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Research Article

COMPARATIVE STUDIES ON POCK MORPHOLOGY AND ANTIGENIC PROPERTIES OF *AVIPOXVIRUSES*

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Abstract- During the present study five isolates *i.e.*, three of fowlpox virus (FPV), one of turkey pox virus (TPV) and one of pigeonpox (PPV) was collected from naturally pox infected chickens, turkey and pigeon in different outbreaks attended in various districts of Chhattisgarh state. Virus was propagated in chicken embryo upto 20 serial passage to ensure the successful adaptation of each virus to chorioallantoic membrane (CAM). Appearance of pock lesions and concentration of virus was inconsistent during initial passage (from 1 to 3 passage), in later passages (10 to 20 passage) the low degree of variation in macroscopic appearance of pock lesions was observed, also the pock morphology was consistent irrespective of *Avipoxvirus* (APV) strain. Partially purified field isolates of one each of FPV, TPV, PPV and vaccine strain of FPV (Vac) were subjected to Western blotting and the protein profile of five field isolates was compared with that of commercial FPV vaccine strain. All field strain of APVs, except PPV showed common polypeptide bands with size of 22 kDa, 35 kDa, 39 kDa, 48 kDa, 62 kDa, 65 kDa, 70 kDa and 91 kDa. While protein profile PPV showed extra bands with size of 27 kDa. These bands were also found in FPV (Vac) but 22 kDa was missing.

Keywords- *Virus, Passage, Chorioallantoic membrane, Pock, Polypeptide band*

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Introduction

Genus *Avipoxvirus* (APV) is a cluster of poxviruses infecting fowl, turkey, pigeon and many wild birds that may vary in their pathogenicity, host specificity and degree of cross-relationship. Fowlpox virus (FPV) represents the type species of genus APV subfamily *Chordopoxvirinae* of family *Poxviridae* [1]. Natural infection in susceptible birds occurs in cutaneous or diptheritic form or both [2]. Host specificity is considered to be one of the important criteria for differentiation of APVs. At present, the exact number of existing APV species, strains and variants is unknown since very often the new isolates continued to be identified from number of avian species. Poxviruses are ubiquitous and it is debatable that how poxvirus infection has been transmitted and globally dispersed among wild and domestic birds. Fowlpox (FP) is of major importance and as the poultry population increased along with turkey and pigeons, other APV infections *i.e.*, turkeypox (TP) and pigeonpox (PP) has also gained considerable economic importance. Losses due to major FP outbreaks are largely attributed to mortality, drop in egg production, meat condemnations and also to an unexpected vaccination failure, particularly in layers [3]. FPV is highly infectious for chickens and turkey rarely for pigeons and not all for ducks and canaries. The turkeypox virus (TPV) is virulent to ducks [4]. Although it is assumed that though APVs are strongly species specific, FPV was also found associated with outbreaks in turkey [5] which reveals the fact that FPV is emerging pathogen of turkey. In India, turkeys are frequently affected and several outbreaks of TP have been reported [6]. TPV was considered more or less similar to FPV but different from other APVs namely, pigeonpox virus (PPV) and canarypox virus (CPV) whose genomes have been characterized. The authors were inconclusive about whether TPV possess definite biological differences with other APVs. Mortality and morbidity related to PPV infection may be high in pigeons. There are relatively less reports of PPV in India. The preliminary studies on first report of PPV in Chhattisgarh and isolation of field strain of PPV was done by Khan, 2014 [7].

It revealed that the field strain of PPV can be conveniently adapted and propagated successfully in both chorioallantoic membrane (CAM) of developing chicken embryo (CE) and chicken embryo fibroblast (CEF) cell culture. By and large the route of transmission, viral virulence and host susceptibility to the infecting APV strain might be responsible for the clinical sign of pox disease and also decide appearance of either cutaneous or diptheritic form of avian pox. Macroscopic appearance of pocks on CAM by different APVs is one of the formost criteria to describe proliferative nature of lesion as a result of consistency in viral growth after its adaptation to chicken embryos. Immunoblotting or Western blotting cannot be used as routine diagnostic test. However, it has a potential to establish standards for evaluation of immunity due to various FPV, TPV and PPV candidate vaccine strains on the basis of immunity, inactivation trials and cross-immunization studies. There are many reports on composite study on comparison of APVs isolated from different birds from the same geographic area as well as diverse regions in India and abroad. Such study has not, so far, been done on field isolates of APV from Chhattisgarh. Thus, the research on comparison of APVs obtained from different birds from various districts of Chhattisgarh are required to establish the data about phylogenetic status, the pathogenicity to chicken, the adaptability to grow and propagate on CAM of CE, the degree of immunogenic proteins of local strains of APVs isolated from natural outbreaks. So the aim of the study was to study the pattern of *Avipoxvirus* growth on chicken embryo along with antigenic properties of the viruses using Western blotting.

Materials and methods

Viral isolates

Virus samples as a dry scab were collected from clinically infected birds. Three isolates namely FPV-1, 2, 3 and one isolate designated as TPV-1 was isolated from diseased chickens and turkey respectively in different outbreaks occurred in

Table-1 Pock morphology produced by APVs

Virus isolate	Appearance of Pock lesion various passage of CAM			
	1-3th passage	4- 5th passage	10th passage	20th passage
FPV-1	Thick oedematous swelling at first passage. Few white pocks with oedematous thickening of CAM at 3rd passage level.	Clear distinct pock seen and comparatively more in number. Size of individual pock was 2-3 mm diameter	Many single and diffused pock in wider area.	Small pock (0.5-2 mm) with slight oedematous thickening of CAM
FPV-2	CAM thickening in first two passages. Pock started to appear at 3rd passage. The size of individual pock was 2-4 mm diameter.	Clear distinct pock with oedematous swelling. Single as well as diffused pock observed.	Single as well as diffused pock many in number in wider area	Very small faint pock and size of individual pock ranged 0.5-2 mm.
FPV-3	Typical pocks few in number observed initially which increased in subsequent passages. Pocks were white single and diffused. Individual pock ranged 2-3 mm.	Typical white pocks in increased number.	Typical white pocks in increased number, wider area, single and diffused pock seen.	Faint small pocks in small area generally scatter. Size of individual pock ranged 0.5-2 mm
TPV-1	Very thick necrosis of centrally inoculated area at first passage and then typical white pocks of larger size in 3rd passage. Size of individual pock was 3-5 mm diameter.	Pock number increased, single as well as diffused pock seen.	Pock was observed in wider area. Single and diffused pock seen	The opacity of pocks increased. Pocks number decreased and of relatively smaller size 1-2 mm
PPV-1	Membrane thickening and oedematous swelling observed at initial first two passage.	Distinct Pock with size of 2-3 mm, few in number seen. Pock was glistening in appearance, irregular shaped and usually single.	Pocks seen in wider area	The opacity of pocks increased, Pocks number decreased and of relatively smaller size 1-2 mm diameter.

Table-2 Antigenic profile analysis of *Avipoxviruses* by immunoblotting

Molecular Size (kDa)	FPV-1	FPV-2	FPV-3	TPV-1	PPV-1	Control	Vaccine strain	Remark
91	+	+	+	+	+	-	+	Common to all
70	+	+	+	+	+	-	+	Common to all
65	+	+	+	+	+	-	+	Common to all
62	+	+	+	+	+	-	+	Common to all
48	+	+	+	+	+		+	Common to all
39	+	+	+	+	+	-	+	Common to all
35	+	+	+	+	+	-	+	Common to all
27	-	-	-	-	+		+	Found in PPV and vaccine
22	+	+	+	+	+	-	-	Not in vaccine

*FPV (*Fowlpov virus*), TPV (*Turkeypox virus*), PPV (*Pegion pox virus*), + presence of antigen, - absences of specific protein

Fig-1 Oedematous thickening of CAM infected by FPV-1 at 1st passage

Fig-2 Thick Oedematous swelling of CAM infected by TPV-1 at 1st passage

various district of Chhattisgarh state. A field isolate of pigeonpox virus (designated as PPV-1) was identified as PPV from Durg (Khan, 2014) was maintained in the Veterinary Microbiology Department, Anjora. Virus was revived by serial passage in chicken embryos and used in the present study. Lyophilized live FPV vaccine from Venkateshwara Hatcheries Private Limited, Pune was also used in the study.

Virus isolation on embryonated chicken egg

Dry scabs collected from each of the field outbreaks of pox in chickens and turkey were used for virus isolation. In case of PPV field strain the CAM suspension of PPV field strain (stored at -20 C) was used instead of dry scabs. Ten percent suspension of scabs in PBS was made using pastle and mortar for each isolates individually. After three cycles of freezing and thawing suspensions were centrifuged for 15 min at 1500 rpm and then supernatant were collected. Supernatant was filtered in syringe filter of 0.45 µm average pore diameter and 0.2 ml of filtrate (inoculums) was used for virus isolation.

Fig-3 Slight thickening of CAM infected by PPV-1 at 1st passage

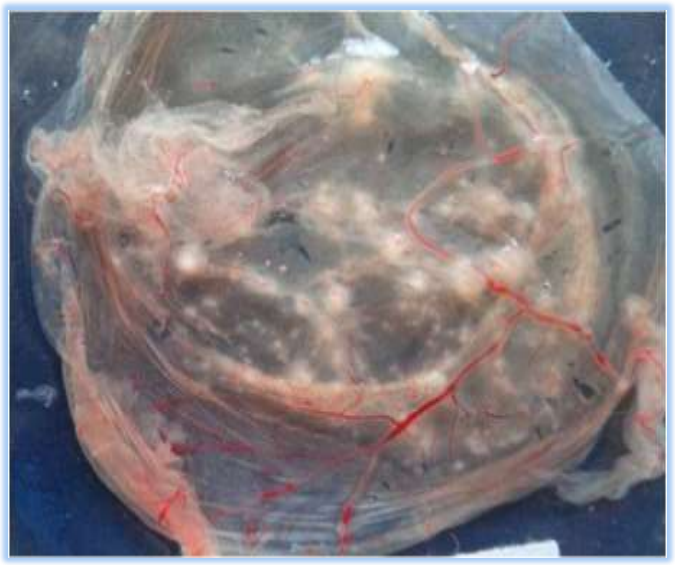

Fig-4 CAM infected by FPV-1 at 3rd passage showing diffused and single pock

Fig.5 CAM infected by TPV-1 at 3rd passage showing large size pocks

Fig-6 CAM infected by PPV-3 at 3rd passage showing pock lesions

Fig-7 FPV-3 infected CAM showing diffused and single pock at 1st passage

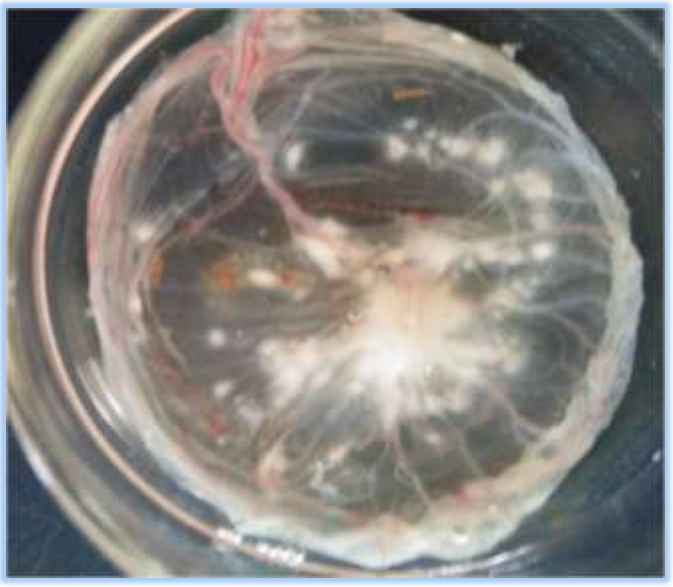

Fig-8 FPV-3 infected CAM showing diffused and single pock at 3rd passage

Fig-9 CAM infected by FPV-1 at 10th passage showing pock on wider area

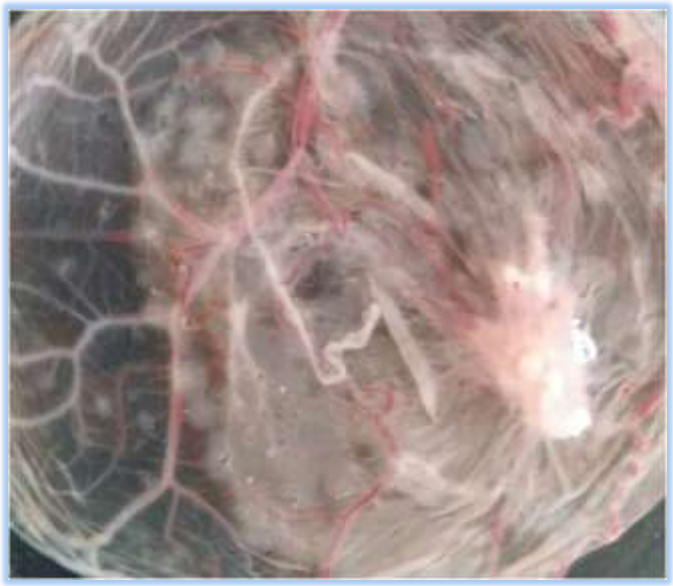

Fig-10 CAM infected by FPV-3 at 10th passage showing pocks on wider area

Fig-11 CAM infected by TPV-1 at 10th passage showing pocks in wider area

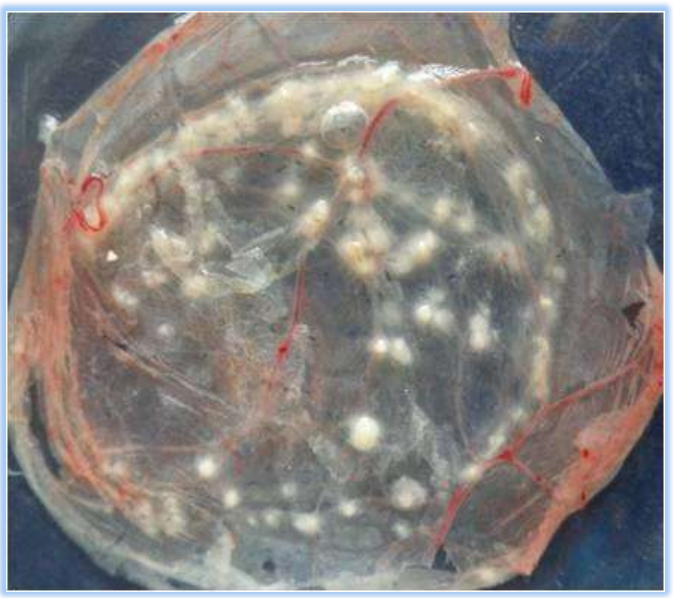

Fig-12 CAM infected by PPV-1 at 10th passage showing pock in wider area

Fig- 13 CAM infected by FPV-1 at 20th passage showing small size pocks

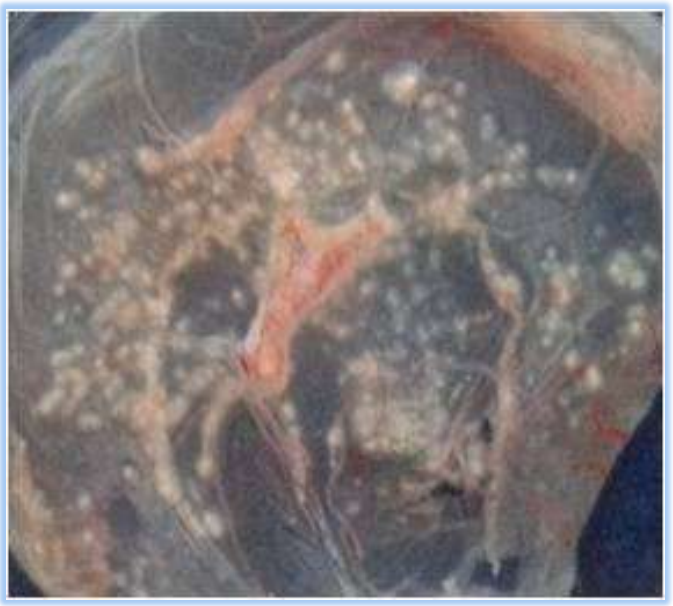

Fig-14 CAM infected by FPV-3 at 20th passage showing small size pocks

Fig-15 CAM infected by TPV-3 at 20th passage showing small size pocks

Fig-16 CAM infected by PPV-1 at 20th passage showing small size pocks

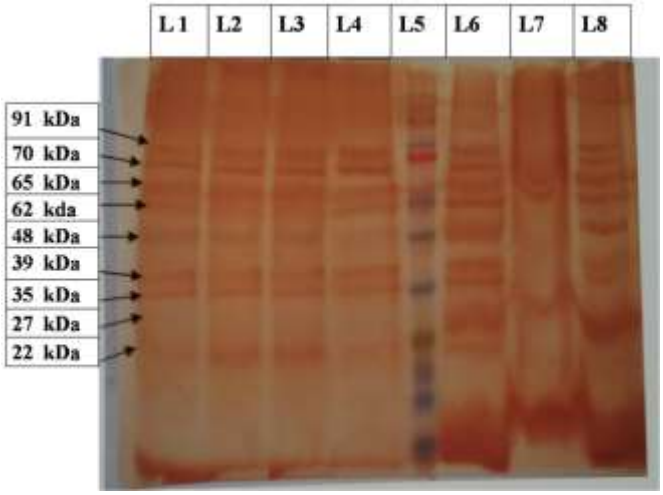

Fig-17 PVDF membrane showing viral polypeptide of partially purified lysates from uninfected CAM, and infected CAM. # Lane 1: FPV-1, Lane 2: FPV-2, Lane 3: FPV-3, Lane 4: TPV-1, Lane 5: protein ladder (11-245 kDa), Lane 6: PPV-1, Lane 7: Uninfected CAM, Lane 8: FPV (Vac). The molecular mass of antigen shown on the left hand of the PVDF membrane

One vial of FPV vaccine strain (1000 dose) was dissolved in 1 ml phosphate buffered saline (pH 7.4) and was used as a reference FPV strain for inoculation in chicken embryos (*i.e.*, 0.1 ml of dose was equal to 100 field doses per egg). Virus was isolated by inoculating 0.2 ml of virus inoculums on 10 days old chicken embryos as per dropped CAM method described by [8]. The inoculated eggs were incubated at 37°C for 5 days. After 5 days, live embryos were transferred to 4ºC chamber for chilling and then CAM was harvested. Development of pock lesions on CAM was examined. The diameter of each pock in mm, colour, consistency and other changes were recorded. Membranes not showing pock lesions/showing pock lesions in the first passage were given further passages. The virus was adapted in CAM of chicken embryos upto 20 passage.

Preparation of APV specific hyperimmune serum in rabbits

Hyperimmune serum against one of field isolates the representative viruses namely, FPV-1 and FPV vaccine was raised in rabbits individually. In a primary dose, 1 ml of 10 percent suspension of infected CAM (at 20th passage level) emulsified with equal volume of Freund's Complete Adjuvant was given sub-cutaneously. On day 14 and 28 booster dose with Freund's Incomplete Adjuvant was given. Control group consisting of 2 rabbits was inoculated in similar manner by using uninfected CAM. A week after the last vaccination test serum was obtained from each rabbit.

Western blotting

Preparation of viral antigen

Partially purified viral antigen was prepared from virus infected CAM (at 5th passages). Five field isolates of APVs *i.e.*, FPV-1, FPV-2, FPV-3, TPV-1, PPV-1 and FPV egg adapted vaccine strain (FPV-Vac) were used in the study. Also, virus uninfected CAM was taken as control. All samples and control CAM were processed in separate tubes. Ten mg clear pocks lesions were taken in 2 ml centrifuge tube containing 100 µl ice cold lysis buffer and 1µl mammalian cocktail protease inhibitor and homogenize. All lysates were centrifuged at 12000 rpm for 15 min at 4ºC. The resulting supernatant was collected and protein concentration was measured in Qubit® flourometer (Thermofischer) using Qubit® protein assay kit (Thermofischer) as per the manual provided in kit.

Preparation of SDS-PAGE

The apparatus, glass plates, comb and spacers were cleaned thoroughly and cassette was prepared. Twelve percent resolving gel solution and six percent stacking gel solution was used for preparing SDS-PAGE. Each test sample including uninfected CAM was mixed with equal volume of 2x Laemmli sample buffer and kept into water bath at 95ºC for 7 min then kept into ice for 2 min. 30 µl of different protein samples were loaded into each individual lane along with 4 µl prestained protein marker.

Electrophoresis

Initially, SDS-PAGE was run at low voltage (80 V, 500 mA, 40 min) and then higher voltage (120 V, 500 mA for 120 min) applied till the end of electrophoresis.

Blotting

Following gel electrophoresis, the separated protein mixture was transferred to a solid support for further analysis. Gel was separated carefully from glass plate and trimmed upto level of marker then kept into transfer buffer immediately. Polyvinylidene fluoride (PVDF) membrane was cut with the same dimensions of gel and was dipped into methanol for 15 min. Filter papers and sponges were dipped into transfer buffer. Sandwich was prepared in transfer apparatus in sequential manner by putting two sponges, two filter paper, gel, PVDF, two filters paper and two, sponges respectively. Air bubbles if any were completely removed and extra liquid were squeezed out. The transfer apparatus was filled with transfer buffer and relocated into transfer tank containing chilled Mili Q water with ice. The electrodes were placed on top of the sandwich in such a way that PVDF membrane should be in between the gel and a positive electrode. The assembly was covered by ice packs to ensure efficient protein separation and electric current (310 mA) was applied for 120 min.

Transfer apparatus was removed from tank and both the gel and PVDF membrane was removed carefully.

Blocking

To prevent nonspecific protein interaction the PVDF membrane was incubated in blocking reagent (5 percent skimmed milk powder) for 1 hr at room temperature.

Incubation of primary antibody

The PVDF membrane was made to react with Rabbit anti-fowlpox antibody diluted upto 1:350 in blocking solution. The membrane was incubated with 7 ml diluted primary antibody and kept at 8º C for overnight. It should ensure that PVDF membrane dipped completely in diluted antibody solution.

Incubation with secondary antibody

After incubation of primary antibody, the PVDF membrane was washed four times with PBST each for 10 min. HRP linked donkey anti rabbit secondary antibody was diluted upto 1: 1000 in PBST. PVDF Membrane was incubated with 7 µl diluted secondary antibody for 1:30 hr at room temperature.

Target antigen detection

After incubation with secondary antibody, the PVDF membrane was washed thrice with 0.1 percent PBST each for 5 min. PVDF membrane was put in freshly prepared substrate (DAB solution) and incubated at room temperature for 5-10 min for target antigen detection. The membrane was rinsed with distilled water to stop the reaction of substrate. It was then placed on filter paper to air dry. The specific protein was detected as a band in the PVDF membrane.

Results and discussions

Virus isolation in chicken embryos

In the present study five samples *i.e*., FPV-1, FPV-2, FPV-3, TPV-1 and PPV-1 were isolated in chorioallantoic membrane of 10 days chicken embryos by dropped CAM method upto 20th passages successfully. The virus can be easily isolated from cutaneous form by embryos inoculation [9-12] and via other routes like bird inoculation and by cell culture techniques [13, 14].

Virus growth on CAM

Virus growth was observed on inoculated CAM by all sample used in the study. There was generalized thickening of membrane at initial passage and white opaque raised area of necrosis called pock noticed onto virus infected CAM. Focal as well as diffuse pocks were noticed in CAM at different passage levels. Oedematous thickening of virus inoculated CAM was observed at first passage by FPV-1 [Fig-1], FPV-2, TPV-1 [Fig-2] and PPV-1 [Fig-3], whereas distinct clear pocks were observed at third passage [Fig-4], [Fig-5], [Fig-6]. Diffused as well as single pock few in number were observed in CAM at first passage by FPV-3 [Fig-7], more distinct and clear lesion in wider area was noticed in subsequent passages [Fig-8]. There was little difference in pock morphology (*i.e*., colour, size, number and density) produced by these viruses were mentioned in [Table-1]. FPV-1, FPV-2 and FPV-3 produced white round pock which have centrally dense and light peripheral area of necrosis. The size of pock was 2-3 mm for FPV-1 and FPV-3. FPV-2 produced comparatively larger size pock 2-4 mm in diameter. TPV-1 produced larger pock with size of 3-5 mm. In case of PPV-1 produced medium size pock (2-3 mm diameter) have oval to irregular shape. Each pock was dense yellowish and glistening in appearance. Pocks number increases gradually with subsequent passages [Fig-9], [Fig-10], [Fig-11], [Fig-12]. Diffused and disperse pocks were noticed in infected CAM simultaneously. After 15th passage size of pocks became smaller [Fig-13], [Fig-14], [Fig-15], [Fig-16] and mostly of scatter even opacity of each pock increased that could be the pathogenicity of viruses were decreased. Mortality or retarded growth in inoculated embryos was not observed during the study. Our finding was similar with Reddy *et al*., [15] reported typical pocks on inoculated CAM at first passage. Masola *et al*., [16] noticed focal proliferations started to be visible during the third passage CAM by pigeonpox virus. Two to three pocks about 1 mm in diameter were observed and marked proliferative pock lesions were observed at the fourth passage. The pocks were increased in number and size ranging from 1-2 mm in diameter. Most of them had coalesced to form large mass. Differences in pock morphology in CAM by different *Avipoxvirus*es were consistent with other published reports of pox cases. Prukner-Radovcic *et al*., [17] observed different pox lesions on CAM *e.g*., metastatic for chicken poxvirus, diffuse lesions caused by fowl poxvirus of turkey, and thick focal lesion of pigeon poxvirus. Yadaw *et al*., [18] found oedematous thickening and diffused pock lesions in CAM at higher dilutions (10^{-4}/10^{-5}) of second passage level, while clear and distinct pock lesions at the lower dilutions (10-1/10-2) of the same passage level. Large sized diffused pocks having diameter of about 4-6 mm were observed in case of FPV, QPV and PPV, while TPV isolates produced small sized (2-3 mm) distinct thickened and oedematous pocks. Karim *et al*., [19] found single oedematous and diffused pock lesions at the site of inoculation at first passage in CAMs, while on the second passage, the pock lesions appeared little clearer. Very clear, rounded opaque and separated typical pock lesions showed at third passage. The fourth and fifth passages lesions were numerous and small in size. They also observed inoculated chicken embryos started to diefrom the third day post inoculation and all embryos died at the 6th days post inoculation during the first passage. In the second passage, the death started from the second day post inoculation and completely died five days post inoculation. All the 3rd, 4th, and 5th passages of inoculated chick embryos died at day four post inoculation. Although pock morphology was not consistent feature somewhat it depends on virus isolates, concentration of virus and number of passages. Virus growth on CAM in various passage indicated that these viruses were adapted onto CAM.

Detection of antigenic profile of APVs by western blot

The viral protein profile was studied to identify the viral antigen among field isolates partially purified virus suspensions. They were subjected to SDS-PAGE. The protein concentration in each well was 7.47µg/ml, 11.5µg/ml, 13. 3µg/ml, 15.4µg/ml, 9.53µg/ml and 7.47 µg/ml for FPV-1, FPV-2, FPV-3, TPV-1, PPV-1 and vaccine strain respectively. After SDS PAGE electrophoresis protein is transferred to PVDF membrane. Immunoblotting using specific serum raised against vaccine strain of FPV (Vac) and one of field isolate FPV-1 was used. The protein profile among five field isolates (FPV-1, FPV-2, FPV-3, and TPV-1 and PPV-1) and FPV (Vac) is summarized in [Table-2] and depicted in [Fig-17]. All field isolates of APVs except PPV showed common polypeptide band with size of 22 kDa, 35 kDa, 39 kDa, 62 kDa, 65kDa, 70 kDa, and 91 kDa. While protein profile of PPV revealed one extra band with size of 27 kDa. These bands were also found in FPV (Vac) but band for 22 kDa was missing. Uninfected CAM was used as negative control did not show any similar band. Similar work was also done by Mockett *et al*., [20] they have detected 30 structural polypeptides in the FP virion. The relative molecular mass 91 kDa, 64 kDa, and 58 kDa were most abundant viral protein but not major immunogens. Polypeptides of 91 kDa, 72 kDa, 62 kDa, 50 kDa, 48 kDa, 45 kDa, 37 kDa, 35 kDa, 33 kDa, 27 kDa, 24 kDa and 23 kDa were major immunogenic protein. Boulanger *et al*., [21] detected three immunodominant FPV proteins *i.e*., 39 kDa core protein, a 30 and 35 kDa protein doublet, and an abundant 63 kDa protein. Our findings are in conformity to those reported by Tadese *et al*., [22] compared viral immunogenic proteins of field isolates and vaccine strain by immunoblot analysis using homologous and heterologous antisera. Only minor antigenic difference was observed. Similar work was also done by Singh *et al*., [23] those found 60 kDa, 46 kDa, 42 kDa, and 39 kDa protein in various field isolates of FPV and vaccine strain of FPV along with PPV by using two monoclonal antibodies indicated that one monoclonal antibody can react with one or more than one anigen. Minor antigenic differences were observed by Awad *et al*., [24] among field isolates of FPV, DPV, PPV through immunoblotting analysis against chicken anti FPV. Similar work was also done by another worker [25-28]. Our study showed less number of protein band as compared to other worker which may be because of the used antigen was only partially purified so the appropriate concentration for particular protein antigen was not achieved besides the difference in various strain, pathogenicy and the method of protein sample preparation.

Conclusion

Avianpox virus isolation, adaptation as well as propagation upto 20 serial passage

circulating in Chhattisgarh. Virus growth on CAM of ECE at 20 passage level was observed in form of consistency in pock morphology. Western blotting revealed slight difference in protein profile of different APVs except PPV which shows extra protein band of 27 kDa as shown by ECE adapted FPV vaccine strain. Also, one band 22 kDa was missing in vaccine strain.

Application of research: Differentiation of *Avipoxvirus*es isolated from infected chicken, turkey and pigeon to conclude weather these isolates are same or different and affecting various host.

Research Category: Veterinary Microbiology

Abbreviations
FPV- *Fowlpox virus*
TPV- *Turkeypox virus*
PPV-*Pigeonpox virus*
CAM- Chorioallantoic membrane
kDa- Kilo daltan
APV- *Avipoxvirus*
FPV (Vac)- Fowlpox vaccine
TP- Turkeypox
CPV- *Canarypox virus*
CE- Chicken embryo
ECE- Embryonated chicken egg
SDS-PAGE- Sodium dodecyle sulphate polyacrylamide gel
PVDF- Polyvinylidene fluoride membrane
PBST- Tween phosphate buffered saline
HRP- Horseradish peroxidise
DAB- Diaminobenzidine
QPV- *Quailpox virus*

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Research project name: PhD Thesis

Author's contribution: S.D. Hirpurkar designed the experiment. Varsha Rani Gilhare carried out the experiment and drafted the final manuscript. Asit Jain helped in analysis and Nidhi Rawat helped in the sample collection.

Author statement: All authors read, reviewed, agree and approved the final manuscript.

Conflict of interest: None declared

Ethical Approval: Approved by Institutional Animal Ethics Committee, College of Veterinary Science and Animal Husbandry, Anjora, Durg, C.G., 491001 dated on 03/05/2016

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