**Molecular typing of fowl adenovirus associated with gizzard erosion in commercial layer grower chicken in Tamil Nadu**

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Received: 14 October 2019; Accepted: 11 November 2019

**ABSTRACT**

The present study was undertaken to characterize fowl adenovirus associated with commercial layer grower chicken showed gizzard erosion. Ninety four commercial layer grower chicken flocks from Namakkal districts of Tamil Nadu had shown reduced feed intake, reduced weight gain, uneven growth and mortality of 0.3 to 7.7%. On postmortem examination of affected birds showed mild to severe gizzard erosion, blackish discoloration of gizzard contents, pale liver and no major lesions were seen in other organs. Total DNA was extracted and 897 bp fowl adenovirus specific hexon gene was amplified by PCR. Out of 94 flocks screened seven flocks were found positive of fowl adenovirus. Chicken embryo liver cell culture was prepared to isolate field fowl adenovirus from suspected flocks. Concurrent infection of chicken anaemia virus (CAV) was also screened by PCR for 419 bp VP2 gene of CAV and found that all the seven flocks which were PCR positive for FAdV also found positive for CAV. Sequencing and phylogenetic analysis of 897 bp FAdV hexon gene revealed that, it was belonged to FAdV serotypes 2 and 3 of species D.

**Keywords:** Commercial layer grower chicken, Fowl adenovirus, Gizzard erosions, PCR, Phylogenetic analysis, Sequencing

**MATERIALS AND METHODS**

**Sample collection:** From October 2016 to September 2018, 9 to 13 weeks old commercial layer grower chicken flocks showed mortality, uneven growth, dullness and reduced feed intake in and around Namakkal district, Tamil Nadu, India. Liver and gizzard samples were collected from 94 commercial layer grower flocks for disease diagnosis and further analysis.

**Histopathological examination:** After necropsy examination, the liver and gizzard tissues collected from affected chicken were fixed with 10% formalin. The formalin fixed tissues were processed by paraffin wax embedding method for tissue sectioning and were stained with haematoxyline and eosin (H&E) stain (Bancroft and Stevens, 1996). The H&E stained slides were read under microscope and histopathological changes were recorded.

**Virus isolation:** For virus isolation 10% suspension of pooled liver and gizzard tissue homogenates were prepared with sterile phosphate buffered saline with pH 7.2 and freeze thawed three times. The homogenates were centrifuged at 2,000 × g for 10 min, the supernatant was treated with antibiotic and antimycotics (100 units of penicillin G, 100 µg of streptomycin and 0.25 µg of amphotericin B) and kept for 1 h at room temperature. After centrifugation at 2,000 × g for 10 min, the supernatant was filtered through
0.45 µm Millipore membrane filter and used for virus isolation. Primary chicken embryo liver cell (CELi) cultures were prepared from 13 to 15 days old embryonated chicken eggs as per the method described by Barua and Rai (2003) with slight modifications.

**DNA extraction and Polymerase chain reaction:** The DNA was extracted from pooled liver and gizzard tissues from each flock by using DNA extraction kit (Catalog No.51304, Qiagen, USA). The quantity and purity of DNA was assessed by Nanodrop™ (Thermo scientific, USA).

The polymerase chain reaction of fowl adenovirus specific 897 bp hexon gene was carried out as per Meulemans et al. (2001) with slight modification in cycle condition. The primer sequence used in this study was: forward 5’CAARTTCCAGRCAGACGGT 3’ and reverse 5’TAGTGATGMC5GCAGATCATCAT 3’. The PCR was carried out in a final volume of 20 ml containing 10 µl of 2x Red dye master mix (Amplicon, USA) (consisting of 0.05 units/µL Taq DNA polymerase, 150 mM Tris HCl (pH 8.5), 40 mM (NH₄)₂ SO₄, 4.0 mM Mg²⁺,0.4 mM of...
Table 1. Commercial layer grower flock details for fowl adenovirus (FAdV) and Chicken anaemia virus (CAV) confirmation

| Farm number | Age (wks) | PCR positives | Farm number | Age (wks) | PCR positives |
|-------------|-----------|---------------|-------------|-----------|---------------|
| 1           | 12        | Negative      | 48          | 10        | Negative      |
| 2           | 12        | Positive      | 49          | 9         | Negative      |
| 3           | 9         | Negative      | 50          | 10        | Negative      |
| 4           | 11        | Negative      | 51          | 10        | Negative      |
| 5           | 10        | Negative      | 52          | 10        | Negative      |
| 6           | 12        | Negative      | 53          | 10        | Negative      |
| 7           | 9         | Negative      | 54          | 11        | Negative      |
| 8           | 9         | Negative      | 55          | 10        | Negative      |
| 9           | 9         | Negative      | 56          | 8         | Negative      |
| 10          | 9         | Negative      | 57          | 10        | Negative      |
| 11          | 11        | Positive      | 58          | 9         | Negative      |
| 12          | 11        | Positive      | 59          | 9         | Negative      |
| 13          | 11        | Positive      | 60          | 11        | Negative      |
| 14          | 13        | Positive      | 61          |           | Negative      |
| 15          | 9         | Positive      | 62          | 11        | Negative      |
| 16          | 10        | Positive      | 63          | 12        | Negative      |
| 17          | 9         | Negative      | 64          | 9         | Negative      |
| 18          | 6         | Negative      | 65          | 9         | Negative      |
| 19          | 9         | Negative      | 66          | 10        | Negative      |
| 20          | 12        | Positive      | 67          | 11        | Negative      |
| 21          | 11        | Negative      | 68          | 13        | Negative      |
| 22          | 12        | Negative      | 69          | 12        | Negative      |
| 23          | 8         | Negative      | 70          | 11        | Negative      |
| 24          | 11        | Negative      | 71          | 10        | Negative      |
| 25          | 9         | Negative      | 72          | 10        | Negative      |
| 26          | 9         | Negative      | 73          | 11        | Negative      |
| 27          | 9         | Negative      | 74          | 10        | Negative      |
| 28          | 13        | Negative      | 75          | 12        | Negative      |
| 29          | 11        | Negative      | 76          | 11        | Negative      |
| 30          | 12        | Negative      | 77          | 9         | Negative      |
| 31          | 10        | Negative      | 78          | 12        | Negative      |
| 32          | 9         | Negative      | 79          | 10        | Negative      |
| 33          | 14        | Negative      | 80          | 12        | Negative      |
| 34          | 11        | Negative      | 81          | 10        | Negative      |
| 35          | 11        | Negative      | 82          | 9         | Negative      |
| 36          | 10        | Negative      | 83          | 11        | Negative      |
| 37          | 12        | Negative      | 84          | 9         | Negative      |
| 38          | 10        | Negative      | 85          | 11        | Negative      |
| 39          | 9         | Negative      | 86          | 12        | Negative      |
| 40          | 11        | Negative      | 87          | 10        | Negative      |
| 41          | 13        | Negative      | 88          | 10        | Negative      |
| 42          | 7         | Negative      | 89          | 12        | Negative      |
| 43          | 10        | Negative      | 90          | 9         | Negative      |
| 44          | 12        | Negative      | 91          | 11        | Negative      |
| 45          | 8         | Negative      | 92          | 11        | Negative      |
| 46          | 12        | Negative      | 93          | 10        | Negative      |
| 47          | 11        | Negative      | 94          | 11        | Negative      |

Each dNTP, 0.2% Tween 20 and Inert red dye and Stabilizer, each 1 µl of forward and reverse primer (10 pmol/µl), 3 µl of DNA and 5 µl of nuclease free water. The reaction was carried out in a thermal cycler (Multigene optimax, Labenet, USA) with initial denaturation at 94°C for 10 min, denaturation at 94°C for 5 min, annealing at 62°C for 30 sec, extension at 72°C for 2 min followed by 35 cycles final extension of 72°C for 10 min. The agarose gel was prepared with 1.5% agarose containing ethidium bromide 1.5 µl/25 ml TAE buffer and amplified 897 bp hexon gene PCR products were visualized UV gel documentation system. Concurrent infection of chicken anaemia virus infection with FAdV was also screened by PCR for 419 bp VP2 gene of CAV as per Ottiger (2010).

Sequencing and phylogenetic analysis: The four purified PCR products were cloned in pTZ57R/T cloning vector and the recombinant clones were confirmed by hexon gene specific PCR and subjected for sequencing. The both
forward and reverse hexon gene nucleotide sequences were aligned using BioEdit version 7.0 sequence alignment editor. Homology searches were conducted using the NCBI program BLAST and FAdV reference serotype sequences were retrieved from the GenBank data base and phylogenetic analysis of the nucleotide sequences of hexon gene was performed with maximum likelihood method with Taimura 3 parameter model using MEGA version 7.0.

RESULTS AND DISCUSSION

Fowl adenoviruses are ubiquitous, relatively stable in the environment and are often isolated from chicken populations. They can cause various pathologies, in nature. The present study indicates an outbreak of fowl adenovirus associated gizzard erosions in commercial layer grower chicken flock. The clinical signs observed in this study were dullness, reduced feed and water intake, reduction in weight gain, uneven growth and mortality of 0.3 to 7.7% between 9 and 13 weeks of age group. On necropsy examination, affected birds showed pale and slightly enlarged liver, mild to severe gizzard erosion and blackish discolouration of gizzard contents. No major gross lesions were observed in lungs, bursa of Fabricius and spleen of FAdV infected birds. Clinical signs and postmortem findings observed in this study was well supported by the findings of Bulbule et al. (2016) who recorded FAdV infection in 6 to 13 weeks of commercial layer grower chicken in India. The variation in mortality percentage might be due to age, breed, immune status, viral load during infection, concurrent infection and involvement or variation of serotypes in FAdV infection. On histopathological examination of gizzard sections revealed that the disruption and hyalinization and focal disruption of gizzard muscle fibres and mononuclear cells infiltration. Moderate degenerative changes with foamy cytoplasm and acidophilic intranuclear inclusions in hepatocytes were also noticed. Histopathological gizzard lesions observed in this study were similar to those reported previously in chickens naturally or experimentally infected with FAdV (Ono et al. 2001, Okuda et al. 2001). For virus isolation with CELi cells showed cytopathic effect (CPE) after third passage. The CPE indicated the presence of vacuole and honey comb appearance in third passage at 24 h post infection followed by cell rounding, clumping, detachment and floating of cells. The FAdV field isolates were well adopted and isolated in CELi cells. Many researchers (Jadhao et al. 2003, Soumyalekshmi et al. 2014, Trivedi et al. 2018) had used CELi cells for isolation of FAdV and observed similar findings as that of our present study.

In the present study, the FAdV associated with gizzard erosions was confirmed by amplification of 897 bp fragment containing the L1 loop of hexon gene. Out of 94 commercial layer grower chicken flocks screened against FAdV, seven flocks from Namakkal district of Tamil Nadu were found positive by PCR for FAdV. The per cent PCR positivity for FAdV field isolates from commercial layer grower chicken was and 7.4% respectively. The similar study was conducted by (Lim et al. 2012, Shade et al. 2013, Bulbule et al. 2016) because the hexon protein is the major surface protein of adenovirus, on which type, group and subgroup specific antigenic determinants were located (Russel, 2009). Hence hexon gene was selected for PCR amplification and detection of FAdV genome. Primer pair Hexon A and Hexon B was able to amplify conserved regions in the two pedestal regions adjacent to loop 1 variable region which enables to amplify all the serotypes of FAdV (Meulemans et al. 2001). Choi et al. (2012) stated that immunosuppression before or concurrently with FAdV infection served as an important factor for developing clinical presentations. In this study, we could found all the FAdV positive flocks had shown PCR positive for 419 bp VP2 gene of chicken anaemia virus (CAV). Similar findings were reported by Bulbule et al. 2016, Niu et al. 2017, Chitradevi et al. 2018.

Genotyping of FAdV associated with gizzard erosions were carried out by (Choi et al. 2012, Mase et al. 2014). In our study, four samples were selected and subjected for sequencing and phylogenetic analysis revealed that three isolates (Genbank accession number LC483158.1, LC483159.1, LC483160.1) grouped into serotype 2 (Belgium isolate - F339915, India - KR152221, Austria - AM407391, Belgium - AF508947 and Italy - HM592282) and only one isolate (Genbank accession number LC483161.1) showed close relationship with serotype 3 of FAdV species D of Belgium isolates (Belgium-AF508948) and all these isolates were comes under FAdV species D (Fig 1). This is in agreement with Bulbule et al. (2016) who characterized FAdV isolates associated with gizzard erosion (GE) in commercial layer birds in India and phylogenetic analysis of the hexon loop L1 gene revealed the presence of FAdV serotypes 1,4,2,3 and 11. Similarly Niczyporuk et al. (2013) confirmed field FAdV isolates from chicken in Poland by PCR specific for hexon gene encoding L1 loop and phylogenetic analysis of sequence revealed that all the isolates belonged to five species (FAdV A-E) and eight serotypes (FAdV 1, 2, 4, 5, 7, 8a, 8b and 11) whereas Xia et al. (2017) also studied phylogenetic analysis of hexon loop 1 gene of FAdV isolates from China and found that 4.5% of isolates were grouped into FAdV serotype 2. Based on the sequencing and phylogenetic analysis of FAdV isolated gizzard erosions in commercial layer grower chicken revealed the presence of FAdV serotype 2 and 3. In conclusion, fowl adenovirus serotype 2 and 3 was involved in causing gizzard erosions in commercial layer grower chicken and the presence of immunosuppressive chicken anaemia virus may aggravate the disease condition.

ACKNOWLEDGEMENTS

The authors thank the Tamil Nadu Veterinary and Animal Sciences University for providing facilities to carry out the work.

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