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
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

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.
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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...
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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REFERENCES

1. Ackermann, M. and Engels, M. 2006. Pro and contra IBR-eradication. Vet. Microbiol. 113: 293–302. [Medline] [CrossRef]
2. Corner, A. H., Greig, A. S. and Hill, D. P. 1967. A histological study of the effects of the herpesvirus of infectious bovine rhinotracheitis in the lactating bovine mammary gland. Can. J. Comp. Med. Vet. Sci. 31: 320–330. [Medline]
3. Del Médico Zajac, M. P., Ladeifa, M. F., Kotsias, F., Muytkens, B., Thiry, J., Thiry, E. and Romera, S. A. 2010. Biology of bovine herpesvirus 5. Vet. J. 184: 138–145. [Medline] [CrossRef]
4. Delhon, G., Moraes, M. P., Lu, Z., Afonso, C. L., Flores, E. F., Weiblen, R., Kutish, G. F. and Rock, D. L. 2003. Genome of bovine herpesvirus 5. J. Virol. 77: 10339–10347. [Medline] [CrossRef]
5. Diel, D. G., Almeida, S. R., Brum, M. C. S., Dezengrini, R., Weiblen, R. and Flores, E. F. 2007. Acute and latent infection by bovine herpesvirus type 5 in experimentally infected goats. Vet. Microbiol. 121: 257–267. [Medline] [CrossRef]
6. Favier, P. A., Marin, M. S., Morán, P. E., Odeón, A. C., Verna, A. E. and Pérez, S. E. 2014. Latency of bovine herpesvirus type 5 (BoHV-5) in tonsils and peripheral blood leukocytes. Vet. J. 202: 134–140. [Medline] [CrossRef]
7. Jones, C., da Silva, L. F. and Sinani, D. 2011. Regulation of the latency-reactivation cycle by products encoded by the bovine herpesvirus 1 (BHV-1) latency-related gene. J. Neurovirol. 17: 535–545. [Medline] [CrossRef]
8. Marin, M. S., Quintana, S., Leunda, M. R., Recavavaren, M., Pagnuco, I., Spáth, E., Pérez, S. and Odeón, A. 2016. A new method for simultaneous detection and discrimination of Bovine herpesviruses types 1 (BoHV-1) and 5 (BoHV-5) using real time PCR with high resolution melting (HRM) analysis. J. Virol. Methods 227: 14–22. [Medline] [CrossRef]
9. Médici, K. C., Alfieri, A. A. and Alfieri, A. F. 2000. Prevalência de anticorpos neutralizantes contra o herpesvírus bovino tipo 1, decorrente de infeção natural, em rebanhos com distúrbios reprodutivos. Cienc. Rural 30: 347–350. [CrossRef]
10. Nyaga, P. N. and McKercher, D. G. 1979. Pathogenesis of bovine herpesvirus-1 (BHV-1) infections: interactions of the virus with peripheral bovine blood cellular components. Comp. Immunol. Microbiol. Infect. Dis. 2: 587–602. [Medline] [CrossRef]
11. OIE—World Organization for Animal Health Infectious bovine rhinotracheitis/infectious postural vulvovaginitis. 2010. Manual of diagnostic tests and vaccines for terrestrial animals. p. 17. Chapter 2.4.13, OIE Terrestrial Manual.
12. Probst, U., Wyler, R., Kühn, U., Ackermann, M., Bruckner, L., Müller, H. K. and Ehrensperger, F. 1985. [Excretion of IBR virus, especially in milk, in experimentally infected cows]. Schweiz. Arch. Tierheilkd. 127: 723–733 (in German). [Medline]
13. Puente, R., Campos, F. S., Furtado, A., Torres, F. D., Franco, A. C., Maisonnave, J. and Roche, P. M. 2016. Comparison between DNA detection in trigeminal nerve ganglia and serology to detect cattle infected with bovine herpesviruses types 1 and 5. PLoS One 11: e0155941. [Medline] [CrossRef]
14. Rissi, D. R., Oliveira, F. N., Rech, R. R., Pierzan, F., Lemos, R. A. A. and Barros, C. S. L. 2006. Epidemiologia, sinais clínicos e distribuição das lesões encefálicas em bovinos afetados por meningoencefalite por herpesvírus bovino-5. Pesqui. Vet. Bras. 26: 123–132. [CrossRef]
15. Schudel, A. A., Carrillo, B. J., Wyler, R. and Metzler, A. E. 1986. Infections of calves with antigenic variants of bovine herpesvirus 1 (BHV-1) and neurological disease. Zentralbl. Veterinarmed. B. 33: 303–310. [Medline]
16. Silva, A. M. 2013. Detecção, isolamento e caracterização molecular de herpesvírus bovino tipos 1 e 5 de bovinos do Estado de Goiás, Brasil. Thesis (PhD). Universidade Federal de Goiás. https://ppgca.evz.ufg.br/upo/67/0/Tese2013_Adriana_Moraes.pdf [accessed on February 9, 2017].
17. Wang, J., O’Keefe, J., Orr, D., Loth, L., Banks, M., Wakeley, P., West, D., Carol, R., Ibata, G., Van Maanen, K., Thoren, P., Isaksen, M. and Kerkhofs, P. 2008. An international inter-laboratory ring trial to evaluate a real-time PCR assay for the detection of bovine herpesvirus 1 in extended bovine semen. Vet. Microbiol. 126: 11–19. [Medline] [CrossRef]
18. Wellenber, G. J., van der Poel, W. H. M. and Van Oirschot, J. T. 2002. Viral infections and bovine mastitis: a review. Vet. Microbiol. 88: 27–45. [Medline] [CrossRef]