RESEARCH ARTICLE

Development of a Single-Step SYBR Green based Real-Time PCR Assay for Identification and Quantification of Fowl Pox Virus in Chicken

Sanganagouda K.[1*], Sabha Kounin[1], Nagraja K.[1], Basavaraj Sajjanar[2], Amitha Rena Gomes[1], Aditya Prasad Sahoo[3], Alamelu M.S.[1]

Abstract

Fowlpox is a re-emerging and economically significant viral disease in poultry. Fowlpox is a complex virus contains a double-strand DNA genome of approximately 300 kb and largest among all pox viruses. In the present study, a clinical case of fowlpox virus (FPV) infection was investigated and a Real-Time PCR (RT-PCR) assay was developed for confirmatory diagnosis of fowl pox and quantification of viral load. Clinical sample was collected from infected bird reared in the organized poultry farm management system and the viral infection was confirmed using conventional PCR amplification of the P4b gene of fowl pox virus. Following that, a single-step SYBR green based Real-Time PCR assay was developed by targeting the same gene of fowlpox virus. The fowl pox viral load in the infected bird was found to be 7.33 ± 0.044 (Log Mean ± SD). The lower detection limit achieved was 134 copies per microliter (µL) with a corresponding threshold cycle (Ct) value of 32.59. Thus, the single-step SYBR green based RT-PCR assay targeting the P4b gene of fowl pox virus reported in this study was highly sensitive, specific and reproducible for the detection and quantitation of fowl pox virus nucleic acids.

Key words: Fowl pox, P4b gene, Real-Time PCR, SYBR green, Viral load.

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Introduction

Fowlpox (FP) is a highly contagious viral disease that affects domestic and wild birds of all ages, sexes, and breeds (Weli and Tryland, 2011). It is characterised by proliferative lesions in the skin that advance to form nodules (cutaneous form), lesions in the upper respiratory and gastro intestinal tracts (diphtheritic form), and in the internal organs (systemic form) (Tripathy, 2022). The causative agent Fowl pox virus (FPV), is double stranded (ds) (Afonso *et al.,* 2000) DNA virus that belongs to the family *Poxviridae* and subfamily *Chordopoxvirinae* (Luschow *et al.,* 2004; Matos *et al.,* 2022). Replication and maturation of the virus occur in the cytoplasm of the host cell (Andrew *et al.,* 2011; Yeo *et al.,* 2019). There are various unresolved questions about FPV, despite many studies over period of 150 years. First, several questions about the genetic characteristics of FPV remain unanswered because of their large genome size (260 to 320 kbp) (Andrew *et al.,* 2011; Yeo *et al.,* 2019). Comparative genetic identification, a core protein of 75.2 kDa is encoded by the avipox P4b protein gene. Among avian pox viruses (APVs), P4b (fpv 167) is a conserved gene that produces orthologs of the P4b core protein of the vaccinia virus (Nahed *et al.,* 2023). To detect APV infection, amplification of P4b fragment is unique of FPV by polymerase chain reaction (PCR) (Nahed *et al.,* 2023).

PCR is well established traditional method of virus identification. However, it is costly, laborious and time-consuming to perform (Manarolla *et al.,* 2010). Alternatively, Real time or quantitative PCR has gained wider acceptance over conventional PCR because it is more rapid, sensitive and reproducible (Mackay *et al.,* 2002). Few real time PCR assays have been described for detection and quantifying of FPV in clinical samples using TaqMan chemistry (Baek *et al.,* 2020; Hui-hui *et al.,* 2015; Ugwu *et al.,* 2023). However, SYBR green based real time PCR assay has the advantages of being more cost-effective, easy to design, precise and yielding a more linear decay plot than a TaqMan PCR assay (Zhang *et al.,* 2011;

[1]Institute of Animal Health and Veterinary Biologicals, Hebbal, Bengaluru-560024, Karnataka, India

[2]ICAR-Indian Veterinary Research Institute, Hebbal, Bengaluru-560024, Karnataka, India

[3]National Institute on Foot and Mouth Disease, Bhubaneswar, Odisha, India

Corresponding Author: Sanganagouda K, R&D Center, Institute of Animal Health and Veterinary Biologicals, Hebbal, Bengaluru-560024, India. e-mail: drpatilvet@gmail.com

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Abera *et al.,* 2014; Tajadini *et al.,* 2014; Peng *et al.,* 2018). The SYBR green based P4b gene Real-Time PCR for molecular diagnosis and viral load quantification in infected material and cell culture will significantly influence the diagnosis and control of fowlpox in chickens. The present study was focused on developing a single-step SYBR green based Real-Time PCR assay for the detection and quantification of FPV in clinical samples.

Materials and Methods

Ethical Approval

In the present study no animal experiment was conducted as the clinical samples were obtained from the outbreak investigations, hence no ethical committee approval was required. All laboratory work was conducted at the Institute of Animal Health and Veterinary Biologicals Laboratory, Bangalore, India

Viruses, Vaccine, Clinical Samples Collection and Processing

The live strain of fowl pox vaccine was procured from Hester Biosciences Limited, Mehsana, Gujarat, India. A biopsy sample (nodular tissue) was collected under local anaesthesia from the infected bird exhibiting FPV symptoms during the outbreak from an organized poultry farm, in Koppal district, Karnataka. The nodular tissue weighing 100 mg was triturated using a sterile mortar and pestle and suspended in phosphate-buffered saline (PBS) containing antibiotic and antimycotic solution (100 units of Penicillin, 0.1 mg of Streptomycin and 0.25 µg of Amphotericin B, Sigma-Aldrich, USA). The tissue homogenate was centrifuged at 9170 x g for 10 min at 4°C. The clear supernatant fluid was collected, frozen at -20°C, thawed 3 times, and centrifuged at 9170 x g for 10 min at 4°C. A volume of 2 mL of the supernatant was collected in sterile tubes and stored at –20°C until used.

Viral DNA Extraction

DNA was extracted from nodular tissue biopsy homogenate supernatant using the DNeasy Blood & Tissue kit (Cat no-69504, QIAGEN, Germany) following the manufacturer's instructions with some modifications as per the protocol described by Nomfundo *et al.,* (2023). Further, the DNA samples purity and concentration were estimated using the micro-volume UV spectrophotometer (Eppendorf, USA) by measuring absorbance at 260 nm and 280 nm using elution buffer as blank.

Primer Design and Synthesis

The forward and reverse primers were designed to amplify partial region of virion core protein P4b gene which is conserved across different avian pox viruses. For this purpose, we choose sequences of flamingo pox virus available in the NCBI GenBank database (Accession number: NC 036582). The designed primers (Forward_P4b_F: 5'-CGTACATCCAAGGTCCCATTT-3' and Reverse _P4b_R: 5'-TTCGATAGTACCACGGGTAGAG-3') having amplicon size of 415 bp, subsequently validated by OligoAnalyzer 1.2 and commercially synthesized (Eurofins Genomics, Bangalore, India) were used. Similarly, the Real Time PCR primers were designed the Forward 5'-TCTAAAGCCTGTAAAGCACTCTC-3' and Reverse 5'- ACTCTCCAGTAATAGTAAACGGAAC-3' in such a way that both the forward primer and reverse primer fall within the sequence frame of the P4b gene of amplicon size 415 bp.

Fowl Pox Virus Confirmation

The presence of the fowl pox virus in the outbreak investigated sample was confirmed with primer sets developed/designed from the gene virion core protein (P4b gene). the PCR reactions were prepared in a reaction volume of 25 µL containing 12.5 µL of 2x DreamTaq green PCR Master Mixture (Thermo Scientific, USA), 5.5 µL nuclease-free water (NFW), 5.0 µL of the extracted DNA template and 1.0 µL of each primer of 10 µM concentration. The PCR reactions were conducted under the following thermal conditions, including an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation (94 °C for 60 sec), annealing (60 °C for 30 sec), elongation (72 °C for 30 sec) and final elongation (72°C for 2 min). After that, the PCR products were resolved using 1.5% agarose gel containing 0.5 µg/ SYBR Safe DNA gel stain (Invitrogen, USA) along with 100 bp DNA ladder (Sigma-Aldrich, USA) in 1X Tris-Boric acid-EDTA (TBE) buffer at 100 V for 45 to 60 min. Further gel images were captured and analyzed under GelDoc Go Imaging System (Bio-Rad, USA) (Abdelfattah *et al.,* 2021).

Generation of Standards for Real-Time PCR

The amplicon size of 415 bp of fowl pox vaccine was bulk produced (~100 µL) by conventional PCR and purified using a QIAquick PCR purification kit (Cat no. 28104). The DNA sample's purity and concentration were obtained using the micro-volume spectrophotometer (Eppendorf, USA) at 260/280 nm. Later tenfold dilutions of the standards were prepared from the vaccine DNA sample up to 10^{-5} dilutions. Clinical sample, positive control and negative control were prepared in duplicates.

SYBR Green-based Real-Time PCR Assay

The Real-Time PCR reactions were prepared as per the method described previously with some modification (Abera *et al.,* 2014). Briefly, in a total volume of 20 µL containing 10 µL of SSO advance universal SYBR green Supermix (Bio-Rad, USA), 1 µL of each forward and reverse primers, 7.0 µL of nuclease-free water and 1.0 µL of template DNA. The Real-Time PCR reactions were carried out in CFX96 Real-Time PCR system (Bio-Rad, USA) using above mentioned PCR primers. Thermal cycler conditions were set as follows: 1 cycle at 98°C for 3 min, followed by 39 cycles at 98°C for 15 sec and

57°C for 30 sec using automatic settings for threshold and baseline. Appropriate positive and negative controls were also included in the assay.

Absolute Quantification of Viral Load in Nodular Tissue

Real-time-PCR reaction was performed in duplicate for test sample and also for positive and negative controls. In order to determine the viral load in the nodular tissue, absolute quantification of the Fowl pox sample was performed. In brief, the purified P4b gene amplicon was quantified (Nanodrop, Eppendorf) to calculate the viral copy number. A purified PCR product was used to make a standard curve belonging to the viral target sequence. Linear standard curves (efficiency 95-100%) were then generated by serial diluting purified viral DNA PCR product of P4b gene to 10^{-5} folds. This was followed by plotting Ct values against copy number values (log_{10}). Then, the viral copy numbers were estimated by using the Ct values and comparing them with the linear equation of the standard curve.

Statistical Analysis

The normality of the Ct values obtained with these methods was tested with the Kolmogorov-Smirnov test and the homogeneity of variances with Leven's test. T-test for paired samples was run to evaluate the significance of differences between the Ct values obtained.

Results and Discussion

Fowl Pox Virus Infection Detected in Chicken by Conventional and Real-Time PCR

In the present study, the FPV infected clinical sample was collected from Koppal district, Karnataka. The specific primers were constructed/designed from the genomic sequence of the flamingo pox virus isolate by targeting the P4b gene and confirmed by conventional PCR assay with an amplicon size of 415 bp (Fig.1). The affected bird had multifocal raised, grey or tan, crusty, verrucous irregular nodules around the eyelids, wattle, legs and comb.

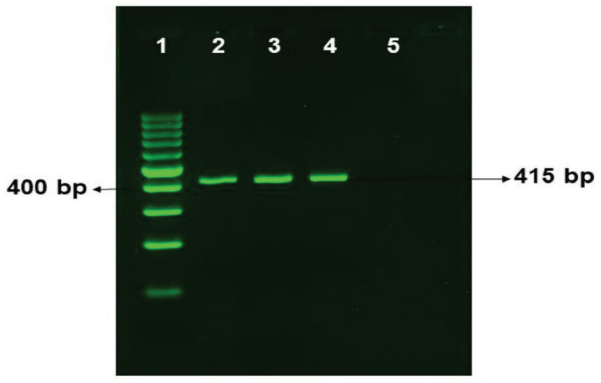

Fig. 1: PCR amplification of P4b gene: lane 1- 1kb DNA ladder; lane 2- Fowl pox vaccine (positive control); lane 3- Clinical sample; lane 4- CAM passage-1; lane 5- No template (Negative control).

Melting Curve Analysis

To evaluate the specificity of PCR products amplified, a melting curve analysis was performed by detecting primer-dimers and non-specific products. The specificity of Real-Time PCR amplified products was determined by only a single peak visible in the melting peak profile (Van der Velden *et al.*, 2003; Zhang *et al.,* 2011). In this study, there was no evidence of non-specific amplification in the melting curve of each sample, indicating a high specificity of the SYBR green Real-Time PCR assay for the detection of FPV in infected nodular tissue (Fig. 2, 3).

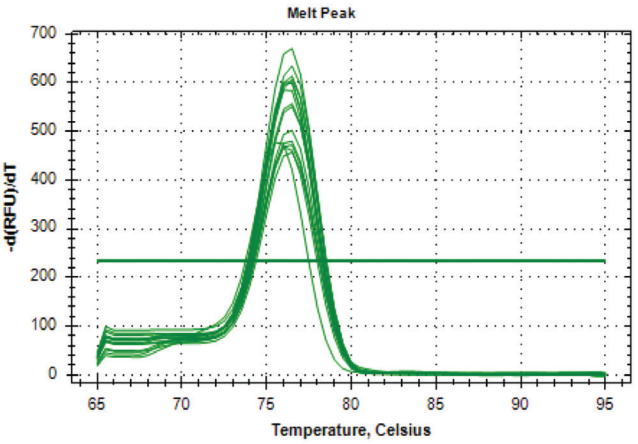

Fig. 2: Specificity of P4b gene based on real-time PCR through Melting curve analysis for P4b gene

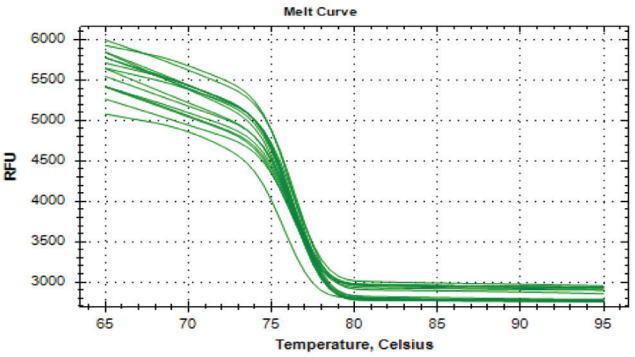

Fig. 3: Specificity of P4b gene based on real-time PCR through Temperature curve analysis for P4b gene

Standard Curve and Detection Limit Analysis

The Fowl pox P4b gene was amplified having an amplicon size of 415 bp. Further, the amplified product was purified. The purified product having DNA concentration 60 ng per µL was used to prepare the standards previously described (Abera *et al.,* 2014). Briefly, A series of 10-fold dilutions of the live attenuated fowl pox vaccine DNA starting from 1.34×10^{10} to 1.34×10^{6} copies per µL were prepared to construct a standard curve. A linear regression relationship between the dilutions and the threshold cycle (Ct) values was observed (Fig. 4). The lower detection limit achieved was 134 copies per µL with a corresponding Ct value of

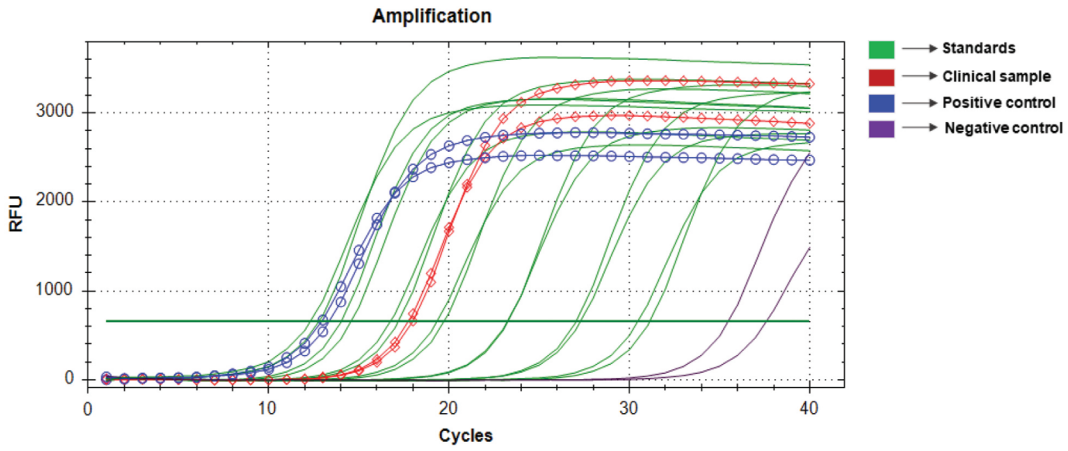

Fig. 4: Amplification plot for 10-old serial dilutions ranging from 1.34 ×10^{10} to 1.34 ×10^{6} copies per µL P4b gene amplicon.

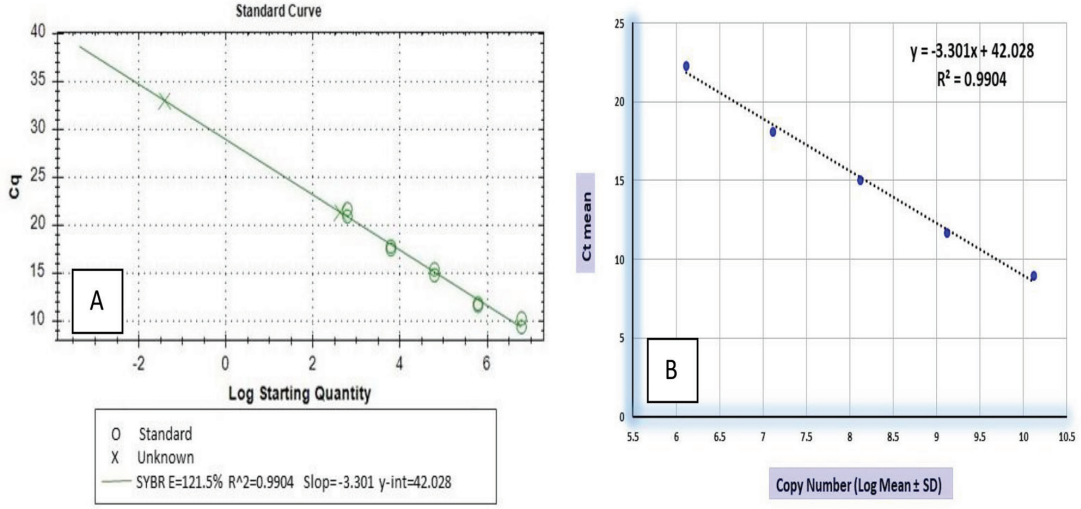

Fig. 5: Standard curve of P4b gene based Real-Time PCR assay (A). (Efficiency, E=121.5%, Correlation coefficient, R^2=0.9904, Slop= -3.301 y-Intercept=42.028). (B) X axis: Copy number (Log Mean ± SD), Y axis: Ct Mean.

32.59. This can be useful in detecting low levels of FPV and confirming the early stage of FPV infection where the virus titres are very low.

For accurate quantification of PCR products, the slope of the standard curve obtained with 10-fold dilutions should approach -3.3 in theory, but a slope from -3.1 to -3.6 was acceptable in practice (Van der velden *et al.,* 2003; Zhang *et al.,* 2011). Furthermore, the corresponding correlation coefficient (R^2) theoretical value should be more than 0.95 (Van der velden *et al.,* 2003). The correlation coefficient and the reaction efficiencies of the standard curve generated in the current study were 0.9904 and 121.5%, respectively (Fig 5.) which was close to the theoretical slope range of -3.1 to -3.6 reported by Zhang *et al.,* (2011).

Fowlpox Virus Loads Detected in Nodular Tissue

Nodular tissue from a clinical sample was used for optimizing the Real-Time PCR technique. The acquired Ct values and related standard concentrations were then displayed against a logarithmic scale. A linear regression curve was subsequently generated using the data points, and the trend line slope was calculated using the formula y = -3.301x + 42.028 (Fig.4) with a reference to y = mx+c. Where x is concentration of the target, y is Ct value, m is the regression coefficient or slope and c is the intercept. Model regression equation indicates the slope is -3.301 and concentration or copy number of target gene in unknown sample is calculated according to the Ct value through equation. Finally, efficiency was estimated to be 121.5% using the equation E = 10(-1/-3.301) in relation to (E = -1+10(-1/slope). The Fowl pox viral load in the infected bird was found to be 7.33 ± 0.044 (Log Mean ± SD). This reflects the very high viral load in the infected birds. The Real-Time PCR assay also demonstrated a high degree of specificity for avian pox viruses. Whereas there was no cross-reaction with other Pox viruses such as, Goatpox, Sheep pox and Lumpy skin disease viruses.

The biggest disadvantage of employing DNA binding dyes such as SYBR Green is that it detects any double-stranded DNA generated during PCR (Abera *et al.,* 2014). Several methodologies were used in this study to cross-check the specificity of the newly developed assays by employing different approaches such as, (I) By validating the precise predicted size of the amplicon, a 95 bp product was obtained, (II) the production of a constant melt peak at 83.68 ± 1.77 (Mean ± SD), (III) PCR product sequence analysis, which revealed high similarity with FPV isolate sequences submitted in GenBank (OQ469498). Similarly, several researchers developed one-step SYBR green Real-Time RT-PCR for quantifying Bovine Viral Diarrhoea (BVD) virus type-1 and haematologic malignancy (Van der velden *et al.,* 2003; Zhang *et al.,* 2011; Abera *et al.,* 2014).

Conclusion

The SYBR green-based one-step Real-Time PCR assay was developed and has been shown to be sensitive, rapid, easy to handle, quantitative and specific for the diagnosis and quantification of Fowlpox virus (FPV). These features make it an excellent tool for laboratory identification of FPV in cell culture supernatant as well as acute-phase disease. The high degree of sensitivity and specificity observed with the clinical samples suggested that the assay should be a useful tool for the quantification of FPV load and epidemiological surveillance in birds. An early and precise diagnosis will also be extremely useful to undertake suitable vaccination programs and control measures at the earliest.

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