Enteric viruses in Brazilian turkey flocks: Single and multiple virus infection frequency according to age and clinical signs of intestinal disease


*Department of Pathology, University of São Paulo, 05508-900, São Paulo, Brazil; and †Department of Infection Biology, Institute of Global Health, University of Liverpool, Leahurst Campus, Neston, South Wirral, CH64 7TE, UK

ABSTRACT Poult enteritis complex has been associated with enteritis and reduction in growth rates in commercial turkeys worldwide. Intestinal samples from 76 turkey flocks from different Brazilian states affected or not with intestinal disorders were evaluated for the presence of adenovirus groups 1 and 2 (TAV), astrovirus types 1 and 2 (TAstV-1 and TAstV-2), turkey coronavirus (TCoV), reovirus, rotavirus, and avian nephritis virus (ANV) using PCR. The percentage of positive samples was categorized according to the geographic origin, age of the flocks, and presence of clinical signs of intestinal disease. The percentage of samples that were positive for at least one virus was 93.4%, whereas the percentage of samples that were positive for more than one virus was 69.7%. An average of 3.20 viruses per sample was detected in turkeys in the growing phase of the production cycle (1 to 4 wk of age). The TAstV-1 and TCoV were the most frequently observed viruses in growing phase turkeys and occurred simultaneously in 85% of these samples. In turkeys in the finishing phase of development (5 to 18 wk), a lower average number of viruses was observed (2.41), and the most frequent viruses isolated in these turkeys were TAstV-1 (57.1%) and rotavirus (51.8%). Overall, every virus was detected more frequently in growing phase turkeys than in finishing phase turkeys with the exception of TAV. Samples from flocks exhibiting clinical signs of intestinal disease showed a higher rate of positivity, and TAstV-1, TAstV-2, and TCoV were the most frequently occurring viruses in this cohort. Birds without clinical signs most frequently harbored TAstV-1 and rotavirus. Future studies should focus on the description and elucidation of the role of each virus, as well as the pathogenic and immunological implications of the different combinations of viruses in turkeys.

Key words: turkey, enteric virus, poult enteritis complex, poult enteritis syndrome, polymerase chain reaction

INTRODUCTION

Poult enteritis complex describes infectious intestinal diseases affecting turkeys, and these diseases are characterized by mild to severe enteritis, decreased growth rates, low rates of feed consumption, and high mortality. Poult enteritis complex diseases have a significant economic impact because they compromise weight gain and feed intake of the flock and generally lead to secondary infections in affected turkeys, which require treatment with increasingly expensive medications (Saif, 2008).

Enteric disorders that affect turkey flocks were first reported by Pomeroy and Fenstermacher (1937) in the form of hemorrhagic enteritis. In the 1950s, an enteric disease called bluecomb disease was widely reported (Peterson and Hymas, 1951; Pomeroy and Sieburth, 1953; Sieburth and Johnson, 1957). Clear observation of an etiological agent was first done by Fujisaki et al. (1969), who demonstrated the presence of reoviruses in the feces of affected poult using electron microscopy. In the subsequent 10 yr, adenoviruses, astroviruses, coronavirus, picornaviruses, and rotaviruses were also observed in the intestinal contents of diarrheic turkeys using electron microscopy (Panigrahy et al., 1973; Carlson et al., 1974; Tolin and Domermuth, 1975; Bergeland et al., 1977; Imada et al., 1979; McNulty et al., 1980).

Several studies have revealed the occurrence of multiple turkey infections by adenovirus, astrovirus, coronavirus, reovirus, and rotavirus, which produce different clinical manifestations depending on whether they occur as single or multiple infections (Reynolds et al., 1987a; Sellers et al., 2004; Day et al., 2007; Pantin-Jackwood et al., 2008a; Saif, 2008; Jindal et al., 2010a).
Different infectious agents or combinations thereof can affect the intestines of the turkey to different extents and cause enteritis and reduction in growth rates. Examples include poult enteritis syndrome and poult enteritis and mortality syndrome; the latter is more severe and causes a high mortality rate within the flock (Saif, 2008).

The development of PCR assays for the rapid and specific detection of enteric viruses has been the basis of several recent studies on the occurrence of viruses that cause such diseases in turkeys (Villarreal et al., 2006; Pantin-Jackwood et al., 2007; Jindal et al., 2009b; Pantin-Jackwood et al., 2008b; Jindal et al., 2010a).

The objective of this study was to determine the prevalence of the various enteric viruses that are present in Brazilian turkey flocks, as well as any correlations that may exist between the different viruses and the geographic origin of the flocks, the age of the flocks, and the presence of clinical signs of disease.

**MATERIALS AND METHODS**

**Sample Collection**

From 2005 to 2009, 76 intestinal samples were obtained from healthy and diseased commercial turkey flocks located in 3 different states in Brazil (Santa Catarina, Minas Gerais, and Goias). Diseases flocks were defined as flocks that presented diarrhea and mortality. When possible, the turkey farms from which the samples were acquired were surveyed, and instructions for the collection of samples were provided to standardize sample acquisition.

The samples consisted of turkey intestines that were collected and pooled from 5 birds at random per house. The turkeys were killed by cervical dislocation. Samples were collected from healthy and diseased flocks including turkeys that ranged in age from 1 to 18 wk. Three samples included no information on the health status of the flock. After collection, intestinal samples were preserved at −20°C and were sent to the Avian Pathology Laboratory at the University of Sao Paulo, Sao Paulo, Brazil, where they were processed and analyzed for their intestinal content.

The age of the flocks was categorized according to the production cycle, which was either the growing phase (1 to 4 wk) or the finishing phase (5 to 18 wk). The most commonly reported clinical signs were diarrhea, mucoid or hemorrhagic feces, mortality, low weight gain and feed intake, impaired growth, nervousness, and prostration.

**DNA and RNA Extraction**

The intestinal contents of the samples were pooled and homogenized, and 0.25 g of the pooled samples was added to 1.0 mL of Tris/calcium buffer (0.1 M Tris/HCl and 1.5 mM CaCl₂, pH 7.3), homogenized for 30 min, and centrifuged for 10 min at 8,000 × g (Munford, 2007). Total DNA and RNA were extracted from 250 µL of the supernatant using the Brazol reagent (LGC, São Paulo, Brazil) according to manufacturer’s instructions. As a negative control, ddH₂O purified by the MilliQ water system (Millipore Corporation, Billerica, MA) was substituted for the intestinal extract. Extracted DNA and RNA were stored at −80°C until further analysis.

**PCR for Adenovirus**

Previously described primer sets were used for PCR amplification of an 897-bp fragment from the hexon gene of group 1 adenoviruses (turkey adenovirus or TAV) and a 1,647-bp fragment of the hexon gene of group 2 adenoviruses (hemorrhagic enteritis virus or HEV; Alvarado et al., 2007; Hess et al., 1999, respectively). Reference strains included a TAV field strain that was isolated from a Brazilian flock and confirmed by sequencing (GenBank FJ360748) and the attenuated HEV vaccine, Dindoral SPF (Merial Animal Health, Campinas, Brazil). The DNA amplification was conducted in a total volume of 50 µL. For TAV amplification, 5 µL of extracted DNA was added to a PCR mixture composed of 1 × PCR Buffer (Invitrogen), 0.2 mM of each dNTP, 0.5 µM of each primer pair (Hexon A and Hexon B, Table 1), 1.5 mM MgCl₂, and 2.5 U of Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA). For HEV detection, 5 µL of the extracted DNA was added to a PCR mixture containing 1 × PCR Buffer (Invitrogen), 0.2 mM of each dNTP, 0.5 µM of each primer (HEV1F and HEV1R, Table 1), 2 mM MgCl₂, and 4 U of Taq DNA polymerase (Invitrogen). The PCR amplification was conducted in a Biometra DNA thermocycler (Biometra GmbH, Goettingen, Germany) for all viruses under investigation. The cycling parameters for TAV amplification were as follows: 5 min at 94°C, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. The cycling parameters for HEV amplification were as follows: 3 min at 94°C, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and 30 s.

**Reverse-Transcription PCR for Astrovirus**

The primers used for reverse-transcription (RT)-PCR of the polymerase gene of the turkey astrovirus type 1 (TAstV-1) and the avian nephritis virus (ANV) were described by Day et al. (2007). The primers used for RT-PCR of the polymerase gene of the turkey astrovirus type 2 (TAstV-2) were described by Koci et al. (2000a). For RT-PCR reactions, reverse transcriptase was used to produce cDNA, which was followed by RT-PCR for the amplification of a 251-bp fragment of TAstV-1, a 473-bp fragment of ANV, or an 802-bp fragment of TAstV-2. Reference strains included a TAstV-1 field strain that was isolated from a Brazil-
ian flock that was confirmed by sequencing (GenBank HQ157559), an ANV field strain that was generously provided by Erica Spackman from the Southeast Poulf Research Laboratory, USDA, Agricultural Research Service in Athens, Georgia. The TAstV-2 field strain that was isolated from a Brazilian flock.

Reverse transcription reactions were performed for each virus with 7 µL of extracted RNA that was denatured at 95°C for 5 min and added to the reverse transcription mix containing 1× First Strand Buffer (Invitrogen), 1 mM of each dNTP, 10 mM dithiothreitol, 1 pmol of each primer, and 200 U M-MLV reverse transcriptase (Invitrogen) for a final reaction volume of 20 µL. The parameters for the RT reaction included an incubation step at 45°C for 60 min, followed by an incubation step at 72°C for 10 min. For the detection of ANV and TAstV-1, 4 µL of the cDNA was amplified by PCR reactions that also consisted of 1× PCR Buffer (Invitrogen), 0.2 mM of each dNTP, 0.6 µL of each primer (T1pol 1F, T1pol 1R, ANV-F, and ANV-R, according to the targeted virus, Table 1), 2 mM MgCl₂, and 5 U of Taq DNA polymerase (Invitrogen) in a final volume of 50 µL. The cycling parameters for amplification were as follows: 5 min at 95°C, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min.

For TAstV-2 amplification, 4 µL of the cDNA was added to the PCR mix containing 1× PCR Buffer (Invitrogen), 0.2 mM of each dNTP, 0.5 µM of each primer (Mkpol10 and Mkpol11, Table 1), 1.5 mM MgCl₂ and 2.5 U of Taq DNA polymerase (Invitrogen) in a final volume of 50 µL. The cycling parameters for amplification were as follows: 3 min at 94°C, then 35 cycles of 94°C for 1 min, 56°C for 1 min and 30 s, and 72°C for 1 min.

RT-PCR for Turkey Coronavirus

The primers used for targeting the 3′ untranslated region (UTR) of the turkey coronavirus (TCoV) have been previously described by Cavanagh et al. (2002) and were used for the amplification of a 179-bp fragment. The attenuated TCoV vaccine strain MASS I SPF H120 (Pfizer Animal Health, São Paulo, Brazil) was used as a reference. The RT reaction conditions used for TCoV were identical to the RT reaction for astrovirus detection. The PCR reaction was conducted using 5 µL of cDNA added to a PCR reaction mixture containing 1× PCR Buffer, 0.2 mM of each dNTP, 0.5 µM of each primer (UTR 41 and UTR 31, Table 1), 1.5 mM MgCl₂, and 2.5 U of Taq DNA polymerase in a final reaction volume of 50 µL. The cycling parameters for amplification were as follows: 3 min at 94°C, then 35 cycles of 94°C for 1 min, 56°C for 1 min and 30 s, and 72°C for 1 min and 30 s. A nested PCR step was performed by adding 5 µL of the PCR product to a reaction mixture identical to the first PCR reaction and subjected to an amplification cycle identical to the first PCR reaction.

<p>| Table 1. Primer sequences and amplicon sizes for the reverse-transcription PCR and PCR reactions |
|-------------------------------|-------------|-------------|-----------------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Amplicon size (bp)</th>
<th>Reference</th>
<th>Target virus</th>
<th>Primer name</th>
<th>Primer sequences</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>897</td>
<td>Alvarado et al., 2007</td>
<td>Adenovirus group 1</td>
<td>Hexon A</td>
<td>5′CAA RTT CAG RCA GAC GGT 3′</td>
<td></td>
</tr>
<tr>
<td>641</td>
<td>Day et al., 1999</td>
<td>Adenovirus group 1</td>
<td>Hexon B</td>
<td>5′TAG TGA TGM CGS GAC ATC AT 3′</td>
<td></td>
</tr>
<tr>
<td>1,647</td>
<td>Hess et al., 1999</td>
<td>Hemorrhagic enteritis virus</td>
<td>HEV1F</td>
<td>5′TAC TGC TGC TAT TTG TTG TG 3′</td>
<td></td>
</tr>
<tr>
<td>473</td>
<td>Day et al., 2007</td>
<td>Astrovirus type 1</td>
<td>T1polF</td>
<td>5′AGC TYA TGM GGT TCT TTC YYG 3′</td>
<td></td>
</tr>
<tr>
<td>473</td>
<td>Day et al., 2007</td>
<td>Astrovirus type 1</td>
<td>T1polR</td>
<td>5′GAT GGT GGG TAG CCT ATT GTG TTC 3′</td>
<td></td>
</tr>
<tr>
<td>802</td>
<td>Koci et al., 2000a</td>
<td>Astrovirus type 2</td>
<td>Mkpol10</td>
<td>5′TGG CGG CGA ACT CCT CAA CA 3′</td>
<td></td>
</tr>
<tr>
<td>802</td>
<td>Koci et al., 2000a</td>
<td>Astrovirus type 2</td>
<td>Mkpol11</td>
<td>5′AAT AAG GTC TGC ACA GGT CG 3′</td>
<td></td>
</tr>
<tr>
<td>473</td>
<td>Day et al., 2007</td>
<td>Avian nephritis virus</td>
<td>ANV-F</td>
<td>5′GYT GGG CGC YTC YTT YGA YAC 3′</td>
<td></td>
</tr>
<tr>
<td>473</td>
<td>Day et al., 2007</td>
<td>Avian nephritis virus</td>
<td>ANV-R</td>
<td>5′CRT TTG CCC KRT ART CTT TRT 3′</td>
<td></td>
</tr>
<tr>
<td>179</td>
<td>Cavanagh et al., 2002</td>
<td>Turkey coronavirus</td>
<td>UTR 11</td>
<td>5′GCT CTA ACT CTA TAC TAG CCT A 3′</td>
<td></td>
</tr>
<tr>
<td>179</td>
<td>Cavanagh et al., 2002</td>
<td>Turkey coronavirus</td>
<td>UTR 31</td>
<td>5′GGG CGT CCA AGT GCT GTA CCC 3′</td>
<td></td>
</tr>
<tr>
<td>179</td>
<td>Cavanagh et al., 2002</td>
<td>Turkey coronavirus</td>
<td>UTR 41</td>
<td>5′ATG TCT ATC GCC AGG GAA ATG TC 3′</td>
<td></td>
</tr>
<tr>
<td>1,120</td>
<td>Pantin-Jackwood et al., 2008a</td>
<td>Reovirus</td>
<td>S4-F13</td>
<td>5′GTG GGT GTT GGA GTT TCC CG 3′</td>
<td></td>
</tr>
<tr>
<td>1,120</td>
<td>Pantin-Jackwood et al., 2008a</td>
<td>Reovirus</td>
<td>S4R1133</td>
<td>5′TAC GCC ATC CTA GCA GCA GCA CC 3′</td>
<td></td>
</tr>
<tr>
<td>630</td>
<td>Pantin-Jackwood et al., 2008a</td>
<td>Rotavirus</td>
<td>NSP4-F30</td>
<td>5′GTG CGG AAC AAA GAI GAA GCA C 3′</td>
<td></td>
</tr>
<tr>
<td>630</td>
<td>Pantin-Jackwood et al., 2008a</td>
<td>Rotavirus</td>
<td>NSP4-R660</td>
<td>5′GTT GGG GTA CCA GGG ATT AA 3′</td>
<td></td>
</tr>
</tbody>
</table>
**RT-PCR for Reovirus and Rotavirus**

The RT-PCR was used for the detection of an 1,120-bp fragment of the S4 reovirus gene and a 630-bp fragment of the NSP4 rotavirus gene using primer sets previously described by Pantin-Jackwood et al. (2008a). As a reference strain for rotavirus, a rotavirus field strain that was isolated by Erica Spackman from the Southeast Poul Research Laboratory, USDA, Agricultural Research Service, Athens, Georgia, was used. As a reference strain for reovirus, the attenuated reovirus vaccine strain NOBILIS REO 1133 (Merck Animal Health, São Paulo, Brazil) was used.

The RT reaction conditions for both reovirus and rotavirus were identical to the conditions used for astrovirus. The PCR reactions were performed by adding 4 µL of cDNA to a PCR reaction mixture containing 1× PCR Buffer, 0.2 mM of each dNTP, 0.6 µM of each primer (S4-F13 and S4R1133 for reovirus; NSP4-F30 and NSP4-R660 for rotavirus, Table 1), 1.5 mM MgCl₂ and 5 U of Taq DNA polymerase in a final volume of 50 µL. The cycling parameters for amplification were as follows: 5 min at 95°C, then 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min.

**Analysis of RT-PCR and PCR Products**

The RT-PCR and PCR products were visualized by UV trans-illumination after separation by electrophoresis in a 1.5% agarose gel immersed in Tris-borate-EDTA (45 mM Tris-borate, 1 mM EDTA) and stained with Blue-Green Dye (LGC, São Paulo, Brazil). The size of the amplified products was estimated by comparison with a 100-bp DNA Ladder (Invitrogen) as a molecular size marker.

**Statistical Analysis**

Descriptive statistics were used to evaluate the positivity of the samples for each virus, and the percentage of positive and negative samples was plotted against the information provided on the age and health status of the flocks. Variance analysis and Tukey’s test were used in the Minitab software (Minitab 16.1.0, 2010 version, Minitab Inc., State College, PA) to determine the degree of significance of any differences observed in the number of virus families detected per sample when adjusted for the age and health status of the birds. The cutoff for significance was set at a P-value ≤0.05.

**RESULTS**

**Occurrence of Enteric Viruses**

A high percentage of samples (93.4%) tested positive for at least one virus with 23.7% of samples harboring a single virus and 69.7% of samples harboring multiple viruses. Intestinal samples most often harbored 3 to 4 viruses per sample with viruses from the astrovirus family (TAstV-1, TAstV-2, or ANV, or a combination of these) being present in all of the samples that tested positive for viruses. The TAstV-1 was the most commonly identified virus in the samples (64.5%), followed by TCoV (55.3%), rotavirus (52.6%), TAstV-2 (44.7%), ANV (35.5%), reovirus (7.9%), and TAV (5.3%), as described in Table 2. The HEV was not detected. The frequencies of distribution of individual and multiple viral infections are displayed in Table 2.

**Geographic Origin**

Most of the intestinal samples were collected from turkey farms within the State of Santa Catarina (43 samples), followed by Goias (26 samples) and Minas Gerais (7 samples). A significantly higher (P < 0.05) average number of viruses per sample was observed in samples that originated in Santa Catarina (3.14 ± 1.33) compared with the samples from Goias (1.73 ± 1.48). Samples that harbored 3 to 4 viruses were most frequently observed in samples from Santa Catarina, whereas in Goias, intestinal samples typically harbored a single virus per sample (10/26), and rotavirus was the most frequent virus detected among these samples (4/26). Comparisons between samples from Minas Gerais and either Santa Catarina or Goias did not reach statistical significance. Data regarding the geographic distribution of intestinal viruses are shown in Table 3.

Most of the samples were collected during the fall (30 samples) and winter (24 samples). During the fall months, most of the samples were collected from Santa Catarina (19 positive samples), whereas 8 samples...
Table 3. Frequencies of individual and multiple virus infections detected in the samples according to the age of the flocks and geographic distribution

<table>
<thead>
<tr>
<th>Viruses detected¹</th>
<th>Frequency by state²</th>
<th>Frequency by age of the flock (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GO</td>
<td>MG</td>
</tr>
<tr>
<td>TAvV-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAstV-1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>TAstV-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCoV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotavirus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reovirus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1TAV = adenovirus group 1; TAstV-1 = astrovirus type 1; TAstV-2 = astrovirus type 2; ANV = avian nephritis virus; TCoV = turkey coronavirus.

2GO = Goias; MG = Minas Gerais; SC = Santa Catarina; negative result (−).
were collected from Goias (7 positive samples and 1 negative sample) and 3 samples were collected from Minas Gerais (3 positive samples). During the winter months, most of the samples were collected from Goias (14 positives and 4 negatives), but only 6 samples were collected from Santa Catarina (6 positives). In the summer months, most of the samples were collected from Santa Catarina (11 positives), and only 4 samples were collected from Minas Gerais (4 positives). All samples that were collected in the spring months originated from Santa Catarina. No negative samples were found during the summer and spring months.

### Age of the Flocks

Twenty samples were collected from turkeys aged between 1 and 4 wk (growing phase), and all samples from turkeys in the growing phase were reported to have come from flocks that showed clinical signs of intestinal disease. Within growing phase samples, most of the samples (65%) harbored 3, 4, or 5 viruses per sample, and TAstV-1 (85%) and TCoV (60%) were the most frequently detected families. Fifty-six samples ranged in age from 5 to 18 wk (finishing phase). Twenty-eight finishing phase turkeys showed no clinical signs of intestinal disease, 25 came from diseased flocks, and 3 did not have a description of the health status of the turkey or the flock.

The TAstV-1 (57.1%), rotavirus (51.8%), and TCoV (44.6%) were the most frequently detected virus families within intestinal samples from finishing phase flocks. Samples that originated from finishing phase flocks also showed a high frequency of single virus detection (15/56) despite showing clinical signs of intestinal disease. With the exception of TAV, all of the viruses occurred at a higher frequency in growing phase flocks than in finishing phase flocks. The results regarding correlations between virus occurrence and flock age are shown in Tables 4 and 5.

Variance analysis revealed a statistically significant difference between the isolation of viruses in growing phase flocks versus finishing phase flocks ($P = 0.047$). A higher average number of viruses were also observed in the growing phase samples ($3.20 \pm 1.36$) than in finishing phase samples ($2.41 \pm 1.55$).

### Presence of Clinical Signs

All 45 samples that originated from flocks showing clinical signs consistent with enteric disease were positive for at least one virus. The frequencies of viruses identified in the order of decreasing occurrence were TAstV-1 (73.3%), TCoV (71.1%), TAstV-2 (64.4%), and rotavirus (57.8%). Of the 28 samples from flocks that showed no clinical signs of disease, 23 were positive for at least 1 virus and 5 were negative. The most frequent viruses identified from this cohort were TAstV-1 (46.4%) and rotavirus (39.3%).

<table>
<thead>
<tr>
<th>Clinical signs</th>
<th>No. information</th>
<th>Absent</th>
<th>Present</th>
<th>Frequency of single-agent infection and coinfection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative samples</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Frequency</td>
<td>1 (15)</td>
<td>3 (25)</td>
<td>7 (53)</td>
<td>4 (30)</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>16</td>
<td>11</td>
<td>10</td>
</tr>
</tbody>
</table>

Percentages (in parentheses) were calculated based on the number of flocks.

### Table 4. Frequencies of clinical signs and numbers of associated viruses according to the age of the flocks

<table>
<thead>
<tr>
<th>Age</th>
<th>Number of samples</th>
<th>Clinical signs</th>
<th>Negative samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to 4 wk</td>
<td>20</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5 to 18 wk</td>
<td>56</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td>55</td>
<td>24</td>
</tr>
</tbody>
</table>
With the exception of reovirus, all viruses were detected less often in flocks that showed no clinical signs of disease compared with diseased flocks. The TAstV-2 and TCoV were detected in almost 40% fewer flocks that showed no clinical signs of disease than diseased flocks. The TAV was detected only in diseased birds. Three samples did not have the associated information on signs of disease. Data regarding the correlations between viruses and clinical signs of disease are displayed in Table 6.

**DISCUSSION**

The observed high percentage of turkey intestinal samples that tested positive for viral infection parallels previous studies conducted in the United States using electron microscopy and PCR (Saif et al., 1985; Pantin-Jackwood et al., 2008a; Jindal et al., 2010b). These previous studies have revealed the presence of enteric viruses during all of the growth phases of the turkey. Studies performed by Jindal et al. (2010a) demonstrated viral intestinal infection until 9 wk of age, and Pantin-Jackwood et al. (2007) showed evidence of intestinal infection until 12 wk of age.

The clinical signs reported in the surveys (diarrhea, mucoid or hemorrhagic feces, high mortality, low weight gain and feed intake, impaired growth, nervousness, and prostration) were consistent with enteric syndromes described by several authors (Pomeroy and Sieburth, 1953; Sieburth and Johnson, 1957; Gross and Moore, 1967; Yu et al., 2000; Carver et al., 2001; Cavanagh, 2005). Classified by the state from which they were isolated, the frequencies of virus-positive intestinal samples in descending order according to Brazilian states are Parana (46%), Santa Catarina (16%), Minas Gerais (16%), Goias (13%), and Rio Grande do Sul (9%; Annual Report of Brazilian Union for Poultry Production, 2009).

A higher average number of virus families was detected in flocks in the growing phase of development, and all of these positive samples were collected from turkeys that presented clinical signs of intestinal disease. The high rate of virus detection most likely reflects a greater susceptibility of this population of turkeys to enteric viruses during the first weeks of development due to an immature intestinal epithelium (Reynolds et al., 1987a; Ismail et al., 2003; Saif, 2008; Jindal et al., 2009a). Epithelial maturation occurs when the intestinal lining of young poults, which, at the nascent stages of development, is accustomed to digesting lipid- and protein-rich yolk, transition to digesting commercial feed rich in carbohydrates, lipids, and protein (Uni, 2006). Uni et al. (1995, 1999) demonstrated that the introduction of the commercial diet causes an increase in the number and length of villi. Upon exposure to commercial feed, the duodenum develops by d 7 posthatch, and the jejunum and the ileum develop by d 14 posthatch. At the cellular level during intestinal development, functional enterocytes replace proliferative enterocytes that com-
prise the villi, gradually restricting the actively proliferating area on the intestinal crypts (Uni et al., 1998).

In our study, the high frequency (81.6%) of viruses from the astrovirus family (TAstV-1, TAstV-2, or ANV) parallels observations of the frequency of astroviruses in turkey intestinal samples in the United States, in which astroviruses have been reported in 89.5 and 100% of the samples analyzed (Pantin-Jackwood et al., 2007, 2008a). Pantin-Jackwood et al. (2008a) showed that TAstV-2 occurred more frequently than TAstV-1, which was also reported by Jindal et al. (2010b). In contrast, our study found a higher frequency of TAstV-1 than TAstV-2. Our study also revealed the presence of ANV (35.5%), which has been found in turkey flocks (12.5%) by Pantin-Jackwood et al. (2008a).

All virus families were more frequently found in association with other viruses than they were as individuals, and viruses of the astrovirus family were always identified in the presence of other virus families. The high frequency of coinfections (69.7%) is consistent with previous studies (Saif et al., 1985; Pantin-Jackwood et al., 2008a; Jindal et al., 2009a, 2010b).

Viruses of the TCoV family were frequently identified in the samples regardless of the presence of signs of enteritis. However, enteritis-associated viruses of the TCoV family were found at a lower frequency in our study (55.3%) than in other Brazilian studies that have shown the presence of poult enteritis and mortality syndrome in 80% (Villarreal et al., 2006) and 78.9% (Silva et al., 2009) of samples that were positive for TCoV. Coinfection with viruses of the Astroviridae family and TCoV family viruses was the most frequent association observed in this study. Yu et al. (2000) demonstrated that coinfection with viruses of the astrovirus family and the TCoV family produced a more severe infection with a higher mortality rate compared with single infections with either of these viruses. Coinfections with TAstV-2 and TCoV have been associated with poult enteritis and mortality syndrome (Reynolds et al., 1987b; Koci et al., 2000b; Ismail et al., 2003; Villarreal et al., 2006; Silva et al., 2009), but a causative relationship between coinfection with TAstV-1 and ANV and enteric syndrome has not been established, although several studies have shown that astrovirus infections are the most common among the enteric viruses detected in poult intestines (Saif et al., 1985; Reynolds and Saif, 1986; Reynolds et al., 1987a; Pantin-Jackwood et al., 2008a).

The TCoV was also detected in flocks that did not show signs of disease (28.6%), all of which were in the finishing phase of development. However, Pantin-Jackwood et al. (2007, 2008a) and Jindal et al. (2010b) did not detect TCoV viruses in turkeys afflicted with poult enteritis syndrome. These results suggest that TCoV infection may be prevented by the more mature immune system of turkeys in the finishing phase and explains the presence of TCoV in the intestines of turkeys beyond 5 wk of age that do not show signs of intestinal disease. The TCoV was found less often in young turkeys that did not show signs of intestinal disease than in diseased young turkeys. Loa et al. (2002) reported a protective IgA response in the intestines of 10-d-old turkeys that prevented TCoV infection for at least 9 wk. Pomeroy et al. (1975) also reported an IgA immune response that was protective against TCoV infection for 6 mo.

With the exception of TAV, we observed a higher incidence of single and multiple virus infections that were associated with clinical signs of intestinal disease in turkeys in the growing phase of development compared with turkeys in the finishing phase of development. This finding is likely due to the immature intestinal epithelium present in the growing phase turkeys, which harbors a more immature mucosal immune system. Beal et al. (2006) have suggested that the intestinal defense against enteric pathogen colonization depends on the complete development of the gastrointestinal-associated lymphatic tissue (GALT). Furthermore, Befus et al. (1980) have shown that Peyer’s patches, which comprise part of the GALT, develop fully by wk 16. Bar-Shira et al. (2003) also demonstrated that an adaptive immune response begins by wk 2 of development, and soon after d 17 of development, lymphocytes appear within the lamina propria and reach a maximum concentration by wk 8 (Yason et al., 1987; Vervelde and Jurissen, 1993). Therefore, early in the development of turkeys, there are no bacterial microbiota in poult intestines to protect from external pathogen colonization (Nurmi and Rantala, 1973).

The observed frequency of rotavirus (52.6%) isolation is consistent with the results of Pantin-Jackwood et al. (2008a), who detected rotavirus in 69.7% of intestinal samples from both normal and diseased turkey flocks. Jindal et al. (2010b) demonstrated a high percentage of samples positive for rotavirus (93%) in flocks with poult enteritis syndrome. Although rotavirus was iden-

### Table 6. Frequencies of viruses detected in association with clinical signs of intestinal disease

<table>
<thead>
<tr>
<th>Clinical signs</th>
<th>Number of flocks</th>
<th>Number of viruses detected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>45</td>
<td>TAV: 4 (8.9) TAv1: 33 (73.3) TAv2: 29 (64.4) ANV: 17 (37.8) TCoV: 32 (71.1) Reovirus: 3 (6.7) Rotavirus: 26 (57.8)</td>
</tr>
<tr>
<td>Absent</td>
<td>28</td>
<td>TAV: 0 (0) TAv1: 13 (46.4) TAv2: 5 (17.8) ANV: 7 (25) TCoV: 8 (28.6) Reovirus: 3 (10.7) Rotavirus: 0 (0)</td>
</tr>
<tr>
<td>No information</td>
<td>3</td>
<td>TAV: 0 (0) TAv1: 3 (100) TAv2: 0 (0) ANV: 3 (100) TCoV: 2 (66.7) Reovirus: 0 (0) Rotavirus: 3 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td>TAV: 4 (5.2) TAv1: 49 (64.4) TAv2: 34 (44.6) ANV: 27 (35.5) TCoV: 42 (54.7) Reovirus: 6 (7.8) Rotavirus: 40 (52.6)</td>
</tr>
</tbody>
</table>

1 TAV = adenovirus group 1; TAstV-1 = astrovirus type 1; TAstV-2 = astrovirus type 2; ANV = avian nephritis virus; TCoV = turkey coronavirus.
tified most frequently in growing phase turkeys showing signs of intestinal disease (55%), turkeys in the finishing phase regardless of the presence of intestinal disease also showed a high percentage of positivity (51.8%). Several previous studies have demonstrated the presence of rotavirus in older birds (Pantin-Jackwood et al., 2007; Woolcock and Shivaprasad, 2008; Jindal et al., 2009a).

Similarly to other viruses, most of the rotavirus-positive samples were also positive for other viruses, and only 6 rotavirus infections were single-agent infections. Yason and Schat (1986) demonstrated that turkeys inoculated with rotavirus resulted in diarrhea but not mortality, suggesting that other infectious agents might synergize with the rotavirus infection to exacerbate intestinal disease. Several studies have suggested that a coinfection with astrovirus and rotavirus, a commonly detected coinfection in our study, is important for the development of enteritis in pouls (Reynolds et al., 1987a,b). However, Jindal et al. (2010a) demonstrated that a coinfection with TAvS-2 and rotavirus is not sufficient to cause enteric syndromes in turkeys regardless of age, which suggests that an initiating agent is necessary for the development of enteritis. Virus-specific antibodies have been shown to control different viral infections in older turkeys as demonstrated by Mukibi-Muka and Jones (1999), who showed that a specific IgA response against reovirus could be detected in the intestines of birds as young as 3 wk of age.

A temporal study that spans several years is necessary to define the environmental and climatic impact on the geographic distribution and occurrence of enteric viruses in farm animals. Our study revealed a higher rate of virus detection during the fall and winter months, which is consistent with a study performed by Silva et al. (2009) that focused on the southeast region of Brazil. Data from Minas Gerais did not show a significant correlation between the frequencies of certain viruses and the weather or the geographical distribution. Because of the small number of samples (7) from Minas Gerais, the results were not statistically robust enough to identify correlations.

Reovirus was detected in 7.9% of samples, which is similar to results from Jindal et al. (2010a) that revealed the presence of reovirus in 10.4% of flocks that did not show signs of intestinal disease. However, Pantin-Jackwood et al. (2007) did not detect reovirus in pouls that did not show signs of intestinal disease. Jindal et al. (2010b) showed that reovirus occurs in 45.5% of turkeys that come from flocks that display poult enteritis syndrome. Similarly to a report by Pantin-Jackwood et al. (2008a), reovirus was never found as a single-agent infection. The exact role that reovirus infection plays during enteric syndromes is not completely understood; however, reovirus has been directly observed by electron microscopy in flocks with poult enteritis and mortality syndrome (Deshmukh et al., 1969; Wooley and Gratzek, 1969).

At least one virus was detected within flocks that presented clinical signs of enteritis, with the most frequently observed number of distinct viruses ranging from 3 to 4. This observation has been reported by Jindal et al. (2010b), who showed that most of the intestinal samples from flocks affected with poult enteritis syndrome were simultaneously positive for between 2 and 3 other viruses. Our results also demonstrated viral intestinal colonization in birds that did not display signs of intestinal disease (23 of 28 samples), which is consistent with several previous studies (Reynolds et al., 1987a; Pantin-Jackwood et al., 2008a; Jindal et al., 2010a). However, the rate of coinfection in turkeys with no signs of intestinal disease occurred at a much lower rate than turkeys that displayed signs of intestinal disease. Almost half of the samples from turkeys without intestinal disease had single virus infections (11 of 23 positive samples). Both of these findings were demonstrated by Jindal et al. (2010a), who, despite only studying coinfections among TAstV-2, TCoV, reovirus, and rotavirus, suggested that viral coinfection may be relevant in the development and intensification of intestinal disease.

Group 1 adenovirus was detected in 5.3% of the samples and was more frequently identified in turkeys in the finishing phase. Group 1 adenovirus has not been found in intestinal samples in studies performed in the United States (Pantin-Jackwood et al., 2007), and a clear role has not been defined for this virus in the development of enteritis. Group 2 adenovirus was not detected in any of our samples or by Pantin-Jackwood et al. (2008a).

Few Brazilian studies exist that analyze the effect of enteric viruses on the intestines of turkey flocks (Vilarreal et al., 2006; Bunger et al., 2009; Silva et al., 2009). Moreover, correlations have not been established between age or the presence of intestinal disease and single and multiple infections by TAV, HEV, TAstV-1, TAstV-2, TCoV, reovirus, and rotavirus.

Molecular methods of virus detection such as genome sequencing are more specific and sensitive diagnostic methods, and they have effectively supplanted traditional methods such as electron microscopy (Jindal et al., 2010b). In this study, we successfully used PCR and RT-PCR to identify the presence of specific virus families within intestinal samples (Hess et al., 1999; Koci et al., 2000a; Cavanagh et al., 2002; Alvarado et al., 2007; Day et al., 2007; Pantin-Jackwood et al., 2008a). Recently, metagenomic studies have been used for the establishment of the intestinal DNA profile of enteric pathogens and microbiota (Day et al., 2010).

In general, clinical signs of intestinal disease are not always readily apparent, as signs such as diarrhea can be difficult to identify (Trampel et al., 1983; Jindal et al., 2010a).

Our results have provided an initial description of the viruses typically found in Brazilian turkey flocks. Our study warrants future investigations aimed at under-
standing the pathogenic aspects of single- and multiple-agent infections by different enteric pathogens, including viruses, bacteria, and protozoa. The fact that viral pathogens occur at a higher frequency in turkeys in the growing phase of development reveals the importance of a clear comprehension of the adaptive mucosal immunity of the intestine, as well as the importance of the innate immune response, in single and multiple infections.

ACKNOWLEDGMENTS

This study was sponsored by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazilia-DF, Brazil) and FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil). We thank Erica Spackman (Southeast Poultry Research Laboratory, USDA, Agricultural Research Service, Athens, GA) for providing the reference strains that were used as positive controls.

REFERENCES


