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Detection and characterisation of a new astrovirus in chicken and turkeys with enteric and locomotion disorders

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Abstract

In this paper, we report the unexpected discovery of a new virus in samples from chicken and turkey flocks with clinical disorders such as tenosynovitis, enteric problems, or runting and/or stunting-like conditions. Since 1987, several virus isolation attempts on samples from these flocks resulted in the same macroscopic characteristic lesions in embryonated SPF eggs, being mortality with bright red discoloration of legs and wing-tips, a swollen dark-red liver and oedema. Initial work suggested presence of an agent with characteristics of a non-enveloped RNA virus. Further work, which is described in this paper, showed that the isolated strains formed a new group of avian nephritis viruses (ANV), which is genetically and antigenically distinct from known avian astroviruses (AAstVs). Inoculation of a representative strain (isolate 19) of this new group of ANV, provisionally named avian nephritis virus-3 (ANV-3), in SPF layer chicks resulted in diarrhoea, runting and stunting and even mortality.

Introduction

Astroviruses are small, round, non-enveloped, positive sense-RNA viruses measuring 28-30 nm in diameter. The name astrovirus comes from 'astron' (Greek for star) describing the star like morphology visualised by electron microscopy (Madeley & Cosgrove, 1975).

Astroviruses cause, in most species, problems such as gastroenteritis and growth depression. To date two different astrovirus species have been identified in chickens. Avian nephritis virus (ANV-1) was originally isolated from a 1-week-old healthy broiler chick by Yamaguchi *et al.* (1979) and has now been classified as an astrovirus after complete sequencing of the viral genome (Imada *et al.*, 2000). This virus causes mild growth depression, interstitial nephritis and sometimes even mortality, especially after infection of 1-day-old SPF chickens. Most sensitive for ANV-1 are 1-day-old SPF chicks but the virus is found in all ages.

Antibodies against ANV have been detected in different parts of the world, suggesting a broad distribution (Koci & Schultz-Cherry, 2002). A second serotype of ANV (ANV-2) has been reported by several Japanese groups (Takase *et al.*, 1989; Shirai *et al.*, 1992).

Baxendale & Mebatsion (2004) reported the isolation and characterisation of another astrovirus named chicken astrovirus (CAstV) which was isolated from broilers exhibiting runting and stunting-like problems. The virus and antibodies against it have been detected in broilers and broiler breeders in Europe and the USA (Baxendale & Mebatsion, 2004; Pantin-Jackwood *et al.*, 2006; 2008; Day *et al.*, 2007; Smyth *et al.*, 2009; Todd *et al.*, 2009b). An experimental infection of 1-day-old chicks with CAstV resulted in mild diarrhoea and distension of the small intestine. Recently, Todd *et al.* (2009a) described the identification of an antigenically and genetically different CAstV which had been reported earlier as enterovirus like virus (ELV) isolates FP3 and 11672 (Spackman *et al.*, 1984). These viruses were originally isolated in the UK from flocks exhibiting early broiler mortality or hatchability problems.

Here, we report the isolation and characterisation of a new avian astrovirus isolate that is antigenically and genetically different from the known avian or chicken astroviruses. The virus was mainly found in chicken and turkey flocks with enteric and locomotion problems. Upon infection, specific lesions were found in embryonated eggs and inoculation of 3-week-old SPF layer chickens resulted in severe interstitial nephritis, runting and stunting like-problems and even mortality. This avian astrovirus (isolate 19), represents a novel group of ANVs, which is tentatively named avian nephritis virus-3 (ANV-3).

Materials and Methods

Virus isolation from field samples. Collected tissues were pooled (0.5 to 1.0 g), mixed and homogenised in buffer containing antibiotics. The tissues included in each sampling are indicated in Table 1. Next, the suspension was centrifuged at 2000g and subsequently filtered (0.45µm). The resulting fluid was inoculated either into the allantoic cavity of 8 or 9 day-embryonated SPF eggs, into the yolk sac of 5 day-embryonated SPF eggs, or onto the chorioallantoic membrane of 9 day-embryonated SPF eggs. The inoculated eggs were incubated at 37°C with 40 to 60 % humidity and were candled daily. Embryonic death occurring within one day of inoculation was considered to be non-specific. Up to 5 passages were made. In Table 1 a description is found of 10 isolates selected from a large number collected over the years. The representative isolate of this group is referred to as isolate 19.

Titration on embryonated chicken eggs. Serial 10-fold dilutions of the suspension of isolate 19 were inoculated into the yolk sac of six-day-old embryonated SPF chicken eggs. Subsequently, the eggs were incubated for 7 days at 37°C and 40-60% humidity. Mortality occurring within 24h was considered non-specific. Embryos dying from 2 to 7 days post inoculation (d.p.i) were inspected for the presence of specific lesions. The infectivity titre calculation was based on the method of Spearman & Kärber (Finney, 1952) and was expressed as median embryo infective dose (EID₅₀) per ml.

RNA extraction and RT-PCR analysis. Viral RNA was extracted using QIAamp[®] Viral RNA Mini Kit (Qiagen) and reverse transcription (RT) was performed using Ready-To-Go[™] You-Prime First-Strand Beads (GE Healthcare), both according to the manufacturer's instructions. For PCR 2µl of cDNA was used in a 96-well thermal cycler (Mastercycler

Gradient, Eppendorf). The reaction mixtures contained 1 μ m forward and reverse primers (Figure 1), in 50 μ l of PCR buffer (0.2mm of each dNTP (HT Biotechnology), 1U of Taq DNA polymerase (HT Biotechnology), 10mM Tris-HCl (pH 9.0), 1.5mM MgCl₂, 50mM KCl, 0.1% Triton X100 and 0.01% (w/v) gelatin). The PCR program included: 30 sec at 95°C followed by 45 cycles of 30 sec at 95°C, 30 sec annealing temperature (Figure 1) and 40 sec at 72°C, followed by 10 min at 72°C. For the nested PCR, a similar PCR was performed using 2 μ l of PCR product and 30 PCR cycles. The PCR products were visualized by agarose gel electrophoresis and purified using the QIAquick® Gel Extraction kit (QiaGen™) according to the manufacturer's instructions.

DNA sequencing. DNA sequencing was performed by standard PCR cycle-sequencing, using a Big Dye® Terminator Ready Reaction Mix (ABI Prism™) and an ABI Prism™ 310 automated sequencing apparatus, all according to the manufacturer's instructions. Typically 20 to 70ng of DNA (purified PCR product) was used in a reaction with 8 μ l Big Dye® Terminator Ready Reaction Mixture and 0.5 μ m primer in 20 μ l. The primers used for sequencing were the forward F-II (5'-AAA GGK AAG ACD AAG ARR RAC MG-3') and the reverse R-II-3 (5'- TCG CCT TCT GGA AGG TCT TCA-3') primers, which amplified a fragment of 469 bp that corresponded to nucleotides 2171-2640 of the ANV1 G4260 genome. The cycle sequencing PCR program contained 25 cycles of: 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C. Samples were purified using DyeEx® Spin columns (QiaGen™), according to the manufacturer's instructions and resuspended in 40 μ l. Sequence determination was performed using ABI Prism® 310 Genetic Analyzer with Data Collection version 1.0.4 and Sequence Analysis version 3 software.

Inoculation of chickens. An animal experiment was conducted to (1) attempt to reproduce the disease signs found in the field and re-isolate the virus (compliance with Koch's postulates), (2) to observe whether the virus would spread to sentinel birds and (3) to produce polyclonal antiserum against the inoculated avian astrovirus. For these purposes, 20 individually labeled 3-week-old SPF layer type chickens (both sexes) were transferred to a negative pressure isolator. Standard food and drinking water were available *ad libitum*. Fifteen chickens were inoculated with embryo homogenate of isolate 19 by three routes: 0.2 ml each by the ocular and intramuscular routes (10^5 EID₅₀/0.2 ml), as well as 0.5 ml ($10^{5.4}$ EID₅₀) by the oral route. The remaining 5 chickens were not inoculated and served as sentinels to observe spreading of the virus. Animals were inspected daily for clinical signs of disease and/or mortality. At 7 d.p.i. 5 chickens were bled and submitted for *post-mortem* (PM) and histological examination (Table 2). At necropsy, samples of duodenum, liver, thymus, kidney, tendon, bursa and pancreas were taken for histological examination. Kidney samples were also used for detection by RT-PCR. PM and histological examination was also performed on chicks that showed severe illness or died during the experiment. The remaining (inoculated) birds were boosted at 37d.p.i. and bled at 58 d.p.i. The sera were pooled and used as antiserum in the immunofluorescence assay.

Immunofluorescence assay. Microtitration plates containing chicken embryo kidney (CEK), chicken embryo liver (CEL) or LMH cells [a chicken hepatoma cell line] (Kawaguchi, *et al.*, 1987) were inoculated with isolate 19, ANV1 or CAstV. After incubation at 37°C in 5% CO₂ atmosphere for two days, the plates were fixed and incubated with dilutions of the chicken antisera raised against the different astrovirus strains, chicken serum samples from the field, or PBS as negative control. Following incubation for 1h at 37°C in a moist atmosphere, the plates were washed with PBS. One hour after incubation with goat anti-chicken IgG-FITC

conjugate (Nordic™) the plates were washed with PBS and mounting buffer was added (PBS/glycerol). A positive signal was the detection of fluorescence, correlating with specific antibodies against the virus-infected cells.

Seroprevalence field study. A small-scale seroprevalence study was performed by testing sera from 10 broiler flocks and 12 layer flocks that were chosen randomly from the routine submissions to the GD Animal Health Service Laboratory. No clinical data were available for these flocks. From each flock, 7 to 30 blood samples were collected and tested for the presence for specific antibodies by immunofluorescence against ANV-1, CAstV and isolate 19 in virus infected LMH cells or chicken embryo liver (CEL) cells.

Results

Virus isolation from field samples and initial typing. Since 1987, an unknown agent has been isolated from a variety of clinical disorders from chicken and turkey flocks in The Netherlands, Germany and the United Arab Emirates (Table 1). Most of these flocks were broiler or turkey flocks with enteric problems and/or arthritis and tenosynovitis resulting in diarrhoea, reduction in feed conversion and growth, and/or swelling and discomfort of the joints (especially the hock) and tendons of the legs resulting in considerable economic damage. The unknown agent had also been isolated from sick broiler breeder and layer flocks.

In most cases involvement of a reovirus was suspected as this virus is well known for causing malabsorption, arthritis and tenosynovitis. However, the isolates were neither reovirus nor any other known pathogen including adenovirus, infectious bursal disease virus, infectious bronchitis virus, paramyxovirus, orthomyxovirus, infectious laryngotracheitis virus,

and chicken anaemia virus (data not shown). Also, bacteria including mycoplasma could be excluded. The isolates caused characteristic macroscopic lesions in SPF embryos, namely bright red discoloration of legs and wing-tips, a swollen dark-red liver and oedema. As the embryo abnormalities did not decrease with further passages, a toxic agent could also be excluded. Treatment of these isolates with high concentrations of acid and chloroform did not influence the infectivity. It was concluded that the infectious agent was most likely a non-enveloped RNA virus.

Identification of the novel avian astrovirus. PCR and sequence analysis experiments were performed to identify the infectious agent present in the field samples. Positive PCR results were obtained using the degenerate general astrovirus specific RT primer 17 (Figure 1) in combination with primer sets 20/21 and 23/24 to detect respectively all avian astroviruses or more specifically the ANVs. These results indicated that isolate 19, and all other strains as listed in Table 1, belonged to the family of Astroviridae. However, the PCR was negative using primer set 27/28 specific for chicken astroviruses, indicating that isolate 19 and the other field strains were genetically different from the CAstV strains as described by Baxendale & Mebatsion (2004).

A specific PCR was developed after analysis of the sequence data of isolate 19 and the other field strains (see below). This PCR, using primers 29 and 30, gave positive results with samples of all field isolates mentioned in Table 1, whereas RT-samples prepared from ANV1 and CAstV were consistently found to be negative. The sizes of these PCR products were approximately 260 bp.

The 10 strains were sequenced and aligned using as reference the sequence of isolate 19. Sequences of the corresponding parts of ORF 1a from other astroviruses available in GenBank were also aligned, for instance ANV1, turkey astrovirus 1 (TAsTV1), turkey

astrovirus 2 (TAsV2), sheep astrovirus, mink astrovirus and human astrovirus (GenBank accession numbers are listed in Table 1). Only ANV1 (nt 2207 – 2607) showed a significant nucleotide sequence identity of 80% (Table 1). Of the other astroviruses, only TAsV1 had a detectable, but hardly significant, nucleotide sequence identity of 56%. The sequence homology of the other 9 field strains varied from 88 to 98% compared to the sequence of isolate 19. When all field strains were compared with ANV1 (Table 1) a sequence homology of 80-82% was observed between each of the new isolates and ANV1. A multiple alignment, using isolate 19 as a reference, of nucleotide sequences and deduced amino acid sequences of the ORF 1a region of the 10 isolates and ANV1 is listed in Figures 2 and 3 respectively.

Remarkably, an insert of 12 nucleotides in the ORF 1a, present in all 10 field strains, distinguished these novel AAsVs from ANV1 and other types of known astroviruses. For all 10 isolates, the 12 nucleotide insert in ORF 1a was located between nucleotides corresponding to the nucleotides 2485 and 2486 of the reference ANV1 strain (Imada *et al.*, 2000). The nucleotide sequence of the 12 nucleotide insert was not identical for all the isolates, however size and location were. The consensus of the nucleotide sequence of the 12 nucleotide insert was: 5' - TCYGGDMARYYT -3'. A dendrographic tree of the results of these nucleotide sequence alignments is presented in Figure 4.

Infection of chickens by the novel avian astrovirus. Inoculation of isolate 19 into 3-week-old SPF layer chickens resulted in clear clinical signs, macroscopical and histopathological lesions, detection of the astrovirus in the kidneys of the chickens and development of an antibody response. The clinical signs observed were diarrhoea (1 inoculated, 1 sentinel bird) and severe depression (2 inoculated, 2 sentinel birds). In total 2 chickens died (1 inoculated bird at 21 d.p.i., 1 sentinel bird at day 28), and another 2 severely ill birds (1 inoculated, 1 sentinel) were killed humanely for animal welfare reasons at day 23. Histopathology was

performed on 5 birds at 7 d.p.i. and all the birds that died during the experiment (Table 2). The kidneys of these birds showed severe interstitial nephritis and tubular degeneration (7/8) as the most prominent signs. The villi in the duodenum were blunted and fused (9/9) and the thymus and bursa showed lymphocytolysis and apoptosis (8/9). Acinar atrophy (7/9) was detected in the pancreas; no abnormalities were observed in the tendon. Horizontal infection to sentinel birds that showed disease signs demonstrated the virulence and infectivity of astrovirus isolate 19. The kidney samples of all 5 inoculated birds (Table 2) were RT-PCR positive at 7 d.p.i., demonstrating the presence of the virus in the kidneys of the inoculated birds.

Immunological relationship between ANV1, CAstV and isolate 19. CEK or CEL cells were infected with the novel avian astrovirus isolate 19, ANV1 and CAstV and the immunological cross-reactivity with the chicken antisera raised against isolate 19, ANV1 and CAstV was investigated. The results of these immunofluorescence assays are listed in Table 3. Each of the 3 antisera showed high titres of $>12 \log_2$ to the specific astrovirus species they had been raised against and no or only very minor cross-reactivity (all titres $< 5 \log_2$) against the other astroviruses.

Seroprevalence field survey. The results of the small scale field survey for the presence of isolate 19, ANV1 and CAstV specific antibodies in serum samples from broiler and layer flocks from The Netherlands with no or unknown clinical signs are listed in Table 4.

Antibodies specific for ANV1 were found in 1 broiler flock and 4 layer flocks, whereas antibodies specific for isolate 19 were present in a high number of broiler (8/10) as well as layer (7/12) flocks. Birds positive for ANV1 were not necessarily positive for isolate 19. In all

flocks tested, antibodies specific for CastV were present and in some cases more than 80% of the birds tested were positive.

Discussion

In this paper, we report the discovery of a new astrovirus present in chicken and turkey flocks with clinical disorders such as arthritis, tenosynovitis, enteric problems, or runting and/or stunting-like conditions. Strains of this formerly unknown virus caused the same, macroscopic, characteristic lesions upon infection of embryonated SPF eggs, being mortality with bright red discoloration of legs and wing-tips, a swollen dark-red liver and oedema. The presented work showed that these strains formed a new type of AAstVs, which is genetically and antigenically distinct from known AAstVs.

The genetic information of 10 of these strains showed that the closest (yet distinguishable) relatives were ANV-1 (80-82% nucleotide sequence similarity in nucleotides 2207 - 2607 of ORF1a) and TAstV1 (about 56% nucleotide sequence similarity in nucleotides 2450 – 2856 of ORF1a). The presence of an insert of 12 nucleotides in the ORF 1a, present in all 10 isolates, distinguished the novel AAstVs from ANV-1 reference strain G4260 and any other type of known astrovirus. Further work is needed to reveal the association of the 12 nucleotide insert in ORF 1a and the immunological and pathobiological differences observed between these groups of astroviruses. The fact that the insert consists of 12 nucleotides, thus 4 triplets which does not disturb the translational frame, suggests that the encoded 4 additional amino acids may have a role in the non-structural proteins that are expressed by ORF 1a. Independently from its biological meaning, the insert can be used as a diagnostic, positive

genetic marker for this novel group of viruses. However, it would be very useful to acquire more knowledge about the ORF1a sequence of more ANV-1 strains.

The antigenicity of isolate 19 of the novel group of ANVs was markedly different from ANV-1 and CAstV as was shown by the immunofluorescence analysis. The antiserum directed against the representative isolate 19 of the novel group was not recognized by ANV1 or CAstV and vice versa. At this moment, the antigenic relationship between isolate 19 and each of the other 9 strains detected here has yet to be investigated. Therefore it cannot yet be excluded that the new genetic group of ANVs could consist of multiple antigenic types. Further work is needed on this aspect.

Isolate 19 of the new group of ANVs, which has mainly been detected in chickens and turkeys suffering from runting and stunting like and/or locomotory disease, induced similar signs of disease upon inoculation of healthy 3-week-old SPF layer chickens, namely diarrhoea and runting and stunting like symptoms, and could be detected by RT-PCR from the diseased chickens, thereby fulfilling Koch's postulates. The clinical tenosynovitis, which was seen regularly in the broilers and poults from the field was not seen in the experimentally infected 3-week-old SPF layers. The isolation of these new avian astrovirus strains from chickens and turkeys with acute tenosynovitis and arthritis without the isolation of any other detectable pathogen suggests that they also might play a role in leg disorders. Possibly the outcome of the experimental infection was influenced by the type of chickens used in this experiment, as the acute tenosynovitis and arthritis leg disorders were all from broilers and turkeys. Another possible factor could be the difference in the conditions of SPF chickens in an isolator and the multiple infective pressure obtained under field conditions. Therefore, more experiments are needed to clarify the role of these AAstVs in tenosynovitis and arthritis.

Isolate 19 also spread from the inoculated to the sentinel birds, showing its virulence and infectivity. The pathogenicity of isolate 19, as a representative of the novel astrovirus,

was further demonstrated by the mortality and histopathological findings of the infected 3-week-old SPF birds, which were much more severe than those described for infections with CAstV strains. Experimental infections of 1-day-old and 5-day-old SPF layer chickens with CAstV caused mild diarrhoea and except for some areas of limited damage at the base of the villi of the small intestine in some birds, no histological visible damage in liver, kidney, spleen, thymus or bursa was observed (Baxendale & Mebatsion, 2004). McNeilly *et al.* (1994) used enterovirus-like virus ELV-4 strain 612, later (Todd *et al.*, 2009a) typed as a CAstV closely related to the isolate of Baxendale & Mebatsion (2004), to infect commercial 1-day-old broilers with maternal antibodies against the virus and found only a growth depression of 11.3, 20.4 and 9.6% respectively in the three trials that were performed. Experimental infections of 1-day-old SPF layer chickens with CAstV strain 612 caused only very mild histological changes in the intestine and liver, and mild to moderate lesions in the kidney (Smyth *et al.*, 2007). Experimental infections of 1-day-old SPF layer chickens with CAstV strain FP3 that is genetically and antigenically distinguishable from strain 612 (Todd *et al.*, 2009a), originally known as ELV-3, (McNulty *et al.*, 1990) caused only mild to moderate histological changes in the intestine, pancreas and kidney (Smyth *et al.*, 2007). An experimental infection with the FP3 strain caused no clinical signs in 1-day-old broilers (McNulty *et al.*, 1990) with an unknown level of maternal antibodies against FP3.

The pathogenicity of isolate 19 seems to be broader than is reported for ANV-1. Many studies have shown that the pathogenicity of ANV-1 is limited to the kidney of chickens, in contrast to the damage observed in kidney, pancreas, duodenum, thymus, bursa and liver that was seen after infection with isolate 19 (Imada, 2008). This pathogenicity of isolate 19 was observed after infection of 3-week-old SPF layers, whereas several strains of ANV-1 caused considerable mortality in 1-day-old SPF chicks but not in older birds (Imada *et al.*, 1981; Frazier *et al.*, 1990; Narita *et al.*, 1990; Shirai *et al.*, 1991a; Shirai *et al.*, 1991b; Reece *et al.*,

1992; Shirai *et al.*, 1992). These differences can be explained by the different type of ANV strains, by strain of bird, route of inoculation or level of maternally derived antibodies; other experimental conditions should also be taken into account (Mandoki *et al.*, 2006; Reynolds & Schultz-Cherry, 2008). It would also be very interesting to compare the pathogenicity of more strains of the ANV-3 group.

Further work is needed to determine the prevalence and relevance of this new group of AAstVs for the international poultry industry. Almost all isolates so far tested were obtained from Dutch and German chickens and turkeys, with the interesting exception of one isolate from the United Arab Emirates. The virus has been detected almost every year since 1987 from clinically affected Dutch and/or German poultry which could indicate that the virus might well be of significance. A limited field survey in The Netherlands indicated that antibodies specific for isolate 19 are found regularly in broiler as well as layer flocks, suggesting that a field infection has occurred with a virus related to isolate 19. Regarding the resistant nature of astroviruses, it would not be surprising if the virus were to be present in more areas of the international poultry industry, as has been shown for other AAstVs.

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Table 1. Characteristics of 10 new avian nephritis virus isolates compared to ANV-1 and other astrovirus species.

Isolate reference	Data on flocks from which isolates were obtained				Year of isolation	% sequence identity (ORF1a)			Presence of 4 amino acid insert	Genbank accession number
	Species	Type	Age	Tissue		nt ^a (with ANV1)	nt ^b (with isolate19)	AA ^c (with isolate19)		
19	Chicken	Layer	36 weeks	Trachea	2004	80	100	100	Yes	FJ940720
637	Chicken	broiler	12 days	Tendon and hock-cartilage	2005	82	90	97	Yes	FJ959032
686	Chicken	broiler	10 days	Various organs	2005	80	88	97	Yes	FJ959035
714	Chicken	broiler	30 days	Caecal tonsils and pancreas	2005	82	89	97	Yes	FJ959036
715	Chicken	broiler	30 days	Caecal tonsils and pancreas	2005	80	98	99	Yes	FJ959037
1736	Chicken	broiler	- ^d	Tendon and tibia	1987	81	89	96	Yes	FJ959038
2383	Turkey	poults	-	Joint and synovial fluid	1988	81	88	94	Yes	FJ959033
2388	Turkey	poults	-	Tendon and tibia	1988	80	88	93	Yes	FJ959034
7279	Chicken	--	-	Pancreas	2000	82	89	96	Yes	FJ959039
161317	Chicken	Layer	36 weeks	Trachea	2004	81	89	97	Yes	FJ959040
ANV-1 ^e						100	80	88	No	AB033998 (nt: 2207-2607)
TAstV1						ND ^f	56	Nd	No	Y15936 (nt: 2450-2856)
CAstV										EU669000
TAstV2										AF206663
SAstV						ND	<50	<40	No	Y15937
MAstV										AY179509
HAstV1										Z25771

^aNucleotide sequence identity (ORF1a) between ANV1 and the 10 isolates.

^bNucleotide sequence identity between isolate 19 (ORF1a) and the other isolates.

^cAmino acid sequence identity between isolate 19 (ORF1a) and the other isolates and other known astroviruses.

^d- : not known.

^eANV-1 = avian nephritis virus 1, TAstV1 = turkey astrovirus 1, TAstV2 = turkey astrovirus 2, SAstV = sheep astrovirus, MAstV = mink astrovirus and HAstV = human astrovirus.

^fNot determined.

Table 2. *Clinical signs, histopathology, and ANV-3 RT-PCR results after inoculation of isolate 19 (avian nephritis virus type 3) in 3-week-old SPF birds*

Histopathology results for 5 birds killed at 7 d.p.i. ^a and birds that died during the experiment				Histology results for 5 sentinel birds		
Organ	Histopathology	5 killed 7 d.p.i	1 died 21 d.p.i.	1 killed ^b 23 d.p.i.	1 killed ^b 23 d.p.i.	1 died 28 d.p.i.
Kidney	Severe interstitial nephritis and tubular degeneration	5/5 ^c	1/1	0/1	1/1	NT ^d
Pancreas	Apoptosis	5/5	0/1	0/1	0/1	1/1
	Acinar atrophy	5/5	1/1	0/1	0/1	1/1
Duodenum	Blunted and fused villi	5/5	1/1	1/1	1/1	1/1
Thymus	Lymphocytolysis	4/5	1/1	1/1	1/1	1/1
Bursa	Lymphocytolysis	5/5	1/1	1/1	1/1	1/1
Liver	Multifocally mononuclear aggregation	5/5	1/1	NT	1/1	0/1
Tendon	Abnormalities	NT	NT	0/1	0/1	0/1
Kidney	ANV-3 RT-PCR ^e	5/5	0/1	0/1	0/1	0/1

^a Days post infection.

^b Humanely killed for animal welfare reasons.

^c Number affected/number examined.

^d Not tested.

^e RT-PCR specific for avian nephritis virus-3.

Table 3. Immunological relationship between the astroviruses ANV-1, CAstV and isolate 19.

Antiserum	Immunofluorescence assay with viruses			
	ANV1	CASTV	Isolate 19	CEK/CEL ^a
anti-ANV-1	>12 ^b	<5	<5	<5
anti-CAstV	<5	>12	<5	<5
anti-isolate 19	<5	<5	>12	<5
SPF serum	<5	<5	<5	<5

^a None infected cells.

^b Reciprocal titres in the immunofluorescence assay expressed as log₂.

Table 4. Detection by immunofluorescence of antibodies to ANV-1, CAstV and ANV-3 isolate 19 in serum samples collected from broiler and layer flocks with no, or unknown, clinical signs in The Netherlands in 2009.

Flock type	Flock	Age in days (d) or weeks (w)	Antibodies detected to		
			ANV-1	CAstV	Isolate 19
Broiler	1	44d	1/30 (3%) ^a	19/30 (63%)	6/30 (20%)
Broiler	2	34d	0/24	13/22 (59%)	1/27 (4%)
Broiler	3	41d	0/9	1/7 (17%)	4/10 (40%)
Broiler	4	41d	0/23	8/22 (36%)	0/25
Broiler	5	41d	0/29	14/30 (47%)	0/30
Broiler	6	36d	0/26	12/25 (48%)	6/27 (22%)
Broiler	7	35d	0/30	24/30 (80%)	2/30 (7%)
Broiler	8	38d	0/25	11/24 (46%)	4/25 (16%)
Broiler	9	38d	0/28	10/28 (36%)	11/28 (39%)
Broiler	10	40d	0/21	18/22 (82%)	1/30 (3%)
Layer	A	29w	0/14	8/14 (57%)	2/14 (14%)
Layer	B	23w	0/14	8/14 (57%)	0/14
Layer	C	29w	2/15 (13%)	12/15 (80%)	0/14
Layer	D	28w	1/12 (8%)	3/12 (25%)	0/12
Layer	E	28w	0/10	7/10 (70%)	1/10 (10%)
Layer	F	28w	0/14	3/14 (21%)	1/14 (7%)
Layer	G	28w	1/14 (7%)	13/14 (93%)	3/14 (21%)
Layer	H	28w	0/14	10/14 (71%)	1/14 (7%)
Layer	I	28w	0/11	3/11 (18%)	0/11
Layer	J	28w	0/15	6/15 (40%)	0/15
Layer	K	28w	3/13 (23%)	9/13 (69%)	4/13 (31%)
Layer	L	28w	2/15 (13%)	6/14 (43%)	1/15 (7%)

^a Number of positive sample/number of birds tested per flock (percent positive samples in flocks with positive sera).

Figure legends

Figure 1. Schematic representation of the avian astrovirus genome and information on and location of the PCR primers used. (A) Schematic representation of the avian astrovirus genome. The four different primer sets and the primer used for reverse transcription (RT) are indicated by arrows and nucleotide numbers. The astrovirus genome consists of ORF-1a, ORF-1b and ORF-2. Nucleotide numbers in the boxes indicate the start and stop sites. (B) Primer names, genome location, sequence, specificity for the known avian astroviruses, product length (in base pairs: bp) and annealing temperature (°C) used for the PCR reaction, and PCR results with isolate 19 are indicated.

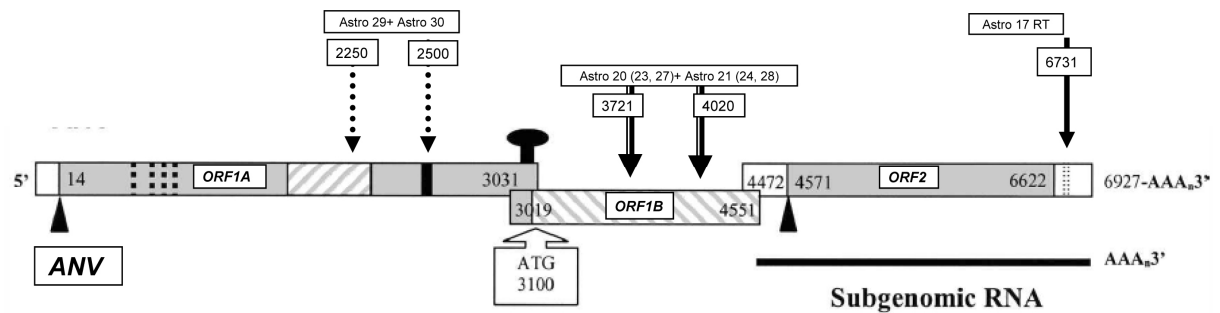
Figure 2. Nucleotide sequence alignment of the ORF-1a gene (nucleotides 2207-2608,) sequences of the novel avian nephritis virus-3 isolates and the avian nephritis virus-1. The 12 nt insert specific for the new isolates is indicated in bold.

Figure 3. Amino acid sequence alignment of the deduced sequence of the ORF-1a gene of the novel avian nephritis virus-3 isolates and avian nephritis virus-1. The 4 amino acid insert specific for the new isolates is indicated in bold.

Figure 4. Phylogenetic tree of the ORF1A sequence of the avian nephritis virus-3 isolates and reference sequences. Shown is a phylogenetic tree of the 407 nucleotide long sequences of the ORF-1a of all 10 isolates of the newly isolated avian nephritis viruses, ANV-1 and turkey astrovirus-1 (TAsTV1). All GenBank accession numbers are indicated in Table 1. The phylogenetic tree was constructed by the neighbour-joining method and bootstrapped a 1000 times using Bioedit and Mega4.1. Values are indicated in the tree.

Figure 1

A



B

Primer	Location	Sequence 5'->3'	Specificity	Length product (bp)	Annealing temperature	PCR product isolate 19	
Astro 20	FW ^a	ORF1b	TGG HCM CCY TTY TTY GGH G	ANV-1, CAstV and ANV3	300	48	+ ^c
Astro 21	REV ^b	ORF1b	RTT RTC MAC DGT KGT DGA RWA YTG				
Astro 23	FW	ORF1b	TGG ACC CCC TTC TTT GGT	ANV-1 and ANV-3	300	46	+
Astro 24	REV	ORF1b	ATT ATC AAC AGT TGT TGA AAA TTG				
Astro 27	FW	ORFf1b	TGG ACC CCC TTC TTT GGT GG	CAstV	359	55	- ^d
Astro 28	REV	ORF1b	TGY TTR CGG TGG AGC CAT GC				
Astro 29	FW	ORF1a	GTY CTY ACC GAR GAR GAR TAY C	ANV-3	260	51	+
Astro 30	REV	ORFf1a	AAD GTT ATY CTC CTA RGB TKH C				
Astro 17	RT ^e	3'-UTR ^f	TCG WTS CTA CYC	astrovirus	NA		

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^a FW: forward.

^b REV: reverse.

^c+ PCR product present.

^d - No PCR product detected.

^e RT: reverse transcription.

^f 3'-UTR: 3'-untranslated region.

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Isolate19 1 CACAAAACCTAAATCGATCTCAAAGCTGCCTTCATGAAAACAAAAGTCCTTACCGAAGAAGAATACCGTC
637 1 ..T.....A.....T.....C.....T...
686 1 ..T.....A.....T.....C.....G..T...
714 1 ..T.....A.....T.....C.....G..T...
715 1 .....
1736 1 ..T...C.....A.....T...T..C.....T...
2383 1 .....C.....A.....T...T..C.....T...
2388 1 ..T.....T..G.....T..C.....T...
7279 1 ..T..G..C..G.....T...T.....T...
161317 1 ..T.....A.....T.....C.....T...
ANV1 2207 ..T..G..C..G.....T..G.....T.....C.....T..C..

Isolate19 71 GGTTAGAGGAAGAAGGCTTCTCAAAGATGAGATTAAGAGATCGTGGACAATCTGCGAGAACAAGCCTG
637 71 ...G...G...T..T.....C.....T.....T..T..G..G...
686 71 ...G...G...T..T.....C.....T.....T..T..G..T..
714 71 ...G...G...T..C.....C.....T.....T..T..G..G..T..
715 71 .....
1736 71 ...G...T...G.....C.....T.....C..T.....T..
2383 71 ...G...T.....C.....C.....C..C.....
2388 71 ...G...T.....C..G.....T.....C..C.....T..
7279 71 ...G...T..T.....C.....T.....T..C..G..G...
161317 71 ...G...G...T..T.....C.....T.....T..T..G..G...
ANV1 2277 ...G...A..T.....C..G..C...T.....CA...G..G..

Isolate19 141 GATCGATTACCAGAATCAGCTAGATGAAGAAGGTGATGATGACTGGTATGAGCAAATGACTGAAGATCAA
637 141 ..T...T...C..A..T.....G.....
686 141 ..T..C..T...C..A..T.....G.....C.....
714 141 ..T..C..T...C..A..T.....G.....A.....G.....
715 141 .....C.....
1736 141 ..T..C...C..A..T.....A.....
2383 141 ..T..C...C..A..T.....A.....C...
2388 141 ..T..C...T.....C.....
7279 141 ..T...C..A..T.....G.....A.....
161317 141 ..T..C..T...C..A..T..C...G...C...T.....
ANV1 2347 ..C...C..T...C..A..T.....C..A.....GAA..G...

Isolate19 211 AGAATTAATGATGAGATTGATAAGCAAATTGAGCAAGACCTTGAAGATCGAGGAGAATGGTATGGCCAGT
637 211 ..G..C.....A.....C.....T.....
686 211 ..G.....C..A...C...G..C.....T.....C.....T.....
714 211 ..G..C.....A.....C.....T.....
715 211 .....C.....
1736 211 ..G..C.....A.....C...G...A...T...G.....C.....
2383 211 ..G..C.....A.....GG..T.....T.....G.....T...
2388 211 ..G..C.....A.....G.....GAGT.....T..C..C.....
7279 211 ..G..C.....A.....C..A.....A.....T.....T..C.....
161317 211 ..G..C.....A.....C.....T.....
ANV1 2417 .....C..A...CC.AA.C...AAG...C..G...A...T.....T...-

Isolate19 281 CTGGGAACCTAGGAGGATAACCTTTAAACAGAGACGATGCTTCGCTTCATTACGCTTGGTCGACAACA
637 281 ...AC.....C..G..A..G.....A...C..A.....
686 281 ...AC.....C..A..G.....T.....C..A.....G...
714 281 ..C..TC.....C..G...T.....T..C..A.....G...
715 281 .....T.....G.....G.....
1736 281 .....TC.....G.....A...G.....
2383 281 ...A...T...A.....C..GG.A.....T.....G...
2388 281 ...A..G.....A.....C..GG.A.....A..C..GA...
7279 281 ...TC.....T...G...G..A.....C..G...
161317 281 ...AC.....C..G..A..G.....A...C..A.....
ANV1 2486 -----AA.....C..G..A.....T.....A...C..G...

Isolate19 351 ACAAATAGCCACAATTTTCATTTCTGATGGCTATGAAGATAGAGCTGAAGAACTCTATAATAA
637 351 .....C.....G.....T...
686 351 .....C.....G..G...T...C...
714 351 .....C.....G.....
715 351 .....
1736 351 .....C...T.....C.....T.....T..T..C...
2383 351 ...T..CG...T..C...C..A...T.....T...
2388 351 .....C.....T..G.....A...T...C...
7279 351 .....CG.....T.....TC..C...
161317 351 .....C.....G.....T...
ANV1 2345 ...GG..G...TG..C.....T...G..C.....C...

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Figure 2. Nucleotide sequence alignment of the ORF-1a gene (nucleotides 2207-2608,) sequences of the novel avian nephritis virus-3 isolates and the avian nephritis virus-1. The 12 nt insert specific for the new isolates is indicated in bold.

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Isolate19      1  HKTKSISKAAFMTKVLTEEEYRRLEEEGFSKDEIKEIVDNLREQAWIDY
637            1  .....
686            1  .....
714            1  .....
715            1  .....
1736           1  .....
2383           1  .....
2388           1  .....R.....
7279           1  ...A.....
161317         1  .....
ANV1-orf1a     1  ...A.....T...D.....L..

Isolate19      51  QNQLDEEGDDDWYEQMTEDQRINDEIDKQIEQDLEDGRGEWYGQSGKPRRI
637            51  .....D.....Q....
686            51  .....D.....Q....
714            51  .....D.....Q....
715            51  .....S....
1736           51  .....D.....Q....
2383           51  .....V.....R.....L...
2388           51  .....V.....R...S...D.....
7279           51  .....D.....Q....
161317         51  .....D.....Q....
ANV1-orf1a     51  .....E.....Q..QN..R.....---.K.

Isolate19      101 TFKQRAMLRFIQLGRQQIATISFPDGYEDRAEELYN
637            101 .....T.....F.
686            101 .....T.....F.
714            101 .....T.....
715            101 .....
1736           101 .....T...L.....F.
2383           101 ..E.....HT.....F.
2388           101 ..E.....C.....IF.
7279           101 .....T.....F.
161317         101 .....T.....F.
ANV1-orf1a     97 .....V..V.....
    
```

Figure 3. Amino acid sequence alignment of the deduced sequence of the ORF-1a gene of the novel avian nephritis virus-3 isolates and avian nephritis virus-1. The 4 amino acid insert specific for the new isolates is indicated in bold.

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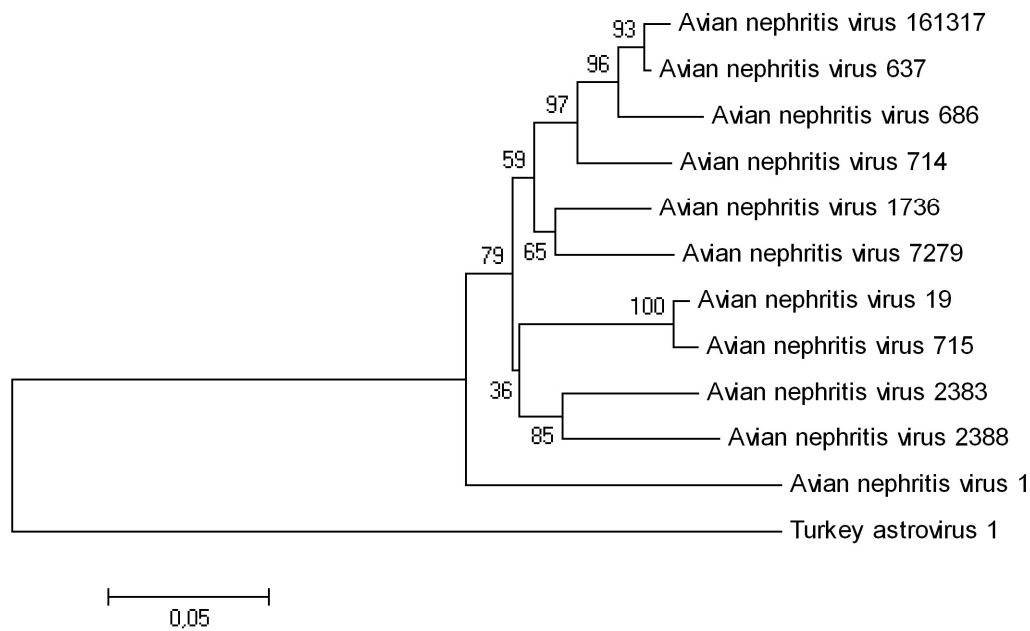


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