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Isolation and Identification of Avian Nephritis Virus from Commercial Broiler Chickens

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ABSTRACT

The present study was undertaken to investigate the incidences of nephropathy encountered in young chicks of certain commercial broiler flocks in the states of Telangana, Andhra Pradesh, Tamil Nadu and Maharashtra. The mortality and growth retardation associated with the condition was a major concern, necessitating a thorough study to establish the etiology. Detailed necropsy of the dead chicks revealed marked inflammation of the kidneys along with gout. Mortality percentage recorded was up to 20%. Samples were collected from the affected flocks for isolation and identification of the causative agent. The presence of ANV RNA was detected using reverse transcriptase-polymerase chain reaction and 42 kidney samples (43.75%) were found to be ANV positive while all were IBV negative. The positive samples were successfully propagated in Specific Pathogen Free chicks and embryonated eggs as was evident from the changes observed and further confirmation using RT PCR. The nucleotide sequence of the representative ANV isolate was determined and showed high identity with ANV sequences in the NCBI database. In the constructed phlyogenetic tree, the ANV isolates were clustered together distinctly separate from the related Chicken Astro virus. This investigation confirms the involvement of ANV in cases of gout, nephritis and related mortality in commercial broiler flocks.

Keywords: Avian nephritis virus, gout, nephropathy, phylogeny, RT PCR, SPF chicks

Avian nephritis virus has been known to infect chickens causing a disease condition characterized by diarrhoea, retarded growth, tubulonephrosis, interstitial nephritis, uricosis (gout) and even death (Mandoki *et. al*., 2006). First description of ANV isolation dates back to 1976 when the virus could be identified in rectal contents of apparently normal broiler chickens in Japan (Yamaguchi *et al*., 1979). Originally, ANV was considered as an avian enterovirus or an enterovirus-like virus. However, sequence analysis of the viral genome revealed molecular properties and gene organization consistent with the Astroviridae family (Imada *et al.*, 2000), which consist of small round and non-enveloped viruses, typically 28 to 30 nm in diameter (Matsui and Greenberg, 2001). ANV is placed within the genus AvAstrovirus of the family but is genetically and antigenically different from the other three members of the genus namely- chicken, duck and turkey Astro-viruses (Koci and Schultz-Cherry, 2002).

ANV isolates differ in pathogenicity therefore the disease manifestation range from subclinical infection to death (Koci and Schultz-Cherry, 2002). Chickens less than two weeks of age are predominantly affected while older chicks exhibit a lower susceptibility to clinical disease (Gough and McNulty, 2008). A resistance to disease reportedly develops after the first month of life (Imada *et al.*, 1981). High rates of mortality are observed in severe clinical cases resulting in severe economic losses (Hewson *et al*., 2015). There are atleast three different serotypes of ANV (ANV-1, ANV-2 and ANV-3) though existence of more serotypes cannot be ruled out since difficulties in tissue

culture isolation have prevented further characterisation of the diversity of ANV serotypes (Shirai *et al*., 1992; de Wit *et al*., 2011; Todd *et al*., 2011). Moreover, convenient serological diagnostics are not widely available.

Histological changes observed in clinical cases include interstitial nephritis which 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). Apart from this, chicken astrovirus (CAstV) has also been implicated in gout cases observed in commercial broilers of India (Bulbule *et al.*, 2013). Establishing ANV infection is therefore not possible without specific diagnostic techniques (Cavanagh *et al*., 1997). The occurrence of ANV infection has been reported from various parts of the world like Europe, Australia, USA, Japan and China (Imada *et al*., 1980; Mándoki *et al*., 2006; Day *et al*., 2007; Hewson *et al*., 2010; Zhao *et al*., 2011a) along with India. The clinical significance and economic implication of ANV is yet to be fully explored.

During 2013-14, several outbreaks involving baby chick nephropathy were observed in Indian commercial broilers resulting in growth retardation and mortality up to 20%. Bulbule *et al*. (2014) described that development of gout can be initiated by multiple factors and the important infectious causes include nephropathogenic infectious bronchitis virus (IBV) and avian nephritis virus (ANV). The present study was carried out to confirm and characterize the causative agent to establish the emergence of ANV as a major deterrent to the growth of the Indian poultry industry. Further studies of the prevalent ANVs would provide insights on the strategies to be employed for curbing such infections.

MATERIALS AND METHODS

Sample collection

Few farms in the states of Telangana, Andhra Pradesh, Tamil Nadu and Maharashtra which reported gout and nephritis related mortality were selected for the investigation. Detailed necropsy was performed and in farms having multiple sheds samples were collected separately. Kidney samples from the same shed were pooled. A total of 96 samples were collected aseptically in phosphate buffered glycerol and sent to the laboratory maintaining cold chain. The details of sample collection are included in Table 1. 20% (w/v) suspensions were prepared in phosphate buffered saline for isolation and other confirmatory studies.

Table 1: Details of sample collection

Place of collection	No of farms visited	Total no. of samples
Pune, Maharashtra	4	11
Hyderabad, Telangana	15	50
Anaparthy, Andhra Pradesh	9	20
Namakkal, Tamil Nadu	9	15
Total	37	96

RT PCR for detection of ANV

RNA extraction

Viral RNA was extracted from the kidney suspensions by Trizol LS (Life science) method as per the manufacturer's protocol. The purity and quantity of extracted RNA was checked by using Nanodrop (Thermo).

RT-PCR for ANV

Extracted viral RNAs were used in subsequent reverse transcription for synthesizing cDNA. Briefly, the mix containing 5 µl RNA, 1 µl of Random Hexamer primer and 0.8 µl dNTPs, 4 µl of RT buffer, 1 µl M-MULV reverse transcriptase enzyme (200 u/µl), 0.5 µl Ribolock RNase inhibitor (40U/µl), 1.2 µl DMSO and 6.5 µl nuclease free water, was subjected to the following cycling conditions- 10 min at 25°C, 60 min at 42°C and 10 min at 72°C. Finally held at 10°C and stored at −20°C for PCR amplification.

For detection of ANV the ORF1b part of Polymerase gene (255 bp) was amplified using specific set of primers described by Bidin *et al*. (2011). Primer sequence: Forward- CTTCTTTGGTGGACT GGATAA G Reverse- CCT TCTTGACATGAGTTACCTC

DNA amplifications were carried out in a total volume of 25 µl containing 100-150 nano gram viral cDNA, 1 µMol each primer and 1 U Taq DNA polymerase (Thermo) using the following conditions: initial denaturation at 95°C for 3 mins, 30 cycles of denaturation 94°C, 30 Sec; annealing

56°C, 1 min and extension 72°C, 1 min. A final extension step was performed at 72°C for 8 min.

RT-PCR for Infectious Bronchitis Virus (IBV)

For detection of IBV in the kidney suspensions, cDNA synthesis was performed as described earlier. PCR amplification was done using the set of primers described by Jahantigh *et al.* 2013 (Primer sequence: Forward-CACTGGTAATTTTTCAGATGG, Reverse-CCTCTATAAACACCCTTGCA)

PCR mix composition was same as that used for ANV PCR. The cycling conditions included an initial denaturing step of 95°C for 3 mins, followed by 30 cycles of denaturation at 94°C for 30 secs, annealing at 54°C for 1 min and extension at 72°C for 1 min. There was a final extension step at 72°C for 8 mins.

Sequencing

In order to authenticate the amplicon obtained, the PCR product of one representative positive sample was submitted to Bioserve Pvt. Ltd. Hyderabad for sequencing along with respective primers.

Phylogenetic analysis

Data retrieved from sequencing was analyzed using CLUSTAL OMEGA software and phylogenetic tree was constructed.

Virus isolation

The samples which were found positive for ANV by RT-PCR analysis were selected for isolation and pathogenicity assessment in day old SPF chicks. Briefly, the chicks were inoculated with 0.2 ml of kidney suspension and observed at regular intervals for any clinical manifestations. Dead chicks were thoroughly examined and kidney samples were collected for further confirmation using PCR. Similarly, the positive samples were also inoculated into 10 day old embryonated SPF eggs by allantoic route. The eggs were incubated for a period of 5 days and were candled daily for detection of any mortality.

Detection of antibodies against IBV

Sera samples of the SPF chicks which survived the experimental infection with ANV were analysed for presence of antibodies against IBV in order to rule out any concurrent infection. Two set of sera samples were collected from each bird at 7 and 21 days post infection and tested by IBV ELISA kit (Idexx) following manufacturer's instructions.

RESULTS AND DISCUSSION

Gross lesions

In the farms, the affected chicks appeared dehydrated and depressed. On necropsy, the major lesions included gout and nephritis with markedly swollen kidneys (Fig. 1).

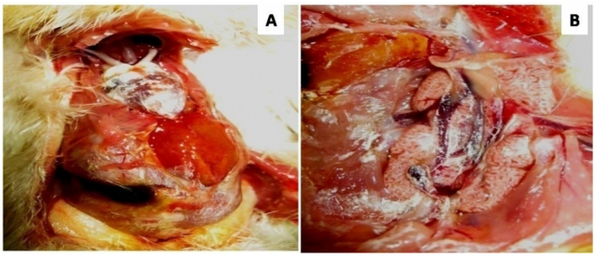

Fig. 1: Commercial broiler chicks infected with Avian Nephritis virus exhibiting gout (A) and swollen kidneys (B)

These observations are comparable to those described by Ghodasara Priya *et al*. (2015) including renal damage and visceral uricosis. Earlier studies have also reported severe uricosis, interstitial nephritis and even death in infected baby chicks (Koci and Schultz-Cherry, 2002). The mortality observed among the infected chicks was around 20 percent. In this context, Ghodasara Priya *et al*. (2015) had reported a lower mortality percentage ranging from 3 to 8% in different affected flocks with 6% average mortality. Differences in the virulence of ANV strains might influence the clinical manifestation of the nephritis. ANV strains isolated from baby chick nephropathy have reportedly shown differences in mortality and in lesions under experimental conditions (Shirai *et al*., 1992).

Detection by RT-PCR

Out of the total 96 samples, 42 (43.75%) were found to be positive for ANV. In the positive samples Polymerase gene of ANV could be amplified to obtain 255 bp PCR

products (Fig. 2). However, in case of IBV RT-PCR, all the 96 samples were found to be negative (Fig. 3).

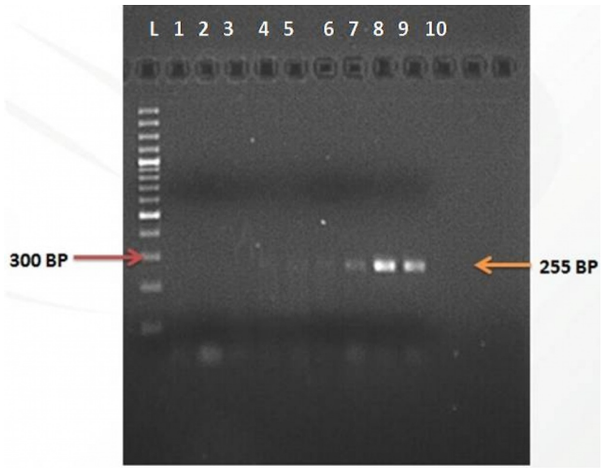

Fig. 2: ANV-specific amplicons visualized by agarose gel electrophoresis. Lane L: Ladder; lanes 1 to 9, samples; lane 10: negative control (SPF chick kidney)

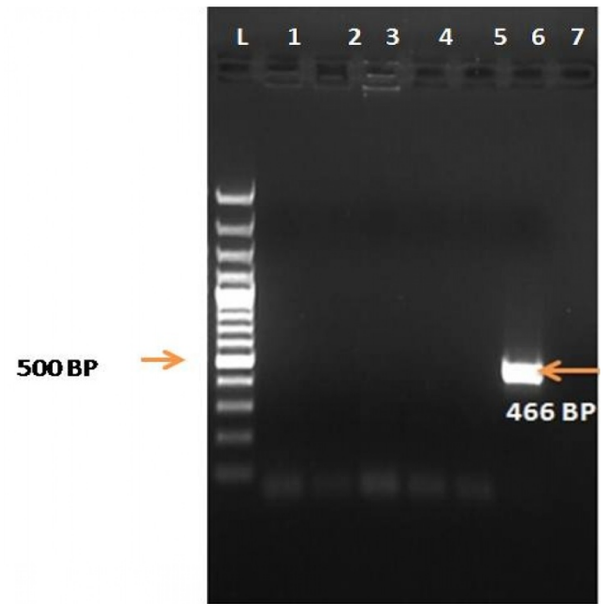

Fig. 3: IBV-specific amplicons visualized by agarose gel electrophoresis. Lane L: Ladder; lanes 1 to 5: samples; lane 6: positive contro (IBV vaccine); lane 7: negative control (SPF chick kidney)

In a similar study the presence of ANV was confirmed in 36/56 (64%) kidney samples from clinical outbreaks of nephritis and gout in Hungary (Mandoki *et al*. 2006). Use of RT PCR for detection of ANV has also been described by other workers (Ghodasara Priya *et al*. 2015, Mandoki *et al*., 2006, Todd *et al*., 2010). RT PCR, therefore, could be suitably employed for detection of ANV from clinical samples and enabled confirmatory differential diagnosis of the cause of gout and nephropathy as was evident from the absence of IBV in the same samples.

Sequencing and phylogenetic analyses

The nucleotide sequence determined from the representative amplicon was deposited to the GenBank under the accession number KM212267.1. By NCBI BLAST database analysis, highest identity of 98% was observed with the nucleotide sequence of the polymerase gene region of the ANV isolate bearing accession number KT376417.1. Similarly, more than 90% identity was observed with different ANV isolates from India and other parts of the world (Table 2).

Table 2: Nucleotide identity percentage between KM212267.1 and few selected published sequences in the NCBI gene bank based on BLAST searches

Isolate I.D	Accession number	Place of collection	Identity with KM212267.1
ANV/1059/2011/HR	KT376417.1	Haryana, India	98 %
DE-CK-SEP ANV-651-2005	HQ188692.1	USA	96 %
CRO-393	JX083350.1	Croatia	94 %
Kr/ADL 112770-ANV	KC593392.1	South Korea	92 %
ANV 1 China	HM029238.1	China	93 %

Results of sequencing confirmed that the amplicons were ANV specific. Phylogenetic tree constructed on the basis of partial polymerase gene sequences reinforced the results obtained by sequencing as the ANV isolates were found sharing a close relationship. The sequence of the ANV selected for this study was in the same cluster with the ANV isolates of India and China while the Chicken Astro viruses (CAstV) formed a separate cluster (Fig. 4).

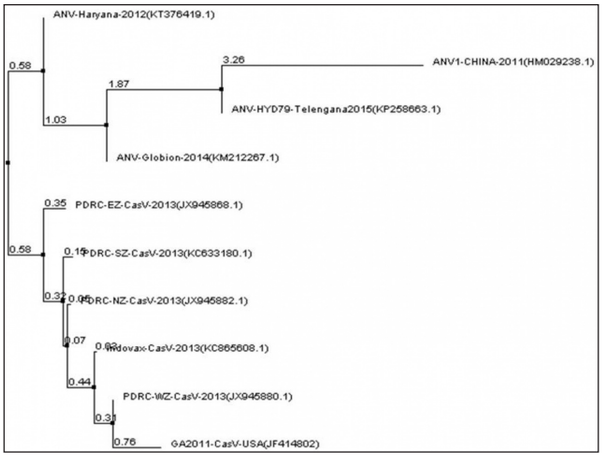

Fig. 4: Phylogenetic tree of ANV and Chicken Astrovirus based on partial polymerase gene sequences. The tree was constructed with Clustalomega using neighbour-joining method and 10 bootstrap replicates

The ANV group was further subdivided into two distinct branches wherein the sequence of our isolate was found to be closely related to the sequence of another isolate from Telangana, India. The other branch consisted of an isolate from Haryana, India. Our finding is comparable to an earlier report where phylogenetic analysis using partial ORF1b nucleotide sequences showed that the avian astroviruses formed four groups namely TAstV-1-like, TAstV-2-like, CAstV-like, and ANV-like viruses (Zhao *et al.*, 2011b). Chicken astrovirus have been known to share some sequence identity with ANV but the isolates of this virus differ antigenically and serologically from ANV (Baxendale and Mebatsion, 2004). Furthermore, studies have described that strains of three phylogenetic groups, designated ANV-1, ANV-2 and ANV-3 were found to differ antigenically from each other and belonged to three different serotypes as detected by indirect immunofluorescence(IF) or serum neutralisation (SN) tests (Shirai *et al*., 1992; de Wit *et al.,* 2011).

Virus isolation

In the SPF chicks inoculated with the ANV PCR positive samples, mortality was recorded up to 96 hours post infection. Marked swelling of the kidneys with urate deposition over the viscera was observed during necropsy (Fig. 5). All the kidney samples collected from the dead chicks were found to be positive for ANV and negative for IBV in RT-PCR. Birds that survived the infection exhibited poor weight gain in comparison to the uninoculated control group. Sera samples collected from these birds were found to be negative in IBV ELISA.

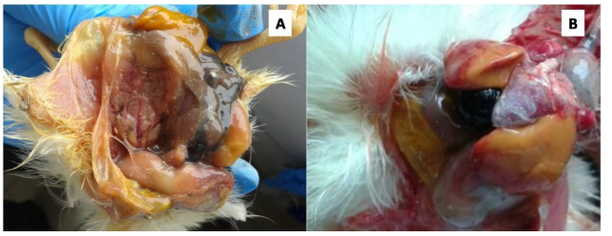

Fig. 5: Changes observed in experimentally infected SPF chicks (A) Swollen kidneys with urate deposition (B) Heart showing urate deposition

In embryonated eggs, ANV specific RNA could be detected in the kidney samples from the second passage onwards. The infected embryos exhibited dwarfism and haemorrhages were seen in parts of the body (Fig. 6). Urate deposits were evident on the kidneys, heart and other viscera.

Fig. 6: Dwarfism and haemorrhages observed in embryos infected with ANV

In the present study, attempts of virus isolation were successful in both SPF chicks and embryonated eggs. The changes observed in the experimentally infected chicks are in accordance with Imada *et al*. (1979) who had described that experimental inoculation of 1-day-old

specific pathogen free (SPF) chicks with ANV resulted in diarrhoea, weight loss, mortality and interstitial nephritis. In embryonated SPF eggs, comparable lesions were observed from the second passage and were confirmed by performing RT PCR analysis of the kidney samples. ANV reportedly grows well in embryonated chicken eggs (Takase *et al*., 1994). and in one study Ghodasara Priya *et al*. (2015) observed pale swollen kidneys with chalky deposits and dilated ureters in the embryos after three passages.

The findings of this study draw attention to the significance of ANV as a cause of gout and nephropathy in commercial broiler flocks of India. In the present investigation, RT PCR, virus isolation and ELISA were carried out to confirm the presence of ANV and thereby rule out the involvement of Nephropathogenic IBV which has been commonly involved in gout cases of chickens. Our study establishes ANV induced mortality in broiler chicks which has been responsible for considerable loss to the farmers. Hewson *et al*. (2015) opined that although ANV has been isolated frequently from commercial broilers in many countries, very little is known about its impact on poultry health and productivity largely due to the lack of convenient serological tests for detection and monitoring of ANV. Moreover, studies suggest difficulties associated with characterization of the diversity of ANV serotypes (Shirai *et al*., 1992; de Wit *et al*., 2011; Todd *et al*., 2011). In India, vaccination and other control measures against ANV infections in poultry are limited, if any. Very few studies concerning ANV are available from India (Ghodasara Priya *et al*., 2015, Bulbule *et al*., 2013). A comprehensive research is essential to gather valuable phylogenetic data and characterize the ANV strains circulating in India. This in turn will enable development of efficacious vaccines and vaccination strategies to combat the threat posed by ANV infection to the Indian poultry industry.

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