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RESEARCH ARTICLE

Detection and Phylogenetic Analysis of the Tegument Protein Gene of Malignant Catarrhal Fever Virus from Clinical Cases of Cattle and Sheep in the Central Balkan Region

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ABSTRACT

This study aimed to describe the clinical course of SA-MCF and the duration of viremia in surviving cattle and to perform the phylogenetic analysis of the tegument protein gene of OvHV-2 in cattle in the Central Balkan. A farm housing two heifers and 7 sheep with a confirmed SA-MCF case were selected for the investigation. For the estimation of the length of viremia and the virus shedding, the animals were sampled repeatedly, weekly for two months. For the phylogenetic analysis, a retrospective study was performed on 21 samples from cattle, and 7 samples from sheep, from the Central Balkan. In the blood samples of the survived heifer, the OvHV-2 genome was detected until week 7, in corneal swabs, the OvHV-2 genome was detected until week 6, and in nasal swabs until week two of the study. A retrospective study revealed that out of 21 tested cattle, OvHV–2 was detected in 15 (71.4%), and out of 7 tested sheep, three (42.9%) were positive. The sequenced samples show the highest percentage of similarity with the strains from Brasil KJ658293.1 (100%) and Germany HM216475.1 (100%).. Since there is a variety of different clinical signs similar to other notifiable diseases such as BVD, IBR, and FMD, there is a clear benefit in including SA-MCF in the differential diagnosis in cattle. As mixed farming in the Central Balkan is practiced, implementing SA-MCF monitoring in passive surveillance would allow a better understanding of the disease, ascertaining its prevalence and could provide new information regarding SA-MCF epidemiology.

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INTRODUCTION

Malignant catarrhal fever (MCF) is a fatal disease in cattle and other even-toed ungulates (Mlilo *et al.*, 2015). The disease is caused by a gammaherpesvirus of the family *Herpesviridae,* genus *Macavirus.* The International Committee for the taxonomy of viruses recognizes 9 different member species of the family *Macavirus* of which 5 cause clinical diseases known as MCF: Alcelaphine gammaherpesvirus 1 (AlHV–1), Alcelaphine gammaher-pesvirus 2 (AlHV–2), caprine gammaherpesvirus 2 (CpHV-2), Hippotraginae gammaherpesvirus 1 (HiHV-1) and ovine gammaherpesvirus 2 (OvHV-2) ("ICTV," 2022; Russell *et al.*, 2009a). The two most studied forms of the disease occurring in cattle are wildebeest–associated malignant catarrhal fever (WA–MCF) caused by AlHV-1, present mainly in susceptible animals in Africa and zoos and sheep-associated malignant catarrhal fever (SA–MCF) caused by OvHV–2 occurring in cattle worldwide (Li *et al.*, 2014; Russell *et al.*, 2009a). These two viruses are highly similar, with 72 genes in total (Hart *et al.*, 2007), of which 50 genes approximately are conserved among all gammaherpesviruses (Russell *et al.*, 2014). Around ten unique genes that characterize viruses that cause MCF have been described (Russell *et al.*, 2009b, 2013). Even though the viruses are highly conserved, different novel genes have been discovered, present in some MCF viruses, but not in others, such as Ov 2.5, Ov 8.5, and Ov 9.5 in OvHV-2 or A 9.5 in AlHV-1 (Russell *et al.*, 2013, 2014). SA–MCF in cattle is a sporadic disease mainly transmitted by housing

sheep and cattle together, with sheep being the natural host of the virus and shedding it through nasal and ocular discharges, but without exhibiting any clinical signs (Russell *et al*., 2009a). Adolescent sheep shed the virus intermittently and represent the main source of infection for other susceptible species (Li *et al*., 2004). The length of the incubation period varies from 2 weeks up to 9 months depending on the infection dose and the exposure period (Gailbreath *et al*., 2010). Cattle are considered dead-end hosts for the virus. Disease signs and severity are influenced by species, with more susceptible animals (deer, bison) having a much shorter and more acute disease course, while cattle appear to show a longer disease course and have more obvious symptoms (Klaus Osterrieder, 2017; Russell *et al*., 2009b). The most typical form in cattle is the so-called head and eye form, with signs including fever, depression, mucopurulent ocular, and nasal discharge, corneal opacity, lymphadenopathy, diarrhea and death consequently (Russell *et al*., 2009a). The disease is spread worldwide and the economic impact of the disease varies greatly, depending on geographical location, housing and breed. Though SA-MCF in European breeds of cattle appears to be a low–morbidity disease, occurring at erratic intervals (Li *et al*., 2014), there are no published data regarding SA-MCF infection in Serbia, besides a case report (Spasojevic *et al*., 2008) and only a handful of published papers in the region. Thus the main aims of this study were to describe the clinical course of the disease and the duration of viremia in surviving cattle and to perform the phylogenetic analysis of the tegument protein gene of OvHV-2 in the population of cattle in the Central Balkan region.

MATERIALS AND METHODS

A farm housing two heifers and seven sheep with a confirmed SA-MCF case was selected for the investigation of the disease. MCF was confirmed as a part of a differential diagnostic panel for foot and mouth disease (FMD), bluetongue (BT), and infectious bovine rhinotracheitis (IBR). One heifer was reported to have clinical signs including fever, anorexia, mucopurulent discharge from the eye, erosion on the nasal plane, seromucous discharge from the nose, and lymphadenopathy with intense salivation. The before mentioned heifer survived the infection and, based on the reported clinical signs, symptomatic treatment included drugs based on glucocorticosteroids (dexamethasone), wide-spectrum antibiotics (tetracyclines), drugs based on calcium, and drugs based on glucose and saline solution. The diseased heifer had been in cohabitation with seven sheep and one other susceptible ruminant in which no signs were reported. For the estimation of the length of viremia and the virus shedding, the animals were sampled repeatedly, weekly for two months.

Samples included corneal and nasal swabs and whole blood samples. Sheep were sampled twice, at the beginning and the end of the study.

For the purpose of the phylogenetic analysis, we performed a retrospective study on 21 samples of cattle, and seven sheep samples originating from different geographical locations in Serbia, the entity of the Republic of Srpska within Bosnia and Herzegovina and Montenegro (Fig. 1) collected from 2015 to 2022 and stored at sample

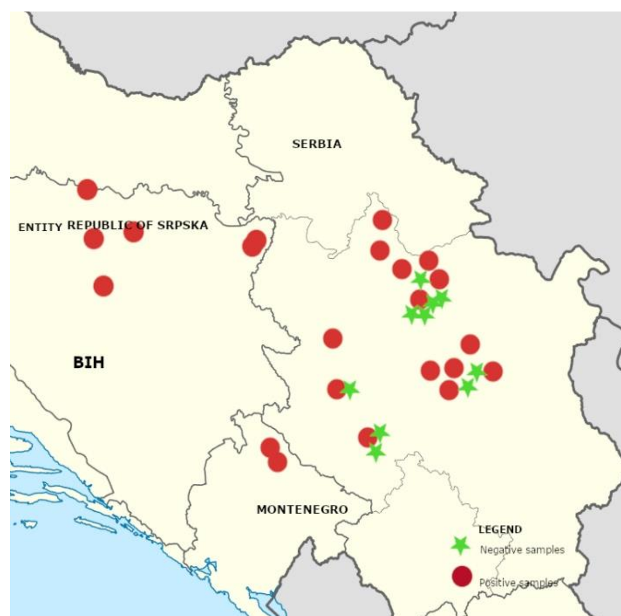

Fig. 1: Map of the Central Balkan region with negative and positive samples Legend: ★ Negative samples; ● Positive samples.

bank at Institute of Veterinary Medicine of Serbia, Belgrade. The samples were submitted for the detection of BT disease.

Corneal swabs and nasal swabs were immersed in 1 ml of PBS, vortexed, and centrifuged at 4.000 rpm for 10 minutes. The obtained supernatants and whole blood samples were used for the extraction of the DNA using a commercial IndiSpin Pathogen Kit (Indical, Germany), following the manufacturer`s instructions For the detection of the MCF genome, polymerase chain reaction (PCR) using primers (Baxter *et al*., 1993) for amplification of the tegument protein gene within ORF75, was performed. The reaction mixture included 11 µl of DreamTaq HotStart Master Mix (DreamTaq Hot Start PCR Master Mix (2x), Thermo Scientific, USA), 1.1 µl of each 10 mM primers, 2 µl of DNA template, and the rest was supplemented with PCR pure water up to 20 µl. The temperature profile included initial denaturation for 15 min at 95°C, followed by 34 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, followed by a final extension at 72°C for 7 min. The amplified PCR products (422 bp) were visualized by electrophoresis on 1.5 % agarose gel stained by ethidium bromide. PCR products were purified using GeneJET PCR Purification Kit (ThermoFisher Scientific, USA) and sequenced at LGC, Biosearch Technologies, Germany by the Sanger sequencing method. The consensus sequences were obtained using the Staden package 2003, and the phylogenetic tree was constructed in Mega X software using the Neighbour-Joining method and the Maximum Composite Likelihood model, with the bootstrap resampling at 1000 replicates, with uniform rates among sites (Tamura *et al*., 2004; Kumar *et al*., 2018).

RESULTS

Concerning the diseased heifer, the OvHV-2 genome was detected in blood samples until week seven of the study, whereas in corneal swabs the OvHV-2 genome was

Table 1: Results of the part of the study regarding the length of the viremia in the infected animals, and the length of the virus secretion from the infected heifer.

Week	Sample type	Species and Results								
		Cow	Heifer	Sheep 1	Sheep 2	Sheep 3	Sheep 4	Sheep 5	Sheep 6	Sheep 7
1	Nose swab	-	+	nt	nt	nt	nt	nt	nt	nt
	Corneal swab	-	+	nt	nt	nt	nt	nt	nt	nt
	Full blood	-	+	+	+	+	-	-	-	-
2	Nose swab	-	+	nt	nt	nt	nt	nt	nt	nt
	Corneal swab	-	+	nt	nt	nt	nt	nt	nt	nt
	Full blood	-	+	nt	nt	nt	nt	nt	nt	nt
3	Nose swab	-	-	nt	nt	nt	nt	nt	nt	nt
	Corneal swab	-	+	nt	nt	nt	nt	nt	nt	nt
	Full blood	-	+	nt	nt	nt	nt	nt	nt	nt
4	Nose swab	-	-	nt	nt	nt	nt	nt	nt	nt
	Corneal swab	-	+	nt	nt	nt	nt	nt	nt	nt
	Full blood	-	+	nt	nt	nt	nt	nt	nt	nt
5	Nose swab	-	-	nt	nt	nt	nt	nt	nt	nt
	Corneal swab	-	+	nt	nt	nt	nt	nt	nt	nt
	Full blood	-	+	nt	nt	nt	nt	nt	nt	nt
6	Nose swab	-	-	nt	nt	nt	nt	nt	nt	nt
	Corneal swab	-	+	nt	nt	nt	nt	nt	nt	nt
	Full blood	-	+	nt	nt	nt	nt	nt	nt	nt
7	Nose swab	-	-	nt	nt	nt	nt	nt	nt	nt
	Corneal swab	-	-	nt	nt	nt	nt	nt	nt	nt
	Full blood	-	+	nt	nt	nt	nt	nt	nt	nt
8	Nose swab	-	-	nt	nt	nt	nt	nt	nt	nt
	Corneal swab	-	-	nt	nt	nt	nt	nt	nt	nt
	Full blood	-	+	nt	nt	nt	nt	nt	nt	nt
9	Nose swab	-	-	nt	nt	nt	nt	nt	nt	nt
	Corneal swab	-	-	nt	nt	nt	nt	nt	nt	nt
	Full blood	-	-	-	-	-	-	-	+	-

Legend: + positive for the OvHV-2 genome, - negative for the OvHV-2 genome, nt – not tested for the OvHV-2 genome.

detected until week 6, and in nasal swabs until week two of the study. Samples collected from the other susceptible heifer were negative for the duration of the study for OvHV-2. Two out of seven sheep were positive at the initial testing. However, by the end of the study, the two previously positive sheep were negative, while another sheep previously negative became positive (Table 1).

A retrospective study revealed that out of 21 tested cattle, OvHV–2 was detected in 15 (71.4%), and out of seven tested sheep, three (42.9%) were positive for the OvHV-2 genome. From the above-mentioned animals, 20 were sampled in Serbia of which 10 were positive for the OvHV – 2 genome, 6 were sampled in the Republic of Srpska of which all were positive for the OvHV-2 genome, and two were sampled in Montenegro, and both were positive for the OvHV-2 genome. Good-quality nucleotide sequences of the OvHV-2 tegument protein gene were obtained from 10 samples and were submitted to the NCBI GenBank under accession numbers MZ927729-MZ927736, and OP820491-OP820492. The sequences were compared with other sequences from NCBI (Table 2). All sequences used were trimmed to 314 base pairs. The sequenced samples show the highest percentage of similarity with the strains from Brasil KJ658293.1 (100%) and Germany HM216475.1 (100%). (Fig. 2). The number of substitutions per site between sequences in the alignment of OvHV-2 ranged from 0.00 - 0.40, while the divergence within the sequenced samples ranged from 0.00 – 0.02.

Table 2: Sequences from the NCBI which were used for comparison in the phylogenetic study.

Accession number	Species	Year	Country of origin
MK552112.1	Cattle	2014	USA
HM216475.1	Goat	2007	Germany
HM216476.1	Deer	2008	Germany
HM216481.1	Goat	2017	Turkey
HM216483.1	Bison	2008	Germany
JQ780444.1	Cattle	2008	Brasil
KC123170	Sheep	2012	Brasil
KF017577.1	Cattle	2013	India
KF017579.1	Cattle	2012	India
KJ658293.1	Cattle	2013	Brasil
KR092147.1	Sheep	2014	India
KX060582.1	Sheep	2016	Canada
L05908.1	Cattle	1993	USA
MF289492.1	Sheep	2017	India
MF977714.1	Sheep	2017	India
MN393474.1	Cattle	2019	Turkey
MN419919.1	Cattle	2019	Turkey
MN419920.1	Sheep	2019	Turkey
MN419921.1	Sheep	2019	Turkey
MT253536.1	Cattle	2017	Turkey
MZ221210.1	Sheep	2021	Brasil
MZ927729	Cattle	2016	Serbia
OP820491	Cattle	2022	Serbia
OP820492	Sheep	2022	Serbia
MZ927730	Cattle	2016	Serbia
MZ927731	Cattle	2021	Serbia
MZ927732	Cattle	2019	Republic of Srpska
MZ927733	Cattle	2015	Serbia
MZ927734	Cattle	2018	Serbia
MZ927735	Cattle	2020	Serbia
MZ927736	Cattle	2016	Serbia

DISCUSSION

SA-MCF is a viral disease of cattle with a high fatality rate and an unpredictable onset of clinical signs in incidental hosts. Furthermore, it is the most often differentially diagnosed disease in cattle in Serbia in IVMS when ruling out transboundary diseases such as FMD and BT. Viremia in sheep can occur year-round, with peaks of viral shedding at different intervals, connected to a variety of external factors (Hüssy *et al*., 2002; Li *et al*., 2004). However, only a few papers have been published regarding the length of viremia in infected cattle, probably because of a very high lethality rate.

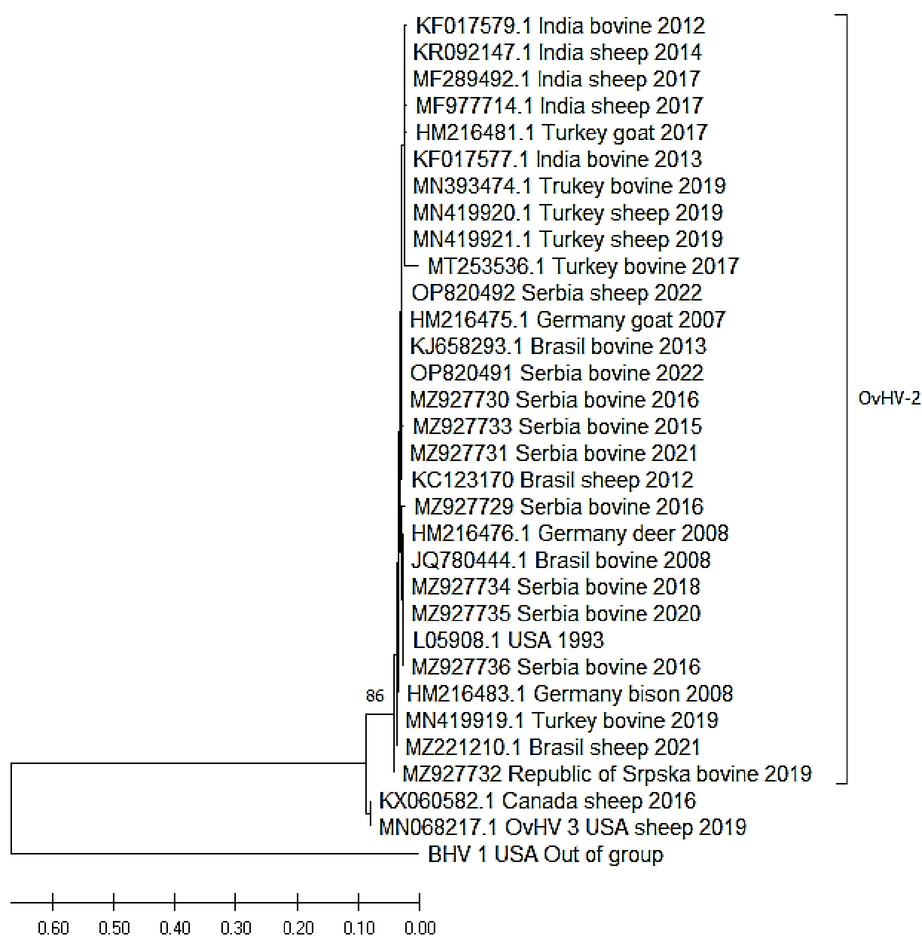

Fig. 2: Phylogenetic tree: Phylogenetic tree illustrating genetic relationships between Serbian strains of OvHV-2 from 2015-2022, and strains from the NCBI (Table 2.). Phylogenetic analysis was conducted in Mega X software using the Neighbour-Joining Method and the evolutionary distances were calculated using the Maximum Composit Liklehood model. The analysis involved 31 nucleotide sequences from different geographical locations. Branches corresponding to partitions reproduced in less than 70% bootstrap replicates were collapsed. The percentage of replicate trees over 70% in which taxa clustered together in the bootstrap test is shown next to the branches.

Even more, clinical cases in cattle were described to occur years after contact with the virus, despite the seroconversion. The results of our study support the multifactorial dependence of clinical cases as on the same backyard holding at which 3 sheep and one heifer were detected positive, there was another susceptible heifer, in which neither clinical signs were reported, nor OvHV-2 DNA was detected (Russell *et al.*, 2009b; Li *et al.*, 2014). The infected heifer was treated symptomatically and survived the infection making a full recovery, although still hasn't been artificially inseminated.

The infected heifer was tested for OvHV-2 every week to determine the length of viremia, and the shedding of the viral DNA. Also, the other heifer was tested to determine the onset of the infection and to estimate the incubation period. Viral DNA was detected in the infected heifer in corneal swabs, nasal swabs and blood for the first two weeks after the initial confirmation, after which nasal swabs were negative for the duration of the study. Corneal swabs remained positive for the OvHV-2 genome for seven weeks, and blood was positive for eight weeks for the OvHV-2. Findings regarding the shedding period for cattle although not significant for viral transmission (Li *et al.*, 2014), do shed a light on the disease course in surviving individuals.

Despite, that both animals were reported to be housed within the same structure, the other susceptible heifer had no reported clinical signs, and was negative for the duration of the study. This suggests that besides environmental stress factors, other individual factors contributing to the disease occurrence should also be investigated. The incubation period for SA-MCF can last up to 9 months and in this instance, the negative heifer could be infected without exhibiting clinical signs or the virus multiplying at the detectable level. Although in the current study setup, if the animal were to develop clinical signs at a later date it would be impossible to determine when the animal became infected.

Concerning sheep, viral DNA was detected in three sheep 6 weeks after the first report of the disease. In the final week of the study, a sheep that was previously negative turned positive. Suggesting that the virus is still circulating within the herd or that the sheep is latently infected with the virus, but was not in the viremia stage of the infection for the majority of the study. Of course, this does not exclude the possibility that a low-level persistent viremia exists, but it is under the detection limit of the PCR protocol.

Although SA-MCF has been present in Serbia (Spasojevic *et al.*, 2008), no molecular or phylogenetic studies have ever been done before with a similar situation in the entire region (Turk *et al.*, 2010; Hristov and Peshev, 2016; Hristov *et al.*, 2017). Therefore, the tracking and comparison of different virus variations are difficult. Although AlHV-1 and OvHV – 2 share a high degree of similarity, notable differences have been recorded such as a lack of the IL – 10 gene in AlHV-1 while it exists in Ov 2.5 and Ov 3.5 (Hart *et al.*, 2007). For this study, a tegument protein gene was targeted with a hemi-nested protocol previously described by Baxter *et al.* (1993) encoded by the ORF 75 and 33. The tegument protein gene represents a conserved part of the OvHV-2 genome and allows the detection of the virus in both cattle and sheep (Oğuzoğlu *et al.*, 2020). The nucleotide sequences of the tegument protein gene obtained from this study and sequences of other OvHV-2 strains showed little variation

among them. The strain MZ927733 showed the highest similarity with strain OP820491. These two strains originated from the same area, and both from bovine hosts, although seven years apart. Interestingly the sequence (OP820492) obtained from a sheep held in the same backyard holding as the diseased cattle (OP820491), clustered together with an isolate from Turkey (MT253536.1) from 2017. The MK552112.1 BHV – 1 strain was added as an out-of-group sequence.

Since there has been a limited number of research papers on SA-MCF, there is a necessity for further research to be done for a better understanding of the circulating virus strains. Since there is a variety of different clinical signs similar to other notifiable diseases such as bovine viral diarrhea (BVD), BTV, IBR, and FMD, there is a clear benefit in including SA-MCF in the differential diagnosis in cattle. As mixed farming in the Central Balkan region is often practiced, implementing SA-MCF monitoring in passive surveillance would allow a better understanding of the disease, ascertaining its prevalence, and could provide new information regarding SA-MCF epidemiology.

Conflict of interest: The authors declare they have no conflicts of interest. All authors of the paper have read and approved the final version of the manuscript submitted and all have made substantive contributions to the work.

Ethics Statement: The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required, the samples used in the study originated from our sample bank.

Authors contribution: DG and VM designed the study and drafted the manuscript. ZSZ carried out molecular tests. MN participated in the sequence alignment and phylogenetic study. BM and BK and MĐ contributed to the interpretation of the results. OS and DL supervised the findings of this work. All authors interpreted the data, critically revised the manuscript for important intellectual contents, and approved the final version.

Data availability statement: The data is available at the request of the corresponding author.

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