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Phylogenetic diversity of avian nephritis virus in Hungarian chicken flocks

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Phylogenetic diversity of avian nephritis virus in Hungarian chicken flocks

Summary

Avian nephritis virus (ANV) infection was detected in 4- to 22-day-old chickens collected on Hungarian farms between 2002 and 2005. The animals suffered from diarrhoea, growth retardation, runting-stunting syndrome, and 2-6% mortality was reported. Tubulonephrosis, interstitial nephritis and uricosis (gout) was diagnosed. The presence of ANV RNA was detected in chicken carcasses using RT-PCR. The virus was demonstrated in 69% of the investigated farms. The nucleotide sequence of the amplification products (corresponding to part of the genome that encodes the GP1 protein) was determined and phylogenetic analysis was performed. The nucleotide sequences showed 76 to 86% identity to the reference strain isolated in 1976 in Japan. The constructed phylogenetic tree indicates high diversity of the Hungarian ANV sequences, regardless their origin and year of sample collection. Analysis of the putative amino acid sequences encoded by the partial GP1 sequences also revealed high diversity of the virus. Even samples collected at the same farm, at the same time but from different flocks, differed in nucleotide and putative amino acid sequences. The possible effects of the sequence diversity on the pathogenicity, antigenicity and diagnostics of ANV are discussed.

Introduction

Avian nephritis (AN) is a disease of chickens caused by the avian nephritis virus (ANV), and characterized by diarrhoea, retarded growth, tubulonephrosis in severe cases, interstitial nephritis, uricosis (gout) and finally death (Shirai *et al.*, 1991a).

Since besides non-infectious causes, such as mycotoxins, chemicals, abnormal protein metabolites, vitamin A deficiency, dehydration (Sályi, 1999) some other viral diseases (i.e. infectious bronchitis virus - IBV) are also able to initiate renal failure (Siller, 1981), nephropathy and gout, the differential diagnosis of ANV infections is not possible without a specific diagnostic method (Cavanagh *et al.*, 1997).

Avian nephritis virus belongs to the family *Astroviridae*, genus *Astrovirus* (Imada *et al.*, 2000). It infects only chickens, but antibodies against the virus have also been detected in turkey flocks (Connor *et al.*, 1987). Day-old chicks are the most susceptible to the disease (Imada & Kawamura, 1997), but some of the characteristic lesions develop in older animals up to 4 weeks of age (Imada *et al.*, 1981). Infected baby chicks suffer severe uricosis, interstitial nephritis and often die (Koci & Schultz-Cherry, 2002). In histological sections degeneration, necrosis and desquamation of the epithelial cells in the proximal convoluted tubules are seen. The affected epithelial cells contain variously sized eosinophil inclusion bodies as the virus replicates in them (Maeda *et al.*, 1979). There is usually moderate interstitial infiltration by lymphocytes and fibrosis may also be developed in the later stages.

Although several research groups have examined the virus and its effects on the chicks, the exact predisposing factors, the process by which ANV causes the disease is not understood. The susceptibility to ANV increases if the chickens are immunosuppressed (Narita *et al.*, 1990).

ANV was first isolated in chicken kidney cell (CKC) cultures from rectal contents of apparently normal, 1-week-old broiler chickens in Japan in 1976 (Yamaguchi *et al.*, 1979). At that time it was thought to be a picornavirus causing a sub-clinical disease (Imada *et al.*, 1979;

Maeda *et al.*, 1979). Later, after studies on the genome structure of the virus, it turned out to be similar to that of the astroviruses, and sequence analysis confirmed that ANV is a member of the family *Astroviridae* (Imada *et al.*, 2000).

Astroviruses are small, non-enveloped viruses, typically 28 to 30 nm in diameter. They have characteristic five-pointed or six-pointed star-like morphology, observed by negative staining electron microscopy (EM). Only 10% of the particles may exhibit the typical morphology, making it difficult to accurately identify astroviruses using direct EM, especially when there are very few viral particles present (Koci & Schultz-Cherry, 2002). The genome consists of a single positive-stranded RNA, but lacks a helicase motif typical of other positive-strand RNA viruses.

The ANV genome includes 6,927 nucleotides and a polyA tail. It has three open reading frames (ORFs). The first ORF (ORF 1a) encodes a serine protease, and the second ORF (ORF 1b) an RNA-dependent RNA polymerase. The third ORF (ORF 2) most likely encodes the virion structural proteins 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 (Imada *et al.*, 2000).

Astroviruses have been identified in different species. Phylogenetic analysis based on aligning available full-length genome sequences (four out of eight human astroviruses, one feline, one porcine, one ovine and three avian astroviruses) has revealed two deep-rooted groups: the mammalian, with the ovine astrovirus being an outlier, and the avian astroviruses (Lukashov & Goudsmit, 2002). Several studies have focused on the clustering and genotyping of the human astroviruses based on shorter and longer sequences from basically all ORFs. Among avian astroviruses, the two turkey astrovirus strains (TAsV-1 and TAsV-2) clustered together and separately from the ANV genome. Analyses of the ORF1a region of these

astroviruses resulted in trees showing similar topology to those constructed after aligning the ORF1b and the ORF2 regions (Lukashov & Goudsmit, 2002).

Another study involving recently isolated avian astroviruses revealed a new chicken astrovirus (CAstV) which shows some sequence identity with ANV, but the isolates of this virus are antigenically and serologically distinct from the ANV (Baxendale & Mebatsion, 2004).

In Hungary, researchers suspected the presence of viral nephritis as far back as the 1970s. At that time the causative agent was identified as a nephrotoxic strain of the infectious bronchitis virus, which also may cause nephritis in young chickens (Tanyi & Sári, 1970).

Recently new cases of baby chick nephropathy emerged in Hungarian chicken flocks. ANV was detected in kidney samples from chickens diagnosed with acute nephritis and gout, using reverse transcription-polymerase chain reaction (RT-PCR) (Mándoki *et al.*, 2006). In the last four years we diagnosed avian nephritis virus infections in several chicken farms from all over Hungary (Mándoki *et al.*, 2005). The aim of this study was to reveal the genetic diversity of ANV in Hungarian chicken flocks and to demonstrate their relationships with the Japanese reference strain.

Materials and methods

Animals and sample collection. Carcasses were sent for diagnostic dissection from Hungarian chicken farms between 2002 and 2005. Flocks were suffering 2-6% mortality among 4-22 days old chicks. After the dissection, kidney samples were collected with separate sterile tools to avoid cross-contamination, and were stored in 8% neutral buffered formaldehyde solution or at -80°C for histological and PCR examination, respectively.

Histopathology. Organs, mainly kidneys showing pathologic changes and parts of the intestinal tract from the same animals were submitted for histopathologic examinations. Specimens were fixed in neutral, buffered 8% formaldehyde solution, routinely processed, embedded in paraffin. Five µm sections were cut with a Reichert sled-type microtome (Vienna, Austria) and were stained with hematoxylin-eosin (HE) according to standard methods (Bancroft & Stevens, 1996).

RNA extraction and RT-PCR. The tissue samples were homogenized in 1.5 ml sterile phosphate-buffered saline (PBS) using sterile ceramic mortars and sterile quartz sand. After sedimentation of the cell debris by centrifuging at 6000×g for 10min, RNA was extracted from the supernatant with QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic (5'-AGATACGCTTGCTCGTCTTG-3') and complementary (5'-CCTCTAACCGGCGATATTCT-3') primers were designed on the non-structural polyprotein (GP1) of ANV annealing between positions 1678-1697 and 2266-2285 of the reference strain (NC_003790), respectively. The oligonucleotides were synthesized by Agricultural Biotechnology Center (Gödöllő, Hungary). RT-PCR was performed using the QIAGEN OneStep RT-PCR Kit (Qiagen). Each 50µl reaction mixture contained 10µl of 5×buffer (the final MgCl₂ concentration was 2.5mM), 0.4mM of deoxynucleoside triphosphate (dNTP) mix, 20U Ribonuclease Inhibitor (Fermentas, Vilnius,

Lithuania), 40pmol of the forward and reverse primers, 2µl of enzyme mix, and 5µl of template RNA. Reverse transcription was performed at 50°C for 30 min, followed by heat denaturation at 95°C for 15 min, and 40 cycles of amplification composed of heat denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and elongation at 72°C for 1 min. The reaction was finished by incubation for 10 min at 72°C. RT-PCRs were carried out in an Omnigene thermal cycler (Hybaid, USA). A positive and negative control sample was also added to each run. Negative control was prepared with sterile water as a template. For the positive control we used the reference strain kindly provided by Dr. Vilmos Palya (CEVA-Phylaxia, Budapest, Hungary). Following the RT-PCR reaction, 15µl of the amplicons were electrophoresed in a 1.2% Tris borate-EDTA buffered agarose gel containing 5µg/ml ethidium bromide, at 8V/cm for 60 min. The bands were visualized by UV transillumination at 302nm wavelength and photographed by a Kodak DS Electrophoresis Documentation and Analysis System using the Kodak Digital Science ID image analysis software version 1.6. The size of the amplified product was estimated by using GeneRuler 100 bp DNA Ladder molecular size marker (MBI Fermentas, Vilnius, Lithuania).

Sequencing and sequence analysis. Eleven positive samples were selected for sequence analysis. After electrophoresis, the amplicons were cut out from the gel and DNA was extracted with the QiaQuick Gel Extraction Kit (Qiagen). Fluorescence-based direct sequencings were performed in both directions on the amplicons at the Agricultural Biotechnology Center (Gödöllő, Hungary) employing an ABI 3100 genetic analyzer (AppliedBiosystems, Foster City, CA, USA). Nucleotide sequences were identified by BLAST search against gene bank databases (<http://www.ncbi.nlm.nih.gov/BLAST/>). The two direction sequences were compiled and aligned to the complete genome sequence of ANV using the Align Plus 4 software (Scientific & Educational Software, Cary, NC, USA).

Phylogenetic trees of the nucleic acid and putative amino acid sequences were established by using sequence data from 11 positive samples. Phylogenetic analyses were performed with the help of the ClustalX (multiple sequence alignment) program; the reference strain from Japan (NC_003790) was used as outgroup to root the trees. Blocks of sequence data leading to 465 bp were used for the analysis. The phylogenetic trees were constructed by neighbor-joining with two parameters distance matrix (Kimura, 1980) using the PHYLIP program. The bootstrap values are replicated 1,000 times and computed with PAUP.

Results

Post mortem examination. 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, visceral uricosis; thin, white, chalk-like, urate salt deposition on the serous membranes and in the subcutaneous connective tissue (gout). The kidneys seemed pale and enlarged as signs of acute tubular nephrosis and the ureters were dilated, filled with uric acid crystals (Figure 1.). On the surface of the kidneys tubular pattern could be observed due to the deposition of urate in the tubules. No deposition was seen in the joints.

Histopathology. In the HE sections, severe cases of tubulonephrosis with non-inflammatory regressive changes in the tubular epithelial cells were diagnosed. Focal mononuclear cellular infiltration in the renal interstitium was also seen and degeneration, necrosis and desquamation of the epithelial cells of the proximal convoluted tubules were observed. As a reaction to this feature also mild interstitial nephritis was seen with infiltration of histiocytes,

lymphocytes and plasma cells. Cytoplasmic inclusion bodies were localized in the necrotized and also in some desquamated tubular epithelial cells (Figure 2.).

RT-PCR. RNA samples from the homogenized kidney tissue were checked by RT-PCR method. The primer pair was designed to a non-structural protein coding region of the genome. The calculated size of the RT-PCR product was 607 bp. The specificity of the primers was first tested against a positive reference strain of the ANV (NC_003790). The reaction resulted in distinct product with the previously calculated size (Figure 3.). All together 56 samples from 13 chicken farms were submitted to RT-PCR investigations for ANV (Mándoki et al, 2005). The viral nucleic acid was detected in 36 samples of 9 farms. Amplification products never occurred in the negative controls.

Sequence analyses. The nucleotide sequences of the 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, Imada *et al.*, 2000) 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 to 93% identity between the investigated Hungarian viruses and the reference ANV strain (accession number NP_620617).

The phylogenetic tree constructed on the basis of ANV nucleotide sequence alignments indicates high diversity of the Hungarian genotypes (Figure 4.). Two main clusters are formed; one contains two sequences obtained from the same flock in 2005; while the other

contains all the remaining Hungarian ANV sequences involved into the analysis. This latter group is sub-divided into two distinct branches: one contains two, closely related sequences from samples collected in 2003, and the other is composed of seven sequences from ANV-positive samples collected between 2002 and 2005, in which further clusters are formed by sequences of ANV genotypes collected in different years. However, the phylogenetic analysis of the deduced amino acid sequences show slightly different clustering (Figure 5.). In this tree the viruses from the same flock from 2005 and two sequences from 2003, which were in different clusters in the nucleotide tree, are forming a monophyletic cluster, and separated from the remaining sequences. The distribution of the remaining sequences also differs from the nucleotide tree. The putative amino acid sequences of the investigated genome fragment of three viruses collected in 2003, 2004 and 2005 are identical, while a further sample collected in the same farm as the latter one in 2003, is separated from them. Bootstrap resampling analyses support most of the clustering both the nucleotide and amino acid trees.

Discussion

The cases described in this study occurred naturally. The chickens died due to visceral uricosis, the histological investigations revealed nephroso-nephritis. The dissected carcasses belonged to different age groups, from 4-day-old to around 3-week-old animals. All the flocks examined by us suffered from diarrhea, retarded growth, renal malfunction and experienced higher losses during breeding than usual.

Although data in the literature suggest that ANV infection produces nephritis only in day-old chickens (Imada & Kawamura, 1997), our findings show that viral nucleic acid was present in up to three-week-old birds showing weak rearing results. The virus infection has an effect on the renal function by destruction of some tubules and causing a consequent

inflammation characterized by infiltration with mononuclear cells influencing the rearing results of the chickens. Our observations supported previous literature data describing that usually few animals die after the infection, but the others 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 (Shirai *et al.*, 1989).

Investigations of the samples revealed the presence of ANV in 69% of the investigated farms, and therefore it seems that the virus is relatively common in the Hungarian chicken populations. During the development of PCR-based diagnostic assay, several primer-pairs were designed and tested on the first samples with suspected ANV infection (data not shown).

Within these attempts only primers annealing the non-structural protein region could consequently detect ANV in the samples. The phylogenetic analysis of the Hungarian genotypes revealed very high diversity of ANV. 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. Also, 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. 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 are originated from the same hatchery. Thus, ANV exhibits so high mutation ratio that it is not possible to trace the origin of the infection in a flock, using phylogenetic methods.

The phylogenetic analysis of the deduced amino acid sequences indicates that several mutations are silent, and the viruses belonging to different genotype groups may show identity in their phenotypes (e.g.: Imr2/03, Sze1/04, Za11/05). On the other hand, differences were found on the protein sequences of viruses collected in the same farm in the same time (e.g.: Sza1/05 and Sza2/05, or Imr1/03 and Imr2/03; Figure 5.)

The high level of sequence divergence may influence the sensitivity of the PCR based diagnostic techniques via mutations in the primer annealing regions. Yet, due to the difficulties of isolation of ANV in cell cultures, the application of RT-PCR was found an appropriate alternative method in the ANV diagnostics.

Mutation derived protein changes might have effect on the pathogenicity or antigenicity of the virus as well. Animal experiments and clinical observations report different levels of pathogenicity of ANV depending on keeping conditions and age of the chicken host (Frazier *et al.*, 1990; Imada *et al.*, 1981; Imada *et al.*, 1983). Besides these factors, differences in the virulence of ANV strains might also influence the clinical manifestation of the nephritis. Shirai *et al.* (1991b and 1992) report that ANV strains isolated from baby chick nephropathy show differences in mortality and in lesions under experimental conditions. Based on the sequence diversity, such differences in the pathogenicity are also likely between the Hungarian ANV strains. 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.

Beyond the pathogenicity mutations influence the antigenical properties of the viruses. At least two distinct serotypes of ANV have been reported so far (Shirai *et al.*, 1991b and 1992). Unfortunately, only partial nucleotide sequence information is available in the GenBank from the "ANV-2" serotype (AB_046864). Because this region represents the structural polypeptide gene, its relationship to the Hungarian genotypes could not be investigated within this study. The antigenic diversity of the virus should be taken into consideration during the development of potential vaccines against avian viral nephritis.

To our knowledge, this is the first time that different ANV strains from natural cases were phylogenetically compared. Because the RT-PCR method described in this study detected relatively different genotypes from high proportion of chicken samples suffering in

gout, it would be beneficial to employ this technique for the testing and phylogenetic comparison of further strains from all over the world. Further investigations are needed on the genes coding other ORFs, especially on the capsid protein region of the AN virus. These studies would provide data on the location of variable and conserved parts of the genome and would help later the more exact analysis of the evolution of avian nephritis virus.

A more comprehensive phylogenetic analysis could provide valuable additional data to clarify the role of ANV in renal malfunctions of chickens.

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Figure legends

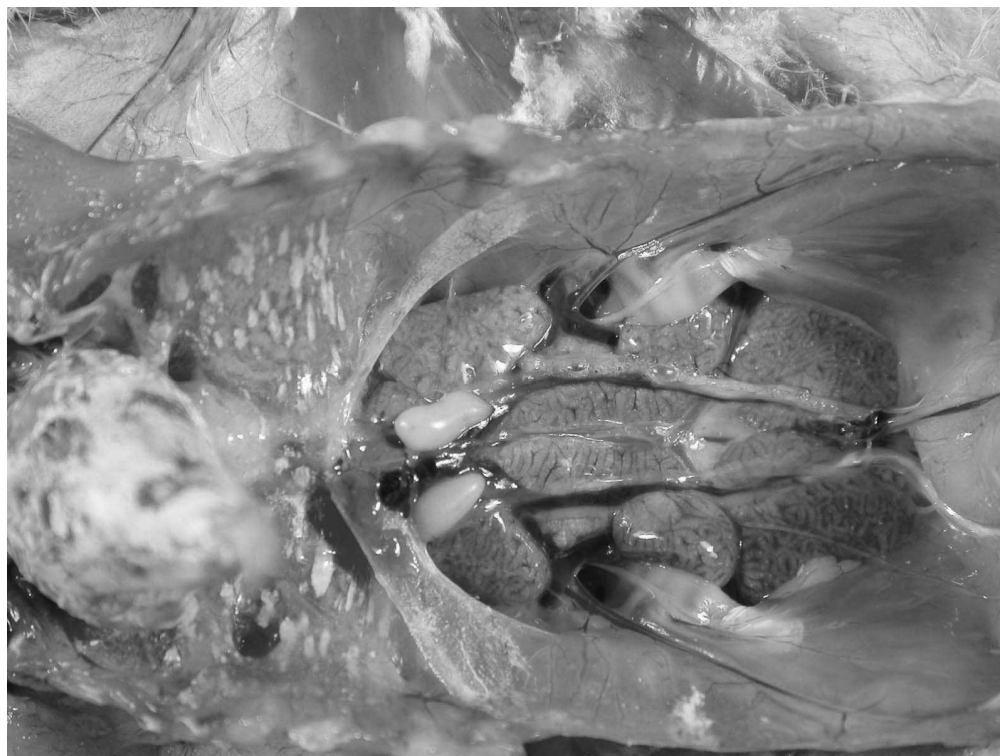
Figure 1. *Uricosis. Chalklike urate crystals are deposited on the epicardium of the heart, under the pleura and in the subcutaneous connective tissue. Swollen, pale, enlarged kidneys showing prominent tubular pattern. The ureters on both sides are dilated and fully packed with urate salts.*

Figure 2. *Degenerated tubules and mild interstitial nephritis among normal kidney parenchyma. The affected epithelial cells are necrotized and desquamated in the lumen of the tubule. Some of the epithelial cells contain cytoplasmic inclusion bodies (arrows).*

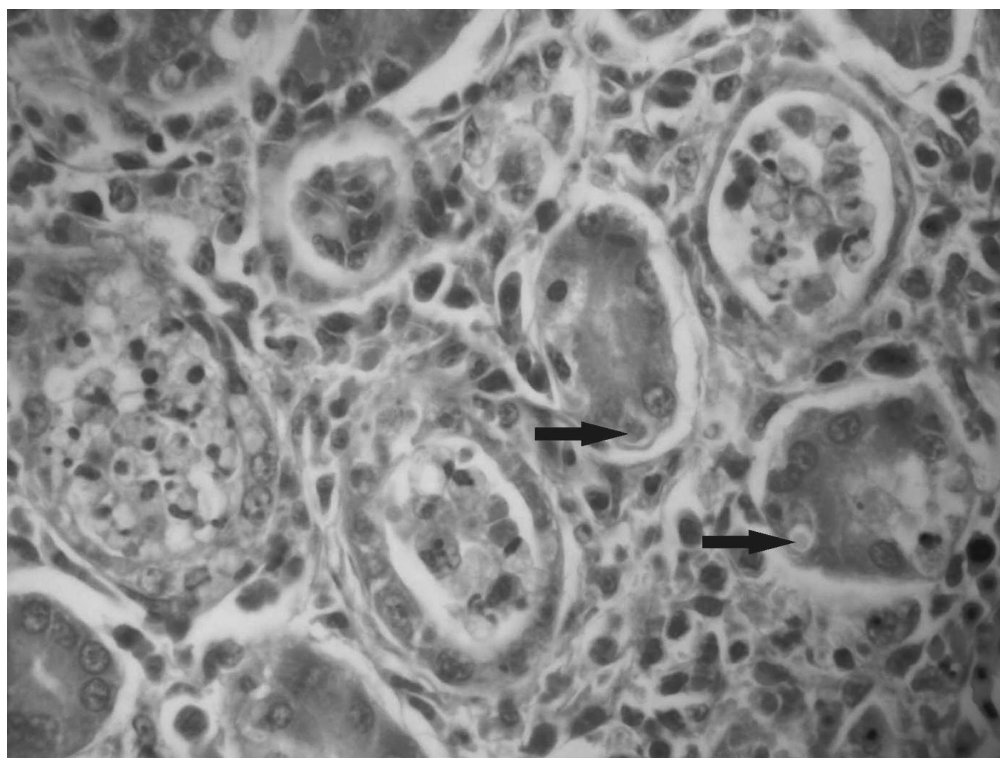
Figure 3. *AVN specific amplicons visualized by agarose gel electrophoresis. Lane M: molecular weight marker; Lane 1-10: samples; Lane 11: positive control; Lane 12: negative control. Lanes 5, 8, 9, 10 show the ANV-specific 607 bp product, while lanes 1, 2, 3, 4, 6 and 7 show no amplified product and were considered negative.*

Figure 4. *Phylogenetic relationship of the investigated strains based on the examined region of the Hungarian ANV strains and the reference strain. Three-letter codes indicate the farm, first number is the serial number of the sample, followed by / and the year of collection (20..). The reference ANV strain (NC_003790, Imada et al, 2000) was used as outgroup. Bar on the left demonstrates the genetic distance. Internal labels represent the bootstrap values of 1000 replicates.*

Figure 5. *Phylogenetic relationship of the putative amino acid sequence of the investigated strains based on the examined region of the Hungarian ANV strains and the reference strain. Codes are indicated at Figure 4. The reference ANV strain (NP_620617, Imada et al, 2000) was used as outgroup. Bar on the left demonstrates the genetic distance. Internal labels represent the bootstrap values of 1000 replicates.*



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