RESEARCH ARTICLE

Sokoto Journal of Veterinary Sciences

(P-ISSN 1595-093X: E-ISSN 2315-6201)

http://dx.doi.org/10.4314/sokjvs.v23i4.4

Okafor *et al.*/*Sokoto Journal of Veterinary Sciences*, **23**(4): 243 - 255.

Immunogenicity and safety of chicken embryo fibroblast-adapted fowlpox vaccine

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Publication History:
Received: 12-04-2025
Revised: 30-10-2025
Accepted: 03-11-2025

Abstract

The use of the chorioallantoic membrane (CAM) route for the propagation of fowlpox virus (FPV) is the method applied in the production of potent and effective fowlpox vaccine at the National Veterinary Research Institute (NVRI), Vom. However, production insufficiency has led to an inadequate supply of vaccine to meet farmers' demands. This has left a large number of susceptible and unvaccinated flocks with outbreaks of the disease being reported in Nigeria. This study adapted FPV to Chicken embryo fibroblast (CEF) cell culture. It assessed the immunogenicity and safety of the resulting vaccine in comparison to a locally produced CAM-based FPV vaccine. The FPV was propagated on CAM and CEF of 9–12-day-old developing chicken embryos. The vaccine harvests were subjected to quality checks, titrated, and used to vaccinate experimental birds via the wing web to monitor for "takes," seroconversion, and safety. The FPV was successfully adapted to CEF as demonstrated by the attainment of 80–90% cytopathic effect within 71–115 hours in the different passages. Both the CEF and CAM-adapted vaccine harvests were highly replicative, producing titres of above $10^{6.0}$/ml $TCID_{50}$, but higher titres of $10^{8.25}$/ml $TCID_{50}$ were recorded in the CEF-adapted vaccines. Experimentally vaccinated birds showed 100% "takes" within 3–4 days post-vaccination, with no adverse effects recorded. This research reported successful development and adaptation of FPV to CEF that was safe and immunogenic, with the potential for its use in the production of a safe and immunogenic vaccine for fowlpox prevention and control in poultry, which may lead to self-sufficiency in fowlpox vaccine production.

Keywords: Cell culture adaptation, Chicken embryo fibroblasts, Fowlpox virus vaccine, Immunogenicity, Safety

Introduction

Fowlpox is recognised as a widespread poultry disease caused by a DNA virus belonging to the genus Avipoxvirus in the family *Poxviridae*. Avipoxviruses are usually species-specific, with different variants affecting various hosts. For example, the fowlpox virus (FPV) mainly infects chickens and, to a lesser extent, turkeys. Other avipoxviruses, such as duckpox virus, turkeypox virus, and pigeonpox virus, infect ducks, turkeys, and pigeons, respectively (Tripathy & Reed, 2008). The development of proliferative lesions and scabs on the skin, along with diphtheritic lesions in the upper respiratory and digestive tracts, are characteristic signs of this progressively spreading disease (Tripathy & Reed, 2020; WOAH, 2023). Although these viruses are closely related and can cause similar clinical signs, they are typically limited to their respective avian hosts due to species-specific barriers. Despite this host specificity, natural and experimental infections have been reported in a wide range of domestic and wild birds, including ducks, geese, pheasants, quails, canaries, pigeons, and hawks (Bwala *et al.,* 2015; Giotis & Skinner, 2018; Giotis & Skinner, 2021). The incidence and severity of avipoxvirus infections vary depending on climatic conditions, husbandry practices, hygiene, and the extent of vaccination implementation (WOAH, 2023). Fowlpox is recognised as a disease of economic importance by the World Organisation for Animal Health (WOAH), and it remains a significant concern in poultry production due to its effects on growth, egg production, and susceptibility to secondary infections. The mortality rate and economic losses in younger birds suffering from the diphtheritic form can be higher than in the cutaneous form, sometimes reaching up to 50%. Mortality from the cutaneous form can also be high, especially when lesions develop around the eyes, which impair the bird's vision and ability to feed (Tripathy & Reed, 2020). Fowlpox has been controlled in most developed countries but remains a problem in many developing nations, including Nigeria, largely due to poor hygiene, contaminated instruments and surfaces, and vector transmission via biting insects such as mosquitoes, other flies, and poultry red mites (*Dermanyssus gallinae*) (Tripathy & Reed, 2020). Nigeria experiences a high prevalence of fowlpox, primarily affecting backyard and free-range flocks, but it can also infect chickens and turkeys raised intensively (Adene & Fatumbi, 2004; Meseko *et al.,* 2012; Meseko *et al.,* 2017).

There are no definitive treatments for fowl pox; thus, control and prevention mainly depend on maintaining good hygiene and timely vaccination, which remains a potent and the most effective way of preventing and controlling the disease in domestic birds. Thus, a wide variety of commercially available vaccines have been tested in birds of different age ranges with varying degrees of success and different levels of protection (Woodward & Tudor, 1973; Baxi & Oberoi, 1999). These fowlpox vaccines which are mostly live vaccines are produced either by inoculation onto chorioallantoic membranes (CAM) of 9- to 12-day-old developing chicken embryos or cell cultures of avian origin. Several cell cultures have previously been employed in the propagation of fowlpox virus, including chicken embryo kidney cells, primary chicken embryo fibroblasts (CEF), chicken embryo dermis cells, or the permanent quail cell line QT-35 (Schnitzlein *et al.,* 1988).

Fowlpox vaccine in most countries is propagated on CAM, but in modern veterinary vaccinology, vaccines derived from cell culture are now widely used (Yusifova, 2021; WOAH, 2023). The immunogenicity, efficacy, and safety of cell culture-adapted fowlpox in comparison to fowlpox vaccines of other origins have been investigated, yielding diverse results (Baxi & Oberoi, 1999; El-Mahdy & Mikheal, 2014; Wambura & Mzula, 2017; Radwan & Mikhael, 2020). However, the overall reports have consistently indicated positive outcomes for the cell culture-adapted fowlpox vaccines. These vaccines, whether administered alone or in combination with other vaccines, have demonstrated effectiveness and safety with several biological advantages of the cell-culture-adapted vaccines, including product uniformity, homogeneity, and stringent bacterial and fungal sterility (El-Zein *et al.,* 1974).

Barahona & Hanson (1968) reported that the embryo's fibroblast is one of the cells that can be used for the multiplication of fowlpox viruses; however, Vero cells have also been used to propagate this virus. Several studies have evaluated chicken embryo fibroblast cell culture-adapted fowlpox vaccines and reported them to be safe, with a high ability to induce a protective immune response in vaccinated birds (Baxi & Oberoi, 1999; Yusifova, 2018; Sarma *et al.,* 2019; Khalili *et al*., 2020). In addition, the use of cell culture-adapted vaccines for fowlpox has been reported to be more economical and productive compared to CAM-adapted vaccines whose production is more time-consuming, thus numerous producers began manufacturing vaccines using cell systems (Yusifova, 2021). The use of the CAM route for the propagation of the fowlpox virus (WOAH, 2023) has been the method applied in the production

of the fowlpox vaccine at the National Veterinary Research Institute (NVRI), Vom, and the vaccine has been effective in the prevention and control of fowlpox in poultry flocks. However, the quantity produced is grossly inadequate to meet the demand of farmers and are thus supplemented by import. The imported vaccines are still inadequate and are highly exorbitant and out of the reach of poor small scale poultry farmers. This leads to poor vaccine coverage with large number of susceptible unvaccinated flocks; thus, outbreaks and infections of flocks are regularly being reported in Nigeria (Odoya *et al.,* 2006). However, there are generally no information on either the production or study on cell culture-adapted fowlpox vaccines in Nigeria. Furthermore, given cases of reported outbreaks of fowlpox in vaccinated flocks, it has become imperative to develop vaccines against fowlpox that have strong immunogenic qualities (Yusifova, 2017; 2018; 2021). Thus, intending to develop a more potent vaccine in enough quantity to meet the demand of the Nigerian poultry industry, this study adapted fowlpox vaccine virus to CEF and assessed its safety and immunogenicity.

Materials and Methods

Ethical approval for the experiment

Ethical approval for the use of sentinels in this experiment was obtained from the NVRI Animal Use and Care Committee (AEC/02/141/23) before the commencement of the experiment.

Propagation of the vaccine virus

The vaccine virus was propagated both in chicken embryo fibroblast (CEF) and chorioallantoic membrane (CAM) of Specific Antibody Negative (SAN) eggs obtained from the National Veterinary Research Institute, Vom, poultry farm. The Primary fibroblast cells were prepared from 9-12-day-old chicken embryos. The chicken embryo fibroblast cell culture for the propagation of the vaccine virus was produced in-house and the working virus seed was inoculated in both suspension and on monolayer as described by Khalili and colleagues (2020). The CAM propagation of the vaccine virus was carried out by inoculating approximately 0.1 ml of the seed virus onto the CAMs of 9- to 12-day-old developing chicken embryos as recommended by WOAH (2023).

Master Seed Virus (MSV) preparation of virus

Two different commercial vaccine vials (BIOVAC and IZOVAC) were procured from a reputable vendor in Jos, both of which were live attenuated fowlpox vaccines propagated on CAM from SPF eggs. Each vial of 1000 doses of the freeze-dried live vaccine was reconstituted in 2 ml of sterile Hanks Minimum Essential Medium (HMEM), giving a titer of 10^3 EID_{50}/dose. The resulting constituent was used as the working seed for the vaccine production.

Chicken embryo fibroblast (CEF) cell culture

Cell culture was done using primary fibroblast cells obtained from 9 to 12-day-old chick embryos of SAN flock as described by Cunningham (1966); Hernandez & Brown (2010) and Cotter *et al.* (2017). Briefly, the limbs and heads of the harvested embryos were cut off and the embryos were macerated and washed several times with phosphate buffered saline (PBS) solution of pH 7.4 and two changes of trypsin solution. The macerated tissue pieces were further digested with trypsin Hanks balanced salt solution (THBSS) using a magnetic stirrer and magnetic apparatus. The digestion process was repeated several times until the embryo was fully digested. The digestion process was halted by adding fetal bovine serum (FBS). The suspension containing the digested cells was then filtered through a coarse muslin gauze. The resulting filtrate was centrifuged at 2500 rpm for 20 minutes to precipitate cells. The resulting supernatant was decanted, leaving the cells at the bottom. The sedimented cells were resuspended in HMEM in a 1: 350 dilution. A liter of the medium was supplemented with 10% Newborn calf serum and 10mls of 1% penicillin-streptomycin to prevent infection. 5 ml of fibroblast cell suspensions were cultured in sterile 25 ml cell culture flasks for the production of monolayer fibroblast cells and incubated at 37°C. Both suspension and monolayer culture flasks were prepared for virus inoculation.

Adaptation of FPV in CEF cell culture

The suspended CEF primary cells in HMEM media were inoculated with 0.2ml virus suspension and incubated at 37°C. For the production of Monolayer CEF, the primary cells were cultured and incubated in HMEM media for 12-24 hours at 37°C. After 70-80% confluency of the cells had been obtained, the media was poured out and 0.2ml of virus suspension was inoculated into mono-layered CEF cells and incubated at 37°C for 45mins - 1 hour for the adsorption of the virus to take place after which non-adsorbed virus was decanted and a maintenance medium (HMEM containing 0.1% antibiotics and 2% Newborn calf serum) was added, incubated at 37°C and observed daily for 3-5days until the cytopathic effect (CPE) reached between 80 and 90% when the vaccines were frozen and harvested. Harvested vaccines were

then subjected to quality checks, including sterility and titer determination as described by Soleimani (2022) and WOAH (2023), and were thereafter passaged in CEF cells for up to five passages.

Vaccine titration
The titers of the MSV and the Fowlpox virus vaccines were obtained by 2 methods: 50 per cent or median Embryo Infectious Dose (EID_{50}) and Median Tissue Culture Infectious Dose ($TCID_{50}$) recommended by Reed & Muench (1938), Khalili *et al.* (2020) and WOAH (2023).

Median Embryo Infectious Dose - EID_{50} of CAM-based vaccines
Specific antibody-negative (SAN) embryonated chicken eggs were inoculated via the CAM route with 0.2 ml of the ten-fold serial dilution of the virus suspension. Five (5) embryonated eggs (9-12 days old) were inoculated for each dilution ($10^1 – 10^{10}$). The embryonated eggs that were found dead within 24 hours post inoculations (PI) were not considered. The embryos that died after 24 hours and those that survived were examined on day 5 PI for evidence of viral activity/infection (presence of pock lesions or generalized thickening of CAM). The EID_{50} was calculated using the Reed & Muench (1938) method.
Median Tissue Culture Infectious Dose of CEF vaccines
Primary CEF cells were prepared in 24-well microplates, and virus dilution from 10^{-1} to 10^{-10} was prepared; 5 wells were allocated to each dilution. An equal volume (1ml) of media and virus suspension was added to each well, while one well remained as a control. The plates were incubated (37℃; 5% CO_2) and monitored daily for CPE and on the fifth day post inoculation. The TCID50/ml was calculated using the Spearman- Karber formula (M = xk + 1 / 2d-drl / n), as described by Spearman (1908) and Kärber (1931).

Vaccine compounding
Some portion of the harvests from the cell culture-adapted vaccine of the various passages were compounded with the requisite excipients, which included peptone, gelatin and antibiotics. The resulting mixture was then dispensed into sterile vaccine vials, partially stoppered and freeze-dried. The freeze-dried vaccines were then subjected to quality assurance checks and titration.

Safety test
For the safety test, three groups of 5 birds each were vaccinated with CEF-adapted vaccine with half field dose (25µl), field dose (50µl), and 10 times (10x) field doses (500µl). CAM-based vaccine was administered to two groups of 5 birds each for field dose (50µl), and 10 times field dose (500µl). The CAM-based half-field dose group were not included due to shortage of space, coupled with the fact that data already exist where mostly field doses are administered to chickens in poultry farms. The CEF-adapted vaccine half-field dose was included in this study because it is a new experimental vaccine. A control group of 5 birds were maintained without vaccination. The groupings and vaccination schedule are outlined in Table 1.
The experimental birds were vaccinated by piercing the wing web with a two-pronged needle dipped into each vaccine type. The vaccinated chickens were then observed daily for 7–10 days for evidence of ‘takes’ (A ‘take’ consists of swelling of the skin or the formation of a scab at the site of vaccination which is considered evidence of successful vaccination), and adverse effects/clinical disease attributable to the vaccines. The birds were further monitored for 5 weeks and were bled weekly to check for seroconversion.

Antibody decay monitoring
For this purpose, the 30 birds above were bled at regular intervals as outlined in Table 1. To monitor for the fowlpox-specific antibody seroconversion and the antibody profile, the weekly post-vaccination sera were stored for analysis at -20°C until analyzed by Enzyme-Linked Immunosorbent Assay (ELISA) using Chicken Fowlpox Virus Antibody (FPV-Ab) ELISA Kit as described by Khalili *et al.* (2020). Due to the shortage of ELISA plates, only a representative sample from each group was analyzed, while the remaining sera were also subjected to analysis by Agar gel immunodiffusion (AGID) test.

Enzyme-Linked Immunosorbent Assay (ELISA)
An FPV antibody ELISA kit (Shanghai Melson Medical) was used to analyze sera randomly selected from samples of each group of different doses and different weeks from the vaccinated and control groups according to the manufacturer’s instruction and the World Organization for Animal Health (WOAH, 2023). Results were read at 450nm wavelength and recorded. The cut off optical density (OD) was calculated according to the manufacturer’s instructions and recorded. 3-4 representative samples were selected from each group per week for ELISA analysis.

Agar Gel Immunodiffusion (AGID) Test
For this purpose, known FPV antigen-containing

Table 1: Experimental design showing the different groupings and their treatment/ durations

Groups	Weeks						
	1	2	3 (7 DPV)	4 (14 DPV)	5 (21 DPV)	6 (28 DPV)	7 (35 DPV)
A1 (5 birds)	Stabilize & bleed	Vaccinate (CEF vac) half dose	Bleed all birds	Bleed all birds	Bleed all birds	Bleed all birds	Bleed all birds
A2 (5 birds)	Stabilize & bleed	Vaccinate (CEF vac) std dose	Bleed all birds	Bleed all birds	Bleed all birds	Bleed all birds	Bleed all birds
A3 (5 birds)	Stabilize & bleed	Vaccinate (CEF vac) 10x std dose	Bleed all birds	Bleed all birds	Bleed all birds	Bleed all birds	Bleed all birds
B2 (5 birds)	Stabilize & bleed	Vaccinate (CAM vac) std dose	Bleed all birds	Bleed all birds	Bleed all birds	Bleed all birds	Bleed all birds
B3 (5 birds)	Stabilize & bleed	Vaccinate (CAM vac) 10x std dose	Bleed all birds	Bleed all birds	Bleed all birds	Bleed all birds	Bleed all birds
Control (5 birds)	Stabilize & bleed	No vaccination	Bleed all birds	Bleed all birds	Bleed all birds	Bleed all birds	Bleed all birds

Key: DPV = Days post-vaccination, A1 (group that received CEF-adapted vaccine - half field dose); A2 (group that received CEF-adapted vaccine - field dose); A3 (group that received CEF-adapted vaccine - 10 times field dose); B2 (group that received CAM vaccine - field dose); B3 (group that received CAM vaccine - 10 times field dose); and control (unvaccinated group)

control samples (Charles River Laboratories) were used for FPV antibody detection in selected serum samples. Agarose gel of 1.5% was prepared and poured into petri dishes. When the gel was set, a gel puncher was used to punch the plates, creating a central well and 6 peripheral wells. The known antigen samples were placed in the central well, while the serum samples to be analyzed were placed in the peripheral wells and labelled appropriately. The plates were then incubated at 37℃ and the results of precipitation lines were read within 24-72 hrs. Post-vaccination serum samples that could not be included in the ELISA due to limited test wells were analyzed with AGID. A total of 62 sera were analyzed via this technique. The assay was carried out as described by WOAH (2023).

Results

Development of primary cell line and vaccine virus propagation

The primary cell line was successfully developed from primary fibroblast cells of 9-12-day-old chick embryos, and a cell count of 125 × 10^4 CEF cells/ml was obtained. The primary cell line was used for the propagation of the vaccine virus and also for the titration of the harvests and the freeze-dried vaccines. 75cm^2 Corning® cell culture flasks (plug seal cap type) were used for virus propagation. Vaccine virus was successfully propagated in CEF and CAM, which showed evidence of virus propagation indicated by the appearance of cytopathic effect (CPE- including cell rounding and syncytia formation) on CEF cell culture (as shown in Plate I) and thickening of the CAM (as shown in Plate III). The virus propagation and passages using cell culture were performed in both suspension and monolayer, and freezing and harvest of products in all 5 passages were only done after 80 – 90% CPE was observed in each passage. The lack of CPE in the control, evident in the appearance of a confluent monolayer of elongated cells is shown in Plate II, and the different timelines for the termination of incubation of the different passages on the attainment of 80 – 90% CPE are indicated in Table 2. This study showed that 80 - 90% CPE was obtained quickly in the monolayer culture passages, indicated by the shortened termination times of incubation required for the monolayer culture (with the shortest termination time being 45hrs), compared to the suspension culture (with the shortest termination time of 71hrs 14mins).

Immunogenicity of the CAM and CEF-adapted vaccines

The vaccine virus seeds: BIOVAC (Seed 1) and IZOVAC (Seed 2) inoculated and propagated on CAM and the various passages in CEF cell culture as well as the freeze-dried harvests (seed 2) titers were determined

and presented in Table 3 showing discontinued passage of the vaccine seed 1 at the end of the third passage with titres of $10^{6.5}$/ml and $10^{6.7}$/ml for suspension and monolayer cultures respectively.

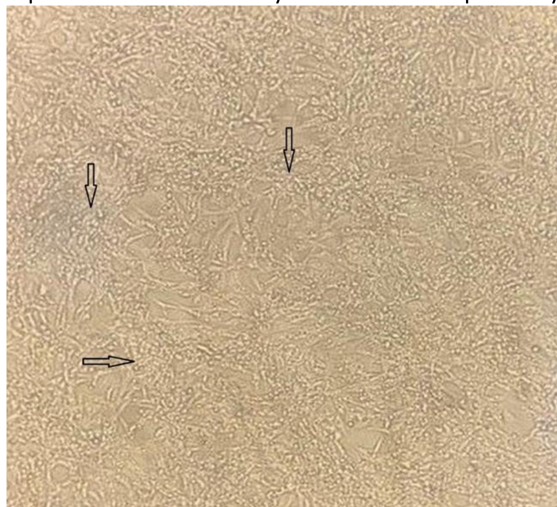

Plate I: CEF culture inoculated with fowlpox virus showing CPE (rounding of cells and formation of syncytia, indicated by black arrows) (passage 2 cells)

Plate II: Uninoculated Control CEF culture without CPE at the termination of incubation (confluent elongated cells indicated by black arrows)

Whereas vaccine seed 2 yielded titres as high as $10^{8.2}$/ml in both suspension and monolayer cultures, as well as high titres after freeze-drying, with termination at the 5th passage at a titre of $10^{7.7}$/ml. The ELISA results of the 3-4 representative serum samples from each group, including chickens vaccinated with the varying doses of CEF-adapted and CAM vaccines as well as the control group over the 5-week observation period, are presented in Table 4. The 79 samples randomly selected and analyzed, were all positive with OD readings above the cut off value of 0.216. The negative control gave OD readings of 0.066, which is below the critical cut off OD value of 0.216 as provided by the manufacturer of the kit (values lower than 0.216 were regarded as negative results). On the other hand, the positive control gave OD readings of 3.310 and 3.251 for the ELISA test, which confirmed their positivity (values higher than or equal to 0.216 were considered to be positive for FPV antibody). Chickens vaccinated with CEF-adapted vaccine generally had higher OD values (as high as 0.298, 0.347 and 1.430 in groups A1, A2 and A3,

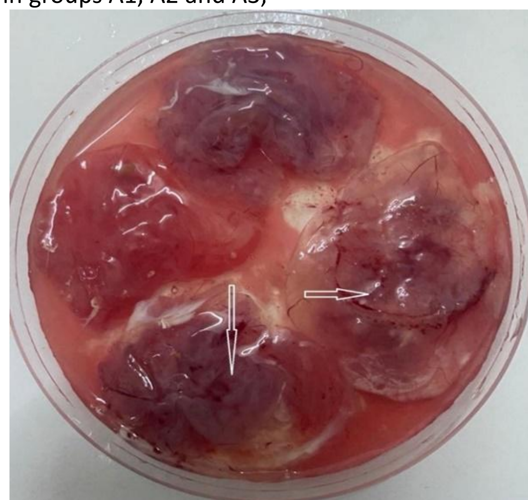

Plate III: Thickened CAM due to pocks formation on FPV-infected CAM (5 days PI), (indicated by white arrows)

Table 2: Termination time of incubation of virus propagation in CEF cell cultures after attaining 80%-90% CPE

Cell Culture Type	Cell Culture Passages				
	1	2	3	4	5
Suspension	115hrs	85hrs 22mins	71hrs 14mins	93hrs	90hrs
Monolayer	85hrs 5mins	45hrs	48hrs	69hrs	60hrs

Key: CPE = Cytopathic Effect, CEF = Chicken Embryo Fibroblast Cell Culture

Table 3: Titers $TCID_{50}$ of different passages of fowlpox vaccine harvest and freeze-dried vaccines grown in CEF cell culture

Vaccine Type	Culture type	Culture Passages				
		1	2	3	4	5
Vaccine Seed 1	Suspension	$10^{6.5}$/ml	$10^{6.5}$/ml	$10^{6.5}$/ml	DC	DC
	Monolayer	$10^{6.5}$/ml	$10^{6.5}$/ml	$10^{6.7}$/ml	DC	DC
Vaccine Seed 2	Suspension	$10^{6.7}$/ml	$10^{6.7}$/ml	$10^{7.2}$/ml	$10^{8.0}$/ml	$10^{8.2}$/ml
	Monolayer	$10^{7.5}$/ml	$10^{7.0}$/ml	$10^{7.2}$/ml	$10^{8.2}$/ml	$10^{7.7}$/ml
Freeze Dried Vaccine (Seed 2)	Suspension	$10^{6.5}$/ml	$10^{6.5}$/ml	$10^{6.5}$/ml	$10^{7.0}$/ml	$10^{7.2}$/ml
	Monolayer	$10^{7.5}$/ml	$10^{6.5}$/ml	$10^{6.5}$/ml	$10^{7.2}$/ml	$10^{7.7}$/ml

Keys: Vaccine Seed 1 – Biovac; Vaccine Seed 2 – Izovac; DC = Discontinued because of the low titers of harvests from passages 1 – 3. $TCID_{50}$ = Median Tissue Culture Infectious Dose; CEF = Chicken Embryo Fibroblast Cell culture.

respectively), signifying the presence of higher antibody titers as shown in Table 4.

Eighteen samples (29%) analyzed with AGID were positive for FPV antibody as presented in Table 5. The result showed all the groups having positive results except the unvaccinated control group and pre-vaccination baseline samples. The treatment group A2 which received a field dose of CEF-adapted vaccine had 5 positive samples, with the highest percentage positivity (50%), while the treatment group A3 which received a 10x field dose of CEF-adapted vaccine produced a lower percentage positivity (30%) with 3 positive samples. The highest percentage positivity across all the vaccinated groups was observed in the 5th week of vaccination, with a total of 6 positive samples (60% positivity).

Safety of CAM and CEF-adapted vaccines

All vaccinated birds (100% of 25) showed “takes” within three to four days after vaccination, as shown in Plate IV. The unvaccinated control group (5 birds) did not show any evidence of “takes”. Additionally, the safety of the vaccines was confirmed across different doses, with no adverse effects or clinical signs observed during the five-week monitoring period after vaccination.

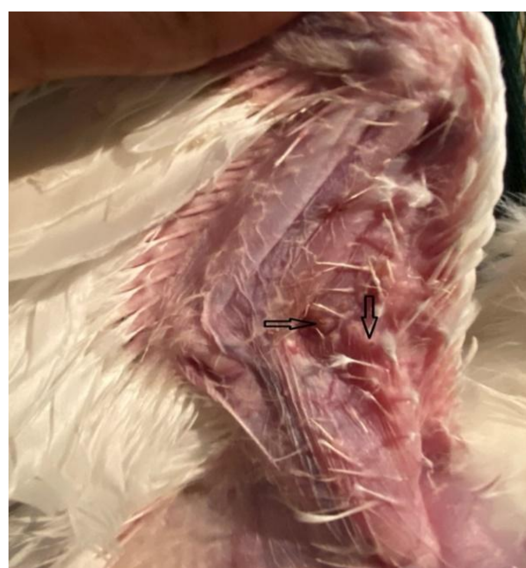

Plate IV: Vaccinated bird with CEF-adapted vaccine showing evidence of “takes” (indicated by black arrows)

Discussion

In this study, vaccine seed originally propagated on CAM was adapted to primary chicken embryo fibroblasts (CEF) cell culture. The adaptation of the vaccine virus to CEF cell culture in this study yielded positive results, producing CPE within 3-5 days, as reported in a similar study by Khalili *et al.* (2020). However, in this study, CPE was observed in some of the passages in less than 3 days.

The monolayer cultures in most of the passages were observed to have taken a shorter time for attaining 80-90% CPE compared to the suspension cultures. This could be attributed to the advantage of the adsorption process in which the monolayer overlying medium is removed before the monolayer is inoculated with the suspension of viral organisms as opposed to the direct and immediate inoculation done during the suspension culture. The inoculation of the viral suspension onto the monolayer allows for wide surface contact between the virus and the cells. In addition, some studies have reported a delayed

Table 4: ELISA result showing the optical density (OD) readings of FPV antibody values post-vaccination for 5 weeks

Groups	Weeks (OD Values) Post-Vaccination (PV)					Manufacturer's OD Reference Values
	1	2	3	4	5	
A1 Samples	0.274	0.262	0.265	0.249	0.260	< 0.216 – Neg
	0.298	0.290	0.283	0.267	0.260	≥ 0.216 – Pos
	0.248	0.286	0.272	0.265	0.291	
A2 Samples	0.263	0.286	0.298	0.347	0.278	< 0.216 – Neg
	0.281	0.239	0.305	0.307	0.302	≥ 0.216 – Pos
	0.263	0.254	0.233	0.321	0.280	
A3 Samples	0.528	0.291	0.275	0.260	0.276	< 0.216 – Neg
	0.258	0.270	0.238	0.273	0.273	≥ 0.216 – Pos
	1.430	0.260	0.257	0.278	0.217	
B2 Samples	0.288	0.260	0.253	0.292	0.301	< 0.216 – Neg
	0.277	0.277	0.276	0.660	0.279	≥ 0.216 – Pos
	0.292	0.269	0.257	0.273	0.383	
B3 Samples	0.254	0.256	0.236	0.260	0.589	< 0.216 – Neg
	0.248	0.235	0.250	0.273	0.304	≥ 0.216 – Pos
	0.295	0.277	0.261	0.278	0.227	
Control (non-vaccinated) Group			0.280		0.272	< 0.216 – Neg
			0.272		0.242	≥ 0.216 – Pos
ELISA Kit Neg. Control			0.066			< 0.216 – Neg
			0.066			≥ 0.216 – Pos
ELISA Kit Pos. Control			3.310			< 0.216 – Neg
			3.251			≥ 0.216 – Pos

Key: A1 (the group that received CEF half field dose vaccination), A2 (the group that received CEF field dose vaccination), A3 (the group that received CEF 10x field dose vaccination), B2 (the group that received CAM field dose vaccination), B3 (the group that received CAM 10x field dose vaccination), NA (not applicable)

Table 5: Pre- and post-vaccination serum samples analyzed by Agar Gel Immunodiffusion Test

Weeks		Groups (Number/Positive)						% Positivity
		A1	A2	A3	B2	B3	Cx	
Pre-Vaccination (BL)		2/0	2/0	2/0	2/0	2/0	2/0	0.00
Post-Vaccination	1	2/0	2/0	2/0	2/0	2/0	NA	0.00
	2	2/1	2/2	2/0	2/0	2/0	NA	30.0
	3	2/2	2/1	2/1	2/0	2/0	NA	30.0
	4	2/0	2/1	2/1	2/1	2/2	NA	50.0
	5	2/0	2/1	2/1	2/2	2/2	NA	60.0
PV % Positivity		30.0	50.0	30.0	30.0	40.0	00.0	

Key: A1 (received half field dose of CEF-adapted vaccine), A2 (received field dose of CEF-adapted vaccine), A3 (received 10x field dose of CEF-adapted vaccine), B2 (received field dose of CAM vaccine), B3 (received 10x field dose of CAM vaccine), Cx (Un-vaccinated control group), BL (Pre-vaccination baseline samples), NA (not applicable)

appearance of CPE with both FPV field isolates and vaccine strain. The CPE were only observed at the 3rd passage in a similar study conducted by Gilhare *et al.* (2015), while Khalili *et al.* (2020) observed CPE at the 8th passage. However, in this study, CPE occurred at the first passage with a titer of $10^{6.5}$ $TCID_{50}$/ml and $10^{7.7}$$TCID_{50}$/ml for the BIOVAC and IZOVAC vaccine seeds, respectively. This difference could be attributed to the nature and difference in the virus strain used in the cultures, as it has been reported by Khalili *et al.* (2020) that not all strains can show CPE at the first passage. In addition, a progressive increase in the titers with increased passages was observed, which could be indicative of a successful adaptation of the virus to the CEF cell culture, as similar results were obtained in the study by Khalili *et al.* (2020).

The vaccination of the broiler chickens with various dilutions of CEF and CAM-based vaccines was also successful, as "takes" were observed post-vaccination in all (100%) of the vaccinated birds, which is indicative of a successful vaccination. Previous studies by Sarma & Sharma (1988), Islam *et al.* (2008) and Sarma *et al.* (2019) documented similar results. Generally, checking for post-vaccination "take" is considered one of the best methods for evaluating pox immunity in vaccinated chickens because the properly administered efficacious vaccine is expected to show "takes" in 99-100% of the vaccinated chickens, as previously reported by Cookson (1996); Barreda (2016) and Sarma *et al.* (2019). In addition, the over five weeks of monitoring of the vaccinated birds, including groups vaccinated with ten times the standard field dose, showed no adverse reactions, death, or development of clinical signs of the disease as a result of the vaccine viruses. There was no reversion to virulence or other safety issues in any of the chickens vaccinated with different doses. These findings are consistent with reports by Shil *et al.* (2007); Sarma *et al.* (2019), Radwan & Mikhael (2020) and WOAH (2023), and support the recommendations of the WOAH. Thus, the vaccines were found to be safe under experimental conditions as administering of the vaccine viruses at a much higher dose did not adversely affect the safety of the vaccines since all the vaccinated chickens remained healthy and were as active as the unvaccinated control birds. Certain studies have also been carried out to compare CAM-adapted and cell culture-adapted fowlpox vaccines, some of which have shown cell culture-adapted fowlpox vaccines to be better than the former, as described in studies by Baxi & Oberoi (1999) and Khalili *et al.* (2020). The findings of some studies also indicated that the cell culture-based vaccines performed better than the CAM-derived vaccines. This was seen in the early observation of pox "takes" in vaccinated birds and the higher antibody titer as shown by ELISA results in birds vaccinated with cell culture-based vaccines compared to the CAM-based vaccines. The plausible reason is the higher antigenic titer as a result of the higher production rate and quality of CEF-adapted viral vaccine compared to the traditional CAM method of production.

The seroconversion of vaccinated birds across the groups was monitored by using ELISA and AGID to analyze the post-vaccination serum samples. Although the ELISA result indicates seroconversion in the different groups, the baseline pre-vaccination serum samples indicated the presence of antibodies when analyzed with ELISA (giving OD values higher than the 0.216 critical cut-off), but were negative for antibodies when analyzed with AGID (indicated by the absence of precipitation lines in the test agar plates). However, the fact that the known negative controls were negative and the known positive controls were also positive validates the test. The discrepancy of the presence of antibodies in the baseline pre-vaccination sera could have been a result of the presence of maternally-derived antibodies (MDA) in the chicks, even though the baseline pre-vaccination sera were sampled at 4 weeks of age, a period where MDA, if present, would have declined to negligible and non-protective levels as suggested by Islam *et al.* (2008). Generally, MDA is higher in day-old chicks and gradually declines below a positive level within 10–15 days of age, as earlier reported by AI-Natour *et al.* (2004) and Akhter *et al.* (2008). This was, however, not the case in this study as the birds had high MDA at four weeks.

Amongst the various diagnostic techniques used for the detection of immune responses, AGID (Agar Gel Immunodiffusion) and ELISA (Enzyme-linked Immunosorbent Assay) are generally recommended for the determination of the immune status of individual birds or populations post-vaccination. However, despite the recommendations, these two diagnostic tests have also been reported to have their limitations according to the OIE Terrestrial Manual (2018). ELISA is considered to be a non-species-specific test (Buscaglia *et al*., 1985); however, some studies have shown ELISA to be highly sensitive but less specific when compared to AGID (Buscaglia *et al.,* 1985; Mockett *et al.,* 1987). In another study by Adebajo *et al.* (2012), which used AGID to determine the seroprevalence of fowlpox antibodies in an area, the resulting prevalence figures were lower and did

not agree with a previous study by Ohore *et al.* (2007), which reported higher prevalence figures using the ELISA technique. This discrepancy associated with ELISA was attributed to its high sensitivity and lower specificity, making it prone to false-positive results, as reported by Buscaglia *et al.* (1985) and Adebajo *et al.* (2012).

This discrepancy also occurred in this study, with all samples analyzed by ELISA showing the presence of antibodies, whereas samples from the same groups analyzed using AGID only showed 29% positivity to fowlpox antibodies. The baseline pre-vaccination sera samples that tested positive with ELISA were negative or showed the absence of fowlpox-specific antibodies with AGID. This suggests that while ELISA is more sensitive, it may be less specific compared to AGID. Although maternally derived antibodies are a likely explanation, another plausible reason could be false positives due to non-specificity of the ELISA assay. Nevertheless, the AGID analysis showed progressive positivity after the first week post-vaccination, with the highest positivity observed in the fourth and fifth weeks post-vaccination.

There was also a higher positivity percentage in the group vaccinated with a standard dose of the CEF-adapted fowlpox vaccine compared to other vaccinated groups. The ELISA analysis similarly showed higher antibody titers (represented by increased optical density values) in the CAM-adapted and CEF-adapted vaccinated groups during the fourth and fifth weeks post-vaccination for the standard field and 10× doses. This finding aligns with a report by Verma *et al.* (2015), which demonstrated increasing virus titers with progressive passage in both CAM and CEF systems. The trends observed in this study also correlate with previous reports by Sarma & Sharma (1988) and Saini *et al.* (1990), which confirmed that fowlpox antibody levels significantly increase up to four weeks post-vaccination before beginning to decline.

It is important to acknowledge that some serum samples were not analyzed using ELISA due to budgetary constraints. Although representative samples from all groups and time points were analyzed with ELISA, this constraint may have reduced the robustness of the serological dataset. Despite this limitation, the use of validated controls and consistency in trends across ELISA and AGID results support the reliability of the findings. Future studies should aim to expand testing capacity for more robust analysis of post-vaccination sera.

In addition, chickens vaccinated with CEF adapted vaccine generally had higher OD values signifying the presence of higher antibody titer which aligns with the findings of some studies by Siccardi (1975) and Baxi & Oberoi (1999) where birds vaccinated with the cell culture-adapted vaccine showed better protection in contrast to birds vaccinated with chicken embryo-adapted vaccines or where cell culture adapted vaccines were shown to be more effective. Cell culture-adapted (CEF) vaccine has been previously reported in studies by El-Zein *et al.* (1974) and Yusifova (2021) to have higher biological properties than a conventional vaccine prepared on the chorioallantoic membrane of embryonated eggs, and are thus likely to provide more protection than the conventional CAM-derived fowlpox vaccine, but it was reported by Baxi & Oberoi (1999) that both the CAM-adapted and CEF-adapted vaccines would elicit adequate immune responses as Fowlpox vaccine produced in embryonic chickens has the disadvantage of low level of infectious activity in chickens. Given this, the search for fowl pox virus vaccine adapted to a cell culture with high infectious and immunogenic properties becomes critical as reported by Yusifova (2018, 2021).

In modern veterinary medicine, vaccines based on cell cultures have found widespread use as previously reported by Yusifova (2021); WOAH (2023). It was reported by Khalili *et al*. (2020) that Fowlpox vaccine is also being manufactured using chicken embryo fibroblast (CEF) culture in many countries, and Yusifova (2018) reported that the cultivation of fowl pox virus in chicken embryo cell cultures is common in preparing biomass for vaccine production. Thus, the use of cell culture-adapted vaccines for fowlpox has been reported to be more economical and productive compared to CAM-adapted vaccines, whose production is more time-consuming; thus, many manufacturers were reported by Yusifova (2021) to have begun producing vaccines based on cell systems. Thus, the use of different cell cultures and different strains of viruses by researchers to improve and increase vaccine effectiveness has been ongoing (Yusifova, 2017; 2021). The need to develop more potent vaccines in enough quantity to meet the demand of the Nigerian poultry industry is very important.

In conclusion, this research successfully demonstrated the development and adaptation of the Fowlpox virus to a primary cell line of CEF for the propagation and titration of a vaccine virus. The study involved multiple passages in both suspension and monolayer cultures, resulting in effective virus adaptation to CEF for vaccine production, with notable cytopathic effects observed. The vaccine

virus, propagated in both CAM and CEF cultures, exhibited high titers, indicating successful production. Immunogenicity and safety assessments of CEF-adapted and chorioallantoic membrane (CAM) vaccines in broiler chicks revealed the induction of "takes" without adverse effects, confirming the vaccine's safety. Vaccines produced in CEF were immunogenic, safe, and produced no untoward clinical signs or adverse effects in vaccinated chickens. Overall, these findings support the safety, and immunogenic potential of the developed CEF-adapted vaccine, laying a solid foundation for its potential application in controlling fowl pox virus infections in poultry. The application and production of fowlpox vaccine by cell culture can be a step towards the self-sufficiency of this type of vaccine in Nigeria. From the results obtained in this study, it becomes imperative that further studies be carried out on a larger scale to establish the efficacy, safety, and potency of CEF-adapted fowlpox vaccines.

Acknowledgements

Adene DF & Fatumbi O (2004). Case Review and Lesson on Poultry Diseases Control in South West Nigeria. In: *Poultry Health and Production, Principles and Practice*. Stirling Horden Publishers (Nig) Ltd, Ibadan, Oyo State, Nigeria. Pp 191-200.

Al-Natour MQ, Ward LA, Saif YM, Stewart-Brown B & Keck LD (2004). Effect of different levels of maternally derived antibodies on protection against infectious bursal disease virus. *Avian Diseases,* **48**(1): 177-182.

Akhter AT, Khan MSR, Paul NC, Kafi A, Shil NK & Siddiqu MP (2008). Persistence of maternally-derived antibody in selected group of chicks to fowl pox virus. *Bangladesh Journal of Microbiology,* **25**(1): 57-59.

Barahona HH & Hanson RP (1968). Plaque enhancement of Newcastle disease virus (lentogenic strain) by magnesium and diethlaminoethyle dextran. *Avian Diseases,* **12**(1): 151-158.

Barreda CB (2016). Relationship between values of fowlpox ELISA and the presence of "Takes" after vaccination. *Avian Diseases,* **60**(1): 67-69.

Baxi MK & Oberoi, MS (1999). Comparative evaluation of cell culture-adapted and chicken embryo-adapted fowl pox vaccine strains. *Avian Diseases,* **43**(1): 16–21.

Buscaglia C, Bankowski RA & Miers L (1985). Cell-culture virus-neutralization test and

The authors acknowledge the African Union Commission for awarding the grant for this research through the Pan African University Life and Earth Sciences Institute (including Health and Agriculture), Ibadan, Nigeria. We also acknowledge the Viral Vaccine Production Unit of the National Veterinary Research Institute (NVRI), Nigeria for the laboratory support and the Regional Laboratory for Animal Influenza and Transboundary Animal Diseases NVRI for the serology.

Funding

Funding for this research was provided by the African Union Commission through the Pan African University Life and Earth Sciences Institute (including Health and Agriculture), Ibadan, Nigeria.

Conflict of Interest

The authors declare that there is no conflict of interest.

References

enzyme-linked immunosorbent assay for evaluation of immunity in chickens against fowlpox. *Avian Diseases,* **29**(3): 672–680.

Bwala DG, Fasina FO & Duncan NM (2015). Avian poxvirus in a free-range juvenile speckled (rock) pigeon (*Columba guinea*). *Journal of South African Veterinary Association*, **86**(1): e1-e4.

Cookson KC (1996). Evaluation of pox takes in commercial egg type pullets. *Proceedings of the forty-seventh North Central Avian Disease Conference*. NCADC, Minneapolis, Minnesota. Pp 73-76.

Cotter CA, Earl PL, Wyatt LS & Moss B (2017). Preparation of cell cultures and vaccinia virus stocks. *Current Protocols in Molecular Biology,* doi.10.1002/cpmb.33.

Cunningham CH (1966). *A Laboratory Guide in Virology*. Sixth edition. Burgess Publishing Company, Minneapolis. Pp 60.

El-Mahdy SS & Mikheal C (2014). Efficacy of fowl pox vaccines against Egyptian isolated strain during 2012. *Veterinary World,* **7**(9): 656-660.

El-Zein A, Nehme S, Ghoraib V & Hasbani S (1974). Preparation of fowl pox vaccine on chicken-embryo-dermis cell culture. *Avian Diseases,* **18**(4): 495-506.

Gilhare VR, Hirpurkar SD, Kumar A, Naik SK & Sahu T (2015). Pock forming ability of fowl pox virus isolated from layer chicken and its

adaptation in chicken embryo fibroblast cell culture. *Veterinary World,* **8**(3):245-250.

Giotis ES & Skinner MA (2018). Spotlight on avian pathology: fowlpox virus. *Avian Pathology,* **48**(2): 87–90.

Giotis ES & Skinner MA (2021). Fowlpox Virus and Other Avipoxviruses (Poxviridae). In: Encyclopedia of Virology DH Bamford, M Zuckerman, editors, fourth edition, volume 2. Oxford: Academic Press. Pp 343–348

Hernandez R & Brown DT (2010). Growth and maintenance of chick embryo fibroblasts (CEF). *Current Protocols in Microbiology*, doi.10.1002/9780471729259.mca04is17.

Islam MR, Khan MSR, Islam MA, Kayesh MEH, Karim MR, Gani MO & Kabir A (2008). Comparative efficacy of imported fowl pox virus vaccine with locally produced one in backyard chicks. *Bangladesh Journal of Veterinary Medicine,* **6** (1): 23–26.

Ka¨rber G (1931). Beitrag zur kollektiven behandlung pharmakologiseher reihenversuche. *Archiv für experimentelle Pathologie und Pharmakologie* **162**(4): 480–483.

Khalili GM, Khalesi B, Ghaderi M, Taghizadeh M, Shahkarami MK, Motamed N, Karimi & Razakani H (2020). Evaluation and optimization of chick embryo fibroblasts for production of a fowl pox vaccine based on cell culture. *Iranian Journal of Virology,* **14**(2): 6-15.

Meseko AC, Shittu I, Bwala DG, Joannis TM & Nwosuh CI (2017). Fowlpox virus from backyard poultry in Plateau State Nigeria: isolation and phylogeny of the P4b gene compared to a vaccine strain. *Nigerian Veterinary Journal,* **38** (2): 124-128.

Meseko CA, Shittu SI & Akinyede O (2012). Seroprevalence of fowl pox antibody in indigenous chickens in Jos North and South council areas of Plateau state, Nigeria: implication for vector vaccine. *International Scholarly Research Notice, Veterinary Science,* doi.10.5402/2012/154971.

Mockett APA, Southee DJ, Tomley FM & Deuter A (1987). Fowlpox virus: Its structural proteins and immunogens and the detection of viral-specific antibodies by ELISA. *Avian Pathology,* **16**(3): 493–504.

Odoya EM, Abegunde A, Agyogbo BG, Omatainse SO, Gwankat E & Okpara UG (2006). Outbreak of turkey pox disease in fowl pox vaccinated poults in Vom, Plateau State of Nigeria. *African Journal of Clinical and Experimental Microbiology,* **7**(2): 136-138.

Ohore OG, Emikpe BO, Oluwayelu DO, Adeyemi RO & Ockiya MA (2007). Seroprofiling of antibodies to fowl pox in commercial and indigenous chickens in Southwestern Nigeria. *Journal of Advanced Veterinary and Animal Research,* **6**(5): 697–701.

OIE (Office Internationales Epizooties) Terrestrial Manual (2018). Fowl Pox, http://www.oie.int/en/standard-setting/terrestrial-manual/access-online/. retrieved 22-12-2023.

Radwan A & Mikhael C (2020). Comparative Evaluation on the efficacy of embryonated chicken egg adapted and tissue culture pigeon pox vaccines against the local virulent strain. *Journal of Applied Veterinary Sciences,* **5**(2): 87-93.

Reed LJ & Muench H (1938). A simple method of estimating fifty per cent endpoints. *American Journal of Tropical Medicine and Hygiene,* **27**(3): 493–497.

Saini SS, Sodhi SS, Maiti NK & Sharma N (1990). Immune response to chicks to fowlpox vaccination. *The Indian Veterinary Journal,* **67**(8): 689-693.

Sarma DK & Sharma SN (1988). Oral immunization of chicks with fowpox virus vaccines. *Indian Journal of Animal Science,* **58**(7): 743-747.

Sarma G, Kersting BA & Spina G (2019). Field safety and efficacy of a unique live virus vaccine for controlling avian encephalomyelitis and fowlpox in poultry. *Veterinary World,* **12**(8): 1291–1298.

Schnitzlein WM, Ghildyal N & Tripathy ND (1988). A Rapid method for identifying the thymidine kinase genes of avipoxviruses. *Journal of Virological Methods,* **20**(4): 341–352.

Shil NK, Rahman MS, Paul S, Cha SY, Jang HK & Song HJ (2007). Vaccination studies against fowl pox in chickens. *Korean Journal of Poultry Science,* **34**(4): 253-257.

Siccardi FJ (1975). The addition of fowlpox and pigeon pox vaccine to marek's vaccine for broilers. *Avian Diseases,* **19**(2): 362-365.

Soleimani S (2022). A Review of the establishment of the seed lot system in the production of biological products and its importance. *Archives of Razi Institute*, **77**(6): 2023–2035.

Spearman CI (1908) The method of 'right and wrong cases' ('constant stimuli') without Gauss's

formulae. *British Journal of Psychology,* **2**(3): 227–242.

Tripathy DN & Reed WM (2008). In: *Diseases of Poultry* (YM Saif editor), twelfth edition. , Ames, IA: Blackwell Publishing Company. Pp 291–307.

Tripathy DN & Reed WM (2020). Pox. In: *Diseases of Poultry* (DE Swayne). Thirteenth edition. John Wiley & Sons, Inc., Hoboken, NJ, USA & John Wiley & Sons Ltd, Oxford, UK. Pp 364–381.

Verma RK, Joshi RK, Joshi N, Gangwar, AK & Shukla S (2015). Study on histopathological changes induced by fowl pox virus (FPV) in chorioallantoic membrane (CAM) of developing chicken embryo and immunological characterization of the egg adapted and cell culture grown virus. *Journal of Pure Applied Microbiology,* **9**(2): 1349-1354.

Wambura PN & Mzula A (2017). A novel rapid direct haemagglutination-inhibition assay for measurements of humoral immune response against non-haemagglutinating Fowlpox virus strains in vaccinated chickens. *Heliyon,* **3**(10): 00428.

WOAH (2023). *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, twelfth edition. https://pilvarad.com/wp-content/uploads/2024/07/oie-book-2023.pdf, retrieved 22-12-2023.

Woodward H & Tudor DC (1973). The immunizing effect of commercial pigeon pox vaccines on pigeons. *Poultry Science,* **52**(4): 1463-1468.

Yusifova K (2017). Features of accumulation in cellular systems of EYP and FEC virus of chickens in comparative aspect. *Proceedings of XVI International Scientific and Practical Conference Modern Trends of Agricultural Production in the World Economy*, Kemerovo. Pp 194-198.

Yusifova K (2018). Improvement of specific prevention against diseases of birds in the collection: Actual problems and innovative solutions. In: *APC. Proceedings of the international scientific-practical conference held at Baku, Azerbaijan*. Pp 158-167.

Yusifova K (2021). The use of cell systems for the manufacture of vaccine against fowl pox. *Research in Agricultural and Veterinary Sciences,* **5**(1): 19-23.