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Development of an RT-PCR diagnostic test for an avian astrovirus

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Abstract

Astroviruses are small round viruses that cause enteric disease in the young of several species. Detection and diagnosis of astrovirus infection in non-human hosts relies heavily on electron microscopy and fluorescent antibody tests. Recently, our laboratory isolated and sequenced an avian astrovirus from poult enteritis mortality syndrome affected turkeys. These studies describe the development of RT-PCR methods, which specifically detect regions of the viral capsid and polymerase genes, and demonstrate their use in detecting astrovirus infection in commercial turkey flocks. © 2000 Published by Elsevier Science B.V.

Keywords: Turkey astrovirus; RT-PCR; Poult Enteritis Mortality Syndrome

1. Introduction

Astroviruses are small round viruses (27–30 nm) that cause enteric disease in several animal species, including humans (Matsui and Greenberg, 1996). Detection of astrovirus in most species is limited to electron microscopy (EM), and fluorescent antibody detection. Both of these methods are time intensive and vulnerable to differences in interpretation. EM suffers from low sensitivity and usually detects virus only during peak shed times. Additionally, EM may fail to detect the characteristic star-like surface projections if the sample is not processed correctly (Caul and Appleton, 1982). Fluorescent antibody tests may fail to recognize astroviruses of different serotypes (Matsui and Greenberg, 1996). Furthermore, virus isolation by cell culture is limited to bovine, feline, porcine, and human astroviruses (Lee and Kurtz, 1981; Woode et al., 1984; Harbour et al., 1987; Shimizu et al., 1990).

Little is understood about astrovirus infection in turkeys (McNulty et al., 1980; Reynolds and Saif, 1986; Reynolds et al., 1987; Thouvenelle et al., 1995a), and detection has been dependent on EM and fluorescent antibody tests. We recently isolated and sequenced an avian astrovirus associated with Poult Enteritis Mortality Syndrome (PEMS) (Koci et al., 2000). This turkey astrovirus (TastV) isolate causes enteritis, thymic and bursal

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atrophy, and mortality in young turkeys (manuscript in preparation).

In this study primers were designed from both highly conserved and potential variable regions of the first completely sequenced TAstV genome (accession number: AF206663). Fig. 1 shows a schematic of the astrovirus genome and the relative location of these primers within the genome. The astrovirus genome is a positive sense RNA virus, which contains 3 open reading frames, (ORF) 1a, 1b, and 2. ORF 1a codes for a serine protease as well as other unidentified non-structural proteins (Willcocks et al., 1999). ORF 1b codes for the highly conserved viral polymerase, and ORF 2 codes for the more variable viral capsid protein (Koci et al., 2000; Koonin and Dolja, 1993). Therefore, RT-PCR protocols were developed specific to regions within both ORF 1b and 2. This allows for the detection of TAstV isolates in commercial turkey flocks closely related to the PEMS-associated astrovirus using primers specific to the capsid gene, or detection of different astrovirus serotypes using polymerase specific primers.

2. Materials and methods

Oligonucleotide primers MKCap8 (TCATCATCCTCTCACACTGG) and MKCap19 (AGCAGCAGTAGGTGGCAGTG), produce an 849 base pair (bp) amplicon from within the viral capsid gene (Fig. 1). Primers MKPol10 (TGGCGGCGAACTCCTCAACA) and MKPol11 (AATAAGGTCTGCACAGGTCG), produce an 802 bp fragment overlapping the viral polymerase ORF (Fig. 1). Both primer sets were used in the RT-PCR detection of TAstV. Total RNA was isolated from the intestines of astrovirus inoculated and control specific pathogen free (SPF) turkey embryos, experimentally infected and sham infected turkey poult intestines, as well as the intestines and feces from commercial turkey flocks suffering from an enteric disease using the TRIzol® total RNA isolation reagent (Life Technologies™, Rockville, MD). First strand cDNA was synthesized by incubating 1 μg of the extracted total RNA with 20 pmol of reverse primer (MKCAP8 or MKPOL10) in a 20 μl reaction containing 15 units of SuperScript Reverse Transcriptase (Life Technologies™), and 20 pmol of dNTPs at 42°C for 60 min. An aliquot (2 μl) of the first strand product was used as template for amplification in a 50 μl reaction containing, 10 pmol of primers (MKCAP8 and MKCAP19, Fig. 2A or MKPOL10 and MKPOL11, Fig. 2B), 20 pmol of dNTPs, 1.5 mM $MgCl_2$, and 1.5 units of *Taq* DNA polymerase (Life Technologies™). Amplification, performed in a Perkin–Elmer 2400 DNA thermalcycler, involved an initial denaturing step at 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1.75 min, and finished with a final extension set at 72°C for 2.5 min. PCR products were electrophoresised in a 1.1% agarose gel in TAE buffer and visualized by ethidium bromide staining and ultra violet irradiation (Sambrook et al., 1989). The PCR products were purified by Qiaquick® PCR purification system (Qiagen®, Valencia, CA) and sequenced (Smith et al., 1986) (Applied Biosystems) to confirm amplification of the intended gene (data not shown).

Using primer pair MKCAP8 and MKCAP19 (Fig. 2A) we amplified an 849 bp product from the total RNA of astrovirus-infected turkey poults from our laboratory model and from commercial

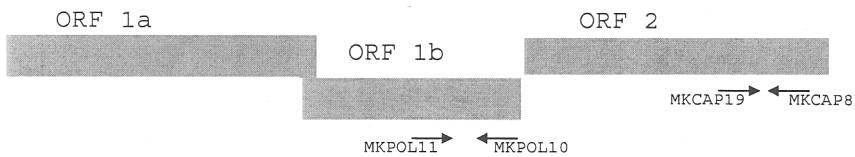

Fig. 1. Representative schematic of astrovirus genomic organization, and location of primers used. ORF 1b codes for the viral polymerase and ORF 2 codes for the capsid protein. Primers MKPOL 11 and MKPOL 10 produce an 802 bp fragment from the 3′ end of ORF 1b. Primers MKCAP 19 and MKCAP 8 produce an 849 bp fragment from the 3′ end of ORF 2.

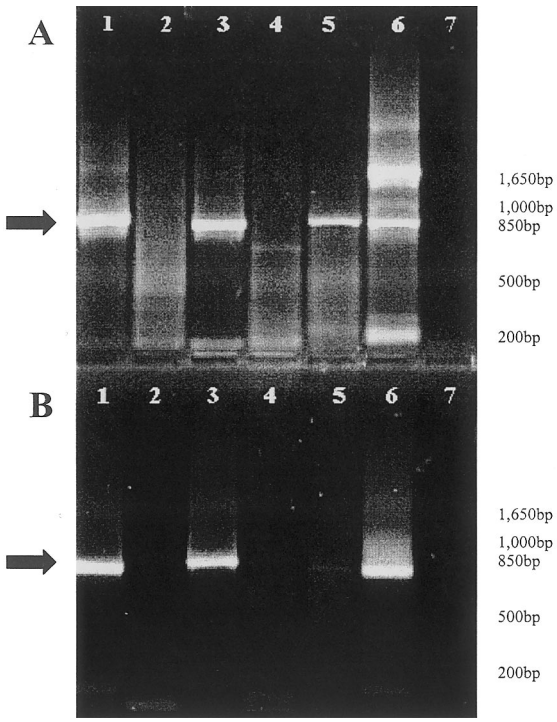

Fig. 2. Amplification and detection of 849 bp amplicon from the capsid region of TAstV using primers MKCAP19 and MKCAP8 (A), and 802 bp amplicon from the TAstV polymerase gene using primers MKPOL 10 and MKPOL 11 (B). Lanes: (1) TAstV inoculated SPF turkey embryo intestine; (2) control SPF turkey embryo intestine; (3) experimentally infected turkey poult intestine; (4) sham infected turkey poult intestine; (5) intestine of turkey poult from field flock with enteritis; (6) feces of turkey poult from field flock with enteritis; (7) no template control.

turkey flocks which where suffering from an enteric disease. There was no 849 bp PCR amplification product in the control SPF embryo and uninfected turkey poult reactions. The sequence of the field products were determined to be ~ 85% similar to the amplicon from experimentally infected poults and SPF embryos (data not shown).

Because of possible immune pressures on the capsid gene, which could lead to multiple serotypes, primers were also designed to the viral polymerase gene. This gene is highly conserved (relative to the capsid gene) (Poch et al., 1989). Primer set MKPOL10 and MKPOL11 produced an 802 bp amplicon in the astrovirus inoculated SPF embryo intestines, experimentally infected turkey poults, as well as from feces and intestines of commercial turkey flocks (Fig. 2B). The amplified product from the field samples were determined to be ~92% similar to the poults and SPF embryos infected experimentally (data not shown).

Both of these primer sets were analyzed for their specificity to TAstV. Several viruses of enteric origin were used to examine cross-reactivity (Fig. 3). RNA was isolated, as described above, from the intestines of coronavirus positive turkey poults (supplied by Dr H. John Barnes, College of Veterinary Medicine, North Carolina State University), avian encephalomyelitis virus (AEV), avian nephritis virus (ANV), and bovine enterovirus (supplied by National Veterinary Services Laboratory, Ames, IA). In addition DNA was isolated from goose parvovirus infected cells (supplied by National Animal Disease Center) using DNAzol® reagent genomic DNA isolation reagent (Life Technologies™, Rockville, MD). RT-PCR reactions were performed as described above, and PCR of goose parvovirus utilized the same conditions as the other viruses, however, 100 ng of total DNA was used as the template for these reactions. Fig. 3A shows that the primers MKCAP8 and MKCAP19 only produced the expected 849 bp amplicon with the astrovirus inoculated SPF embryo intestines RNA. The same is true with primers MKPOL10 and MKPOL11 (Fig. 3B); the 802 bp amplification product is only detected in the astrovirus inoculated SPF embryo intestines. These data show these primers specifically detect the presence of TAstV.

3. Results and discussion

This is the first report, to our knowledge, of a turkey astrovirus specific RT-PCR test. Little is known about the prevalence of astrovirus in turkeys, or the frequency of TAstV disease. There are few published reports of astrovirus disease in turkeys (McNulty et al., 1980; Reynolds and Saif, 1986; Thouvenelle et al., 1995a,b). However, this

may be a function of the tools currently available for the diagnosis of astrovirus infections. The virus we have sequenced was isolated from turkey poults suffering from PEMS (Koci et al., 2000). PEMS has caused severe problems in turkey flocks throughout the southeastern United States (Barnes and Guy, 1997). Currently we have detected TAstV in PEMS affected commercial turkey flocks from several states within the Southeast. Additionally, we have detected TAstV in commercial turkey flocks throughout the United States that suffer from enteritis and mortality, but are currently not defined as 'PEMS-affected flocks'. We are presently using these protocols to survey healthy and diseased turkey flocks to understand the distribution of astroviruses, its importance in poultry diseases, and its role in PEMS.

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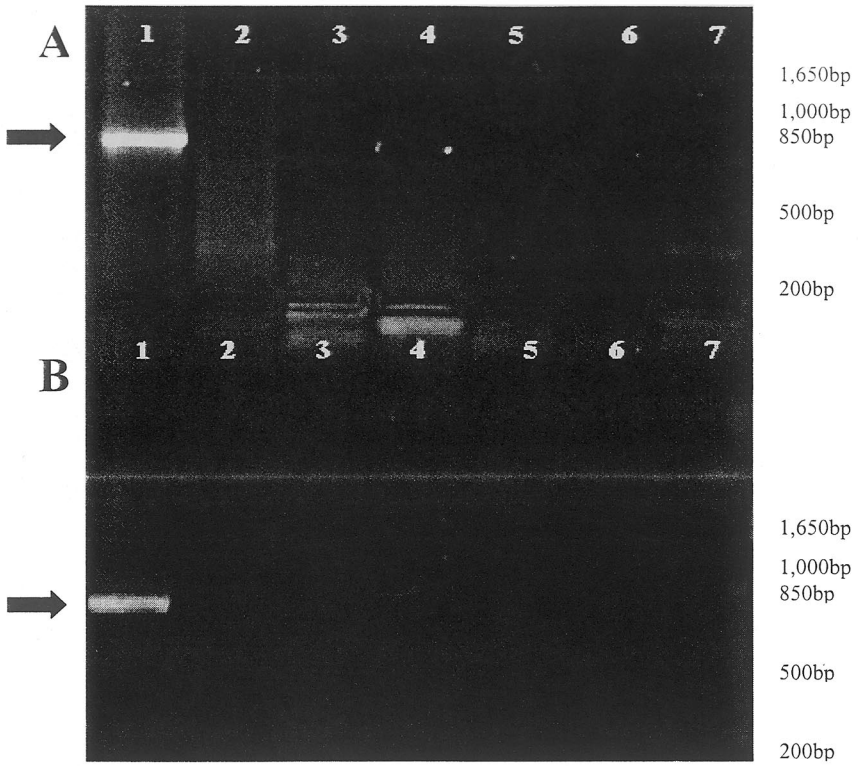

Fig. 3. Specificity of TAstV RT-PCR. Lanes: (1) astrovirus inoculated SPF embryo intestine; (2) control SPF embryo intestine; (3) turkey coronavirus positive poult intestines; (4) avian encephalomyelitis virus; (5) avian nephritis virus; (6) bovine entrovirus; (7) goose parvovirus. Detection of 849 bp amplicon using primers MKCAP8 and MKCAP19 (A) and 802 bp amplicon using primers MKPOL10 and MKPOL 11 (B) is only seen in inoculated SPF embryo intestines.

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