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Uterine infection with bovine herpesvirus type 4 in dairy cows

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Contents

Diseases of the reproductive tract are a frequent problem in dairy herds. Herpesviruses are uterine pathogens also involved in other clinical diseases; for example, bovine herpesvirus type 4 BoHV-4 induces abortion, enteritis, metritis, pneumonia and vaginitis, but it can also be detected in healthy cows. The role of BoHV-4 in the development of clinical endometritis (CE) or subclinical endometritis (SE) has not clearly been described. Therefore, the objective of this study was to describe the prevalence of uterine BoHV-4 infection and its relationship with clinical, bacteriological and cytological findings in dairy cows 20–30 days after calving. The experiment was performed as a completely randomized block design, with farm (n = 10) as blocking criterion and with cow (n = 397) as the experimental unit. Logistic regression models were used to assess the effect of BoHV-4 infection on CE, SE and reproductive performance. Proportion of cows infected with BoHV-4 was 5.8% (n = 23/397). BoHV-4 was isolated in 11.0% (n = 12/109), 4.8% (n = 4/84) and 3.6% (n = 7/194) of cows diagnosed as CE, SE or healthy, respectively. A logistic model revealed that BoHV-4 infection showed a tendency to increase the risk for CE (AOR = 2.17; p = .10) but significantly reduced both, the odds for artificial insemination within 80 days post-partum (dpp) (AOR = 0.37; p = .035) and for pregnancy within 200 dpp (AOR = 0.13; p = .004). Furthermore, BoHV-4 infection increased the chance for intrauterine infection with *Trueperella pyogenes* (AOR = 5.55; p < .001) and vice versa (AOR = 5.79, p < .001). In conclusion, BoHV-4 infection is associated with reduced chances for insemination and pregnancy by 200 dpp and showed a trend to be associated with increased risk for CE. Furthermore, BoHV-4 and *Trueperella pyogenes* infections are strongly related.

1 | INTRODUCTION

Bacterial contamination of the bovine uterus occurs commonly within the first weeks after calving. The persistence of pathogenic bacteria can lead to uterine infection and then to clinical diseases such as puerperal metritis <21 days after calving or clinical endometritis (CE) ≥21 days after calving (Sheldon, Lewis, LeBlanc, & Gilbert, 2006). Furthermore, the inflammation may only be detectable by cytology and defined as subclinical endometritis (SE, Sheldon et al., 2006). The diagnosis of SE is based on the proportion of polymorphonuclear cells (PMN) and epithelial cells in the endometrial sample (Kasimanickam et al., 2004; Madoz et al., 2013).

The species *bovine herpesvirus 4* (BoHV-4) occurs worldwide and is part of the genus *Rhadinovirus*, subfamily *Gammaherpesvirinae*, family *Herpesviridae*. It is known that BoHV-4 has a tropism to lymphoid cells (Egyed, Ballagi-Bordány, Bartha, & Belák, 1996). Like other herpesviruses, BoHV-4 infects the host persistently. A latent infection can be reactivated, for example, by glucocorticoid treatment (Dubuisson et al., 1989).

The role of BoHV-4 in the aetiology of reproductive disorders is not fully understood because the virus has been isolated not only from diseased cattle (abortion, enteritis, pneumonia and vaginitis [reviewed by Morán, Pérez, Odeón, & Verna, 2015 and Chastant-Maillard, 2015]) but also from healthy one (Graham et al., 2005; Lin et al., 1997).

Furthermore, BoHV-4 infection was found in cows with signs of metritis (Monge, Elvira, Gonzalez, Stiz, & Wellenberg, 2006; Nikolin et al., 2007; Donofrio, Franceschi, Capocefalo, Cavirani, & Sheldon, 2009; De Boer, Zheng, Buddle, & McDougall, 2014).

The role of BoHV-4 in the aetiology of CE is still unclear. Frazier et al. (2001) described the effect of BoHV-4 on the occurrence of CE, whereas Fábián, Makrai, Sachse, Szeredi, and Egyed (2008) found no association between the presence of BoHV-4 in the uterus and uterine histological or bacteriological findings. Therefore, the latter authors suggested that BoHV-4 plays a minor role in the pathogenesis of bovine endometritis. The interaction between bacterial-initiated PGE_2 synthesis and rapid BoHV-4 replication, however, was suggested as mechanism for the development of uterine disease (Donofrio et al., 2008). Thus, it is likely that BoHV-4 might contribute as a cofactor in combination with uterine pathogens such as *Escherichia coli* and *Trueperella pyogenes* to the development of endometritis (Donofrio et al., 2008; Morán et al., 2015; Szenci et al., 2016).

Despite Gür and Dogan (2010) found a greater prevalence of BoHV-4 in repeat breeder cows compared to cows with a maximum of two inseminations, there is limited information about the role of BoHV-4 in subfertility (Chastant-Maillard, 2015). Recently, Szenci et al. (2016) hypothesized that BoHV-4 is a secondary pathogen in cows with endometritis and might contribute to prolonged calving intervals.

Thus, the objectives of this study were to describe the prevalence of uterine BoHV-4 infection, its relationship with clinical, bacteriological and cytological findings, as well as effects of BoHV-4 on the reproductive performance in dairy cows.

2 | MATERIALS AND METHODS

This study was approved by the institutional ethics committee and the national authority according with § 8 of Law for Animal Experiments, Tierversuchsgesetz-TVG (BMWF-68.205/0105-II/3b/2011). This publication is part of a research project of which some parts have been published previously (Prunner, Wagener, Pothmann, Ehling-Schulz, & Drillich, 2014), describing the overall prevalence of CE and SE, risk factors for uterine diseases and effects (excluding BoHV-4 infections) on reproductive performance.

2.1 | Study farms

The study was conducted on 10 dairy farms (herd size: 31–223) in Austria between June 2011 and April 2012. The majority of cows were Simmental, but also Holstein–Friesian and Brown Swiss were kept on the farms. The average milk yield was 8,568 kg per lactation (range 6,264–10,826 kg). Cows were milked twice a day. Rations were based on grass silage, maize silage and hay, supplemented with concentrates and minerals. The farmers were interviewed about calving assistance (no intervention vs. assisted calving) of examined cows during the researchers' visit. The voluntary waiting period was set at 40–50 dpp on eight farms or was not defined. Cows were bred by artificial insemination (AI) after observed oestrus in all herds. No timed breeding protocols (e.g. OvSynch) were used. Pregnancy diagnosis was performed by transrectal palpation and ultrasonography of the uterus and its contents by the local veterinarians.

2.2 | Study design

A total of 400 cows were enrolled in the study. Sample collection was performed between 20 and 30 dpp. Cows with caesarean section, fetotomy or any systemic antibiotic treatment between calving and sample collection were excluded from the study to avoid false-negative bacteriological findings.

Vaginoscopic examination was performed in cows before samples were collected for bacteriological, virological and cytological examinations. Examination and collection of samples has been performed in the same way as described in detail by Prunner, Wagener et al. (2014). In brief, an autoclaved speculum for cows or heifers (Hauptner and Herberholz, Solingen, Germany) was introduced into the vagina up to the cervix. The vaginal mucus was classified into score 0–3 according to Williams et al. (2005): score 0 = healthy cows without or with clear discharge; score 1 = mucus containing flecks of white or off-white pus; score 2 = less than 50% pus; and score 3 = more than 50% of white or off-white pus.

Two intrauterine samples were collected with the cytobrush technique. The cytobrush (Gynobrush, Heinz Herenz, Hamburg, Germany; 20 mm in length and 7 mm in diameter) was fixed on a metal rod of 65 cm length and protected by a plastic catheter and a plastic sleeve. The brush was inserted through the cervix into the uterus by transrectal guidance. Thereupon, the brush was pushed forward of the plastic catheter, rolled slightly along the uterine wall and retracted into the catheter. For cytology, the brushes were rolled on microscope slides, fixed and stained (Hemacolor, Merck, Darmstadt, Germany). A total of 300 cells were counted under a microscope by ×400 magnification, and the proportion of PMN and endometrial cells was determined. The threshold value for the diagnosis of SE was set at 5% PMN in cows with vaginal discharge score 0 (Baranski, Podhalicz-Dzięgielewska, Zdunczyk, & Janowski, 2012; Kaufmann, Drillich, Tenhagen, Forderung, & Heuwieser, 2009; Plöntzke, Madoz, de la Sota, Drillich, & Heuwieser, 2010). The bacteriological analysis was applied according to Prunner, Pothmann et al. (2014). In brief, the brushes were placed in PBS solution, transported to the laboratory of the Vetmeduni Vienna, rolled on different agar plates (Oxoid, Columbia Sheep Blood Agar, Hampshire, UK; Oxoid, MacConkey, Hampshire, UK) and incubated for 48 hr at 37°C under aerobic conditions. Afterwards, the different bacteria were purified by restreaking on agar plates. For analyses by Fourier transform infrared (FTIR) spectrometry, isolates were cultivated on tryptic soy agar (Oxoid, TSA, Hampshire, UK) suspended in sterile deionized water and dried on a ZnSe sample holder. Afterwards, the measurement was carried out by FTIR spectrometer (Tensor 27, Bruker Corporation, Billerica, MA, USA). Infrared spectral data were analysed using the OPUS software (version 5.5; Bruker Corporation) and compared with reference databases, which comprise about 800 species. For virological examination, the brushes were put in microtubes with 1 ml

of RNAlater (RNAlater, Sigma-Aldrich Chemistry, Steinheim, Germany) and frozen subsequently after sample collection at −80°C. The analysis of samples was performed all at once at the Research Centre Vetcore of the University of Veterinary Medicine, Vienna. There, the cytobrushes including the majority of the cellular material were removed from the tubes and placed in 500 µl lysis buffer (Buffer AL, Qiagen, Hilden, Germany) and 20 µl proteinase K (Qiagen, Hilden, Germany). Samples were mixed and incubated at 56°C for 1 hr. Subsequently, DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) starting from step 3 of the manufacturer's protocol for animal blood.

As the viral glycoprotein B (gB) gene is one of the most conserved proteins among herpesviruses (Franceschi, Capocefalo, Cavirani, & Donofrio, 2013; Goltz et al., 1994), this gene was chosen as the target region for the BoHV-4 qPCR assay. BoHV-4 gB nucleotide sequences were obtained from the NCBI Genbank database (NCBI accession number AJ609274.1, AJ617687.1, AJ617688.1, EU055543.1, GQ246863.1, GQ246864.1, GQ246865.1, GQ246866.1, GQ246867.1, GQ375280.1, JN133502.1, JX644988.1, JX644989.1, NC_002665.1) and used as references for primer design using the Primer Express 2.0 software (Life Technologies, Foster City, USA). The final BoHV-4 gB qPCR primers and the TaqMan probe were located on homologous regions of the currently available gB sequences (forward: 5′-CACCTCTTCCACAACAACATCAA-3′; probe: 5′-FAM-TCAT TAGCTRCCTCTCCCCAGAACACGT-BHQ1-3′; reverse: 5′-TACTGG TACCYTGATTATCAGTGGAT-3′). The qPCR assay was validated with bovine DNA (EDTA blood) from BoHV-4-free animals as negative controls. Additionally, a dilution series over 5 log decades was prepared from DNA of a lyophilized virus preparation and used for the generation of standard curves. Out of these, the PCR reaction efficiency was calculated using the formula described by Klein et al. (1999). The determined PCR efficiency for BoHV-4 gB was 97.12%. The qPCR analyses were carried out in 25 µl reaction including 5 µl DNA template, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 1× buffer B2 (Solis BioDyne, Tartu, Estonia), 300 nM of each primer, 200 nM probe and 1 unit HOT FIREPol DNA polymerase (Solis BioDyne, Tartu, Estonia). The temperature profile initiated with 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. All samples were analysed in duplicates on a ViiA 7 real-time PCR instrument (Life Technologies, Foster City, USA). As a control for sample collection and successful DNA extraction, an additional qPCR was performed targeting the bovine GAPDH-gene (forward: 5′-TTGTCTCCTGCGACTTCAACA-3′; reverse: 5′-GTGGTCGTTGAGGGCAATG-3′; NCBI accession number NM_001034034.2). GAPDH qPCR was carried out with the DNA binding dye EvaGreen (Biotium, Hayward, USA). Reaction conditions have been described (Ertl & Klein, 2014). Samples were excluded if no GAPDH DNA was detected.

2.3 | Data management and statistical methods

All results were recorded on case report forms and transferred into spreadsheet software (Excel 2010, Microsoft Office Corporation, Redmond, WA, USA). The full data set contained animal specific data (parity, calving assistance), clinical findings (discharge score, uterine health status), results of the bacteriological, cytological and virological examinations and reproductive performance. The statistical analyses were performed with SAS (version 9.2; SAS Institute Inc., Cary, NC, USA).

A completely randomized block design was used with farm (n = 10) as blocking criterion and with the cow as the experimental unit. Statistical significance was set at $p < .05$, and a trend for significance was set at $p < .10$. The logistic model assessing the risk factors for CE, SE, odds for artificial insemination by 80 dpp (AI80) and pregnancy by 100 and by 200 dpp (PRE100 and PRE200) included the random effect of farm and the fixed effects of season at parturition (winter vs. spring vs. summer vs. fall), parity (1 vs. ≥2), calving (normal vs. moderate difficulty vs. severe difficulty), back fat thickness (BFT; in mm as continuous predictor), BoHV-4 (No vs. Yes), *E. coli* (No vs. Yes), *T. pyogenes* (No vs. Yes) and their second-order interactions. Models were analysed with PROC GLIMMIX (SAS, 2003) with binomial distributions and logit link functions. Modelling was performed using a manual backward elimination method with an exclusion criteria set at $p > .15$ (except for BoHV-4 that was forced to remain in the model). In addition, logistic models assessing the risk for infection with BoHV-4, *E. coli* and *T. pyogenes* were performed. These models included the same random and fixed factors previously described with the exception that each infection was removed from the model when it was used as the response variable.

Calving-to-first service and calving-to-conception intervals were analysed with PROC PHREG (SAS, 2003). Cox's proportional hazards regression models included the same fixed effects as the logistic models. Modelling was performed using a manual backward elimination method with an exclusion criteria set at $p > .15$. Time intervals (median ± 95% confidence interval, CI) for days to first service and days to conception were obtained from PROC LIFETEST (SAS, 2003).

3 | RESULTS

3.1 | Descriptive statistics

Cytobrush samples were collected from a total of 400 cows. Three samples had to be excluded from the analyses because of low DNA amounts on the cytobrush. Thus, overall, 397 cows (primiparous 28.2%, n = 112; multiparous 71.8%, n = 285) were included in the final analysis. Twenty-three samples (5.8%) were positive for BoHV-4. These BoHV-4 positive samples were detected in four of the ten farms. The herd level prevalence of BoHV-4 in the four positive farms was 2.7% (n = 1/37), 2.7% (n = 1/37), 11.1% (n = 5/45) and 11.5% (n = 16/139), respectively. BoHV-4 was isolated in 11.0% (n = 12/109), 4.8% (n = 4/84) and 3.6% (n = 7/194) of cows diagnosed as CE, SE or healthy, respectively (missing information about uterine cytology in 10 cows). The mean proportion of PMN in BoHV-4-positive cows was 14.9% compared with 2.0% in negative cows.

3.2 | Analysis of risk factors

The overall percentage of cows with CE was 27.5% (109 of 397). The risk factors for CE are shown in Table 1. BoHV-4 was not significantly

TABLE 1 Logistic model assessing the risk for clinical endometritis in dairy cows (n = 397)

		n/N[a]	%	AOR	95% CI	p-Value
Calving difficulty[b]	No	53/230	23.0	Ref[c]		.020
	Moderate	43/122	35.3	2.07	1.24–3.45	
	Severe	13/44	29.6	1.41	0.65–3.04	
BoHV-4	No	97/374	25.9	Ref		.103
	Yes	12/23	52.2	2.17	0.85–5.49	
T. pyogenes	No	76/346	22.0	Ref		<.001
	Yes	30/51	58.9	5.20	2.75–10.00	
BFT[d]	–	–	–	1.15	1.01–1.30	.031

[a]n/N = number out of total number.
[b]Information is missing from one cow.
[c]Ref = reference (AOR = 1).
[d]Back fat thickness (BFT): AOR given for each increase of 2.5 mm in BFT.
AOR = adjusted odds ratio.
Predictor variables and their second-order interactions were removed from the table when $p > .15$.

associated with the risk for CE (AOR = 2.17; $p = .103$) although the percentage of cows with CE was 52.2% in BoHV-4-positive cows and 25.9% in BoHV-4-negative cows. Infection with *T. pyogenes* increased the risk for CE (AOR = 5.20; $p < .001$; 58.9% vs. 22.0% for cows with or without *T. pyogenes*, respectively). Calving difficulty had also an effect on the risk for CE ($p = .02$), particularly for those cows receiving moderate calving assistance (AOR = 2.07). Finally, with every increase of 2.5 mm in BFT, the risk for CE increased by 15% ($p = .03$). The remaining predictors had no effect ($p > .15$).

The risk factors for SE are shown in Table 2. Season had an effect on the risk ($p = .009$) given that percentage of affected cows were lower in fall and spring than in summer and winter. With every increase of 2.5 mm in BFT, the risk for SE decreased by 18% ($p = .009$). The remaining predictors had no effect ($p > .15$).

Results of the logistic models for factors affecting reproductive performance parameters are shown in Table 3. BoHV-4 infection reduced the odds for artificial insemination within 80 dpp (AOR = 0.37; $p = .035$) given that percentage of inseminated cows was 43.5% vs. 74.1% for cows positive and negative for BoHV-4, respectively. An infection with *T. pyogenes* also reduced the odds for AI80 (AOR = 0.38; $p = .005$; 51.0% vs. 75.4% for cows with or without *T. pyogenes*, respectively). The odds for AI80 showed a tendency to be lower in parity ≥2 (AOR = 0.61; $p = .094$) and was lower in cows with severe calving difficulty (AOR = 0.40; $p = .028$). *T. pyogenes* reduced the odds for PRE100 (AOR = 0.54; $p = .053$; 37.2% vs. 50.9% for cows with or without *T. pyogenes*, respectively). Also, season showed a tendency to affect the odds for PRE100 ($p = .088$), with summer and spring showing lower rates than winter. BoHV-4 infection had no effect on the odds for PRE100 (AOR = 0.87; $p = .753$; 43.5% vs. 49.5% for cows positive and negative for BoHV-4, respectively). The remaining predictors had no effect ($p > .15$). BoHV-4 infection reduced the odds for PRE200 (AOR = 0.13; $p = .004$) given that percentage of pregnant cows was 80.0% vs. 94.7% for cows positive and negative for BoHV-4, respectively. The remaining predictors had no effect ($p > .15$).

T. pyogenes infection decreased the hazard of pregnancy (AHR = 0.66; 95% CI = 0.46–0.95; $p = .025$) and increased the calving to conception interval by 24 days (median and [95% CI]) 107 (86–132) vs. 83 (77–91) for cows with and without *T. pyogenes*, respectively.

BoHV-4 infection increased the risk for *T. pyogenes* infection (AOR = 5.55; 95% CI = 2.23–13.81; $p < .001$) given that the prevalence of *T. pyogenes* was 39.1% (9 of 23) vs. 11.2% (42 of 374) for cows positive and negative for BoHV-4, respectively. *E. coli* also increased the risk for *T. pyogenes* infection (AOR = 2.47; 95% CI = 1.23–4.97; $p = .011$) given that the prevalence of *T. pyogenes* was 22.4% (15 of

TABLE 2 Logistic model assessing the risk for subclinical endometritis in dairy cows (n = 387)

		n/N[a]	%	AOR	95% CI	p-Value
Season	Winter	28/112	25.0	Ref[b]		.009
	Spring	4/29	13.8	0.45	0.13–1.55	
	Summer	43/156	27.6	1.15	0.65–2.01	
	Fall	9/90	10.0	0.33	0.15–0.74	
BoHV-4	No	80/364	22.0	Ref		.713
	Yes	4/23	17.4	0.81	0.27–2.48	
BFT[c]	–	–	–	0.82	0.71–0.95	.009

[a]n/N = number out of total number.
[b]Ref = reference (AOR = 1).
[c]Back fat thickness (BFT): AOR given for each increase of 2.5 mm in BFT (as continuous predictor).
AOR = adjusted odds ratio.
Predictor variables and their second-order interactions were removed from the table when $p > 0.15$.

TABLE 3 Logistic model assessing the risk factors for insemination by 80 dpp and for pregnancy by 100 and 200 dpp in dairy cows (n = 397)

				AOR	95% CI	*p*-Value
		n/N[a]	%	Inseminated by 80 dpp		
Parity	1	87/112	77.7	Ref[b]		.094
	≥2	200/285	70.2	0.61	0.34–1.09	
Calving difficulty[c]	No	167/230	72.6	Ref		.028
	Moderate	93/122	76.2	1.25	0.71–2.22	
	Severe	27/44	61.4	0.40	0.18–0.89	
BoHV-4	No	277/374	74.1	Ref		.035
	Yes	10/23	43.5	0.37	0.15–0.93	
T. pyogenes	No	261/346	75.4	Ref		.005
	Yes	26/51	51.0	0.38	0.20–0.74	
				Pregnant by 100 dpp		
Season	Winter	65/113	57.5	Ref		.088
	Spring	13/30	43.3	0.57	0.24–1.36	
	Summer	69/160	43.1	0.54	0.39–0.88	
	Fall	48/94	51.1	0.77	0.44–1.33	
BoHV-4	No	185/374	49.5	Ref		.753
	Yes	10/23	43.5	0.87	0.36–2.10	
T. pyogenes	No	176/346	50.9	Ref		.053
	Yes	19/51	37.2	0.54	0.29–1.04	
				Pregnant by 200 dpp		
BoHV-4	No	287/303	94.7	Ref		.004
	Yes	12/15	80.0	0.13	0.03–0.52	

[a]*n*/*N* = number out of total number.
[b]Ref = reference (AOR = 1).
[c]Information is missing from one cow.
AOR = adjusted odds ratio.
Predictor variables and their second-order interactions were removed from the table when $p > .15$.

67) vs. 10.9% (36 of 330) for cows with or without *E. coli*. *T. pyogenes* increased the risk for BoHV-4 infection (AOR = 5.79; 95% CI = 2.33–14.35; $p < .001$) given that prevalence of BoHV-4 was 17.7% (9 of 51) vs 4.1% (14 of 346) for cows with or without *T. pyogenes*. *T. pyogenes* increased the risk for *E. coli* infection (AOR = 2.31; 95% CI = 1.17–4.54; $p = .016$) given that prevalence of *E. coli* was 29.4% (15 of 51) vs. 15.0% (52 of 346) for cows with or without *T. pyogenes*.

4 | DISCUSSION

The objectives of this study were to describe associations between clinical, cytological and bacteriological findings and the occurrence of BoHV-4 in cows at 20–30 dpp. We hypothesized that intrauterine infections with BoHV-4 increase the risk for CE or SE and lead to impaired reproductive performance of affected animals. To the best of our knowledge, this is the first study investigating clinical, bacteriological, cytological and virological findings together in uterine samples of dairy cows during the post-partum period.

This is the first study that described the intrauterine prevalence of BoHV-4 in Austrian dairy farms. The overall prevalence of BoHV-4 in 397 uterine samples (5.8%) was at the lower range of previously published data on seroprevalences by Bilge Dagalp et al. (2012), who found an average prevalence of 47.2% and 5.4% in problem and normal herds, respectively. The range of prevalence could be affected by farm-specific conditions or management-related factors. Thus, farm was included in our logistic model as a random factor to strengthen the generalization of our results.

Analyses of risk factors for CE and SE were performed by logistic models with several factors included. An infection with *T. pyogenes* was a significant risk factor for CE, whereas BoHV-4 showed a tendency to increase the prevalence of CE, and both pathogens were found as mutual risk factors. The co-isolation of BoHV-4 with uterine bacteria has previously been shown (Fábián et al., 2008; Nak et al., 2011; Szenci et al., 2016). *T. pyogenes* is generally accepted as a key risk factor for CE (Williams et al., 2005; Sheldon, Cronin, Goetze, Donofrio, & Schuberth, 2009) and has been discussed in detail previously by this group (Prunner, Pothmann et al. 2014; Prunner, Wagener et al. 2014; Wagener, Prunner, Pothmann, Drillich, & Ehling-Schulz, 2015). Fábián et al. (2008) identified BoHV-4 DNA in 27 of 31 infertile cows but found no correlation between BoHV-4 infection and histological or bacteriological findings. Our study partly supports the hypothesis by

these authors that BoHV-4 was unlikely to be a major pathogen in CE, but the role of BoHV-4 as a pathogen in bovine reproductive disorders still needs further studies (Chastant-Maillard, 2015; Frazier et al., 2001). With regard to the total number of cows positive for BoHV-4, it can be speculated whether a greater number of positive cows would confirm the trend towards an association with CE. Recently, Szenci et al. (2016) described BoHV-4 as a secondary pathogen in cows with vaginal purulent discharge 158 ± 95 dpp. It can also be hypothesized but not proven with our study design that cows with bacterial intrauterine infection are immunologically debilitated and, hence, the reactivation of a persistent BoHV-4 infection is facilitated.

The relationship between BoHV-4 infection and SE has not been elucidated. It can be assumed that an infection with BoHV-4 has an effect on the endometrial immune response, as demonstrated *in vitro* models (Donofrio et al. 2010). However, although the proportion of PMN was higher in BoHV-4-positive cows, the logistic model did not identify BoHV-4 as a risk factor for SE. It can be discussed that a concomitant bacterial infection causes an increase in PMN rather than a viral infection. A significant relationship between SE and uterine pathogens, however, has not been found in this and previous studies (Madoz, Giuliodori, Migliorisi, Jaureguiberry, & de la Sota, 2014; Prunner, Pothmann et al. 2014; Prunner, Wagener et al. 2014).

Season and increase of back fat thickness were determined as risk factors for SE in this study. With increasing body condition, the risk for SE decreased, indicating that the metabolic status in association with the uterine immune response plays a major role in the pathology of SE (Gabler et al., 2009; Galvao et al., 2010). This is in contrast to studies that did not find body condition of cows as a risk factor for SE (Plöntzke et al., 2010; Cheong, Nydam, Galvão, Crosier, & Gilbert, 2011; Prunner, Wagener et al. 2014).

Although BoHV-4 was not a significant risk factor for CE and SE, we found that BoHV-4 infection significantly reduced the risk for AI within 80 dpp and the risk for pregnancy within 200 dpp. Only limited information is available about the effect of BoHV-4 infections on reproductive performance. Gür and Dogan (2010) found that repeat breeder cows were more often found with anti-BoHV-4 antibodies than cows with a maximum of two inseminations. Szenci et al. (2016) hypothesized that BoHV-4 infections contribute to prolonged calving intervals. To our best knowledge, this is the first study that described effects of BoHV-4 infection on the odds of insemination and pregnancy in a logistic model, including other potential risk factors. Although our study described associations between BoHV-4 infection and reproductive performance parameters, it has to be noted that the design of our study does not allow any conclusion about cause–consequence effects.

Other risk factors for insemination or pregnancy that were significant in this study, that is parity, calving assistance, season and *T. pyogenes*, have been analysed in previous studies but not consistently been significant (Kaufmann, Drillich, Tenhagenm, & Heuwieser, 2010; Plöntzke et al., 2010; Prunner, Wagener et al. 2014).

In conclusion, this study revealed new information about uterine BoHV-4 infection and its associations with CE, SE and reproductive performance of dairy cows. From the results of this study, BoHV-4 can be regarded as cofactor for CE but not as a primary pathogen. However, the odds for AI and pregnancy were lower in BoHV-4-positive cows, suggesting a role in impaired reproductive performance on herd level. Further studies are necessary to investigate potential interactions of BoHV-4 within the uterine microbiota.

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CONFLICTS OF INTEREST

The authors disclose any financial and personal relationships with other people or organizations that could inappropriately bias or influence their work.

AUTHORS CONTRIBUTIONS

SK analysed the data of this study and drafted the manuscript. IP designed the study, conducted the trial, analysed data and drafted the manuscript. MG analysed the data, performed statistical tests and drafted the manuscript. MD designed and supervised the study, analysed data and drafted the manuscript.

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