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The investigation of the presence of bovine herpesvirus type 4 (BoHV-4) in cows with metritis in a dairy herd

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Summary: To investigate the occurrence of bovine herpesvirus type 4 (BoHV-4) infection in a dairy herd including cows with metritis, various methods were used for the diagnosis BoHV-4 in vaginal discharge samples. Blood serum samples were obtained from 368 cows for serodiagnosis of BoHV-4 infection. Of 368 serum samples, 256 (69.6%) were found positive for BoHV-4 specific antibodies. For the virological study, vaginal discharge samples of 55 cows with metritis were used and tested for BoHV-4 using polymerase chain reaction (PCR), virus isolation and immunofluorescence techniques. Twenty-nine percent (16/55) of the vaginal discharge samples obtained from cows with metritis were found positive for BoHV-4 DNA by PCR. In this study, the presence of BoHV-4 was determined in cows with metritis in post-partum period in a dairy herd.

Key words: Antibody, bovine herpesvirus type 4, metritis, polymerase chain reaction, sequence analysis.

Bir süt sığırcılığı işletmesinde metritisli ineklerde BoHV-4 ün varlığının araştırılması

Özet: Metritis olgularının gözlendiği bir sütçü sığır sürüsünde, örneklenen vajinal svaplarda farklı tanı yöntemleriyle BoHV-4 infeksiyonunun varlığı araştırıldı. BoHV-4 ün serolojik tanısı amacıyla 169 adedi metritis semptomları gösteren, 199 adedi ise metritisli hayvanlarla birlikte barındırılan sağlıklı görünümlü hayvanlar olmak üzere toplam 368 adet sığırdan kan serum örneği alındı. Virolojik kontrol amacıyla, metritisli 55 adet inekten alınan vajinal svap örnekleri polimeraz zincir reaksiyonu, virus izolasyonu ve immunfloresan tekniği ile BoHV-4 yönünden araştırıldı. 368 serum örneğinin 256 adedi (%69.6) BoHV-4 spesifik antikorları yönünden pozitif bulundu. Seropozitiflik oranları, metritisli ve metritisli hayvanlarla birlikte barındırılan sağlıklı görünümlü hayvanlarda sırasıyla, %69.8 ve %69 olarak tespit edildi. Metritisli hayvanlardan alınan vajinal svap örneklerinin %29 (16/55)'unun BoHV-4 DNA'sı yönünden PCR tekniği ile pozitif olduğu saptandı. Bu çalışmada, bir süt sığırcılığı işletmesinde bulunan doğum sonrası dönemdeki metritis problemli ineklerde BoHV-4 ün varlığı belirlenmiştir.

Anahtar sözcükler: Antikor, bovine herpesvirus tip 4, dizin analizi, metritis, polimeraz zincir reaksiyonu.

Introduction

Bovine herpesvirus type 4 (BoHV-4) belongs to the family *Herpesviridae*, subfamily *Gammaherpesviridae* and species *Rhadinovirus*. BoHV-4 has no close biological and virological relationship to other known herpes viruses of the family *Bovidae* (1, 16).

The virus has been identified in the respiratory tracts of infected animals in cases of vulvovaginitis, endometritis, mastitis, abortion and also from apparently healthy cattle (2, 4, 7, 11, 12, 19). The role of BoHV-4 in infections occured the respiratory and genital tract has been studied by several researchers and the virus has been reported to be responsible for post-partum and chronic metritis problems alone or with other pathogens (12, 18). Like other herpesviruses, BoHV-4 have been persistently infected cattle and latent virus can be reactivated by glycocorticoid treatment and other stress factors (5). The latency of BoHV-4 can establish in lymphoid tissues and a prolonged viremia associated with the mononuclear cells (15). Recently, BoHV-4 were isolated from three vaginal discharge from cows with post-partum metritis where some cows had BoHV-4 spesific neutralizing antibodies (14). This persistent infection is also characterized by constant presence of circulating antibodies. The antibodies against herpesviruses are not fully protective against reinfections (15). Unknown natural stimuli are also able to induce reactivation since infectious virus infrequently recovered from latently infected animals (5). One natural stimulus could be parturation. Cows infected at various stages of gestation showed post-partum metritis (18). Post-partum metritis is often associated with a prolonged excretion of BoHV-4 for several weeks to months (12, 18).

In the present study, it is aimed to investigate the presence of BoHV-4 infection in cows with metritis in post-partum period. In addition, the diagnostic values of

different techniques used for the BoHV-4 detection in vaginal discharge samples was compared.

Materials and Methods

Herd history and clinical materials: In a dairy herd including 800 milking cows, located in the Marmara district of Turkey, an increase in the post-partum metritis, despite the antibiotic treatment was observed. A combined conventional vaccine including BoHV-1, Bovine Viral Diarrhea Virus (BVDV), Parainfluenza Virus 3 (PIV-3), Bovine Respiratory Syncitial Virus (BRSV) and Haemophilus somnus (Virashild somnus, Novartis, USA) had been used in this dairy herd for 7 years.

Blood serum samples were obtained from 368 cows for serodiagnosis of BoHV-4 infection with enzyme-linked immunosorbent assay (ELISA). Of these blood samples 169 were collected from cows showing symptoms of post-partum metritis, 199 of them were collected from clinically healthy cows that were housed together with the cows with post-partum metritis. For the virological study, vaginal discharges samples from 55 cows with metritis were used and tested for BoHV-4 using PCR, VI and IFA techniques.

Blood samples were collected directly into blood tubes with silicon and centrifuged at 1500 g for 10 minutes to separate the serum which was then stored at -20°C until use. Vaginal discharge samples were vortexed and then centrifuged at 1500 g for 10 minutes. The samples were filtrated and then stored at -80°C.

Cell culture and virus: Bovine turbinatae (BT) cell culture was used for propagation of BoHV-4 DN-599 strain, VI and direct IFA techniques. BT cells were grown in Dulbecco's Minimal Essential medium (DMEM) (Gibco, Gibco Laboratories, USA) supplemented with 5% foetal calf serum (Gibco, Gibco Laboratories, USA), and antibiotics (100 Units Penicilline-G and 0.1 mg dihydro-streptomycin/ml). BoHV-4 DN-599 strain was used as control viruses for PCR and IFA techniques. Reference virus strain was kindly provided by Dr.G.J.Wellenberg (Lelystad, Netherlands).

Indirect ELISA: A commercial indirect ELISA (BioK 066, BioX, Belgium) used for the detection of antibodies against the BoHV-4 was carried out according to the guidelines of the manufacturer.

VI technique: Vaginal discharge samples were inoculated (100 µl) onto the BT monolayer. The monolayers were incubated at 37^{o}C in an atmosphere with 5%CO_2 and controlled daily for appearence of cytopathic effect (CPE). The monolayers were passaged at weekly intervals for a total of three passages. The supernatants of all cell culture were examined for BoHV-4 virus by direct immunofluorescence technique.

IFA technique: IFA technique was applied as described by Nettleton et al (13). Briefly, the supernatants of cell cultures were individually inoculated onto the BT monolayer cultures grown in 24 well plates. At the end of 3rd day of incubation, the cell cultures were fixed with formol for 10 minutes and stained by using fluorescein-isothiocyanate (FITC) conjugate (Bio028, BioX, Belqium). The results were read by an immunofluorescence microscope (Nikon, Japan). BoHV-4 strain DN-599 and non-inoculated BT cells served as positive and negative controls, respectively.

DNA extraction and PCR technique: DNA extractions from vaginal discharge samples were carried out described by Sambrook et al. (17). For detection of BoHV-4 genome a gB specific PCR as describe elsewhere (8) was used. The primer pair (B1:5'-CCCTTCTTTACCACCACCTACA3') and (B2:5'-TGCCATAGCAGAGAAAC AATGA-3') produced a 615 bp fragment.

The detection of BoHV-4 DNA was performed with the use of the BoHV-4 gB as described by Wellenberg et al (20) with some modifications. Briefly, 3µl of DNA was subjected to thermocycling in a 30µl reaction mixture. The reaction mix contained 2.5U Taq Polymerase, 3.5mM dNTP mix, each primers 10 pmol, 1.5 mM $MgCl_2$, 1X PCR buffer and 6% DMSO. Thermal cycling conditions were 6 min at 96^{o}C followed by 40 cycles of 56^{o}C 45 sec,72^{o}C 2 min, and 95^{o}C 1 min, followed by a final 10 min extension at 72^{o}C for both primer sets. From the final reaction, 3-5 µl the amplified PCR products was analyzed by 1% agarose gel containing ethidium bromide. The gels were read for specific size bands by UV transillumination.

Sequence analysis: The amplified gB PCR products were purified with a kit (Roche, Germany). Nucleotide sequences of the amplified products were detected with in Beckman Coulter CEQ8000 Genetic Analysis system. The sequence data were then subjected to multiple alignment and phylogenetic analysis using MegAlign of the DNAStar Lasergene v7.0 software. The sequence comparisons were obtained for each isolate versus that of BoHV-4 gB segment sequence obtained from the GenBank.

Results

ELISA: Out of 368 blood sera tested, 256 (69.6%) showed BoHV-4 antibody by ELISA. Seventy percent of samples (118/169) obtained from cows with post-partum metritis, and 69% of the samples (138/199) from clinically healthy cattle housed together with the cows with post-partum metritis had antibody against BoHV-4. Serological results of these cows positive to BoHV-4 were also given by Bilge-Dağalp et al (3).

VI and IFA technique: While BoHV-4 has been isolated from 7 (12.7%) of 55 vaginal discharge samples, 21.8% (12/55) of tested samples were found positive by

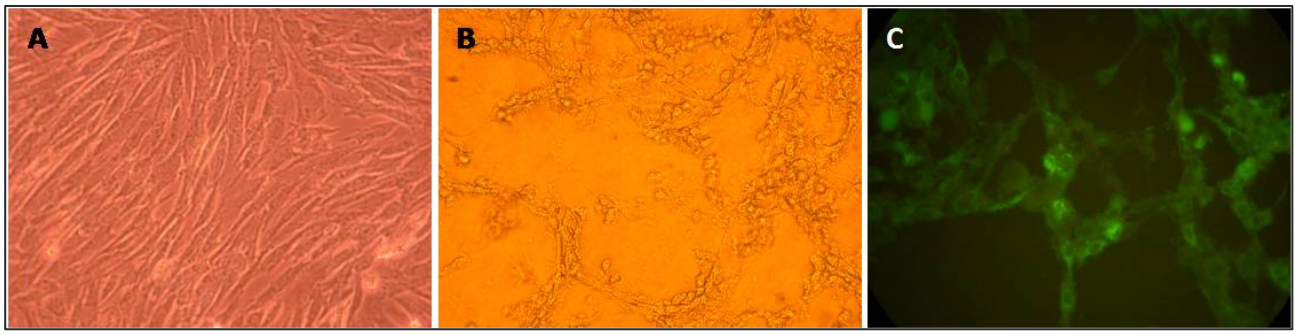
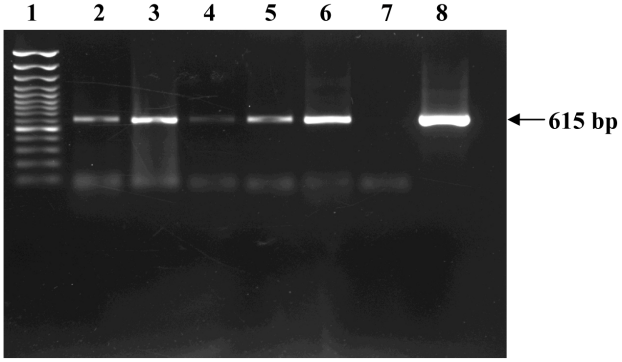


Figure 1. A. Cell control (Bovine Turbinata) X 400, B. The morphological changes of the field isolate K.339 in cell culture (2th passage) X 400 (4th day), C. The field isolate K.339 in immunofluorescent test X 800.
Şekil 1. A. Hücre kontrol (Bovine turbinate) X 400, B. Saha izolatı K.339 un (2. pasaj aşamasında) hücre kültüründe oluşturduğu morfolojik değişimler (CPE) X400 (4. gün), C. Saha izolatı K.339 un immunfluoresan testi görüntüsü X800.

IFA technique (Table 1). CPE induced by a BoHV-4 field strains on BT cell line were shown in Figure 1.B. The isolates were identified by PCR and IFA technique. The appearance of BoHV-4 field strains using IFA technique was also given in Figure 1.C.

Table 1. The individual data of animals detected positive for BoHV-4 using PCR
Tablo 1. PCR ile BoHV-4 pozitif olarak saptanan hayvanların bireysel verileri

Animal no	Age	BoHV-4 Ab	gB PCR	VI	IFA
K67	4	-	+	+	+
K68	2	+	+	-	-
K72	3	+	+	+	+
K73	4	-	+	-	-
K74	8	+	+	-	-
K2	6	+	+	-	-
K8	2	+	+	-	+
K15	6	+	+	-	+
K20	5	-	+	+	+
K22	2	+	+	+	+
K24	2	+	+	+	+
K255	3	+	+	-	+
K288	3	+	+	-	+
K339	2	+	+	+	+
K79	2	+	+	-	+
K40	4	+	+	+	+
Total		13	16	7	12



Line 1. 100 bp DNA ladder
Lines 2-6. Positive samples
Line 7. Negative sample
Line 8. DN-599 (BoHV-4 reference strain)

Figure 2. The result of BoHV-4 gB PCR in vaginal discharge samples.
Şekil 2. Vajinal svap örneklerinin BoHV-4 gB PCR sonuçları.

PCR: The amplification of BoHV-4 specific 615 bp fragment from DNA of test samples and positive control virus BoHV-4 DN-599 were described as positive reaction. Out of vaginal discharge samples 29.1% (16/55) were found to be positive for BoHV-4 (Table 1).

Sequence and phylogenetic analysis: K-339 virus isolate was amplified with primer pairs designed for a 615 bp region of the B gene (Figure 2). The sequences of isolates K-339 is available in Genbank, accession number EU055543. The sequences compared in this paper have

Table 2. An identity/percentage comparison between an isolate (K.339) sequence and published BHV-4 sequences
Tablo 2. BoHV-4 K-339 izolatının GenBankasında yeralan diğer dizinlerinin karşılaştırılması

	K339	AJ617687	AJ609274	AJ617688	Z15044	AF318573	DN599
K339	ID	0,994	0,994	0,994	0,992	0,992	0,990
AJ617687	0,994	ID	1,000	1,000	0,998	0,998	0,996
AJ609274	0,994	1,000	ID	1,000	0,998	0,998	0,996
AJ617688	0,994	1,000	1,000	ID	0,998	0,998	0,996
Z15044	0,992	0,998	0,998	0,998	ID	1,000	0,998
AF318573	0,992	0,998	0,998	0,998	1,000	ID	0,998
DN599	0,990	0,996	0,996	0,996	0,998	0,998	ID

been submitted to GenBank, with accession numbers AJ617688 (UK), AJ609274 (UK), AJ617687 (UK), AF318573 (Mainland Europe), Z15044 (Mainland Europe) and DN-599 (The reference strain of BoHV-4) as indicated in Table 2, Figure 3.

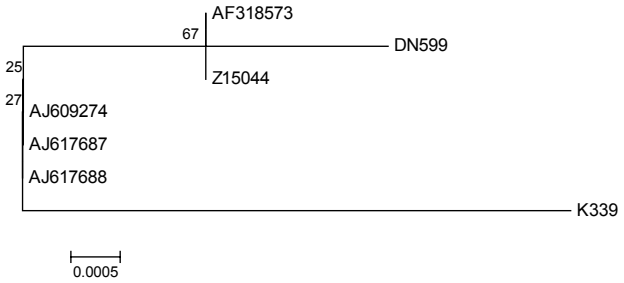


Figure 3. Neighbour Joining analysis of a Turkish BoHV-4 isolate (K .339)
Şekil 3. Türkiye'den izole edilen BoHV-4 saha suşunun (K-339) filogenetik analizi

Discussion and Conclusion

In this study, 29.1% of the vaginal discharge samples from cows with postpartum metritis were found to be BoHV-4 DNA-positive. The rate of positivity in tested vaginal discharges was highest for gB-PCR (29.1%), which was followed by IFA (21.8%) and VI techniques (12.7%). Overall seroprevalence of BoHV-4 is estimated as 69.6%. Of cattle with metritis and healthy animals housed together with the cattle with metritis in the postpartum period, approximately 70 percent were seropositive for antibodies versus BoHV-4. Although the prevalence of antibodies to BoHV-4 in healthy animals is generally high, the evidence of BoHV-4 related clinical signs have not been detected. These antibodies in healthy cattle were concluded to be formed before the time of sampling. However, due to the highest rates of seropositive animals BoHV-4 infection have been circulated subclinically in this herd at a relatively high level.

The pathogenesis of BoHV-4 infection was questioned due to the isolation of BoHV-4 from healthy individuals, and from cattle with a wide variety of clinical signs (12, 19). The virus has been isolated from cattle with genital problems (metritis, mastitis) as well as healthy animals. Several studies established a strong relationship between BoHV-4 antibody and fertility problems encountered (4,12, 18). In contrast to studies reported by Fabian et al (6), Frazier et al (7) and Monge et al (12) reported that BoHV-4-related post-partum metritis cases are an important problem in the United States and Spain, respectively (7). In this study were pointed that there is an interaction between the BoHV-4 infection and postpartum metritis. However, it should be remembered that other factors (viruses, bacteria, etc.) that might have influences on the high rate of metritis cases. In this study, we mainly planned to investigate the presence of BoHV-4 in cattle with metritis. BVDV was not investigated in vaginal discharge samples, because, cattle has been vaccinated with a combined conventional vaccine for along time. But, we tested all vaginal discharge samples for BoHV-1, because the sampled animals might be latently infected or reinfected in spite of vaccination for BoHV-1 in this herd. No positive samples for BoHV-1 were detected using PCR and IFA techniques (unpublished data).

Virological examination of BoHV-4 infection was done using three different techniques. Positivity rates of these techniques, namely gB PCR, VI and IFA were 29.1%, 12.7% and 21.8%, respectively. It is noted that all of the samples detected as positive with either VI or IFA for BoHV-4 were also positive with gB PCR. Similarly, Wellenberg et al (20) reported that the gB primer was sensitive enough to detect only 2 to 10 BoHV-4 DNA molecules in a sample and that the sensitivity of PCR method with gB primers and virus isolation were estimated as 93% and 61%, respectively. It is known that the gB primer has been used because of the conserved nature of this gene (20). In addition the gB is essential in the BoHV-4 infection (9) and constitutes an important component of the virion particle (10). Overall thirteen of 16 animals infected with BoHV-4 were seropositive, only 3 animals were found seronegative (Table 2). These cows were not re-examined for seroconversion. Our opinion is that, these cows (n=3) sampled after first calving had primary infection. Otherwise, it is possible that the seropositivity detected in the others is associated with the reactivation of latent infection because of their age and lasting circulation of the infection.

The nucleotide sequence of a field isolate (K-339) had 99% percent nucleotide identity with sequence of 5 various BoHV-4 isolates (accession numbers AJ617688, Z15044, AJ609274, AJ617687, AF318573) identified as European strain like Movar 33, in GenBank and DN-599 of which the sequence analysis was performed in this study (Table 2). The Neighbourhood Joining analysis indicated that isolate K-339 is genetically more close to the viruses reported from UK rather than those from mainland Europe (Figure 3). The identity matrix of the sequences between Turkish and UK isolates was recorded as 99.4%. On the other hand, DN-599 (e.g reference strain of BoHV-4) was found in a separate cluster with two BoHV-4 viruses from mainland Europe.

Data shown that BoHV-4 is common and one of the possible aetiological agent causes the metritis in sampled animals in the post partum period. In this study, the molecular characterization of BoHV-4 has reported for the first time in Turkey. According to the results, the Turkish BoHV-4 isolates are closely related to strains from UK, but relatively different from mainland Europe. Further studies need to be planned based on the

investigation of other agents causing metritis, and also BoHV-4 and the isolation and molecular characterisation of the BoHV-4 field strains from cows with clinical outcome except metritis.

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