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BOVINE HERPES VIRUS QUANTIFICATION BY qPCR IN THE BLOOD OF ASIMPTOMATIC LATE-TERM COWS

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Abstract

Bovine herpesvirus-1(BoHV-1) infections can be asymptomatic nonetheless it can also cause systemic illness in young calves and several diseases in adult cattle, including infectious bovine rhinotracheitis, infectious pustular vulvovaginitis and abortions. BoHV-1 can also establish recurrent life-long latent infections after primary infection. For viral load detection and quantification, we analysed blood samples from 19 asymptomatic pregnant cows belonging to three different breeds (Montbéliard, Holstein and Romanian Black Spotted). Viral DNA extraction from plasma was performed using the Nucleic Acid Extraction or Purification Reagent Kit (Medicalsystem Biotechnology Co., Ltd. Ningbo, China), and the Auto-Pure 32A automatic extractor (Allsheng Instruments Co., Ltd. Hangzhou). The BHV-1 putative fibronectin binding protein Genesig Advanced Kit (Primerdesign Ltd, UK) was used for qPCR amplification. The qPCR reactions were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). After analysis of the amplification curves, only one sample out of 19 was positive with a viral load of 4×10^3 copies/mL of blood. The affected animal was asymptomatic, which emphasizes the need for recurrent testing of transmissible infectious pathogens, and increasing biovigilance for minimizing eventual economical losses.

Key words: *Bovine herpesvirus-1, cow, quantitative PCR, late-term pregnancy, detection.*

INTRODUCTION

Bovine herpesvirus-1 (BoHV-1) infection is a common occurrence in ruminants' populations worldwide (Lopes et al., 2019). According to the International Committee on Taxonomy of Viruses (2023), BoHV-1 is a member of *Varicellovirus* genus, included Herpesviridae family with three subtypes BoHV-1.1, BoHV-1.2a and BoHV-1.2b. Being a double-stranded DNA virus spread to Europe in the late 1960s and early 1970s (Graham, 2013), BoHV-1 causes infectious bovine rhinotracheitis (IBR) in both domestic and wild ruminants, affecting the respiratory and reproductive systems (Oberto et al., 2023). On the genital tract, this virus is the causative agent for infectious pustular vulvovaginitis and balanopostitis, temporary infertility, embryonic death, abortions and generalized disease in newborn calves (Murkan, 2019). Furthermore, the virus is known to induce immunological suppression, conjunctivitis, encephalitis, and a decrease in milk production (Marin et al., 2016).

BoHV-1 establishes latency in the trigeminal ganglion or pharyngeal tonsils following primary infection, or in the sacral ganglia following genital infection causing the animals to remain carriers and potential disseminators for the rest of their lives (Muylkens et al., 2007; Ostler, 2023). Stress linked with parturition, transport, animal movement and mixing, inclement weather, simultaneous infection, poor husbandry or food, overcrowding, or following corticosteroid treatment can all cause reactivation (Raaperi et al., 2014; Narayan et al., 2018). Additionally, the number of animals in the latent infection phase is much higher than the number of animals exhibiting clinical signs, this fact making it easy to be transmitted and difficult to be controlled or eradicated (Lopes et al., 2019). All of these problems

associated with BoHV-1 made it one of the most economically damaging pathogens. Thus, Can et al. (2016) registered a financial loss of 509 USD, due to the high abortion rate as a result of this infection and the average cost of infection was estimated at about 379 USD.

Most of BoHV-1.1 strains were isolated from respiratory tract infections or abortion cases, while BoHV-1.2 strains were commonly identified in genital organ lesions (Ostler, 2023). Cell cultures, histopathological examinations, serological testing, polymerase chain reaction (PCR), immunohistochemistry (IHC) and immunofluorescence (IF), Western blot, enzyme linked immunosorbent assay (ELISA), and electron microscopy were all used to diagnose BoHV-1 infections, the only accurate distinguishing criterion being viral DNA analysis by restriction endonuclease fingerprinting (Muylkens et al., 2007). Quantitative polymerase chain reaction (Q-PCR) is a method by which the amount of the PCR product can be determined, in real-time, and is very useful for investigating gene expression (Narayan et al., 2018). Based on this, the current study aimed to trace the presence of the virus in cows' blood as a source of BoHV-1, in order to obtain a germfree plasma required for the transfusion of newborn calves in need.

MATERIALS AND METHODS

Ethical statement

The authors of this study respected all rights of animals' welfare in correlation to European Union and National legislation (Directive 2010/63/UE; Law 34/2014), and none of them suffered during any of the implied procedures.

Sample collection

Blood samples were collected via coccygeal vein from 19 asymptomatic pregnant cows from three different breeds (Montbéliard n = 7, Holstein n = 4, and Romanian Black Spotted n = 8) belonging to 3 commercial dairy farms located in Ilfov county, Romania, using 18 G needles and BD Vacutainer K2 EDTA (Plymouth, UK) collection tubes. Furtherly, the specimens were centrifuged at 1500× *g* for 15 min at 4°C, and plasma was harvested and frozen at −80°C until DNA extractions.

DNA extraction from plasma

Viral DNA extraction from plasma was performed using the Nucleic Acid Extraction or Purification Reagent Kit (Medicalsystem Biotechnology Co., Ltd. Ningbo, China), and the Auto-Pure 32A automatic extractor (Allsheng Instruments Co., Ltd. Hangzhou) following manufacturer's guidelines.

Detection and quantification of BoHV-1 by qPCR

For qPCR amplification the Bovine herpesvirus 1 putative fibronectin binding protein Genesig Advanced Kit (Primerdesign Ltd, UK) based on TaqMan principle was used. The amplification reactions were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with CFX Maestro Software.

Amplification of the target pathogen was detected using specific primers and a FAM fluorochrome-labelled probe complementary to the pathogen target gene. Also, to confirm a valid DNA extraction, the kit included a mixture of primer and a FAM fluorochrome-labelled probe that detects an endogenous gene. Therefore, it wasn't possible to multiplex with BoHV-1 detection.

In order to carry out the PCR reactions, two reaction mixtures are prepared, one containingprimers and probes complementary with the pathogen target gene and the second, containing primers and probes complementary with the endogenous control (a specific gene in the bovine genome) according to Table 1.

Table 1. PCR reaction components

Reagents	Per reaction (µl)
2xSsoFast Advanced Universal Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA)	10
Target gene/endogenous control primer and probe	1
Nuclease-free water	4
DNA sample	5

For quantitative analysis, a calibration curve was performed by serial dilutions (1, 10, 10^2, 10^3, 10^4 and 10^5 DNA copies/µL) using the positive control template provided by the kit.

The qPCR run protocol settings are shown in Table 2.

Table 2. qPCR time and temperature steps

Stage	Time	Temperature	Cycle	Signal scan
Enzyme activation	1 min	95°C	1	-
Denaturation	10 sec	95°C	50	-
Annealing/ elongation	60 sec	60°C		FAM
Final elongation	5 min	60°C	-	-

The CFX Maestro Software performed automatically the calibration curve and calculated the concentration of the samples in DNA copies/µL.

RESULTS AND DISCUSSIONS

Regarding both domestic and wild ruminants, BoHV-1 has generally been considered a serious threat to the upper respiratory system and reproductive function. Therefore, it is crucial to streamline this pathogen's diagnosis in order to higher in-farm eradication chances.
In the current investigation, we used the qPCR approach on a total of 19 cows belonging to three different commercial farms.
The amplification profile curves for BoHV-1 DNA standards with concentrations of 10^5, 10^4, 10^3, 10^2, 10, and 1 copies/µL are shown in Figure 1, and the calibration curve used for the quantification of the number of BoHV-1 DNA copies/µL in the samples is shown in Figure 2.

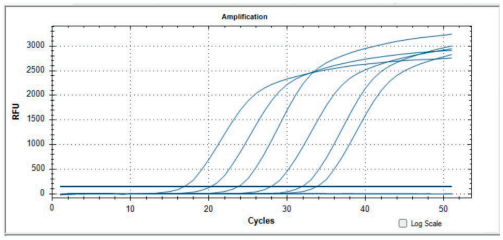

Figure 1. The amplification profile curves corresponding to 10^5, 10^4, 10^3, 10^2, 10, and 1 BoHV-1 DNA copies/µL standards

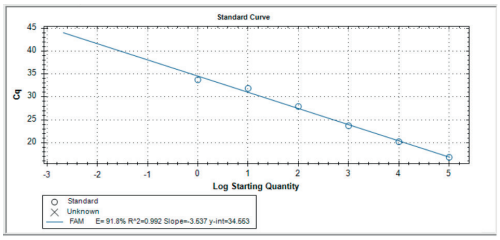

Figure 2. The calibration curve $Cq = f(log(\,copies\ DNA/\mu L))$, where Cq represents the quantification cycle

The amplification profile curves of the examined samples are displayed in Figure 3. Only one sample belonging to a Holstein female, which had a viral load of $4x10^3$ copies/mL of blood, was positive, as it can be observed in Figure 3.

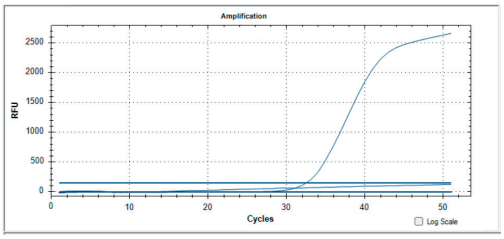

Figure 3. The amplification profile curves of the 19 examinated blood samples using primers and FAM fluorochrome-labelled probe complementary to the putative fibronectin binding protein gene of BoHV-1 genome

The Figure 4 displays the amplification profile curves of the 19 examinated blood samples using primers and FAM fluorochrome-labelled probe complementary to endogenous control, showing that all examined samples' extracted DNA are of high quality and are enough for pathogen detection.

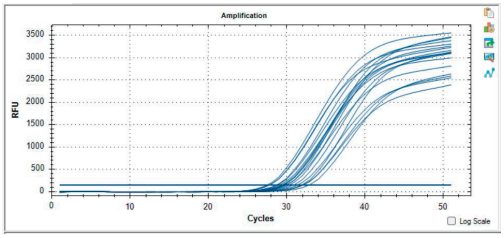

Figure 4. The amplification profile curves for blood samples evaluated using complementary primers and probes to the endogenous control

Various molecular techniques have been developed in recent years for the differentiation of bovine alphaherpesviruses that are closely related to BoHV-1. Numerous multiplex PCR techniques, including traditional and real-time techniques, have been created.
Thus, BoHV-1 can be isolated not just from blood samples (Jithin et al., 2019), but also from sperm, uterine lavage, aborted foetus (Malla et al., 2018) or respiratory tract (Marin et al., 2016).
Relatively recent, Hanna Ferreira et al. (2018) demonstrated that milk seems to be a suitable

sample for the viral nucleic acid detection, as a more sensitive test compared to the serological method, for the latent BoHV-1 infection diagnosis has been described.

In order to distinguish BoHV-1 DNA, Oliveira et al. (2009) presented a nested PCR that amplified BoHV-1 DNA in the first round before running two type-specific PCRs. The authors have nonetheless shown that some samples may have viral DNA quantities below the PCR detection limit. In addition, this method is a nested PCR that necessitates the use of agarose gels in a manner similar to all other conventional PCRs, and additional precautions must be made to avoid cross-contamination.

Comparatively to traditional PCR, real-time qPCR significantly decreased the chance of contamination. Being more sensitive than traditional PCR, it provides a quick, trustworthy, and quantitative testing approach (Diallo et al., 2011).

To check for false-negative results brought on by unsuccessful nucleic acid extraction or the presence of inhibitory components in the reaction, it is crucial to utilize an internal/endogen control. By employing such an internal/endogen control, it was ensured that DNA extracts from "difficult" samples were PCR-competent, preventing the reporting of false-negative findings and boosting assay robustness.

The investigations on the Balkan region are not very thorough, although BoVH-1 or related pathogens from blood samples were highlighted in Romania (Aniță et al., 2017) and Bulgaria (Peshev, 2021) with the addition of one Serbian paper which referred to nasal swabs specimens (Nišavić et al., 2018). Therefore, reported incidence rates were variable and method-dependent 63.60% (Aniță et al., 2017), 37.5% (Peshev, 2021), 3.6% (Nišavić et al., 2018). Nevertheless, the latter cited research papers used less sophisticated techniques (ELISA and classical PCR).

Based on their research efforts, Lopes et al., (2019) deliberated that the uterus is a viral replication target. Additionally, detecting BoHV-1 in the uterus, oviduct and ovaries of tissue samples obtained from cows, they mentioned that the placenta and uterine tissue may be the source of the spread, leading to foetal infection and abortion.

El-Mohamady et al. (2020), after three successive passages for BoHV-1 isolation, showed a clear cytopathic effect in 8 (20%) out of 40 sperm samples. One year later, using the same sample type , Untari et al. (2021) indicated that there is no infection in the semen of 27 bulls while using PCR detection in Indonesia.

Recently, El-Mayet et al. (2022) used the qPCR proved that BoHV-1 latent infections in female calves can be reactivated by stress after progesterone exposure.

In the present work, the viral DNA extraction and the real time qPCR assay protocols were validated. This assay was highly sensitive and could detect the BoHV-1 genome even when DNA from only 10^3 viral particles/mL blood was used.

Regarding host bloodstream invasion, little is known about the behaviours of several alpha-herpesviruses. Further testing will be done on this protocol capacity to identify and quantification BoHV-1 in blood, milk and sperm samples taken from different dairy farms.

Bovine alpha-herpesvirus DNA can be quickly, sensitively, and specifically detected using real-time qPCR. It can also be helpful for the simultaneous detection of different bovine alpha-herpesvirus variants. This method is a great resource for identifying these viruses in cattle for both research reasons or epidemiological surveys.

CONSLUSIONS

The qPCR method demonstrates golden standard potential for identifying BoHV-1 positive animals despite the small sample size of this investigation.

From the standpoint of financial losses, early detection of BoHV-1 positive individuals is essential for dairy farm management. More than that, it is crucial to carry out screening programmes using the qPCR approach, especially if a vaccination program is established in the farm.

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