

PRESENCE OF THE VIRAL DNA IN *OOPHOROUS CUMULUS* COMPLEXES OF COWS CLINICALLY HEALTHY AND NATURALLY INFECTED BY BOVINE HERPESVIRUS 1 (BOHV1)

(Presença do DNA viral em complexos *cumulus oóphorus* de vacas clinicamente saudáveis e naturalmente infectadas pelo Herpesvirus bovino 1 (BoHV1))

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RESUMO

O objetivo deste estudo foi verificar a presença do DNA do herpesvirus bovino 1 (BoHV1) em Complexos *Cumulus Oóforus* (COCs) e sangue de vacas naturalmente infectadas. Os COCs foram obtidos de 15 doadoras por meio de aspiração folicular guiada por ultrassom (OPU). A extração do DNA foi realizada em um *pool* de COCs de todas as aspirações de uma mesma doadora e no sangue total. Após este procedimento, seguiu-se a realização das reações de *Nested*-PCR. O DNA do BoHV1 foi identificado no *pool* de COCs de três doadoras soropositivas, não sendo detectado em nenhuma das amostras soronegativas. Entretanto, não foi encontrado DNA viral em amostras sanguíneas, mesmo nas oriundas de animais soropositivos. Todas as amostras de sangue e COCs foram submetidas à *Nested*-PCR para a detecção do BoHV5, não sendo encontrado nenhum resultado positivo. Os resultados obtidos permitem concluir que o DNA viral pode estar presente em estruturas ovarianas de vacas infectadas naturalmente pelo BoHV1.

Palavras-chave: ovócitos; *Nested*-PCR; infecção viral

ABSTRACT

This study was carried out to verify the presence of DNA of the bovine herpesvirus 1 (BoHV1) in *Cumulus Oophorus* Complexes (COCs) and blood from the cows naturally infected by BoHV1. COCs from 15 donors were obtained through Follicular Aspiration Guided by Ultrassom (OPU). The DNA extraction was accomplished in a *pool* of COCs of all aspirations from the same donor and in the total blood. After this procedure, the *Nested*-PCR reactions were accomplished. The DNA of BoHV1 was identified in the *pool* of COCs from three serum-positive donors, but it was not detected in none of the serum-negative samples. However, no viral DNA was found in sanguine samples, even in those from the serum-positive animals. All samples were subjected to *Nested*-PCR for detection of BoHV5, but no positive result was found. The obtained results allow concluding the viral DNA could be present in ovarian structures of the cows naturally infected by BoHV1.

Keywords: oocytes; *Nested*-PCR; viral infection

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INTRODUCTION

The interaction between gametes and embryos with pathogens has been thoroughly studied, as being related to viral agents with interference into reproduction. In this context, the BoHV1 causing Infectious Bovine Rhinotracheitis (IBR) is distinguished (Bielanski & Dubuc, 1994; Guerin et al., 1997; Vanroose et al., 1999; Makarevich et al., 2007).

The BoHV1 is a virus presenting cosmopolitan occurrence. It is associated to significant losses in productivity of the meat and dairy livestock, as generating a considerable economical loss due to breathing and reproductive problems. The disease is usually subclinical and characterized by viral latency, a moment at which no expressions of the viral antigens happens, besides occurrence of the interrupted replication (Jones et al., 2011). However, the following occurrences are frequent abortions from fourth to eighth month under gestation, estrus repetition at regular or irregular intervals, natimortality, perinatal mortality, reduced fertility and temporary infertility due to uterine infection (Takiuchi et al., 2005).

Concerning to contamination of the oocyte, an aspect to be considered is the presence of the cytoplasmic prolongations of the corona radiata cells. Those cytoplasmic prolongations cross the pellucida zone (ZP), as arriving to perivitelline space (Hyttel, 1987). Those projections form communications with the cytoplasmic membrane of the oocyte, as establishing an important link in passage of the chemical components that regulate the

maturation of the oocyte. Those conditions suggest that BoHV1 can use those filaments to facilitate its itinerary by ZP (Vanroose et al., 2000).

This study was carried out to verify the presence of DNA of the bovine herpesvirus 1 (BoHV1) in *Cumulus Oophorus Complexes* (COCs) and blood from the cows naturally infected by BoHV1.

MATERIAL AND METHODS

Fifteen adult bovine females, crossbred Holstein x Gir at different stages of the estrus cycle were used. Those animals came from herds which were not vaccinated against BoHV1 in order to avoid the interference of the vaccination into results.

For selection of the oocyte donor cows, the blood samples were collected. The serum neutralization was accomplished in microplates according to the methodology proposed by the "Manual of Diagnostic Tests and Vaccines for Terrestrial Animals" (OIE, 2015). A second sanguine sample was collected from each animal and placed into tubes containing the anticoagulant, in order to accomplish the molecular detection by *Nested-PCR* technique.

The follicular aspirations were accomplished after the restraint of the animals and intercoccigeous epidural anesthesia. For this procedure, the ultrasound device (DPS Mindray DP-2200VET), equipped with microconvex transducer adjusted at eight MHz frequency and coupled to an intravaginal guide, was used. Some needles 40x10 (19G) and vacuum pump with 80mmHg pressure, as corresponding to a flow of 14mL

water/min were used. The follicles larger than two millimeters were punctured and the collected material were transported in plastic 50mL tubes (Falcon), as containing 5mL saline solution buffered (PBS), added with 1000UI heparin at 37°C. The aspirated content was washed in PBS in the embryo collection filter with 80µ mesh (Millipore). COCs were traced in stereoscopic microscope and transferred to another plate containing Talp-hepes for their maintenance. The COCs were conditioned in microtubes and later frozen (-20°C), for subsequent detection of the viral DNA through *Nested-PCR*.

The detection of the viral DNA through *Nested-PCR* was accomplished to evaluate the presence of BoHV1. In this context, the *Nested-PCR* for BoHV5 was also accomplished, as taking into account the possibility to occur the crossed reaction by serum neutralization.

The extraction of the total DNA was accomplished in COCs, as indicated by the manufacturer of the extraction Kit "SV Wizard Genomic"

(Promega®), in order to use them in the molecular assay. The used oligonucleotides and the protocol for the accomplishment of the *Nested-PCR* reactions standardized from the description by Campos et al. (2009). Each reaction was constituted by a final volume of 25µL, as containing 1µL total DNA of each sample, 2µL of each one of the oligonucleotides (20 pMol), 2.5µL of DMSO, 12,5µL of Go Taq® Green Master Mix 2x (Promega®) and ultrapure water (Millipore®), as amplifying a DNA fragment of 162 base pairs. Aliquots of the reactions were subjected to horizontal electrophoresis in 1% agarose gel.

RESULTS

The serum-positive animals did not present the DNA of BoHV1 in the samples under analysis in blood. However, positive reaction by *Nested-PCR* (Figure 1) was observed in *pool* of COCs of three serum-positive animals (3/12). Concerning to BoHV5, the viral DNA was not detected in any sample under analysis.

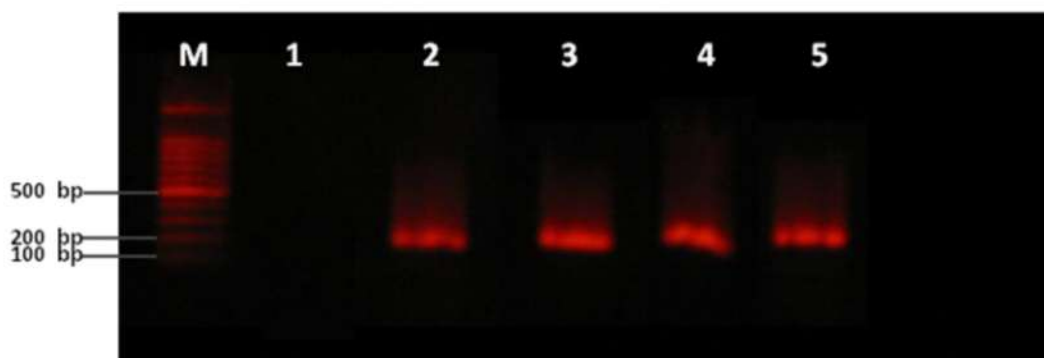


Figure 1. Product from *Nested-PCR* in 1% agarose gel, for COCs of cows serum-positive to BoHV1 (M: marker; 1: negative control; 2: positive control; 3, 4 and 5: positive COCs samples).

DISCUSSION

In the present experiment, the *Nested*-PCR technique was adopted for detection of the viral DNA, since it is more sensitive in comparison with other techniques for detection of BoHV1, such as viral isolation, fluorescence and serology (Takiuchi et al., 2005).

According to expectation, the serum-positive animals did not present the DNA of BoHV1 in the samples, either in blood and COCs. However, positive reaction by *Nested*-PCR was observed in the *pool* of COCs from three serum-positive animals (3/12).

The serum-positive animals revealing the viral DNA presented no clinical symptomatology of the disease. This condition invalidates the hypothesis presented by Oliveira (2007). According to this author, the serum-positive animals presenting no clinical symptoms are improbable transmitters of BoHV1 through COCs and follicular liquid.

Similarly to the present research, Oliveira (2007) found negative COCs in naturally infected animals, under low stressful conditions. However, this researcher did not find the viral DNA in the serum-positive animals, a fact not observed in the present work. This might have happened because the *Nested*-PCR, used in the present experiment, is a more sensitive method in relation to conventional PCR used by Oliveira (2007). So, the *Nested*-PCR is the most appropriate technique for molecular research in structures with low viral load (Takiuchi et al., 2005). Other aspect to be considered is the infection of the genital organs and the

viral excretion are associated to acute phase of the disease, which is extremely short in infected animals under natural conditions (Jones et al., 2011).

According to Singh et al. (1983), the infectiousness level observed in animals which were artificially contaminated is much higher than in the naturally infected animals. In studies with the virus added under culture conditions, the viral DNA was detected in samples of COCs, follicular liquid, spermatozoids and embryos (Bielanski & Dubuc, 1994; Vanroose et al., 1999; Ferreira et al., 2005; Marley et al. 2008).

In the present experiment, the fact to find the viral DNA in the COCs of the naturally contaminated animals rather indicates the virus could be adhered in the *cumulus* cells, ZP or it is inside the oocyte. The ZP of the immature oocytes and embryos are very irregular, with the presence of several pores, flaws and projections (Vanroose et al., 2000). In this context, Vanroose et al. (2000) affirm that particles with size around 180-200 nm (approximate size of the viral particles of BoHV1) can cross a part of ZP, as arriving to more internal layers.

The absence of the viral DNA in blood is also associated to short period of the acute manifestation of the disease in the naturally infected animals. During the reinfection periods, the virus can be found in blood or inside leucocytes. Therefore, the virus reactivation occurring at moments of stress or immunosuppression can lead to its propagation through blood stream. Thus, the latency mechanism, that is characteristic of the subfamily

Alphaherpesvirinae mainly in the trigemio and sacral ganglions (Jones et al. 2011), explains the results obtained in sanguine samples.

Besides BoHV1, there is also a concern with the possible transmission of BoHV5 due its great genomic similarity with BoHV1, that was observed by Abdelmagid et al. (1995). According to Chowdhury (1995), both BoHV5 and BoHV1 present 85% genomic similarity. So, it is possible that BoHV5, after viral reactivation, to have tropism by the genital organs, as leading to endangerment in the genital organ as observed for BoHV1. So, all samples were analyzed by *Nested*-PCR for BoHV5 in order to verify the occurrence of samples contaminated by this virus type. However, no polluted sample was found.

The present results contrast with those observed by Silva-Frade et al. (2010), who found the DNA of BoHV5 in oocytes by the PCR technique. However, those authors experimentally infected the oocytes, a fact that was not accomplished in the present experiment.

CONCLUSIONS

The results allow concluding the viral DNA could be present in ovarian structures of the cows which were naturally infected by BoHV1. So, the serum-positive animals presenting no clinical symptomatology can be potentially transmitters of BoHV1 through COCs.

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