Original Research

Pathological Changes and Molecular Detection of Avian Nephritis Virus from Commercial Broiler Chicken

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Abstract

Gout is a metabolic disorder that occurs due to multiple causes including management, nutrition and infection. Avian nephritis virus (ANV) has been associated with nephropathy in commercial broilers. Hence the present study reports detection of ANV by reverse transcriptase polymerase chain reaction (RT-PCR) and its associated pathomorphology in gout affected commercial broiler from Puducherry and Tamilnadu during March 2015 and October 2015. Gout was recorded grossly in 24/120 (20%) and microscopically in 28/120 (23.33%) cases. Out of the 18 samples screened for ANV, 2 (11.11%) samples were found to be positive by RT-PCR. On microscopical examination of kidney moderate to severe dilatation of tubules and focal to multifocal interstitial mononuclear cells infiltration were observed. Isolation and identification of ANV is complicated and time consuming, hence the RT-PCR used in the present study can be a potential diagnostic tool for screening of ANV in the cases of gout and nephropathy in chickens.

Key words: Avian Nephritis Virus, Broiler Chicken, Gout, Pathology, RT-PCR

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Introduction

Nephropathy and subsequent gout is a metabolic disorder resulting in hyperuricemia and is a frequent cause of mass losses in the poultry. Gout is multifactorial in origin which can be infectious, nutritional, toxic, poor management or possibly a combination of factors. Bulbule *et al*. (2013) reported several outbreaks of gout in commercial broilers in India during 2011 and 2012, causing up to 40% mortality and in these cases, the managemental and nutritional causes were not involved. Among the infectious causes the specific viral diseases often associated with kidney damage and gout are infectious bronchitis virus, avian nephritis virus

and chicken astro virus. Avian nephritis virus (ANV) is associated with acute, highly contagious, but typically subclinical disease in chicken. The disease is characterized by diarrhoea, retarded growth, tubulonephrosis, interstitial nephritis, uricosis (gout) and finally death (Mandoki *et al*., 2006b). The etiological agent, Avian Nephritis Virus belongs to the family *Astroviridae* and genera *Avastrovirus*. The family Astroviridae is divided into two genera: *Mamastrovirus* (mammalian astroviruses) and *Avastrovirus* (avian astroviruses). In chickens, two astrovirus species, namely chicken astrovirus (CAstV) and avian nephritis virus, which are antigenically and genetically distinct, have been detected.

Avian nephritis virus was first isolated from the rectal contents of a 1-week-old, apparently normal, broiler chicken (Yamaguchi *et al*., 1979). Serological testing has shown that ANV infections are widespread in commercial chicken in Japan (Imada *et al*., 1980), Belgium (Decaesstecker and Meulemans, 1991), Hungary (Mándoki *et al*., 2006a) and Nigeria (Oluwayelu *et al*., 2012). In a cross sectional study carried out in India, the incidence of avian nephritis was recorded to 36.4% (Bulbule *et al*., 2013). Ghodasara *et al.* (2015) reported the pathomorphology, isolation and detection of Avian Nephritis Virus by RT-PCR from field outbreak in commercial broiler in Raipur, Chhattisgarh. Sukumar and Sumitha (2016) and Gogoi *et al.* (2017) also detected the avian nephritis virus (ANV) from commercial broiler flocks in various parts of south India.

Occurrence of ANV infections has been reported from different parts of the world, however the clinical significance and economic implication of the disease is unclear largely due to the lack of convenient diagnostic tests. Additionally, majority of the diseases/conditions end up finally as gout, differential diagnosis of ANV infections become either difficult or not possible merely on the basis of the clinical and morphological features. It is also difficult to isolate the virus in cell culture due to its comparatively poor growth in primary chicken cells. In addition, antisera specific to antigenically different ANVs for detection of virus antigen by immuno-staining methods are not widely available. Nucleic acid based method like RT-PCR has been widely used for the confirmatory diagnosis of ANV (Mándoki *et al*., 2006b). The present study reports the gross and microscopic lesions and detection of ANV by RT-PCR in commercial broilers from field outbreaks from Tamil Nadu and Puducherry.

Materials and Methods

The study was carried out on the broiler kidney samples collected between March 2015 and October 2015 from various farms located at Udumalpet, Coimbatore, Salem and Puducherry. A total of 240 kidney samples (both left and right) from 120 broiler birds were collected for further examination. The age of the birds varied from day-old to six weeks. Representative tissue samples fixed in 10% neutral buffered formalin and in absolute alcohol (gout) were processed by paraffin embedding technique for microtomy and 4-5 µm thick sections were prepared and stained by routine haematoxylin and eosin (H&E) for detailed

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histopathological studies as described by Luna (1968). Parallel sections were also stained by DeGalantha method for gout. For molecular diagnosis kidney samples were collected and stored at -20c.

Extraction of RNA from Tissues

RNA from eighteen kidney samples was extracted with TRI reagent according to the manufacturer's instructions (SIGMA-ALDRICH). About 30 mg of tissue sample was weighed and triturated with 1ml of TRI reagent for about 10 minutes. The cell lysate was vortexed and homogenized for about 5-10 minutes. The samples were allowed to stand for 5 minutes at room temperature and once again vortexed after adding chloroform (0.2 ml per ml of tri reagent). The mixture was centrifuged at 12000 x g for 15 minutes at 4˚C. From this, the resulting aqueous phase was transferred to a fresh tube and added 0.5 ml of isopropanol (per ml of tri reagent), vortexed and allowed to stand for 5-10 minutes at room temperature. The mixture was once again centrifuged at 12000 x g for 10 minutes at 4˚C which resulted in the formation of RNA pellet on the sides and bottom of the tube. The pellet was washed by adding 1 ml of 75% ethanol (per ml of tri reagent) and air dried. Later it was dissolved by adding 200µl of DEPC treated water and kept at 65˚C for 15-20 minutes to facilitate dissolution. RNA samples were stored at -80˚C until further use. The quality of RNA was checked by using Agarose Gel Electrophoresis (Rio *et al*., 2010) and by measuring the ratio of A260/A280.

The following set of primer was used for the detection of Avian Nephritis Virus as described by Todd *et al*. (2010). One step m-MuLv RT-PCR kit (Merck specialities Pvt. Ltd. India) was used for the preparation of reaction mixture.

Table 1: Primer sequence for the detection of ANV

Region of Amplification	Primer Sequence (5'-3')	Size
3'-UTR of ANV genome (Nucleotides 6715 to 6896)	Forward : ACGGCGAGTACCATCGAG Reverse : AATGAAAAGCCCACTTTCGG	182 bp

The PCR reaction (total volume 25 µl) contained 3 µl of RNA, 12.5 µl of 2X RT PCR mix, 0.5 µl of RT Enzyme mix, 0.5 µl of RNAse inhibitor, 1 µl (100pmol/µl) of each primer and 6.5 µl of Ultra-pure water. The reaction was carried out in a thermocycler (Eppendorff Inc, USA), with an initial Reverse Transcription at 45˚C for 30 minutes followed by Primary denaturation 94˚C for 2 minutes, Denaturation at 94˚C for 30 seconds, Annealing at 57˚C for 40 seconds, Extension at 72˚C for 30 seconds and final extension at 72˚C for 5 minutes. The amplified products were checked by agarose (1%) gel electrophoresis at 100 volts for 45 minutes.

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Result and Discussion

Avian nephritis virus positive birds showed swollen kidneys, prominent ureter and visceral gout (Fig. 1). The other noticeable findings observed was presence of chalky white masses surrounding the heart, kidneys, liver and other organs (Fig. 2). Hence in the present study, broilers birds that had lesions of gout and severe nephrosis were included for further study. In the present study, the occurrence of gout was more in birds less than 3 weeks (18/28, 64.28%). Grossly, gout was recorded in 24/120 (20%) cases.

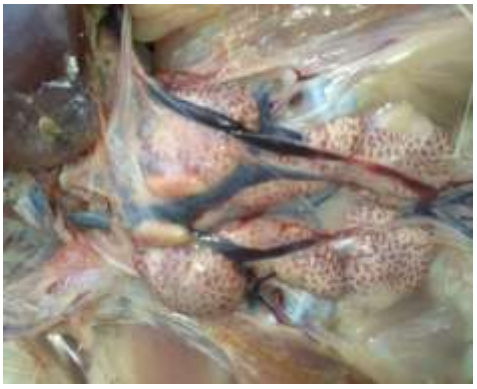

Fig. 1: Swollen kidneys with chalky white deposits.

Fig. 2: Chalky white deposits noticed in the pericardial sac and surface of liver.

Microscopically, gout was recorded in 28/120 (23.33%) cases and in these cases, kidneys showed severe damage of tubules characterized by moderate to severe tubular dilation, necrosis with large deposits of radiating fine needle shaped crystals in the tubular lumen and in the interstitum (Fig. 3). The crystals appeared black against a yellow background on De Galantha staining (Fig. 4). On microscopical examination of kidneys, large to moderate, focal to diffuse interstitial mononuclear cells infiltration were observed (Fig. 5). In some cases, interstitial nephritis characterized by multifocal perivascular and periglomerular lymphoplasmocytic infiltration with follicular pattern (Fig. 6).

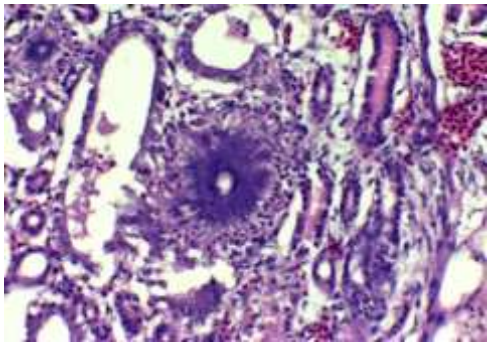

Fig. 3: Kidney- Presence of fine needle shaped urate crystals in tubules.
H&E x 400

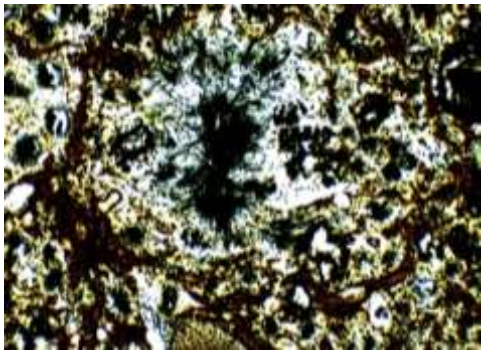

Fig. 4: Kidney-Fine needle shaped black urate deposits. De Galantha x 200

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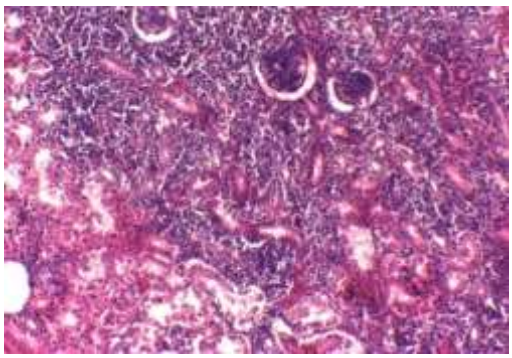

Fig. 5: Kidney- Diffuse infiltration of lympho-plasmacytic cells. H&E x 200

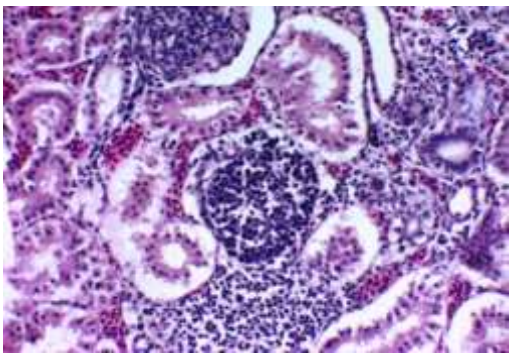

Fig. 6: Kidney-Peri glomerular aggregation of mononuclear cells. H&E x 400

The gross and histopathological features observed in the present study concurred with the observations of earlier workers (Ansar *et al*., 2004; Feizi *et al*., 2012 and Auda, 2013). Infiltration of lymphoid cells and formation of lymphoid germinal centers in affected tissue is considered to be one of the local immune responses to viral infection (Ghodasara *et al*., 2015). Histolopathological changes observed in clinical cases of ANV include interstitial nephritis that can also be encountered in infections caused by some strains of avian infectious bronchitis virus (Reece *et al.*, 1992; Imada, 2008; Hewson *et al.,* 2010)**.** Mandoki *et al*. (2006a) also observed that the histological investigations of ANV are not informative enough to differentiate between the possible causes of gout in young chickens.

Fig. 7: Agarose (1%) gel electrophoresis of RT-PCR product (182 bp) of 3' UTR of ANV genome. Lane 2&7: Negative, Lane 3&6: Positive, Lane 4: Positive control, Lane 5: 100 bp DNA ladder, Lane 8: Negative control

Isolation and identification of ANV is complicated, time consuming and require special cell lines and ANV specific antiserum for the assay (Yamaguchi *et al*., 1979). In addition, antisera specific to antigenically different ANVs for use in the detection of virus antigen by immuno-staining methods are not widely

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available. Nucleic acid based techniques such as RT-PCR can be used as potential tool for routine and confirmatory diagnosis for ANV (Mandoki *et al*., 2006b). For the molecular detection of ANV a total number of 18 kidney samples from birds less than 3 weeks of age with characteristic lesions of gout were used. Out of 18 samples, 2 (11.11%) samples were found to be positive for RT-PCR (Fig. 7).

Bulbule *et al.* (2013) observed higher incidence of ANV (36.4%). Ghodasara *et al.* (2015) reported the pathomorphology, isolation and detection of avian nephritis virus by RT-PCR from field outbreak in commercial broiler in Raipur, Chhattisgarh. Sukumar and Sumitha (2015) from Tamilnadu detected the avian nephritis virus (ANV) from 2 out of 5 kidney samples of broiler birds died of gout, diarrhoea, runting-stunting syndrome. Gowthaman *et al*. (2015) also detected the presence of ANV in 2 out of 21 commercial broiler flocks. Recently, Gogoi *et al.* (2017) studied the incidences of nephropathy in young chicks of certain commercial broiler flocks in the states of Telangana, Andhra Pradesh, Tamil Nadu and Maharashtra and found that 42 out of 96 kidney samples (43.75%) were found to be ANV positive. Mandoki *et al*. (2006b) from Hungary confirmed the presence of ANV in 36/56 (64%) kidney samples from clinical outbreaks of nephritis and gout by RT-PCR, based on primers located within open reading frame (ORF) 1a, which encodes the non-structural viral protease. Day *et al*. (2007) detected the ANV in 61/109 (56%) enteric samples of chicken by using an alternative RT-PCR based on the use of degenerate primers located within ORF 1b (RNA polymerase). In the present study, the primer targeting untranslated region (UTR) of the ANV genome between nucleotides 6715 to 6896 were used. The target region was present in the conserved region of capsid protein ORF 2 sequence. According to Todd *et al*. (2010) the primers designed based on this sequence was reported to be highly sensitive with the product size of 182 bp.

Conclusion

The present study highlighted the prevalence of ANV in the broiler chicken. Visceral gout was the most common gross and microscopic feature of ANV infected birds. The possibility of other viral agents like infectious bronchitis, infectious Bursal disease and chicken astro viral infections cannot be ignored. PCR is the most preferred diagnostic method for the detection of ANV in birds.

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