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Investigation of Rotavirus infection in sheep using serological and molecular techniques

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ABSTRACT

In this study, serological and molecular research was conducted on the Rotavirus infection in domestic breeds of sheep at 2–3 years of age. The sheep included in the study were raised on small scale family units of less than 20 sheep per unit, in central Kars province and its districts (Susuz, Arpaçay, Kagizman and Selim) in the Northeast Anatolia region of Turkey. The blood and fecal samples were collected randomly from 450 sheep. They were analyzed for the presence of Rotavirus and the antibody against the virus using enzyme-linked immunosorbent assay (ELISA). The highest seropositive ratio (73.46%) was found in central Kars province. The seroprevalence of Rotavirus in sheep raised in the Kars region was determined to be 55.33%. Rotavirus was not detected in fecal samples with ELISA. Molecular detection of Rotavirus from fecal samples was done by reverse transcription polymerase chain reaction (RT-PCR) technique using specific generic primers for VP6 protein. Rotavirus could not be detected in RT-PCR. The data that were obtained showed that the infection spreads on small scale family farms. Based on this information, recommendations were made for controlling Rotavirus infection.

Key words: C-ELISA, Rotavirus, RT-PCR, Sheep.

INTRODUCTION

Rotaviruses cause enteric infections that are subclinical in adult humans and many animal species, but that can cause death in newborn humans and animals. Rotaviruses are resistant to environmental conditions and feces are a significant cause of spreading of infection (Hysser and Estes, 2008). Rotavirus causes high morbidity and mortality leading to increased economic losses at the farms (Andres *et al.,* 2007; Gokce and Erdogan, 2009). Rotaviruses are classified in the genus Rotavirus of the subfamily Sedoreovirinae in the family Reoviridae (Carsten, 2009). The genome of the virus is a double-stranded RNA with 11 segments. The virus is having icosahedral symmetry, is 70 nm in diameter and is non- enveloped. It has a three-layered capsid consisting of an outer capsid, an inner capsid and a core. VP7 (glycoprotein) and VP4 (protease-sensitive protein) in the outer capsid stimulate the production of neutralizing antibodies. Based on the differences in the genetic regions that encode these proteins, rotaviruses are classified into the G and P genotypes (Estes and Kapikian, 2007). To date, 27 G genotypes and 37 P genotypes have been identified in humans and various animal species (Abe *et al.,* 2011; Alkan *et al.,* 2012; Badaracco *et al.,* 2013). Infections in sheep and lambs are caused by strains of ovine rotavirus in serogroup A (Kaminjolo and Adesiyun, 1994; Munoz *et al.,* 1996) and serogroup B (Holland, 1990; Theil *et al.,* 1995).

Laboratory diagnosis involves the detection of Rotavirus using electron microscopy, latex agglutination and antigen ELISA techniques. When there is a need to examine a large number of samples, latex agglutination and ELISA is a rapid and inexpensive diagnostic method. The specificity and sensitivity of these tests is over 90% (Mayameei *et al.,* 2010). Furthermore, numerous PCR methods are high sensitivity, and rapid results. The reverse transcription polymerase chain reaction (RT-PCR) is employed using generic or type-specific primers.At present, the VP4, VP6 and VP7 regions are most often used (Alkan *et al.,* 2012; Falcone *et al.,* 1990; Garaicoechea *et al.,* 2006; Gazal *et al.,* 2011). In recent years, it was announced that circulating Rotavirus in small ruminants might be new subgroup in Turkey. But there was no further extensive investigation in Turkey to determine the genetic type of Rotavirus.

Kars region's economy is primarily based on animal husbandry and about 80 % of the population rely on it for their livelihood. The aim of this study was to identify the seroprevalence and viroprevalance of Rotavirus infection in sheep raised in the Kars region in northeastern Turkey using ELISA and RT-PCR. This study is significant because

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it obtained the first data on this infection in sheep in the area under study, and it is the first study ever done on this infection in sheep in northeastern Turkey.

MATERIALS AND METHODS

Ethics Statement: This research was conducted after the approval of Mehmet Akif Ersoy University Animal Testing Local Ethics Council (Approval Number: MAKU HADYEK-2014/12-81).

Geographical area: This study was conducted in Kars province and its vicinity, in Turkey. The Kars region, located in north-eastern Turkey (43.05° E and 40. 36° N), which is the most important livestock production area in Turkey, is mountainous and has a cold climate. In total, five foci were selected to represent the Kars region (central Kars province, and Arpaçay, Kagizman, Selim and Susuz districts) (Fig. 1). Sample size was calculated as three hundred and eighty four using a confidence level of 95% and confidence interval (CI) of 5% and considering the sheep number of Kars Region as approximately five hundred and forty three thousand (data were obtained from the Kars Province Directorate of Ministry of Food, Agriculture and Livestock).

Serum samples: Blood samples were collected from 450 local sheep randomly, without clinical signs of the disease between May to October in 2015, although 384 samples would have been sufficient. The age of the animals varied from 2 to 3 years. Blood samples were taken from the jugular veins of the animals. Blood tubes (without EDTA) were centrifuged at 3,000 × g for 10 min, and the serum samples were transferred to sterile tubes and stored in "20 °C until analyzed.

Fecal samples: Samples were collected from 450 local sheep randomly, without clinical signs of the disease between May to October in 2015. The age of the animals varied from 2 to 3 years. Collected fecal samples were kept at -80 ºC until analyzed.

Competitive Enzyme Linked Immunosorbent Assay (C-ELISA): ELISA (Rotavirus Elisa Kit Cat.Nr.Bio K 126, Jemelle-Belgium) used for the detection of antibodies against Rotavirus was carried out according to the manufacturer's instructions. At the end of the test, optical densitieswere measured with an ELISA reader (Epoch, BIO-TEK, USA) at 450 nm absorbance according to the instructions in the test procedure.The mean optical densities of the positive (OD pos) and negative sera (OD neg) and those of all the samples (OD samples) were calculated. For each tested sample and the positive serum, the percent inhibition was calculated (%inhib) by means of the following formulas:

% inhibition of sample = [(ODneg – ODsample) ÷ ODneg] x100
% inhibition of positive = [(ODneg – ODpos) ÷ODneg] x100

Direct ELISA: A commercial ELISA kit (Rotavirus Elisa Kit Cat.Nr.Bio K 067, Jemelle-Belgium) detecting Rotavirus antigen in fecal samples was used in the study according to the manufacturer's instructions. Optical densities were measured with an ELISA reader at 450 nm absorbance, according to the instructions in the test procedure. Samples giving values equal to or greater than 0.150 were accepted as positive and values less than 0.150 as negatives.

RNA extraction and Reverse transcriptase polymerase chain reaction (RT-PCR): From fecal samples, viral RNA was extracted using a Viral Nucleic Acid Extraction Kit

Fig 1: Geographical positioning of the Kars region in which the study was performed.

(Vivantis, Malaysia), according to the manufacturer's recommendation. The follow-up cDNA synthesis was carried out using a first strand cDNA synthesis Kit (Fermentas, Lituhania) as described by the manufacturer's protocol, using random primers. The reaction mixture was incubated first at 25 °C for 10 mins, followed by a second incubation at 37 °C for an hour and then 70°C for 10 min, for inactivation on Moloney murine leukaemia virus reverse transcriptase (MMLV-RT). For diagnosis, primers were chosen for the VP6 genetic region, targeting the internal capsid protein that is in a genetic region that is protected from mutational variation. Amplification of cDNA by PCR was performed using the primer pairs VP6-F (5′- GAC GGV GCR ACT ACA TGG T -3′) and VP6-R (5′- GTC CAA TTC ATN CCT GGT GG -3′). The primers flank a 379 bp DNA fragment for Rotavirus (Iturriza-Gomara *et al.,* 2002). PCR was carried out in a total volume 50 µL containing 5 µL of cDNA to the master mix, 4.5 µL of 10 X reaction buffer (100 mM Tris-HCl pH 8.3 and 500 mM KCl), 2 µL of 50 mM MgCl2, 20 pmole from of each of the primers, 1 µL of 10 mM dNTP mix, and 0.2 µL of 5 IU Taq DNA polymerase (MBI, Fermentas, Lithuania), 35.3 µL of RNAse free distiled water. The PCR was initiated by a single denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 50 °C, extension for 1 min at 72 °C. The mixture was then incubated, as a single step for 10 min at 72 °C for final extension . Ten microlitres of each PCR product were analyzed on 1% agarose (Prona, Spain) gel containing ethidium bromide (Sigma, USA). NCDV (G6P[1]) strain was used as positive control for Rotavirus. Nuclease free water was used as negative control.

Statistical Analysis: The statistical evaluation of the seropositivity rates determined for the sheep with respect to the study sites (foci) was made using the Minitab 14.0 Inc. (State College, PA, USA) and with the chi-square (χ^2) test. P values less than 0.05 (P<0.05) were considered to be statistically significant.

RESULTS AND DISCUSSION

C-ELISA and statistical analysis: A total of 450 serum samples were examined for antibodies against Rotavirus. Out of these, 249 (55.33%) samples were found positive. Seropositivity rate ranged from 45.94 to 73.46%. Seroprevalence for Rotavirus was the highest (73.46%) in central Kars province followed by Arpaçay (47.05%), Kagizman (46.73%), Susuz (46.15%) and Selim(45.94%). There was a statistically significant difference in Rotavirus seroprevalence in centralKars province(73.46%) compared with other four districts (Table 1). There was no significant difference in the prevalence of antibodies against Rotavirus between Susuz, Arpaçay, Kagizman and Selim districts.

Direct ELISA and RT-PCR: In this study, Rotavirus antigen was not detected in 450 fecal samples using direct ELISA. RT-PCR technique was used to determine the presence of Rotavirus nucleic acid in fecal samples. Specific PCR products of 379 base pairs were not detected in fecal samples (Fig. 2).

Rotaviruses are one of the causes of enteritis in lambs. They cause significant economic losses at sheep

Table 1: Distribution of sheep blood sera according to the different foci

Location (Focus)	Number of tested animals	Number of seropositive animals	Number of suspect animals	Seropositivity (%)
Kağızman	92	43	0	46.73[b]
Arpaçay	85	40	2	47.05[b]
Central Kars Province	147	108	3	73.46[a]
Susuz	52	24	0	46.15[b]
Selim	74	34	0	45.94[b]
Total	450	249	5	55.33

a, b: Differences between the study sites shown with different superscripts are statistically significant (P<0.05).

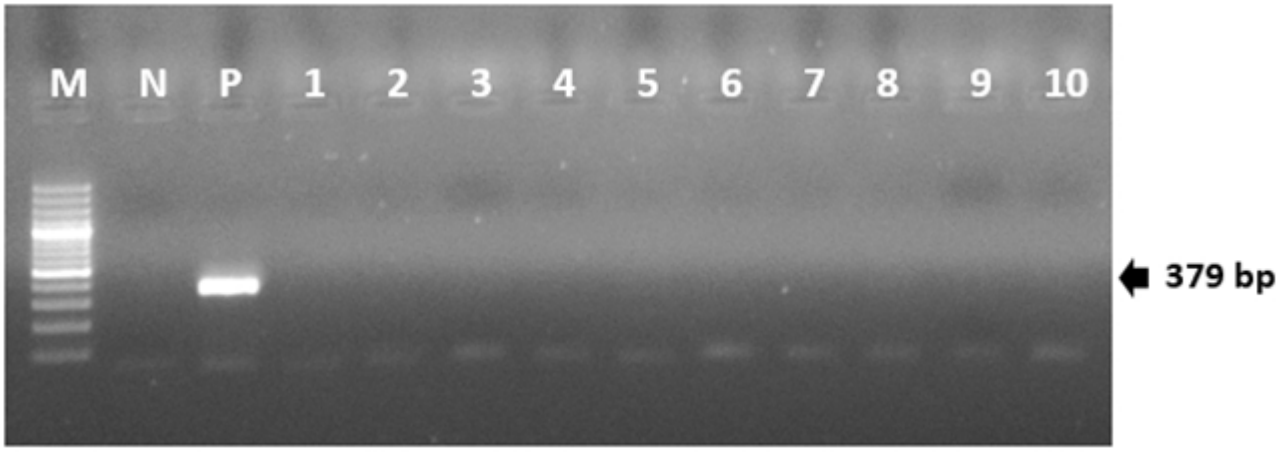

Fig 2: The results of Rotavirus RT-PCR in fecal samples.M: 100 bp. DNA Ladder, N:Negative control, P:Positive control (379 bp), 1-10: Fecal samples used for the study

farms. Few studies have been done on rotavirus infections in sheep in Turkey and around the world. Ovine rotaviruses have been identified as a cause of diarrhea in newborn lambs in England, Japan, America (Theil *et al.,* 1995; Makabe *et al.,* 1985; Chasey and Banks, 1986; Schoenian, 2007), Egypt (Khafagi *et al.,* 2010) and India (Gazal *et al.,* 2011; Wani *et al.,* 2004).

In Turkey, Burgu *et al.* (1999) employed ELISA and polyacrylamide gel electrophoresis (PAGE) to test fecal samples from 175 sheep and 96 lambs, 40 of which had diarrhea and 56 of which appeared healthy. They also investigated the presence of the rotavirus antibody with a microneutralization test conducted using the Bovine Rota Virus Northern Ireland 75/447 strain on blood serum samples from 298 sheep at 6 different sheep farms. In conclusion, the existence of rotavirus infections could not be serologically and virologically determined.

Sheep and cows are kept together on farms in the province and districts of Kars, where the study was conducted. Yildirim *et al.* (2008) identified rotavirus infections in cattle on farms in the same region where this study was conducted. The fact that rotavirus infections have been identified in cattle in this region leads to the conclusion that it is likely that sheep have rotavirus infections that were passed on by cattle. Since this study found a seropositive rate of 55.33% on small-scale family farms in the area of Kars, this proves the presence of subclinical rotavirus infections in adult sheep. This percentage is higher than that reported by Burgu *et a*l. (1999). Furthermore, the identification of a high percentage of seropositive results (73.46%) from animals in central Kars can be explained by the historically large number of neonatal diarrhea cases on farms in this region. For this reason, we think that the epidemiology of rotavirus infections in adult sheep should be evaluated together with cases of diarrhea in newborn lambs.

Electron microscopy, latex agglutination test (LAT), virus isolation, direct ELISA and RT-PCR techniques are widely used to identify rotavirus antigens in fecal samples (Khafagi *et al.,* 2010; Al-yousif *et al.,* 2001; Gumusova *et al.,* 2007; Ali *et al.,* 2008; Dhama *et al.,* 2009; Mayemeei *et al.,* 2010). In a study of lambs and goat kids with diarrhea in Egypt, Khafagi *et al.* (2010) identified a 12.3% prevalence of rotavirus in lambs with diarrhea using the LAT and ELISA methods. Similarly, Wani *et al. (*2004) employed the ELISA and RNA-PAGE methods to test fecal samples collected from lambs with diarrhea in India's Kashmir region and reported that the prevalence of the Group A rotavirus was 25%. Furthermore, Gazal *et al.* (2011) gathered fecal samples for three years from lambs with diarrhea in the Jammu and Kashmir region of India, finding that the prevalence of Group A rotavirus was 13.2% with the LAT technique, 9.8% with the RNA-PAGE technique and 10.4% with the RT-PCR technique. The rotavirus antigen was identified in fecal samples from 4 goat kids using ELISA when severe diarrhea and high death rates due to infection occurred on two goat farms in Turkey. The subjects were diagnosed with rotavirus using RT-PCR and after the etiological agent was isolated, and G and P typing (G8P[1]) of the virus was conducted. (Alkan *et al.,* 2012).

A previous study comparing RT-PCR with ELISA test for the detection of rotavirus infection found RT-PCR to be more sensitive compared to the ELISA (Gutiérrez-Aguirre *et al.,* 2008; Mukhopadhya *et al.,* 2013). Also, RT-PCR technique is more sensitive than the ELISA technique, particularly for identifying low concentrations of rotavirus in fecal samples. However, in this study, specific amplicons could not be identified after RT-PCR testing was done using specific generic primers for the VP6 genetic regions in fecal samples.

In this study Rotavirus antigen could not be detected by both Ag-ELISA and RT-PCR from the fecal samples. This may be due to the fact that the fecal samples were collected from healthy/non-symptomatic sheep (Tate *et al.,* 2013). As a local disease causing agent Rotaviruses have a short viraemia phase or none at all. For this reason antigen can mostly be found in animals with diarrhea (Chitambar *et al.,*2008). It is important to note that in studies concerning detection of Rotavirus nucleic acid, the virus did not specifically belong to sheep. A study conducted by Ciarlet *et al.* (2008) virus was genotyped G8P[14] (VP7/VP4) and believed to belong to human or cattle species; Gazal *et al.*(2011) in India indicated in their study that G6P[11] belong to cattle; Galindo-Cardiel *et al.* (2011) detected G8P[1] and concluded that this type is dominant in sheep. Thus this suggests that different regions may have different dominant genotypes of Rotavirus. It should be noted from the previous studies that viruses obtained from sheep originated from cattle or human species. Rotavirus infections in sheep bear a potential risk for cattle by crossing interspecies barrier where cattle and sheep are bred together. For this reason vaccination for cattle with region type specific vaccine may be recommended for endemic regions; since no vaccine is available for sheep, vaccines for cattle may be used in sheep. Similarly one vaccine used in babies (Rotashield) contains monkey strains and another vaccine (RotaTeq) contains cattle strains (Dennehy, 2008). When choosing vaccine strain, determining dominant genotypes in the geographical region under concern is the important factor.

Recommendations for preventing rotavirus infections include good care and diet, ensuring that newborns consume colostrum and milk, following hygiene and sanitation rules and conducting vaccination programs at farms where the infection occurs frequently.

In conclusion, the results obtained in the present study showed that Rotavirus infection is common in the

Northeast part of Turkey, which is the most important cattle and sheep production area in Turkey. The seroprevalence of Rotavirus in sheep raised in the Kars region was determined to be 55.33%. The serological data that were collected in this study indicate the presence of Rotavirus infection (probably subclinical) in adult sheep, and although the Rotavirus antigen was not identified in adult sheep, the data show that the percentage of seropositive animals in small scale family farms needs to be evaluated as an epidemiological indicator.

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