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Presence of gammaherpesvirus BoHV-4 in endometrial cytology samples is not associated with subclinical endometritis diagnosed at artificial insemination in dairy cows

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ABSTRACT

In the past, bovine herpesvirus 4 (BoHV-4) has been suggested to be associated with metritis and endometritis. However, not many field studies investigated the association between BoHV-4 and subclinical endometritis (SCE). In the present study, the association between the intrauterine presence of BoHV-4 and SCE diagnosed during artificial insemination (AI) was examined on two dairy farms in Belgium. An immunoperoxidase monolayer assay (IPMA) and an enzyme-linked immuno sorbent assay (ELISA) were used to screen the serum for anti-BoHV-4 antibodies. A SYBR green based one step real time qPCR was used to detect and quantify BoHV-4 (ORF20) in nasal, uterine and vaginal samples collected at AI. A reverse transcription qPCR (RT-qPCR) was used to detect mRNA (gB) as proof of a productive BoHV-4 infection. BoHV-4 was detected in 39.4% (farm A)/23.8% (farm B) of the nasal samples, 48.5% (farm A)/19.0% (farm B) of the uterine samples and 51.5% (farm A)/42.9% (farm B) of the vaginal samples. Active replication was only detected in farm A in 38.5% of the BoHV-4 positive nasal samples and in 5.9% positive cases of the vaginal samples. The prevalence of SCE diagnosed at AI was 45.5% and 42.9% in farm A and farm B, respectively. The presence of SCE was associated with a reduced pregnancy outcome at artificial insemination (AI) ($P < 0.001$). The occurrence of SCE at AI was not associated with the presence of latent or productive BoHV4 infections in the uterus nor in the vagina and nose ($P > 0.05$).

1. Introduction

Bovine herpesvirus 4 (BoHV-4) is a member of the genus *Rhadinovirus* of the subfamily *Gammaherpesvirinae* which can be found in cattle worldwide (Zimmermann et al., 2001). The virus was isolated for the first time in Europe from calves and was associated with respiratory disease and keratoconjunctivitis (Bartha et al., 1966). Up till now, it has been identified in cattle with a variety of clinical signs including skin lesions (House et al., 1990; Reed et al., 1977), tumours (Kaminjolo et al., 1972), mastitis (Kalman et al., 2004), metritis (Parks and Kendrick, 1973), and in clinically healthy individuals (Belak and Palfi, 1974; Goyal and Naeem, 1992; Luther et al., 1971). More recently, the virus was detected in bovine semen from an artificial insemination (AI) center (Egyed et al., 2011; Moran et al., 2013). Unlike most *gammaherpesviruses*, BoHV-4 is able to replicate *in vitro* in a broad range of species (Donofrio et al., 2002). However, until now, BoHV-4 has not been clearly recognized as the primary etiologic agent of any specific disease entity. Interestingly, *in vivo* studies have shown that monocyte/macrophage lineage cell are a possible site of persistent/latent BoHV-4 infection (Donofrio and van Santen, 2001). Experimental infection of bulls indicated that BoHV-4 can be reactivated from latent stage with dexamethasone (Dubuisson et al., 1989).

Uterine infections cause major economic losses in the dairy industry, affecting approximately half of all dairy cattle after parturition, provoking subfertility by disrupting uterine and ovarian functions (Sheldon et al., 2009). When endometritis occurs without the presence of clinical signs, it is designated as 'subclinical endometritis' (SCE). Subclinical endometritis is a highly prevalent disease (25–35%),

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characterized by the absence of clinical signs of endometritis, but having a profound effect on fertility (Sheldon et al., 2006). Subclinical endometritis is defined as an increased percentage of polymorphonuclear neutrophils (PMNs) in the uterine lumen as detected by cytologic techniques such as cytobrush, low volume lavage or cytotape (Gilbert et al., 2005; Kasimanickam et al., 2004; Pascottini et al., 2017). Advantage of cytotape among other cytologic techniques is the possibility to sample cows during insemination using ordinary material and with high cytology standards.

The decades-long debate about the direct or indirect role of BoHV-4 in clinical signs of cattle is still active. The unsuccessful reproduction upon experimental infections and frequent isolations of the virus out of cattle with various or even no specific clinical signs, made its direct causative role unclear (Egyed et al., 2011). BoHV-4 has been suggested to be a contributor in the bovine postpartum uterine disease (Parks and Kendrick, 1973). Szenci et al. could only detect a correlation between BoHV-4 and reproductive disorders when a co-infection with the bacterium *Histophilus somni* (Szenci et al., 2016).

The objectives of the present study were to describe the prevalence of BoHV-4 latent and productive infections in two dairy farms in the Flemish region of Belgium and to examine the potential association between the presence of SCE and the pregnancy outcome with an active BoHV-4 uterine replication at the moment of insemination.

2. Materials and methods

2.1. Study design

This study was conducted in two dairy farms (A and B) located in the Flemish region of Belgium from February until September 2016. A total of 54 Holstein-Friesians (21 primiparous and 33 multiparous), housed in free stall barns and milked twice daily, were included. Cows were fed with a total mixed ration according to their production level. Herd level inclusion criteria were mainly based on the farmer's willingness to collaborate. Cow level inclusion criteria were: healthy Holstein-Friesian cow presenting standing estrus and offered for a first insemination after calving. Cows that had developed any type of postpartum uterine disease such as metritis or CE during the voluntary waiting period (VWP) were excluded from the study.

All inseminations were done after the VWP, which was set at 55 days in milk (DIM). A total of 54 Holstein-Friesian cows (33 from farm A, 21 from farm B) were inseminated and sampled after spontaneous heat expression using the cytotape device.

Vaginal and nasal samples were taken using a Cytobrush Plus GT (Cooper Surgical, Berlin, Germany). To do so, a cytobrush adapted to an insemination stylet covered with a 22"-long individually wrapped (Agtech, Manhattan, KS, USA) was introduced deep in the vagina with the help of a clean gloved hand. Once the fornix was reached, the cytobrush was released, rotated twice, re-covered, and removed from the genital tract. To acquire the nasal sample, a clean cytobrush was introduced in the previously wiped right nostril of the cow (approximately 6 cm deep). Before the cytobrush was removed, it was rotated twice. In both cases (vaginal and nasal samples) after sampling, the cytobrushes were introduced in 15 ml Falcon conical centrifuge tubes containing 6 ml of sterile PBS solution, and placed in a refrigerated container for transportation. Lastly, a whole blood sample was taken from the coccygeal vessels into sterile glass vacuum blood collection tubes without anticoagulant (BD Vacutainer Precision Glide, Becton Dickinson, Franklin Lakes, NJ).

Once at the laboratory facilities, all cytologic tubes were vortexed for 3 min in order to suspend and homogenize the cellular material of the sample in the PBS. In the cytotape tube, the tip of the AI sheath was removed and 3 subsamples (1 ml each) were allocated in 3 Eppendorf Safe-lock micro centrifuge tubes (1.5 ml), correspondingly identified, and placed in a −70 °C freezer. The remaining 3 ml were centrifuged at 700 × *g* for 5 min. After discarding the supernatant, a drop of the pellet was positioned on a microscope slide and spread on the glass slide with the tip of the sampler. After air drying, slides were stained with a modified Wright-Giemsa (Diff-Quick, Fisher Diagnostics, Newark, DE, USA) staining. Slides were conventionally evaluated by light microscopy (Kyowa Optical, Tokyo, Japan). A total of 300 nucleated cells were counted at 400× magnification to identify individual cell types (PMNs and endometrial epithelial cells). The PMN counts were expressed as a percentage of the total number of nucleated cells counted. Subclinical endometritis cut-off point was set at PMN ≥ 1. The vaginal and nasal cytobrush samples were also allocated in Eppendorf Safe-lock micro centrifuge tubes (1.5 ml; triplicates), correspondingly identified, and placed in a −70 °C freezer. The whole blood samples were centrifuged at 1500 × *g* for 15 min, the serum separated in aliquots and stored at −70 °C.

2.2. Serological examination

BoHV-4-specific serum antibody titers were determined by an in-house immunoperoxidase monolayer assay (IPMA) which was described previously (Wellenberg et al., 1999). Briefly, Vero cells were seeded in 96-well cell culture plates, inoculated with 50 µl of the V.test strain of BoHV-4 and incubated for 48 h at 37°C and 5% CO_2. Subsequently, the culture medium was discarded and the cells were washed with 100 µl PBS/well. The plates were dried at 37°C for 1 h and kept at 20°C until use. Plates were thawed and then fixed in 4% paraformaldehyde for 10 min at room temperature. Plates were washed twice with PBS and a solution of 1% H_2O_2. Inactivated serum samples (56°C, 30 min) were used at a starting dilution of 1/40. They were diluted fourfold with dilution buffer in a V-plate. 50 µl of each serum dilution were transferred into the wells of the 96-well plates and incubated at 37°C for 1 h. Plates were washed three times and 50 µl of 1/400 goat anti-cow IgG H&L HRP were added to each well and incubated for 1 h at 37°C. Plates were washed three times and 50 µl of a substrate solution of 3-amino-9-ethylcarbazole in 0.05 M acetate buffer, pH 5, with 0.05% H_2O_2 was added to each well, incubating at room temperature for 20 min. Then, the reaction was blocked by replacing the substrate by acetate buffer and the results were determined by examination with a microscope. Furthermore, the IPMAs have been performed with cells infected with BoHV-1, BoHV-2, BoHV-5, EHV-1, EHV-2, EHV-5 and BoHV-4 for both BoHV-4 positive and negative sera. Non-infected Vero cells were used as control. Additionally, an indirect enzyme-linked immuno sorbent assay (ELISA) was performed, following the instruction of the manufacturer (BIO-K-312, Bio-X Diagnostics, Belgium).

2.3. Preparation of DNA standards and SYBR green based one step qPCR for the detection of the ORF20 gene

DNA of each sample was extracted using a commercially available kit (QIAamp® cador Pathogen Mini Kit, Cat. 54106, Qiagen), according to the manufacturer's instructions. Subsequently, DNA concentration was determined by spectrophotometry at an absorbance of 260 nm. The sequence of the ORF20 gene of BoHV-4 (GenBank, accession number JQ838046) was used for the design of the primers. A PCR was performed as previously described (Egyed et al., 1996). The outer primers (ORF20_F and ORF20_R) were used to flank a 567-bp fragment. DNA extracted from a purified BoHV-4 strain and distilled water served as positive and negative controls, respectively. The amount of cDNA was determined using the Nanodrop 2000 Spectrophotometer (Thermo Scientific). DNA standards were stored in single-use aliquots of 10 µL (6 ng/µL) volume at -70°C until their use. The ENDMEMO online web tool was used to calculate the number of DNA copies per microliter. The qPCR primers were designed using the online web Primer 3 plus tool. The sequences of the primers for qPCR targeting ORF20 are depicted in Table 1. In order to set up a standard curve, the concentration of DNA was measured as described above, and further serially ten-fold diluted

Table 1
Primers used for PCR and Qpcr.

Primer name	Gene	Primer sequence	Amplicon size
ORF20_F	ORF20	5′-GTTGGGCGTCCTGTATGGTAGC-3′	576 nt
ORF20_R	ORF20	5′-ATGTATGCCCAAAACTTATAATATGACCAG-3′	
qPCR_fw	ORF20	5′-GCTTTGTACCACTTGAGCTT-3′	167 nt
qPCR_rv	ORF20	5′-CACATCCCAACAACGCACTA-3′	
gB-T7_F	gB	5′-*TAATACGACTCACTATAGGG*CCCTTCTTTAC CACCACCTACA-3′	615 nt
gB_R	gB	5′-TGCCATAGCAGAGAAACAATGA-3′	
RT-qPCR_fw	gB	5′-ACCACCTCTTCCACAACAACATCAAC-3′	124 nt
RT-qPCR_rv	gB	5′-TGGTACCCTGATTATCAGTGGATGGC-3′	

T7 promoter sequence indicated in italics.

in nuclease free water (Gibco). Real time-qPCR reaction mixtures (20 µL) consisted of 10 µL Precision OneStep qPCR Mastermix with SYBR Green and ROX (Primer Design), 200 nM (0.4 µL/reaction) of qPCR_fw and 50 nM (0.1 µL/reaction) of qPCR_rv primer which have been optimized, 6.5 µL nuclease free water and 3 µL of standard DNA template or H_2O. Reaction mixtures were loaded in MicroAmp Optical 96-well reaction plates (Applied Biosystems), sealed with MicroAmp Optical Adhesive Films (Applied Biosystems), and experiments were performed in a StepOnePlus apparatus (Applied Biosystems). An enzyme activation step at 95 °C for 8 min was followed by 40 cycles, each 10 s at 95 °C and 60 s at 60 °C. Afterwards, a first-derivative melting curve analysis was performed by heating the mixture to 95 °C for 15 s, then cooling to 60 °C for 1 min, and heating back to 95 °C at 0.3 °C increments. Results were analyzed using the StepOnePlus Software version 2.2. The baseline was set automatically, and the threshold was placed manually in the exponential phase of the amplification reaction. Melt curve analysis and agarose gel electrophoresis were performed to assess specificity of the reactions. Amplification efficiency was determined by running a standard curve over a linear dynamic range (LDR) from 5 log10 copies/reaction to 1 log10 copies/reaction in 1:10 dilution steps. Each dilution point of the standard curve was analyzed in triplicate and also three non-template control reactions (nuclease free water) were included in each experiment.

2.4. Preparation of gB mRNA standards for absolute quantification by an RT-qPCR

RNA of samples were extracted using a commercially available kit (QIAamp® cador Pathogen Mini Kit, Cat. 54106, Qiagen), according to the manufacturer's instructions. Template DNA was removed by treatment of the reaction mixture with 2U DNase I (Sigma Aldrich, St. Louis, USA). One-step reverse transcription (RT)-PCR (Invitrogen, Merelbeke, Belgium) was conducted, using the primer gB-T7_F which incorporate a T7-promotor sequence at the 5′ and the primer gB_R (Table 1), generating a 615bp fragment. Afterwards, the RT-PCR product was analyzed by electrophoresis on a 1.2% agarose gel and purified with the Nucleospin Gel and PCR-Clean up kit (Macherey-Nagel, Düren, Germany). *In vitro* transcription was performed using the T7 RNA Polymerase-PlusTM Enzyme Mix (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA), according to the manufacturer's instructions. DNase I (Sigma Aldrich, St. Louis, USA) was used to remove the template DNA. The *in vitro* generated RNA was purified using the RNeasy Mini Kit (Qiagen). Electrophoresis on a 1.2% agarose gel was performed to assess the size of the transcript (615 bp). Synthetic RNA standards were stored in single-use aliquots of 10 µL volume at -70°C until their use. Primers RT-qPCRm_fw and RT-qPCRm_rv (shown in Table 1) were used for detecting the mRNA of gB gene. RT-qPCR reaction mixtures (20 µL) consisted of 10 µL Precision OneStep qRT-PCR Mastermix with SYBR Green and ROX (Primer Design), 50 nM (0.1 µL/reaction) of RT-qPCR_fw and 200 nM (0.4 µL/reaction) of RT-qPCR_rv primer which have been optimized, 6.5 µL nuclease free water and 3 µL of standard mRNA template or H_2O. The reactions were performed in a StepOnePlus apparatus (Applied Biosystems). An enzyme activation step at 55°C for 10 min and 95 °C for 8 min were followed by 40 cycles, each 10 s at 95 °C and 60 s at 60 °C.

The specificity of both primers for ORF20 and gB mRNA was determined by testing the following viruses: alphaherpesviruses including bovine herpesvirus 1 (BoHV-1), bovine herpesvirus 2 (BoHV-2), bovine herpesvirus 5 (BoHV-5) and equine herpesvirus 1 (EHV-1); gammaherpesviruses including equine herpesvirus 2 (EHV-2) and equine herpesvirus 5 (EHV-5). Furthermore, additional MDBK infection assays were performed to mimic the latency and reactivation status of the virus. A transcription blocking agent (Actinomycin D (ActD, Invitrogen)) was used to block the transcription of BoHV-4, mimicking the latency status. MDBK cells that were infected with BoHV-4 without Actinomycin D were included as samples for a fully productive infection.

2.5. Statistical analyses

All on farm collected data and results from laboratory analyses were transferred to a Microsoft Excel (Microsoft Corp., Redmond, WA) work file. Initially, data exploration and re-organization were done with the PivotTables function (Microsoft Excel). Statistical analyses were done using the R version 3.3.0 (R Core Team, Vienna, Austria). The function summary of the R code system (package Base) was used for the descriptive analyses, considering the cow as the unit of interest.

The function glmer of the package lme4 (Bates et al., 2015) was used to build logistic regression models to determine the association between the pregnancy outcome of each AI with other fixed effects. The responsive variable of the models (AI outcome) was binomial, with cows classified as being pregnant or not pregnant. The fixed effects were: parity, days in milk at AI, presence or absence of SCE at AI, presence or absence of BoHV4 ORF20 in the uterus, nasal mucosa and vaginal mucosa, and presence or absence of BoHV4 ORF20 either in the uterine, nasal or vaginal mucosa. First, univariable models were constructed to identify fixed effects associated ($P < 0.2$) with the pregnancy outcome. The final multivariable model was built by manual stepwise backward elimination and only risk factors (fixed effects) with P-values < 0.05 were retained. Also, logistic regression models were computed in order to find associations between the presence of SCE at AI (positive or negative) with all the fixed effects previously mentioned. After identifying univariable risk factors ($P < 0.2$) associated with SCE, a final multivariable model was built ($P < 0.05$) by manual stepwise backward elimination. For all the models, farm was included as random effect. All results are expressed as odds ratios with their respective 95% confidence intervals.

Table 2
Detailed information collected from cows in farm A.

Animal			Histopathology	Serological test		Nasal swabs		Uterine swabs		Vaginal swabs		Pregnancy
Number	Parity	DIMatAI	PMN%	ELISA	IPMA	ORF20*	gB mRNA*	ORF20	gB mRNA	ORF20	gB mRNA	
17	2	51	0	positive	640	1.9×10^3	negative	negative	NT	negative	negative	empty
4	4	53	0	positive	640	5.2×10^4	2.8×10^5	2.0×10^2	negative	3.3×10^4	negative	empty
28	2	58	0	positive	640	1.1×10^4	6.7×10^3	1.6×10^3	negative	2.2×10^3	negative	empty
1138	1	54	0	positive	160	negative	NT	negative	NT	4.0×10^3	negative	empty
19	3	52	0	positive	160	1.5×10^4	negative	2.1×10^2	negative	2.2×10^3	negative	empty
104	4	133	0	positive	40	2.5×10^2	6.8×10^3	6.8×10^3	negative	3.0×10^2	negative	empty
103	5	63	0	positive	160	negative	NA	1.9×10^2	NA	1.8×10^2	NA	pregnant
63	4	54	0	positive	160	2.3×10^4	2.2×10^4	5.4×10^2	negative	1.6×10^4	negative	pregnant
13	4	54	0	positive	160	1.8×10^4	negative	1.0×10^3	negative	4.2×10^3	2.7×10^3	pregnant
94	2	127	0	positive	640	4.0×10^4	NA	7.0×10^3	negative	1.1×10^4	negative	pregnant
5	1	58	0	positive	2560	negative	negative	negative	NT	1.3×10^2	NA	pregnant
20	1	63	0	positive	160	negative	NT	negative	NT	1.6×10^2	negative	pregnant
24	2	61	0	positive	2560	negative	NT	negative	NA	negative	NA	pregnant
46	2	68	0	positive	160	negative	NT	negative	NT	negative	NT	pregnant
56	2	62	0	positive	640	negative	NT	negative	NA	5.0×10^2	negative	pregnant
74	1	51	0	positive	640	negative	negative	negative	NA	negative	NT	pregnant
77	1	56	0	negative	< 40	negative	NT	negative	NA	negative	NT	pregnant
25	2	58	0	positive	160	1.8×10^2	NA	negative	NT	negative	NT	pregnant
65	3	59	1	positive	160	negative	NT	negative	NT	negative	NT	empty
31	3	69	1	positive	2560	2.6×10^3	9.5×10^3	5×10^2	NA	1.1×10^5	NA	empty
89	2	54	4	positive	160	negative	NT	negative	NT	negative	NT	empty
88	2	60	1	positive	40	negative	NT	negative	NT	negative	NT	empty
84	2	77	3	positive	160	negative	NA	2.7×10^5	NA	3.9×10^2	negative	empty
95	2	56	4	positive	160	2.2×10^3	NA	4.4×10^4	NA	4.6×10^2	negative	empty
22	3	53	21	positive	40	2.4×10^5	NA	1.8×10^3	NA	1.8×10^2	NA	empty
6	1	52	7	positive	10240	negative	NA	1.1×10^4	negative	1.8×10^2	negative	empty
79	4	90	1	positive	640	5.8×10^3	negative	4.3×10^2	negative	negative	NT	empty
2	2	54	8	positive	10240	negative	NA	negative	NA	negative	NT	empty
37	1	80	5	positive	160	negative	NT	1.7×10^2	negative	negative	NT	empty
38	2	63	3	positive	160	negative	NT	negative	NA	negative	NT	empty
51	2	54	21	positive	640	negative	negative	negative	NA	negative	NT	empty
97	1	51	5	positive	640	negative	NT	negative	NT	negative	NT	empty
33	2	50	1	positive	160	negative	NT	1.7×10^2	negative	negative	NT	pregnant

DIMatAI: Days in milk at artificial insemination, PMN%: Percentage of polymorphonuclear cell, NA: not available, NT: not tested.
* copies/ml.

3. Results

3.1. Occurrence of SCE at AI

To determine the prevalence of SCE and its eventual association with BoHV-4 infection at the moment of insemination, endometrial cytology samples were acquired from two commercial dairy farms. Three samples were discarded due to missing data. Thus, 54 cows (A:33/B:21) were included; 24.2% (8/33) primiparous and 75.8% (25/33) multiparous cows in farm A and 61.9% (13/21) primiparous and 38.1% (8/21) multiparous cows in farm B. All cows were sampled at the first AI attempt and the average DIM at sampling/insemination was 67.1 ± 20. The conception rate was 39.4% (13/33) in farm A and 38.1% (8/21) in farm B. The SCE prevalence on farm A was 45.5% (15/33), and 42.9% on farm B (9/21), as shown in Tables 2 and 3.

3.2. High seroprevalence of BoHV-4 in the two herds

To check for past BoHV-4 infections in the two herds, the seroprevalence was determined by both IPMA and ELISA. Firstly, the antibodies against BoHV-4 from 54 serum samples were tested by IPMA as described in the methods. The IPMA is specific for the detection of BoHV-4 antibodies, no cross-reaction was observed for all of the tested virus strains. BoHV-4 specific antibody titers greater than or equal to 40 were regarded as positive. The BoHV-4 seroprevalence was 96.9% (n = 32) in farm A, and 90.5% (n = 19) in farm B (Table 2 and Table 3). In addition, an ELISA was performed. As shown in Table 2 and Table 3, the percentage of BoHV-4 seropositive animals by ELISA was 96.9% (n = 32) in farm A, which is exactly the same as determined by IPMA and was 85.7% (n = 18) in farm B, which is one animal less compared to the results obtained with the IPMA.

3.3. Lack of association between the presence of SCE and BoHV-4 DNA

PCR is a commonly used method to detect BoHV-4 DNA in epidemiological studies. For this study, a SYBR green-based one step qPCR method for detection of BoHV-4 ORF20 gene was established. The amplification efficiency of the qPCR assay was 97.6%, the slope of the standard curve was -3.38, according to the slope of the exponential phase in the amplification chart. Melt curve analysis showed amplification of a specific product with a melting peak at 80.8 °C, which was confirmed by the agarose gel electrophoresis analysis. Amplification was not observed in the non-template control. The standard curve had a wide dynamic range (10^1-10^5 copies/reaction) with a high linear correlation (R^2 = 0.997) between the cycle threshold (Ct) value and template concentration (data not shown). DNA extracts from samples were analyzed in duplicate reactions. Quantification of the viral DNA copies was possible if the Ct values of both reactions fell within the LDR of the RT-qPCR assay. Samples with a specific melt curve were considered positive but not quantifiable when the Ct value of the reactions fell at Ct values higher than the lowest peak of the LDR. All samples collected from the two farms were tested with the successfully established SYBR green-based qPCR assay. The diagnostic results showed that (i) in farm A, 39.4% (13/33) of the nasal samples, 48.5% (16/33) of the uterine samples and 51.5% (17/33) of the vaginal samples were positive; (ii) in farm B, 23.8% (5/21) of the nasal samples, 19.0% (4/21) of the uterine samples and 42.9% (9/21) of the vaginal samples were positive. All individual results are shown in Tables 2 and 3. The viral genome copies

Table 3
Detailed information collected from cows in farm B.

Animal			Histopathology	Serological test		Nasal swabs		Uterine swabs		Vaginal swabs		Pregnancy
Number	Parity	DIMatAI	PMN%	ELISA	IPMA	ORF20*	gB mRNA*	ORF20	gB mRNA	ORF20	gB mRNA	
1224	1	58	0	positive	640	1.7×10^2	negative	negative	NT	7.0×10^4	negative	empty
174	1	50	0	positive	160	4.4×10^3	negative	9.2×10^2	negative	3.3×10^4	negative	empty
232	1	97	0	negative	< 40	negative	NA	negative	NA	negative	NA	empty
114	3	83	0	positive	160	negative	negative	negative	NT	negative	NT	empty
98	1	60	0	positive	640	negative	NT	negative	NT	negative	NT	empty
107	1	53	0	positive	640	3.8×10^3	NA	negative	NT	negative	NT	empty
71	1	52	0	positive	160	negative	NT	negative	NT	5.0×10^3	negative	pregnant
67	6	96	0	positive	640	negative	NA	3.5×10^2	NA	negative	NA	pregnant
137	2	94	0	positive	160	negative	NT	1.1×10^3	negative	negative	NT	pregnant
149	1	71	0	positive	640	negative	NT	negative	NT	1.8×10^2	negative	pregnant
241	1	98	0	positive	640	negative	NT	negative	NT	negative	NT	pregnant
240	1	107	0	positive	640	negative	NT	negative	NT	negative	NT	pregnant
204	2	51	3	positive	640	negative	NT	negative	NT	1.6×10^4	negative	empty
25	3	52	8	negative	< 40	negative	negative	negative	NA	negative	NA	empty
84	2	99	1	positive	640	negative	NT	4.2×10^2	negative	6.7×10^2	negative	empty
170	1	54	3	negative	40	negative	NT	negative	NT	negative	NT	empty
45	1	54	2	positve	40	negative	NA	negative	NT	negative	NT	empty
59	4	88	8	positve	40	6.3×10^2	negative	negative	NT	2.5×10^2	negative	empty
109	1	54	7	positve	640	negative	NT	negative	NT	negative	NT	empty
95	7	92	1	positive	160	negative	NT	negative	NT	2.2×10^2	negative	pregnant
90	1	87	3	positive	160	2.9×10^4	NA	negative	NT	9.0×10^3	negative	pregnant

DIMatAI: Days in milk at artificial insemination, PMN%: Percentage of polymorphonuclear cell, NA: not available, NT: not tested.
* copies/ml.

both in farm A and farm B were depicted in Fig. 1. Our results did not show an association between BoHV-4 and SCE, which might be a further proof for the lack of direct pathogenicity of BoHV-4.

3.4. BoHV-4 gB mRNA was detected in nasal and vaginal samples of animals from one farm

To further analyze if a productive BoHV-4 infection is associated with SCE, an RT-qPCR detecting gB mRNA level of BoHV-4 was developed. RT-qPCR primers were listed in Table 1. RT-qPCR efficiency is 103.8%, which was derived from the slopes of the standard curves. Melt curve analysis showed amplification of a specific product with a melting peak at 81.1 (data not shown).

No amplification was observed for all of the tested viruses with both PCRs based on the melt curve (data not shown). MDBK cells were collected for both qPCR and RT-qPCR at different time points (0 hpi, 3 hpi, 6 hpi, 9hpi, 12 hpi and 24 hpi) after mock treatment or treatment with Actinomycin D. The gB mRNA quantity was first detected at 12 hpi ($3.5\log_{10}$/ml) and further increased at 24 hpi in the mock treated MDBK cells. No detectable mRNA was found in the Actinomycin D treated MDBK cells (Fig. 2). Therefore, the ORF20 gene positive samples were further analyzed by the newly established RT-qPCR assay for quantifying the mRNA expression of gB. In farm A, 5 out 13 (38.5%) samples from nasal samples and 1 out of 17 (5.9%) samples from vaginal samples were positive. No positive reactions were found from uterine samples. No gB mRNA positive samples were found in farm B.

3.5. Analysis of risk factors

Logistic regression models were fitted mainly to assess the effects of SCE with fertility, and BoHV-4 presence with SCE. The only risk factor associated with a reduced pregnancy outcome at AI was the presence of SCE ($P < 0.001$). None of the other risk factors had a significant effect on the pregnancy outcome ($P > 0.05$). The occurrence of SCE at AI was not associated with the BoHV4 presence in the uterus nor in vagina or nose ($P > 0.05$). Neither of the tested fixed effects were associated with SCE diagnosed at AI ($P > 0.05$), as demonstrated in Tables 4 and 5.

4. Discussion

Based on a previous large field study, a relatively high SCE prevalence (27%) during AI was observed in Belgium dairy farms, with a significant negative effect on fertility (Pascottini et al., 2017). To investigate whether there is an association between BoHV-4 infection and SCE, two farms in the East Flemish region of Belgium were analyzed more in depth. The seroprevalence was firstly tested with IPMA and ELISA. The BoHV-4 seroprevalence was 96.9% in farm A and 90.5% in farm B with the IPMA and 96.9% in farm A and 85.7% in farm B with the ELISA. These results indicate that BoHV-4 was highly prevalent in the two Flemish dairy farms. The IPMA and ELISA results were quite similar, suggesting that both techniques are suitable to detect BoHV-4 antibodies in serum. In order to determine the presence of latent/productive BoHV-4 infections, a new pair of primers used for SYBR green one step qPCR was designed for detection and quantification of the ORF20 gene of BoHV-4. Samples collected from nose, uterus and vagina of 54 cows from two different farms were analyzed. A relatively higher frequency of positive samples was observed in the vagina compared to nasal and uterine samples. To examine if the BoHV-4 infection was in a productive state (primo-infection or reactivation), another RT-qPCR was established for detection of the mRNA of gB. Only 5 out of 13 ORF20 gene positive nasal samples and 1 out of 17 ORF20 gene positive vaginal samples from farm A were gB mRNA positive. No positive samples were detected in farm B. This result indicates that BoHV-4 was latent in the majority of the BoHV-4 infected animals and that viral shedding occurred mainly *via* nasal secretion.

BoHV-4 has been described mainly as a secondary pathogen in reproductive disorders of cattle (Donofrio et al., 2008; Fabian et al., 2008). It is assumed that BoHV-4 has a negative effect on the endometrial immune response, upon a bacterial infection of the uterus. Furthermore, bacterial infections are believed to reactivate latent BoHV-4 (Donofrio et al., 2010, 2007; Donofrio et al., 2008). However, previous studies failed to associate the presence of SCE with bacterial infection (Baranski et al., 2012; Madoz et al., 2014). SCE is considered as a dysregulated uterine inflammation caused by a previous uterine infection by for instance BoHV-4. In this context, a recent *in vitro* study demonstrated that BoHV-4 replicates in the epithelial cells of the reproductive tract and is able to hijack leukocytes to invade through the

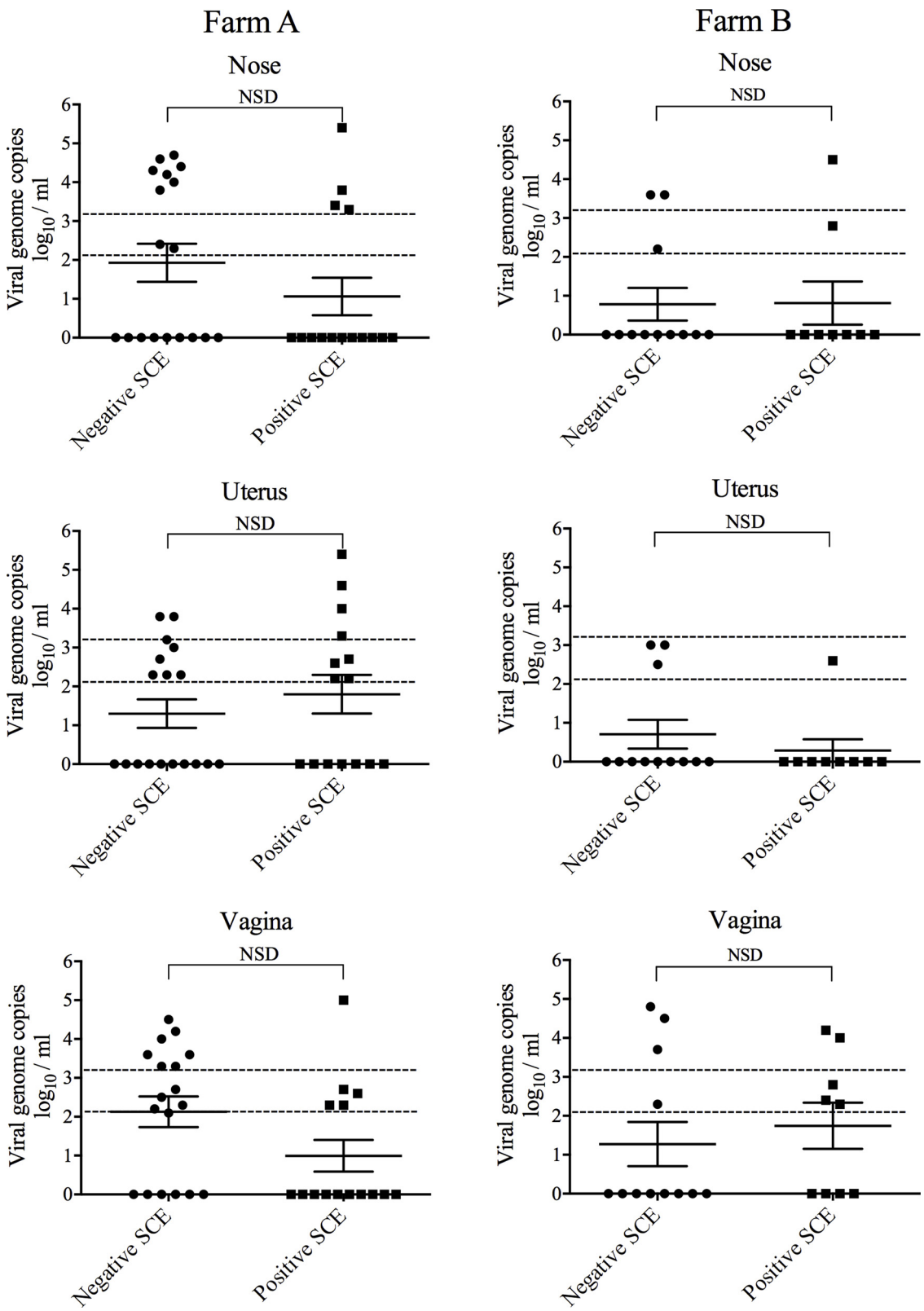

Fig. 1. Comparison of number of copies of BoHV-4 ORF20 from samples (nose, uterus and vagina) between cows with SCE and without SCE. The upper dotted line represents the quantification limit and the bottom line represents the detection limit. NSD: not significantly different.

basement membrane (Yang et al., 2017). Results of the present study did not support our initial hypothesis of BoHV-4 as a persistent agent causing uterine inflammation since no association was found between SCE and BoHV-4 infection. This is in line with another similar study, where a significant relationship between SCE and BoHV-4 has not been found (Klamminger et al., 2017; Szenci et al., 2016).

SCE was associated with a reduced pregnancy outcome. It was reported that around 50% of cows are positive for SCE at 40–60 days postpartum by applying endometrial cytology, which is strikingly similar to our results (Gilbert et al., 2005). In endometrial cytology

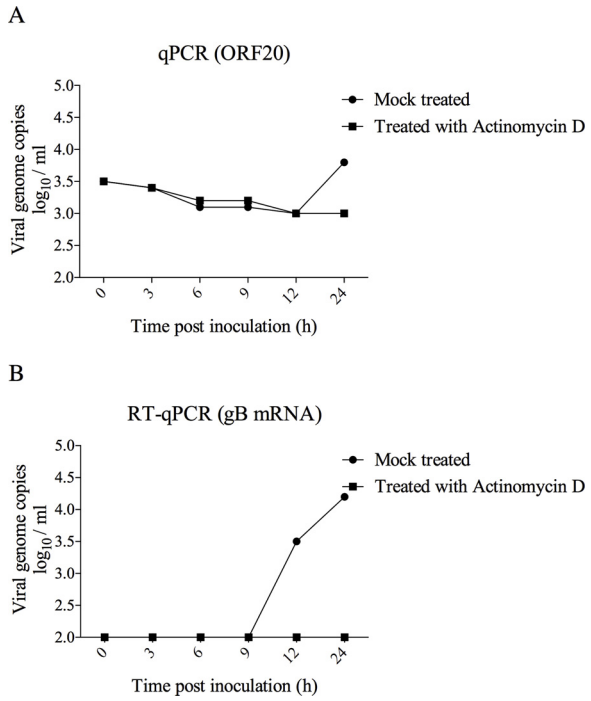

Fig. 2. BoHV-4 infection on MDBK cells after mock or Actinomycin D treatment. The MDBK cells were seeded at a concentration of 2×10^5 cell/ml in a 24 well-plate. MDBK cells were inoculated with BoHV-4 strain V.test at a multiplicity of infection (MOI) of 1. At 3 hpi, cells were treated with Actinomycin D (ActD, Invitrogen) (5 µg/ml) or mock treated. Afterwards, the cells were collected at 0, 3, 6, 9, 12 and 24 hpi. DNA and RNA were extracted from the collected cells. A qPCR (A) with ORF20 primers and an RT-qPCR (B) with gB primers were performed.

Table 4
Results of the univariable model of risk factors associated with the pregnancy outcome.

Variable		Odds ratio	95% CI	P-value
Lactation[1]	1	Reference	–	–
	≥2	0.76	0.25-2.33	0.63
DIM at AI[2]		1.02	0.99-1.05	0.16[b]
SCE[1]	Negative	Reference	–	–
	Positive	0.09	0.02-0.39	0.001[a]
BoHV-4 nose[1]	Absence	Reference	–	–
	Presence	0.48	0.14-1.63	0.24
BoHV-4 uterus[1]	Absence	Reference	–	–
	Presence	0.77	0.25-2.42	0.65
BoHV-4 vagina[1]	Absence	Reference		–
	Presence	1.32	0.44-3.95	0.62
BoHV-4	Absence	Reference	–	–
nose/uterus/vagina[1]	Presence	1.84	0.57-5.95	0.31

[a] and [b] risk factors with P < 0.05 and < 0.2, respectively; CI confidence interval, AI artificial insemination.
BoHV-4: BoHV-4 ORF20 positive.
[1] Categorical variable.
[2] Continuous variable.

samples acquired during AI, results were also similar (Pascottini et al., 2017), but the SCE was slightly higher in the present study. This may be due to uterine sampling at the first AI attempt (just after the end of the VWP) which is a risk factor associated with SCE diagnosed at AI.

Multiple studies have been carried out to clarify the role of BoHV-4 in the cattle industry. Therefore, a rapid, sensitive and reliable method is required to screen large numbers of samples for DNA of BoHV-4 in cattle. In the past, a PCR assay was developed to detect BoHV-4 glycoprotein B gene and a nested PCR for the detection of the ORF20 gene (Egyed et al., 1996; Wellenberg et al., 2001). With these tests, no difference between latent and productive infection could be made. To solve this problem, a SYBR green based one step qPCR was set up to identify and quantify the ORF20 gene of BoHV-4 and an RT-qPCR to identify and quantify the mRNA of BoHV-4 gB. A novel combination of qPCR and RT-PCR was used in order to make a difference between latent and productive BoHV-4 infections. Because only a productive infection can influence certain pathophysiological processes, we have developed that combination of qPCRs to discriminate. The presence of mRNA of BoHV-4 can be related with an active BoHV-4 replication. Latent and productive BoHV-4 infection was detected in the respiratory and reproductive tracts of the cows. However, it was not possible to figure out which cells (epithelial cells, fibroblasts, leukocytes) were latently/actively infected.

Table 5
Results of the univariable model of risk factors associated with SCE diagnosed at AI in dairy cows.

Variable		Odds ratio	95% CI	P-value
Lactation[1]	1	Reference	–	–
	≥2	2.12	0.68-6.61	0.19[a]
DIM at AI[2]		0.99	0.96-1.02	0.36
BoHV-4 nose[1]	Absence	Reference	–	–
	Presence	0.5	0.15-1.62	0.25
BoHV-4 uterus[1]	Absence	Reference	–	–
	Presence	1.04	0.34-3.15	0.95
BoHV-4 vagina[1]	Absence	Reference		–
	Presence	0.63	0.21-1.85	0.39
BoHV-4	Absence	Reference	–	–
nose/uterus/vagina[1]	Presence	0.51	0.17-1.55	0.23

BoHV-4: BoHV-4 ORF20 positive.
[a] risk factor with P < 0.2, CI confidence interval, AI artificial insemination.
[1] Categorical variable.
[2] Continuous variable.

As reported before, periodical reactivation into the lytic cycle is necessary to complete the life cycle of the virus (Laichalk and Thorley-Lawson, 2005). Detection of viral transcriptional activity at the portal site of virus replication may be a better parameter than finding genetic material in virus diagnostics. The gB is one of the most conserved glycoproteins among the herpesvirus family. The previous study has demonstrated that gB is indispensable for a lytic replication of BoHV-4 (Franceschi et al., 2013). Therefore, the mRNA of gB was detected by RT-qPCR to demonstrate an active replication of BoHV-4. This can be the result of a primo-infection or reactivation. Our study revealed that replication of BoHV-4 was more prominent in the respiratory tract than in the genital tract. Therefore, the respiratory tract may play the most important role in BoHV-4 shedding and transmission. Furthermore, we did not find mRNA of gB in any of the uterine samples, which indicates that BoHV-4 was not actively replicating in the uterus. To the best of our knowledge, this is the first study using RT-qPCR to detect a productive replication of BoHV-4 *in vivo*.

Based on the results from our study, the occurrence of SCE at AI was not associated with the BoHV-4 presence and replication status in the uterus nor in the vagina and nose. Since no evidence of direct BoHV-4 and SCE were observed in this study, the direct pathogenicity of BoHV-4 in cattle needs further investigation.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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