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Detection of Astrovirus, Coronavirus and Haemorrhagic Enteritis Virus in Turkeys with Poult Enteritis Mortality Syndrome in Turkey

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This study was carried out to investigate the presence of turkey astrovirus 2 (TAstV-2), turkey coronavirus (TCoV) and haemorrhagic enteritis virus (HEV) by molecular methods in cloacal swabs collected from both clinically healthy turkey flocks and those associated with poult enteritis mortality syndrome (PEMS) in Turkey. In the reverse transcriptase polymerase chain reaction (RT-PCR) examination of 230 cloacal swabs collected from 23 turkey flocks associated with PEMS, TAstV-2 was detected in 13.4% (31/230) of the animals and in 43.4% (10/23) of the flocks. In addition, this virus was found in two turkeys originating from one of the four clinically healthy flocks. On the other hand, neither TCoV nor HEV were detected in any of the turkey samples examined in this study. In the partial sequence analysis of four randomly selected DNA samples, 96% nucleotide identity was observed between our strains and reference Turkey astrovirus isolated from turkeys in Italy between 2000 and 2004 (sequence accession number DQ381378.1).

Key words: enteritis viruses, poult enteritis mortality syndrome, PEMS, turkey

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Introduction

Poults enteritis complex (PEC) is a common term that includes all infectious intestinal diseases of young turkeys such as coronaviral enteritis of turkeys, maldigestion syndrome, runting and stunting syndrome of turkeys, poult malabsorption syndrome, poult enteritis and mortality syndrome (PEMS), spiking mortality of turkeys, poult enteritis syndrome (PES) and turkey viral enteritis. (Barnes *et al*., 2000; Jindal *et al*., 2014). The complex is characterized by enteritis, moderate to marked growth depression, retarded development, impaired feed utilization; poor feed conversion efficiency, and sometimes increased mortality (Jindal *et al*., 2014).

A number of viruses (Astrovirus, Calicivirus, Coronavirus, Haemorrhagic Enteritis Virus, Reovirus, Picornavirus, Picobirnavirus, Enterovirus and Rotavirus), bacteria (*Escherichia coli* and species of Salmonella, Clostridium, Campylobacter and Enterococcus) and protozoa (coccidia, cryptosporidium) have been detected in PEMS-affected flocks (Barnes *et al*., 2000; Jindal *et al*., 2014). Astrovirus is an important cause of enteritis in humans and animals. Turkey astrovirus (TAstV) was first identified in 1980 in the United Kingdom from diarrheic turkey poults (McNulty *et al*., 1980) and subsequently reported from the United States (where it was named as TAstV-1) (Saif *et al*., 1985; Reynolds and Saif, 1986). Later, another turkey astrovirus (TAstV-2), genetically distinct from TAstV-1, was identified and characterized (Schultz-Cherry *et al*., 2000). TAstV-2 isolate (N/96) has been reported to bear many similar features to Human Astrovirus (Koci and Schultz-Cherry, 2002).

The most important infectious disease caused by TAstV is the enteric disease affecting the digestive tract of commercial poults which are reported to result in more economical losses than those affecting any other systems worldwide (Schultz-Cherry *et al*., 2000; Pantin-Jackwood *et al*., 2008).

Turkey coronavirus (TCoV) (mud fever, bluecomb diseases, coronaviral enteritis of turkeys) infects turkeys of any age, morbidity being close to %100, and mortality varying from 10 to 50% or more, being the highest in young birds. The virus has been detected only in epithelium of the in-

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testinal tract. It was first described in the state of Washington in the 1940s and rose to prominence following outbreaks in Minnesota in 1951. Some 20 years later, it was demonstrated that the causative agent was a coronavirus (Cavanagh *et al*., 2001). TCoV is also among the pathogens associated with PEMS, and had significant economic importance in the US during the 1990s (Goodwin *et al*., 1995, Barnes and Guy, 1997). Two manifestations of the syndrome have been described as "spiking mortality of turkeys", and less severe "excess mortality of turkeys" (Cavanagh *et al*., 2001).

Only group II avian adenovirus of haemorrhagic enteritis virus (HEV) has economic impact in turkeys. It has been identified in the majority of the countries where turkeys are raised intensively (Arbuckle *et al*., 1979). HEV is ubiquitous, and most commercial turkeys acquire infection with the virus from the environment. Haemorrhagic Enteritis (HE) is a disease of turkeys 4 weeks of age and older (Pierson and Donermuth, 1997). Clinical outbreaks are characterized by intestinal haemorrhages accompanied with immunosuppression.

When compared with most of the countries worldwide, turkey production in Turkey can be regarded as not very widespread but this is growing sector in Turkey. However, there is little quantitative information about infectious diseases that have significant effect on turkey breeding. In particular, no data are available about the significance of PEMS associated viral enteritis in turkey population of the country. This study was therefore carried out to investigate the presence of TAstV, TCoV and HEV by generic polymerase chain reaction (PCR) assays in cloacal swabs collected from both clinically healthy turkey flocks and those associated with PEMS.

Materials and Methods

Sample Collection

A total number of 270 cloacal swab samples were collected from different turkey flocks belonging a commercial turkey company located in the north of Turkey between April and July 2014. The turkeys were white feathered Californian breeds at the ages ranging from 11 to 119 days.

Of the cloacal swab samples, 230 were from 23 different turkey flocks associated with PEMS and the remaining 40 samples were collected from 4 turkey flocks of clinically healthy turkeys. The samples were originated from a total of 27 different turkey flocks, each flock being represented with 10 samples. Each flock sampled in this study had the capacity of approximately 7000 turkeys.

In the clinical examination of the animals within the flocks, various symptoms such as severe diarrhea, moderate to marked growth depression, retarded development, impaired feed utilization; poor feed conversion efficiency were recorded in 23 flocks which were linked with PEMS. The turkeys in the remaining four flocks seemed to be apparently healthy.

Cloacal swab samples within Stuart Transport Medium were stored in cool boxes and transported to the laboratory within two days. Each swab sample was processed separately.

RNA Extraction

Cloacal swabs were homogenized in one ml phosphate buffered saline (0.01M PBS). Following spinning at 800 g for 10 min, the supernatant was transferred to another microtube and was recentrifuged at the above conditions. Then, the upper aliquot of the suspension was used for RNA and DNA extraction.

Total RNA was extracted directly from 150 μl of the supernatant using EZ-10 Spin Column (Bio Basic Inc., Canada) according to the manufacturers' instructions. The extracted RNA was resuspended in 50 μl of distilled water containing 0.1 % diethyl pyrocarbonate (DEPC).

DNA Extraction

300 μl of the supernatant was transferred into a microtube and routine procedures were followed to extract the DNA (Cetinkaya *et al*., 2002).

Reverse transcription- polymerase chain reaction (RT-PCR) Astrovirus

The primers used for the detection of TAstV in this study were evaluated previously (Koci *et al*., 2000) (Table 1). The screening of the flocks for TAstV was performed by employing a one-step RT-PCR kit (Qiagen, Hilden, Germany) combined with the primer pair of MKPol 10 /MK Pol 11 which produces an approximately 802-bp fragment in positive samples.

One-step RT-PCR was performed in a TC 512 Temperature Cycling System (Techne, Staffordshire, UK) in a

Table 1. **Primer sequences and lengths of PCR amplification products**

Gene	Primer	Oligonucleotide sequence (5′ -3′)	Fragment Length (bp)	Reference
Polymerase	MKPOL10	TGGCGGCGAACTCCTCAACA	802	Koci *et al*., 2000
	MKPOL11	AATAAGGTCTGCACAGGTCG		
Hexon	HEV 1F	TACTGCTGCTATTTGTTGTG	1647	Hess *et al*., 1999
	HEV 1R	TCATTAACTCCAGCAATTGG		
UTR	UTR 41	ATGTCTATCGCCAGGGAAATGTC	266	Cavanagh *et al*., 2001
	UTR 11	GCTCTAACTCTATACTAGCCTA		
UTR	UTR 41	ATGTCTATCGCCAGGGAAATGTC	179	Cavanagh *et al*., 2002
	UTR 31	GGGCGTCCAAGTGCTGTACCC		

reaction volume of 25 μl. RT-PCR was carried out as one RT cycle at 42℃ for 1 h, followed by enzyme inactivation at 94℃ for 3 min, then 35 cycles of denaturation at 94℃ for 30 s, annealing at 56℃ for 1 min. and extension at 72℃ for 2 min. The final extension cycle was done at 72℃ for 10 min. The amplified products were detected by staining with ethidium bromide (0.5 mg/m*l*) after electrophoresis at 80 V for 2 h (7 V/cm) in 1.5% agarose gels.

Coronavirus

A nested PCR was performed for TCoV. Firstly a one-step RT-PCR kit (Qiagen, Hilden, Germany) combined with the primer pair of UTR-41 /UTR-11 (Cavanagh *et al*., 2001) which produces an approximately 266-bp fragment in positive samples was employed. Then, the nested PCR using primer pair of UTR-41 /UTR-31 (Cavanagh *et al*., 2002) which produces an approximately 179-bp fragment in positive samples was conducted.

RT-PCR was carried out as one RT cycle at 45℃ for 1 h, followed by 10 minutes at 72℃, 94℃ for 3 min for initial denaturation, then repeated 35 cycles of denaturation at 94℃ for 30 s, annealing at 48℃ for 1 min. and extension at 72℃ for 2 min. with a final extension cycle at 72℃ for 10 min. The nested PCR was performed at the above conditions.

Hemorrhagic Enteritis Virus

The primers used for the detection of HEV in this study were also evaluated previously (Hess *et al*., 1999) (Table 1). The screening of the flocks for HEV was performed by employing PCR combined with the primer pair of HEV 1F /HEV 1R (*Hexon* gene) which produces an approximately 1647 bp fragment in positive samples. PCR reaction conditions were described by Hess *et al*., 1999.

Positive and Negative Controls

Infectious bronchitis vaccine H120 (supplied from Bornova Veterinary Control and Research Institute, Izmir) and HE Vaccine (Merial, France) were used as positive controls for TCoV and HEV assays, respectively and distilled water was used as negative controls in all the assays. As we were unable to find a reference strain for Astrovirus at the beginning of the study, four field samples which were detected to be positive for TAstV by both RT-PCR and sequence analysis were used as positive controls during the assays.

Sequence and Phylogenetic Analysis

Four randomly selected RT- PCR products amplified with MKPol 10 /MK Pol 11 primers for TAstV-2 were submitted to a sequencing company (Pendik Veterinary Control and Research Institute, Istanbul, Turkey) for partial DNA sequence analysis. The phylogenetic tree of the sample determined as TAstV-2 (2014/TAstV/BL. 1) was constructed by using the neighbour-joining method in the PHYLIP sequence program.

Statistical Analysis

A chi-squared test was used to compare the results obtained from PEMS associated flocks and healthy flocks, and at different age groups; $P<0.05$ was considered statistically significant.

Results

In the RT-PCR examination of 230 cloacal swab samples collected from turkey flocks associated with PEMS, TAstV-2 was detected in 13.4 % (31/230) cloacal swabs representing 43.4% (10/23) turkey flocks. In addition, this virus was found in two turkeys originating from one of the four clinically healthy flocks, representing the proportion of 25% and 5% at flock and animal bases (Table 2). The difference between the detection rates obtained from PEMS associated and healthy flocks was not significant ($p>0.05$). The overall positivity for TAstV-2 was calculated as 12.2% (33/270) and 40.7% (11/27) at animal and flock bases, respectively. When the results were considered according to different age groups, 15 positive samples were detected at the ages ranging from 11 to 28 days (growing phase) in the examination of 50 samples from five flocks. The positivity was obtained from 18 samples at the ages ranging from 29 to 119 days (finishing phase) in the examination of 220 samples from 22 flocks. The differences by age was found to be statistically significant ($p<0.05$) (Table 2). On the other hand, neither TCoV nor HEV were detected in any of the turkeys examined in this study.

Four randomly selected TAstV-2 positive samples were

Table 2. **Detection of Turkey Astrovirus-2 from cloacal swabs in turkey flocks**[a]

Flock Status[b]	Number of flocks			Number of samples			Age Status (days)					
							11-28			29-119		
	+	N	%	+	N	%[c]	+	N	%[d]	+	N	%[d]
PEMS	10	23	43.4	31	230	13.4	15	50	30	16	180	8.9
Healthy	1	4	25	2	40	5		—		2	40	5
TOTAL	11	27	40.7	33	270	12.2	15	50	30	18	220	8.2

[a] All the samples were negative for TCoV and HEV.
[b] Each flock had the capacity of approximately 7000 turkeys.
[c] $p>0.05$
[d] $p<0.05$
+ Number of positives
N: Total number
PEMS: Poult enteritis mortality syndrome

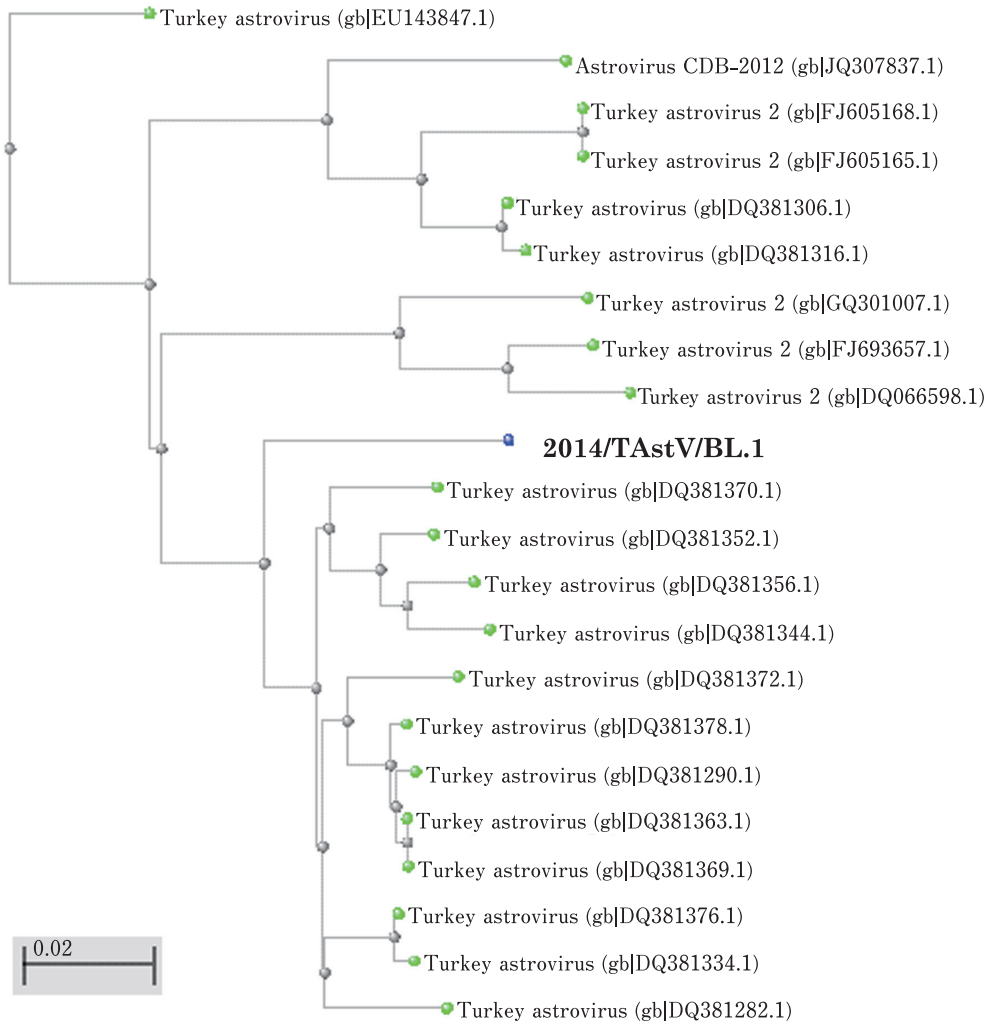

Fig. 1. **The phylogenetic tree of the sample determined as TAstV-2 (2014/TAstV/BL.1) constructed by neighbor-joining method in the PHYLIP sequence program according to 0.75 maximum sequence.**

investigated further by partial sequence analysis using the amplicons yielding a specific product in the RT-PCR combined with primers specific for *polymerase* gene. PCR products were sequenced on both forward and reverse strands, using the ABI 310 Genetic Analysis System. The sequences of four products amplified with RT-PCR in this study revealed 100% nucleotide identity. The sequence identity was between 90% and 96% with the same gene region of TAstV-2 obtained from the GenBank database. The highest nucleotide identity was detected with Turkey astrovirus 2654/04 *polymerase* gene (sequence accession number DQ381378.1) which was isolated from turkeys in Italy between 2000 and 2004 (Cattoli *et al*., 2007). The phylogenetic analysis of one sample (2014/TAstV/BL. 1, Accession number: KM875548) by the neighbour-joining method in the PHYLIP sequence program confirmed it as TAstV-2 (Fig. 1).

Discussion

There is a paucity of information about the aetiology of PEMS in turkey population of Turkey. Although many studies toward the investigation of PEMS have been reported elsewhere particularly in countries with intensive turkey breeding such as the USA and Brazil (Pantin-Jackwood *et al*., 2006; da Silva *et al*., 2009; Jindal *et al*., 2010; Mor *et al*., 2013; Moura-Alvarez *et al*., 2014), to the best of author's knowledge no study has been conducted on this subject in Turkey so far. The aim of this study was therefore to determine the presence and frequency of TAstV-2, TCoV and HEV in both PEMS-associated and clinically healthy meat-type turkeys.

High proportions ranging from 59% to 100% have been reported for the prevalence of astrovirus in various European countries (Domanska-Blicharz *et al*., 2014). When com-

pared to the above mentioned proportions, the frequency of TAstV-2 in PEMS-associated turkey flocks was lower with 43.4% in this study. This difference might be due to sample type, age, sampling time or weather conditions. In the present study, the positivity rate obtained from turkeys at growing phase (11−28 days) was significantly higher than that obtained at finishing phase (29−101 days). This is in agreement with the results of previous studies (Pantin-Jackwood *et al*., 2006). On the other hand, in a study carried out by Domanska-Blicharz *et al*. (2014) which reported 94. 1% for the prevalence of TAstV-2, the highest positivity was reported in turkey flocks at the ages of 5 to 9 weeks.

There is a lack of information about the pathogenicity of astrovirus. Likewise other studies (Pantin-Jackwood *et al*., 2006; Moura-Alvarez *et al*., 2013, Domanska-Blicharz *et al*., 2014), TAstV-2 was detected in a healthy flock in this study, and the difference between the detection rates of this virus from cloacal samples obtained in PEMS associated and healthy flocks was not statistically significant. Also, a weak correlation has been reported between presence of astrovirus and health status of turkey flocks (Domanska-Blicharz *et al*., 2014). The detection of TAstV-2 in healthy birds has arisen strong suspicion about the presence of strains with different virulence factors (Jindal *et al*., 2010; Domanska-Blicharz *et al*., 2011). In a comparative pathogenicity of TAstV-2 obtained from turkey flocks affected with PES and from clinically normal birds, Mor *et al*. (2011) reported that that TAstV-2 from PES birds may be more pathogenic than those from apparently healthy poults. The overall growth depression by TAstV-2 from PES and clinically healthy as compared to control was 16% and 2%, respectively. There is therefore an urgent need to develop effective typing methods in order both to have a better understanding of the pathogenic features and to characterize serotypes of astrovirus circulating in poultry (Guix *et al*., 2005).

There are two antigenically and genetically different types of astrovirus and, TAstV-2 has been reported at higher proportions in turkey flocks when compared to TAstV-1, with the exception of a few studies such as one carried out in Brazil which reported TAstV-1 at a higher rate (Moura-Alvarez *et al*., 2013). Therefore, only MKPOL10/MKPOL 11 primer pare specific to TAstV-2 was employed to investigate the presence of astrovirus in turkey samples in the current study.

In previous studies, the most frequently detected agent in turkeys with PEMS has been reported as TAstV-2 which was usually coincided in the format of co-infection with other agents rather than individually. It can therefore be commented that the severity of astrovirus infection by only itself would be limited when compared to co-infection with other agents (Saif *et al*., 1985; Pantin-Jackwood *et al*., 2008).

A 100% identity was obtained between the four randomly selected positive samples in the phylogenetic analysis which confirmed the isolates of the present study as TAstV-2. In the comparison of the results with the sequence data obtained from GenBank database for *polymerase* gene revealed the highest nucleotide identity with the strains (sequence accession number DQ381378.1) isolated in Italy between 200 and 2004 (Cattoli *et al*., 2007). This cluster could be explained by geographical proximity.

Other PEMS associated agents examined here, namely TCoV and HEV, could not be detected in any of cloacal swab samples. There may be a number of plausible explanations for this. Symptoms in turkeys with PEMS could be associated with other agents such as reovirus, rotavirus, small round viruses, parasites or bacteria. Alternatively, the number of samples examined in this study was limited or these agents might have little significance in the turkey population of the country. In a study conducted by Pantin-Jackwood *et al*., 2008, although the presence of TAstV-2 was reported as 100%, no positivity was detected for TCoV and HEV, similar to our study. On the other hand, the presence of rotavirus and reovirus were reported at the proportions of 69.7% and 45.5%, respectively in the same study. In another study, while TCoV and Group1 adenovirus were not reported in turkeys with PEMS, various rates ranging from 15% to 70% were noted for the presence of astrovirus, reovirus and rotavirus (Jindal *et al*., 2012). The fact that the investigation of rotavirus and reovirus in PEMS-associated turkeys was beyond the scope of the current study can be interpreted as a deficiency. On the other hand, the presence of TCoV and HEV has been reported at different rates in both PEMS-associated and clinically healthy turkey flocks in various studies (Carver *et al*., 2001, Culver *et al*., 2006; Villarreal *et al*., 2006; Palya *et al*., 2007; Teixeira *et al*., 2007; Domanska-Blicharz *et al*., 2010; Lojkic *et al*., 2010).

It is known that viral enteritis due to the agents associated with PEMS can cause important disorders such as poor feed conversion rate and marked growth depression which result in great economical losses in meat-type turkeys. The absence of effective vaccines for the important agents like TAstV-2 and TCoV is one of the major constraints against the control of PEMS associated viral enteritis. The detection of TAstV-2 in clinically healthy turkeys, as reported in the current study and by other researchers, needs to be clarified. Large-scaled studies are therefore required to obtain comprehensive data which will help us to better understand the etiology of PEMS-cases, to develop effective vaccines and improve biosecurity procedures

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Conflict of Interests: The authors declare that there is no conflict of interest.

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