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Demonstration and quantification of ovine herpesvirus 2 in Croatia - a case report

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ABSTRACT

Ovine herpesvirus-2 (OvHV-2), a gammaherpesvirus (genus *Rhadinovirus*) causes a severe disease known as sheep-associated malignant catarrhal fever (SA-MCF) in certain ruminants, such as cow, deer, bison and water buffalo. Suspected cases of SA-MCF in cows without identification of the agent have been reported in Croatia in the past. In June 2005, on a farm in northwest Croatia, where 17 Simmental diary cows and 2 heifers shared stables and meadows with sheep, a 13 month-old heifer showed symptoms reminiscent of SA-MCF, including anorexia, high fever, nasal discharge, and neurological symptoms, such as ataxia, tremor, convulsions and hyperesthesia. The animal died within 14 days. Gross necropsy findings were sharply demarcated erosions on mucosal surfaces, including the tongue, oral mucosa, esophagus, abomasum, jejunum, colon, caecum and urinary bladder. Histopathology revealed extremely severe perivascular and intramural arterial infiltrations with mononuclear cells, mostly lymphocytes. These lesions were seen in almost every organ, especially the brain and lungs. Formaldehyde fixed samples from the brain, cerebellum, spleen and lymph nodes were obtained and subjected to DNA extraction procedures. Fluorogenic real-time PCR (Polymerase chain reaction) amplification specific to OvHV-2 DNA was performed and OvHV-2 DNA was detected in the brain, cerebellum and spleen, as well as in the lymph nodes. These data indicate that the animal had been infected with OvHV-2, the agent of SA-MCF. For the first time, OvHV-2 was identified and quantified in a Croatian heifer as the causative agent of SA-MCF.

Key words: ovine herpesvirus-2, *Gammaherpesvirinae,* sheep-associated malignant catarrhal fever, real-time PCR, heifer

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Introduction

Malignant catarrhal fever (MCF) is an acute, generalized and often fatal disease of cloven-hoofed animals caused by ovine closely-related members of *Gammaherpesvirinae* family. The disease is characterized primarily by high fever, lymph node swelling, and widespread inflammation of mucosal surfaces, while lymphoproliferation and vasculitis are the main histological lesions (SIMON et al., 2003). Two major epidemiological forms of MCF are defined; one is known as wildebeest-associated MCF (WA-MCF) reflecting the fact that the causative agent (Alcelaphine herpesvirus 1) exists in nature as an endemic subclinical infection in wildebeest. The second epidemiological form is described as sheep-associated malignant catarrhal fever (SA-MCF) and is caused by the closely-related ovine herpesvirus 2 (OvHV-2) (PLOWRIGHT, 1990). Wildebeest-associated MCF is restricted to the African continent, e.g. to areas inhabited by wildebeest, while sheep-associated MCF occurs worldwide. Though never successfully propagated *in vitro,* there is substantial epidemiologic, serologic and molecular evidence that all varieties of domestic sheep represent a reservoir of OvHV-2 (KIM et al., 2003; LI et al., 2005). Moreover, data recently published strongly suggest that OvHV-2 replication is localized to the respiratory tract of shedding sheep (CUNHA et al., 2008). The virus can be transmitted to susceptible, poorly adapted ruminants, such as domestic cattle, deer, wild ruminants and, as recently reported, to pigs as well (SCHULTHEISS et al., 2000; ALBINI et al., 2003). SA-MCF usually appears sporadically and affects only one or a few animals, but occasionally significant herd outbreaks occur (BEREZOWSKI et al., 2005). Suspected cases of SA-MCF in cows without identification of the agent have been detected in Croatia, especially in northwest areas, where cows sometimes share stables and meadows with sheep. A wide spectrum of clinical manifestations has been detected during the past decades, ranging from the acute form, in which minimal changes were observed prior to death, to more noticeable cases, characterized by high fever, bilateral corneal opacity, profuse catarrhal discharges from the eyes and nares, necrosis of the muzzle and erosion of the buccal epithelium. So far, diagnosis has normally been achieved by clinical observation and the detection of characteristic histopathological changes.

This paper describes a case of SA-MCF in a 13 month-old heifer and subsequent diagnostic findings, including identification and quantification of OvHV-2 DNA in different organs.

Materials and methods

Assessment of epidemiological data and clinical signs. In June 2005, on a farm in northwest Croatia, where 17 Simmental dairy cows and 2 heifers shared stables and meadows with several sheep, a 13 month-old heifer showed symptoms reminiscent of SA-MCF; anorexia, high fever, bilateral corneal opacity, profuse catarrhal discharges from

the eyes and nares, necrosis of the muzzle (Fig. 1) and erosion of the buccal epithelium. Due to anorexia, there was also a significant weight loss. Neurological symptoms, such as ataxia, tremor, convulsions and hyperesthesia preceded the death of animal, which occurred 14 days after the onset of the disease.

Pathological investigation and material sampling. An autopsy was performed and organ samples were taken for histopathological analysis. After formalin fixation, the samples were embedded in paraffin and cut in 5 μm thick slices, which were deparaffinized and stained using the routine haematoxylin-eosin method.

Formaldehyde fixed samples from the brain (73a, non-identified region), cerebellum (73b), spleen (73c, 73d), and lymph nodes (73e, 73f) were obtained. Samples of 25 mg each from the brain, cerebellum, and lymph nodes, and 10 mg from the spleen were washed twice with phosphate buffered saline (PBS) before DNA extraction.

Real-time PCR. DNA was extracted from tissues using the QIAamp DNA Mini Kit (Qiagen, Basel, Switzerland) according to the protocol of the supplier. In order to reduce viscosity, the lyzed sample was transferred to a QIAshredder column (Qiagen) and centrifuged at room temperature for 5 minutes (ALBINI et al., 2003). The DNA was eluted in a volume of between 70 and 100 μL. Fluorogenic real-time PCR amplification specific for OvHV 2 DNA was performed in duplicate wells with 10 μL template or with a 1:10 dilution of it, using the primers, probe, reaction mix and thermal cycle conditions essentially as described previously (HUSSY et al., 2001). A synthetic 72mer consisting of the primary target sequences was used as a positive control. Negative controls consisted of one sample without added DNA (negative control 1) and one sample containing the elution from an unloaded column (negative control 2).

Results

Pathology and histopathology. Macroscopically, the lymph nodes were swollen. Sharply demarcated erosions were found on most mucosal surfaces, including the tongue, oral mucosa, oesophagus, abomasum, jejunum, colon, coecum and urinary bladder. Hemorrhagic punctuations were found on the larynx (Fig. 2), trachea, pericardium, endocardium and gall bladder. The epicardium of the right atrium was thickened and covered with fibrinoso-haemorrhagic exudates. Congestion and oedema with bluish opacity were noted in the eyes (Fig. 3). Sharply demarcated necrotic skin lesions were also seen on the thorax. The brain was congested and the lungs were oedematous. Histopathologicaly, the most important finding was very significant perivascular and intramural arterial infiltrations with mononuclear cells, mostly lymphocytes (Fig. 4).

Real-time PCR. In order to detect OvHV-2, DNA was extracted from several tissues and subjected to real-time PCR. The results are shown in Table 1. OvHV-2 DNA was detected in the brain, cerebellum and spleen as well as the lymph nodes, indicating that

the animal had been infected with OvHV-2, the agent of sheep-associated malignant catarrhal fever.

Table 1. Detection of OvHV-2 DNA in samples from heifer with suspected MCF.

Sample	Ct value	Interpretation
Positive control	29. 31[a]	OvHV-2 DNA detected
Negative control 1	Not applicable[b]	Not detected
Negative control 2	Not applicable	Not detected
Brain (73a)	26. 26	OvHV-2 DNA detected
Brain (73a) 1:10	33. 32	OvHV-2 DNA detected
Cerebellum (73b)	Not tested	
Cerebellum (73b) 1:10	32. 31	OvHV-2 DNA detected
Spleen (73c)	28. 28	OvHV-2 DNA detected
Spleen (73c) 1:10	27. 28	OvHV-2 DNA detected
Spleen (73d)	26. 27	OvHV-2 DNA detected
Spleen (73d) 1:10	26. 26	OvHV-2 DNA detected
Lymph node (73e)	Not applicable	Not detected
Lymph node (73e) 1:10	24. 24	OvHV-2 DNA detected
Lymph node (73f)	27. 27	OvHV-2 DNA detected
Lymph node (73f) 1:10	25. 24	OvHV-2 DNA detected

[a]Ct values from two independent wells are shown; [b]If a Ct value has not been detected until cycle 40 of the real-time PCR reaction, the corresponding sample is considered to be negative. The term "Ct value" is therefore not applicable for negative samples.

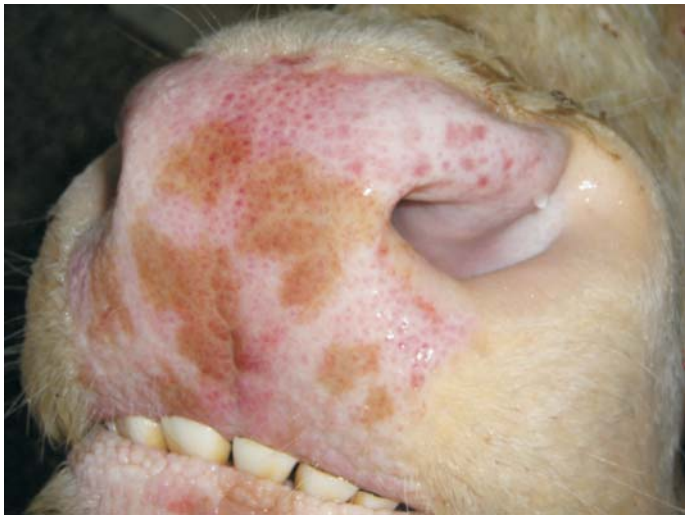

Fig. 1. Necrosis on the muzzle

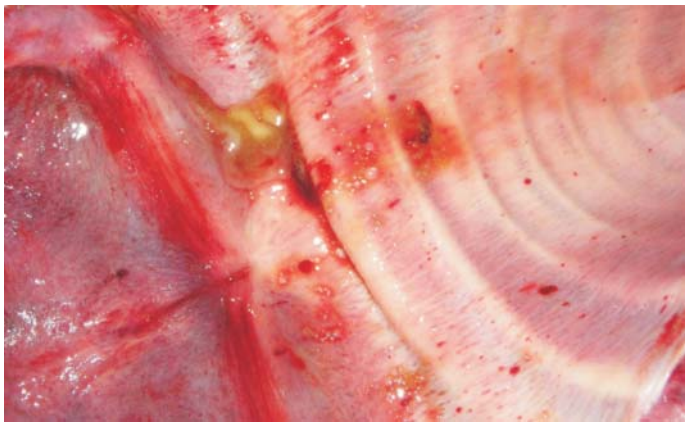

Fig. 2. Hemorrhagic punctuations on the larynx

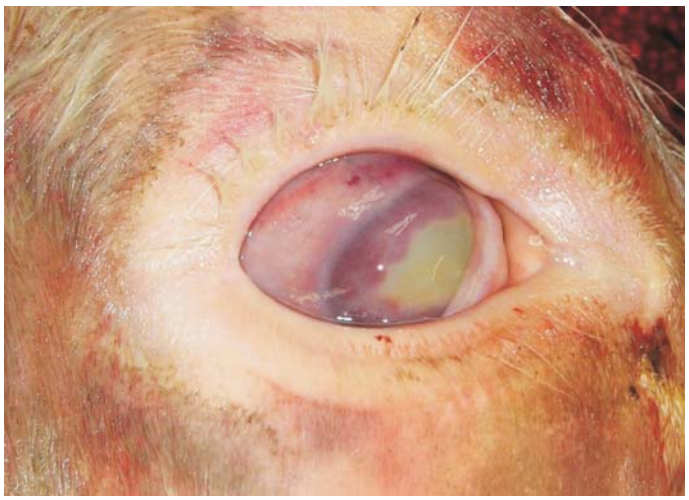

Fig. 3. Congestion, oedema and advanced corneal opacity in the eye

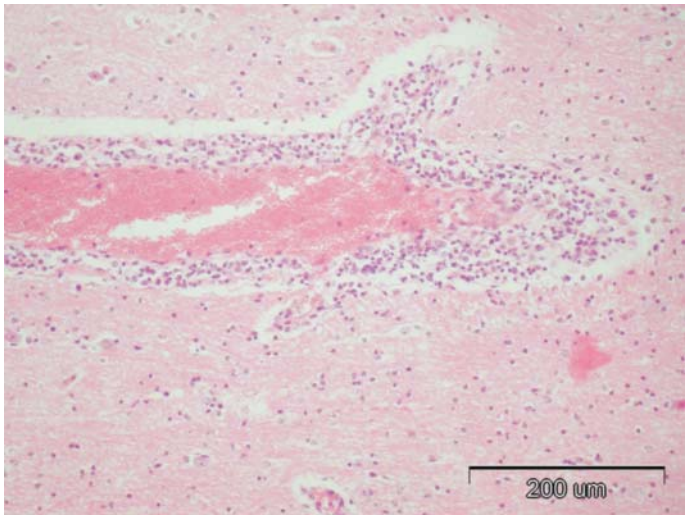

Fig. 4 . Severe cerebral vasculitis characterized by predominately mononuclear cells infiltration. H&E

Discussion

Suspected cases of SA-MCF in cows without identification of the agent have been reported in Croatia, especially in northwest areas where cows usually share stables and meadows with sheep. In this case, the epidemiological data also revealed close contact between the heifer and sheep. Clinical findings were consistent with those already described in the literature (ANONYM., 2008), but since clinical findings and gross pathological examination cannot be relied on, as they can be extremely variable, histopathological examination of a variety of tissues was performed. The discovery of significant perivascular and intramural arterial infiltration of mononuclear cells, mostly lymphocytes, is characteristic of SA-MCF and provides a more certain diagnosis.

It must be emphasised that SA-MCF is one of the few known infectious diseases for which the etiological agent has never been isolated. Proof of OvHV-2 infection relies solely on the presence of antibodies in reactions in which AVH-1 infected cells are used as antigen and on the detection and amplification of viral DNA in various clinical materials, such as the peripheral blood leukocytes of clinically affected animals as well as fresh tissues and paraffin-embedded samples collected post mortem (LI et al., 1994; HŰSSY et al., 2001). Since the detection of viral DNA, various highly specific, sensitive PCR (Polymerase chain reaction) methods have been employed worldwide in studies of the disease in clinically affected animals and natural hosts.

Fluorogenic real-time PCR amplification specific to OvHV-2 DNA was performed in our study and OvHV-2 DNA was detected in the brain, cerebellum, and spleen, as well as in the lymph nodes. The detection of OvHV-2 DNA in a variety of tissues from animals with an MCF-like disease clearly demonstrates that these animals had been infected with OvHV-2. Since OvHV-2 is found only on extremely rare occasions in healthy animals (MŰLLER-DOBLIES et al., 2001a, 2001b), causative involvement of OvHV-2 in the observed disease is extremely likely. However, inhibition of the PCR reaction in undiluted samples has previously been observed (ALBINI et al., 2003). Therefore it was not surprising that one undiluted sample reacted negatively, whereas the Ct values obtained from the 1:10 dilution were often equal to or even lower than Ct values obtained from undiluted DNA. The PCR method, especially real-time PCR, emerged as a robust test that can be employed to detect viral DNA in various clinical materials, such as peripheral blood leukocytes of clinically affected animals, as well as fresh tissues and paraffin-embedded samples collected post mortem (BAXTER et al., 1993; LI et al., 1995; HŰSSY et al., 2001). Finally, it could be concluded that, for the first time, using fluorogenic real-time PCR, OvHV-2 was identified and quantified in one Croatian heifer as the causative agent of SA-MCF.

References

ALBINI, S., W. ZIMMERMANN, F. NEFF, B. EHLERS, H. HÄNI, H. LI, D. HŰSSY, M. ENGELS, M. ACKERMANN (2003): Identification and quantification of ovine gammaherpesvirus 2 DNA in fresh and stored tissues of pigs with symptoms of porcine malignant catarrhal fever. J. Clin. Microbiol. 41, 900-904.

ANONYMOUS (2008): Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Malignant catarrhal fever. 5th ed. OIE, Paris, Chapter 2.4.15.

BAXTER, S. I. F., I. POW, A. BRIDGEN, H. W. REID (1993): PCR detection of the sheep-associated malignant catarrhal fever. Arch. Virol. 132, 149-159.

BEREZOWSKI, J. A., G. D. APPLEYARD, T. B. CRAWFORD, J. HAIGH, H. LI, D. M. MIDDLETON, B. P. O'CONNOR, K. WEST, M. WOODBURY (2005): An outbreak of sheep-associated malignant catarrhal fever in bison (*Bison bison*) after exposure to sheep at a public auction sale. J. Vet. Diagn. Invest. 17, 55-58.

CUNHA, W. C., L. T. TRAUL, N. S. TAUS, J. L. OAKS, D. O'TOOLE, C. M. DAVITT, H. LI (2008): Detection of ovine herpesvirus 2 major capsid gene transcripts as an indicator of virus replication in shedding sheep and clinically affected animals. Virus Res. 132, 69-75.

HŰSSY, D., N. STAUBER, C. M. LEUTENEGGER, S. RIEDER, M. ACKERMANN (2001): Quantitative fluorogenic PCR assay for measuring ovine herpesvirus 2 replication in sheep. Clin. Diagn. Lab. Immunol. 8, 123-128.

KIM, O., H. LI, T. B. CRAWFORD (2003): Demonstration of sheep-associated malignant catarrhal fever virions in sheep nasal secretions. Virus Res. 98, 117-122.

LI, H., D. O'TOOLE, O. KIM, L. OAKS, T. B. CRAWFORD (2005): Malignant catarrhal fever-like disease in sheep after intranasal inoculation with ovine herpesvirus. J. Vet. Diagn. Invest. 17, 171-175.

LI, H., D. T. SHEN, D. O'TOOL, D. P KNOWLES, J. R. GORHAM, T. B. CRAWFORD (1995): Investigation of sheep-associated malignant catarrhal fever virus infection in ruminants by PCR and competitive inhibition enzyme-linked immunoadsorbant assay. J. Clin. Microbiol. 8, 2048-2053.

LI, H., D. T. SHEN, D. O'TOOL, D. P. KNOWLES, J. R. GORHAM, T. B. CRAWFORD (1994): Competitive inhibition enzyme-linked immunoadsorbant assay for antibody in sheep and other ruminants to a conserved epitope of malignant catarrhal fever virus. J. Clin. Microbiol. 7, 1674-1679.

MŰLLER-DOBLIES, U. U., J. EGLI, B. HAUSER, H. LI, M. STRASSER, F. EHRENSPERGER, U. BRAUN, M. ACKERMANN (2001a): Malignant catarrhal fever in Switzerland: 2. Evaluation of the diagnosis (in German). Schweiz. Arch. Tierheilkd. 143, 581-591.

MŰLLER-DOBLIES, U. U., J. EGLI, H. LI, U. BRAUN, M. ACKERMANN (2001b): Malignant catarrhal fever in Switzerland. 1. Epidemiology. Schweiz. Arch. Tierheilkd. 143, 173-183.

PLOWRIGHT, W. (1990): Malignant catarrhal fever virus. In: Virus Infections of Ruminants (Dinter, Z., B. Morein, Eds.). Elsevier Science Publishers, Amsterdam, pp. 123-150.

SCHULTHEISS, P. C., J. K. COLLINS, T. R. SPRAKER, J. C. DeMARTINI (2000): Epizootic malignant catarrhal fever in three bison herds: differences from cattle and association with ovine herpesvirus-2. J. Vet. Diagn. Invest. 12, 497-502.

SIMON, S., H. LI, D. O'TOOLE, T. B. CRAWFORD, J. L. OAKS (2003): The vascular lesions of a cow and bison with sheep-associated malignant catarrhal fever contain ovine herpesvirus 2-infected $CD8^+$ T lymphocytes. J. Gen. Virol. 84, 2009-2013.

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SAŽETAK

Ovčji herpesvirus 2 (OvHV-2) iz roda *Rhadinovirus*, potporodice *Gammaherpesvirinae* uzročnik je zarazne korice goveda u nekih vrsta domaćih i divljih preživača. Dosad su u Republici Hrvatskoj zabilježeni mnogi slučajevi sumnje na pojavu zarazne korice u goveda temeljeni na kliničkoj metodi dijagnosticiranja, ali bez dokaza uzročnika bolesti. U lipnju 2005. godine na jednoj farmi u sjeverozapadnoj Hrvatskoj zabilježena je pojava zarazne korice goveda u jedne junice, simentalske pasmine, u dobi od 13 mjeseci, koja je bila smještena u istoj staji s još 17 mliječnih krava i jednom junicom simentalske pasmine te manjim stadom ovaca. Životinja je naglo oboljela uz znakove gubitka apetita, visoke vrućice, seroznog iscjetka iz nosa te pojavom znakova središnjega živčanoga sustava, ataksije, tremora, grčeva i hiperestezije. Životinja je uginula 14. dan od početka prvih znakova bolesti. Razudbom je utvrđena prisutnost oštro ograničenih erozija na sluznici jezika, usne šupljine, jednjaka, sirišta, tankoga, debeloga i slijepoga crijeva te mokraćnoga mjehura. Histopatološki je gotovo u svakom organu, a posebno u mozgu i plućima, utvrđena jaka perivaskularna i intramuralna infiltracija arterija mononuklearnim stanicama, većinom limfocitima. Iz formalinom fiksiranih uzoraka mozga, maloga mozga, slezene i limfnih čvorova izdvojena je DNK i podvrgnuta fluorogenoj real-time PCR amplifikaciji specifičnoj za OvHV-2. OvHV-2 dokazan je i kvantificiran u svim pretraživanim organima. Dobiveni rezultat upućuje na to da je uginula životinja bila zaražena s OvHV-2 što je ujedno i prvi dokaz uzročnika zarazne korice goveda u Hrvatskoj.

Ključne riječi: ovčji herpesvirus-2, *Gammaherpesvirinae*, zarazna korica goveda, lančana reakcija polimerazom