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NOTE

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Latent *bovine herpesvirus* 1 and 5 in milk from naturally infected dairy cattle

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ABSTRACT. *Bovine herpesvirus* 1 and 5 (BoHV-1 and -5) are antigenically and genetically related and can establish latent infection. We aimed to analyze the applicability of the milk sample to detect latently BoHV-infected cattle. BoHV-1 non-vaccinated clinically healthy cows from five dairy cattle herds (herd 1, n=24; herd 2, n=39; herd 3, n=39; herd 4, n=36; herd 5, n=70) were studied. We confirmed the presence of BoHV-1, and for the first time, BoHV-5 in the milk of naturally infected dairy cattle.

KEY WORDS: *bovine herpesvirus* 1, *bovine herpesvirus* 5, milk, naturally infected dairy cattle, virus latency

Bovine herpesvirus 1 (BoHV-1), the causative agent of infectious bovine rhinotracheitis (IBR), is widely shed in beef and dairy herds of all ages worldwide [1]. In comparison, *bovine herpesvirus* 5 (BoHV-5) has a more limited geographic distribution, mainly in Brazil [15] and Argentina [16], causing bovine meningoencephalitis.

Both BoHV-1 and BoHV-5 are genetically and antigenically related *Alphaherpesviruses* [4]. They can remain dormant until viral reactivation because of host immunosuppression [8]. Their ability to maintain a latent infection occurs in neural and non-neural sites, such as peripheral blood leukocytes [7]. Similar to blood, milk also contains leukocytes [2], and therefore, it could be a useful sample type for analysis of viral shedding, but there are still few studies about its use. There are previous studies that showed BoHV-1 isolation from milk samples [13, 19], but there is no study about BoHV-5 in milk. As milk is easy to collect and could be a BoHV transmission route among animals, it is important to confirm the role of milk sampling as a method to detect BoHV infection in dairy cattle.

The aim of this work was to evaluate the possibility of using milk to detect latent BoHV-1 and BoHV-5 in naturally infected dairy cattle through viral molecular analysis.

This study was performed in the Zona da Mata region, Minas Gerais−Brazil, and it was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Viçosa. The sample size was calculated by Epi Info® software (version 3.5.1−Centers for Disease Control and Prevention (CDC), Atlanta, GA, U.S.A.), with a 95% confidence level and 5% confidence interval, based on a design prevalence rate of 12% at minimum, according to a previous study that reported BoHV-1 and BoHV-5 coinfection prevalence [17]. Blood and milk samples were collected from non-BoHV-1-vaccinated and clinically healthy cows from five dairy cattle herds (herd 1, n=24; herd 2, n=39; herd 3, n=39; herd 4, n=36; and herd 5, n=70). These 208 cows were chosen at the authors' convenience.

We utilized the virus neutralization test (VNT) and nested polymerase chain reaction (N-PCR) assay to classify the animals as virus (BoHV-1 and BoHV-5)-positive or virus (BoHV-1 and BoHV-5)-negative. Although the VNT does not distinguish the

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Table 1. Polymorphisms of glycoprotein B of bovine herpesvirus

Isolate	GenBank accession	810 [aa residue]	858	876	879	Specie	Herd
Reference	NC_001847	T [270D]	T	A	A	BoHV1	-
S2	KM252874	T [270D]	T	A	A	BoHV1	1
S3	KM252875	T [270D]	T	A	A	BoHV1	1
S4	KM252876	T [270D]	T	A	A	BoHV1	1
S6	KM252877	T [270D]	T	A	A	BoHV1	2
S7	KM252878	T [270D]	T	A	A	BoHV1	2
S8	KM252879	T [270D]	T	A	A	BoHV1	2
S11	KM252882	T [270D]	T	A	A	BoHV1	2
S12	KM252883	T [270D]	T	A	A	BoHV1	3
S13	KM252884	T [270D]	T	A	A	BoHV1	3
S14	KM252885	T [270D]	T	A	A	BoHV1	3
S15	KM252886	T [270D]	T	A	A	BoHV1	3
S17	KM252887	T [270D]	T	A	A	BoHV1	4
S19	KM252888	T [270D]	T	A	A	BoHV1	4
S20	KM252889	T [270D]	T	A	A	BoHV1	4
S21	KM252890	T [270D]	T	A	A	BoHV1	4
S26	KM252894	T [270D]	T	A	A	BoHV1	5
S27	KM252895	T [270D]	T	A	A	BoHV1	5
Reference	NC_005261	G [270E]	G	G	G	BoHV5	-
S9	KM252880	G [270E]	G	G	G	BoHV5	2
S10	KM252881	G [270E]	G	G	G	BoHV5	2
S23	KM252891	G [270E]	G	G	G	BoHV5	5
S24	KM252892	G [270E]	G	G	G	BoHV5	5
S25	KM252893	G [270E]	G	G	G	BoHV5	5

The columns indicate the polymorphic sites of the glycoprotein B gene. The substitutions in the protein are indicated between brackets. The reference isolates correspond to the reference genomes of BoHV1 and BoHV5 available at the GenBank RefSeq database.

immune response between BoHV-1 and BoHV-5, it is the test of choice for antibody detection against BoHV according to the OIE [12]. Serum sample preparation and the VNT assay were performed according to OIE instructions [12] using the BoHV-1 LA viral strain and MDBK cell line.

There are several methods to discriminate BoHV-1 and BoHV-5, such as high-resolution melting (HRM) analysis [9], multiplex real time PCR [5], restriction site mapping of viral DNA, cross-neutralization tests, and monoclonal antibody reactivity [3]. As each technique has advantages and disadvantages, we utilized automatic sequencing and N-PCR assays because they are low cost and well established techniques in the scientific community. Moreover, N-PCR is a very sensitive and specific technique [6], and is recommended in situations where there is a very low quantity of DNA, which is the case during latent infection. We performed milk DNA extraction (Wizard® SV Genomic DNA Purification System, Promega Corp., Madison, WI, U.S.A.) following the manufacturer's instructions to perform the N-PCR assay. BoHV-1 and BoHV-5 DNA were amplified by oligonucleotides as previously described [6].

Milk samples were submitted to sequencing and polymorphism analyses. The contiguous sequences were assembled using CLC Genomics Workbench version 6.5.1 (CLC Bio, Aarhus, D.K.) and submitted to GenBank. For comparison purposes and polymorphism analysis, the sequences of 16 strains of BoHV-1 and 2 strains of BoHV-5 were downloaded from GenBank and aligned using MAFFT version 7.130. In order to determine the type of the virus in the infected animals (BoHV-1 and/or BoHV-5 infection), we performed a polymorphism analysis. Despite the fact that BoHV strains share highly conserved sequences of glycoprotein B, we found three synonymous substitutions and one non-synonymous substitution that allowed for the differentiation between BoHV-1 and BoHV-5 strains (Table 1). Analyzing the sequences of BoHV, we found that BoHV-1 strains have an aspartate (D) residue on position 270 of glycoprotein B, while BoHV-5 strains have a glutamate (E) residue.

To quantify the viral load contained in milk samples, real time PCR assays were performed under universal conditions using previously described primers [18] for the milk samples with positive result in N-PCR assays (herd 1, n=9; herd 2, n=16; herd 3, n=18; herd 4, n=27; herd 5, n=30). The samples presenting a cycle threshold higher than 38 were considered negative.

As a result, all herds showed positive results for VNT (herd 1=54.16%; herd 2=23.07%; herd 3=51.28%; herd 4=44.44%; and herd 5=67.14%), as shown in previous studies [1, 10]. Furthermore, we observed for the first time a wide frequency of positivity of BoHV-1 and/or BoHV-5 DNA in milk among the herds through N-PCR analysis (herd 1=37.5%; herd 2=41.02%; herd 3=46.15%; herd 4=75.0%; and herd 5=42.85%). However, any clinical signs of BoHV-1 or BoHV-5 were reported, and all animals maintained normal production on the farms. Thus, these facts indicate that those animals that tested positive were latently infected (BoHV is in the host, but there is no replication, antigen production, or clinical signs) [8]. Previous studies showed latency of BoHV-1 [11] and BoHV-5 [7] in peripheral blood leukocytes. Considering the leukocytes in milk, this information strengthens our suggestion of

	Herd 1		
	N-PCR-positive	N-PCR-negative	Total
VNT-positive	5	8	13
VNT-negative	4	7	11
Total	9	15	24

	Herd 2		
	N-PCR-positive	N-PCR-negative	Total
VNT-positive	3	6	9
VNT-negative	13	17	30
Total	16	23	39

	Herd 3		
	N-PCR-positive	N-PCR-negative	Total
VNT-positive	7	13	20
VNT-negative	11	8	19
Total	18	21	39

	Herd 4		
	N-PCR-positive	N-PCR-negative	Total
VNT-positive	12	4	16
VNT-negative	15	5	20
Total	27	9	36

	Herd 5		
	N-PCR-positive	N-PCR-negative	Total
VNT-positive	19	28	47
VNT-negative	11	12	23
Total	30	40	70

	All herds (1, 2, 3, 4, 5)		
	N-PCR-positive	N-PCR-negative	Total
VNT-positive	46	59	105
VNT-negative	54	49	103
Total	100	108	208

Fig. 1. Contingency tables showing comparison between results obtained from the virus neutralization test (VNT) on sera samples and from nested polymerase chain reaction (N-PCR) on milk samples in herds 1, 2, 3, 4, and 5. Kappa index to check the agreement between both diagnostic tests: herd 1=0.020; herd 2 = −0.079; herd 3 = −0.228; herd 4=0.0; herd 5 = −0.062; herds 1, 2, 3, 4, 5 = −0.086.

using milk samples to detect latent infection in dairy cows.

The Kappa index was measured to check the agreement between the VNT test and N-PCR assay for each herd, and all the herds as a whole, respectively. The Kappa indices found were: herd 1=0.020, herd 2 = −0.079, herd 3 = −0.228, herd 4=0.0, herd 5 = −0.062, and herds 1, 2, 3, 4, 5 = −0.086 (Fig. 1). These results imply that there are low degrees of agreement between these two tests. The possible reasons for samples presenting a positive VNT/negative N-PCR and negative VNT/positive N-PCR analysis might be the low viral load, which cannot be detected by the N-PCR assay and low antibody level that cannot be detected by the VNT, respectively. Correspondingly, a previous study also showed a low Kappa index (k=0.13) when working with the VNT and nucleic acid detection in the trigeminal ganglia (*post mortem* analysis) [14]. In our study, we provide an *in vivo* analysis. In the future, it is also important to measure the Kappa index between blood and milk samples for each test.

Furthermore, because of the low viral load (shown below), the detection of anti-BoHV-1 and/or BoHV-5 antibodies, and the absence of clinical signs of herpesvirus infection in the animals, it is clear that there was latent infection. In this case, the detection of antibodies against herpesviruses indicates that the animals had latent infection, meaning that there was antibody production during an acute infection at some time in the past [14].

Even though VNT is the test of choice for antibody detection against BoHV according to the OIE [12], it is not designed, by itself, to detect latently infected animals. It is necessary to develop more sensitive serological methods and standardization of the molecular assay, which has higher sensitivity [14] and would also be important to detect the latent infection. In order to diagnose BoHV latent infection *in vivo*, molecular assays should be performed on tissues such as peripheral blood leukocytes, which are non-neural sites of latent infection maintenance and can be collected from blood and milk [2]. The advantage of considering milk samples is the practical and non-invasive *in vivo* collection.

We detected both BoHV-1 and BoHV-5 strains in milk from naturally infected dairy cattle (Table 1): the number of BoHV-1 strains identified in each herd was 3, 4, 4, 4, and 2 for herds 1 to 5, respectively. Meanwhile, the number of BoHV-5 strains was 2 and 3 for herds 2 and 5, respectively. Both types of BoHV were found in herds 2 and 5. A previous study reported BoHV-1 shedding into milk from experimentally infected dairy cows [13]; however, to our knowledge, the present study is the first report of BoHV-5 detection in milk from naturally infected dairy cattle.

There was a measurable amount of BoHV-1 and/or BoHV-5 DNA in all herds (herd 1=18.04 [SD=9.84]; herd 2=12.33 [SD=6.04]; herd 3=22.69 [SD=7.99]; herd 4=41.42 [SD=7.91]; herd 5=11.1 [SD=5.41] DNA copies/ml of milk) (Fig. 2). The low

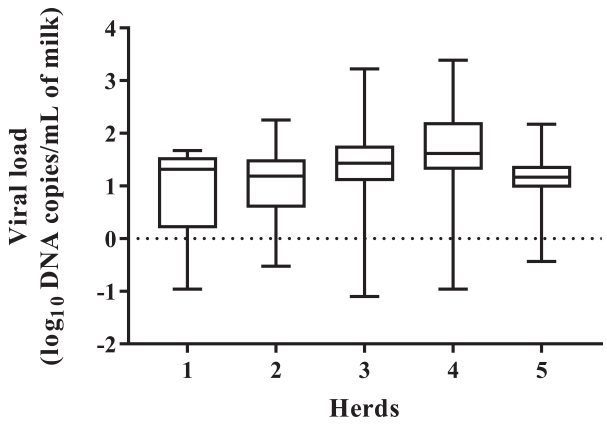

Fig. 2. BoHV-1 and/or BoHV-5 load represented by $\log_{10}$ DNA copies/m*l* of milk in each herd (1, 2, 3, 4, 5). Herd 1=18.04 [SD=9.84]; herd 2=12.33 [SD=6.04]; herd 3=22.69 [SD=7.99]; herd 4=41.42 [SD=7.91]; herd 5=11.1 [SD=5.41] $\log_{10}$ DNA copies/m*l* of milk.

loads observed in the present study imply latent infection (no viral replication).

Our findings are important to further understand BoHV infection in naturally infected animals. Milk seems to be a suitable sample for the viral nucleic acid detection as a more sensitive test compared to the serological method, especially for the diagnosis of latent infection. Moreover, our analysis highlighted the presence of BoHV-5 in cattle milk for the first time and confirmed BoHV-1 in the same type of sample, indicating that it is an important source of virus shedding.

CONFLICT OF INTEREST. The authors declare there is no conflict of interest regarding this study.

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