

## The Use of Real-Time PCR (qPCR) for the Diagnosis of Bovine Herpes virus 5 in Formalin-Fixed, Paraffin-Embedded Bovine Brain Samples Identified as Bovine Unspecific Encephalitis

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### Abstract

**Background:** Bovine herpes virus 5 (BoHV-5) is a neurovirulent alpha herpes virus that causes fatal meningoencephalitis and huge economic losses in the livestock and associated industries worldwide; its diagnosis in the laboratory is essential for disease control, immunologic surveillance and effective monitoring programs in endemic areas.

**Materials and Methods:** In this study, brain samples from 21 Colombian cattle that died of neurological disease, were negative for rabies and were diagnosed with nonspecific encephalitis were processed by real-time PCR (qPCR) in the laboratory of virology at the Institute of Biosciences, Sao Paulo State University, Brazil, to detect BoHV-5 genetic material in tissue preserved in paraffin blocks. Tissues were considered positive for BoHV-5 if amplification occurred at or before 30 PCR cycles, inconclusive if amplification occurred between 30 and 40 cycles and negative otherwise.

**Results and Conclusion:** Two cases were positive, demonstrating that qPCR may be useful for confirming herpes encephalitis in acid formalin-fixed tissues with an inconclusive diagnosis.

**Keywords:** Bovine Herpes virus 5; Real-Time PCR; Diagnosis; BoHV-5

**Abbreviations:** qPCR: Real-time PCR; BoHV-5: Bovine Herpes Virus 5; BoHV-1: Bovine Herpes virus 1; gC: Glycoprotein C; Us9: Protein Us9;  $\alpha$ -tif: Protein  $\alpha$ -tif; BSE: Bovine Spongiform Encephalopathy; vCJD: Creutzfeldt-Jakob Disease; ICA: Colombian Agricultural Institute

### Background

Bovine herpes virus 5 (BoHV-5), known as bovine encephalitis herpes virus, is a neurovirulent alpha herpes virus that causes fatal meningoencephalitis in calves and significant economic losses in the cattle industry. This virus may also be associated with bovine herpes virus 1 (BoHV-1), which commonly affects the respiratory and reproductive systems of the same host [1-3]. Molecularly, the envelope of BoHV-5 is formed by the glycoproteins gB, gC, gD, gE, gG, gK, gH, gI, gL and gM and the proteins Us9 and  $\alpha$ -tif. The gC protein has been associated with the introduction of the virus in the brain, and gD is the receptor that facilitates binding to the host cell, initiating a replication process similar to those of other alpha herpes viruses [4-8]. Although present in both BoHV-1 and BoHV-5, the gE amino acid sequence differs significantly between these viruses, causing a major difference in the nervous system invasion pattern and latency of BoHV-1 and BoHV-5 [4,6]. The Us9 protein of BoHV-1 is crucial for reactivation in the trigeminal ganglia, and the same protein in BoHV-5 has an important role in viral transport from olfactory receptor neurons to the olfactory bulb [9]. From an epidemiological standpoint, a viral neutralization test against BoHV-1 can be useful for suspected infection with BoHV-5 [10]; however, because they share many structural, biological, antigenic and molecular characteristics, cross-reactions may occur between the two viruses, which hinder the evaluation of their serological prevalence [11-13].

BoHV-5 is transmitted by direct contact with contaminated

aerosols, fomites or even semen [14]. It can affect animals of any breed, sex and age, but calves are more susceptible [1,15]. There is a high mortality rate; however, some animals recover [16].

BoHV-5 infection is clinically characterized by depression, anorexia, isolation from the flock, serous ocular secretion and nasal discharge, drooling, muscle tremors that are most evident in the head and neck, hyperesthesia and loss of vision [17-19]. Infection also leads to walking in circles, ataxia, trismus, hypotonia of the tongue, difficulty in drinking water, nystagmus, bruxism, catatonias, prolonged decubitus, pedaling movements and death [1,11,20].

Many etiological agents, namely bacteria, viruses, toxins, pesticides, and nutritional and metabolic imbalances (mineral deficiency or toxicity), may cause nervous disorders in cattle; BoHV-5 is often misdiagnosed as herpetic meningoencephalitis, resulting in inadequate epidemiological analysis [21]. Among the diseases to be considered in the differential diagnosis of BoHV-5 are rabies, polioencephalomalacia/thiamine deficiency, bacterial meningitis, acute lead poisoning, botulism and bovine spongiform encephalopathy (BSE) [14,22,23].

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**Received** July 09, 2013; **Accepted** October 10, 2013; **Published** October 14, 2013

**Citation:** Pedraza-Ordoñez F, Yamatogi RS, Araujo Jr JP, Rocha NS, Alessi AC (2013) The Use of Real-Time PCR (qPCR) for the Diagnosis of Bovine Herpes virus 5 in Formalin-Fixed, Paraffin-Embedded Bovine Brain Samples Identified as Bovine Unspecific Encephalitis. J Veterinar Sci Technol 4: 145. doi:10.4172/2157-7579.1000145

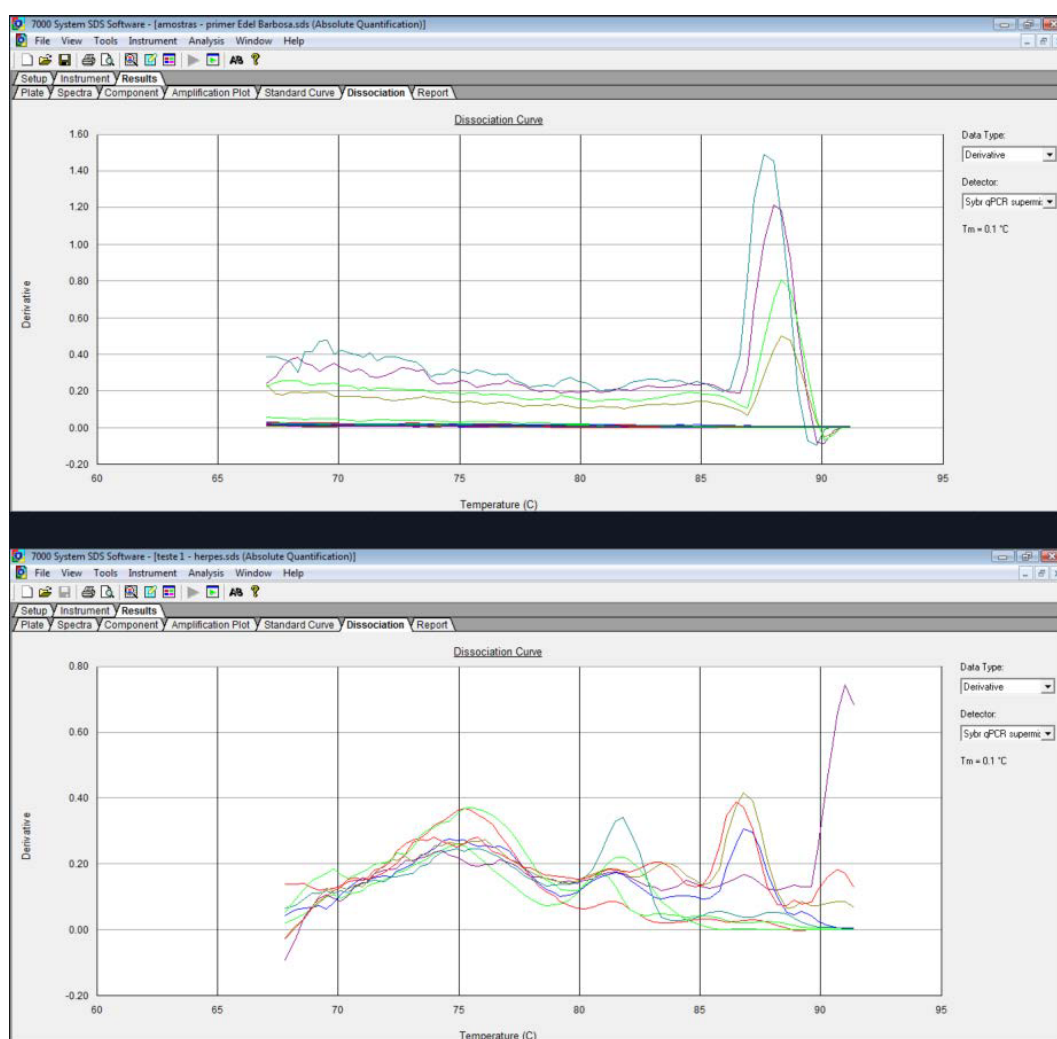
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Histopathologically, intranuclear inclusion bodies in astrocytes and neurons, although not always found, can help diagnose of BoHV-5 [16,24]. More sophisticated techniques, such as viral isolation from brain samples and nasal secretions, the use of fluorescent antibodies and immunohistochemistry, can confirm the diagnosis of the disease, but the quality of the sample and the use of preservatives prior to its arrival to the laboratory can affect the accuracy of these assays [13,25]. The quality of the diagnosis is usually directly related to the quality of samples submitted to the diagnostic laboratory; the correct specimens must be collected, preserved and submitted to the laboratory in a timely fashion [26,27]. In many cases, formalin is used to fix the tissue, and although formalin fixation is excellent for routine histopathology, it usually inactivates the virus and hinders its detection using molecular tests because it alters the reactivity of antigens and masks antigenic determinants [20]. Molecular techniques, such as polymerase chain reaction (PCR), can be used to detect the virus and differentiate it from BoHV-1 in field samples or laboratory experiments [14,28]. The purpose of this study was to use real-time PCR (qPCR) to identify the bovine encephalitis herpes virus in natural cases of bovine nervous disease that were negative for rabies and were identified in the laboratory as nonspecific encephalitis.

## Materials and Methods

### Paraffin-embedded brain tissue

A retrospective study was conducted using twenty-one formalin-fixed, paraffin-embedded brain samples from Colombian cattle with non-suppurative encephalitis. Paraffin blocks of tissue from cases that occurred between 2000 and 2009 were obtained with permission from the National Veterinary Diagnostic Laboratory of the Colombian Agricultural Institute (ICA) in Bogotá and Montería. The samples were fixed in 10% formalin prior to embedding, but it was not clear how long the materials had remained in the fixative; estimates ranged from several days to over one year. The samples were processed by conventional methods for histological analysis and stained with hematoxylin-eosin. The samples were selected from two hundred and ten cases negative for rabies disease (by indirect immunofluorescence test) using histopathological criteria compatible with BoHV-5 infection, such as perivascular cuffing, mononuclear cell infiltration, areas of malacia and gitter cells (macrophages) and intranuclear inclusion bodies. The cases analyzed were Zebu or Zebu cross-breed males and females, aged between six months and five years, with clinical symptoms of



**Figure 1: Real-time PCR (qPCR) for Bovine Herpesvirus 5 (BoHV-5) using two different primers.** The upper panel shows positive cases identified by qPCR with primers suggested by Gomes et al. (2002), including two controls and two samples. The lower panel shows qPCR using the primers suggested by Campos et al. (2009). Note the number of nonspecific peaks, which preclude the positive identification of the cases analyzed.

neurological disease. A post-mortem diagnosis of non-suppurative encephalitis was possible with microscopic examination.

### Real-time PCR (qPCR)

**6.2.1 DNA extraction:** The Illustra™ Tissue & Cells genomic Prep Mini Spin Kit (GE Healthcare Life Sciences, Pittsburgh, Pennsylvania, USA) was used for the extraction of total genetic material from paraffin-embedded tissues according to the manufacturer's instructions. To evaluate a possible cross-reaction, two blank reactions (no reagent, only samples) were obtained from each group of 10 paraffin tissue sections. Finally, the DNA concentration was determined by reading the optical density at 260 nm.

**Sample processing:** We tested two primer pairs to detect BoHV-5: sense, TACGGACTGCCGGATTAACA, and anti-sense, GTCACCACTA CCACCGCCGCCA ACAT, as suggested by Gomes et al. [31]; and sense, PF 5'-GTGGAGCGCCGCTTCGC-3', and anti-sense, PR 5'-TATCGCGGAGAGCAGGCG-3', as suggested by Campos et al. [30]. Of the 21 DNA samples extracted from the bovine brain tissues, five had already been evaluated with positive results for BoHV-5 by nested PCR [34].

For qPCR, we used 400 nmol of each primer, 10 µL of 2X Power SYBR® Green PCR Master Mix (Promega, USA), 4 µL of DNA extract from samples, and nuclease-free water to a final volume of 20 µL. The reaction conditions were as follows: 95°C/10 minutes for the initial denaturation, 40 amplification cycles (95°C for 15 s for denaturation and 60°C for 1 minute for annealing and extension), and a melting curve analysis. The fluorescence data were collected during the annealing and extension steps, and the threshold cycle numbers (CT) were determined using an ABI PRISM® 7300 Sequence Detector (Applied Biosystems, USA) and SDS software version 1.2.3 (Sequence Detection Systems 1.2.3, 7300 Real Time PCR System; Applied Biosystems, USA). As a negative control, the sample was replaced with nuclease-free water; as

a positive control, a DNA sample extracted from tissue with positive immunohistochemistry and nested PCR results was used. All of the reactions were performed in duplicate.

The β-actin gene (GenBank: NM 173979.3) was included as an internal control to demonstrate the presence of bovine DNA in the samples and verify the efficiency of the extraction process.

**Analytical sensitivity and specificity assay:** For analytical sensitivity assay, a DNA from cell culture containing 10<sup>7</sup> TCID<sub>50</sub> (tissue culture infectious doses) was extracted according to described above. Ten-fold serial dilutions of purified DNA were used as template in qPCR. The analytical specificity assay was made using 4 agents: Canine Herpes virus type 1, *Neospora caninum*, Human Herpes virus type 4 - Epstein Barr and Human Herpes virus type 8 - Kaposi Sarcoma.

### Results and Discussion

qPCR has revolutionized the laboratory diagnosis of human diseases [29], but to our knowledge, it has not yet been used for the diagnosis of bovine herpes virus. In the present study, BoHV-5 DNA was detected by qPCR in two samples among all of the cases analyzed, demonstrating the usefulness of this technique as an aid in the diagnosis of BoHV infection in cattle with clinical symptoms of neurological disease, even in samples that are stored in acid formalin for long periods. The primers suggested by Campos et al. [30] did not perform well and presented several nonspecific peaks for detection of the virus; therefore, they were not used in this work. However, the primers proposed by Gomes et al. [31] resulted in well-defined peaks in four positive cases (including the two controls). In the analytical sensitivity the qPCR showed positive results until 1 TCID<sub>50</sub>. In specificity assay no agent tested was positive. The difference in performance between the primers can be related to several factors, such as the amplification mix used, the quality of the extracted genetic material or even the characteristics of the initiator. The comparison between the two types of primers is shown in Figure 1.

Case	Age (months)	Sex	Municipality	Micro	Macro	Nested PCR*	qPCR
0014	>24	No data	Planeta Rica	No significant lesions	No data	-	-
0105	60	Female	La Ceja	Perivascular cuffing	Bruxism, ptyalism	-	-
0151	30	Male	Tuluá	Inclusion bodies	Blind, recumbency	-	-
0212	24	Male	Montería	Non suppurative meningoencephalitis	Recumbency, opisthotonos	Positive BoHV-5	Positive BoHV-5
0303	48	Male	Valledupar	No data	Hyperexcitability, no menace reflex	-	-
0310	6	Male	Bucaramanga	Meningitis	Ptyalism, bruxism, circling	Positive BoHV-5	-
0324	8	Male	Valledupar	Congestion, hemorrhage	Found dead	Positive BoHV-5	-
0413	19	Female	Palmira	Meningitis and congestion	circling, recumbency	-	-
0669	>24	No data	Montería	Edema, congestion	No data	-	-
0853	>24	No data	Tierra Alta	Perivascular cuffing, gliosis	No data	-	-
0854	>24	No data	Ciénaga de Oro	Gliosis, edema, congestion, perivascular cuffing	No data	-	-
0878	18	Male	Valledupar	Perivascular cuffing	Recumbency, opisthotonos	Positive BoHV-5	-
0904	23	Female	La Dorada	Meningitis	Blind, circling, recumbency	-	-
0945	>24	No data	Puerto Libertador	Edema, congestion, hemorrhage	No data	-	-
0949	>24	No data	Montelíbano	Edema.	No data	-	-
1014	9	Female	Sincelejo	Perivascular cuffing	Ptyalism, bruxism, recumbency	Positive BoHV-5	Positive BoHV-5
1029	51	Female	Puerto Tejada	Meningitis	Found dead	-	-
1044	42	Male	Valledupar	Non suppurative meningoencephalitis	circling, recumbency, opisthotonos	-	-
1697	>24	No data	Montería	Gliosis, edema, congestion, malacia	No data	-	-
1824	>24	No data	Montería	Gliosis, edema, satellitosis	No data	-	-
0454	>24	No data	No data	Edema, gitter cells, congestion, perivascular cuffing	No data	-	-

\* Pedraza et al. (2010) [28].

**Table 1:** Major clinicopathological findings in cattle affected with nonspecific encephalitis identified as BoHV-5 positive by qPCR.

PCR has been used several times to determine the presence of virus in tissue from in natural cases maintained in paraffin blocks. In a retrospective study, Ely et al. [32] found seven positive cases of BoHV-5 and one positive case of BoHV-1 by PCR in thirty-two cases classified as non-specific encephalitis and negative for rabies. Ferrari et al. [33] detected fifteen positive samples by PCR in twenty naturally infected cattle in which half of the brain was used fresh for virus isolation and the other half was fixed in 10% buffered formalin for an average of one week prior to PCR. Gomes et al. [31] found two positive cases among three samples tested by nested PCR. Pedraza et al. [34] found five positive cases among fifteen samples tested using nested PCR (Table 1).

In our study, possibly because the samples were kept in acid formalin for very long periods, the amplification of viral DNA was limited in cases with morphological alterations characteristic of infection with bovine herpes virus. The two positive cases might correspond to samples that were maintained in formalin for a short time or that contained a large amount of virus, which would have facilitated its detection in qPCR. Another important result was the difference between tests. Two samples tested positive for BoHV-5 by qPCR, while five tested positive by the nested PCR performed by Pedraza et al. [34]. This difference may be due to several factors, including the greater sensitivity of nested PCR compared to qPCR. In the previous study, the first product of the nested PCR showed no positive results, and positivity was detected in the five cases only in the second amplification [34]. Because two positive results were achieved by qPCR in a single amplification (suggesting good test sensitivity), using nested qPCR could identify a larger number of positive cases.

Since bovine spongiform encephalopathy (BSE) and its subsequent association with variant Creutzfeldt-Jakob disease (vCJD) were identified in the last century, the socio-economic and public health importance of research in bovine encephalitis has increased [35]. The diagnosis of the causes of illness or death affecting cattle is of fundamental importance for the rapid implementation of preventive methods; however, there are often challenges in sending samples to the laboratory. In Brazil, for example, in a study of 496 cases of nervous disease in cattle, it was impossible to achieve a conclusive diagnosis due to poor sample collection or preservation in 68.35% of the cases [22]. This limitation, coupled with the fact that it is difficult to serologically differentiate between BoHV-1 and BoHV-5, has caused the under-diagnosis of bovine herpetic encephalitis in several regions of South America [10]. Several studies have proposed the use of molecular techniques to help differentiate between the two viruses, including PCR, which is specific and practical and has the advantage of allowing retrospective studies of samples fixed in formalin and preserved in paraffin [3,32,36,37]. Other techniques, such as immunohistochemistry, have also been suggested as useful tools in cases that were closed without a specific diagnosis [25,38-40].

In this work, we showed that qPCR is a useful technique for the molecular detection of BoHV-5 in formalin-fixed and paraffin-embedded brain samples. We propose its use for the identification of positive cases that have a diagnosis of nonspecific encephalitis, thus contributing to the accurate epidemiological mapping of bovine encephalitis in several regions of the world.

## Conclusions

BoHV-5 DNA was detected by qPCR in two samples among all of the cases analyzed, demonstrating the usefulness of this technique as an aid in the diagnosis of BoHV infection in cattle with clinical symptoms of neurological disease, even in samples that are stored in acid formalin

for long periods. The identification of BoHV-5 positive cases that have a diagnosis of nonspecific encephalitis, contributes to the accurate epidemiological mapping of bovine encephalitis in several regions of the world.

## Author's Contributions

Francisco Pedraza-Ordoñez: Data collecting and writing of the manuscript.

Ricardo Seiti Yamatogi, Joao Pessoa Araujo Jr.: qPCR analysis and critical review of the manuscript.

Noeme Sousa Rocha and Antonio Carlos Alessi: Analysis and interpretation of data, making and critical review of the manuscript.

## Competing Interest

None of the authors of the present document has conflict of interest for its publication.

## Acknowledgements

The authors would like to extend their gratitude to Dr. Dario Mogollon of the Colombian Agricultural Institute (ICA) for providing the samples and Prof. Dr. Alexandre Secoron Borges of UNESP, Botucatu, Brazil, for providing the bovine qPCR internal control.

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