Isolation of Reticuloendotheliosis Virus from Chorio-allantoic Membrane of SPF Chicken Eggs inoculated with Fowl Pox Virus

Nilabja Roy Chowdhury[1], Bimalendu Mondal[2], Sanchay K. Biswas[3], Apratim Maity[1], Kunal Batabyal[4] and Subhasis Batabyal[1]*

[1]*Department of Veterinary Biochemistry, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal & Fishery Sciences, Kolkata, West Bengal, India.*
[2]*Division of Virology, ICAR-Indian Veterinary Research Institute, Mukteshwar, Dist. Nainital, Uttarakhand, India.*
[3]*Joint Directorate, Centre for Animal Disease Research and Diagnosis, ICAR- Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India.*
[4]*Department of Veterinary Microbiology, Faculty of Veterinary & Animal Sciences, West Bengal University of Animal & Fishery Sciences, Kolkata, West Bengal, India.*

**Corresponding author at: Department of Veterinary Biochemistry, Faculty of Veterinary & Animal Sciences, West Bengal University of Animal & Fishery Sciences, Kolkata, West Bengal, India.*
E-mail: batabyals2009@gmail.com.

Veterinaria Italiana 2023, **59** (4), x-xx. doi: 10.12834/VetIt.3164.21331.2
Accepted: 24.05.2023 | Available on line: 31.12.2023

Keywords
Egg-Inoculation, Fowlpox, Reticuloendotheliosis, Retrovirus, Virus isolation.

Summary
Fowl Pox Viruses (FPV) infect chickens and turkeys giving rise to pock lesions on various body parts like combs, wattles, legs, shanks, eyes, mouth etc. The birds, affected with FPV, also show anemia and ruffled appearance which are clinical symptoms of Reticuloendotheliosis. Interestingly, the field strains of FPV are integrated with the provirus of Reticuloendotheliosis Virus (REV). Due to this integration, the infected birds, upon replication of FPV, give rise to free REV virions, causing severe immunosuppression and anemia. Pox scabs, collected from the infected birds, not only show positive PCR results upon performing FPV-specific ***4b core protein*** gene PCR but also show positive results for the PCR of REV-specific ***env*** gene and ***FPV-REV 5'LTR junction***. Homogenized suspension of the pock lesions, upon inoculating to the Chorio-allantoic Membrane (CAM) of 10 days old specific pathogen-free embryonated chicken eggs, produces characteristic pock lesions in serial passages. But the lesions also harbor REV mRNA or free virion, which can be identified by performing REV-specific ***env*** gene PCR using REV RNA from FPV-infected CAMs. The study suggests successful replication and availability of REV mRNA and free virion alongside the FPV virus, although the CAM is an ill-suited medium for any retroviral (like REV) growth and replication.

Introduction

Fowl Pox Virus (FPV) is a prototypical member of the *Avipoxvirus* genus under the *Chordopoxvirinae* subfamily belonging to the *Poxviridae* family (Skinner, 2008). Poxvirus diseases of poultry and other domestic birds (canaries and pigeons) have a worldwide economic impact (Welker *et al*., 1998; Afonso *et al*., 2000). It causes loss of egg production in layer birds, reduced growth rates in broiler birds, and blindness and lethality in acute cases. Nevertheless, the FPV virus infects chickens and turkeys. It is known to cause two forms of the disease, namely dry pox (cutaneous form) and wet pox (diphtheritic form), associated with different routes of infection (Tripathy and Reed, 2013). The cutaneous form, the most common, occurs following infection by biting arthropods serving as mechanical vectors for viral transmission. The disease is characterized by rapid inflammation, followed by scab formation. Also, the desquamation of degenerated epithelial tissues is evident with a predisposition to cellulitis. The diphtheritic form involves droplet infection of

Please refer to the forthcoming article as: Roy Chowdhury *et al*. 2023. Isolation of Reticuloendotheliosis Virus from Chorio-allantoic Membrane of SPF Chicken Eggs inoculated with Fowl Pox Virus. Vet Ital. doi: 10.12834/VetIt.3164.21331.2.

the mucous membrane of the upper respiratory tract. The prognosis of this form is poor due to asphyxiation of the respiratory tract, which leads to lethality.

Compellingly, FPV field strains are integrated with either full-length or near full-length provirus of Reticuloendotheliosis Virus (REV) (Singh *et al*., 2003; Biswas *et al*., 2011; Niewiadomska and Gifford, 2013; Joshi *et al*., 2019; Woźniakowski *et al*., 2018). REV is a member of the genus *γ-Retrovirus*, under the family *Retroviridae*. It is known to cause severe immunocompromising in young birds including lymphoid and non-lymphoid tumor formation, atrophy of reticuloendothelial organs (like bone marrow, spleen, liver and kidney), anemia and abrogation of immune cells. These symptoms are collectively known as the Runting Syndrome (Okoye *et al*., 1993; Cheng *et al*., 2006; Biswas *et al*., 2011; Etienne and Emerman, 2013). Also, Nakanuke disease is seen due to REV infection in birds showing malformed feathers (Woźniakowski *et al*., 2018). In FPV-infected birds, not only the clinical pox lesions seen but also signs of REV infection are depicted.

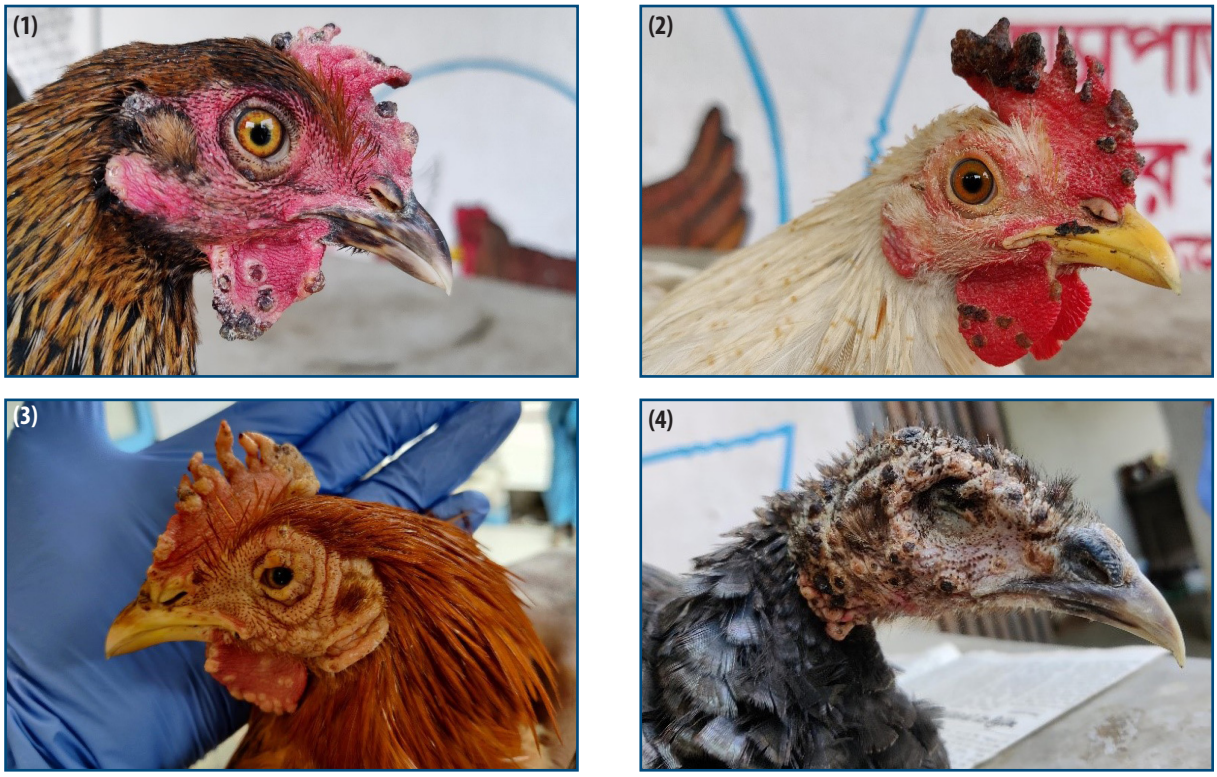
Chorio-allantoic membrane (CAM) of Specific Pathogen free (SPF) embryonated chicken eggs (ECE) are suitable medium for FPV culture (Gilhare *et al*., 2015). Pock lesions are observed after inoculating FPV into SPF ECEs by dropped CAM method. The lesions can be hemorrhagic petechiae or white diphtheritic in nature with unusual thickening of the CAM (Gilhare *et al*., 2015). The dropped CAM method of inoculation is still used to culture FPV field strains and to produce commercial Fowl Pox vaccines (OIE, 2013).

There are several reports of the integration of REV provirus into the FPV genome and identification of REV free virions in FPV-infected birds showing clinical symptoms of both viral infections. But no distinct study shows REV free virions originating from FPV itself. As there are incidences that segments of REV provirus can also integrate into the host bird genome and recur during FPV infection, a study defining the origination of REV from the FPV genome fills the void. In this study, local isolation of REV RNA is depicted upon inoculation of FPV field strains in SPF ECEs via dropped CAM method.

Materials and methods

Collection of Field Samples from Fowl Pox Outbreak

Field samples of Fowl Pox and Turkey Pox outbreaks were obtained from the Barasat-I block Veterinary Hospital, West Bengal, India. The birds showed characteristic pock lesions on combs, wattles, legs and surrounding areas of beak and eyes. In the case of young birds, severe anemia and feather deformity were seen (Fig 1-4).



Figures 1-4. *1, 2 & 3: Chickens showing pock lesions on combs, wattles and areas surrounding the eye. 4: Pock lesions of pox affected turkey.*

All the fowl pox-affected birds were not vaccinated with fowl pox vaccines. No pharyngeal lesions or ocular lesions were seen in all those birds. The lesions were procured from the hospital in sterile sample vials and stored at -20°C.

DNA extraction from the pock lesions

The pock lesions were triturated and homogenised in phosphate buffer solution to make 10% suspension. GeneJET Viral DNA/RNA Purification Kit, Thermo Scientific™ was used to extract DNA from the prepared suspension. DNA extraction protocol, provided with the kit, was followed to extract the DNA. Extracted DNA was eluted with 50µl Nuclease Free Water.

Polymerase Chain Reaction for FPV Gene

Fowl pox specific PCR was done to amplify and identify the ***4b core protein*** gene of FPV. The primers used in the reaction were adopted from the works of Lee and Lee (Lee and Lee, 1997). The primer sequences for this particular reaction were as follows:

- Forward Primer (P1F):
  5′ – **CAG CAG GTG CTA AAC AAC AA** – 3′
- Reverse Primer (P2R):
  5′ – **CGG TAG CTT AAA CGC CGA ATA** – 3′

The reaction mixture was of 50 µl total volume containing 10mM Tris-HCl (pH: 8.0), 50mM KCL, 0.08% NP 40, 1.5mM MgCl2, 200µM of each dNTP, 5 µl DNA, 0.2 µM Primers and 1.5U Taq Polymerase (Thermo Scientific). The whole reaction was carried out in a thermal cycler maintaining thermal conditions as follows:

Primary Denaturation	94°C		**5 minutes**
Subsequent Denaturation	94°C	30 cycles	**1 minute**
Annealing	58°C		**1 minute**
Amplification	72°C		**1 minute**
Final Amplification	72°C		**10 minutes**

10µl of PCR products (expected to be 578 bp for the positive sample) were separated in 1% agarose gel electrophoresis.

Polymerase Chain Reaction for REV and its integration site in FPV genome

Amplification of the ***env*** gene present (present within the range of 5999 to 6806 bp) within the REV provirus was carried out using the following sets of primers as depicted by Davidson & Malkinson (1996):

- Forward Primer (P3F):
  5´ - **TGA CCA GGC GGG CAA AAC C** - 3´
- Reverse Primer (P4R):
  5´ - **CGA AAG GGA GGC TAA GAC T** - 3´

Identification of 5′ ***Long Terminal Repeats (LTR)*** junction region of the REV provirus with the FPV was carried out by using the following sets of primers as depicted by Wang *et al*. (Wang *et al*., 2006):

- Forward Primer (P5F):
  5´ - **ACC TAT GCC TCT TAT TCC AC** – 3′
- Reverse Primer (P6R):
  5′ – **CTG ATG CTT GCC TTC AAC** – 3′

10µl of each of the PCR products (expected to be 807bp and 370bp respectively for the positive samples) were separated in 1% agarose gel electrophoresis. In both cases, the PCR annealing temperatures were 52°C.

Egg Inoculation of viruses and subsequent harvesting

Serially diluted 10% pock scab-tissue suspensions of 20µl were inoculated into the 10 days old SPF embryonated chicken eggs via dropped CAM method. The eggs were incubated at 37°C for another 5 days maintaining a 60% relative humidity.

The CAM tissues which showed swelled areas and pock lesions (haemorrhagic and diphtheritic whitish lesions) were collected and triturated with 1X Phosphate Buffer Solution to make 10% homogenized suspension in the post-incubation period. DNA extraction of the harvested CAMs was done for performing FPV-specific ***4b core protein*** gene PCR to make sure of the presence of FPV in the CAM tissue. Subsequent passages of the viruses were conducted afterward.

RNA extraction from CAM suspension and DNase I treatment

RNA extraction from the 10% CAM suspensions was done using TRIzol solution with subsequent precipitation with isopropanol followed by RNA pellet dissolution with nuclease-free water. Briefly, 200µl of CAM suspension was digested with 1ml TRIzol solution. The addition of 200µl chloroform & subsequent centrifugation gave rise to a clear supernatant aqueous phase containing the RNA. Precipitation of RNA with Isopropanol and subsequent washing with 75% ethanol precipitated RNA pellet. The pellet was dissolved in 30µl nuclease-free water. The dissolved RNA was treated with DNase I, RNase-free, Thermo Scientific™ to destroy the DNA impurities. Briefly, the DNase reaction buffer, DNase

I was added to the RNA and was incubated at room temperature for 30 minutes. This was followed by the addition of 50 mM EDTA. Subsequent heating of the mixture at 65°C for 10 minutes deactivated the DNase without degrading the RNA.

The RNA was then again precipitated with isopropanol and washed with 75% ethanol and dissolved in 30µl nuclease-free water.

Preparation of cDNA and performing REV-specific env PCR

cDNA was synthesized from the DNase I, RNase-free treated RNA, extracted from the samples by the following protocol:

a. Mixture A was made by adding 10.5 µl RNA and 2 µl random hexamer primers, subsequently heating to 90°C for 5 minutes. This was followed by snap-chilling of the mixture.

b. Mixture B was made by adding the following components: 5X RT Reaction Buffer (4 µl), Riboblock RNase Inhibitor (0.5 µl), 10mM dNTP Mixture (2 µl), RevertAid RT (200U/µl) (Thermo Scientific) (1 µl).

c. Mixture A and Mixture B were added and were kept in the following thermal conditions: 25°C for 10 minutes, 42°C for 1 hour, 80°C for 5 minutes and holding at 4°C.

This was followed by a REV-specific ***env*** PCR where the expected 807bp product was separated in 1% agarose gel electrophoresis.

Results

The presence of FPV was confirmed by FPV-specific **4B** core gene PCR using pox scab samples from a chicken and a turkey. Both the samples showed amplicons of identical sizes (578bp) with the positive control (Fig. 5) upon performing gel-electrophoresis. The scabs were also positive for REV-specific ***env*** gene PCR (Fig. 6, lane 1-3) and **FPV-REV 5'LTR junction-specific** PCR (Fig. 6, lane 5-7).

The scab samples, once confirmed to be FPV positive, were inoculated to the CAMs of SPF ECEs. This gave rise to hemorrhagic and whitish-diphtheritic lesions on the infected CAMs (Fig. 7 & 8).

RNA was extracted from the CAMs showing pock lesions and cDNA was synthesized subsequently. REV-specific ***env*** PCR revealed the presence of ***env*** mRNA of REV (Fig. 9).

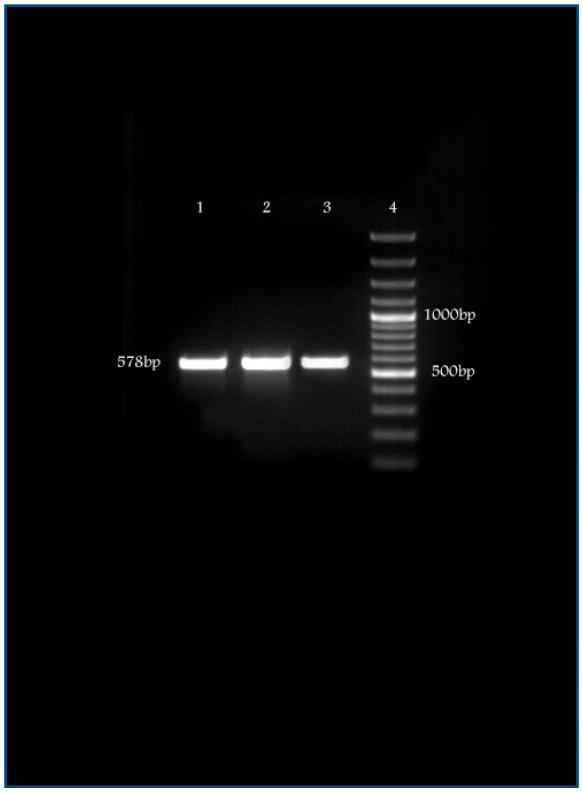


Figure 5. *Cropped agarose gel of FPV-specific Polymerase Chain Reaction of 4b core gene (578bp). Lane 1: Positive Control; Lane 2: Fowl Pox Scab Sample; Lane 3: Turkey Pox Scab Sample. Lane 4: 100bp DNA ladder.*

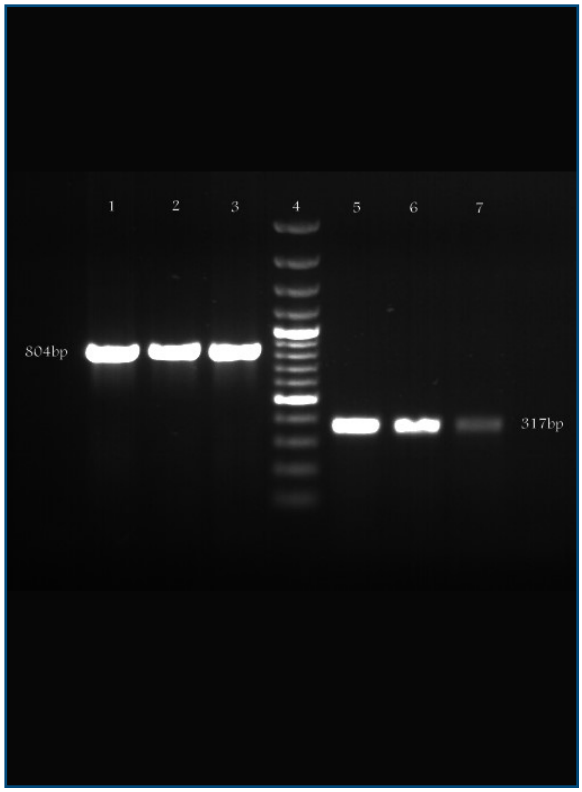


Figure 6. *Cropped agarose gel of REV-specific Polymerase Chain Reaction of env gene (Lane 1-3) (807 bp) and FPV-REV 5' LTR junction region (Lane 5-7) (370bp). Lane 1 & 5: Positive Control; Lane 2 & 6: Fowl Pox Scab Sample; Lane 3 & 7: Turkey Pox Scab Sample. Lane 4: 100bp DNA ladder.*

Veterinaria Italiana 2023, **59** (4), xx-xx. doi: 10.12834/VetIt.3164.21331.2

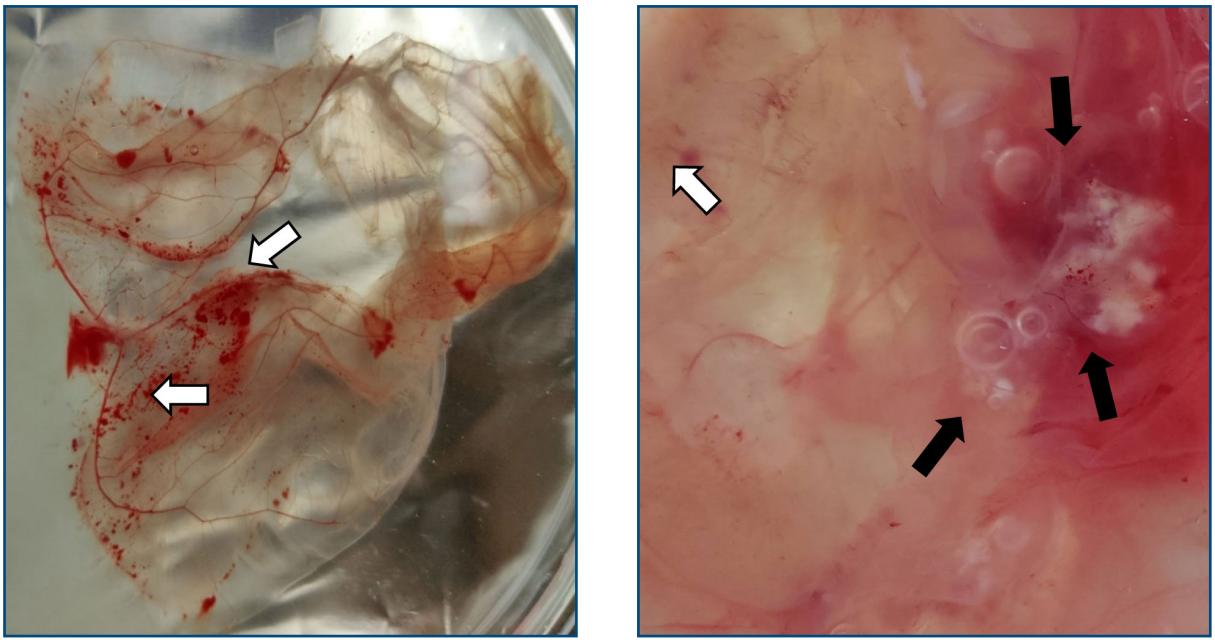


Figure 7-8. *Pock lesions on CAM of ECEs inoculated with FPV field strains on 2nd and 3rd passages respectively. White Arrows: Haemorrhagic Pock Lesions; Black Arrows: Characteristic Whitish diphtheritic pock lesion.*

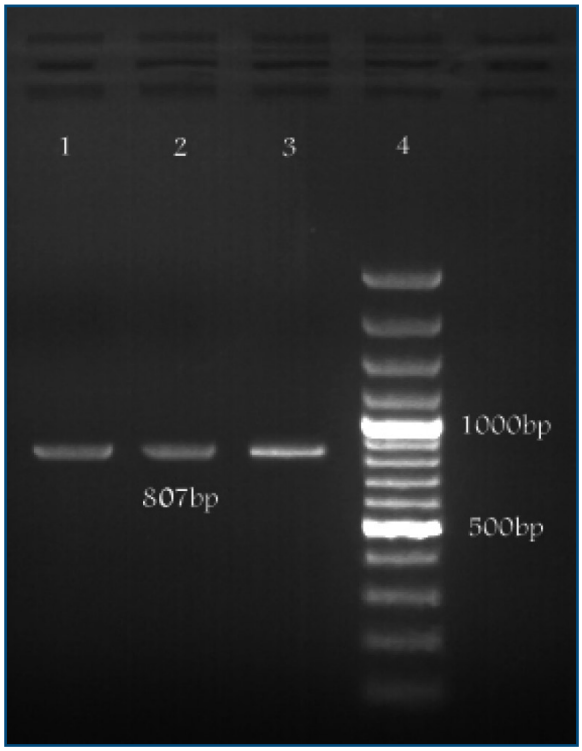


Figure 9. *Cropped agarose gel of REV specific Polymerase Chain Reaction of env gene from the synthesised cDNA from CAM pock lesion RNA (Lane 1-3) (807bp). Lane 1: Positive Control; Lane 2: Fowl Pox Scab Sample; Lane 3: Turkey Pox Scab Sample. Lane 4: 100bp DNA ladder.*

Discussion

FPV affected birds in this study showed characteristic cutaneous pock lesions on combs, wattles, beak and leg areas. They also displayed symptoms like anemia and deformed feathers which are characters of Reticuloendotheliosis infection. This can be justified by the fact that REV provirus uses its integration in the FPV genome to get transmitted from one bird to another. This also signifies vigorous spread of FPV, especially in the endemic regions, due to co-replication of REV which leads to marked immunosuppression of the host birds. Now, whether REV free virion is produced in the epithelial tissues (site of predilection for FPV) or reticuloendothelial tissues (site of predilection for REV) during viremic phases of FPV infection, was an open question. This study reports about possible local replication of REV along with the FPV. As discussed, positive results for env gene were shown upon doing RT PCR of CAM of ECEs inoculated with FPV. This clearly indicates that REV RNA was produced in those pock lesions which is indicative to the formation of REV free virion though being an ill-suited medium for REV's growth (Li *et al*., 2020).

Thus, this study indicates that REV mRNA or free virion production from FPV genome is a locally occurring event, i.e., occurring in the FPV predilection sites.

A few things were left out to evaluate is the possible epigenetic involvement of REV provirus which is integrated between ORF 201 and ORF 203 in the FPV genome (Fig. 10), where the provirus itself represents ORF 202. We were not able to check if the REV provirus also acts as an alternate enhancer segment, is an open question. Also, the fact that commercial vaccine strains of FPV still contains the junction of FPV and REV 5' LTR region is a matter of interest because it is reported that these LTRs

can also serve their epigenetic functionality in the host genome (Todd *et al*., 2019) which might be beneficial for the FPV for its replication strategy and natural selection (Li *et al*., 2020).

Nevertheless, we hereby report, for the 1st time, that REV free virion is originated during and from the FPV replication stages in the host tissues favorable to FPV's replication condition, using an in-ovo approach.

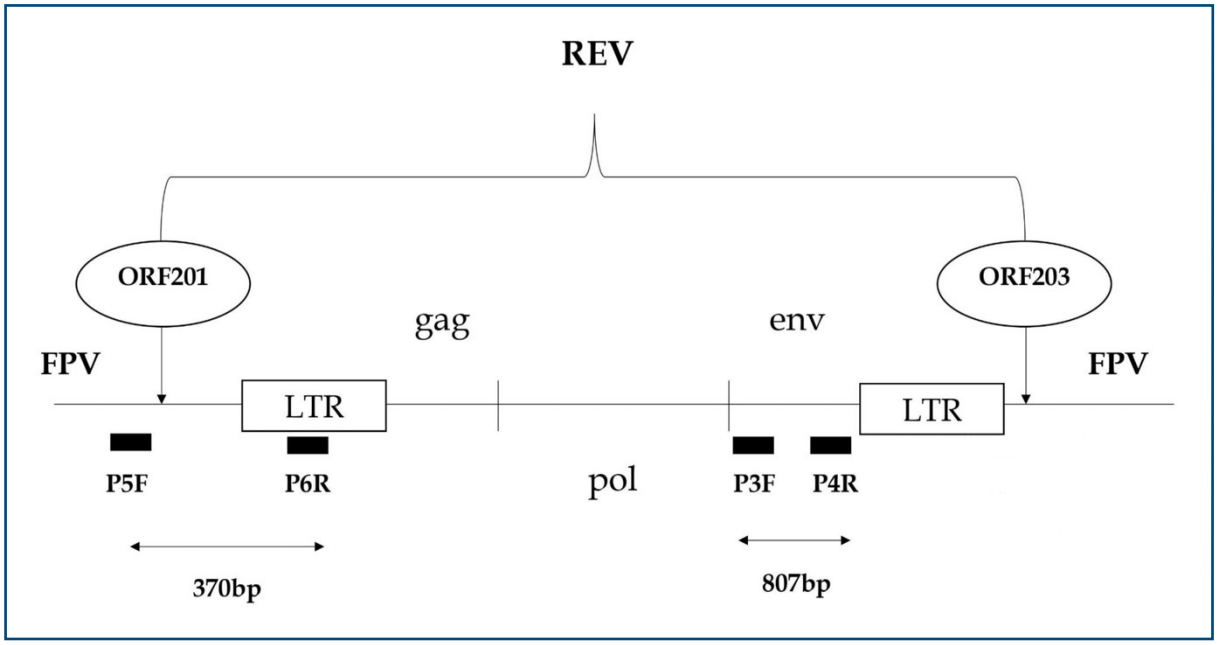


Figure 10. *Integration of REV provirus in the FPV genome. The full-length REV provirus spans from ORF 201 to ORF 203 of the FPV genome. The primer pairs depicted here are P5F & P6R (amplifies the 5' LTR flanking region of the FPV, amplicon size: 370bp); P3F & P4R (amplifies the env gene of REV provirus, amplicon size: 807bp).*

References

Afonso C.L., Tulman E.R., Lu Z., Zsak L., Kutish G.F. & Rock D.L. 2000. The Genome of Fowlpox Virus. *J Virol*, **74**, 3815–3831.

Biswas S.K., Jana C., Chand K., Rehman W. & Mondal B. 2011. Detection of fowl poxvirus integrated with reticuloendotheliosis virus sequences from an outbreak in backyard chickens in India. *Vet Ital*, **47**, 147–53.

Cheng W.H., Huang Y.P. & Wang C.H. 2006. Serological and virological surveys of reticuloendotheliosis in chickens in Taiwan. *Journal of Veterinary Medical Science*, **68**, 1315–1320.

Davidson I. & Malkinson M. 1996. A non-radioactive method for identifying enzyme-amplified products of the reticuloendotheliosis proviral env and LTR genes using psoralen-biotin labelled probes. *J Virol Methods*, **59**, 113–119.

Etienne L. & Emerman M. 2013. The Mongoose, the Pheasant, the Pox, and the Retrovirus. *PLoS Biol*, **11**, e1001641.

Gilhare V.R., Hirpurkar S.D., Kumar A., Naik S.K. & Sahu T. 2015. Pock forming ability of fowl pox virus isolated from layer chicken and its adaptation in chicken embryo fibroblast cell culture. *Vet World*, **8**, 245–250.

Joshi L.R., Bauermann F. V., Hain K.S., Kutish G.F., Armién A.G., Lehman C.P., et al. 2019. Detection of Fowlpox virus carrying distinct genome segments of Reticuloendotheliosis virus. *Virus Res*, **260**, 53–59.

Lee L.H. & Lee K.H. 1997. Application of the polymerase chain reaction for the diagnosis of fowl poxvirus infection. *J Virol Methods*, **63**, 113–119.

Li K., Liu Y., Xu Z., Zhang Yu, Yao Y., Nair V., et al. 2020. Prevention of Avian Retrovirus Infection in Chickens Using CRISPR-Cas9 Delivered by Marek's Disease Virus. *Mol Ther Nucleic Acids*, **21**, 343–353.

Niewiadomska A.M. & Gifford R.J. 2013. The Extraordinary Evolutionary History of the Reticuloendotheliosis Viruses. *PLoS Biol*, **11**.

OIE. 2013. FOWL POX. (https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.10_FOWLPOX.pdf accessed on).

Okoye J.O.A., Ezema W. & Agoha J.N. 1993. Naturally occurring clinical reticuloendotheliosis in Turkeys and chickens. *Avian Pathology*, **22**, 237–244.

Singh P., Schnitzlein W.M. & Tripathy D.N. 2003. Reticuloendotheliosis Virus Sequences within the Genomes of Field Strains of Fowlpox Virus Display Variability. *J Virol*, **77**, 5855–5862.

Skinner M.A. 2008. Fowlpox Virus and Other Avipoxviruses. *In* Encyclopedia of Virology Elsevier, 274–284.

Todd C.D., Deniz Ö., Taylor D. & Branco M.R. 2019. Functional evaluation of transposable elements as enhancers in mouse embryonic and trophoblast stem cells. *Elife*, **8**.

Tripathy D.N. & Reed W.M. 2013. Pox. *In* Diseases of Poultry Wiley, 333–349.

Wang J., Meers J., Spradbrow P.B. & Robinson W.F. 2006. Evaluation of immune effects of fowlpox vaccine strains and field isolates. *Vet Microbiol*, **116**, 106–119.

Welker P., Grabbe J., Grützkau A. & Henz B.M. 1998. Effects of nerve growth factor (NGF) and other fibroblast-derived growth factors on immature human mast cells (HMC-1). *Immunology*, **94**, 310–7.

Woźniakowski G., Frant M. & Mamczur A. 2018. Avian reticuloendotheliosis in chickens – an update on disease occurrence and clinical course. *J Vet Res*, **62**, 257–260.