Molecular Characterization of an Avian Astrovirus

MATTHEW D. KOCI,1,2 BRUCE S. SEAL,1 AND STACEY SCHULTZ-CHERRY1*

Southeast Poultry Research Laboratory, U.S. Department of Agriculture-Agricultural Research Service, Athens, Georgia 30605,1 and Department of Medical Microbiology, College of Veterinary Medicine, University of Georgia, Athens, Georgia 306042

Received 21 December 1999/Accepted 27 March 2000

Astroviruses are known to cause enteric disease in several animal species, including turkeys. However, only human astroviruses have been well characterized at the nucleotide level. Herein we report the nucleotide sequence, genomic organization, and predicted amino acid sequence of a turkey astrovirus isolated from pouls with an emerging enteric disease.

Astroviruses are small nonenveloped, positive-sense RNA viruses, which are distinct from other small round, positive-stranded RNA viruses such as caliciviruses and picornaviruses (5, 30, 39). Astroviruses have been reported to cause enteric disease in mammalian and avian species (2, 3, 9, 11, 14, 16, 18, 22, 26–28, 31, 35, 36, 38, 40, 41; R. E. Gough, M. S. Collins, E. Borland, and L. F. Keymer, Letter, Vet. Rec. 114:279, 1984), and is one of the major causes of diarrhea in infancy and childhood (17). Astroviruses also cause outbreaks of enteric disease in turkey pouls. Astrovirus was first reported as a cause of gastroenteritis and mortality of turkeys 6 to 11 days of age in 1980 by McNulty et al. (28). Since then, there have been sporadic reports of astrovirus outbreaks in turkeys, mostly related to enteritis and growth depression (31). Sequence information has been reported from a previous turkey astrovirus (T AstV) isolate (21) (now referred to as T AstV-1 for clarity). However, this sequence is limited to 476 nucleotides (nt) from the 3′ end of the genome. Only the human astroviruses (H AstV) have been sequenced completely.

H AstV has been reported to contain approximately 6,800 nt and to be organized into three open reading frames (ORFs), ORF 1a, 1b, and 2. From the 5′ end of the genome, ORF 1a codes for the nonstructural proteins identified as a serine protease, transmembrane helices, and a nuclear localization signal, respectively (7). ORF 1b codes for the RNA-dependent RNA polymerase (RDRP). ORFs 1a and 1b overlap by approximately 70 nt. ORF 1b is brought into frame by a retrovirus-like frameshift sequence that produces a stem-loop (25). At the 3′ end of the genome is ORF 2, which codes for the capsid protein and is followed by an untranslated region and poly(A) tail. ORF 2 is transcribed into a subgenomic message of approximately 2,500 nt (7). In these studies we report the isolation and molecular characterization of a T AstV from pouls with an emerging enteric disease of turkeys (33). Sequence analysis of this isolate has established that the T AstV genome contains 3 ORFs, each with predicted gene products, molecular characteristics, and organization consistent with previously reported astroviruses.

The putative astrovirus was isolated from the thymus and intestines of turkey pouls affected with poult enteritis mortality syndrome (Fig. 1) (33). Briefly, the thymus and intestines from infected turkey pouls (provided by H. John Barnes, North Carolina State College of Veterinary Medicine) were homogenized, filtered (0.2-μm-pore size; Fisher Scientific, Norcross, Calif.) and inoculated into the yolk sac of 20-day-old specific-pathogen-free (SPF) turkey embryos as described (33). At 5 days postinoculation intestines, intestinal fluid, and bursas were removed. After three passages the filtered tissue homogenate was centrifuged in a Sorvall fixed-angle rotor for 3 h at 23,425 × g, and the viral pellet was resuspended in 1 ml of 0.5 M Tris–0.25 M EDTA (TE) buffer, pH 7. The suspension was overlaid on a 27–37% CsCl gradient and centrifuged for 15 h at 130,000 × g in an SW28 rotor (L8-60M Ultracentrifuge; Beckman). A faint viral band, above the gradient layer, was removed with a syringe, brought to 15 ml with TE buffer, and centrifuged again at 23,425 × g for 3 h in a fixed-angle rotor. The purified viral pellet was resuspended in 200 μl of phosphate-buffered saline and a 50 μl aliquot of purified virus was negatively stained (15) and analyzed using a JEM-1210 transmission electron microscope (JEOL, Inc., Tokyo, Japan) at the College of Veterinary Medicine Electron Microscopy Laboratory, University of Georgia (Fig. 1).

Total RNA was isolated from purified virus using TRIzol Total RNA Isolation Reagent (Life Technologies, Rockville, Md.) as previously described (6). An initial cDNA library was synthesized (12) using the SMART cDNA Library Construction Kit (Clontech Laboratories, Inc., Palo Alto, Calif.) and then cloned using the TOPO-XL cloning system (29) according to manufacturer’s directions (Invitrogen, San Diego, Calif.). Clones were screened using Luria-Bertani medium containing kanamycin (13). Approximately 25 clones, of varying length, were sequenced as previously described (34) and analyzed using DNASTAR (Madison, Wis.) and GeneWorks 2.3 (IntelliGenetics, Mountain View, Calif.) programs. These sequences were compared to reported sequences in the GenBank database using the basic local alignment search tool (BLAST) (1), and phylogenetic analysis was completed using parsimony (37). Two clones (p25.5 and p25.6) were identified as having putative amino acid similarities to T AstV and H AstV capsid protein (Fig. 2). These clones represented the last 1.5 kb of the 3′ end of the astrovirus genome and contained the poly(A) tail. Gene-specific primers were created using PRIMER2 software (Scientific and Educational Software, Stateline, Pa.) and were used to synthesize a series of cDNA libraries using the 5′ RACE System for rapid amplification of cDNA ends (8) (version 2.0; Life Technologies). Each cycle of subsequent cDNA synthesis was designed to overlap with its predecessor by at least 200 nt. Figure 2 illustrates the astrovirus genome and the sequencing strategy used. Each new clone, upstream of the previous sequence, was analyzed by BLAST individually. This was undertaken to reduce the chance of bias towards astrovirus when incorporated into the growing consensus data. Three or
more clones from each 5' RACE reaction were sequenced at least three times to generate one overall consensus sequence for TaStV. Finally, RNA was isolated from the intestines of experimentally infected turkey poults, and TaStV was resequenced directly from the reverse transcription-PCR (RT-PCR) product. Briefly, 1 μg of total RNA was incubated with 20 pmol of reverse primer, 20 pmol of each deoxynucleoside triphosphate, and 15 U of Superscript Reverse Transcriptase (Life Technologies) in a 20-μl reaction mixture for 60 min at 42°C. An aliquot (2 μl) of the first-strand product was amplified in a 50-μl reaction mixture containing 20 pmol of each primer, 20 pmol of each deoxynucleoside triphosphate 1.5 mM MgCl₂, and 1.5 U of Taq polymerase (Life Technologies). Amplification was performed in a Perkin-Elmer 2400 DNA thermal cycler, and products were then purified using the Qiaquick PCR purification system (Qiagen, Valencia, Calif.) and sequenced. There was no significant difference between the sequence of the cloned cDNA and the RT-PCR products. The 5' terminus of the virus was confirmed by synthesizing replicate 5' RACE RT-PCR products using primers of different distances from the suspected end (all of the primers used were within 1 kb of the suspected end). These products were electrophoresed in a 1% agarose gel to confirm that each reaction yielded an amplicon of the expected size. These products were purified and sequenced as above.

Analysis of the complete, 7,325-nt, TaStV sequence identi-
The amount of similarity between the sequence of this turkey isolate and the mammalian sequences was not surprising, as avian viruses can be quite different from their mammalian counterparts. However, we were surprised at the limited similarity observed between this isolate and TAstV-1. The previously reported sequence was used by Jonassen et al. to identify a region conserved among astroviruses within the 3' noncoding sequence (21). This conserved region was not identified in our isolate, and furthermore, primers designed from the conserved region failed to produce RT-PCR product from our isolate.

The reason for this lack of similarity is not understood. However, TAstV may represent a different serotype since antibodies against the TAstV-1 isolate failed to recognize our virus by Western blot analysis (data not shown).

The predicted amino acid sequence of the entire TAstV capsid protein was also compared to published astrovirus sequences. However, only the feline astrovirus (FAstV) and HAstV capsid proteins have been completely sequenced. These sequences were used to determine amino acid similarities (Table 1) and phylogenetic relationships (Fig. 3). A heuristic search was completed with midpoint rooting. The phylogenetic tree in Fig. 3 shows that astroviruses clustered in one main branch, while the hepatitis E virus and Norwalk virus sequences clustered together as another branch.

The amount of similarity between the sequence of this turkey isolate and the mammalian sequences was not surprising, as avian viruses can be quite different from their mammalian counterparts. However, we were surprised at the limited similarity observed between this isolate and TAstV-1. The previously reported sequence was used by Jonassen et al. to identify a region conserved among astroviruses within the 3' noncoding sequence (21). This conserved region was not identified in our isolate, and furthermore, primers designed from the conserved region failed to produce RT-PCR product from our isolate.

The reason for this lack of similarity is not understood. However, TAstV may represent a different serotype since antibodies against the TAstV-1 isolate failed to recognize our virus by Western blot analysis (data not shown).
specific to base positions 588 to 979 of ORF 2. This probe was synthesized by PCR, using gene-specific primers, clones 25.5 (Fig. 2), and digoxigenin-11-dUTPs and then hybridized to the membrane, and detected by chemiluminescence following the manufacturer’s instructions (Roche Molecular Biochemicals, Indianapolis, Ind.). The detected RNAs correspond to the reported size of the astrovirus genome and subgenomic RNA (20). There is also a faint third band visible beneath the subgenomic RNA. The identity of this band is not known.

These data represent the first complete sequence and molecular characterization of a nonhuman astrovirus, to our knowledge. This is based on the identification of sequence similarities to astrovirus as well as the identification of several distinctive astrovirus properties. These comparisons showed TAstV to be 7,325 nt [excluding the poly(A) tail] in length, to contain three ORFs, and to produce one subgenomic RNA of approximately 2.7 kb. Each ORF coded for proteins with limited amino acid similarity to HAstV. The predicted proteins within this viral genome are designated as a serine protease, an RDRP, and a viral capsid protein. Also detected was a retrovirus-like frameshift signal with potential secondary structure in the genome. Each of these elements is consistent with other sequenced astroviruses and establish this newly described virus as being a TAstV.

This TAstV was isolated from turkeys affected by an emerging disease, which is characterized by enteritis, high mortality, growth depression, lymphoid atrophy, and immunosuppression. It is because of this lymphoid atrophy and immunosuppression that we first examined the thymus as the source of disease agents, which led to the isolation and subsequent molecular characterization of this novel virus (33). This virus can readily be detected in the intestines, thymus, and bursa of infected poults, using both electron microscopy and RT-PCR (unpublished observation). More importantly, when naïve poults are given purified TAstV they exhibit many of the disease signs, including enteritis, mortality, lymphoid atrophy, and immunosuppression (unpublished data).

**Nucleotide sequence accession numbers.** The nucleotide sequence of TAstV was submitted to GenBank and was given the accession number AF206663. The isolates used in this study for nucleotide and amino acid comparison to TAstV were
REFERENCES


