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Isolation of First Local Coranavirus from Cattle with Winter Dysentery in Turkey

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Abstract: Winter dysentery (WD) is a very contagious disease of cattle characterized by profuse diarrhea. The precise etiology of disease is still not fully elucidated. Aim of the presented study was investigation of the causative agents of disease in a herd affected by WD and to evaluate routine haematological and biochemical parameters of cows with WD. This study was carried out on blood and fecal samples collected from 12 cows showing typical signs of disease during an outbreak of WD in Bursa, Turkey. Samples were analysed for the presence of bovine coronavirus (BCoV), Bovine viral diarrhea (BVD), Eimeria oocysts and Campylobacter spp. using ELISA, RT-PCR, flotation technique and culture. Faecal samples were inoculated in HRT cell cultures for virus isolation. Isolated viruses were identified as coronaviruses from 25% of the samples by ELISA, PCR and serum neutralisation tests. Campylobacter spp. was isolated from 33.3% cows with dysentery. BVD and Eimeria oocysts were not detected in any of the samples.

Results of the presented study indicates that BcoV along with Compylobacter spp. may be the primary agent of WD in cows. However negative results for BCoV and Campylobacter spp of 58 % of feacal samples indicates that etiology of the disease is still not fully elucidated and pathogens other than BCoV and Campylobacter could also be involved in pathogenesis of the disease

Key Words: Campylobacter, cattle, coronavirus, winter dysentery.

Türkiye'de Sığırlarda Kış Dizanterisinde İlk Lokal Coranavirusun İzalasyonu

Özet: Kış dizanterisi sulu ishal ile karakterize sığırların çok bulaşıcı bir hastalığıdır. Hastalığın etiyolojisi hala tam olarak aydınlatılamamıştır. Sunulan çalışmanın amacı kış dizanterisi ile enfekte olan sürüde etiyolojik ajanların, rutin hematolojik ve biyokimyasal parametrelerin araştırılmasıdır. Çalışmada Bursa bölgesinde tipik kış dizanterisi bulguları gösteren 12 sığırdan kan ve dışkı örnekleri toplanmıştır. Örnekler, bovine coronavirus (BCoV), bovine viral diarrhea (BVD), Eimeria ookistleri ve Campylobacter spp. yönünden ELISA, RT-PCR, kültür ve flotasyon tekniği ile analiz edilmiştir. Dışkı örneklerinde, virus izolasyonu için HRT hücre kültürü yapılmıştır. Örneklerin%25'inden ELISA, PCR ve serum nötralizasyon yöntemi ile coronavirus identifiye edilmiştir. Dizanterili sığırların %33,3'ünden Campylobacter spp. izole edilmiştir. Hiçbir örnekte BVD ve Eimeria ookistlerine rastlanılmamıştır.

Sunulan çalışmanın sonuçları göstermektedir ki Campylobacter spp ile birlikte BcoV sığırlarda kış dizanterisinin primer etkeni olabilir. Ancak dışkı örneklerinin % 58'i BCoV ve Campylobacter spp yönünden negatiftir ve

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hastalığın etiyolojisi hala tam olarak aydınlatılamadığı için BCoV ve Campylobacter spp dışında başka pathojenlerde hastalığın pathogenezinde rol olabilir.

Anahtar Kelimeler: Campylobacter spp, sığır, coronavirus, Kış Dizanterisi.

Introduction

Winter dysentery is a highly contagious disease which causes acute onset of profuse watery diarrhea in adult cattle primarily during winter[1]. Clinical features include explosive watery diarrhea, profound drop in milk yield, variable anorexia, depression and mild respiratory signs. Disease has a high morbidity but low mortality rates[2,1]. The precise aetiology of winter dysentery is still unclear. However in recent years, a bovine coronavirus (BcoV) along with Campylobacter jejuni has been implicated as the etiological agent[3-8]. Concurrent risk factors, such as changes in diet, cold temperatures, and presence of other microorganisms increases the risk of clinical disease in adult cattle infected by BCV[1].

The aim of this study was to investigate the causative agents of WD. Therefore stool samples of 12 cows of suffering from different degrees of winter dysentery were examined by ELISA, RT-PCR, and flotation technique also routine haematological and biochemical parameters of cows with winter dysentery were evaluated.

Materials and Methods

This report was conducted on a cattle breeding farm in Bursa, Turkey at the beggining of the April. The herd consisted of 40 dairy cows. Clinically, 12 cows were found to be suffering from different degrees of winter dysentery. These animals were from various breeds (8 Holstein Friesian, 4 Brown Swiss) and ages (2-7 years). Body temperature, heart and respiration rates (PR and RR) were determined by clinical examination. 12 healthy cows from same herd, aged between 2 –7 years were selected as a control group for comparison of biochemical and haematological values.

Blood samples were taken by jugular venipuncture from each cow into 10 mL evacuated tubes with EDTA (Ethylenediaminetetraacetic acid) or without anticoagulant, to evaluate routine haematological and biochemical parameters. Haematological parameters, including total white cell count (WBC) and differential, haematocrit rates (HCT), haemoglobin (Hg), erythrocyte and platelet counts were estimated by an automatic analyzer (Cell-Dyne 3500®, Abott Inc., USA). Blood samples without anticoagulant were centrifuged at 3000 r. p.m. for 20 minutes and sera were separated and evaluated within 2 h of collection. Serum biochemistry analyses including potassium (K), sodium (Na), calcium (Ca), phosphorus (P), magnesium (Mg), albumin, alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST), total biluribin, (TBIL) total protein (TP), creatinine (Cre), blood urea nitrogen (BUN) were measured by an auto analyzer (VETSCAN®, Analyzer, Abaxis Inc, USA). Blood samples were serologically evaluated for Bovine Viral Diarrhea (BVD) virus.

Faecal samples collected within sterile container from all affected and control animals for virological, bacteriological and coccidial examination and cooled as soon as possible. Faecal samples were sent to the Veterinary Control and Research Institute (Etlik-Ankara, Pendik-Istanbul / Turkey) for coronavirus and BVD virus isolation. Bacteriogical cultures and flotation examinations of faecal samples were evaluated at the laboratories of microbiology and parasitolgy of the Faculty of Veterinary Medicine in Bursa, Turkey. Faecal samples were analyzed for the presence of Eimeria oocysts by flotation technique with saturated sodium chloride solution (sp. gr. 1.20) as the flotation medium[9]. For the isolation of Campylobacter species, each faecal sample was plated on Butzler-selective medium (Oxoid) which was supplemented with 5% defibrinated sheep blood. The plates were incubated microaerophilically at 42°C in incubation jars, containing a "Gas Pak" (Campy Gen [AGS]Oxoid, microbiology System) for 48 to 96 h. Campylobacter species were identified by colony morphology, Gram staining, growth at 25°C, susceptibility to nalidixic acid and cephalotin, nitrate reduction, H2S in tirple sugar iron (TSI), and the presence of catalase and oxidase[10].

In addition standart culture methods were used to isolate Salmonella from faecal samples. In enrichment step, approximately 1 g feces added into 10 ml of Tetrathionate broth (Difco) containing 0.2 ml of iodine solution, and the mixture was incubated at 42°C for 18 to 24 hours. After incubation, the sample-broth mix-

ture was inoculated onto Brilliant Green agar with novobiocin (BGN; Becton Dickinson) and Xylose Lysine Tergitol 4 (XLT-4) selective media, and both plates were incubated at 37°C for 18 to 24 hours. At the end of incubation period characteristic colonies were subjected to biochemical tests to confirm Salmonella spp. and the results were interpreted in accordance with the recommendations by Quinn et al. (2000). Fecal samples were also inoculated onto 5 % ovine Blood agar plates (Oxoid), Mac Conkey agar (Oxoid), EMB agar (Oxoid) plates and were incubated at 37°C for 24 hours. Suspected colonies were subjected to biochemical tests and other enteric bacteria, such as Enterobacteriaceae were identified[10]. Faecal samples of cows with diarrhea were diluted with 10% D-MEM medium (Dulbecco's minimum essential medium) containing 10µg/ml pancreatine, 100 IU/ml penicillin, 100mg/ml streptomycin and 10 µg /ml partricine. All samples were centrifuged at 3000 rpm for 30 minutes. Obtained supernatants were filtered through 0.22µm cellulose acetate filters and divided in to cryovials and stored at -80 ºC[11-13]. Coronavirus antigen detection ELISA test kit (Bio-X Coronavirus detectition Elisa Kit) was used according to manufacturers instruction.

Human rectal tumour (HRT-18) cell culture which was grown in Dulbecco's minimal essential medium (D-MEM) combined with 10% foetal calf serum was used for isolation of corona virus. For this purpose, 4 faecal supernatants of 1ml were inoculated on monolayer HRT-18 cell cultures. The monolayers were confluent and incubated at 37 C° with CO_2 for 1 hour and washed three times with D-MEM plus pancreatine (10µg/ml) afterwards added to same medium plus pancreatine for propagation of virus and incubated 7 days at 37 C° with CO_2.

HRT-18 cell cultures were prepared in 24 well plates. Isolated virus strains were diluted 10 fold with D-MEM and 200 µl of virus suspension was taken from every dilution and placed into 4 wells. After 1 hour incubation period, all inoculated cell culture wells were washed three times with D-MEM containing pancreatine, added to same medium and incubated for 7 days. CPE formation was observed every day during incubation period. The amounts of viruses were calculated according to Sperman-Kaerber method.

For identification of isolated virus, serum neutralization tests were carried out in 24 well plates containing monolayer HRT-18 cell cultures. Isolated and titrated 200 µl virus samples were dilueted as 100 $TCID_{50}$/0.1ml and mixed with 200 µl coronavirus hyperimmune serum obtained from rabbits immunized with coronavirus Mebus strain, at 37ºC for 1 hour and at the end of the incubation period virus and serum mixtures were inoculated on to HRT-18 cell cultures. After 1 hour incubation period, all inoculated cell culture wells were washed three times with D-MEM containing pancreatine and added to same medium and incubated for 7 days. CPE formation was observed daily during incubation period.

RNA was extracted from HRT supernatant infected with centrifuged 10% fecal suspensions and control samples (dH_2O and noninfected HRT cell supernatant as negative control and Mebus strain of BcoV as positive control) using EZ-RNA total RNA isolation kit according to procedure recommendations by manufacturer (Biological Industries Co., Israel). The RNA pellet was resuspended with 30 µl RNase free H_2O.

RT-PCR procedure was performed as previously described. 2 µl of downstream primer (50 pmol BCV-2 5´-AGA ATG TCA GCC GGG GTA T-3´) was added to a tube containing, 8µl of RNA sample. The tube was incubated at 95^{O} C for 2 min and then cooled on ice for 5 min. RT-PCR mixture containing 10µl sample, 5µl 5x M-MLV Buffer (250 mM Tris-HCl, pH 8.3, 375 mM potassiumchloride, 15 mM Magnesium chloride, 50mM DTT), 2 mM of each of deoxynucleotides, 20U M-MLV reverse transcriptase (Promega), were used in RT-PCR reaction. Reaction was incubated at 37 ^{O}C for 60 min.

PCR procedure was performed as previously described (Cho and others 2001). PCR mixture containing 5 µl the sample cDNA, 5µl 10xPCR Buffer (100 mM Tris-HCl, pH 8.0, 500 mM potassiumchloride, 0.8%Nonidet P40), 2 mM $MgCl_2$, 2 mM of each of deoxynucleotides, 1U Taq DNA polymerase (Fermantes), 50 pmol of each primers (BCV-1 5´-GCC GAT CAG TCC GAC CAA TC-3´) and (BCV-2 5´-AGA ATG TCA GCC GGG GTA T-3´) were used in PCR reaction. The mixture was preheated for 4 min at 94 ^{O}C, subjected to 37 cycles of 1 min at 94 ^{O}C, 1 min at 55 ^{O}C and 2 min at 72 ^{O}C. A final extension step was performed 7 min at 72 ^{O}C. The amplified PCR products were stained with etidium bromide and visualized under ultraviolet light on 1.5% agarose gel. (Figure 1)

The data were expressed as mean ± SE. The significance between the mean values was determined by Student's t-test. For all comparisons, values of $P < 0.05$ were considered significant.

Results

Clinical findings observed in cows suffering from WD were characterized by a sudden onset of watery diarrhea and profound decrease in milk production (40%). Faeces of 10 cows was bloody and was containing mucus. In addition, these cows were severely depressed and moderately dehydrated, mild pale mucosal membranes, tachycardia (range: 92 – 104 beats/ minute, reference range: 60-80 beats/minute), high respiratory rate (range:40- 52 breaths/ minute; reference range: 10- 30 / breaths/minute), normal body temperature (37.8- 38.9 C; reference range: 37.8 -39.2 C), and decreased ruminal motility (range: 3 – 6/ 5 minutes; reference range: 8-12/5 minutes) were other clinical findings observed. Faeces of other two cows were dark green, watery and homogenous with little odour. In these cows, body temperatures (range: 38.2-39.1 C), respiratory rates (range: 24-28 breaths/minute) pulse rates (range: 72-80 beats/ minute) and ruminal motility (range: 8-12/ 5 minutes) were within normal limits. In addition, all animals suffering from WD had serous nasolacriminal discharge and mild cough. Clinical diagnosis of WD was made according to clinical examination.

Table 1. Results of bacterial culture, virological and coccidial isolation in cows with winter dysentery

Tablo 1. Kış dizanterili sığırlarda bakteri kültürü, virolojik ve coccidia izolasyon sonuçları

Number of Cattle	Bacteriologic culture	Virus isolation	Coccidial isolation
1	Campylobacter spp	BcoV	negative
2	negative	negative	negative
3	Campylobacter spp	negative	negative
4	negative	negative	negative
5	Campylobacter spp	BcoV	negative
6	negative	negative	negative
7	negative	BcoV	negative
8	negative	negative	negative
9	negative	negative	negative
10	negative	negative	negative
11	negative	negative	negative
12	Campylobacter spp	negative	negative

Compared to healthy cattle, haematological results including hematocrit rates, erythrocyte counts, haemoglobin concetrations, serum urea, creatinine and total protein concentrations ($p<0.05$) were higher in cattle with winter dysentery. In addition, serum urea, creatinine and total protein concentrations were higher ($p<0.05$) in cattle suffering from WD (Table 2). No Eimeria oocysts were detected in faecal samples of examined animals.

Table 2. Mean (± SE) biochemical and haematological findings in cows suffering from Winter Dysentery (WD)

Tablo 2. Kış dizanterili sığırlarda biyokimyasal ve hematolojik bulgular

Parameters	WD cows (n=12)	Healthy Cows (n=12)
Leukocyte (x 10^3/l)	7.6 ± 1.2	6.9 ± 0.9
Neutrophile (x 10^3/l)	2.9 ± 0.8	3.3 ± 0.3
Lymphocyte (x 10^3/l)	3.8 ± 0.3	3.9 ± 0.2
Eosinophile (x 10^3/l)	0.6 ± 0.2	0.7 ± 0.4
Erythrocyte (x 10^6 /l)	7.4 ± 0.7^a	5.7 ± 0.3^{b*}
Hemoglobin (g/dl)	14.4 ± 2.1^a	10.7 ± 1.3^{b*}
Hemotocrite (%)	42.3 ± 5.6^a	33.4 ± 4.1^{b*}
Urea (mg/dl)	52.4 ± 2.1^a	29.7 ± 3.4^{b*}
Creatinine (mg/dl)	2.1 ± 0.3^a	1.4 ± 0.2^{b*}
AST (IU/L)	124.5 ± 13.1	121 ± 10.2
CK (IU/L)	158.3 ± 12.2	166.2 ± 7.4
Total protein (g/dl)	7.9 ± 0.6^a	7.1 ± 0.4^{b*}
Ca (mg/dl)	9.1 ± 0.4	9.4± 0.7
P (mg/dl)	6.8 ± 0.3	6.6 ± 0.2
K (mEq/L)	5. 1 ± 0.4	4.9 ± 0.7

* Differences between the values on the same row are found to be statistically significant ($p<0.05$)

BcoV was isolated from 3 faecal samples collected from the affected animals and seen CPE focuses at the first passages on HRT cells consisting of syncytial formation and lysis of the infected monolayers and identified by SNT and PCR. The titers of BcoV were found as $TCID_{50}$ $10^{4.5}$/ml, $10^{4.5}$ /ml and $10^{5.0}$ /ml respectively (Figure 1).

A total of 12 faecal samples for bacterial culture were collected from affected cattle and Campylobacter spp. were isolated from 4 of 12 cows suffering from WD (Table-1).

Ten cattle with dehydration were treated with the intravenous administration of 10 to 15 liters of Lactated Ringers solution and 5% Dextrose (Eczacıbası Inc., Istanbul, Turkey) as far as dehydration findings were eliminated. Bread yeast was given orally to all affected cows at a

dosage of 1 gr/kg once a day for 5 days. In addition, levamisole (i.m. 2.5 mg/kg, once a day for 3 days, Actipar®, Alke Inc., Istanbul, Turkey), tylosine (10 mg/kg, i.m, qh12 for 5 days; Tylan®, Lilly-Elanco Inc., Istanbul, Turkey) were administered to all animals with diarrhea.

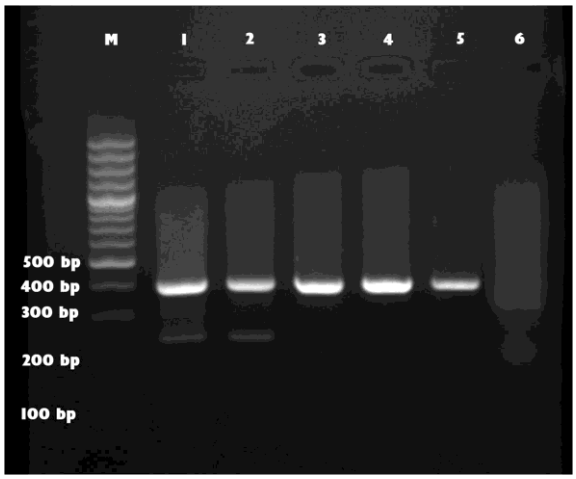

Figure 1. *Agorose jel electrophoresis of PCR products of BcoV field strains*

Şekil 1. *Bco V saha suşunun Agoroz jel eloktroforezi*

M; 100 bp DNA ladder. Line 1; Bovine Coronavirus DNA prepared from infected cell culture fluid of Mebus strain of BcoV Lane 2-5 Bovine Coronavirus DNA prepared from infected cell culture fluid of the sample;, Lane 6; DNA from non-infected HRT cell supernatant

Discussion

Winter Dysentery is a disease affecting mostly household, adult milking cows in postpartum period at winter times. However, cases in the present study were identified in April. Although low mortality rates, disease causes serious economical impact over dairy herds because of loss of body condition and milk yield[1]. Likewise, in the cases presented, substantial decrease of milk yield was observed throughout disease and for up to 6 weeks following recovery.

Winter dysentery is characterized by an acute onset of watery, sometimes blood containing diarrhea and 25-95% production loss. Diarrhea could be accompanied or preceded by nasolacrimal discharge and/or cough. Other possible signs include mild colic, dehydration, depression, a brief period of anorexia and decrease in body condition at certain level[14,1]. Similarly, presented cases show acute onset of watery diarrhea, and a marked decrease in milk production. Furthermore, ten cows had severe watery diarrhea containing blood and mucus and also cows were dehydrated and depressed. Respiratory findings including mild cough and serous nasolacrimal discharge were observed in all cows suffering from WD. It is notified that Bovine coronavirus, which is accepted as the primary etiologic agent of Winter Dysentery, can cause infection of respiratory epithelium in cattle[15,13,16,17]. Therefore, both enteric and respiratory findings could be observed in WD as detected in the cases presented.

Disease was believed to be caused by Campylobacter fetus var. jejuni but incompatibility to isolate bacterium from all cases similar to winter dysentery and inability of bacterium to cause clinical signs similar to WD in experimental infections suggests that bacterium could be secondary pathogen in the course of disease[14,1]. In study presented, a total of 12 faeces samples were collected from the affected cattle for bacterial culture. However Campylobacter spp. was isolated only from 4 of the cows with WD. In addition, a local BcoV strain was isolated from 3 faecal samples collected from same animals. Saif et al. (2008) reported that feces of the 90% of the animals from an outbreak of winter dysentry were BCoV positive[23]. Results of faeces of other animals for bacterial examination were negative. These results indicate that BcoV is probably the primary pathogen of WD however Campylobacter spp. may only contribute to pathogenesis of the disease as a secondary pathogen.

Unlike neonatal diarrheic calves[18-21], there are few reports, associated with intestinal coranavirus infection in adult cows[5,6,22-24]. A bovine coronavirus (BCoV) closely related to the virus that causes diarrhea in neonatal calves, has been implicated as the etiologic agent of WD[4,11,19,15,20]. Evidence for Coronavirus as an etiologic agent of WD is the ability of virus to induce disease similar to winter dysentery, affected cattle seroconvert to BCV and virus and thus virus particles could be isolated from faces of affected cattle[25,13].

Other causes of acute diarrhea, such as BVD, salmonellosis, coccidiosis, always should be considered in clinical diagnosis[14,26,1,27]. Although there are major differences between clinical course of WD and diseases mentioned above all cows were evaluated for BVD, salmonellosis and coccidiosis, eventually results of

examinations of 12 faeces samples for BVD, salmonella spp and eimeria spp. were negative.

None of the results of haematology are pathognomic for winter dysentery. In the present study, serum urea and creatinine concentrations, indicators of glomerular filtration, were mildly high in cattle with dysentery, probably due to dehydration. Similarly, high hematocrit rates, total protein levels, erythrocyte counts and haemoglobin concentrations may be a result of dehydration.

WD has a high morbidity but low mortality rates and features of faeces return to normal in 2-3 days in most animals affected by WD. Treatment for WD is based on providing basic medical support to affected animals[14,28,1]. Clinical improvement was observed in all the animals treated with bread yeast, levamisole, tylosin, lactated Ringers solution and 5% dextrose solution at third day and full recovery of all animals was observed at the end of fifth day of treatment.

Results of this study represent the first local coronavirus from a cattle herd with winter dysentery in Turkey. Winter Dysentery needs to be considered in the differential diagnosis of cattle with clinical signs such as severe watery diarrhea with blood and mucus, dehydration, depression nasolacrimal discharge or cough fever. As etiologic agents in these cases should be taken into consideration, and in preventive measures against winter dysentery, Coronavirus along with Campylobacter spp should be taken into consideration. On the other hand eight negative results of cattle with symptoms of WD indicates that ethiology of the disease is stil not clearly elucidated and different pathogens other than BCoV and Campylobacter could also be involved in pathogenesis of the disease

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