Gammaherpesvirus infections in cattle in Europe

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INHALTSVERZEICHNIS

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3
Gammaherpesvirus infections in cattle in Europe

Macaviruses (subfamily Gammaherpesvirinae) infect ruminants and swine, causing asymptomatic latent infections in reservoir hosts and malignant catarrhal fever (MCF) in susceptible species. It is not known whether infection with one macavirus might protect from infection with another. We therefore assessed cattle from across Europe for infection with bovine herpesvirus-6 (BoHV-6) and gammaherpesviruses of other ruminants that can cause MCF. Lung, spleen, bronchial lymph node (BLN) and tongue were collected from 448 cattle (338 necropsied, 100 slaughtered) in Switzerland, United Kingdom, Finland, Belgium and Germany. Lung and spleen were screened for BoHV-6, OvHV-1, OvHV-2, CpHV-2 and Bison LHV by quantitative PCR. Only BoHV-6 was identified and detected in 32% of the animals, with variable geographical frequency (22%-41%). Infection was detected across all ages and positively correlated with age. There was no significant association with sex and disease entity/aetiology. Viral loads varied between organs, but were overall highest in BLN, with no significant correlation between organs or with any population parameters. The study confirms that BoHV-6 is endemic in cattle across Europe and that infection is not associated with specific disease process or any other disease cause. It supports previous data indicating that BoHV-6 is a commensal of domestic cattle that is not associated with disease processes and that infections with other macaviruses are rare and sporadic.

Keywords: BoHV-6; commensal; gammaherpesvirus; qPCR; European cattle
Gammaherpesvirus-Infektionen bei Rindern in Europa

Macaviren (Subfamilie Gammaherpesvirinae) infizieren Wiederkäuer und Schweine, sie verursachen asymptomatische latente Infektionen im Reservoirwirt und Bösartiges Katarrhalfeber (BKF) in empfänglichen Spezies. Es ist nicht bekannt, ob eine Infektion mit nicht BKF aus lösenden Gammaherpesviren wir bovines Herpesvirus 6 (BoHV-6) Rinder vor BKF schützen kann. Ziel der vorliegenden Studie war, Daten zu Prävalenz, ausschließenden und Ko-Infektionen von Gammaherpesviren in Rindern zu generieren. Es wurden Lunge, Bronchiallymphknoten, Milz und Zunge von 448 Rindern (338 Sektionen, 100 Schlachtungen) in der Schweiz, Grossbritannien, Finnland, Belgien und Deutschland gesammelt. Lunge und Milz wurden mittels quantitativer PCR auf BoHV-6, OvHV-1, OvHV-2, Cp-HV-2 und Bison LHV untersucht. Es wurde ausschließlich BoHV-6 nachgewiesen, mit einer Prävalenz von 32% und variabler geographischer Häufigkeit (22- 42%). Infizierte Tiere waren signifikant älter als nicht infizierte Tiere. Eine signifikante Korrelation mit Geschlecht und Erkrankung/Ätiologie wurde nicht nachgewiesen. Die Viruslast variierte zwischen den Organen, war jedoch im Schnitt im Lymphknoten am höchsten; sie war nicht mit Populationsparametern korreliert. Die Studie bestätigt, dass BoHV-6 bei Rindern in Europa endemisch ist und eine Infektion nicht mit spezifischen Erkrankungen und anderen Krankheitsursachen assoziiert ist. BoHV-6 ist ein Kommensale bei Rindern, Infektionen mit anderen Macaviren sind dagegen selten.

Stichwörter: BoHV-6, Kommensale, Gammaherpesviren, qPCR, Rinder
Gammaherpesvirus infections in cattle in Europe

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Abstract: The genus Macavirus, subfamily Gammaherpesvirinae, comprises ungulate viruses that infect domestic and wild ruminants and swine. They cause asymptomatic latent infections in reservoir hosts and malignant catarrhal fever in susceptible species. Lung, spleen, bronchial lymph node and tongue were collected from 448 cattle (338 necropsied, 100 slaughtered) in Switzerland, United Kingdom, Finland, Belgium and Germany. Animals were screened for bovine herpesvirus-6, ovine herpesvirus-1 and -2, caprine herpesvirus-2 and bison lymphotrophic herpesvirus by quantitative PCR. Only BoHV-6 was detected, with an overall frequency of 32%, ranging between 22% and 42% in the different countries. Infection was detected across all ages, from one day after birth, and was positively correlated with age. There was no evidence of an association of BoHV-6 infection with specific diseases processes. In positive animals, BoHV-6 was detected in all organs with high frequency, consistently in lungs or spleen. Viral loads in organs varied substantially. In BoHV-6 positive gravid cows, organs of foetuses were tested for infection with negative results, indicating that the virus is not vertically transmitted. Our results confirm previous data indicating that BoHV-6 is a commensal of domestic cattle not associated with disease processes and confirm that infections with other macaviruses are rare and sporadic.

Keywords: BoHV-6; commensal; gammaherpesvirus; qPCR; European cattle
1. Introduction

Herpesviruses are enveloped double stranded DNA viruses and cause of numerous human and animal diseases. Primary herpesvirus infection generally results in a productive infection that is subsequently limited by the host immune response, leaving behind latently infected cells that persist in the host [1].

Members of the subfamily Gammaherpesvirinae have a narrow host range and cell tropism. They infect lymphocytes, epithelial cells and endothelial cells [2]; however, latency in B cells is their predominant feature, and some subfamily members cause persistence associated tumour formation [3]. The genus Macavirus comprises nine ungulate gammaherpesviruses. Macaviruses infect domestic and wild ruminants as well as swine, causing asymptomatic infections in reservoir hosts [4]. However, among these are also the so-called malignant catarrhal fever (MCF) viruses, like ovine herpesvirus 2 (OvHV-2), alcelaphine herpesvirus 1 (AIHV-1), AIHV-2, and caprine herpesvirus 2 (CpHV-2), that can cause the fatal disease MCF in susceptible species [4].

The reservoir hosts shed virus into the environment and are capable of transmitting it to susceptible hosts when direct or indirect contact occurs, whereas poorly adapted hosts do generally not shed infectious virus and are therefore considered to be dead-end hosts [4]. Other macaviruses, including bovine gammaherpesvirus 6 (BoHV-6) and the porcine lymphocytic herpesviruses (suid herpesvirus (SuHV) 3, 4 and 5) have not been associated with disease despite being prevalent in cattle and swine [4].

Knowledge on the course of Macavirus infections in reservoir hosts comes from transmission studies using confirmed naturally OvHV-2 infected sheep. These first detected viral DNA in the blood (buffy coat cells), followed by nasal swab, though at least 7 days later. The virus was also found to be excreted transiently with the semen soon after the blood had turned positive [5], and a later study reports low levels of viral DNA in placenta and amniotic fluids of infected sheep [6]. The latter study also specified that naturally infected sheep shed infectious virus sporadically in nasal secretions, but with only short peaks of large amounts [6]. Experimental OvHV-2 infections of sheep complete the picture and have identified the lung as the site of initial viral replication [7]. After this initial lytic replication, OvHV-2 was primarily found as latent virus in infected peripheral blood leukocytes [7]. Our group has investigated the distribution of OvHV-2 by quantitative PCR in naturally infected sheep and detected the virus with high frequency in lungs and mediastinal lymph nodes (81%) as well as the spleen (91%); interestingly, it was also found in the tongue with similar frequency (85%), indicating the potential for shedding also from the oral cavity. Interestingly, we also found the virus in these tissues in infected cattle without MCF, though at lower frequency (tongue: 45%; spleen: 38%; lung: 36%; mediastinal lymph node: 30%) and with substantially lower viral loads [8].

BoHV-6, previously known as bovine lymphotropic herpesvirus (BLHV), was first detected and isolated in the USA from a bovine lymphoma induced by bovine leukaemia virus (BLV). At that time, its role as a co-factor in BLV-associated disease was proposed [9]. Subsequently, BoHV-6 was identified by PCR in cows suffering from postpartum metritis non-responsive to conventional antibiotic treatment in the UK and other European countries [10, 11] and in bovine abortions in Canada [12], suggesting a role in reproductive system disorders. However, experimental data supporting a causative link between BoHV-6 and disease is lacking. In a recent study, BoHV-6 was detected at low copy number in the brain of two cattle suffering from a non-suppurative encephalitis that was found to be causatively linked to parainfluenza virus 5 infection [13]. Previous molecular studies have shown that BoHV-6 infects peripheral blood mononuclear cells (PBMC) and found infection in 52-87% and 30% of healthy adult cattle and calves, respectively, in the USA and Poland [9; 14; 15], indicating that BoHV-6 infection is widespread in cattle populations. Infection seems to occur at a very young age; the study in the USA found 38% of the 2-week-old calves PCR positive for BoHV-6 [9]. BoHV-6 infection is not exclusive to cattle; viral DNA has been detected in three bison in the USA [14] and seven buffalo (Bubalus bubalis) in Brazil [16].
Our group is interested in gammaherpesvirus infections in their natural hosts as opposed to their dead-end hosts, and potential protection from infection by related viruses. As part of this, we wanted to assess cattle for infection with BoHV-6 and gammaherpesviruses of other ruminants of which they are not natural hosts, but that can cause MCF in cattle and other ruminants. Accordingly, we examined a large cohort of randomly selected diseased and necropsied as well as slaughtered cattle from several European countries for (co-)infection with the above viruses, using cattle with MCF as “controls” for dead-end host OvHV-2 infection. We tested the lungs, spleen, bronchial lymph node and tongue since we had previously found these tissues to carry the virus in OvHV-2 reservoir host infection.

BoHV-6 was identified as the only gammaherpesvirus in all non-MCF cattle, and at high frequency, which prompted us to investigate various population parameters and tissue loads at individual animal level, in the attempt to better characterise the virus-host interaction and potential effects of BoHV-6.

2. Materials and Methods

Animals and tissue samples

The study was performed on 448 cattle without MCF that either underwent a diagnostic post mortem examination after they had been euthanized due to clinical disease, or were slaughtered between March 2017 and November 2018. The diagnostic cases had been examined at the Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich (n = 96), the Institute of Animal Pathology, Vetsuisse Faculty, University of Bern (n = 48), Switzerland, the Department of Veterinary Pathology and Public Health, Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, UK (n = 51), the Department of Veterinary Bioscience, Faculty of Veterinary Medicine, University of Helsinki, Finland (n = 34), the Department of Pathology, Bacteriology and Poultry Diseases, Faculty of Veterinary Medicine, Ghent University, Belgium (n = 50), the Institute of Veterinary Pathology, Justus-Liebig-University Giessen (n=14), the State Institute for Chemical and Veterinary Analysis Freiburg (n=35) and the State Veterinary Diagnostic Centre (Staatliches Tierärztliches Untersuchungsamt) Aulendorf (n=20), Germany. A total of 100 slaughtered animals were sampled at the slaughterhouse in Zurich, Switzerland (n=50) and Wigan, UK (n = 50).

From all animals, samples of lung, bronchial lymph node, spleen, and tongue (mucosa and submucosa) were obtained and stored in RNAlater (Sigma®, Life Science) and frozen at -80°C until processing. In gravid cows (n=3), lungs, bronchial lymph node, spleen and tongue from the foetus were also collected.

In addition, samples from the same tissues were collected from four necropsied cattle (of which one was gravid) with confirmed OvHV-2 associated malignant catarrhal fever (MCF). These were included as positive controls for the virus and for general comparison of viral loads. A bison tested positive for bison LHV in lungs, bronchial lymph node and tongue (with viral loads of 65 copies/µL (tongue), 19 copies/µL (bronchial lymph node), and 0.5 copies/µL (lung)) served as positive control for bison LHV, and tissues tested positive for OvHV-1 in a sheep (28,000 copies/µL in the lung) and in a cattle (300 copies/µL in a lymph node) and for CpHV-2 in a buffalo (22,000 copies/µL in the lung) served as positive control for OvHV-1 and CpHV-2 respectively.

For all animals, breed, age, sex, pathological diagnoses and disease aetiology were recorded. For descriptive analyses, animals were grouped into different age ranges. Necropsied animals were grouped according to the primarily affected organ systems, based on recorded pathological diagnoses, as follows: systemic disease and/or multiple organs affected (including, for example, also animals with a diagnosis of cachexia or septicaemia), musculoskeletal system, liver, gastrointestinal, cardiovascular system, body cavities (including, for example, animals with a diagnosis of peritonitis due to a perforating foreign body), respiratory system, reproductive system and other organs affected (including the haemolymphatic systems, the integument, neurological and urinary system). The latter group was created as the number of cases for each organ system was very low (total of 18 cases). Animals where no abnormality had been detected
at necropsy, animals where no further information was available, and the slaughtered animals were each allocated to separate groups. Animals were also grouped according to the disease entity/aetiology, creating the following groups: slaughtered animals, unknown entity, traumatic, toxic, congenital, metabolic, bacterial, parasitic, or viral injury/disease, degenerative or neoplastic disease, organ misalignment, or multifactorial disease.

**Tissue processing and DNA extraction**

Tissue samples (25-35 mg) were subjected to DNA extraction with the Qiagen® DNeasy Blood and Tissue kit (Qiagen, UK) according to manufacturer’s instructions. The eluted DNA was quantified using a NanoDrop® 2000 (Thermo Fischer Scientific Inc., USA) following the manufacturer’s instructions. The DNA was either used directly or stored at -20°C until further use.

**Quantitative polymerase chain reaction (qPCR)**

Quantitative PCR protocols were established for gamma-herpesviruses that have previously been shown to infect cattle, i.e. BoHV-6, OvHV-1 and -2, CpHV-2 and Bison LHV [8, 15, 17].

**Primers and probes.** The primers for preparation of the standard plasmid DNA were designed manually. For the viruses, conserved sequences within the glycoprotein B (gB) gene were selected as a target for the Taqman qPCR using the GeneScript Bioinformatics software (https://www.genscript.com/ssl-bin/app/primer). For OvHV-2, the genome copy number was estimated using OvHV-2 ORF63-specific primers [17], and for BoHV-6, we adopted the published assay based on the gB gene sequence [15]. The possibility of non-specific reactions with other sequences was avoided by using an alignment search tool (BLAST) at the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). The 12s-ribosomal DNA internal genome was used to normalise the qPCR data [18]. Primer and probe sequences as well as gene accession numbers are listed in Table 1. All primers and probes were commercially synthesised (Eurofins).

**Table 1.** Taqman primer and probe sequences used for the qPCR.

| Target [Accession] | Primer/probe sequence 5'→3' | Ann. temp. |
|--------------------|-----------------------------|------------|
| OvHV-1             | F: GTATGGCAGCCGTTAGTTCA     |            |
| [D. Griffiths, G. Russell, unpublished] | R: AAGGCGTAGACGCTTCATCT | 57.3 °C |
|                    | Probe: FAM-CTCGCCTAGCGTCTACAAGCTGTTGC-TAMRA |           |
| BoHV-6             | F: ACCCCGTAAAAGTGATTTACCC   |            |
| [KJ705001]         | R: GTAGTAGCTGATGCTAGCTAGC   | 56.4 °C    |
|                    | Probe: FAM-CAAAAGATCAGAGACGCAAGAG-TAMRA |           |
| OvHV-2             | F: GAGAACAAGCGCTCCCTACTGA   |            |
| [DQ198083]         | R: CGTCAAGCATCTTCATCCAG     | 56 °C      |
|                    | Probe: FAM-AGTGACTCAGACGATACAGCAGCAGCAG-TAMRA |           |
Bison LHV [KX062140] F: GGTTTGCTTCCCTGCTTAAA R: CTCCAAGTCTGCGAGCTGTA Probe: FAM-CTTCCAGCATGGTCCGCC-TAMRA 60 °C

CpHV-2 [NC043059] F: TCAAGAGCAACAGGAACCAG R: CTATGCTGCTCACCACGTTT Probe: FAM-AGGCTGCCAAAGGCGTCCAC-TAMRA

12s-internal DNA [Stahel et al., 2013] F: GCGGTGCTTTATAYCCTTCTAGAG R: TTAGCAAGRATTGGTGAGGTTTATC Probe: VIC-AGCCTGTTCTATAAYCGAT-MGBNFQ 60 °C

FAM: 5′ modification (6-Fluorescein amidite); TAMRA: 3′ modification quencher; VIC: 5′ modification: commercial fluorescent dye, MGBNFQ: 5′ modification (minor groove binder nucleotide quencher); Y: (Pyrimidine) C or T; R: (purine) A or G.

Optimization of qPCR conditions. The reaction mixtures were optimized to contain 7 μl of (2x) TaqMan® universal PCR master mix (Applied Biosystems); the primers and probes were at a concentration of 0.5 pmol in 20 μl total reaction volume. Initially, the optimal annealing temperatures of primers and probes were determined by gradient protocols (DNA Engine Opticon 2, UK) in the range of 55-60°C. The optimal cycling temperature profile in all qPCR assays involved an initial 50°C for 2 min, followed by 13 min at 95°C and 39 cycles of 95°C for 15 sec, finally, 1 min of the optimal annealing temperature (Table 2).

Production of reference plasmids for viral quantification. The constructed reference genes were produced by cloning the PCR products into the commercial vector pCR2.0 TOPO (Invitrogen) according to the manufacturer’s instructions. The initial constructed plasmid was delivered into one vial of chemically competent Escherichia coli (Mach1™ One Shot®, Invitrogen), using the heat-shock method as suggested by the manufacturer. The transformed E. coli was grown to amplify the constructed plasmids that were extracted (Qiagen) and measured using a Qubit fluorimeter (Invitrogen). Normalisation to account for variability in DNA quality and contaminants was performed using primers specific for the ruminant 12s-ribosomal DNA internal genome. Therefore, the exact genome copy number and normalization was determined using limiting dilutions of reference plasmids (see below).

Estimation of assay validity. The intra and inter assay variation was analysed by performing five runs for each reference plasmid in tenfold serial dilutions independently in duplicate starting from 2 x 107 to 2 x 100 copies/2 μl. The assay specificity was determined by cross testing clinical diagnostic samples known to be positive for other herpesviruses. All quantification statistics were analysed by 7500 v 2.3 software (Applied Biosystems) using T-test Mann-Whitney and Fisher’s exact test. The assays were also validated according to MIQE guidelines to include: mean value, standard deviation, coefficients of variation (CV) of the Ct values, and the standard error of means for each.

The data regarding the assay’s repeatability and reproducibility were generated from five identical experiments in which the Ct value of duplicates of each dilution of the standard plasmid was calculated. From these data, it was concluded that these assays were highly specific and sensitive hence the assays were used to quantify viral loads.

Testing of animals. In a first step, the lungs of all animals were tested for the ruminant specific 12s-ribosomal DNA internal gene (reference gene for normalisation of the data) and for all five
selected gamma-herpesviruses. Having only identified BoHV-6 in the lung samples of all animals without MCF, the spleen of all animals was tested for ruminant specific 12s-ribosomal DNA, BoHV-6 and OvHV-2, in a second step. In animals tested positive for BoHV-6 in lungs and/or spleen, bronchial lymph node and tongue were then tested for 12s-ribosomal DNA and BoHV-6.

In cattle with MCF, we applied the same protocol and tested in a first step the lungs for the ruminant specific 12s-ribosomal DNA and for all five selected gamma-herpesviruses. Besides OvHV-2, we detected BoHV-6 in one animal and subsequently tested the remaining organs (spleen, bronchial lymph node and tongue) for OvHV-2 and BoHV-6. Since the BoHV-6 positive animal was gravid, we also tested the spleen and the lung of the foetus for BoHV-6 and all four organs for OvHV-2. In the remaining BoHV-6 positive gravid cows (n=3), lungs and spleen of the foetus were tested for the ruminant specific 12s-ribosomal DNA and BoHV-6.

Statistical analyses
Data were analysed using R version 4.0.5 (2021-03-31). Initially, data were analysed with the Shapiro-Wilk test for normal distribution. Logarithmic transformation was applied to viral counts for all organs to achieve a normal distribution and perform parametric analyses (ANOVA and Pearson’s correlation). For the remaining parameters (age, country of origin, etc) non-parametric analyses were performed using \( \chi^2 \) to examine the association of the parameters with BoHV-6 infection.

3. Results

3.1. Screening of cattle for gammaherpesvirus infection
Screening of the lungs of all test animals without MCF for OvHV-1, OvHV-2, BoHV-6, Cp-HV-2 and Bison LHV revealed infection with BoHV-6 in 115 samples (26%) but did not find any other viruses tested for. The subsequent testing of all spleens for BoHV-6 and OvHV-2 did again only detect BoHV-6, in 107 animals with a positive result in the lungs, and in 27 animals where the lung was negative for BoHV-6 (total of 134 samples; 30%). These results confirmed BoHV-6 infection in a total of 32% (142/448) of the tested non-MCF animals. In addition, one of the four cattle with MCF was found to be co-infected with BoHV-6.

3.2. BoHV-6 infection
BoHV-6 was detected in cattle from all five contributing countries. Table 2A summarises the number and age distribution of animals sampled from each country and the origins of the samples (post mortem examination for diagnostic purposes or sampled at slaughter) and whether BoHV-6 was detected in the collected samples.
The probability to detect BoHV-6 infection in an animal was found to be independent of the country where the sample was collected (\( \chi^2 = 7.3488; p = 0.1186 \)); it was also independent of whether the animal was slaughtered or necropsied (\( \chi^2 = 2.7524; p = 0.097 \)). However, the case cohort collected in Germany comprised by far the lowest proportion of positive cases (22%) whereas the UK cohort had the highest percentage of positive cases (41%). Given the type of sampling (with and without slaughterhouse material) this cannot be considered a reflection of the prevalence in each of these countries.
BoHV-6 positive animals ranged in age from 1 day to 13.62 years, with an average age of 3.86 years (Table 2A). They were significantly older than negative animals (Mann-Whitney test, \( p<0.001 \)) which had an average age of 1.47 years (Table 2B). For further analysis, animals were allocated by age to seven categories, each spanning one year. Figure 1 summarises the age distribution for all tested animals and shows that the proportion of positive animals was increasing with age.
Table 2. Geographical origin of cattle without MCF examined in the present study and the test results for BoHV-6 infection.

A. Age range of the examined cattle. Animals were either diseased and had undergone a diagnostic post mortem examination (“Diagnostics”) or were healthy slaughtered cattle (“Slaughterhouse”).

|                    | Switzerland | UK       | Finland  | Belgium  | Germany  | Total     |
|--------------------|-------------|----------|----------|----------|----------|-----------|
| **Diagnostics**    |             |          |          |          |          |           |
| Age range (average)| 1 d – 12 y  | 2 d - 6 y| 1 d - 10 y| 1 d – 10 y| 1 d – 10 y| 1 d – 13 y|
|                    | (3.1 y)     | (1.46 y) | (2.7 y)  | (1.63 y) | (1.2 y)  | (2.23 y)  |
| BoHV-6 pos        | 43 (30%)    | 17 (33%) | 12 (35%) | 16 (32%) | 15 (22%) | 103 (30%) |
| BoHV-6 neg        | 101         | 34       | 22       | 34       | 54       | 245       |
| Total tested      | 144         | 51       | 34       | 50       | 69       | 348       |

|                    |             |          |          |          |          |           |
| **Slaughterhouse** |             |          |          |          |          |           |
| Age range (average)| 11 mo - 13.6 y | 10 mo - 2.75 y | 1 d - 10 y | 1 d – 10 y | 1 d – 10 y | 1 d – 13 y |
|                    | (2.79 y)    | (1.62 y) | (2.7 y)  | (1.63 y) | (1.2 y)  | (2.22 y)  |
| BoHV-6 pos        | 15 (30%)    | 24 (48%) | 39       |          |          | 39 (39%)  |
| BoHV-6 neg        | 35          | 26       |          |          |          | 61        |
| Total tested      | 50          | 50       |          |          |          | 100       |

|                    |             |          |          |          |          |           |
| **All**            |             |          |          |          |          |           |
| Age range (average)| 11 mo - 13.6 y | 10 mo - 6 y | 1 d - 10 y | 1 d – 10 y | 1 d – 10 y | 1 d – 13 y |
|                    | (3.02 y)    | (1.54 y) | (2.7 y)  | (1.63 y) | (1.2 y)  | (2.22 y)  |
| BoHV-6 pos        | 58 (30%)    | 41 (41%) | 12 (35%) | 16 (32%) | 15 (22%) | 142 (32%) |
| BoHV-6 neg        | 136         | 60       | 22       | 34       | 54       | 306       |
| Total tested      | 194         | 101      | 34       | 50       | 69       | 448       |

Abbreviations: d – days; mo – months; y – years; BoHV-6 pos – PCR positive for BoHV-6 in at least one tested organ; BoHV-6 neg – PCR negative in all tested organs
B. Age and sex of the examined cattle regardless of whether they had undergone a diagnostic post mortem examination or were healthy slaughtered cattle. For 5 animals, the sex was not reported, leading to a total of 443 animals considered for the evaluation.

| BoHV-6 negative | Switzerland | UK | Finland | Belgium | Germany | Total |
|-----------------|-------------|----|---------|---------|---------|-------|
| Age range (average) | 1 d – 12 y (2.07 y) | 2 d - 6 y (1.22 y) | 1 d - 6 y (1.64 y) | 1 d – 9y (11 mo) | 1 d – 6.5y (6 mo) | 1 d – 12 y (1.47 y) |
| Male | 35 | 21 | 6 | 14 | 31 | 107 |
| Female | 101 | 36 | 14 | 20 | 23 | 194 |
| Total | 136 | 57 | 20 | 34 | 54 | 301 |

| BoHV-6 positive | Switzerland | UK | Finland | Belgium | Germany | Total |
|-----------------|-------------|----|---------|---------|---------|-------|
| Age range (average) | 1 mo – 13.6 y (5.24 y) | 2 d - 6 y (2.00 y) | 4 mo- 13 y (4.65 y) | 1 d – 10y (3.12 y) | 1 d – 10y (3.77 y) | 1 d – 13.6 y (3.86 y) |
| Male | 10 | 15 | 1 | 4 | 3 | 33 |
| Female | 48 | 26 | 11 | 12 | 12 | 109 |
| Total | 58 | 41 | 12 | 16 | 15 | 142 |

Figure 1. Age distribution of cattle without MCF examined in the present study, with proportions of BoHV-6 positive and negative animals. Overall, 32% of the tested animals were BoHV-6 positive.

BoHV-6 infection was significantly associated with animal sex ($\chi^2=9.1496$, $p<0.001$), with female animals infected more frequently (36%) than males (24%) (Supplemental Table S1A). There was a significant association of BoHV-6 infection with the animals’ age groups ($\chi^2=95.948$; $p<2.2e-16$), shown in 1-year steps up to >6 years of age, with a significant increase in the proportion of infected animals with age. Based on the Chi2 test (Table 3) it was found that in animals younger than 1 year, the probability of BoHV-6 infection was 0.09 lower than expected, with a goodness of fit test of 9.865 and a p-value of <0.01. On the other hand, older animals (> 6 years), showed a stronger tendency to be positive for BoHV-6 (the probability was 0.04 higher than expected).
Table 3. Frequency distribution of the probabilities for BoHV-6 infection in the different age groups, based on the Chi Square test. Differences (“Expected – Observed”) higher than 0.05 and lower than -0.05 between expected and observed represent an unexpected result, not following the distribution.

| Age group (y) | Expected Neg | Expected Pos | Observed Neg | Observed Pos | Difference Neg | Difference Pos |
|---------------|--------------|--------------|--------------|--------------|----------------|----------------|
| 0<1           | 0.26         | 0.12         | 0.35         | 0.03         | -0.09          | 0.09           |
| 1<2           | 0.15         | 0.07         | 0.15         | 0.07         | 0.00           | 0.00           |
| 2<3           | 0.07         | 0.03         | 0.07         | 0.03         | 0.00           | 0.00           |
| 3<4           | 0.06         | 0.03         | 0.04         | 0.05         | 0.02           | 0.02           |
| 4<5           | 0.05         | 0.02         | 0.03         | 0.04         | 0.02           | -0.02          |
| 5<6           | 0.03         | 0.01         | 0.02         | 0.02         | 0.01           | -0.01          |
| ≥6            | 0.07         | 0.03         | 0.03         | 0.07         | 0.04           | -0.04          |
| All           | 0.68         | 0.32         | 0.68         | 0.32         |                |                |

Abbreviations: y – years; neg – PCR for BoHV-6 negative in all tested organs; pos – PCR for BoHV-6 positive in at least one tested organ

Investigation of the association between BoHV-6 infection and both sex and age group showed that in male animals younger than 1 year a negative result was substantially more frequent than expected (0.11 expected vs 0.61 observed) (Supplemental Table S1B).

Investigation for an association between BoHV-6 infection and the primarily affected organ systems (Supplemental Table S2A) found a significant association ($\chi^2= 25.263; p<0.0042$). The correlation line has the best fit for the following organ systems: body cavities, liver, reproductive system, respiratory system, “others” and “slaughtered animals”. However, there are multiple organ systems involved as well as non-diseased animals, so these apparent associations are likely to be non-specific. There was no significant association between BoHV-6 infection and the disease entity/aetiology (Supplemental Table S2B) as identified in diseased animals during the diagnostic post mortem examination ($\chi^2=8.87, p=0.7141$).

3.3. Viral loads in different organs

All animals tested positive for BoHV-6 in lungs and/or spleen were also tested for the presence of the virus in bronchial lymph nodes (BLN) and tongue. In more than half of the positive cases (n=81; 57%), BoHV-6 DNA was detected in all tested organs. Figure 2 summarises the different combinations of samples where viral DNA was detected and indicates that the combined testing of spleen and lung does reliably detect infection.
Figure 2. Detection of BoHV-6 in the different tested organs of cattle tested positive for the virus (n=142).

Viral copy numbers varied substantially in the different organs and animals, ranging from as few as 0.13-0.45 copies/μL to as much as 47,609 copies/μL in a BLN (Fig. 3). The variation was highest in BLN and spleen whereas it was comparatively limited in lung and tongue (Table 4).

Figure 3. Box plot showing the BoHV-6 copy numbers per μL in each tested organ. Values are expressed as mean±2SD, with outliers (Max and Min), first quartile (q1), median, and third quartile (q3). The graph shows the box plots without the outliers.
Table 4. BoHV-6 copy numbers in the different organs tested by q-PCR.

A. Viral loads in the different organs tested.

|                  | Lung  | Spleen | BLN    | Tongue |
|------------------|-------|--------|--------|--------|
| Mean (copies/μL) | 15    | 73     | 193    | 22     |
| Average (copies/μL) | 196   | 1232   | 1873   | 431    |
| Max (copies/μL)  | 4558  | 46222  | 47609  | 20441  |
| Min (copies/μL)  | 0.13  | 0.14   | 0.45   | 0.41   |
| No tested positive | 115   | 134    | 122    | 102    |
| % tested positive | 81    | 94     | 86     | 72     |

Abbreviations: BLN – bronchial lymph node; copies/μL – BoHV-6 copy numbers/μL; No tested positive – number of animals in which the organ tested positive for BoHV-6 DNA by PCR; % tested positive – percentage of BoHV-6 positive animals in which the organ tested positive.

B. R square value of the correlation found between the different organs based on Log 10 BoHV6 copy numbers.

|          | Lung | Spleen | BLN | Tongue |
|----------|------|--------|-----|--------|
| Lung     | 1    |        |     |        |
| Spleen   | 0.67 | 1      |     |        |
| BLN      | 0.50 | 0.60   | 1   |        |
| Tongue   | 0.46 | 0.47   | 0.50| 1      |

Abbreviations: BLN – bronchial lymph node

Based on the geometric means obtained from the positive organs, the viral load was overall highest in the BLN, followed by the spleen, the tongue and the lung (Table 4).

Transformation to decimal logarithm served to normalize the data and find associations between the viral load found in the different organs using the Shapiro-Wilk Normality test. This did not reveal a significant correlation for viral copy numbers between the different organs (R2 below 0.75). The highest R2 was found between lung and spleen (R2 = 0.67) indicating a high probability to find both organs infected once one was found to be positive. The results are shown in Table 4B and C and in Figure 4.
Analysis of variance indicated that there was no significant association between the viral loads of any of the tissues tested and the different population parameters (age, sex or country of origin, disease category/affected organ system).

In the three gravid BoHV-6 positive cows (aged 6, 7 and 12 years), viral copy numbers in the tested organs/tissue were consistently very low (max. 25.2 viral copies/μL) and thereby substantially lower than average. In none of the gravid cows BoHV-6 DNA was detected in lungs and spleen of the foetus.

3.4. Cattle with MCF

All animals with confirmed MCF carried OvHV-2 DNA in all tested tissues (lung, BLN, spleen and tongue), with highly variable viral copy numbers, ranging from less than 1,000 copies/μL in spleen and BLN in one animal to 77,351 copies/μL in the lung in another. One pregnant cow, a 5 year old Braunvieh with MCF was found to be co-infected with BoHV-6. This animal showed the comparatively highest OvHV-2 copy numbers in all tested organs (lung 77,351 copies/μL; spleen 36,829 copies/μL; BLN 32,312 copies/μL; tongue 10,556 copies/μL) and carried BoHV-6 DNA in all tested organs, though with low viral copy numbers, ranging from 14.63 copies/μL in the lung to 529.74 copies/μL in the BLN. The organs of the fetus (SSL 49 cm; approx. 5 months of pregnancy) carried OvHV-2 with low viral loads: lung 9.52 copies/μL; spleen 4.24 copies/μL; lymph node 2.78 copies/μL, tongue 4.98 copies/μL and were negative for BoHV-6.

An overall comparison of OvHV-2 loads in the tested organs of cattle with MCF with BoHV-6 loads in the same organs of the non-MCF cattle showed that MCF is associated with significantly higher viral OvHV-2 loads than BoHV-6 infection in cattle (Supplemental Table S3).

4. Discussion

In the present study, 448 slaughtered or diseased cattle without MCF were examined for infection with gammaherpesviruses known to infect cattle (OvHV-1, OvHV-2, BoHV-6,
CpHV-2, Bison LHV) in tissues that have previously been shown to carry these viruses [8; 19], using qPCR. The aim was to gather data on the prevalence of infections and evidence of potential protection of animals infected with one gammaherpesvirus from infection by related viruses.

Taqman q-PCR assays were used to detect and quantify the viruses in question. In the present study, it allowed the detection of very low infection levels, down to 1 copy/µL DNA of all viruses tested for.

Interestingly, we only detected BoHV-6, confirming previous studies that cattle are its natural host. The overall prevalence of 32% in our study cohort from countries across Europe is similar to the prevalence previously reported from the USA and Poland [9; 14; 15]. Although our study is based on a large cohort of cattle from five different countries across Europe and thus also from different forms of husbandry and production, no significant difference was identified between the countries with regards to the rate of BoHV-6 infection. A potential breed predisposition could not be fully investigated due to partially incomplete and highly variable information, but a basic screen did not provide any evidence of the latter (data not shown). However, BoHV-6 infection was significantly associated with age, showing an increasing prevalence in older animals. BoHV-6 infection was also associated with sex, as the percentage of positive animals was significantly higher in female cattle. However, it has to be considered that the older age groups had a lower proportion of male animals.

Previous studies on BoHV-6 infection mainly focused on its association with other infections or diseases. Rovnak et al. [9] who were the first to detect the virus, found viral DNA in blood cells of 94% and 87% of cattle seropositive and -negative for BLV, respectively, and in overall 91% of adult cattle and 38% of calves that they had collected from dairy herds in the USA (Colorado, New York, New Jersey) over a 13 year period. Since they also detected the virus in 63% of BLV-positive lymphomas the authors proposed BoHV-6 as a co-factor in the pathogenesis of BLV and proposed the name bovine lymphotropic herpesvirus [9]. Other studies focussed on the association of BoHV-6 with the reproductive tract. Banks et al. [11] examined vaginal swabs and exudates of cows from 13 herds with a history of post-partum metritis (PPM) that did not respond to standard treatments, and detected BoHV-6 in 27% of either sample types. While they considered this as indication of a potential role of BoHV-6 in PPM, they were aware that the correlation could be random as they did not examine any PPM-unaffected herds [11]. Similarly, the present study found an association with pathological processes in the reproductive tract. However, these represented variable changes in both BoHV-6 positive animals (mastitis (n=5), dystocia (n=2), each 1 uterine rupture and spermic granuloma) and BoHV-6 negative animals (mastitis (n=4), metritis (n=2), each 1 metritis and mastitis, retained placenta and endometritis, and uterine rupture). Different from OvHV-2 which we could also detect in foetal tissues in a gravid cow with MCF, BoHV-6 was not detected in lung or spleen of the foetuses in the four BoHV-6 positive gravid cows in the present study, indicating that the virus is not transmitted transplacentally. There is one previous report that described a case of an aborted foetus in Canada in which BoHV-6 was found in brain and lymph nodes [12]. However, the foetus was found to also be infected with Neospora caninum, a protozoan parasite leading to abortion in the earlier phase of pregnancy and inducing placental epithelial necrosis, serum leakage and maternal mononuclear cell infiltration [20]. Destruction of the placental barrier could therefore have allowed BoHV-6 to spread into foetal tissue. However, we found BoHV-6 infection in one of the six 1 day old calves (and overall in 3/45 calves aged between 1 and 15 days), which suggests that infection can occur perinatally and/or shortly after birth. However, the prevalence of infection increased with age, and there were no age-associated differences in viral loads.

More general surveys on BoHV-6 prevalence, similar to the present study, have previously been undertaken in the USA. In 2000, Collins and co-authors detected BoHV-6 in blood leukocytes in 52–79% dairy cows in four different herds in Colorado, USA, backing the older study by Rovnak and coworkers [9]. In the present study, the prevalence of BoHV-6 was substantially lower, ranging between 22% and 41% depending on the country. However, the case selection varied significantly, since we examined animals that were either necropsied due to disease or
slaughtered, whereas the other studies examined healthy dairy cows or samples collected as part of BLV focussed studies [9, 14]. Nonetheless, the present study provides clear evidence that BoHV-6 is endemic in cattle across Europe. We found an association with a range of primarily affected organs, not only the reproductive tract as discussed above, but also, for example, the respiratory tract or the liver, again with heterogenous processes, and also with being a slaughtered animal. However, it does not indicate association with specific diseases at all. BoHV-6 seems to differ from other gammaherpesviruses; for example, ovine herpesvirus 2 which is very widespread in the domestic sheep population with more than 90% of animals reported to be persistently infected [4].

Experimental studies using murine gammaherpesvirus-68 (MHV-68) and OvHV-2 in their natural hosts have suggested that gammaherpesviruses initially replicate in the lungs; Subsequent latency occurs in lymphoid tissues [21, 22]. For BoHV-6, the sites of initial replication and latency have not yet been identified. The present study provides evidence that lungs and lymphatic tissues are involved and confirm the systemic spread of the virus in natural infections.

Further studies are needed to identify the viral target cells in the tissues and to identify the sites of viral persistence/latency. So far, we do not know how BoHV-6 is transmitted, but detection of viral DNA in the tongue where we primarily sampled the epithelium suggests that it might also shed orally, which would be consistent with contact transmission, as known for most herpesviruses. In contrast to studies on OvHV-2 in their natural host where generally low viral loads were detected [5, 19], the present study detected not only highly variable, but partly high BoHV-6 loads in tissues, reaching a maximum of more than 47,000 copies per 100 ng DNA. This variability could be due to the heterogeneity of the tested cattle population and a variable length of infection; animals were infected at an unknown time point and could have been exposed to variable initial viral doses.

The study also included a few MCF cases, to compare OvHV-2 loads in diseased animals as dead-end hosts of the virus. Indeed, all MCF cases exhibited higher mean OvHV-2 loads in the tissues than BoHV-6 loads in non-MCF cattle, though the maximum copy number in spleen and BLN were lower. Also the cattle with MCF were single-infected, with one exception, a gravid cow that was also positive for BoHV-6, but with low viral loads. These results are similar to those of a previous study which found BoHV-6 co-infection in 1/18 cattle and 3/21 bison with MCF (spleen and/or lymph node), but observed no significant correlation [14].

Previous studies have detected OvHV-2 in healthy domestic cattle [19, 23]. To our surprise, we did not detect OvHV-2 or OvHV-1, in any cattle without MCF in the present study, a result which is in contrast to those of previous studies [8, 19, 24, 25]. This discrepancy is most likely not due to the method of testing, as we applied the highly sensitive quantitative for all viruses that was established in the study of Al-Saadi [8]. An explanation could be that the farmers in Europe are by now well aware of the risk of MCF due to contact of cattle with sheep and therefore may attempt to prevent the latter, knowing that this could prevent an MCF outbreak in the farm. The fact that MCF cases were rare among the necropsied cattle during the last few years in all diagnostic laboratories contributing to the study supports this interpretation.

Transmission of OvHV-2 to susceptible animals is predominantly horizontal [4], with nasal dissemination being demonstrated experimentally [26, 27]. Although reports confirming the vertical transmission of OvHV-2 in cattle are sparse, viral DNA has been detected in an asymptomatic calf born to a cow with sheep associated-MCF [28] and in the brain of the foetus of a cow with MCF [29]. In our study we detected OvHV-2 DNA in the organs of a foetus from a gravid cow with MCF, which provides further evidence of vertical transmission of OvHV-2 in naturally infected cattle. In contrast to this, we did not find any evidence of vertical transmission of BoHV-6, since all foetuses from BoHV-6 positive gravid cows were negative for BoHV-6. Consequently, horizontal transmission remains the most likely route of BoHV-6 infection to susceptible animals.
5. Conclusions

BoHV-6 is the only gammaherpesvirus endemic in European cattle populations with relatively high prevalence. There is no evidence that infection is associated with any disease processes, suggesting that BoHV-6 is a commensal in cattle.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: BoHV-6 infection in female and male animals and in the different age groups. Table S2: Frequency of BoHV-6 infection in animals grouped according to the main affected organ system or the disease entity, based on recorded pathological diagnoses. Table S3: Comparison of OvHV-2 loads in lung, spleen, bronchial ln and tongue of cattle with MCF with BoHV-6 loads in the same organs in cattle without MCF.

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Institutional Review Board Statement: The study was undertaken on material collected from slaughtered animals or from animals that underwent a diagnostic post mortem examination. For studies using such material, ethical review and approval is not required.

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Supplementary Table S1. BoHV-6 infection in female and male animals and in the different age groups. The sex was not known for 5 of the 448 examined animals.

A. BoHV-6 infection in male and female animals.

| Sex   | Neg | Pos | All |
|-------|-----|-----|-----|
| Male  | 107 | 33  | 140 |
| Female| 194 | 109 | 303 |
| Total | 306 | 142 | 443 |

neg – PCR for BoHV-6 negative in all tested organs; pos – PCR for BoHV-6 positive in at least one tested organ

B. Frequency of the probabilities for BoHV-6 infection in the different age and sex groups, based on the Chi2 test. Differences (“Expected – Observed”) higher than 0.05 and lower than -0.05 between expected and observed (in bold) represent an unexpected result, not following the distribution.

| Age group (y) | Male Expected | Male Observed | Female Expected | Female Observed |
|---------------|---------------|---------------|-----------------|-----------------|
| 0 <1          |               |               |                 |                 |
| Neg           | 0.11          | 0.01          | 0.61            | 0.06            |
| Pos           | 0.06          | 0.24          | 0.02            | 0.22            |
| Neg           | 0.22          | 0.02          | 0.02            | 0.02            |
| 1 <2          |               |               |                 |                 |
| Neg           | 0.05          | 0.02          | 0.12            | 0.13            |
| Pos           | 0.13          | 0.10          | 0.05            | 0.16            |
| Neg           | 0.16          | 0.05          | 0.05            | 0.05            |
| 2 <3          |               |               |                 |                 |
| Neg           | 0.02          | 0.01          | 0.03            | 0.05            |
| Pos           | 0.05          | 0.02          | 0.10            | 0.03            |
| 3 <4          |               |               |                 |                 |
| Neg           | 0.01          | 0.01          | 0.00            | 0.01            |
| Pos           | 0.01          | 0.03          | 0.03            | 0.06            |
| 4 <5          |               |               |                 |                 |
| Neg           | 0.01          | 0.01          | 0.01            | 0.02            |
| Pos           | 0.02          | 0.03          | 0.04            | 0.05            |
| 5 <6          |               |               |                 |                 |
| Neg           | 0.00          | 0.01          | 0.00            | 0.01            |
| Pos           | 0.01          | 0.02          | 0.02            | 0.04            |
| ≥6            |               |               |                 |                 |
| Neg           | 0.01          | 0.02          | 0.00            | 0.02            |
| Pos           | 0.05          | 0.05          | 0.05            | 0.11            |
Abbreviations: y – years; neg – PCR for BoHV-6 negative in all tested organs; pos – PCR for BoHV-6 positive in at least one tested organ
**Supplemental Table S2.** Frequency of BoHV-6 infection in animals grouped according to the main affected organ system or the disease entity, based on recorded pathological diagnoses.

**A.** Detection of BoHV-6 infection in animals grouped according to the primarily affected organ systems.

| Affected organ system                          | Neg | Pos | All |
|-----------------------------------------------|-----|-----|-----|
| No abnormality detected                      | 6   | 0   | 6   |
| No information available                     | 16  | 2   | 18  |
| Systemic disease and/or multiple organs affected | 36  | 9   | 45  |
| Musculoskeletal                               | 18  | 9   | 27  |
| Liver                                        | 9   | 7   | 16  |
| Gastrointestinal                             | 85  | 23  | 108 |
| Cardiovascular                               | 11  | 4   | 15  |
| Body cavities                                | 8   | 9   | 17  |
| Respiratory                                  | 39  | 21  | 60  |
| Reproductive                                 | 9   | 9   | 18  |
| Others                                       | 8   | 10  | 18  |
| Slaughtered animals                          | 61  | 39  | 100 |
| **Total**                                    | 306 | 142 | 448 |

Others: Due to the low number of cases, animals which showed primary affection of the haemolymphatic, neurological or urinary system or the integument were assembled under this heading.

Abbreviations: y – years; neg – PCR for BoHV-6 negative in all tested organs; pos – PCR for BoHV-6 positive in at least one tested organ.
B. Detection of BoHV-6 infection in animals grouped according to the disease entity or aetiology.

| Disease entity/etiology | Neg | Pos | All  |
|-------------------------|-----|-----|------|
| Slaughtered animals     | 67  | 39  | 106  |
| Unknown                 | 43  | 13  | 56   |
| Trauma                  | 23  | 11  | 34   |
| Congenital              | 5   | 2   | 7    |
| Metabolic               | 6   | 6   | 12   |
| Degenerative            | 2   | 2   | 4    |
| Toxic                   | 2   |     | 2    |
| Bacterial               | 105 | 48  | 153  |
| Parasitic               | 11  | 5   | 16   |
| Viral                   | 10  | 2   | 12   |
| Neoplastic              | 2   | 2   | 4    |
| Organ misalignment      | 15  | 7   | 22   |
| Multifactorial          | 15  | 5   | 20   |
| Total                   | 306 | 142 | 448  |

Abbreviations: y – years; neg – PCR for BoHV-6 negative in all tested organs; pos – PCR for BoHV-6 positive in at least one tested organ
**Supplemental Table S3.** Comparison of OvHV-2 loads in lung, spleen, bronchial ln and tongue of cattle with MCF with BoHV-6 loads in the same organs in cattle without MCF.

|             | Lung           | Spleen         | BLN             | Tongue          |
|-------------|----------------|----------------|-----------------|-----------------|
| MCF (OvHV-2) | 10,063.46      | 15.35          | 13,749.81       | 72.67           |
| Non-MCF (OvHV-2) | 4,430.70      | 193.08         |                  |                 |
| MCF (BoHV-6)  | 17,320.12      | 21.58          |                  |                 |
| Non-MCF (BoHV-6) | 17,320.12      | 21.58          |                  |                 |

| Mean (copies/μL) | 25,135.96 | 195.66 | 16,294.11 | 1,232.27 | 10,530.52 | 1,873.49 | 19,235.23 | 431.10 |
| Average (copies/μL) | 25,135.96 | 195.66 | 16,294.11 | 1,232.27 | 10,530.52 | 1,873.49 | 19,235.23 | 431.10 |
| Max (copies/μL) | 77,350.72 | 4,558.00 | 36,829.21 | 46,222.00 | 32,312.40 | 47,609.00 | 37,262.72 | 20,441.00 |
| Min (copies/μL) | 3,066.22 | 0.13 | 847.59 | 0.14 | 948.26 | 0.45 | 5,037.91 | 0.41 |
| No tested positive | 4 | 115 | 4 | 134 | 4 | 122 | 4 | 102 |

Abbreviations: copies/μL – BoHV-6 or OvHV-2 copy numbers/μL; No tested positive – number of animals in which the organ tested positive for BoHV-6/OvHV-2 DNA by PCR.
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