Indian Journal of Animal Sciences **89**(4): 377–380, April 2019/Article
https://doi.org/10.56093/ijans.v89i4.89092

Molecular detection of integrated reticuloendothelial virus genes in fowlpox virus field isolates and live vaccines of poultry

RANJANI RAJASEKARAN[1], J JOHN KIRUBAHARAN[2], S RAJALAKSHMI[3] and M VIDHYA[4]

Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu 600 007 India

Received: 14 September 2018; Accepted: 18 October 2018

ABSTRACT

Two field isolates (FWPV-W1 and FWPV-W2) obtained from unvaccinated backyard poultry chickens and four commercial live vaccines (FWPV-G, FWPV-V, FWPV-B and FWPV-H) of fowlpox virus origin were isolated on chorio-allantoic membrane (CAM) of embryonated chicken eggs. The CAM tissues infected with FWPV-W1, FWPV-W2, FWPV-G, FWPV-V, FWPV-B and FWPV-H individually were subjected to DNA isolation. The isolated DNA was tested for the presence of P4b gene by PCR to confirm FWPV. Then, each of the field isolates and commercial vaccines were screened for presence of reticuloendothelial virus envelope (REV-*env*) gene and long terminal repeat (LTR) region by PCR. FWPV-W1 isolate was positive for REV-*env* gene (807 bp) and REV-LTR region (370 bp), which confirmed presence of near full-length REV integration in its genome. Whereas, FWPV-W2 isolate was positive for LTR region and negative for REV-*env* gene. This suggested that full-length REV is not present in all FWPV field isolates. All the four commercial live vaccines, were negative for REV-*env* gene. This showed that full-length REV is absent in these commercial vaccines, ensuring safety of the usage of these vaccines in India. However, the commercial vaccines were positive for REV-LTR region, which does not affect the vaccine safety.

Key words: Envelope gene, Field isolate, Fowlpox virus, Live vaccine, LTR region, Reticuloendothelial virus

Fowlpox is caused by fowlpox virus (FWPV), the prototype species of *Avipoxvirus* (APV) genus of *Chordopoxvirinae* subfamily of *Poxviridae* family (www.ictvonline.org). It is a contagious viral disease of poultry that occurs in two major forms, viz. cutaneous form and diphtheritic form. Both the forms could occur either independently or simultaneously. In addition to these two forms of fowlpox disease, a systemic infection could also occur causing increased mortality of birds (Tripathy and Reed 2003). The different forms of the disease are predominantly attributed to virulence of the virus and susceptibility of the host. The virulence of the virus is modified by the integration of reticuloendothelial virus (REV) in FWPV genome, causing immunosuppression, thereby increasing the susceptibility of the host to FWPV infection (Tadese *et al*. 2008).

REV is an oncogenic retrovirus, that gets integrated in FWPV by natural recombination events (Ding *et al*. 2004). REV was detected in various FWPV field isolates and vaccine strains (Hertig *et al.* 1997; Awad *et al*. 2010). The FWPV field isolates carrying REV were found to be more pathogenic to birds, causing immunosuppression and increased mortality (Garcia *et al*. 2003). REV is a potential vaccine contaminant and that was encountered earlier with Marek's disease vaccine. The presence of REV in FWPV vaccines altered the safety and efficacy of the vaccine (Fadly *et al*. 1996). As REV causes immunosuppression in birds, vaccination of birds with REV integrated FWPV vaccines did not provide protection against fowlpox disease. Also, these REV integrated FWPV vaccines caused tumors in vaccinated birds (Fadly *et al*. 1996).

Therefore, to detect REV integration in Indian field isolates obtained from unvaccinated backyard poultry and Indian fowlpox live vaccines, this study was attempted using PCR amplification of REV-envelope gene (Wilhelmsen *et al.* 1984) and PCR amplification of long terminal repeat region (Wang *et al*. 2006). The phenomenon behind this molecular technique is depicted in Fig. 1. The integration of REV into FWPV genome is a natural event (Singh *et al*. 2003), which integrates *gag, pol* and *env* genes flanked on either side by long terminal repeat regions (LTR). Likewise, the elimination of *gag, pol* and *env* genes from FWPV genome is also a natural event, which leaves LTR as remnants. Therefore, PCR amplification of REV-*env* gene depicts the presence of near full-length REV integration, whereas PCR amplification of only the LTR region depicts previous integration of full-length REV that was excluded from FWPV genome eventually (Davidson *et al*. 2008).

Present address: [1]PhD Scholar (dr.ranjanivet@gmail.com), Department of Veterinary Microbiology, [2]Dean (jjohnk@gmail.com), Madras Veterinary College, Chennai, Tamil Nadu. [3,4]Assistant Professor (rajalakshmi.s@tanuvas.ac.in, vidhyamahalingam@gmail.com).

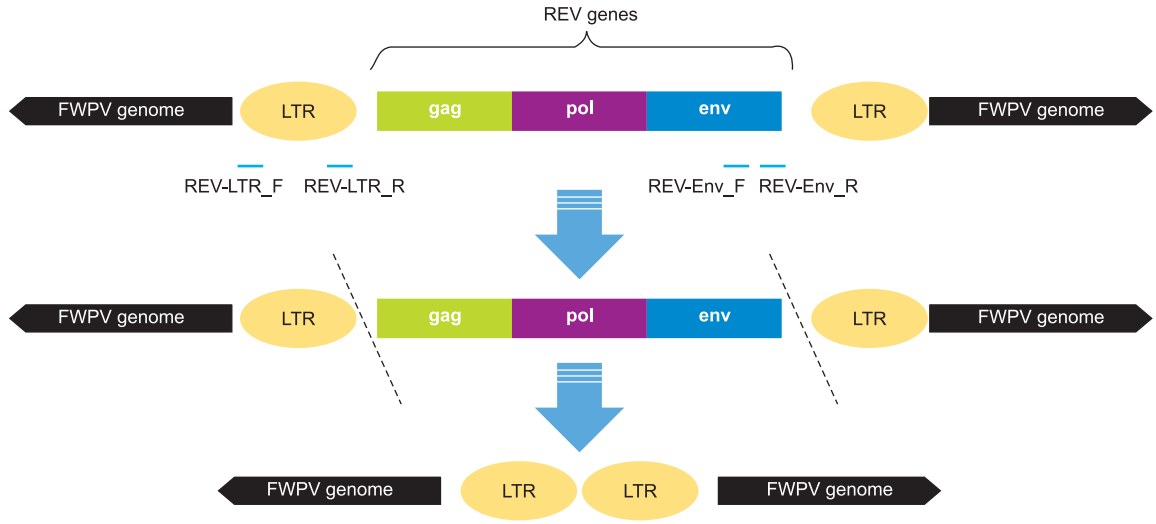

Fig. 1. Schematic representation of the integration of full-length REV in FWPV genome and the presence of LTR remnants after exclusion of REV. Gross representation of primer binding sites of REV-*env* gene and REV-LTR region are shown.

MATERIALS AND METHODS

Viruses: Two FWPV field isolates and four commercial vaccines were used in this study. The field isolates were collected as dried scab materials from unvaccinated backyard poultry chicken in Chennai (FWPV-W1) and Puducherry (FWPV-W2). The vaccines were obtained from Globion Pvt Ltd (FWPV-G), Venky's Pvt Ltd (FWPV-V), BioMed Pvt Ltd (FWPV-B) and Hester Biosciences Pvt Ltd (FWPV-H).

Virus isolation: FWPV-G, FWPV-V, FWPV-B and FWPV-H were re-suspended in sterile phosphate buffered saline (PBS) and 0.1 ml was inoculated onto chorio-allantoic membrane (CAM) of 13-day old specific pathogen free embryonated chicken eggs and was incubated at 37°C for 5 days (Tripathy and Reed 2003). Dried scab material of FWPV-W1 and FWPV-W2 were triturated separately in PBS containing 50 IU/ml pencillin and 50 µg/ml streptomycin. The suspension was clarified by centrifugation at 3,000 rpm for 10 min and 0.1 ml of the supernatant was inoculated onto CAM.

DNA isolation: Total DNA was isolated from CAM tissues infected with FWPV-W1, FWPV-W2, FWPV-G, FWPV-V, FWPV-B and FWPV-H respectively using QIAamp DNA kit (Qiagen, Germany) following manufacturer's instructions. Briefly, the FWPV infected CAM tissue was triturated in mortar and pestle and was lysed using proteinase-K. The lysate was bound to the silica membrane which was washed twice using 75% ethanol and the total DNA was eluted in 50µl of elution buffer. The concentration of DNA was estimated in spectrophotometer using elution buffer as blank.

PCR amplification of P4b gene: Primers described in Table 1 were used for PCR amplification of P4b gene to detect the presence of FWPV in CAM infected with FWPV-W1, FWPV-W2, FWPV-G, FWPV-V, FWPV-B and FWPV-H. The reaction mixture comprised 5 µl of PrimeStar master mix (Clonetech, USA), 0.5 µM of each primer and 50 ng of total DNA in a final reaction volume of 10 µl. The reaction cycle involved an initial denaturation at 98°C/30 sec followed by 35 cycles of denaturation at 98°C/10 sec, 53°C/10 sec and extension at 72°C/30 sec with a final extension at 72°C/5 min. The amplified PCR product was separated by agarose gel (1.5%) electrophoresis stained with ethidium bromide and was viewed under ultraviolet light.

PCR amplification of reticuloendothelial virus genes: Primers described in Table 1 were used for PCR amplification of reticuloendothelial virus (REV) envelope (REV-*env*) gene and LTR region respectively. The reaction mixture of PCR comprised one unit of phusion DNA polymerase, 1.5 mM of $MgCl_2$, 200 mM of each dNTP, 0.5 mM of each primer and 50 ng of total DNA in a final reaction volume of 50 ml. The reaction cycle involved an initial denaturation at 98°C/30 sec followed by 35 cycles of denaturation at 98°C/10 sec, optimum annealing

Table 1. Primers used for P4b gene and REV gene amplification

Gene	Primer sequence (5'–3') (F-Forward; R-Reverse)	Ta (°C)	Product size (bp)	Reference
P4b	F: CAGCAGGTGC TAAACAACAA R: CGGTAGCTTA ACGCCGAATA	53	578	Binns *et al.* (1989)
REV-*env*	F: TGACCAGGC GGGCAAAACC R: CGAAAGGGA GGCTAAGACT	52	807	Wilhelmsen *et al.* (1984)
REV-LTR	F: ACCTATGCC TCTTATTCCAC R: CTGATGCTT GCCTTCAAC	52	370	Wang *et al.* (2006)

temperature/10 sec and extension at 72°C/30 sec with a final extension at 72°C/5 min. The amplified PCR product was separated by agarose gel (1.5%) electrophoresis and purified using Nucleospin Gel and PCR cleanup kit (Machery-Nagel, Germany) as per manufacturer's instructions. The PCR product was eluted in 20 ml elution buffer.

Nucleotide sequencing: The purified PCR elute of REV-*env* gene and REV-LTR region was subjected to nucleotide sequencing. The sequences were aligned using MEGA7.0 software (Kumar *et al*. 2016) and were subjected to BLAST analysis in NCBI.

RESULTS AND DISCUSSION

In present study, two field isolates and four commercial live vaccines of FWPV that was propagated on CAM were positive for P4b gene, which was amplified at 578 bp. P4b gene is the conserved gene of FWPV that is used as a molecular detection tool to confirm the presence of FWPV in a sample (Lee and Lee 1997). Upon confirmation by P4b gene PCR, the field isolates and commercial live vaccines of FWPV under this study were screened for the presence of REV gene by molecular methods. The integration of REV genes into FWPV genome is a naturally occurring recombination event that had occurred in FWPV isolates 50 years ago (Kim and Tripathy 2001). As FWPV genome consists of potential insertion sites to harbour foreign genes, REV genes get integrated into genomic sites of FWPV genome easily (Jones *et al*. 1993). It was observed that the integration of REV genes into FWPV genome had always occurred between open reading frame 201 and 203 (Davidson *et al*. 2008). Though this phenomenon has been positively exploited in FWPV vectored vaccine construction (Paoletti 1996), it has also necessitated the need to screen FWPV field isolates and commercial live vaccines for integration of REV genes. Such screening of FWPV field isolates for integrated REV genes gained importance when the pathogenicity of FWPV in poultry was observed to be altered (Garcia *et al*. 2003). Screening of commercial live FWPV vaccines for integration of REV genes is necessary because REV integrated FWPV vaccines do not provide protection against fowlpox due to the immunosuppressive effects induced by REV in birds (Singh *et al*. 2003). Further, a REV integrated FWPV vaccine would introduce REV infection in birds through vaccination, if full-length REV is present. The LTR remnants present in FWPV do not cause REV infection. Earlier, it was reported that, every FWPV isolate of chicken and turkey carried full-length REV genes (Garcia *et al*. 2003). In this study, only FWPV-W1 was positive for both REV-*env* gene and REV-LTR region amplifying an expected product size of 807 bp (GenBank Accession No: MH365471) and 370 bp (GenBank Accession No: MH365472) respectively. Whereas, FWPV-W2 was negative for REV-*env* gene and positive only for REV-LTR region. Thus, the integration of REV genes in FWPV is not universal (Davidson *et al*. 2008). The commercial vaccines, FWPV-G (GenBank Accession No: MH835451), FWPV-V (GenBank Accession no: MH835452), FWPV-B and FWPV-H were positive for REV-LTR region and negative for REV-*env* gene. The presence of LTR remnants in commercial live vaccines of FWPV has been reported earlier (Moore *et al*. 2000). Though some FWPV live vaccines have been reported for full-length REV contamination (Hertig *et al.* 1997, Awad *et al*. 2010), we report absence of full-length REV in these four commercial vaccines used in India.

In conclusion, the commercial vaccines were negative for full-length REV ensuring safe usage of these vaccines in poultry in India. Among the field isolates, only FWPV-W1 carried full-length REV genes, reconfirming the fact that not all FWPV field isolates would harbour REV genes.

ACKNOWLEDGEMENTS

The authors thank Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai, India for providing financial assistance and necessary facilities for the first author to carry out this research work.

REFERENCES

Awad A M, Abd El-Hamid H S, Abou Rawash A A and Ibrahim H H. 2010. Detection of reticuloendotheliosis virus as a contaminant of fowl pox vaccines. *Poultry Science* **89**(11): 2389–95.

Davidson I, Shkoda I and Perk S. 2008. Integration of the reticuloendotheliosis virus envelope gene into the poultry fowlpox virus genome is not universal. *Journal of General Virology* **89**(10): 2456–60.

Ding J, Cui Z, Yu L, Sun S and Jiang S. 2004. Study on the natural recombinant fowlpox virus containing genomic fragment from REV. *Acta Microbiologica Sinica* **44**: 588–92.

Fadly A M, Witter R L, Smith E J, Silva R F, Reed W M, Hoerr F J and Putnam M R. 1996. An outbreak of lymphomas in commercial broiler breeder chickens vaccinated with a fowl pox vaccine contaminated with reticuloendotheliosis virus. *Avian Pathology* **25**: 35–47.

Garcia M, Narrang N, Reed W M and Fadly A M. 2003. Molecular characterization of reticuloendotheliosis virus insertions in the genome of field and vaccine strains of fowl poxvirus. *Avian Disease* **47**: 343–54.

Hertig C, Coupar B E H, Gould A R and Boyle D B. 1997. Field and vaccine strains of fowlpox virus carry integrated sequences from the avian retrovirus, reticuloendotheliosis virus. *Virology* **235**: 367–76.

Jones D, Isofort R, Witter R, Kost R and Kung H J. 1993. Retroviral insertions into a herpesvirus are clustered at the junction of the short repeat and short unique sequences. *Proceedings of the National Academy of Sciences USA* **90**: 3855–59.

Kim T J and Tripathy D N. 2001. Reticuloendotheliosis virus integration in the fowl poxvirus genome: not a recent event. *Avian Disease* **45**: 663–69.

Kumar S, Stecher G and Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**: 1870–74.

Lee H L and Lee H K. 1997. Application of the polymerase chain reaction for the diagnosis of fowlpox virus infection. *Journal of Virological Methods* **63**: 113–19.

Moore K M, Davis J R, Sato T and Yasuda A. 2000.

Reticuloendotheliosis virus (REV) long terminal repeats incorporated in the genomes of commercial fowl poxvirus vaccines and pigeon poxviruses without indication of the presence of infectious REV. *Avian Disease* **44**: 827–41.

Paoletti E. 1996. Applications of pox virus vectors to vaccination: an update. *Proceedings of the National Academy of Sciences USA* **93**: 11349–353.

Singh P, Schnitzlein W M and Tripathy D N. 2003. Reticuloendotheliosis virus sequences within the genomes of field strains of fowlpox virus display variability. *Journal of Virology* **77**: 5855–62.

Tadese T, Fitzgerald S and Reed W M. 2008. Detection and differentiation of re-emerging fowlpox virus (FWPV) strains carrying integrated reticuloendotheliosis virus (FWPV-REV) by realtime PCR. *Veterinary Microbiology* **127**: 39–49.

Tripathy D N and Reed W M. 2003. Pox. *Diseases of Poultry*. 11th edn, pp. 253–269. (Eds) Saif Y M, Barnes H J, Glisson J R, Fadly A M, McDougald L R and Swayne D E. Iowa State Press Ames, Iowa.

Wang J, Meers J, Spradbrow P B and Robinson W F. 2006. Evaluation of immune effects of fowlpox vaccine strains and field isolates. *Veterinary Microbiology* **116**: 106–19.

Weli S C, Traavik T, Tryland M, Coucheron D H and Nilssen O. 2004. Analysis and comparison of the 4b core protein gene of avipoxviruses from wild birds: evidence for interspecies spatial phylogenetic variation. *Archives of Virology* **149**: 2035–46.

Wilhelmsen K C, Eggleton K and Temin H M. 1984. Nucleic acid sequences of the oncogene v-rel in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-rel. *Journal of Virology* **52**: 172–82.