

## Bovine Herpesvirus-4, A Potential Cause of Mastitis in Canadian Dairy Cows

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**Abstract:** Bovine herpesvirus-4 (BHV-4), genus *Rhadinovirus*, is a member of the family *Herpesviridae*, subfamily *Gammaherpesvirinae* with a worldwide distribution. This study was conducted to investigate the role of the virus in mastitis and/or subclinical mastitis in the Canadian dairy herds. Milk samples from 176 dairy cows were divided into 2 main groups; "A" contained samples with no bacterial isolates, and "B" containing samples either with *Staphylococcus aureus* or *Streptococcus uberis* isolates. Approximately ninety-eight percent of milk samples had antibodies against BHV-4. Among cows with negative bacterial milk cultures, significantly higher titres of BHV-4 antibodies were associated with samples which had high Somatic Cell Counts (SCC), compared to low SCC samples. Viral DNA was detected in 2% of milk samples by the PCR amplification of BHV-4 glycoprotein-B gene. To our knowledge, this is the first report of the presence of BHV-4 in milk of Canadian dairy herds. Further investigation is needed to determine whether BHV-4 is one of primary causative agents in bovine mastitis, or whether the observed correlation of high titres of specific milk BHV-4 antibody with high SCC was due to reactivation of latent BHV-4 infections by primary bacterial insults.

**Key words:** Antibodies, BHV-4, mastitis, PCR, virus

### INTRODUCTION

Bovine herpesvirus-4 is a member of the family *Herpesviridae* with a worldwide distribution among both healthy cows and those with various clinical manifestations (Czaplicki and Thiry, 1998; Gür and Doğan, 2010). The virus is in the *Rhadinovirus* genus of the *Gammaherpesvirinae* subfamily, enveloped with an icosahedral nucleocapsid, 150 nm in diameter, and containing double stranded DNA of 146 kbp (Zimmermann *et al.*, 2001). Antigenically closely related strains of BHV-4 with similar restriction patterns are placed in 2 main groups, European strain (Movar 33/63) and American strain (DN 599) (Fichtelova and Kovarcik, 2010). The clinical manifestations associated with BHV-4 in cattle include pneumonia and keratoconjunctivitis (Fichtelova and Kovarcik, 2010), postpartum metritis (Monge *et al.*, 2006), abortion (Czaplicki and Thiry, 1998; Kirkbride, 1992), mastitis (Izumi *et al.*, 2006; Miyano *et al.*, 2004; Wellenberg, 2002b; Wellenberg *et al.*, 2001), encephalitis, and diarrhoea (Thiry *et al.*, 1990). BHV-4, like all other herpesviruses cause latent infection particularly of macrophages (Donofrio and Santen, 2001; Osorio *et al.*, 1985). Experimental reactivation of the virus by dexamethasone was established in calves (Castrucci *et al.*, 1987). Few reports suggested the immunosuppressive effect of the virus

(Egyed, 2000). The role of BHV-4 in clinical and subclinical mastitis was described for the first time by Wellenberg *et al.* (2000). The virus was detected in 5.9% of cows with normal milk and 37.6% of cows with mastitis by nested PCR (Kálmán *et al.*, 2004). Experimental intramammary infection of cows with BHV-4 resulted in a significant increase in the Somatic Cell Count (SCC) of the milk from the infected quarter (Wellenberg *et al.*, 2002a). Mixed infections of BHV-4 with *Staphylococcus aureus*, *Streptococcus uberis* and *Escherichia coli* were reported in cows with mastitis (Kálmán *et al.*, 2004; Wellenberg *et al.*, 2001; 2002a, b; Zadoks *et al.*, 2001). To our knowledge, this is the first report to describe the detection of BHV-4 nucleic acid and antibodies from cows with mastitis or subclinical mastitis in Canadian dairy cows.

### MATERIALS AND METHODS

Milk samples for this study were selected from a subset of the National Cohort of Dairy Farms data in Canada (Reyher *et al.*, 2011). A total of 176 milk samples were selected from 9846 samples of 1035 cows during the year 2008 and stored at -70°C. Sampling, bacterial isolation, SCC were performed by Maritime Quality Milk, Canada according to the protocol described in (Reyher *et al.*, 2011). Samples were divided into 2 groups; "A"

with no bacterial isolates and group “B” with bacterial isolates (Table 1). Group A was further divided into 2 subgroups; “1” with SCC less than 100,000 cells/mL and “2” with SCC higher than 200,000 cells/mL. Group B was constituted by 2 subgroups according to the isolated bacteria; “3” with *Streptococcus uberis* and “4” *Staphylococcus aureus*. The work was conducted during 2011 at the Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island, Canada.

**Pre-treatment of milk samples:** Milk samples were thawed, an aliquot of 1 mL was centrifuged for 20 min at 4000 × g (Eppendorf 5424 centrifuge, USA) and 400 µL were taken from the middle layer just under the cream layer for BHV-4 antibody analysis. The remaining sample was centrifuged at 14000 rpm for 3 min and the upper 400 µL was discarded. The cell pellet was resuspended in the remaining 200 µL for DNA extraction.

**Antibody detection:** A semiquantitative commercial BHV-4 ELISA kit (Bio-X Diagnostics, Belgium) were used for the detection of BHV-4 antibodies in milk samples. A 100 µL of each undiluted sample was tested in two wells. The first was coated with viral antigen and the other with an antigen-free cell culture lysate. Samples were incubated for 1 h at room temperature. The plate was washed 3 times, a conjugate solution was added and the plate was further incubated for 1 h. After washing the plate, chromagen solution was added and the plate was incubated for 10 min in the dark. A volume of 50 µL of stopping solution was added and the Optical Densities (OD) were read with an ELISA reader (Biotek Synergy Microplate Reader) using a 450 nm filter. The degrees of positivity were scored (+1 to +5) according to the company instructions.

**DNA extraction:** DNA extraction was carried out by using DNeasy Blood and Tissue kit (Qiagen, Canada, CAT# 69504). Approximately 200 µL of the pretreated milk sample was mixed with 20 µL of Proteinase K (Qiagen). For extraction of a known positive BHV-4 virus reference strain (DN599 strain kindly provided by Dr. S.M. Goyal, University Of Minnesota), 50 µL of virus stock was mixed with 20 µL of Proteinase K (Qiagen) and volume was adjusted to 220 µL with PBS. A volume of 200 µL of lyses buffer was added and samples were incubated at 56°C for 10 min. DNA precipitation was carried out by ethanol followed by 2 washing steps and DNA was eluted according to the description of the manufacturer. Extracted DNA products were stored at -20°C.

**Glycoprotein B- PCR (gB-PCR):** Amplification of BHV-4 glycoprotein B gene was carried out as described

by (Wellenberg *et al.*, 2001) with slight modifications. The PCR was performed in a final volume of 20 µL containing 25 pmol of both forward primer gB1 (5'-CCCTTCACCACCACCTACA-3') and reverse primer gB2 (5'-TGC CATAGCAGAGAAACAATGA-3') (Invitrogen, Canada), 2 µL 10× CoralLoadPCR Buffer, 10 µL HotStarTaq Plus Master Mix, 5.2 µL of nuclease-free water (HotStarTaq Plus Master Mix Kit, QIAGEN, Cat# 203643), and 2 µL of extracted DNA. The parameters for PCR instrument was 1 cycle of 95°C for 5 min, then 40 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec and a final elongation step for 7 min at 72°C. Extracted DNA from BHV-4 DN599 reference strain was used as a positive control, and the elution solution instead of template was used as a negative control. The amplified 615 bp PCR product was visualized in a 1.5% agarose gel in 0.5% TBE. Gels were stained with 1 µg/mL ethidium bromide to confirm amplicon size and photographed using UV Light Box and camera.

**Virus isolation:** PCR positive milk samples were submitted for virus isolation. Milk samples were defatted by centrifugation at 1500 × g for 10 min. A volume of 200 µL of the defatted milk was added to 300 µL of Dulbecco’s Minimum Essential Medium (DMEM) (ATCC, 30-2002) and pipetted on a semi-confluent monolayer of BVDV free Bovine Turbinate (BT) cell line (ATCC, CRL-1390). The cell cultures were observed regularly for cytopathic effect and a non inoculated cell BT culture served as negative control (Donofrio *et al.*, 2003; Wellenberg *et al.*, 2000).

**Statistical analysis:** Differences between the ELISA scores of subgroup 1 (control) and each of the comparison groups were assessed using the Two-sample Wilcoxon rank-sum (Mann-Whitney) test. Group differences were considered significant at p< 0.05.

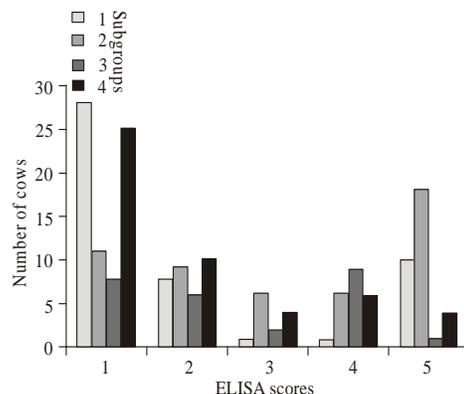


Fig. 1: Score 5 was significantly higher in subgroup 2 compared to other subgroups

Table 1: Groups, subgroups, ELISA scores and PCR results of BHV-4 in milk samples

Group	Subgroup	SCC cells/mL	Bacterial isolate/Quarters	No. of cows	Cows ELISA scores					Antibodies (%)	PCR (%)	
					0	1	2	3	4			5
A	1	<100.000	-ve /all quarters	50	2	28	8	1	1	10	48 (96)	0 (0)
	2	>200.000	-ve/sampled quarter	50	0	11	9	6	6	18	50 (100)	1 (2)
B	3	-	<i>S. uberis</i> /sampled quarter	26	0	8	6	2	9	1	26 (100)	0 (0)
	4	-	<i>S. aureus</i> /sampled quarter	50	1	25	10	4	6	4	49 (98)	1 (2)
Total				176							173 (98.2)	2 (1.3)

## RESULTS AND DISCUSSION

The overall prevalence of antibodies against BHV-4 in the milk samples of 176 cows tested was 98.2%, and this is consistent with the previously reported high seroprevalence of 84 and 79% in 2 Dutch dairy herds (Zadoks *et al.*, 2001). On the other hand, a lesser seroprevalence of 36% was reported in dairy cows with a history of metritis from the United States (Frazier *et al.*, 2002). The BHV-4 antibody ELISA scores (Table 1) were significantly higher in subgroup 2, compared to subgroup 1 ( $p = 0.0002$ ). Thirty six percent of the cows in subgroup 2 had high antibody titre against BHV-4 (Fig. 1). The high SCC in this subgroup might be attributable to the presence of BHV-4 and its antibodies since there were no successful bacterial isolation.

Of the 176 extracted DNA from milk samples, PCR amplification of the BHV-4 glycoprotein-B gene was successful in 2 samples. The expected 615 bp amplicon size was detected in one sample from subgroup 2 and another sample from subgroup 4. All instances BHV-4 genome detections occurred in the group of cows with high SCC, and no such detection occurred among the low SCC cows. In a similar study (Wellenberg *et al.*, 2001); four milk samples were positive by gB-PCR from among 48 clinical mastitis cases and 48 control cows. Despite the high sensitivity and specificity of the gB-PCR assay (Wellenberg *et al.*, 2001), and despite the reported ten times higher sensitivity of BHV-4 detection compared to virus isolation, the number of successfully amplified products might not reflect the actual number of BHV-4 infected samples. This is because milk contains high concentrations of proteins and fats, which are major inhibitors of the PCR assay (Nakayama *et al.*, 2006). Although milk was defatted prior to the extraction in our study, other milk components such as proteinase might have inhibited the PCR amplification in some samples (Powell *et al.*, 1994). Virus isolation from these two PCR positive samples was not successful. This could be due to some degree of virus inactivation due to the storage, as the samples were stored for more than 3 years. Alternatively, isolation could have been unsuccessful if PCR positivity was due to predominance of non infectious virus particles in the PCR positive samples. The observed high titres of specific BHV-4 antibody among the samples of subgroup 2 (high SCC and negative in bacterial culture), and the presence of the BHV-4 genome in one of

these samples, suggest the possible causative role of BHV-4 in subclinical mastitis. As for the results obtained from subgroup 4 (*Staphylococcus aureus* positive), it could not be determined whether there was any relation between BHV-4 and *Staphylococcus aureus*. Previously, a significant association between the BHV-4 seropositive animals and *Staphylococcus aureus* infection was reported (Zadoks *et al.*, 2001).

## CONCLUSION

The significantly higher levels of specific antibody in the milk of subgroup 2 animals, in the absence of bacterial isolation, indicate recent BHV-4 infections, which might explain the high SCC in this subgroup. On the other hand, the lower titres of specific milk antibody in subgroup 4 (*Staphylococcus aureus* positive), together with the successful PCR detection of BHV-4 genome in one of the animals of this subgroup, is suggestive of the role of bacterial infection in recurrence of BHV-4 infection. Further investigations are needed to elucidate whether BHV-4 is one of primary causative agents in bovine mastitis, or whether the BHV-4 reactivation occurs due to primary bacterial insults.

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