

**Szent István University
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**Molecular diagnosis of certain viral diseases of poultry,
detection of avian nephritis virus and phylogenetic comparison
of different Hungarian virus strains**

Brief version of the PhD thesis

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INTRODUCTION

Avian nephritis (AN) is a wide spread viral kidney disease of newly hatched chickens resulting in well recognizable macroscopic lesions. Poultry is prone to develop uricosis, so it is often diagnosed as cause of death. There are several non-infectious and also infectious causes which may play a role in the aetiology of uricosis. The non infectious causes are connected to nutritional or keeping problems (mycotoxin contamination of the feed, inadequate protein or fatty acid composition of the feed, vitamin A deficiency, lack of water supply, cold stable temperature). Among the infectious causes bacterial or viral pathogens may be listed. Infections of certain nephropathogenic strains of infectious bronchitis virus (IBV) result in nephrosis and interstitial nephritis. In Hungary, researchers suspected the presence of viral nephritis already in the 70's. At that time the causative agent was identified as a nephrotoxic strain of the infectious bronchitis virus. The avian nephritis virus (ANV) may also cause severe tubular degeneration in the kidney and finally gout. PM examination of young chickens sent to the Department of Pathology and Forensic Veterinary Medicine indicated that the diagnosed tubulonephrosis and interstitial nephritis might be of viral origin. As any condition which results in nephropathy and renal failure leads to gout, the differential diagnosis of ANV became essential. The aim of the thesis was to develop a fast and reliable method to diagnose ANV in samples sent to our laboratory from different farms experiencing high losses and hence to prove the presence of the avian nephritis virus in Hungary.

The infectious nephritis, caused by the avian nephritis virus is a disease of newly hatched chickens. The infected baby chicks suffer from diarrhea, retarded growth, runting-stunting syndrome; and limited mortality (2-6%) of the flocks is usually reported. Under field conditions, clinical signs associated with this virus infection in broiler chickens vary from none (subclinical) to outbreaks of the so-called baby chick nephropathy. Pathological findings in the dead chicks include nephrosis, interstitial nephritis and visceral urate deposition. Mortality may be influenced by the virulence of the ANV strain, breed of birds, and field conditions. Vertical transmission is not proved yet, the chickens get infected via oral route. The virus then replicates in the epithelial cells of the small intestine. Other organs are affected following viremia showing signs of degenerative changes which are characteristic especially in the kidneys. In the histological sections degeneration, necrosis and desquamation of the epithelial cells in the proximal convoluted tubules are seen. The affected epithelial cells contain various-sized eosinophil inclusion bodies as the virus replicates in

them. There is usually moderate interstitial nephritis, and fibrosis may also develop in the later stages.

The differential diagnosis of ANV infections is difficult or not possible based merely on the morphological and histological investigations, as other diseases of poultry may develop similar changes. The isolation of the virus might be complicated. It usually replicates in embryonic chicken kidney cells, and causes rounding of the cells; but the ANV strains may differ in their ability of in vitro replication. The virus particles can be demonstrated by transmission electron microscopic examination, but the procedure takes days and it is not specific for the ANV as several other viruses show similar appearance. The complete sequence of the reference strain was deposited in the Genebank (NCBI), so molecular diagnostic methods could be used.

Avian nephritis virus belongs to the family *Astroviridae*, genus *Astrovirus*. The genome consists of a single positive-stranded RNA, but lacks a helicase motif typical of other positive-strand RNA viruses. The genome includes 6,927 nucleotides and a polyA tail. It has three open reading frames (ORFs). The first ORF (ORF1a) encodes serine protease and the second ORF (ORF1b) an RNA-dependent RNA polymerase. The third ORF (ORF2) most likely encodes the structural proteins of the virion as a polyprotein precursor. Two different sized RNAs were detected in the ANV-infected cells, indicating that viral proteins are expressed via the production of subgenomic mRNAs.

ANV was first detected in 1976 and was classified to the family *Picornaviridae*. In 2000 the virus was re-ordered as a new member of the family *Astroviridae*. Up to now five avian astroviruses have been placed into the family (duck astrovirus 1, turkey astrovirus 1 and 2, and ANV 1 and 2), but recently three new astrovirus-like viruses were also isolated from chickens. The avian astroviruses exhibit relatively low level of sequence identity with astroviruses of other species; and also considerable variability is seen within the avian astroviruses. There are reports on at least two serotypes of ANV, based on the substantial differences in the genomes and also in pathogenicity of different strains.

The aim of my PhD project was to work out PCR based diagnostic methods, which can be carried out quickly and give specific diagnosis with high efficiency. I was working mainly with the PCR technique, because the PCR method is an appropriate routine diagnostic tool for the rapid and reliable detection of pathogens in samples collected from diagnostic cases. The PCR is also a proper tool for a routine screening of the animal flocks for the presence and the distribution of different infectious diseases.

MATERIALS AND METHODS

Between 2002 and 2005 several carcasses from 25 different Hungarian flocks showing the special pathological changes were sent to our Department for diagnostic examination. According to the anamnesis the losses in the flocks were higher than expected and the remaining animals show retarded growth. Otherwise in the flock no obvious clinical signs of the disease or only general symptoms were observed. In the carcasses sent for dissection different pathological changes were found depending on the age: signs of diarrhea, acute kidney failure or gout. Other diagnostic institutes in Hungary experiencing similar dissection results in baby chicks collected and sent intestinal and kidney samples from 26 different farms. Two frozen samples from suspected ANV cases from 1991 were also subjected to examination.

In order to detect Avian Nephritis Virus (ANV), kidney and intestinal samples from chickens diagnosed with enteritis, acute nephritis and gout were subjected to histopathologic and transmission electron microscopic (TEM) examination.

Other samples were pooled for screening or stored individually for particular organ examinations, then homogenized for subsequent RNA extraction/purification. ANV-specific primer pairs were designed on the 5' non-structural region of the reference strain (GeneBank accession number NC_003790). An RT-PCR assay followed by a nested reaction was developed. After the optimization of the PCR reaction on the reference strain, other kidney samples were also screened. The reactions resulted in distinct products with the previously calculated size, 816bp after the RT-PCR and 324bp after the nested RT-PCR reaction. The nucleotide sequence of the 816bp long amplicon was determined. The later PCR screening resulted in a detection of a specific product in several samples from Hungarian flocks.

Later a PCR method with higher specificity and sensitivity was developed. To minimize the possibility of contamination, a fast and reliable ANV specific single-step RT-PCR based detection method without a nested step has been carried out with a new primer pair positioned also on the ORF1. This reaction resulted in a 607bp long amplicon.

Phylogenetic trees of the nucleic acid and putative amino acid sequences were established by using sequence data from 11 positive samples. Blocks of sequence data leading to 465 bp were used for the analysis.

We designed a primer pair on the ORF2 genom region to analyse the phylogenetic status of the Hungarian samples also on the structural protein region. The reactions resulted in distinct products with the previously calculated size, 408bp after the RT-PCR.

All of our samples were screened for infectious bronchitis virus (IBV), which is another frequent viral pathogen resulting in distinct and recognizable pathological kidney changes. Because of the periodical appearance of uricosis and high losses among baby chicks, the contamination of the vaccines were suspected, therefore as a possible route of infection in hatcheries five commercial IBV vaccines were also checked for the presence of ANV specific nucleic acid.

RESULTS

During the external examination the carcasses showed signs of diarrhea with feces around the cloaca, retarded growth and exsiccation. The major macroscopic changes were signs of renal damage, acute visceral uricosis (gout). No deposition was seen in the joints.

In the HE sections, severe cases of tubulonephrosis with non-inflammatory regressive changes in the tubular epithelial cells of the proximal convoluted tubules were diagnosed. Cytoplasmic inclusion bodies were localized in the desquamated tubular epithelial cells. Focal mononuclear cellular infiltration in the renal interstitium (mild interstitial nephritis) was also observed. The TEM examination revealed the presence of small round, non-enveloped virions of 28-31 nm in diameter in the tubular epithelial cells with icosahedral capsid. The characteristic star-like morphology of the capsid could not have been recognized.

The ANV specific RT-PCR method resulted in distinct amplicons in 33 farms out of 53. The 819 bp RT-PCR-product was detected in kidney, intestine, thymus and pancreas samples of baby chicks showing the pathological lesions. The direct nucleotide sequence of the 684bp section of the RT-PCR product has shown the highest identity with the nucleotide sequence of the targeted genome region of ANV (accession number NC_003790) in BLAST search. The sequence was deposited in the Genbank (accession number AY831433) and was aligned with the complete ANV genome, exhibiting 92% identity between the Hungarian genotype and the ORF1 genome section of the reference virus strain.

The nucleotide sequence of the 607bp amplification products was determined and phylogenetic analysis was performed. The nucleotide sequences of 465bp long regions of the 607bp amplicons were determined and have been deposited into the GenBank under the following accession numbers: DQ327608-DQ327618. They showed the highest identity with the nucleotide sequence of the ORF1 genome region of ANV (accession number NC_003790) in BLAST searches. The sequences were aligned to each other and to the reference strain, the identity levels varied between 76 and 86%. The sequenced genome fragments were translated to amino acids (aa) resulting putative, 154 aa long, partial sequences of the non-structural polyprotein of ANV. The amino acid sequence alignments indicated 88 and 93% a identity between the investigated Hungarian viruses and the reference ANV strain (NP_620617). Even samples collected on the same farm, at the same time, but from different flocks differed in nucleotide and putative amino acid sequences.

DISCUSSION

All examined flocks suffered from diarrhea, retarded growth, renal malfunction and experienced losses higher than usual during rearing. Based on the results of the morphological and histopathological the nephropathy found in the chicken carcasses was thought to be of infectious origin. The chickens died of visceral uricosis, the histological investigations revealed viral nephroso-nephritis. The dissected carcasses belonged to different age groups, varying from 4-day-old to around 3-week-old animals. The possible viruses which may initiate renal failure, are certain nephropathogenic strains of infectious bronchitis virus (IBV) and in the last decade studies indicated that another virus may cause tubulonephrosis and interstitial nephritis, which is the *avian nephritis virus* (ANV). The results of the dissection and the supplementary examinations called our attention to the possible presence of ANV in Hungary.

An ANV specific RT-PCR based detection method was developed to identify the virus. In a survey we detected the specific PCR product in samples collected from different Hungarian flocks. During the development of the PCR-based diagnostic assay, several primer pairs were designed and tested on the samples with suspected ANV infection. Two primer pairs annealing the non-structural protein region and a primer pair annealing to the ORF2 could consequently detect ANV in the samples. The products of the RT-PCR always showed the highest identity to the Japanese reference strain after the direct nucleotide sequence analysis. Although the Hungarian and Japanese chicken populations are separated from each other, the variability amongst the Hungarian genotypes was similar to that found between the Japanese reference strain, and the Hungarian samples. The phylogenetic analysis of the deduced amino acid sequences indicated that several mutations are silent, and the viruses belonging to different genotype groups may show identity in their phenotypes. Differences were also found on the protein sequences of viruses collected in the same farm in the same time. The positive samples used in a phylogenetic analysis were showing high divergence from each other and the reference strain.

In our cases the small intestine samples of the baby chicks up to one week of age were showing strong positivity for the ANV nucleic acid while the kidney samples from the same animals remained negative. We could prove the suspected pathogenesis, because following an oral infection the virus replicates first in the intestinal epithel cells and reaches the other organs (i.e. kidney) after a viraemia. As the losses in the flock did not stop after the first week, we received futher samples from the same farm a few days later. The kidney samples

of the chickens checked from the second batch became positive for the presence of the ANV nucleic acid. Using our new test we proved the presence of the avian nephritis virus in several Hungarian flocks and the results of the screening helped to clarify the occurrence of this viral disease.

According to our diagnostic RT-PCR method developed for the detection of IBV specific nucleic acid, the IBV infection was detected alone in 11%, or together with ANV infection in 33% of the examined chicken flocks. Despite of the pathological findings indicating viral infection in the background of gout, in 26% of the farms involved in the study we could not prove the presence of infectious bronchitis or avian nephritis virus infection. We excluded the vaccine contamination from the possible infection sources as all the vaccine samples turned out to be negative for ANV nucleic acid.

Nephropathy and subsequent gout is a frequent cause of mass losses in the poultry industry. Among the agents playing role in the etiology of the disease the *avian nephritis virus* (ANV) is associated with acute, highly contagious, but typically subclinical disease in chickens. Young chickens are the only animals to develop clinical disease and distinct kidney lesions when exposed to ANV. As the mortality is usually not high the main loss caused by the disease is the stunting of the young chicks. Our findings show that ANV infection produces nephritis not only in day-old chickens, but viral nucleic acid was present in up to three-week-old birds showing weak rearing results. Adult chickens can also be infected without showing any clinical symptoms; however reduced egg production and quality are reported.

This ANV specific RT-PCR based detection method can not only be used for research purposes, but on the other hand it is a quick, specific and reliable screening method for the diagnosis of the disease even in case of this highly mutagenic RNA virus. Based on the sequence diversity, differences in the pathogenicity between the Hungarian ANV strains are also probable. Further investigations could identify the connection between the nephritis and the presence of the virus, to clarify the effect of the genetic diversity of the ANV strains and the predisposing factors in the clinical or pathological manifestation. The phylogenetic analysis of more ANV isolates might allow a better understanding of the virus evolution, as the sequence analysis of this genome is convenient, because it is relatively small and easy to handle.

OVERVIEW OF THE NEW SCIENTIFIC RESULTS

- The presence of the *avian nephritis virus* (ANV), the causing agent of the baby chick nephropathy was proved by morphological and molecular biological methods in Hungary.
- An appropriate routine diagnostic RT-PCR method for the rapid and reliable detection of ANV was developed and tested on chicken samples collected from cases of gout and nephrosis/nephritis. The RT-PCR method described in this study detected relatively different genotypes from high proportion of samples collected from chicken suffering of gout. The application of RT-PCR was found an appropriate alternative differential diagnostic method in the suspected ANV cases.
- The aetiological role of the ANV infection was confirmed in poultry flocks experiencing high losses due to uricosis. The avian nephritis virus infection might more often be the reason for the improper breeding results in chicken industry, than diagnosed.
- Investigations of the samples revealed the presence of ANV in 62% of the investigated farms, and therefore it seems that the virus is relatively common and widely distributed in the Hungarian chicken populations.
- Different ANV strains from natural cases were phylogenetically compared. The phylogenetic tree constructed on the basis of ANV nucleotide sequence alignments indicates high diversity of the Hungarian genotypes. The Hungarian strains collected within a three years period have shown similar genetic distance to each other as to the reference strain isolated about 30 years earlier in Japan. Moreover, in some cases, the sequences of positive ANV samples from the same farm, but from chicken flocks of different age, have shown remarkable differences, even though they originated from the same hatchery.
- Our observations revealed that although the mortality rate is low, the infected chickens suffer from the sub-clinical form of the disease as they are more sensitive to keeping conditions and grow slower than expected causing economical losses to the farmers.
- The infectious bronchitis (IBV) causes similar clinical signs and pathological changes (such as nephros-nephritis) as the ANV in chickens and the co-infection of IBV results in more prominent changes and higher losses in flocks with subclinical ANV infection.

LIST OF PUBLICATIONS

1.1. List of publications related to the thesis

Mándoki Míra, Dobos-Kovács Mihály, Ivanics Éva, Nemes Csaba, Bakonyi Tamás, Rusvai Miklós:
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1.2. List of presentations related to the thesis

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