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Molecular detection of bovine alphaherpesvirus 1 and 5 in the brain of cattle with and without neurological disease

Detecção molecular de alfaherpesvírus bovino 1 e 5 no encéfalo de bovinos com ou sem doença neurológica

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Highlights

Cattle with rabies may be coinfected with BoAHV5.
Cattle affected by non-inflammatory neurological diseases may be infected with BoAHV5.
BoAHV5 DNA in the brain can complicate differential diagnosis of neurologic diseases.

Abstract

The objective of the present study was to verify the presence of DNA of bovine alphaherpesvirus 5 (BoAHV5) and 1 (BoAHV1) in brain samples from healthy cattle slaughtered or that died naturally affected by neurological diseases. The healthy ones were beef cattle (n=30) raised in the north central mesoregion from Paraná, southern Brazil. The sick cattle had a definite diagnosis of rabies (n=38), BoAHV encephalitis (n=25) and other neurological diseases (n=37). Polymerase chain reaction (PCR) assays were performed on fragments of the telencephalon, cerebellum and brain stem to confirm the presence of BoAHV5 and

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BoAHV1 DNA. Bovine alphaherpesviruses were confirmed in cases of BoAHV5 (n=22) and BoAHV1 (n=3) encephalitis. There were no observed coinfections. In rabid cattle, BoAHV5 was confirmed in eight (21%) and BoAHV1 in one (2.6%). BoAHV5 was also present in two (5.4%) cattle with other diseases (botulism and polioencephalomalacia), and in two (6.6%) healthy cattle. It can be concluded that, due to the possibility of latent infection, the presence of BoAHV5 or BoAHV1 DNA in the brain of cattle that died with neurological diseases may complicate the differential diagnosis in some cases.
Key words: BoAHV5. Bovine rabies. Differential diagnosis. Encephalitis. Latent infection.

Resumo

O objetivo do presente estudo foi verificar a presença do DNA de alfaherpesvírus bovino 5 (BoAHV5) e 1 (BoAHV1) em amostras do encéfalo de bovinos clinicamente sadios abatidos ou que morreram acometidos naturalmente por doenças neurológicas. Os sadios eram bovinos de corte (n=30) criados na mesorregião norte central do estado do Paraná, região sul do Brasil. Os bovinos doentes tinham diagnóstico definido de raiva (n=38), de encefalite por BoAHV (n=25) e de outras enfermidades neurológicas (n=37). A reação em cadeia da polimerase (PCR) foi realizada em fragmentos do telencéfalo, cerebelo e tronco encefálico para confirmação da presença de DNA de BoAHV5 e de BoAHV1. A presença de BoAHV foi confirmada nos casos de encefalite por BoAHV5 (n=22) e por BoAHV1 (n=3). Coinfecções não foram observadas. Nos bovinos com diagnóstico de raiva, BoAHV5 foi confirmado em oito (21%) e BoAHV1 em um (2,6%). BoAHV5 também estava presente em dois (5,4%) bovinos com outras doenças (botulismo e polioencefalomalacia). e em dois (6,6%) bovinos clinicamente sadios. Pode-se concluir que, devido à possibilidade de infecção latente, a presença do DNA de BoAHV5 ou de BoAHV1 no encéfalo de bovinos que morreram com doenças neurológicas pode, em alguns casos, dificultar o trabalho de diagnóstico diferencial.
Palavras-chave: BoAHV5. Diagnóstico diferencial. Encefalite. Infecção latente. Raiva.

Introduction

Neurological diseases are important in cattle breeding because they cause considerable economic losses, since most are diagnosed in outbreaks with a high lethality rate (Ribas et al., 2013; Queiroz et al., 2018a). Since most neurological diseases may have similar clinical manifestations, there is constant confusion in establishing the correct diagnosis. Additionally, the neurological dysfunctions observed in the affected animal are directly related to the affected areas of the central nervous system (CNS). They are independent of the disease per se (Borges et al., 2021). Consequently, an adequate differential diagnosis is fundamental to determine the ongoing disease; and acquiring a proper differential diagnosis is challenging. The cause of the neurological disease is most frequently achieved via specific diagnostic laboratory methods after the death of the affected animal.

Several retrospective studies have investigated the occurrence of neurological disease in cattle from diverse geographical regions of Brazil due to the importance of brain diseases in the national surveillance

to determine the etiology of infectious and non-infectious neurological syndromes in cattle. These have included studies from the states of Rio Grande do Sul (Sanches et al., 2000; Barros, 2009; Santos et al., 2018), Paraíba, Pernambuco, and Rio Grande do Norte (Galiza et al., 2010; Souza et al., 2023), Mato Grosso do Sul (Ribas et al., 2013), Goiás (Terra et al., 2018), and Paraná (Queiroz et al., 2018a). These retrospective investigations demonstrated that inflammatory diseases were predominant, with toxic diseases being the second most frequent neurological dysfunction. Collectively, these two processes represented approximately 86% of all cases of neurological disease diagnosed in cattle from different geographical regions of Brazil. Additionally, inflammatory diseases were predominantly due to viral infections (89%), with rabies (56%) and bovine alphaherpesvirus 5 (BoAHV5) encephalitis (20%) being the most frequently diagnosed (Borges et al., 2021).

BoAHV5-associated encephalitis occurs predominantly in cattle from South America (Perez et al., 2002). In Brazil, BoAHV5 is the principal herpesvirus that causes encephalitis in cattle (Claus et al., 2007; Arruda et al., 2010). Less frequently, bovine alphaherpesvirus 1 (BoAHV1) can also cause inflammation in the brain (Silva et al., 2007a,b; Rissi et al., 2008; Queiroz et al., 2018a). Herpesviruses have as a particular and striking characteristic the ability to remain in hosts causing latent infections (Franco et al., 2017). Although the trigeminal nerve ganglia are the main site of latency, latent virus can establish itself in different locations in the brain, as well as in other tissues, as demonstrated through experimental BoAHV5 infection in calves (Vogel et al., 2003; Isernhagen et al., 2011; Cagnini et al., 2015).

The presence of the DNA of these viruses in the brain of cattle that died with neurological disease can, therefore, complicate the work of differential diagnosis. A positive polymerase chain reaction (PCR) does not necessarily implicate a causal relationship between the detection of virus DNA in tissues and the occurrence of encephalitis, requiring additional evidence, such as histopathological lesions, to confirm a causal hypothesis (Rissi et al., 2008). Furthermore, cattle latently infected with alphaherpesviruses may die due to other neurological diseases, as demonstrated with rabies (Rodenbusch et al., 2020) and polioencephalomalacia (David et al., 2007). The authors are unaware of studies demonstrating the presence of latent BoAHV infection in animals affected by other neurological diseases. The objective of the present study was to search for the presence of BoAHV5 and BoAHV1 DNA in the brains of cattle, either healthy or naturally affected by neurological disease.

Material and Methods

Study areas, animals, and sampling

Brain samples were collected from two groups of cattle: a) clinically healthy animals, cattle without any clinical manifestation of disease (n=30), and b) diseased cattle, animals that were naturally affected by neurological diseases (n=100). The brains of the healthy animals were obtained from a slaughterhouse in Londrina, Paraná, southern Brazil. These animals consisted of Nelore

cattle, 2 to 3 years old, that were reared on different farms.

The brains of cattle with clinical manifestations of neurological disease were obtained after routine *post-mortem* evaluations done at two universities: Universidade de Brasília (UnB; n=55) and Universidade Estadual de Londrina (UEL; n=45). Cattle from these two institutions were predominantly beef cattle breeds, mainly Nelore, of varying ages and were clinically examined for neurological disease. Most of these animals died due to disease progression, while few (n=19) were euthanized *in extremis* due to advanced stages of neurological disease.

Specific brain fragments (cerebral cortex, cerebellum, and brain stem) of all cattle from the UEL were collected during routine *post-mortem* evaluations. The fragments were stored separately, and maintained at -80 ºC until used in molecular assays. However, only fragments of the cerebral cortex from cattle derived from UnB were maintained at -80 ºC for molecular analyses. Consequently, brain fragments of all 130 animals were processed for molecular detection of BoAHV1 and BoAHV5.

Neurological diseases diagnosed in cattle

The cattle from UnB were diagnosed with rabies (n=32), botulism (n=8), BoAHV5 encephalitis (n=7), polioencephalomalacia (n=3), bacterial encephalitis (n=2), and BoAHV1 encephalitis, cerebral babesiosis, and nervous eimeriosis (n=1 each). Cattle examined at UEL were diagnosed with BoAHV5 encephalitis (n=15), rabies (n=6), polioencephalomalacia and bacterial encephalitis (n=4 each), hepatic encephalopathy (n=3), botulism, BoAHV1 encephalitis, and malignant catarrhal fever (n=2 each), and chlorpyrifos poisoning, carbamate poisoning, head trauma, cerebral babesiosis, listeriosis, tetanus, and uremic encephalopathy (n=1 each).

The diagnoses of the diseases were based on the combination of clinical, epidemiological, gross and histopathological findings associated with specific laboratory diagnostics. Direct immunofluorescence and intracerebral inoculation in mice were performed with fragments of the brain tissues of all animals with neurological disease, confirming the rabies diagnosis and ruling out this disease in other cases. The confirmation of encephalitis by BoAHV1 or BoAHV5 was carried out by identifying the characteristic histopathological alterations (non-suppurative meningoencephalitis) associated to the detection of the DNA of those agents in brain tissues of affected cattle.

Detection of DNA of BoAHV1 and 5 in brain tissues

The nucleic acids from all brain fragments were extracted using a combination of the phenol/chloroform/isoamyl alcohol and silica/guanidine isothiocyanate methods (Boom et al., 1990; Alfieri et al., 2004). The extracted nucleic acids were then used in PCR assays designed to identify partial fragments of the glycoprotein C gene of BoAHV1 and BoAHV5 as described (Claus et al., 2005), with modifications (Oliveira et al., 2015). The quality of the extraction procedure was assessed using an internal reaction control, based on the ND5 gene

from bovine mitochondrial DNA (Claus et al., 2005). Nucleic acids derived from a previous study were used as positive controls (D'Arce et al., 2002). Nuclease-free water (Invitrogen, Carlsbad, CA, USA) was used as the negative control in all PCR assays; positive and negative controls were included in all molecular assays. All PCR products were separated by electrophoresis in 2% agarose gels, stained with ethidium bromide, and examined under ultraviolet light.

Detection of BoAHV1 and BoAHV5 infections in cattle

An animal was considered infected by BoAHV1 and/or BoAHV5 when viral DNA was identified in any of the brain tissues examined. Coinfections were defined as the concomitant detection of nucleic acids of BoAHV1 and BoAHV5 in brain tissues of the same animal. Frequency distributions for BoAHV1 and BoAHV5-associated infections were established in healthy cattle and those affected by each of the neurological diseases.

Results

Table 1 summarizes the distribution of animals where BoAHV1 or BoAHV5 DNA were identified. DNA of at least one of the BoAHVs was detected in 19.2% (25/130) of the animals examined. Cases of encephalitis caused by BoAHV were more frequently associated with BoAHV5 (22/25; 88%) than BoAHV1 (3/25; 12%). Coinfections by these two viruses were not identified.

When the tissue distribution of BoAHV5-associated encephalitis was evaluated, viral DNA was most frequently detected in the cerebral cortex (20/22; 91%) and brain stem (11/15; 73%), and less frequently in the cerebellum (7/15; 46%). Additionally, in the three cases of BoAHV1-related encephalitis, viral DNA was detected in the cerebral cortex (3/3), the brain stem (1/2), and the cerebellum (2/2).

In this study, cattle diagnosed with rabies were concomitantly infected by BoAHV. Most of these coinfections were associated with BoAHV5 (23.6%; 9/38). In these cases, herpesviruses DNA was most frequently (8/9; 89%) detected within the cerebral cortex. Only one case of coinfection was detected involving rabies virus and BoAHV1.

BoAHV5 DNA was detected in the brains of two healthy cattle (2/30; 6.6%) and in cattle with other neurological diseases (2/37; 5.4%; Table 1). It must be highlighted, that BoAHV5 was detected only in the brainstem of all clinically healthy cattle. Furthermore, BoAHV5 was detected only in the cerebral cortex in one case of cattle botulism and in one case of polioencephalomalacia. BoAHV1 DNA was not detected in the brain tissue sections of any of these animals.

Table 1
Detection of bovine alphaherpesvirus 5 (BoAHV5) and 1 (BoAHV1) DNA in the brain of clinically healthy cattle and cattle with neurological diseases

Neurological disease	n	BoAHV5 positive	%	BoAHV1 positive	%
BoAHV5 encephalitis	22	22	100	0	0
BoAHV1 encephalitis	3	0	0	3	100
Rabies	38	8§	21.0	1§§	2.6
Other neurological diseases	37	2†	5.4	0	0
Botulism	10	1	10	0	0
Polioencephalomalacia	7	1	14.3	0	0
Bacterial encephalitis	6	0	0	0	0
Hepatic encephalopathy	3	0	0	0	0
Cerebral babesiosis	2	0	0	0	0
Malignant catarrhal fever	2	0	0	0	0
Nervous eimeriosis	1	0	0	0	0
Uremic encephalopathy	1	0	0	0	0
Listeriosis	1	0	0	0	0
Carbamate poisoning	1	0	0	0	0
Chlorpyrifos poisoning	1	0	0	0	0
Head trauma	1	0	0	0	0
Tetanus	1	0	0	0	0
Healthy cattle	30	2*	6.6	0	0

§ Viral DNA located in the cerebral cortex of 7 cattle (exam carried out only in this location) and in the cerebellum of 1 cattle
§§ Viral DNA located in the cerebral cortex and absent in the brain stem and cerebellum
† Viral DNA located in the cerebral cortex (exam carried out only in this location)
* Viral DNA located in the brain stem and absent in the cerebral cortex and cerebellum.

Discussion

In this retrospective study, brain tissue samples of 100 cattle that died due to different neurological diseases were investigated for the presence of BoAHV5 and BoAHV1 DNA. We believe the results of this study are an important contribution for the understanding of neurological disease of cattle in Brazil, since we investigated the occurrence of these viral infections in cattle that died due to several neurological diseases and with a defined diagnosis. The authors are unaware of previous studies using a similar evaluation. These findings are important to highlight the demonstration

that the presence of herpesviral DNA in the brain of cattle may be detected despite not necessarily being implicated as the causative agent of the neurological disease, highlighting the importance of the association of genetic material and respective microscopical expected lesions.

The conditions identified during this study may be considered representative of most of the epidemiologically significant neurological diseases of cattle occurrence of in Brazil. Rabies, BoAHV5 encephalitis, botulism, and polioencephalomalacia are the most frequently diagnosed neurological diseases of cattle identified in surveys done within several states in the country (Barros, 2009; Galiza et al., 2010; Ribas et al., 2013; Souza et al., 2023). During this study, rabies and BoAHV5 encephalitis contributed to 60% of all cases diagnosed; these results are consistent with previous observations from the states of Paraná (Queiroz et al., 2018a) and Goiás (Terra et al., 2018), that demonstrated that these two diseases were the predominant neurological diseases diagnosed in cattle from those geographical locations. It must be highlighted that the cattle evaluated in the current study also originated from the states of Paraná and Goiás, where the Nelore breed is largely dominant.

During this study, alphaherpesvirus encephalitis in cattle was more frequently induced by BoAHV5 (22/25; 88%) with comparatively few cases associated with BoAHV1 (3/25; 12%); similar findings were reported in studies done within the states of Paraná (Queiroz et al., 2018a) and Goiás (Blume et al., 2018). It must be highlighted that although BoAHV1 has been detected as a causal agent in naturally occurring cases (Silva et al., 2007a,b; Rissi et al., 2008), BoAHV5 is the main bovine alphaherpesvirus associated to encephalitis in cattle (Claus et al., 2007; Arruda et al., 2010). In the cattle examined in the present study diagnosed with herpetic encephalitis, the DNA of these viruses was observed distributed in the three segments of the brain evaluated, with frequency being more elevated at the cerebral cortex and brain stem; similar diverse distribution of BoAHV5 DNA was observed in experimentally induced BoAHV5 infection in calves (Vogel et al., 2003; Isernhagen et al., 2011; Cagnini et al., 2015). In those studies, this same pattern of distribution could be observed even in clinically healthy, latently infected animals.

In cattle affected by rabies, concomitant infection with bovine alphaherpesviruses, especially BoAHV5, was common, occurring in almost a quarter of cases. Similar results were described in a study conducted in Rio Grande do Sul, in which 21% of rabid cattle were latently infected with bovine alphaherpesvirus (Rodenbusch et al., 2020). As observed in the current study, BoAHV5 DNA was detected in brain tissues in more cases than BoAHV1 DNA. However, unlike the cattle included in the present study, BoAHV1 infection was always accompanied by BoAHV5 infection in cattle reared in Rio Grande do Sul (Rodenbusch et al., 2020).

Studies have shown that BoAHV1 (Batista et al., 2010) and BoAHV5 (Spilki et al., 2003) were isolated from the brains of calves that died from rabies, during which the detected herpesvirus induced active and not latent infections. However, during this study it was not possible to determine if the herpesviruses induced active or latent infection, since virus isolation attempts were

not performed. Additionally, it was also not possible to define a specific distribution pattern of BoAHV5 DNA in brain tissues, since in seven of the eight cattle with coinfections with rabies vírus and BoAHV5, only fragments of the cerebral cortex were available for PCR.

It is worth highlighting the elevated frequencies of coinfections involving rabies virus and BoAHV5 during this study, and an investigation from the state of Rio Grande do Sul (Rodenbusch et al., 2020). However, a possible direct relationship relative to the occurrence of these simultaneous infections is currently unknown. More likely, cattle bearing a latent herpesviral infection may become infected by rabies virus and eventually succumb to rabies. This hypothesis is plausible for regions of Brazil where BoAHV5-associated infections are predominant. As already demonstrated in surveys carried out in the states of Rio Grande do Sul (Campos et al., 2009) and Paraná (Oliveira et al., 2015), an elevated number (25□90%) of healthy cattle carried latent infection with BoAHV5 and/or BoAHV1. However, the frequency of infected animals may vary according to the geographic region within the state (Oliveira et al., 2015). This type of study has not been carried out in other states in Brazil and, therefore, the epidemiological situation is unknown in Goiás and Distrito Federal. However, 22% (7/32) of the rabid cattle included in the present study and raised in both locations were coinfected with BoAHV5, indicating that this virus does, in fact, circulate throughout the bovine population of Goiás and Distrito Federal.

In the healthy cattle studied, latent infections due to BoAHV5 were infrequent (6.6%), with the viral DNA being detected only in the brain stem; similar results (8.3%) were described in healthy cattle from the same geographic mesoregion of the state of Paraná where the current study was done (Oliveira et al., 2015). However, these results must be compared with caution since BoAHV5 DNA was investigated in the brain tissues of cattle in the present study, while the presence of the DNA was evaluated only in the trigeminal nerve ganglia in the previous study (Oliveira et al., 2015). As the trigeminal nerve ganglia are the main site of virus latency (Vogel et al., 2003; Isernhagen et al., 2011), there is a chance that latent infection with BoAHV5 was not detected in some of the 28 healthy cattle included in the present study and may have been considered negative in view of the absence of trigeminal ganglia tissues for examination. This, however, does not compromise the results of the present study, as its purpose was to investigate whether the BoAHV DNA in brain tissues could somehow hinder the interpretation of the results that lead to the definition of the diagnosis.

BoAHV5 DNA was detected within the brains of only two animals affected by other neurological diseases (i.e., botulism and polioencephalomalacia). These results suggest that latent BoAHV5-associated infections do not play a significant role in defining the actual responsible for disease in the cattle population studied. In botulism, the clinical course of the disease and the neuropathological findings can easily differentiate from BoAHV5 encephalitis. Clinically, BoAHV5 encephalitis occurs as a cerebral syndrome, and the clinical manifestations of cortical dysfunctions, such as amaurosis, decreased level of consciousness, changes in behavior and personality, and abnormal postures, among others, are frequently observed since the

onset of the disease; and nonsuppurative meningoencephalitis predominantly at the cerebral cortex is the typical histopathological lesion (Lunardi et al., 2009; Isernhagen et al., 2011; Queiroz et al., 2018a,b). In botulism, on the contrary, a decreased level of consciousness (depression) is frequently observed during the advanced evolution of the clinical disease without any characteristic neuropathological alteration in the brain (Lisbôa et al., 1996; Ribas et al., 2013). However, the clinical differentiation between polioencephalomalacia and BoAHV5 encephalitis is more challenging since both diseases are characterized by signs typical of cortical dysfunction from the onset of the disease (Cunha et al., 2010). Histopathology of the brain finally allows differentiation, as in polioencephalomalacia evidence of inflammation, if present, is more discrete (Sant'Ana et al., 2009; Ribas et al., 2013).

Consequently, due to the elevated rate of coinfections observed in the cattle population evaluated with rabies, BoAHV5 infections could have made it difficult to define the correct diagnosis in these cases. This is of utmost importance since cattle with BoAHV5 encephalitis and rabies can present typical cerebral syndromes, when the rabid cattle have signs of cortical dysfunction since the beginning of the evolution of the disease, and non-suppurative meningoencephalitis occurs in both diseases (Ribas et al., 2013; Queiroz et al., 2018b). However, the presence of the rabies virus in the brain is confirmed by direct immunofluorescence assay and/or intracerebral inoculation in mice or cell culture done in accredited laboratories throughout Brazil. As in Brazil these tests are routinely used for the surveillance of rabies and other encephalopathies in herbivores, as established by the official program of the Ministry of Agriculture and Livestock, the potential confusion with BoAHV5 encephalitis is a minor problem in practice.

Conclusion

The possible occurrence of latent infections due to BoAHV in the brain tissues of cattle that died of neurological disease may complicate the adequate differential diagnosis in the affected animal. Therefore, to increase the probability of success in defining the diagnosis, the results of PCR on nervous tissue must be interpreted with caution, in association with other specific laboratory assays. Finally, confirmation of encephalitis caused by BoAHV1 or BoAHV5 requires the identification of viral nucleic acids within the brain tissues in association with characteristic histopathological findings consistent with these infections.

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Ethics statement

This study was previously approved by the Ethics Committee on the Use of Animals of the Universidade Estadual de Londrina (process number CEEA/UEL 04/2009 and CEUA/UEL 32340.2012.04).

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