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Isolation, characterization and molecular diagnosis of pigeon pox virus and fowl pox virus from field outbreaks in Chhattisgarh

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Abstract
The present study was undertaken to isolate, identify, and characterize pigeon pox virus (PPV) and fowl pox virus (FPV) from field outbreaks in Durg district of Chhattisgarh, India. Virus isolation was carried out using embryonated chicken eggs (ECE) via the chorioallantoic membrane (CAM) route. Distinct pock lesions were produced on CAM, and progressive histopathological changes such as oedema, congestion, necrosis, haemorrhage, and intracytoplasmic inclusions were recorded at 48, bnv72, 96, and 120 hours post-inoculation. Serological assays including agar gel immunodiffusion (AGID) and counter immune-electrophoresis (CIE) confirmed the antigenic identity of field and vaccine strains, showing precipitation bands and complete lines of identity. The haemagglutination (HA) test revealed titres up to 1:640 for PPV and 1:320 for FPV in both field and vaccine strains, indicating stable haemagglutinin activity across passages. Molecular confirmation was achieved through polymerase chain reaction (PCR), where amplification of the conserved P4b gene produced the expected 578 bp product, confirming the presence of avipoxvirus. The study concludes that field and vaccine strains of PPV and FPV are antigenically similar, with stable haemagglutination properties and confirmatory molecular detection, suggesting their suitability for diagnostic and immunological applications.

Keywords: Pigeon pox virus (PPV), Fowl pox virus (FPV), Embryonated chicken egg, Chorioallantoic membrane, Histopathology, Agar gel immunodiffusion (AGID), Counter immune-electrophoresis (CIE), Haemagglutination (HA), Polymerase chain reaction (PCR), P4b gene

1. Introduction
Avipoxviruses are large double-stranded DNA viruses belonging to the family *Poxviridae* and genus *Avipoxvirus*, infecting a wide range of avian species (Moyer, 2000; Van Riper & Forrester, 2007) [17, 31]. Among these, pigeon pox virus (PPV) and fowl pox virus (FPV) are of particular veterinary importance, causing significant economic losses in pigeons and poultry. Clinically, the disease manifests as proliferative cutaneous nodules and, in severe cases, diphtheritic lesions in the upper respiratory and digestive tract, leading to reduced productivity, secondary infections, and mortality (Tripathy & Reed, 2003; Saif *et al.* 2008) [28, 23].
Reports of PPV and FPV outbreaks are widespread, with consistent cases recorded in India (Kaura & Iyer, 1938; Yadav *et al.* 2007; Mohan & Fernandez, 2008; Pazhanivel & Balachandran, 2012) [12, 33, 16, 20] as well as globally (Elamin *et al.* 1980; Weli *et al.* 2004; Rampin *et al.* 2006; Offerman *et al.* 2013) [8, 21, 19]. Virus isolation on the chorioallantoic membrane (CAM) of embryonated chicken eggs remains a gold standard, as avipoxviruses produce characteristic pock lesions that aid in diagnosis and attenuation studies (Brandly, 1941; Cunningham, 1966) [4, 5]. Histopathology of CAM and affected tissues typically reveals oedema, congestion, necrosis, haemorrhage, and intracytoplasmic inclusion bodies, which are pathognomonic for avipoxvirus infection (Woodruff & Goodpasture, 1930; Yadav *et al.* 2007) [32, 33].
Serological tests such as agar gel immunodiffusion (AGID), counter immuno-electrophoresis (CIE), and haemagglutination (HA) are widely used for detection and antigenic characterization of avipoxviruses (Uppal & Nilakantan, 1970; Tamador *et al.* 2001; Gilhare, 2012) [30, 27]. However, these techniques may lack sensitivity compared to molecular tools. The polymerase chain reaction (PCR), targeting the conserved P4b core protein gene, has emerged as a rapid, sensitive, and reliable diagnostic method for avipoxvirus confirmation

(Lee & Lee, 1997; Smits *et al.* 2005; Manarolla *et al.* 2010; Abdallah & Hassanin, 2012; Masola *et al.* 2014) [13, 26, 14, 1, 15]. Despite vaccination programs, sporadic outbreaks of PPV and FPV continue to occur, with reports of vaccine failure and reduced immunity in flocks (Singh *et al.* 2000; Biswas *et al.* 2011) [25, 3]. Continuous isolation and characterization of field strains are therefore necessary to monitor antigenic stability, assess diagnostic tools, and evaluate their suitability as vaccine candidates.

The present study was aimed at isolating and characterizing PPV and FPV from field outbreaks in Chhattisgarh, India, using embryonated chicken eggs, histopathology, serological assays, and PCR. This integrated approach provides a comprehensive understanding of the pathology, antigenic properties, and molecular identity of circulating strains, contributing to improved diagnosis and control of avipoxvirus infections.

2. Materials and Methods

Field material for pigeon pox virus (PPV) isolation was collected from outbreaks in Durg district, Chhattisgarh, by aseptically removing dry scabs from affected pigeons. A departmental reference strain of fowl pox virus (FPV) and commercially available lyophilized vaccine strains of PPV and FPV (Venkateshwara Hatcheries Pvt. Ltd., Pune) were used for comparison. Fertile 10-12 day-old embryonated chicken eggs (ECE) from seronegative flocks were obtained from the Government Poultry Farm, Durg.

For virus isolation, scabs were homogenized in phosphate-buffered saline (PBS, pH 7.0) to prepare a 10% suspension, freeze-thawed, centrifuged, and treated with antibiotics. The supernatant was inoculated onto the chorioallantoic membrane (CAM) of ECE using the dropped CAM method (Cunningham, 1966) [5]. After 5 days incubation at 37 °C, CAMs were harvested, examined for pock lesions, and stored for further analysis.

Histopathology was performed on infected CAMs showing distinct lesions. Tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic examination of intracytoplasmic inclusions and degenerative changes.

Serological identification was carried out using agar gel immunodiffusion (AGID) and counter immuno-electrophoresis (CIE) with hyperimmune sera raised in rabbits against PPV and FPV vaccines. For AGID, antigen and sera were placed in agarose wells on slides and incubated at 37 °C for up to 72 h. For CIE, antigen and antisera were electrophoresed in agarose prepared with barbitone buffer (pH 9.2) until precipitation lines developed.

Haemagglutination (HA) tests were conducted using 1% chicken erythrocytes. Two-fold serial dilutions of CAM suspensions and cell culture supernatants were mixed with RBC suspensions in microtitre plates, and the highest dilution showing agglutination was recorded as the HA titre.

For molecular confirmation, DNA was extracted from infected CAM by the phenol-chloroform method. PCR amplification targeted the conserved P4b gene of avipoxvirus using published primers (Forward: 5'-CAGCAGGTGCTAAACAACAA-3'; Reverse: 5'-CGGTAGCTTAACGCCGAATA-3'), yielding a 578 bp product. PCR was performed in a 25 µl reaction mixture, and amplification products were analyzed by agarose gel electrophoresis and visualized under UV illumination.

3. Results and Discussion

3.1 Isolation, Adaptation and Propagation of PPV in Chicken Embryo

Field samples collected from pigeons and fowls showing characteristic pox lesions were processed for virus isolation. Following inoculation of the field strain of PPV onto the chorioallantoic membrane (CAM) of embryonated chicken eggs, distinct pathological changes were observed. At first passage, generalized white foci with oedematous thickening appeared, while FPV produced localized pock lesions restricted to the inoculated site. On successive passages, PPV lesions measured 2-5 mm in diameter with congestion, haemorrhage, and necrosis, whereas FPV lesions were larger (4-6 mm) but more localized. Notably, only PPV field strains induced haemorrhagic lesions, distinguishing them from FPV. No significant differences were recorded between field and vaccine strains of PPV; however, FPV vaccine strains produced smaller lesions (1-2 mm) compared to field isolates. Progressive lesion development was evident at 48, 72, 96, and 120 hours post inoculation, ranging from mild oedema to severe necrosis with generalized pocks.

Serial passage up to the fifth generation showed that PPV adapted well to embryonated chicken eggs, inducing consistent CAM changes without evidence of attenuation. Virus titration revealed EID_{50} values of $10^{6.3}$/ml for PPV (field) and $10^{6.8}$/ml for PPV (vaccine), while FPV field and vaccine strains showed titres of $10^{5.4}$/ml and $10^{6.1}$/ml, respectively. Subsequent propagation in chicken embryo fibroblast cultures produced mild cytopathic effects in early passages and plaque formation by the third passage. These findings confirm the adaptability and stable pathogenicity of PPV field isolates, with lesion characteristics suitable for use in neutralization and vaccine evaluation studies.

Table 1: Progressive 'pock' lesion formation on CAM infected with PPV

Post inoculation	Morphology of the CAM
48 hr	Slight Oedema and small sized pock (plate 9)
72 hr	Oedema, thickening along with congestion and haemorrhagic small generalized pock lesions (plate 10)
96 hr	Severe Oedema, necrosis along with scattered pock lesions all over the CAM. Most of these lesion were distributed on CAM over an area just below the artificial air sac (plate 11)
120 hr	Oedematous CAM with generalized pock with average size of 2-5 mm in diameter (plate 12)

Table 2: Morphological changes induced by PPV on CAM in serial passages

Level of passage	Morphology of the CAM
1	Severe oedema and small pock
2	Generalized pock and severe oedema along with haemorrhage and necrosis
3	Slight oedema and generalized pock lesion with haemorrhage
4	Slight oedema and generalized pock lesion of 2-5 mm in diameter
5	Generalized pock of 2-5 mm diameter

Table 3 shows the progressive histopathological changes in CAM infected with PPV at different post-inoculation intervals. At 48 hours, oedema and congestion were evident, indicating the initial pathological response of the tissue. By 72 hours, the severity increased with the appearance of haemorrhage, along with oedema, congestion, and necrosis, suggesting rapid viral replication and tissue damage. At 96 hours, oedema, congestion, and necrosis persisted, highlighting sustained pathological alterations. By 120 hours, advanced changes such as oedema, congestion, necrosis, and distinct intracytoplasmic inclusions in lymphocytes were observed, confirming progressive viral involvement and characteristic pathological features of PPV infection.

Histopathological examination of CAM infected with PPV and FPV revealed intracytoplasmic inclusions of variable size, congestion, oedema, necrosis, and haemorrhage (Plate 16 and Plate 17). These findings corroborate earlier studies (Brandly, 1941; Tripathy *et al.* 2000; Rampin *et al.* 2006; Yadav *et al.* 2007; Manarolla *et al.* 2010; Ebrahimi *et al.* 2012; Offerman *et al.* 2013) [4, 33, 21, 19, 14]. Progressive degenerative changes due to viral replication were evident across different post-inoculation intervals (Table 3).

Table 3: Progressive changes in histopathology of CAM infected with PPV

Post inoculation (hrs)	Histopathological changes in CAM due to PPV replication
48 hr	Oedema and congestion (Plate 18)
72 hr	Oedema, haemorrhage, congestion and necrosis (Plate 19)
96 hr	Oedema, congestion and necrosis (Plate 20)
120 hr	Oedema, congestion, necrosis and inclusions in lymphocytes (Plate 21)

Plate 1: Field case of pigeon pox

Plate 2: Field case of fowl pox

Plate 3: Uninfected CAM after 5 days incubation

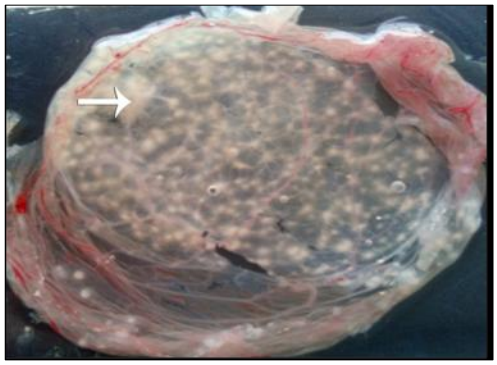


Plate 4: Showing pock lesions on infected CAM (field strain PPV)

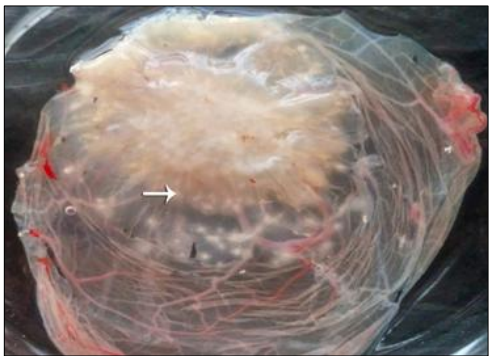


Plate 5: Generalized pock lesions and oedema on infected CAM by field strain PPV

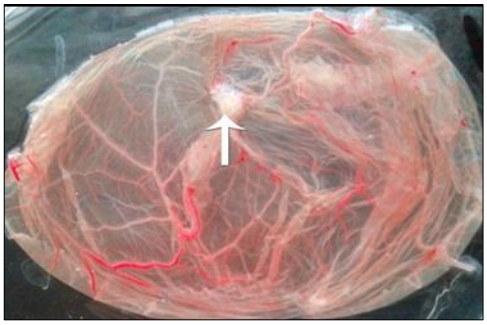


Plate 6: Haemorrhagic pock lesions on infected CAM (field strain FPV)

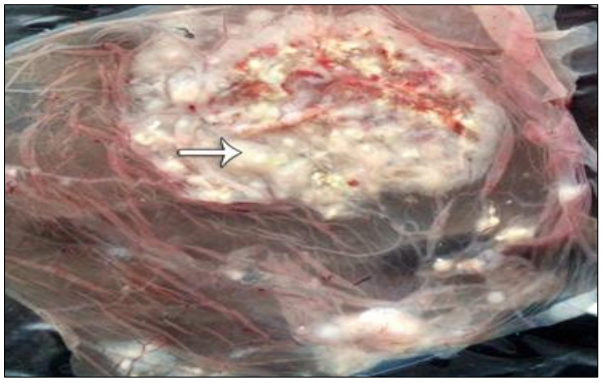


Plate 7: Localized pock lesions on infected CAM (vaccine strain PPV)

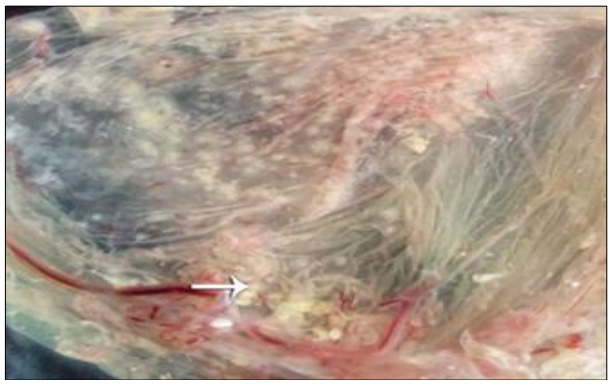


Plate 8: Localized pock lesions with edema on infected CAM (vaccine strain FPV)

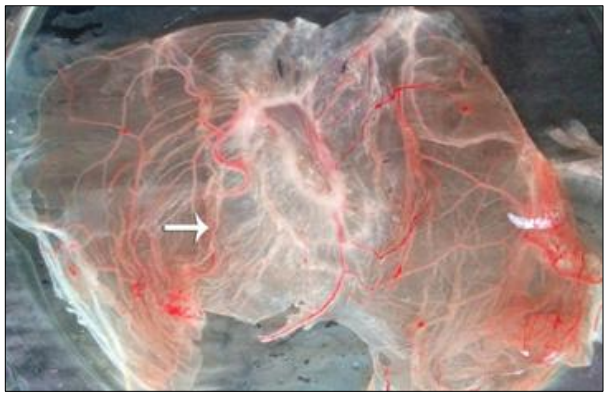


Plate 9: Periodic development of pock lesions on infected CAM after 48 hr (field strain PPV)

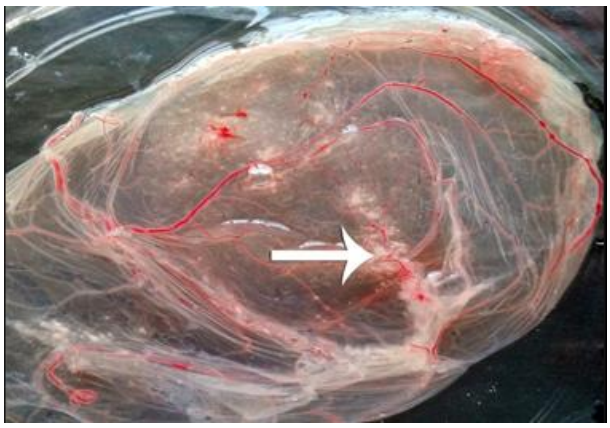


Plate 10: Showing infected CAM after 72 hr (field strain PPV)

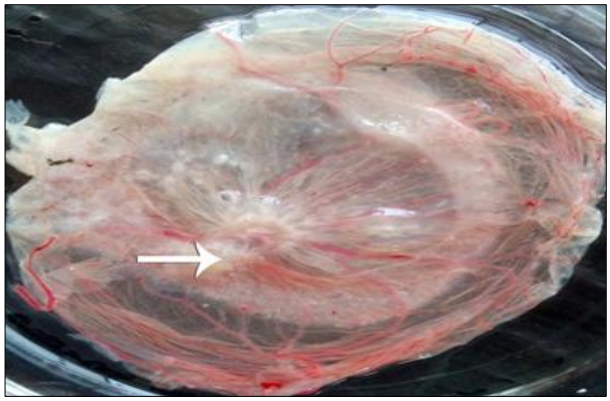


Plate 11: Showing infected CAM after 96 hr (field strain PPV)

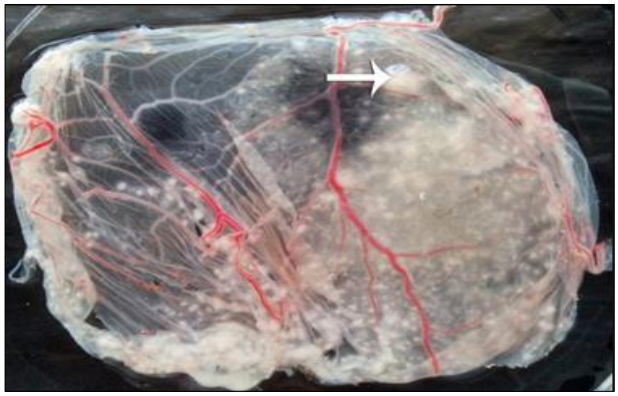
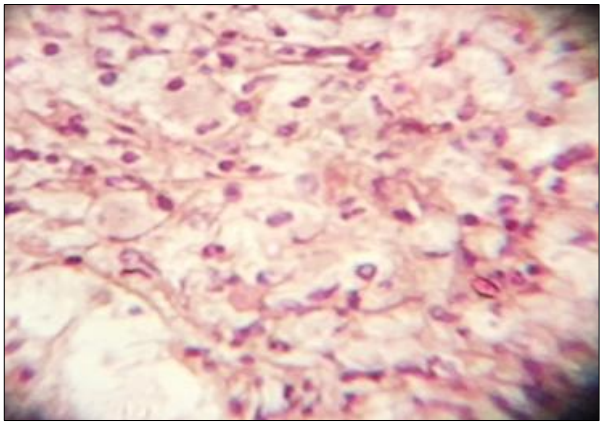


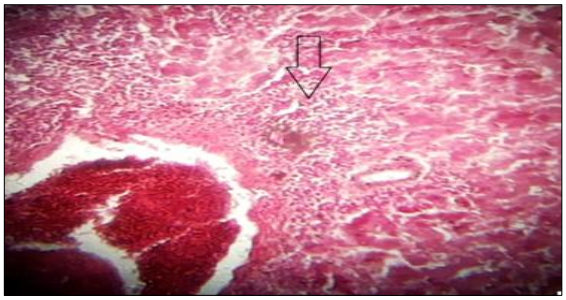
Plate 12: Showing infected CAM after 120 hr (field strain PPV)

Plate 13: Gross lesions of liver Note: Numerous white foci in liver



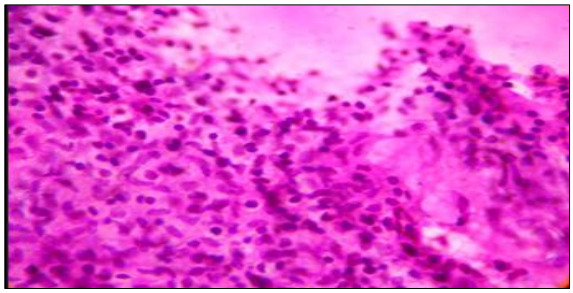
Note: Depicting heavy infiltration of PMN leucocytes and fibrinous exudates in alveoli and interstitial spaces

Plate 14: Histopathology of lungs (40X)



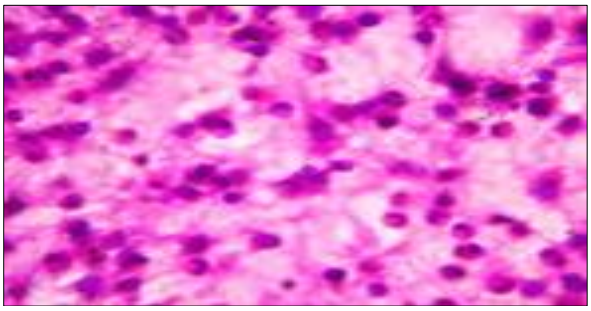
Note: Heavy infiltration of PMN leucocytes

Plate 15: Histopathology of liver (40X)



Note: Intracytoplasmic inclusions

Plate 16: Histopathology of infected CAM (field strain PPV)



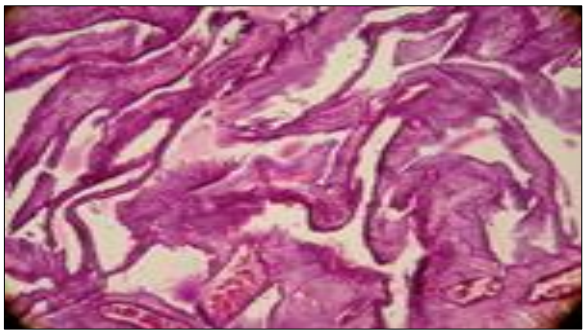
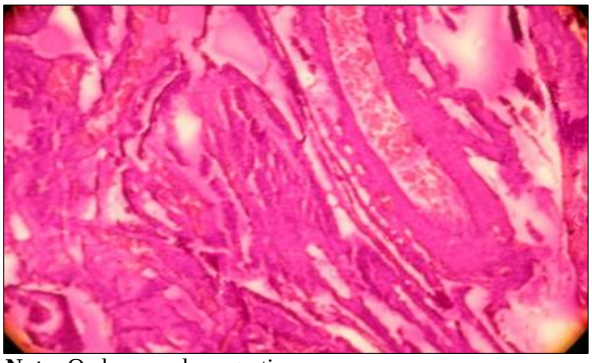
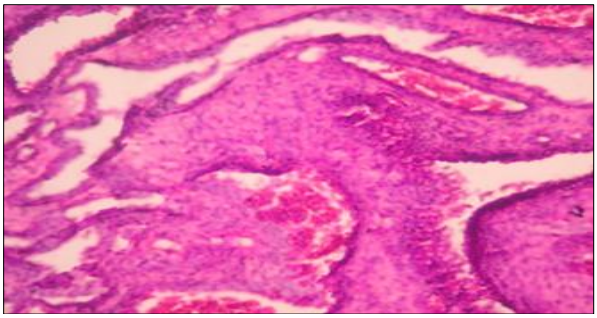


Plate 17: Histopathology of infected CAM (field strain FPV): Intracytoplasmic inclusions and congestion



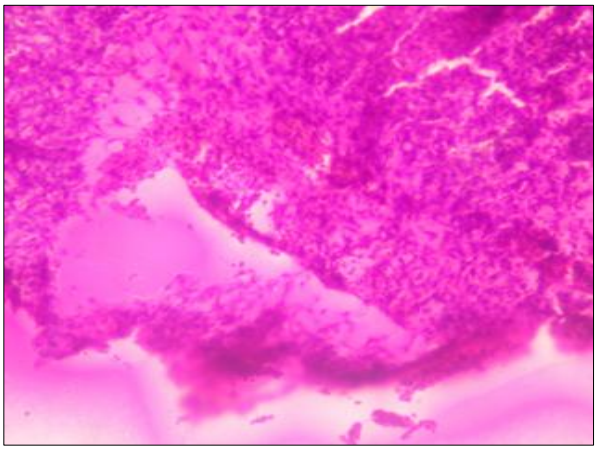
Note: Oedema and congestion

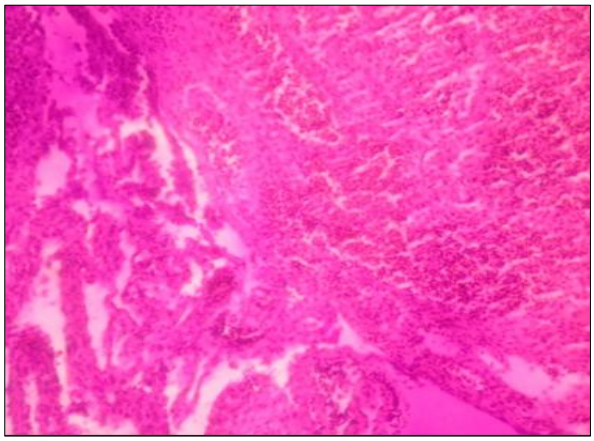
Plate 18: Histopathology of infected CAM after 48hr (field strain PPV)



Note: Oedema, haemorrhage, congestion and necrosis

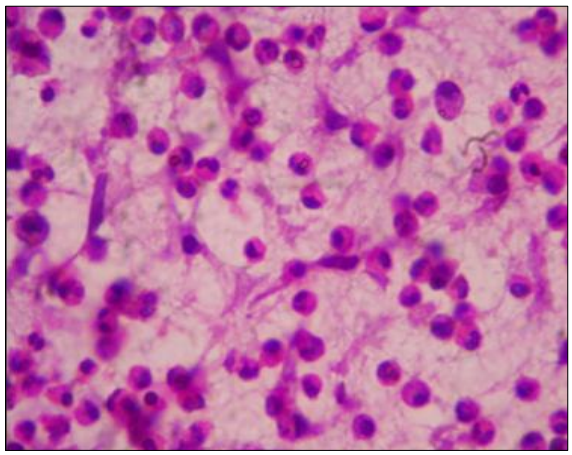
Plate 19: Histopathology of infected CAM after 72 hr (field strain PPV)

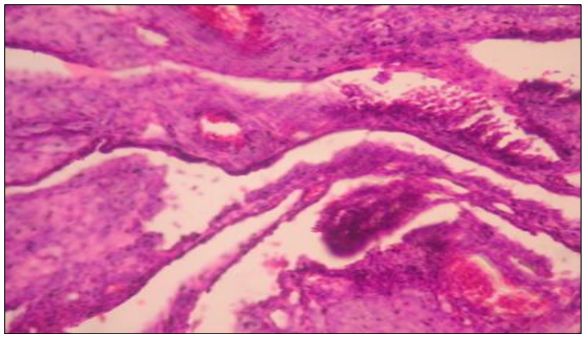




Note: Oedema, congestion and necrosis

Plate 20: Histopathology of infected CAM after 96 hr (field strain PPV)



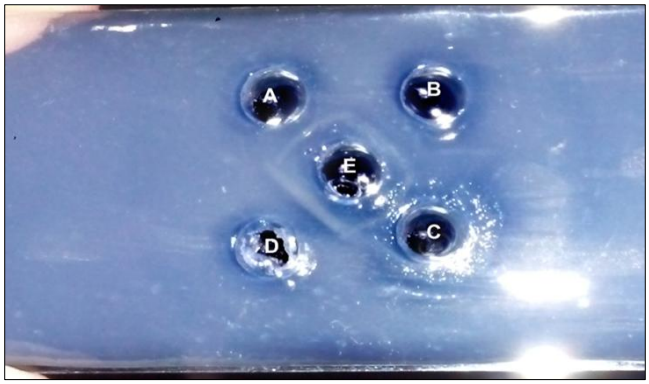


Note: Oedema, congestion, necrosis and inclusions in lymphocytes

Plate 21: Histopathology of infected CAM after 120 hr (field strain PPV)

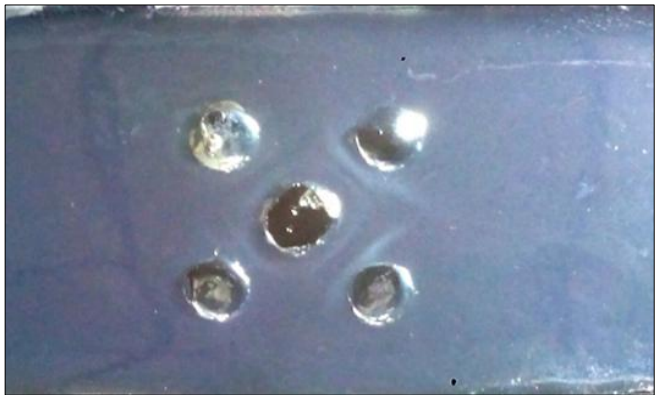
3.2 Agar Gel Immunodiffusion (AGID) Test

Both PPV and FPV field isolates produced clear precipitation bands against hyperimmune serum in AGID, showing a line of complete identity with vaccine strains (Plate 22 and Plate 23). This indicates antigenic similarity between field and vaccine strains. Comparable results were reported by Elamin *et al.* (2004) [9], Gulbahar *et al.* (2005) [11], and Al-Attar *et al.* (2007) [2]. The findings confirm the suitability of AGID as a simple, rapid diagnostic assay for avipoxviruses, consistent with previous reports (Tamador *et al.* 2001; Smits *et al.* 2005; Gilhare, 2012) [27, 26].



Note: Clear precipitation line against PPV hyperimmune sera in central well

Plate 22: Precipitation lines in AGID
A- FPV (vaccine), B- FPV (field), C- PPV (vaccine), D- PPV (field), E- Hyperimmune sera of PPV



Note: Precipitation line against FPV hyperimmune sera in central well

Plate 23: Distinct precipitation lines in AGID
A- FPV (vaccine) B- FPV (field), C- PPV (vaccine), D- PPV (field), E- Hyperimmune sera of FPV

3.3 Counter Immuno-Electrophoresis (CIE)

Field strains of PPV and FPV reacted specifically with their respective antisera, producing distinct precipitation lines within 1 hr in CIE (Plate 24). These observations are in agreement with earlier reports (Sarma & Sharma, 1987; Das *et al.* 1990; Tamador *et al.* 2001; Gilhare, 2012) [6, 27], highlighting CIE's sensitivity and rapidity for serological detection.

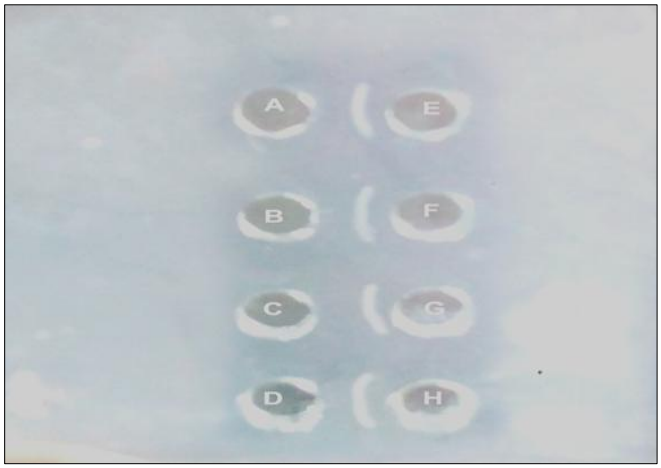


Plate 24: Showing clear precipitation lines in CIE
A- PPV (field) B- PPV (field), C- FPV (field), D- FPV (field)
E, G- Hyperimmune sera of PPV, F, H- Hyper immune sera of FPV

3.4 Haemagglutination (HA) Test

Both field and vaccine strains of PPV and FPV exhibited HA activity. The highest titre of 1:640 was recorded for PPV (field strain) and 1:320 for FPV (field strain) at the third passage (Plate 25).

Table 4 presents the HA titre of field strains of PPV and FPV at different passage levels. At the 2nd passage, the HA titre of PPV was 1:320, while FPV showed a lower titre of 1:160. At the 3rd passage, the titre increased for both viruses, with PPV reaching 1:640 and FPV 1:320, indicating higher antigenic activity with successive passages. Similarly, Table 5 shows the HA titre of vaccine strains of PPV and FPV at the 3rd passage, where PPV exhibited a titre of 1:640 and FPV 1:320, which closely resembled the values observed in field strains, suggesting consistent replication and antigenicity of both vaccine and field strains.

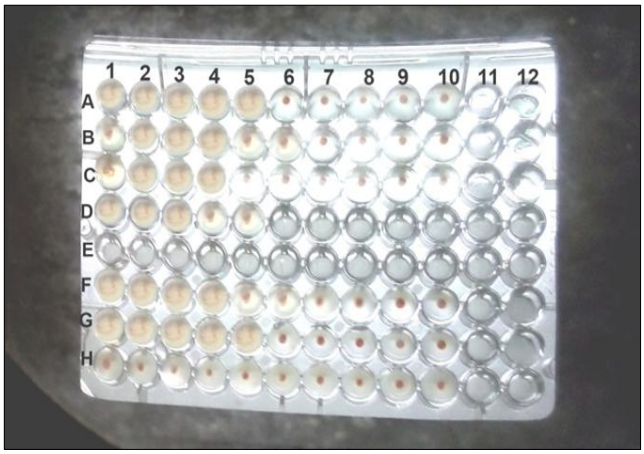


Plate 25: Showing HA titre pattern
A- 2nd passage PPV (field), B- 3rd passage PPV (field), C- 2nd passage FPV (field), D- 3rd passage FPV (field) E- Negative control, F- 2nd passage PPV (vaccine), G- 2nd passage FPV (vaccine) H- positive control

Table 4: HA titre of field strains of PPV and FPV

Level of passage	PPV	FPV
2nd	1:320	1:160
3rd	1:640	1:320

Table 5: HA titre of vaccine strains of PPV and FPV

Level of passage	PPV	FPV
3rd	1:640	1:320

HA titres observed are comparable with the findings of Rouhandeh (1956) [22] and Narain *et al.* (1979) [18], who reported HA activity in avipoxviruses. The constant HA titre of 1:320 in PPV cell culture medium indicates stability of viral haemagglutinin across passages.

3.4 Polymerase Chain Reaction (PCR)

DNA extracted from infected CAM yielded amplification of the conserved P4b gene, producing the expected 578 bp amplicon (Plate 26). Similar results have been reported by Lee & Lee (1997) [13], Smits *et al.* (2005) [26], Rampin *et al.* (2006) [21], Manarolla *et al.* (2010) [14], Abdallah & Hassanin (2012) [1], and Masola *et al.* (2014) [15]. PCR thus proved to be a highly specific and sensitive tool for confirmatory diagnosis of PPV and FPV infections.

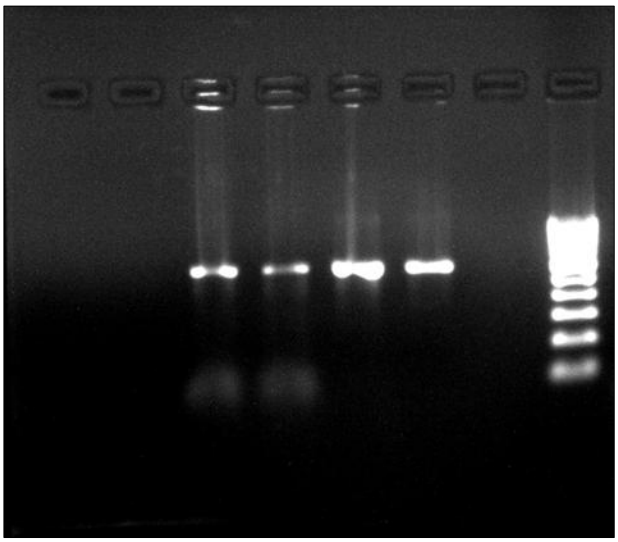


Plate 26: Agarose Gel showing PCR products

Lane 1- FPV (field)
Lane 2- PPV (field)
Lane 3- Positive control (FPV vaccine)
Lane 4- Positive control (PPV vaccine)
Lane 5- Negative control
Lane 6- Ladder (100 bp)

4. Conclusion

The present study successfully isolated and characterized pigeon pox virus (PPV) and fowl pox virus (FPV) from field outbreaks in Chhattisgarh using embryonated chicken eggs, histopathology, serological assays, and PCR. Progressive lesions in CAM, including oedema, congestion, necrosis, haemorrhage, and intracytoplasmic inclusions, confirmed viral pathogenicity, while AGID and CIE demonstrated antigenic similarity between field and vaccine strains. Haemagglutination assays revealed stable titres for both viruses, and PCR targeting the conserved P4b gene produced a 578 bp amplicon, confirming the presence of avipoxvirus. These findings highlight the diagnostic reliability of combining classical and molecular techniques for avipoxvirus detection and emphasize the antigenic stability of field and vaccine strains, which is crucial for effective diagnosis, epidemiological monitoring, and future vaccine development.

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6. Future Scope

- **Genomic characterization:** Whole-genome sequencing of field strains of PPV and FPV to identify genetic variations and evolutionary relationships with vaccine strains.
- **Vaccine evaluation:** Experimental studies to assess the efficacy of existing vaccines against emerging field isolates and to explore the need for updated or recombinant vaccines.
- **Diagnostic advancement:** Development of rapid, field-applicable molecular and serological diagnostic tools for early detection and differentiation of avipoxviruses.
- **Immunopathological studies:** Investigation of host immune responses and pathogenesis in different avian species to better understand virus-host interactions and improve disease management strategies.

7. Conflict of Interest

The author declares no conflict of interest regarding the publication of this research work.

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