Isolation of epizootic hemorrhagic disease virus from sheep in western Turkey

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SUMMARY

Epizootic hemorrhagic disease (EHD) is a systemic viral infection of ruminants, especially deer, characterized by systemic blood circulation disorders and death. Data on the prevalence, clinical and pathological characteristics, and virulence of the infection in sheep are very limited in the literature. In this study, virological details of epizootic hemorrhagic disease virus (EHDV) were investigated in sheep with clinical symptoms such as high fever, edema of the head, particularly under the chin, and lesions in the mouth and nose in the Aydin province of western Turkey. The blood serum samples taken from animals with clinical symptoms were tested for the presence of specific antibodies against bluetongue virus (BTV) and EHDV using competitive ELISA (cELISA) and agar gel precipitation test (AGPT), respectively. In addition, Bluetongue Virus Antigen Capture ELISA (BTACE) kit was used to test for BTV antigens. Supernatants collected from the cell cultures with cytopathogenic effect were subjected to BTACE to test for the presence of BTV antigens. One step RT-PCR was used to examine samples for the presence of BTV and EHDV genomes. No antibodies against BTV and EHDV and no BTV antigens were detected. However, a band of 533 base pairs was detected by RT-PCR in two of the samples, confirming the presence of the EHDV genome. These findings suggest that EHDV infection could be present in sheep in the region and may be the cause of subclinical or atypical symptoms that result in difficulties in the differential diagnosis of other viral infections.

Keywords: Epizootic hemorrhagic disease virus, sheep, Turkey

RÉSUMÉ

La maladie hémorragique épizootique (EHF) est une infection virale systémique des ruminants, en particulier des cervidés, caractérisée par des troubles de la circulation sanguine et de la mortalité. Les données sur la prévalence, clinique et pathologique les caractéristiques et la virulence de l'infection chez les ovins sont peu nombreuses. Les caractéristiques du virus responsable de la maladie ont été étudiées chez les ovins présentant des symptômes cliniques tels que une forte fièvre, un œdème de la tête, en particulier sous le menton, et des lésions dans la bouche et le nez dans la province d’Aydin en Turquie. Le sang et sérum prélevés sur des animaux avec des symptômes cliniques ont été respectivement testés quand à la présence d’antigènes spécifiques contre le virus de la fièvre catarrhale (BTV) et le virus de l’EHF par ELISA compétitif (cELISA) et épreuve de précipitation en gel d’agarose (AGPT). En outre, le kit ELISA de capture de l’antigène de fièvre catarrhale (BTACE) a été utilisé pour tester des antigènes BTV. Les surnageants recueillis à partir de cultures de cellules sur lesquelles ont été observés des effets cytopathogènes ont été soumis au BTACE pour détecter la présence d’antigènes BTV. La présence des virus BTV et EHDV a également été recherchée par RT-PCR. Aucun anticorps contre BTV et EHDV et aucun antigène BTV n’a été détecté. Toutefois, une bande de 533 paires de bases a été détectée par RT-PCR dans deux des échantillons, confirmant la présence du génome de l’EHF. Ces résultats suggèrent que l’infection par le virus de l’EHF peut être présente chez les ovins dans la région et peut être la cause de symptômes atypiques qui conduisent à des difficultés dans le diagnostic d’autres infections virales.

Mots-clés: virus épizootique de la maladie hémorragique, mouton, Turquie

Introduction

Epizootic hemorrhagic disease (EHD), also known as the deer disease, causes death in the deer populations but it is also an important cause of severe clinical symptoms and significant loss of productivity and death in cattle in recent years [15]. Epizootic hemorrhagic disease virus (EHDV) is classified within the Orbivirus genus of the Reoviridae family. The genome of the orbiviruses is generally composed of double-stranded RNA consisting of 10 segments. EHDV and bluetongue virus (BTV) are classified within the same genus and there are similarities in their antigenic and genotypic characteristics [32]. There are 10 known serotypes of EHDV. Transmission is through blood-sucking flies within the genus Culicoides [25].

EHDV generally causes infections in wild ruminants and cattle. The infection, especially in white-tailed deer, causes severe disease. Although it is thought that the infection rarely causes disease in cattle, the infection is seen in the form of severe clinical symptoms and outbreaks in Israel and Turkey in recent years [15, 16, 27]. EHDV infection in cattle and deer begins with fever, loss of appetite, and difficulty in swallowing. Edema can be seen in the face, tongue, neck, eyelids and conjunctiva. Redness, bleeding, and lesions can be seen in the nose, mouth, tongue, pharynx, larynx, and esophagus. In addition, symptoms such as abortion, congenital anomaly, bloody diarrhea, rapid weight loss, apathy, imbalance, and lameness can also be seen in the course of the infection [18]. Pneumonia and respiratory distress may be noted. The animals may recover within a few weeks but the lameness may sometimes become permanent.
It is assumed that sheep is susceptible to EHDV infection but rarely develops clinical symptoms [12, 31]. Goats are not considered susceptible to the infection. Data on the prevalence and virulence of the virus and the clinical and pathological findings in sheep and goats are very limited in the literature. Kedmi et al. [16] failed to detect any symptoms of EHDV infection in sheep during an outbreak in Israel and concluded that sheep had no role in epidemiology of the virus.

The serological presence of EHDV infection in Turkey was first discovered by Burgu et al. [6]. The authors reported seropositivity rates of 0.4% and 0.9% in cattle and sheep, respectively, for EHDV serotype 1 in southern parts of Turkey. The seropositivity for serotype 2 in the cattle and sheep were 6.5% and 4.5%, respectively. Alkan and Dagalp [3] tested 1.342 serum samples collected from cattle for the presence of antibodies against Ibaraki virus (EHDV Serotype 2); only three of the samples (0.22%) from Mугla province of Turkey were positive.

The first EHDV infection with clinical symptoms in Turkey was reported in cattle in Mугla province during the summer of 2007. The infection spread to the nearby Aydin province and resulted in deaths and losses in productivity. EHDV infection was later reported in Izmir, Canakkale, and Istanbul provinces. Reverse transcriptase-Polymerase Chain Reaction (RT-PCR) analysis of the gene encoding the VP2 protein showed that the virus isolated in Turkey was serotype 6 [27]. Albayrak et al. [1] found precipitating antibodies against EHDV in 3.50% of the 399 bovine and 2.43% of 82 gazelle (Gazella subgutturosa subgutturosa) serum samples collected from Aegean, Black Sea and Southeastern Anatolia regions of Turkey. However, no EHDV infection in sheep has been reported in Turkey.

The first blood sample (sample No. 1), was taken for virological examination from one of the three animals brought to the Veterinary Medicine clinics. Samples were taken from three sheep from these herds that were brought to the clinics of the Adnan Menderes University, Faculty of Veterinary Medicine in Aydin province (37°50’N – 27°50’E) in August and December of 2008. The sheep showed symptoms such as high fever, edema of the head, particularly under chin, and mouth and nose lesions. In addition, the sheep were at the latest stages of pregnancy; one of them was reported ill six days and the other ten days ago. In clinical examination of these animals, apathy, fatigue, edema of the head and mouth and nose were seen. Animals’ body temperatures were 40.5, 40.9, and 39.5°C. Based on the information obtained from the owner of the herd of 36 animals, the disease was seen in 6 animals, one of which had already died.

The second and third samples (samples No. 2 and 3) were taken from a case occurred in a herd of 50 head in December of 2008. Based on the information received at the time of clinical examinations, eating and drinking in the herd had stopped, eight of the animals were ill with symptoms such as lameness, abdominal distension, and edema of head and six animals were recovering. The samples 2 and 3 were taken from two ewes that were brought to the clinic in coma. The ewes were at the latest stages of pregnancy; one of them was reported ill six days and the other ten days ago. In clinical examination of these animals, apathy, fatigue, edema of the head, mild cyanosis of the tongue, and hyperemia and lesions in oral mucosa were noted. The first sheep sampled recovered but the other two sheep were euthanized. Blood samples taken from these three sheep showing clinical symptoms were collected in tubes containing kaolin and EDTA.

### IMMUNOLOGICAL METHODS

The presence of EHDV and BTV-specific antibodies in blood serum samples taken from the animals were tested using Agar Gel Precipitation Test (AGPT) (Veterinary Diagnostic Technology, Inc., USA) and competitive ELISA (cELISA; VMRD, Inc., USA), respectively. Presence of BTV antigens in blood samples were investigated by a capture ELISA (BTACE) (New South Wales, Australia) that can detect all 24 serotypes of BTV [14]. The tests were performed following manufacturer’s instructions.

Leukocyte samples isolated from blood samples with EDTA were inoculated in permanent Vero cell cultures and passaged five times for virus isolation. Supernatants of the leukocyte samples showing cytopathogenic effect (CPE) after five passages were subjected to one step Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) to test for the presence of BTV and EHDV genomes. For detection of EHDV, 533 bases of segment 3 were amplified using primers described by Ohashi et al. [23]. Sequence of the EHDV forward and reverse primers were 5'-CAGCCTGTATATCGGATTTG-3' and 5'-TTCCGGAGATACCTCCATTAC-3', respectively. The 5' positions of forward and reverse primers in the segment 3 of the EHDV were 1427 and 1931 bases, respectively. Two primer pairs were used for detection of the BTV genome in One Step RT-PCR. The primers were also described previously [4, 5, 33]. A region of 1157 bases of segment 7 of BTV was amplified using the forward (5'-GTTAAAATCTATAGAGAT-3' and 5'-GTAAAAACTCGTCAAGAG-3') and reverse (5'-GTAAGGTGATCTAAGAG-3' and 5'-GTAAGTTTAAATCGCAAGACG-3') primers. The
5′ positions for the BTV primers were 1 and 1156 of the segment 7, respectively. RNA extraction kit QIAamp Viral RNA Mini Kit (Qiagen, USA) was used to isolate RNA. Reverse transcription and PCR amplification were performed using the One Step RT-PCR kit (Qiagen, USA) following manufacturer’s instructions and modifications reported by Ohashi et al. [23]. Supernatants of the cultures showing CPE were also tested for the presence of BTV antigen using BTACE.

Results and Discussion

Laboratory results from samples taken from three sheep with clinical symptoms are summarized in Table 1. In this study, three of the blood leukocyte and erythrocyte samples inoculated into the permanent Vero cell cultures caused CPE similar to that produced by BTV. Specific antibodies against BTV and EHDV were not detected using cELISA and AGPT, respectively. No BTV antigens were detected in blood leukocytes and erythrocytes using BTACE. The presence of BTV genome was not identified in the supernatants collected from CPE-positive cell cultures using one step RT-PCR. However, a band of 533 bp was seen in the isolates, confirming the presence of EHDV genome.

There are only very few reports of EHDV infection in sheep in the current literature. In general, it has been established that EDHV infection in sheep either does not exist or it occurs very rarely [12, 31]. Some researchers reported that inoculation of material containing EHDV or direct virus inoculation in sheep did not cause any clinical symptoms [9-11, 24], or viremia [30]. EHDV has been isolated from sheep very rarely [21] but this may be due to misdiagnosis resulting from the lack of advanced diagnostic methods and molecular techniques in older studies [7, 13, 22, 29]. In a study conducted in Indonesia, antibodies against EHDV serotype 5 were found in 24% (150 / 808) of the cattle but none of the sheep [26]. No EHDV antibodies were detected in an epidemiological study in sheep in Kazakhstan [17]. Temizel et al. [27] isolated EHDV in cattle in Turkey, but did not report any infections in sheep. Eschbaumer et al. [8] infected 12 East Fresian sheep with EHDV-7 isolated from an outbreak in Israel. The antibody response after inoculation of sheep with the virus from that outbreak was inconsistent. There was no detectable viremia and only a small amount of viral RNA was found in the blood of two sheep. The authors reported that the sheep did not show any symptoms.

Antibodies against EHDV were found in sheep in Oman using AGID and cELISA [2, 28]. Hampy [13] detected antibodies against EHDV in three sheep in Texas, USA. In the American state of Georgia, 29% of sheep (n = 286) and 7% of goats (n = 433) were seropositive for EHDV [22]. In experimentally infected sheep, 65% had fever and one had lung hemorrhage but no other pathological findings were reported. However, the development of neutralizing antibodies in sheep infected with EHDV has been reported [31]. EHDV was isolated from two sheep in Colorado [29]. Serotyping using plaque-inhibition test, genome electropherotyping, ve protein analysis showed that the virus was EHDV type 2. Noon et al. [21] isolated EHDV from two dead bighorn sheep in Arizona, USA.

Antibodies against neither BTV nor EHDV were found in the sheep sampled in our study although there was a period of over six days between the collection date of samples and clinical expression of the disease. The lag of six days should be sufficient for the rising of anti-BT antibody titers above detectable levels. Lack of antibodies against EHDV, which were measured using AGID, could be because the precipitating antibodies were present in later but not in the acute stage of the infection or antibody titers were too low to detect using AGID test at the time of sampling. Apathy, high fever, edema of the head particularly under the chin, and lesions in the mouth and nose were seen in the sheep sampled in our study. These findings are similar to those seen in many diseases including bluetongue and suggest that EHDV maybe circulating in cattle and sheep in this region and maybe sporadically causing a variety of subclinical atypical viral infections, complicating the differential diagnosis.

To our knowledge, the present study is the first report on the isolation of EHDV in sheep in Turkey. It has been shown in previous studies that EHDV could replicate in cultures of pulmonary arterial endothelial cells taken from sheep, deer, and cattle but the virus replicated slower and produced less CPE when in cultured in sheep cells [19]. This study shows that EHDV can be propagated directly in vero cell cultures in contrast to BTV that requires adaptation in embryonated hen’s eggs. Temizel et al. [27] also isolated EHDV by directly inoculating the samples taken from cattle into BHK cell cultures.

Data obtained in this study show that EHDV may be circulating in the sheep and cattle in the region causing

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*Cytopathogenic effect was seen in all cultures inoculated with leucocytes obtained from the three cases.

| Table I: Laboratory results of samples taken from three sheep with clinical symptoms* |
economic losses. Kedmi et al. [16] determined the antibody positivity rate of 35.2% in 114 cattle in an epidemiological study performed after an outbreak of EHDV in Israel in 2006 but found no antibody against EHDV in 66 sheep located nearby. The authors concluded that EHDV infection in sheep was not significant and sheep did not play any roles in epidemiology of EHDV in cattle. In our study, however, isolation of virus in blood of sheep suggests that viremia by EHDV is possible in sheep and that sheep may play a role in epidemiology of the disease. In this context, it can be argued that sheep should not be ignored in the epizootiology and spread of the disease.

Aim of this article was to report that EHDV infections can occur in sheep. However it is unknown whether environmental factor(s) or other synergistic infection(s) may have also been present along with the EHDV infection in these sheep. The pathogenicity and virulence of EHDV in sheep may depend on the serotype of the virus, host characteristics and environmental conditions. Thus, epizootiological, biological and molecular properties of the virus in Turkey should be investigated. In addition, experimental studies using isolates in Turkey on the pathogenesis in sheep are needed.

It is possible that EHDV may cause sporadic atypical symptoms and subclinical infections that may cause difficulties in differential diagnosis of various viral infections. Thus, periodical controls should be carried out in sheep as well and combat and control strategies should be developed accordingly. The fact that the disease was seen in the month of December suggests that the flies carrier of the infection remain active for periods longer than expected. These results have important implications especially for areas such as Aydin where average temperature and humidity remains relatively high even in the winter, creating an environment that supports the growth of blood-sucking flies. It is known that some of the species of Culicoides prefers feeding on sheep [20] and control of these flies may be important in combatting the EHDV infection in sheep.

Conclusion

In this study, EHDV was investigated in samples obtained from three sheep that were brought to the clinic with symptoms similar to EHD. EHDV genome was detected in the supernatants of two of these samples using RT-PCR. To our knowledge, this is the first study that shows isolation of EHDV in sheep in Turkey. The results of this study suggest that EHDV infection can also be seen in sheep and, thus, sheep should not be ignored in epidemiology of EHDV. In addition, the genus Culicoides flies carrying EHDV maybe active even in the winter months in warm and humid regions such as Aydin and the control of these flies in the winter is important.

Reference


