

Notas Científicas

Bovine herpesvirus type 1 in cumulus-oocyte complexes collected from naturally infected cows

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Abstract – The objective of this work was to investigate the presence of bovine herpesvirus type 1 (BoHV-1) in follicular fluid and in cumulus-oocyte complexes (COC) recovered from naturally infected cows but with no clinical signs of the disease. Cows that were seropositive (n=38) or seronegative (n=8, control) to infectious bovine rhinotracheitis were selected after a serum neutralization test in microplates. The presence of the virus was investigated by PCR in COC and in follicular fluid. Viral DNA was not found in any of the samples. The obtained results suggest that serologically positive cows with no clinical signs of the disease offer negligible risk of transmitting BoHV-1 by COC or follicular fluid.

Index terms: *Bos indicus*, follicular fluid, infectious bovine rhinotracheitis virus, transmission risk, viral DNA.

Herpesvírus bovino tipo 1 em complexos cumulus-oócito coletados de vacas infectadas naturalmente

Resumo – O objetivo deste trabalho foi investigar a presença do herpesvírus bovino tipo 1 (BoHV-1) no fluido folicular e em complexos cumulus-oócito (COC) coletados de vacas naturalmente infectadas mas sem sinais clínicos da doença. Vacas soropositivas (n=38) ou soronegativas (n=8, controle) para o vírus da rinotraqueíte infecciosa bovina foram selecionadas por prova de soroneutralização em microplacas. A presença do vírus foi investigada por PCR em COC e fluido folicular. O DNA viral não foi encontrado em qualquer das amostras. Os resultados obtidos são indicativos de que vacas soropositivas, mas sem sinais clínicos da doença, oferecem risco negligível de transmissão do BoHV-1 por COC ou fluido folicular.

Termos para indexação: *Bos indicus*, fluido folicular, vírus da rinotraqueíte infecciosa bovina, risco de transmissão, DNA viral.

Embryo transfer emerged as an alternative for animal germplasm exchange due to its low costs, better logistics, and, mainly, lower sanitary risks, when compared to the trade of live animals (Thibier & Nibart, 1987). However, most of the scientific background supporting the international regulation for embryo transfer was formed before the increase in the use of in vitro embryo technologies in the last decade (Viana et al., 2012). During this period, there was also an alarming reduction in scientific literature about the biological safety of embryo transfer (Blondin, 2014).

The effectiveness of trypsin washing for decontamination of in vitro-produced embryos is

controversial (Wrathall et al., 2006; D'Angelo et al., 2009). Consequently, risk analysis should focus on the chance of contamination of the gametes before embryo production. The development of strategies to investigate the presence of pathogens in the cumulus-oocyte complexes (COC) is, therefore, critical for the safety of in vitro-produced embryos.

Infectious bovine rhinotracheitis (IBR) is a disease caused by bovine herpesvirus type 1 (BoHV-1), and it causes great economic losses both in the dairy and beef industries worldwide (Muylkens et al., 2007). In cattle experimentally infected with BoHV-1, the virus was detected in embryos and follicular fluid; however, the

virus was not found in embryos produced in vitro from dexamethasone-treated seropositive cows (Bielanski & Dubuc, 1994). The results of artificial infections in vitro are also controversial, since the virus was found in the embryos (Givens & Marley, 2008), but the amount of viral particles used is usually much higher than that occurring in natural infections. Moreover, it is very unlikely that a donor would be aspirated during the acute phase of the infection.

The objective of this work was to investigate the presence of BoHV-1 in the follicular fluid and in COC recovered from naturally infected cows but with no clinical signs of the disease.

Naturally infected and seropositive (n=38) or seronegative (n=8) Gir cows (*Bos indicus*) were selected from herds located in the southeastern region of the state of Minas Gerais, Brazil, during 2006–2007. The animals were raised in grazing systems and were never vaccinated for IBR. All cows were also evaluated for the occurrence of clinical signs of the disease, such as fever, abnormal breathing, spontaneous cough, ocular and nasal discharges, flow of saliva, conjunctivitis, and hyperemia of the nasal mucosae; however, none of the cows showed any of these clinical signs.

Blood samples were collected 60 days before and on the day of follicular aspiration. The serological diagnosis of BoHV-1 and of *Bovine viral diarrhoea virus* (BVDV) was performed with the reference serum-neutralization method using the Colorado strain of BoHV-1 (ATCC VR 864) and Madin-Darby bovine kidney (MDBK) cells (ATCC CCL 22). The final point of the reaction was determined as the serum's highest dilution that was able to inhibit 100% of the cytopathic effect induced by BoHV-1 in the MDBK cells. An animal was considered as seropositive when serum titre was ≥ 4.0 .

COC and samples of follicular fluid were recovered by transvaginal ultrasound-guided follicle aspiration. The COC recovered were identified under 40x magnification and morphologically classified as "viable" or "nonviable". A pool of COC and the samples of follicular fluid from each donor were stored in liquid nitrogen.

DNA extraction was performed using the commercial Biosystems DNA extraction kit (Biosystems, Curitiba, PR, Brazil), according to the manufacturer's instructions. Identification of virus particles was performed by the PCR technique. The primers used

and the amplification conditions are described in Alegre et al. (2001). Briefly, reactions were performed in a volume of 50 μL , with 10 μL DNA, *Taq* buffer – 10 mmol L^{-1} Tris HCL pH 9.0, 1.5 mmol L^{-1} MgCl_2 , 200 $\mu\text{mol L}^{-1}$ of dNTPs, 5% glycerol, and 2.5 U *Taq* DNA polymerase (Phoneutria Biotecnologia e Serviços Ltda., Belo Horizonte, MG, Brazil) – and 50 pmol of one of the sense or antisense primers (primer 1: 5'-AGACCCAGTTGTGATGAATGC-3'; and primer 2: 5'-ACACGTCCAGCACGAACACC-3'), which amplify a 183-bp fragment of the BoHV-1 thymidine kinase *gB* gene. A mix of reagents (without DNA) and milli-Q water, as well as a sample of an animal not exposed to BoHV-1, were used as negative controls. Amplified fragments were analyzed by electrophoresis in 1% agarose gel containing Tris-borate-EDTA buffer (0.045 mol L^{-1} Tris-borate, 1.0 mmol L^{-1} EDTA, pH 7.2), at a voltage designed for the gel dimensions (1.0 to 10 V cm^{-1} of gel), and were then stained with ethidium bromide. A molecular weight marker with 100-bp increments 100-bp ladder, (Invitrogen, ThermoFisher Scientific Inc., Waltham, MA, USA) was used as size standard.

The analytic sensitivity of the PCR method was evaluated using negative bovine follicular fluid and oocytes infected with serial ten-fold dilutions of BoHV-1 Colorado strain (ATCC VR-864). Briefly, MDBK cells containing $10^{5.4}$ TCID₅₀ mL^{-1} of BoHV-1 were ten-fold serially diluted with negative bovine follicular fluid and with oocytes from serologically negative cows. The analytic sensitivity of the PCR in COC and in follicular fluid was 10^{-2} TCID₅₀ mL^{-1} . The COC recovery rates in seropositive and seronegative cows were compared by chi-square analysis. The number of COC classified as viable or nonviable for in vitro embryo production was analyzed by analysis of variance (Anova), and means were compared by the t test using the SAS software, version 8.02 (SAS Institute Inc., Cary, NC, USA). Statistical significance was based on a p-value of 5% probability.

The COC recovery rate was similar ($p > 0.05$) between seronegative (control, 81.73%, i.e., 94 of 115 follicles) and seropositive animals (72.25%, i.e., 349 of 483 follicles). The average number of viable (5.12 ± 2.53 vs. 4.47 ± 3.02) and degenerated (6.62 ± 2.55 vs. 4.71 ± 2.75) COC did not differ between the two groups ($p > 0.05$). Although it was not designed for this purpose, the present study indirectly evaluated

the effect of BoHV-1 infection on COC quality (mainly affected by follicular wave status, and follicle dominance or atresia), but the obtained results did not provide any evidence of the detrimental effects of IBR contamination on COC recovered from cows with different viral titres, but showing no clinical signs of the disease. The associations among viral titres, number, and quality grade of the COC recovered are shown in Table 1.

In the present study, all samples of follicular fluid and of COC, either from seronegative or seropositive cows, were negative to BoHV-1 in the PCR reaction. These results are in alignment with the previous reports that in vivo-derived embryos recovered from IBR serologically-positive animals had no effect on pregnancy rates and did not contaminate recipients (Stringfellow & Wrathall, 1995), and also that in vitro-produced embryos from serologically-positive heifers tested negative for BoHV-1 (Bielanski & Dubuc, 1994).

The present study was designed to reproduce the most common situation in the commercial embryo industry practice, in which seropositive donors were infected naturally and showed no clinical signs of the disease when aspirated. Other studies investigated the presence of virus particles in follicular fluid and in COC recovered from animals artificially infected, or naturally infected but in the acute period of the disease (Bielanski et al., 1993; Bielanski & Dubuc, 1994; Stringfellow & Givens, 2000). The level of infectivity in all these experimental models is unlikely to occur in naturally infected animals out of the acute phase of the disease, when the virus spreads and contaminates the

reproductive tract (Guerin et al., 1997). Therefore, the obtained results bring an important contribution to the risk analysis of in vitro-produced embryos.

It is also vital to consider the possibility of false-negative results. This may occur due to PCR sensitivity, which is affected by the primers and procedures used, including the case of BoHV-1 (Marley et al., 2008). In the present study, the analytic sensitivity of the PCR reaction was shown to be high, even in the presence of a very small amount of viruses, as previously reported (Alegre et al., 2001). The speculative possibility of not detecting BoHV-1 in samples with very low concentration of viral particles would also be associated with a contamination potential below the minimum threshold to cause infection, as found for other viruses (Gard et al., 2009). Therefore, the relevance of eventual false-negative tests is low, and the screening of COC and of follicular fluid for BoHV-1 using PCR probably represents an alternative protocol to reduce the potential risk of disease transmission by the transfer of in vitro-derived embryos.

In conclusion, these results suggest that serologically positive cows with no clinical signs of the disease offer negligible risk of transmitting BoHV-1 by COC or follicular fluid.

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Table 1. Associations among viral titres, number, and quality grade of the cumulus-oocyte complexes (COC) recovered by transvaginal ultrasound-guided follicle aspiration from cows naturally infected with bovine herpesvirus type 1 (BoHV-1).

Viral title	Number	Viable COC		Nonviable COC	
		Mean±SD	%	Mean±SD	%
Negative	8	5.12±2.53	43.61 (41/94)	6.62±2.55	56.39 (53/94)
4	4	5.75±6.23	56.09 (23/41)	4.50±1.91	43.90 (18/41)
8	4	4.00±2.70	45.71 (16/35)	4.75±3.10	54.28 (19/35)
16	7	4.71±2.69	56.89 (33/58)	3.57±1.71	43.10 (25/58)
32	3	5.33±2.08	48.48 (16/33)	5.66±4.72	51.51 (17/33)
64	8	3.25±1.48	41.93 (26/62)	4.50±2.07	58.06 (36/62)
128	8	3.65±2.32	41.42 (29/70)	5.12±2.29	58.57 (41/70)
256	4	6.75±3.86	54.00 (27/50)	5.75±5.56	46.00 (23/50)

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