DOI: 10.24099/vet.arhiv.2484

Quantitative profiling of fowl adenoviral load in experimentally infected chicken using SYBR Green real time PCR

Somasundaram Chitradevi[1*], Kuppanan Sukumar[2], Ponnusamy Suresh[3], Gurusamypalayam Amirthalingam Balasubramaniam[4], Duraisamy Kannan[5] and Angamuthu Raja[6]

[1] Veterinary University Training and Research Centre, Coimbatore, Tamil Nadu
Veterinary and Animal Sciences University, Tamil Nadu, India

[2] Department of Veterinary Microbiology, Veterinary College and Research Institute, Namakkal, Tamil Nadu
Veterinary and Animal Sciences University, Tamil Nadu, India

[3] Department of Veterinary Microbiology, Veterinary College and Research Institute, Theni, Tamil Nadu
Veterinary and Animal Sciences University, Tamil Nadu, India

[4] Department of Veterinary Pathology, Veterinary College and Research Institute, Namakkal, Tamil Nadu
Veterinary and Animal Sciences University, Tamil Nadu, India

[5] Department of Poultry Science, Veterinary College and Research Institute, Namakkal, Tamil Nadu
Veterinary and Animal Sciences University, Tamil Nadu, India

[6] Department of Animal biotechnology, Madras Veterinary College, Chennai, Tamil Nadu
Veterinary and Animal Sciences University, Tamil Nadu, India

CHITRADEVI, S., K. SUKUMAR, P. SURESH, G. A. BALASUBRAMANIAM, D. KANNAN, A. RAJA: Quantitative profiling of fowl adenoviral load in experimentally infected chicken using SYBR Green Real time PCR. Vet. arhiv 95, 39-50, 2025.

ABSTRACT

The present study aimed to quantify the fowl adenovirus (FAdV) load in various organs at different time intervals in experimentally infected chicken using SYBR Green based real time polymerase chain reaction (Real time PCR). The SYBR Green based real time PCR was standardized using 590 bp FAdV serotype 11 plasmid standards, and the sensitivity and specificity of the assay was validated. It was found that real time PCR was 100 times more sensitive than conventional PCR. To assess the fowl adenoviral load in different organs, experimental infection was conducted. Ninety-eight day-old specific pathogen free chicks were inoculated with field isolated, PCR confirmed and sequenced FAdV serotype 11 isolate with oral and intramuscular route at a rate of 0.5 ml of $10^{6.5}/TCID_{50}$ except control group. The infected chicks were sacrificed on days 3, 5, 7, 10, 14 and 21 after inoculation. In both the groups, high FAdV copy numbers were found in the caecal tonsil at 5 to 7 days post inoculation, followed by the bursa of Fabricius and the liver, and FAdV copy numbers were seen up to day 21 pi in all the organs, where the kidneys showed the lowest copy numbers when compared to the other organs.

Key words: fowl adenovirus; serotype 11; hexon gene; SYBR Green; real time PCR; quantification of fowl adenovirus

---

* Corresponding author:
Dr. S. Chitradevi, M.V.Sc., Ph.D., Assistant Professor, Veterinary University Training and Research Centre, Coimbatore-641 035, Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu, India, e-mail: chitradevi.dr@gmail.com

ISSN 0372-5480
Printed in Croatia

Introduction

Fowl adenoviruses (FAdVs) are common infectious agents of chicken that cause several disease conditions, such as Inclusion Body Hepatitis (IBH), Hydropericardium Syndrome (HPS), Hepatitis Hydropericardium Syndrome (HHS), Gizzard Erosions (GE), Proventriculitis (PV), Tenosynovitis, and respiratory infections in poultry (McFERRAN and SMYT, 2000). Fowl adenoviruses are classified in the family *Adenoviridae,* genus Aviadenovirus, and include eight species, five of which are the FAdV species consisting of *Fowl aviadenovirus A* (FAdV-1); *Fowl aviadenovirus B* (FAdV-5); *Fowl aviadenovirus C* (FAdV-4 &10) *Fowl aviadenovirus D* (FAdV-2, 3,9 & 11) and *Fowl aviadenovirus E* (FAdV-6, 7, 8a & 8b) (HARRACH et al., 2012). Diagnosis of FAdV infection can be made from observation of gross and histopathological examinations of the liver, virus isolation using embryonated eggs and cell culture, PCR RFLP analysis, sequencing, and phylogenetic analysis. Recently, real time PCR has been shown to be more sensitive than virus isolation in cell culture, and conventional PCR is used for detection and quantification of fowl adenoviral load from various tissues of infected birds (ROMANOVA et al., 2009; GUNES et al., 2012; GRAFL et al., 2013; VERA-HERNÁNDEZ et al., 2016; ABSALON et al., 2017; LI et al., 2018). ROMANOVA et al. (2009) developed a highly sensitive real time PCR assay to detect and quantify FAdV DNA in chicken tissues, using FAdV 9 as a model. Hence, the present study was formulated to find the FAdV load from various tissues of experimentally infected chicken, using SYBR Green based real time PCR.

Materials methods

SYBR Green based real time PCR for 590bp hexon gene of FAdV 11

Amplification of source gene. The Hexon gene amplified from field fowl adenovirus serotype 11 isolate Ind-TN-CB3-2017 (GenBank accession number MK816403.1) maintained at the Department of Veterinary Microbiology, Veterinary College and Research Institute, Namakkal, Tamil Nadu, India, was used as the source of DNA and extracted using a QIAmp tissue extraction kit (Catalog No.51304, Qiagen, USA).

Development of the standard plasmid. Polymerase chain reaction was performed using the 590 bp hexon gene primer with a slight modification to the annealing temperature (MEULEMANS et al., 2001). The PCR was carried out in a final volume of 50 µl with the following cycle conditions: Initial denaturation 95°C, 5 min; denaturation 94 °C, 30 sec; annealing 52°C, 45 min; extension 72°C, 1 min 35 cycles followed by final extension of 72°C, 10 min. The PCR amplified 590 bp hexon gene product was placed in agarose gel under an UV transilluminator, and sliced and purified as per the kit protocol (Favorprep Gel Purification Kit Cat. No. FAGCK 000 FAGCK 001). The 590 bp hexon gene was ligated to pTZ57R/Tvector (2.88kb) according to the Thermoscientific InsTA clone kit #K1213 protocol. The ligated mixture was transformed into DH5α cells and colony PCR was carried out for confirmation of the plasmid DNA for the FAdV from recombinant colonies on LB agar plates.

The plasmid was extracted from an overnight culture of *E.coli* (DH5α) in Luria broth, using a GeneJet Plasmid extraction (Thermo Scientific) kit. The quality and quantity of the purified plasmid DNA was assessed using Nanodrop. The process was repeated in triplicate using Nanodrop to obtain the average concentration, and converted from ng/µl to 10^{10}copies per µl using the Thermo scientific online formula tool, by adding the requisite quantity of nuclease free water.

Serial dilution of plasmid was conducted from 10^{10} to 10^{-2} for the standard plasmid, and PCR was carried to find the level of detection (LOD) in conventional PCR. The last band obtained in the dilution series during the gel electrophoresis of PCR amplified products was considered as the LOD.

The specificity of real time PCR for FAdV detection was assessed by testing positive DNA samples of avian leucosis virus (ALV) with the plasmid DNA template as the positive control, and nuclease free water as the negative control.

SYBR Green based real time PCR and generation of a standard curve using plasmid constructs.

SYBR Green based real time PCR amplification was performed in a Roche Light Cycler 96 (Roche, USA). The total volume for the real time PCR reaction mix was 10 µl (SYBR Green PCR Master Mix (2X) - 5 µl, Forward and Reverse Primer (10 pmol/µl) each 1 µl, serially diluted plasmid DNA 1 µl and Nuclease Free Water - 2 µl) in each well, assayed in duplicate. The real time PCR cycle conditions and primers were used as described by GUNES et al. (2012). No template control (NTC) was used in each run to confirm that there was no contamination in the assay. The known copy number of the target DNA was plotted against the corresponding quantification cycle (Cq) values and the standard curve was constructed. For quantification of the virus in samples of unknown concentration, their Cq values were compared with the standard curve and the numbers of copies of FAdV DNA per reaction mixture were calculated.

Quantification of FAdV load in experimentally infected chicken

Virus Strain. The virus isolate FAdV 11 (Ind-TN-CB3-2017; GenBank accession number MK816403.1) used in this experimental infection was isolated from a field outbreak using chicken embryo liver cells, and PCR confirmed and sequenced. PCR was carried out in extracted DNA to screen for concurrent infections viz., for Marek's disease virus (MDV) *Meq gene,* 184 bp (ABDUL-CAREEM et al., 2006), for Avian leucosis virus (ALV) envelope glycoprotein gp85, 466 bp (OTTIGER, 20) for Reticular endothelial virus (REV) REV LTR, 291bp (OTTIGER, 2010) and for Chicken infectious anemia virus (CAV) the VP2 gene, 419 bp OTTIGER (2010). The FAdV isolate, free from these concurrent infections, was used for the experimental infection. The field isolate was scaled up and $TCID_{50}$ assay of the bulk harvest was done to determine the FAdV titer used in this experimental study.

Calculation of $TCID_{50}$. The $TCID_{50}$ was calculated by seeding the chicken embryo liver cells with 1 x 10^4 cells per well in a 96-well plate containing 100 µl of growth medium with 10 per cent FBS per well. After 24 h of incubation, the cells were infected with 100 µl of a ten-fold dilution of the virus (10^{-1}-10^{-10}) per well. Each dilution had six replicates as well as the controls. The 96-well plate was incubated at 37°C, in a five per cent CO_2 atmosphere, and examined for the development of CPE. The fifty per cent end point ($TCID_{50}$) was calculated as per the method of REED and MUENCH (1938).

Experimental study. All animal procedures were performed in accordance with local ethical regulations and the approval of the in - house Ethics Committee. During the experimental period of three weeks, the chicks were housed in an in - house animal facility unit and efforts were made to minimize their stress.

To study the viral genome load in the organs of the chickens infected with FAdV 11, 98 one-day old specific pathogen free (SPF) chicks were divided into three groups. Forty-two chickens in group I were inoculated orally with 0.5 ml $10^{6.5}/TCID_{50}$ FAdV 11, forty-two chickens in group II were inoculated intramuscularly with 0.5 ml $10^{6.5}/TCID_{50}$ FAdV 11 in the breast muscle, and fourteen chickens in group III were designated as the uninoculated control. The birds were observed daily for mortality and clinical signs for up to 21 days post infection (dpi). Six birds in the treatment groups and two birds in the control group were sacrificed according to the required procedure each on days 3, 5, 7, 10, 14, 17 and 21 after inoculation, and post mortem examination was conducted. Tissue samples, viz., the bursa of Fabricius, caecal tonsils, kidneys, liver, spleen and thymus, were collected and stored at -80°C for DNA extraction.

Quantification of FAdV load. DNA was extracted from pooled samples each with six bursa of Fabricius, caecal tonsils, kidneys, livers, spleens and thymus from the oral and I/M groups on days 3, 5, 7, 10, 14, 17 and 21 post-inoculation using a DNA extraction kit (Catalog No.51304, Qiagen, USA). SYBR Green based real time PCR cycle condition and primers were used in this study, as described by GUNES et al.,(2012). Absolute quantification of the FAdV hexon genome copy numbers from tissue samples was carried out, and Cq values were obtained. Using the statistical data obtained from the light cycler program, a report was generated and exported into an Excel Sheet (Microsoft office) for

further interpretation. Viral copy numbers (VCN) were expressed per 100 ng of tissue DNA. The concentration was expressed in log 10 values. The efficiency of real-time PCR was calculated by 10E (-1/ slope of the standard curve) in the light cycler program. FAdV load on different days post infection (dpi) was calculated from the data obtained from real time PCR assay.

Results

Recombinant clones containing the 590 bp FAdV hexon gene were confirmed by colony PCR (Fig. 1).

The concentration of input pDNA was 44 ng/ µl and the copy number was 1.155×10^{19}copies/ µl. The concentration was adjusted to 10^{10} copies/ µl and plasmids were diluted serially from 10^{10} to 10^{-2}

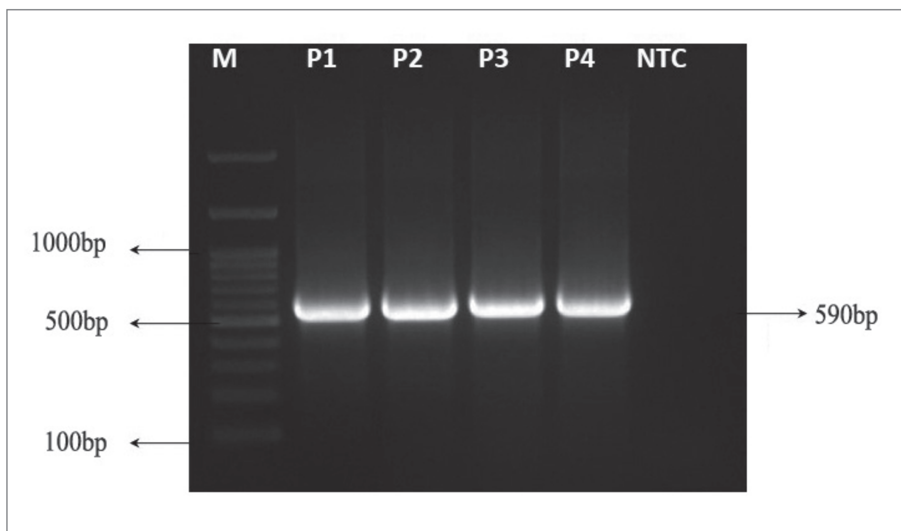

Fig. 1. Agarose gel electrophoresis showing of 590bp amplified PCR product of plasmid hexon gene by colony PCR

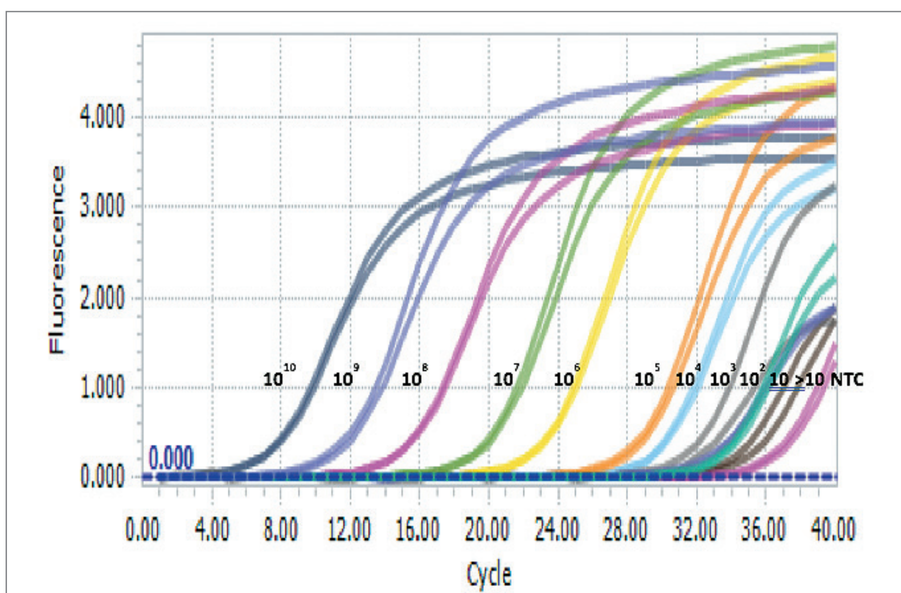

Fig. 2. Amplification plot detection of the 590bp hexon gene by SYBR Green based real time PCR

copies/ µl. The level of detection (LOD) was found to be 10^2 copies in conventional PCR.

SYBR Green based real time PCR was carried out using specific primers. All dilutions were tested in duplicate, and Cq values were obtained for each dilution (Fig. 2). The assay showed linearity with the correlation coefficient (R^2) of 0.999 and -3.56 respectively.

Amplification curves were obtained and the highest dilution in the tenfold dilution series which amplified dependably was defined as the detection limit. It was found that an exponentially amplified curve could be seen in up to less than 10 copies in the qPCR, but 10^2 copies for conventional PCR. The results indicated that the qPCR for plasmid hexon was 100 times more sensitive than conventional PCR.

No mortalities were observed in any of the three groups during the entire study period. Dullness, depression and mild diarrhea were noticed between days 3 and 5 post-inoculation (pi) in two birds in group I and three birds in group II, but not in the control group (Group III). No other major clinical signs were observed.

In the orally infected birds, the bursa of Fabricius showed a viral genome load of 1.99×10^6 at day 3 pi after which there was a slight decline up to day 5 pi and copy numbers increased to a maximum of 5.30×10^6 copies at day 10 pi. A fall in copy numbers was noticed from days 14 to 21 pi. The lowest copy number of 2.79×10^5 copies was observed on day 21 pi. When compared to other tissues, caecal tonsil showed a significantly higher copy number of 1.84×10^7 on day 5 pi and 4.25×10^6 copies were seen up to day 21 pi. There was a steady decline in viral genome copy numbers from 5.45×10^5 to 1.10×10^2 from days 3 to 21 pi in the kidneys. Liver tissues showed the maximum copy number of 6.35×10^6 copies on day 10 pi and the minimum copy number of 1.19×10^5 on day 21 pi. In the spleen, 4.27×10^6 copies were found on day 3 pi, after which the copy numbers declined, and the lowest copies were seen on day 21 pi. Whereas in the thymus the maximum viral load was found on day 3 pi (3.71×10^6 copies), a consistent decrease in viral load was recorded up to day 21 pi (1.31×10^2). With the oral route of inoculation, significantly higher copy numbers were seen in the caecal tonsil on day 5 pi, followed by the bursa of Fabricius and liver on day 10 pi (Fig. 3).

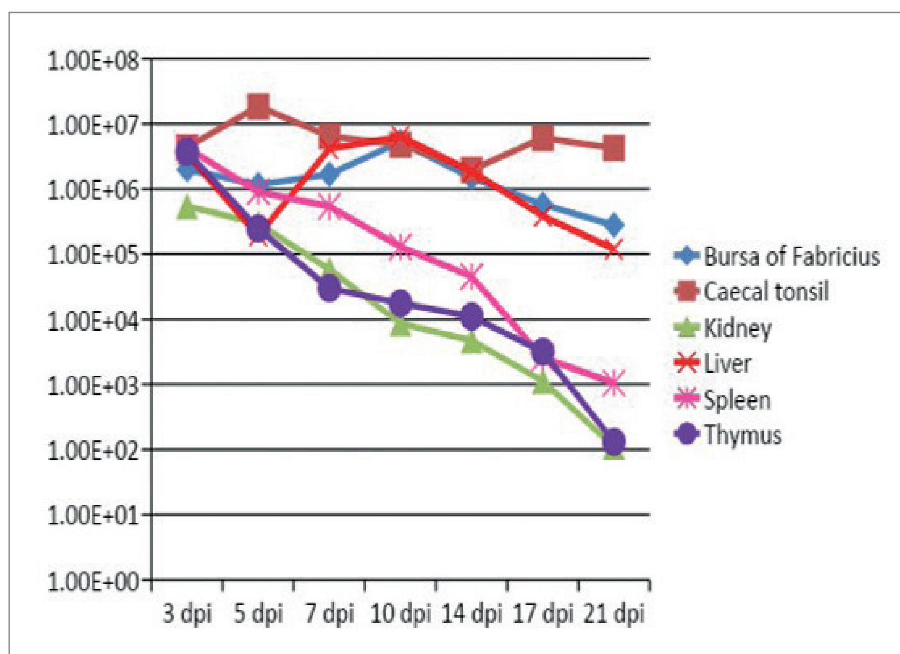

Fig. 3. Fowl adenovirus load (100 ng of tissue DNA) in different organs at different time intervals in orally infected chicken with FAdV serotype 11 isolate

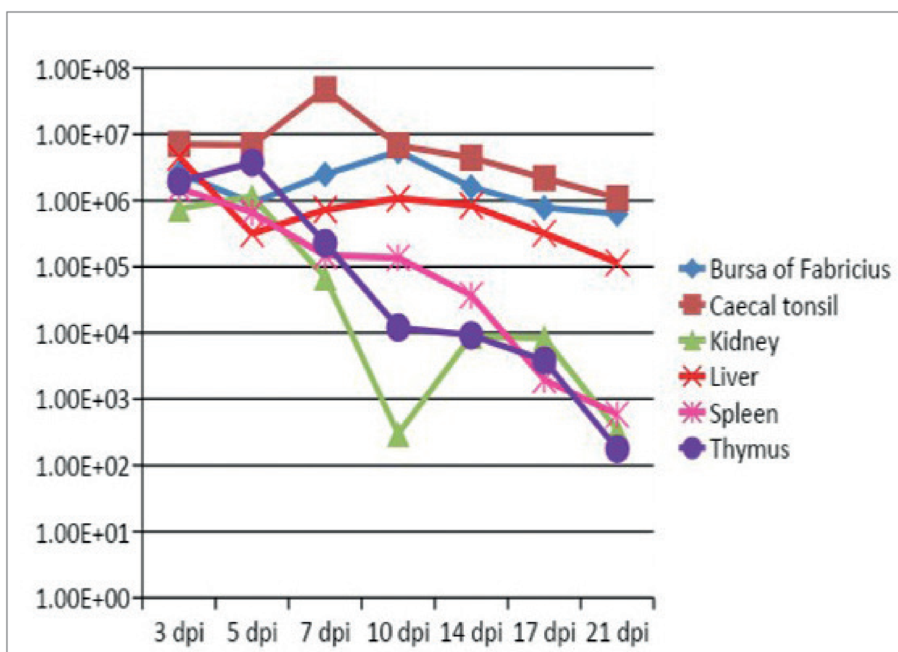

Fig. 4. The fowl adenovirus load (100 ng of tissue DNA) in different organs at different time intervals of intramuscularly (I/M) infected chicken with FAdV serotype 11 isolate

In the intramuscularly infected birds, the bursa of Fabricius showed the maximum copy number of 5.51x10^6 on day 10 pi and the minimum number of 6.28x10^5 on day 21 pi. In the caecal tonsil the highest copy numbers were recorded on day 7 pi at 4.72x10^7. In the kidneys, the maximum of 7.50x10^5 copies were seen on day 3 pi and the lowest copy numbers of 3.43x10^2 on day 21 pi. A steady decline in copy numbers was observed over the remaining days post infection. In the livers also, there was a maximum of 4.51x10^6 and 1.07x10^6 copies present on day 3 pi and day 14 pi. The lowest viral copies were seen at 21st dpi. In the spleens the copy numbers were higher on day 3 pi and thereafter a decreasing trend in copy numbers was observed, and the lowest numbers were seen on day 21 pi. In thymus, the maximum of 3.73x10^6 copies was observed on day 5 pi and the lowest (1.76x10^2) on day 21 pi (Fig. 4).

On the basis of these findings, caecal tonsil and the bursa of Fabricius showed significantly higher FAdV copy numbers than the other organs, and the liver showed a consistent viral load up to day 21 pi. In both the groups, the kidneys showed low copy numbers when compared to the other organs.

Discussion

Fowl adenovirus infection is routinely diagnosed by virus isolation, PCR followed by restriction enzyme digestion or sequencing of the PCR products. In general, PCR has proven to be very sensitive and specific, but it is time consuming in terms of sample handling and post PCR analysis. Real time PCR has gained wide acceptance due to its improved rapidity, sensitivity, reproducibility, and the reduced risk of contamination (MACKAY et al., 2002). Detection by SYBR green is the simplest and least expensive approach since it does not require fluorescent probes against the viral genome (SEGURA et al., 2010). SYBR green based real time PCR was developed and has been used by many researchers for the detection and quantification of fowl adenovirus in different tissues (ROMANOVA et al., 2009; GRGIĆ et al., 2011; GUNES et al., 2012; ABOUZIED et al., 2021; SABARUDIN, et al., 2021; ISLAM et al., 2023).

Fowl adenovirus hexon is the major capsid protein and it contains type, group and subgroup specific antigenic determinants. In hexon gene, loop L1 variable region is flanked by conserved pedestal 1 region, variable region is utilized for amplification of the 590bp product using HexL1-s, and HexL1 primers. STEER et al. (2009) described HexL1-s and HexL1-as primer pair was more sensitive for the detection and classification of all serotypes of FAdV than HEX-SF and HEX-SR primers, which proved to be less sensitive for classification of FAdV using HRM analysis. In the present study, it could be demonstrated that SYBR Green based real-time PCR for detection of FAdVs was more sensitive than conventional PCR using the Hexon HexL1-s and HexL1 primer pair. In this study, a 590bp fragment of FAdV serotype 11 specific hexon gene was cloned to generate the plasmid and the correct insertion of the hexon gene was confirmed by colony PCR. Real time PCR was 100 times more sensitive than the conventional PCR. Not all dilutions of FAdV serotype 11 hexon plasmid were detected by conventional PCR, which were positive in the real time PCR. The results of this study correlate well with ROMANOVA et al. (2009) who carried out detection and quantification of FAdV 9 from infected chickens, and the minimum detection limit of qPCR was 9.4 viral genome copy numbers. They also found that SYBR green based qPCR was 100 times more sensitive than conventional PCR. Similar findings were observed by GUNES et al. (2012) who developed SYBR green based real time PCR for detection of all FAdV species, and real time PCR was 10^4 times more sensitive than conventional PCR.

NICZYPORUK and CZEKAJ (2018) found that the detection limit qPCR assays was eight copies when chicken were experimentally infected with FAdV 8a-6/12j and FAdV 1/A-61/11z strains. The assay showed high specificity for the FAdV serotype 11 when tested with the standard plasmids hexon and non FAdV such as ALV. The primer pairs amplified only the specific product. FAdV and non FAdV viruses showed neither cross amplification nor a detectable fluorescent signal, confirming the specificity of the qPCR assay for FAdV detection as reported by ROMANOVA et al. (2009) and GUNES et al. (2012). On the basis of our study, standardization of real time PCR for the FAdV serotype 11 demonstrates that the real time PCR assay was more sensitive than conventional PCR, and highly specific. The findings of this study are in accordance with earlier reports by ROMANOVA et al. (2009), GUNES et al. (2012) and WANG et al. (2020). They showed it to be ten times to 10^4 times more sensitive, and also a quicker method than conventional PCR techniques used for detection of FAdV.

The FAdV serotype 11 used in this experimental study was isolated from field outbreaks in commercial broiler chicken from Tamil Nadu, India (CHITRADEVI et al., 2021). During the entire experimental study period no mortality was observed in any of the three groups. Between days 3 and 5 pi the birds were dull and depressed, and diarrhea was noticed in groups 1 and 2, but not in the control group. No other major clinical signs were observed. The results of the present study are similar to the findings of GRGIĆ et al. (2011) who found no clinical signs or pathological changes when white ten-day old SPF Leghorn chickens were infected with $2x10^8$ PFU FAdV 8 by oral and intramuscular routes. STEER et al. (2015) also recorded no mortality or clinical signs when the day-old SPF chicks inoculated via the ocular route with FAdV serotype 11. The clinical signs observed between days 3 to 5 pi are in agreement with JADHAO et al. (2003) who recorded the same clinical signs observed between days 3 to 5 pi, when 14 day-old birds were inoculated with a dose of $10^3 TCID_{50}/0.5$ ml of FAdV 4 via the oro-nasal route, but they recorded 40 per cent mortality. The results of this study are in contrast to ZHAO et al. (2015) who observed 8.6 per cent mortality in three week old SPF chicken pathogenically orally inoculated with FAdV serotype 11 (HBQ12) but the inoculated chicks showed depression between days 3 and 15 pi. Mortality commenced from day 4 pi, and the clinical scores of serotype 11 infection were less than serotype 4. In our study, no mortality was found in the day-old chicks inoculated with 0.5 ml $TCID_{50} 10^{6.5}$ by either the oral or the intramuscular route. Although FAdV acted as the primary pathogen in causing IBH, the absence of concurrent infection, differences in en-

vironmental conditions between field and experimental studies, other stress factors associated with conditions on the farm, differences in the susceptibility of the chicken breeds used in the study might influence the outcome of the disease.

For quantification of the FAdV load, in general, 10^2 to 10^7 copies / 100 ng tissue of DNA of FAdV load were found in different organs at different time intervals, irrespective of being in the oral or intramuscular group. The highest copy number of 10^7 copies was found in caecal tonsil in both the groups, whereas the maximum load was reached on day 5 pi in the oral group ($1.84x10^7$) and on day 7 pi in the I/M group ($4.72x10^7$). This finding is well correlated with GRGIĆ et al. (2013) who found the highest FAdV 4 load in the caecal tonsils irrespective of the inoculation route, and a slight increase in copy numbers was noticed in the caecal tonsil followed by the bursa of Fabricius and the liver in the I/M route group. They also found the same result when FAdV 8 was used by GRGIĆ et al. (2011). Similarly ROMANOVA et al. (2009) detected 10^2 to 10^7 copies / 100 ng tissue DNA in the liver, bursa of Fabricius and caecal tonsils, and found a high amount of viral copies in the caecal tonsil a week after inoculation, which was then negative on days 14 and 21 pi when two-week-old White Leghorn chicks were inoculated with FAdV 9 intramuscularly. However in this study the presence of viral load up to day 21 of the experiment might be due to serotype variations, and the age and type of birds used, since FAdV is more common in meat producing birds, and the incidence was maximum at the age of three to six weeks. The presence of viral copies in the bursa of Fabricius and the caecal tonsils may cause FADV associated immunosuppression (STEER et al., 2011; SCHACHNER et al., 2018; WANG et al., 2020)

In our study, on day 3 pi, both the groups showed 10^6 copies, except in the kidneys, and this result is consistent with ROMANOVA et al. (2009) and STEER et al. (2015). There was a slight decline in copy numbers on day 5 pi and they reached a high on day 10 pi in the bursa of Fabricius, the liver, spleen and thymus. However, GUNES et al. (2012) found the highest shedding of FAdV D ($5.89x10^6$) on day 10 pi when comparing FAdV A and B, and no shedding was found on day 35 pi. COOK (1983) indicated that the replication rate of the adenovirus strains reached a peak in the first three weeks post infection, and that these strains had a higher degree of tropism for the digestive tract than the other organs.

After day 10 pi, decreasing copy numbers from 10^6 to10^5 were seen in the bursa of Fabricius and the liver, whereas there were 10^3 copies in the spleen, 10^2 copies in the kidneys and thymus on day 21 pi. This correlates well with NICZYPORUK and CZEKAJ (2018), who found the highest copy numbers of the hexon gene in the gizzard, liver and intestines in the first, second and fourth weeks, and the lowest copy numbers in the third week of the experiment in the kidney, thymus and spleen, when the chickens were infected with the FAdV 1/A-61/11z strain by the intraperitoneal route. ABSALON et al. (2017) experimentally orally infected day-old SPF chicks with 10^6 $TCID_{50}$ of low pathogenic FAdV serotype 11 . The birds were sacrificed on different days post infection, and viral copy numbers were detected in the liver, thymus, spleen, bursa of Fabricius, intestine, caecal tonsils, kidney and proventriculus by real time PCR. It was found that genome copies were detected on day 3 pi and the highest number of genome copies was reached in all the organs on day 6 pi, whereas on day 7 pi the viral copy numbers decreased However, the viral copy numbers could be detected until day 42 of the trial, except in the spleen. In our study, irrespective of the route of inoculation, 10^5 to 10^6 copies were maintained up to day 21 pi in the liver, bursa of Fabricius and caecal tonsil. In the kidneys, spleen and thymus 10^2 to 10^3 copies were seen on day 21 pi. Similarly, GRGIĆ et al. (2013) recorded the shedding of FAdV 4 on cloacal swabs in orally infected birds on day 21 pi, whereas in intramuscularly infected birds it was recorded on day 28 pi. REECE et al. (1985) reported the persistence of FAdV 8 for up to 8 weeks in the caecal tonsils of the parent birds. Virus excretion in broilers peaked at between 4 and 6 weeks of age, most likely due to the decrease in maternal antibodies at that time (McFERRAN, 1981).

Conclusions

The results of this study showed that SYBR green based real time PCR is 100 times more sensitive than conventional PCR. Viral copy numbers were significantly higher in the caecal tonsil, followed by the bursa of Fabricius, liver, spleen, thymus and kidney. The presence of FAdV was recorded in the caecal tonsil, bursa of Fabricius, liver, spleen, thymus and kidney up to 21days pi, irrespective of the route of inoculation. Even though the liver is the primary organ for detection of FAdV, the caecal tonsil and bursa of Fabricius may also be used as samples for diagnosis of FAdV during the early stages of the infection.

Declaration of competing interest

The author(s) declares that there is no conflict of interest.

Acknowledgements

The authors gratefully acknowledge Tamil Nadu Veterinary and Animal Sciences University for providing the facilities to carry out this research work.

References

ABDUL-CAREEM, M. F., B. D. HUNTER, E. NAGY, L. R. READ, B. SANEI, J. L. SPENCER, S. S. SHARIF (2006): Development of a real time PCR assay using SYBR Green chemistry for monitoring Marek's disease virus genome load in feather tips. J. Virol. Methods 133**,** 34-40.
https://doi.org/10.1016/j.jviromet.2005.10.018

ABOUZIED, H., M. SOLIMAN, E. El-SAYED, N. ALI, M. El-TARABILLI (2021): Molecular Investigation of Fowl Aviadenovirus in Broiler Flocks in North Sinai Province. Suez Canal Vet. Med. J, 26**,** 449-467.
https://doi.org/10.21608/SCVMJ.2021.217942

ABSALÓN, A. E., A. MORALES-GARZON, P. F. VERA-HERNÁNDEZ, D. V. CORTÉS- ESPINOSA, S. M. URIBE-OCHOA, L. J. GARCÍA, E. LUCIO-DECANINI (2017): Complete genome sequence of a non-pathogenic strain of fowl adenovirus serotype 11: Minimal genomic differences between pathogenic and non-pathogenic viruses. Virology 501, 63-69.
https://doi.org/10.1016/j.virol.2016.11.006

CHITRADEVI, S., K. SUKUMAR, P. SURESH, G. A. BALASUBRAMANIAM, D. KANNAN (2021): Molecular typing and pathogenicity assessment of fowl adenovirus associated with inclusion body hepatitis in chicken from India. Trop. Anim. Health Prod. 53, 412.
https://doi.org/10.1007/s11250-021-02851-8

COOK, J. K. (1983): Fowl adenoviruses: studies on aspects of the pathogenicity of six strains for 1 day old chicks. Avian Pathol. 12, 35-43.
https://doi.org/10.1080/03079458308436147

GRAFL, B., D. LIEBHART, A. GÜNES, P. WERNSDORF, F. AIGNER, J. BACHMEIER, M. HESS (2013): Quantity of virulent fowl adenovirus serotype 1 correlates with clinical signs, macroscopical and pathohistological lesions in gizzards following experimental induction. Vet. Res. 44, 38.
https://doi.org/10.1186/1297-9716-44-38

GRGIĆ, H., Z. POLJAK, S. SHARIF, É. NAGY (2013): Pathogenicity and cytokine gene expression pattern of a serotype 4 fowl adenovirus isolate. PLoS One 8, e77601.
https://doi.org/10.1371/journal.pone.0077601

GRGIĆ, H., D. H. YANG, É. NAGY (2011): Pathogenicity and complete sequence of a fowl adenovirus serotype 8 isolate. Virus Res. 156**,** 91-97.
https://doi.org/10.1016/j.virusres.2011.01.002

GÜNES, A., A. MAREK., B. GRAFL, E. BERGER, M. HESS (2012): Real time PCR assay for universal detection and quantitation of all five species of fowl adenoviruses (FAdV-A to FAdV-E). J. Virol. Methods 183**,** 147-153.
https://doi.org/10.1016/j.jviromet.2012.04.005

HARRACH, B., M. BENKO, G. W. BOTH, M. BROWN, A. J. DAVISON, M. ECHAVARRIA, M. HESS, M. S. JONES, A. KAJON, H. D. LEHMKUHL, V. MAUTNER, S. K. MITTAL, G. WADELL (2012): Family *Adenoviridae*. In: Virus Taxonomy: Classification and nomenclature of viruses. Ninth report of the International Committee on Taxonomy of Viruses. (King, A. M. Q., M. J. Adams, E. B. Carstens, E. J. Lefkowitz, Eds), Elsevier Inc, NY, USA, pp. 125-141.

ISLAM, M. N., M. RAHMAN, K. RAHMAN, J. ALAM (2023): First Evidence of Fowl Adenovirus Induced nclusion Body Hepatitis in Chicken in Bangladesh. Can. J. Infect. Dis. Med. Microbiol. 2023, 7253433.
https://doi.org/10.1155/2023/7253433

JADHAO, S. J., J. N. DEEPAK, J. M. KATARIA, R. S. KATARIA, A. K.TIWARI, R. SOMWANSHI, P. SANGAMITHRA, K. C. VERMA (2003): Characterization of fowl adenovirus from chicken affected with infectious hydropericardium during 1994-1998 in India. Indian J. Exp. Biol. 41, 321-327.

LI, R., G. LI, J. LIN, S. HAN, X. HOU, H. WENG, M. GUO, Z. LU, N. LI, Y. SHANG, T. CHAI, L. WEI (2018): Fowl adenovirus serotype 4 SD0828 infection causes high mortality rate and cytokine levels in specific pathogen

free chickens compared to duck. Front. Immunol. 9, 49. https://doi.org/10.3389/fimmu.2018.00049

MACKAY, I. M., K. E. ARDEN, A. NITSCHE (2002): Real time PCR in virology. Nucleic Acids Res. 30, 1292-1305. https://doi.org/10.1093/nar/30.6.1292

McFERRAN, J. B. (1981): Comparative diagnosis of viral diseases III. In: Adenoviruses of vertebrate animals. (KURSTAK, E., C. KURSTAK, Eds), Academic Press, NY, USA, pp. 102-165.

McFERRAN, J. B., J. A. SMYTH (2000): Avian Adenoviruses. Rev. Sci. Tech. 19, 589-601.

MEULEMANS, G., M. BOSCHMANS, T. P. BERG, M. DECAESSTECKER (2001): Polymerase chain reaction combined with restriction enzyme analysis for detection and differentiation of fowl adenoviruses. Avian Pathol. 30, 655- 660. https://doi.org/10.1080/03079450120092143

NICZYPORUK, J. S., H. CZEKAJ (2018): A comparative pathogenicity analysis of two adenovirus strains, 1/A and 8a/E, isolated from poultry in Poland. Arch. Virol. 163, 3005-3013. https://doi.org/10.1007/s00705-018-3965-9

OTTIGER, H. P. (2010): Development, standardization and assessment of PCR systems for purity testing of avian viral vaccines. Biologicals 38, 381-388. https://doi.org/10.1016/j.biologicals.2010.01.015

REECE, R. L, D. A. BARR, D. C. GRIX (1985): An investigation of vertical transmission of a fowl adenovirus serotype 8. Aust. Vet. J. 62, 136-137. https://doi.org/10.1111/j.1751-0813.1985.tb07266.x

REED, L. J., H. MUENCH (1938): A simple method for estimating 50 per cent endpoints. Am. J. Epidemiol. 27, 493-497. https://doi.org/10.1093/oxfordjournals.aje.a118408

ROMANOVA, N., J. C. CORREDOR, E. NAGY (2009): Detection and quantitation of fowl adenovirus genome by a real-time PCR assay. J. Virol. Methods 159, 58-63. https://doi.org/10.1016/j.jviromet.2009.02.026

SABARUDIN, N. S., S. W.S HEAU WEI TAN, Y. F. PHANG, A. R. OMAR (2021): Molecular characterization of Malaysian fowl adenovirus (FadV) serotype 8b species E and pathogenicity of the virus in specific pathogen-free chicken. J. Vet. Sci. 22, e42. https://doi.org/10.4142/jvs.2021.22.e42

SCHACHNER, A., M. MATOS, B. GRAFL, M. HESS (2018): Fowl adenovirus-induced diseases and strategies for their control – a review on the current global situation. Avian Pathol. 47, 111-126. https://doi.org/10.1080/03079457.2017.1385724

SEGURA, M. M., M. MONFAR, M. PUIG, F. MENNECHET, S. IBANES, M. CHILLON (2010): A real time PCR assay for quantification of canine adenoviral vectors. J. Virol. Methods 163, 129-136. https://doi.org/10.1016/j.jviromet.2009.09.010

STEER, P. A., J. R.SANDY, D. O'ROURKE, P. C. SCOTT, G. F. BROWNING, A. H. NOORMOHAMMADI (2015): Chronological analysis of gross and histological lesions induced by field strains of fowl adenovirus serotypes 1, 8b and 11 in one day old chickens. Avian Pathol. 44, 106-113. https://doi.org/10.1080/03079457.2015.1007919

STEER, P. A., D. O'ROURKE, S. A. GHORASHI, A. H. NOORMOHAMMADI (2011): Application of high-resolution melting curve analysis for typing of fowl adenoviruses in field cases of inclusion body hepatitis. Aust. Vet. J. 89, 184-192. https://doi.org/10.1111/j.1751-0813.2011.00695.x

STEER, P. A., N. C. KIRKPATRICK, D. O'ROURKE, A. H. NOORMOHAMMADI (2009): Classification of fowl adenovirus serotypes by use of high resolution melting curve analysis of the hexon gene region. J. Clin. Microbiol. 47, 311-321. https://doi.org/10.1128/JCM.01567-08

VERA-HERNÁNDEZ, P. F., A. MORALES-GARZÓN, D. V. CORTÉS-ESPINOSA, A. GALIOTE-FLORES, L. J. GARCÍA-BARRERA, E. T. RODRÍGUEZ-GALINDO, A. TOSCANO-CONTRERAS, E. LUCIO-DECANINI, A. E. ABSALÓN (2016): Clinicopathological characterization and genomic sequence differences observed in a highly virulent fowl *Aviadenovirus* serotype 4. Avian Pathol. 45, 73-81. https://doi.org/10.1080/03079457.2015.1125443

WANG, J., I. ZAHEER, M. K. SALEEMI, X. QI, Y. GAO, H. CUI, K. LI, L. GAO, A. FAYYAZ, A. HUSSAIN, C. LIU, Y. ZHANG, X. WANG, Q. PAN (2020): The first complete genome sequence and pathogenicity characterization of fowl adenovirus 11 from chickens with inclusion body hepatitis in Pakistan. Vet. Microbiol. 244, 108670. https://doi.org/10.1016/j.vetmic.2020.108670

ZHAO, J., Q. ZHONG, Y. ZHAO, Y. X. HU, G. Z. ZHANG (2015): Pathogenicity and complete genome characterization of fowl adenoviruses isolated from associated with inclusion body hepatitis and hydropericardium syndrome in China. PloS ONE 10, e0133073. https://doi.org/10.1371/journal.pone.0133073

Received: 9 October 2023
Accepted: 2 May 2024
Online publication: 31 October 2024

CHITRADEVI, S., K. SUKUMAR, P. SURESH, G. A. BALASUBRAMANIAM, D. KANNAN, A. RAJA: Kvantitativno profiliranje opterećenja peradi adenovirusima na primjeru eksperimentalno inficiranih pilića i uz primjenu SYBR Green bojila za PCR u stvarnom vremenu. Vet. arhiv 95, 39-50, 2025..

SAŽETAK

Cilj istraživanja bio je kvantificirati adenovirusno opterećenje (FAdV) u različitim organima eksperimentalno inficiranih pilića. Isto je učinjeno i s obzirom na različite vremenske intervale od infekcije. Lančana reakcija polimerazom u stvarnom vremenu (RT-PCR) uz upotrebu boje SYBR Green standardizirana je uz pomoć plazmidnog standarda 590 bp FAdV serotipa 11, te su procijenjene osjetljivost i specifičnost testa. Ustanovljeno je da je RT-PCR sto puta osjetljiviji u odnosu na konvencionalni PCR. Kako bi se procijenilo adenovirusno opterećenje u različitim organima peradi, provedeno je eksperimentalno inficiranje 98 jednodnevnih pilića bez specifičnih patogena. Pilići su oralnim i intramuskularnim putem inokulirani sekvenciranim FAdV serotipom 11, potvrđenim PCR-om, u količini od 0,5 mL, koji je sadržavao$10^{6.5}$/$TCID_{50}$, uz izuzetak kontrolne skupine. Inficirani pilići žrtvovani su 3., 5., 7., 10., 14. i 21. dan nakon inokulacije. U eksperimentalnoj i kontrolnoj skupini pronađene su brojne kopije FAdV u cekalnim tonzilama 5 - 7 dana nakon inokulacije, zatim u Fabricijevoj burzi i jetri. Kopije FAdV zabilježene su sve do 21. dana nakon inokulacije u svim organima, s tim da je u bubrezima uočen najmanji broj kopija u usporedbi s drugim organima.

Ključne riječi: adenovirusno opterećenje; serotip 11; gen *hexon*; boja SYBR Green; RT-PCR; kvantifikacija adenovirusa ptica