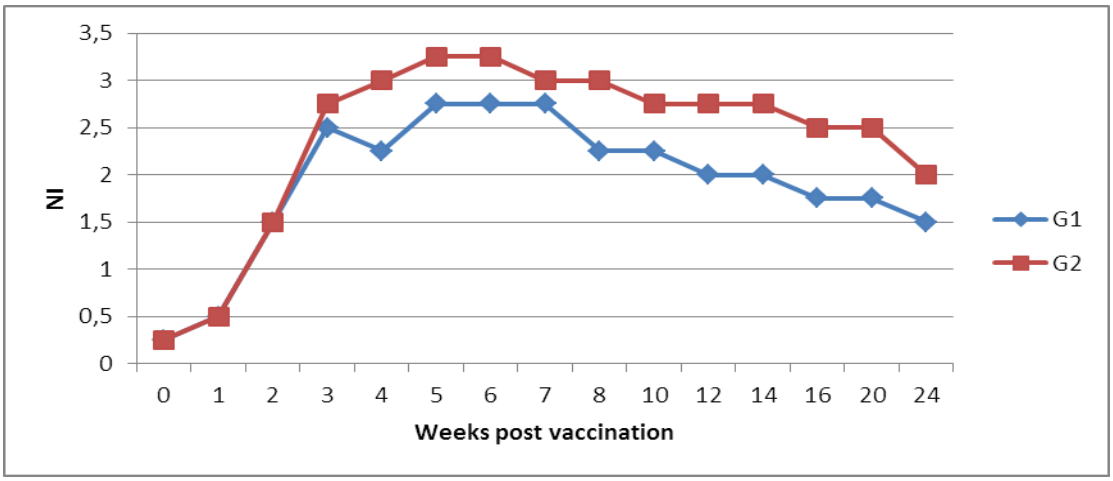

Fig 2: Results of the Neutralizing Index of sera collected from vaccinated pigeons

Table 3: Post challenge reaction (pock lesion) of pigeons vaccinated with TCPP and EAPP vaccines and controlled non-vaccinated

Squabs Group	No. of tested birds	Pock lesion		Protection %
		+ve	- ve	
G1	20	2	18	90
G2	20	0	20	100
G3	20	20	0	0

G1= Cell culture adapted vaccine vaccinated group.
G2= Egg adapted vaccine treated group.
G3= control group (contact and isolated).
+ve = Show clinical signs of disease (cutaneous pock lesion in the un-feathered area of the skin and or diphtheritic lesions in the mucous membrane of oro-pharyngeal mucosa).

Table 4: Results of the Neutralizing Index of sera collected from vaccinated pigeons

Time post-vaccination	G1	G2
0 day	0.25	0.25
7 Days	0.50	0.50
14 Days	1.50	1.50
21 Days	2.50	2.75
28 Days	2.25	3.0
35 Days	2.75	3.25
42 Days	2.75	3.25
49 Days	2.75	3.00
8 Weeks	2.25	3.00
10 Weeks	2.25	2.75
12 Weeks	2.00	2.75
14 Weeks	2.00	2.75
4 Months	1.75	2.50
5 Months	1.75	2.50
6 Months	1.50	2.00

DISCUSSION

Vaccination plays a clue in the modern poultry industry. Without it, the productivity would not have progressed so successfully and as rapidly as it has over the last few decades **(Frank *et al*. 2001a)**. As vaccination is the only means for controlling pigeon pox disease **(Tripathy and Reed, 2001 and 2008)**. Pigeon pox was controlled in Egypt by egg-adapted pigeon pox vaccine (Hungarian strain**) (Helmy *et al*., 1967),** and also the tissue culture adapted vaccine was produced **(Kafafy *et al*.., 2018).**

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. Reported that PPV was adapted to the cell system, and the cell culture vaccine (CEF) has higher biological properties than a conventional vaccine prepared on the chlorioallantoic membrane of embryonated **eggs (Ael-Zein et al., 1974 and Olfat et al., 2005).**

In this study, the tested egg adapted vaccine was titrated in the ECE and showed a titer of $10^{3.6}$ EID_{50}/dose awhile, the tissue culture adapted vaccines were titrated on Vero cells showing a titer of $10^{3.0}$ $TCID_{50}$/dose. Evaluations of the vaccines proved that they were 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 ten field dose showing no adverse effects attributable to the vaccine in agreement with the recommendation of **(OIE, 2018)**.

The inoculated pigeons with the pigeons vaccinated with the tissue culture adapted vaccines showed slight thickening of skin and scales at the site of inoculation in 90% of inoculated birds, no local or general symptoms appeared, while in the pigeons vaccinated with the embryonated egg adapted vaccines showed thickening of the skin and takes at the site of inoculation in 98% of inoculated birds, no local or general symptoms appeared. These results agreed with **Buller and Palumbo (1991)** and **Tripathy and Reed (1997).**

Evaluation of the cell-mediated immune response applied by using a Lymphocyte proliferation test carried out on the whole blood collected from vaccinated pigeons. The results were expressed as optical density (OD) in the table (2) and Fig (1), which showed that their maximum was on 12-day post-vaccination where the tissue culture adapted vaccines was 1.534 and 2.034 for egg adapted vaccines.

Challenge is considered the master test to measure the immunizing capacity of the vaccine against pox infection, so the tested vaccines were tested by challenging the immunity of the vaccinated pigeons with the virulent pigeon pox virus. The results screened in Table (3) indicated that vaccinated pigeons were able to overcome the virus infection with protection percent reached 90% for tissue culture adapted vaccines and 100% for egg adapted vaccines. These results agreed with those of **Frank *et al*..(2001b)** and as mentioned by **Michael (1981) and Soad *et al*. (2007)**.

Humoral immune response or the antibody response to vaccination detected that the immune status of vaccinated pigeons was estimated by serum neutralization test (SNT). In Table (4) and Fig (2), the observed results showed that the neutralizing antibodies reached their maximum neutralizing index (NI) 2.75 for tissue culture adapted vaccine and 3.25 for egg adapted vaccine35 days post-vaccination and still protective till the end of the studies after six months when the tissue culture adapted vaccines was 1.5 and egg adapted vaccines was 2.00.It agreed with **Michael *et al.* (1986)**.

CONCLUSION

In conclusion, from the obtained results, the present study proved that PPVvaccine on tissue culture is satisfactory, but the egg adapted vaccine still more effective. The egg adapted vaccine still potent and preferable in the protection against avipox disease.

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