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# Identification of Turkey Astrovirus and Turkey Coronavirus in an Outbreak of Poult Enteritis and Mortality Syndrome

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## ABSTRACT

This article reports a survey on turkey astrovirus (TAstV) and turkey coronavirus (TCoV) infections with RT-PCR in 17 turkey flocks affected by acute enteritis and two apparently normal turkey flocks located in the Southeastern region of Brazil by PCR (TAstV and TCoV). Seven out of the 17 affected flocks were positive for TAstV and 14 for TCoV, with seven co-infections. In one of the two apparently normal flocks, a TAstV-TCoV co-infection was found. Although a definitive association of these agents and the signs can not be made, the implications of these findings are discussed.

## INTRODUCTION

Poult Enteritis and Mortality Syndrome (PEMS) is considered a multifactorial disease that includes enteropathogenic viruses and bacteria. Most research has focused on turkey astroviruses (TAstV), turkey coronavirus (TCoV) and *Escherichia coli* (Barnes & Guy, 2003). Nevertheless, other viruses such as adenovirus, rotavirus and reovirus and *Salmonella* spp are enterotropic agents with worldwide distribution associated with PEMS and could cause major economic losses to the poultry industry (Barnes & Guy, 2003).

Astroviruses are known as the causative agents of acute gastroenteritis in mammals and turkeys (Kurtz *et al.*, 1977; Bridger, 1980; McNulty *et al.*, 1980; Reynolds *et al.*, 1987a; Leite *et al.*, 1991; Tanaka *et al.*, 1994), as well as acute nephritis in chickens (Imada *et al.*, 2000). In turkeys, infections usually occur during the first four weeks of age (Reynolds *et al.*, 1987b) and are more prevalent than infections by any other enteritis-causing agent in poultry (Reynolds & Saif, 1986; Reynolds *et al.*, 1987a; Saif *et al.*, 1985), leading to a moderate increase in mortality (Koci & Schultz-Cherry, 2002).

Turkey astrovirus (TAstV) is a non-enveloped virus with a single-stranded positive-sense RNA of the family *Astroviridiae*, with a diameter of 25-35 nm. Its genome is 6.5-7.5 kb long with 3 open reading frames (ORFs) that code for the non-structural proteins serine-protease (ORF1a) and RNA-dependent RNA-polymerase (ORF1b) and the structural proteins of the viral capsid (ORF 2) (Koci *et al.*, 2000; Reynolds & Schultz-Cherry, 2003).

Turkey coronavirus (TCoV) is a member of Group 3 of the genus *Coronavirus* of the family *Coronaviridae*; this is an enveloped virus with up to 220 nm in diameter which genome, an 27-kb-long RNA, codes for 4 the structural proteins S, E, M and N. The most important non-structural protein of TCoV, the RNA- dependent RNA- polymerase (Rd Rp), is coded by ORF1b, the most conserved, and plays a major role on viral replication and transcription (Lai & Cavanagh, 1997). As TAstV, TCoV causes a disease of significant economic importance to the turkey industry, called

transmissible enteritis or bluecomb disease, which affects turkeys of all ages. However, clinical signs are more common in the first two weeks of life, usually appearing suddenly, with a high rate of morbidity, depression, anorexia, diarrhea, dehydration and weight loss (Guy, 2003).

TAstV and TCoV co-infections are common in turkeys and cause a highly negative impact on intestinal absorptive functions (Ismail *et al.*, 2003). These co-infections have been suggested as one of the causative factors of PEMS, a disease characterized by increased mortality, severe growth depression and immune dysfunctions (Barnes & Guy, 1997). For this reason, TAstV and TCoV co-infections are suspected to predispose the poults to infections by other "opportunistic" pathogens, such as *E. coli* and other agents (Qureshi *et al.*, 2000; Barnes & Guy, 1999).

Commercial turkey production in Brazil is concentrated in the Southeast region of the country with an annual production of 30 million turkeys and an 8.14% growth rate/time. However, hitherto, there have been no reports of PEMS, nor have astroviruses or coronaviruses been identified in turkeys in Brazil.

The aim of this survey was to elucidate the agents involved in PEMS in Brazilian turkey flocks.

## MATERIALS AND METHODS

### History

From January to February 2004, turkey flocks from different farms located in Southeastern region of Brazil showed 70% morbidity and 30% mortality in a disease that began during the first week of life. It was characterized by severe diarrhea, weight loss, ruffled feathers, prostration, drooping wings and restlessness. The signs persisted up to at least 13 weeks of age.

At post-mortem examination, atrophy of the bursa of Fabricius, loss of intestinal mucosa, enteritis and gas in the gut were evident.

### Samples

Samples from 17 turkey flocks affected by acute enteritis aged 10 to 91 days and 2 apparently healthy flocks aged 49 days were received at the Laboratory of Avian Pathology from the University of São Paulo. Each sample consisted of the whole enteric tract of 5 birds per flock, randomly selected (Table 1). The samples were prepared as 20% suspensions of enteric contents from the entire gut in 0.01M PBS pH 7.4 (PBS) and clarified at 12,000 x g for 30 minutes at 4 °C. The supernatant was collected for analysis.

### Astrovirus survey

Astroviruses were surveyed by an RT-PCR aimed to amplify an 802-bp fragment of the RNA-dependent RNA–polymerase (Rd Rp) gene, with the specific primers MKPol10 (5'TGGCGGCGAACTCCTCAACA3') and MKPol11 (5'AATAAGGTCTGCACAGGTCG3') described by Koci *et al.* (2000), with reactions modified from the original protocol as described below for the optimization of astrovirus detection. Total RNA was extracted with TRIzol reagent (Invitrogen™) according to the manufacturer's instructions from faecal suspensions and from negative (ultra-pure water) and positive [field strain of avian astrovirus detected from a fecal specimen of a turkey identified and available at LABOR and reported by Villarreal *et al.*, 2005.] controls. Next, 7 ml of each RNA was re-suspended in DEPC-treated water and denatured at 95°C for 5 minutes and added to the reverse transcription mix containing 1 x First Strand Buffer, 1 mM of dNTP, DTT 10 mM, 1µM of each primer (MKPol10 e MKPol11) and 200U of M-MLV Reverse Transcriptase (Invitrogen™) to a final reaction of 20 ml. The reverse transcription was carried out at 42°C for 1 hour.

The PCR was performed with the addition of 2 µl of c-DNA to the PCR mix (1 x PCR Buffer (Invitrogen™), 0.2 mM of each dNTP, 0.5mM of each primer (MKPol10 and MKPol11), 1.5 mM $MgCl_2$, 28.25 µl of ultra-pure water and 1.25 U of Taq DNA polymerase (Invitrogen™) to a final reaction volume of 50 µl and submitted to an initial denaturation at 94°C for 3 minute, followed by 35 cycles of 94°C/30 seconds, 56 °C /1 minute and 72°C/2 minutes, with a final extension at 72°C/10 minutes.

### Coronavirus survey

Primers UTR 41 (5'ATGTCTATCGCCAGGGAAAT GTC 3'), UTR 31 (5' GGGCGTCCAAGTGCTGTACCC 3'), and UTR 11 (5' GCTCTAACTCTATACTAGCCTA 3') described by Cavanagh *et al.* (2002) were used to amplify a 179-bp fragment of the 3'untranslated region (3' UTR) of avian coronaviruses and the reactions modified from the original protocol as described below for the optimization of coronavirus detection. Total RNA was extracted with TRIzol reagent (Invitrogen™) according to the manufacturer's instructions from faecal suspensions and from negative (ultra-pure water) and positive (infectious bronchitis virus strain H120) controls. Next, 7 ml of each RNA were re-suspended in DEPC water, denatured at 95°C for 5 minutes and added to the reverse transcription mix containing 1 x First Strand Buffer, 1 mM of each of

dNTP, DTT 10mM, 1µM of each primer (UTR 41 and UTR 11), and 200U of M-MLV reverse transcriptase (Invitrogen™) to a final reaction of 20 µl. Reverse transcription was carried out at 45ºC/ 1 hour, followed by 10 minutes at 72ºC.

The PCR was performed with the addition of 5 µl of c-DNA to a PCR mix containing 1 x PCR Buffer (Invitrogen™), 0.2 mM of each dNTP, 0.5µM of each primer (UTR 41 and UTR 11), 1.5 mM $MgCl_2$, 28.25 µl of ultra-pure water and 1.25 U of Taq DNA polymerase (Invitrogen™) to a final reaction volume of 50 µl and submitted to 94ºC/3' for initial denaturation, followed by 35 cycles of 94ºC/30 seconds, 48ºC /1 minute, and 72°C/2 minutes and a final extension at 72°C/10 minutes.

The nested step was performed with the addition of 5 µl of the PCR product to the nested mix (1 x PCR Buffer (Invitrogen™), 0.2 mM of each dNTP, 0.5µM of each primer (UTR 41 and UTR 31), 1.5 mM $MgCl_2$, 28.25 µl of ultra-pure water, and 1.25 U of Taq DNA polymerase (Invitrogen™)) to a final reaction volume of 50 µl and submitted to the same cycles of the PCR step.

### Haemorrhagic enteritis virus survey

HEV was surveyed by a PCR aimed to amplify a 1647-bp fragment of the HEV *Hexon* gene. DNA was extracted as described by Chomksinsky (1993) and used in the PCR with the specific primers HEV1F: 5´ TACTGCTGCTATTTGTTGTG-3´ and HEV2R: 5´ TCATTAACTCCAGCAATTGG 3´ and reaction conditions described by Hess *et al.*(1999) The EDS-76 strain of avian adenovirus was includes as the positive control and ultra-pure water as the negative control.

### Rotavirus and reovirus survey

The stool sample was searched for rotavirus and reovirus RNAs in PAGE (polyacrylamide gel electrophoresis) according to Herring *et al.* (1982). Total RNA was extracted with phenol/chlorophorm from 20% fecal suspensions in PBS, precipitated with ethanol and resolved in 3.5 %/ 7.5% discontinuous polyacrylamide gel under 20 mA for 2 hours and stained with silver. The NCDV rotavirus strain (White *et al.*, 1970) and reovirus strain S1133 were included as positive and PBS as negative controls.

## RESULTS

Seven out of the 17 affected flocks were positive for TAstV and 14 for TCoV, according to the amplification of the expected DNA fragments of 802 bp and 179 bp respectively, with seven co-infections (Table 1). A TCoV-TAstV co-infection was also found in one of the two apparently normal flocks. The negative controls did not result in amplification of DNA products. Examples of amplified fragments to each virus can be seen in Figure 1.

Furthermore, reovirus, hemorrhagic enteritis virus (adenovirus) or rotavirus were not detected in any sample examined by PCR or PAGE.

As the sampling was not statically designed, but was rather based on casuistic, statistical analysis was not carried out on these results.

**Table 1** – Detection of turkey astrovirus (TAstV) and turkey coronavirus (TCoV) in turkey flocks showing poult enteritis and mortality syndrome.

| | | | | |
|---|---|---|---|---|
| 1 | 19 | Severe Diarrhea | - | - |
| 2 | 19 | Severe Diarrhea | - | + |
| 3 | 14 | Severe Diarrhea | + | + |
| 4 | 14 | Severe Diarrhea | - | - |
| 5 | 49 | Diarrhea , Weigh loss, Ruffled Feathers, Prostration, Nervousness | + | + |
| 6 | 49 | Diarrhea , Weigh loss, Ruffled Feathers, Prostration, Nervousness | + | + |
| 7 | 49 | Diarrhea , Weigh loss, Ruffled Feathers, Prostration, Nervousness | + | + |
| 8 | 51 | Diarrhea | - | - |
| 9 | 13 | Diarrhea , Nervousness | + | + |
| 10 | 19 | Diarrhea , Weigh loss, Ruffled Feathers, Prostration, Nervousness | - | + |
| 11 | 91 | Diarrhea , Weigh loss, Ruffled Feathers, Prostration, Nervousness | + | + |
| 12 | 9 | Diarrhea , Weigh loss, Ruffled Feathers, Prostration, Nervousness | - | + |
| 13 | 10 | Diarrhea , Weigh loss, Ruffled Feathers, Prostration, Nervousness | - | + |
| 14 | 91 | Severe diarrhea , Weigh loss | + | + |
| 15 | 19 | Severe diarrhea , Weigh loss | - | + |
| 16 | 56 | Severe diarrhea , Weigh loss , Prostration | - | + |
| 17 | 56 | Severe diarrhea , Weigh loss , Prostration | - | + |
| 18 | 49 | Normal | - | - |
| 19 | 49 | Normal | + | + |

* No sample was positive for hemorrhagic enteritis virus (HEV), Reovirus either for Rotavirus.

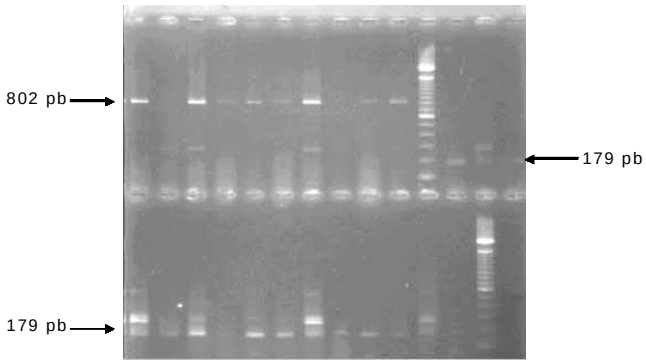


**Figure 1** - Agarose gel stained with ethidium bromide showing the 802bp fragment of the RdRp gene of turkey astrovirus (TAstV) of the eight TAstV- positive samples found in the present study and the 179pb fragment of the 3'UTR of turkey coronavirus (TCoV) of thirteen of the fifteen TCoV-positive samples found in the present study.

## DISCUSSION

Turkey astrovirus and turkey coronavirus co-infections were detected in flocks of turkeys aged 7 to 91 days in a Brazilian turkey-producing region undergoing severe enteritis and high mortality, suggestive of PEMS.

A TCoV infection in turkeys leads to virus replication in the apical portion of the intestinal villi, causing malabsorption, poor digestion, and diarrhea and changing of the intestinal enviroment (Naqi *et al.*, 1971). In contrast, TAstV replicates in the basal portion of the villi and, more rarely, in the crypts, causing osmotic diarrhea (Reynolds & Schultz-Cherry, 2003; Behling-Kelly *et al.*, 2002). Furthermore, infections by these viruses make the enteric tract susceptible to secondary infections by pathogenic bacteria. This type of pathogenesis explains the delayed growth and the diarrhea observed in the animals of this survey.

TCoV infection has been reported in the USA, UK, Canada, and Australia as the causative agent of enteric diseases similar to those reported here (Cavanagh *et al.*, 2002; Dea *et al.*, 1986, Nagaraja & Pomeroy, 1997). TAstV in commercial poultry has a worldwide distribution and causes the most prevalent viral infection in turkeys aged 1 to 5 weeks. This virus often occurs in association with other viruses, mainly rotavirus D, playing a role in PEMS (Reynolds *et al.*, 1987b). Nevertheless, in the present study, birds of several older ages (up to 91 days) were found positive to TAstV, suggesting that the age range of infection of susceptible animals is larger than previously reported.

Experimental co-inoculation of turkeys with TAstV and TCoV led to a more severe clinical response and a high mortality rate when compared to single-inoculations (Yu *et al.*, 2000). In cases of PEMS, mortality is usually high and it is possible that outbreaks of this disease are caused by co-infections with two or more viruses, such as TAsV and TCoV (Yu *et al.*, 2000; Barnes & Guy, 1997).

Thus, in this study, this synergism between coronaviruses and other enterotropic viruses can be suggested as responsible for the severity of the enteric disease observed and for the high mortality rates in the flocks surveyed (Barnes & Guy, 1999). Nevertheless, the viruses reported herein don't allow one to definitely associate these agents with the pathological features described, but may be associated with turkey flocks in which the enteric syndrome was observed.

TAstV and TCoV can be detected in intestinal contents of poults prior to the onset of clinical disease and gross pathologic changes; in the same way, poults in the later stages of astrovirus infection may display clinical signs even without detectable astrovirus particles in their intestinal tract (Reynolds & Schultz-Cherry, 2003). This may explain why some apparently normal flocks such as those in the present study, were positive to TAstV and TCoV and while flocks exhibiting typical signs of TAstV and TCoV infection were negative to these viruses.

Turkey production in Brazil has grown significantly in different regions of the country. Surveillance on the prevalence and distribution of TAstV and TCoV must be carried out in order to assess the risk they pose to the turkey industry in the country. Furthermore, experimental studies using SPF birds are needed on the synergism between these enteropathogens and others such as coccidia and *E. coli*. A more comprehensive molecular characterization of the identified viruses will help understand their genetic diversity and assist in establishing preventive and control measures for the turkey enteric disease.

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