Presence of viral and bacterial organisms in milk and their association with somatic cell counts

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ABSTRACT

About 20 to 35% of milk samples from cows with intramammary infection or high somatic cell count (SCC) are negative on bacteriological culture analysis. However, little is known about SCC in milk of cows infected with viruses. In the first part of our study, we developed a real-time PCR assay for detection of bovine herpesvirus (BHV) 1, BHV2, and BHV4, and bovine viral diarrhea virus (BVDV) in composite quarter milk samples. A total of 1,479 lactating cows of 1,964 cows in the dairy herd were initially selected because these cows had complete SCC data for at least 3 consecutive test results, of which 139 lactating cows from different lactation age groups were selected randomly and studied extensively. Composite quarter milk samples were collected on 3 alternate days and examined for viruses, SCC, and bacteriological analysis. In total, 10, 28, and 0.7% of the composite quarter milk samples from cows were positive for BHV1, BHV2, and BHV4, respectively; BVDV was not detected in composite quarter milk samples. Bovine herpesvirus was not associated with a particular bacterial species. Our study results indicate that cows positive for BHV in composite quarter milk samples alone are less likely to have elevated SCC compared with cows with bacterial intramammary infection; BHV1, BHV2, and BHV4 are probably not major udder pathogens.

Key words: bovine mastitis, bovine herpesvirus, somatic cell count, real-time PCR

INTRODUCTION

Mastitis is the inflammation of the mammary gland caused by microorganisms or trauma to the udder. During the inflammatory response, the immune system of the mammary gland is activated to eliminate the

pathogen (Oviedo-Boyso et al., 2007). The number of somatic cells secreted in milk, referred to as SCC, is an indicator of the inflammatory response to an IMI (Schukken et al., 2003). Somatic cell count thresholds ranging from 100,000 to 300,000 cells/mL have been used to classify uninfected (<100,000 cells/mL) and infected (>300,000 cells/mL) quarters (Deluyker et al., 2005; Berry and Meaney, 2006; Schwarz et al., 2010). In mastitis prevention and control practices, an operational threshold of 200,000 cells/mL is typically used to reduce the diagnostic error to prevent misclassifying an udder with IMI (Schukken et al., 2003).

Mastitis-causing organisms have been categorized as contagious or environmental based on their distinct characteristics of distribution and interaction with the teat and teat duct (Smith and Hogan, 1993). In addition to *Chlamydia* and *Prototheca*, nonbacterial pathogens such as fungi and yeast have also been reported to cause bovine mastitis (Radostits et al., 2000; Roesler et al., 2001). Bacteriological culture analysis is the standard method for identifying IMI. However, culture of quarter milk samples of cows with subclinical mastitis often yields no pathogens (Sargeant et al., 2001). Despite intensive etiological research, around 20 to 35% of clinical cases of bovine mastitis have an unknown etiology (Wellenberg et al., 2002b).

Wellenberg et al. (2002b) reviewed the role of viral infections in bovine mastitis. They observed that under natural cases of bovine mastitis, bovine herpesvirus 1 (BHV1), bovine herpesvirus 4 (BHV4), foot and mouth disease (FMD) virus, and parainfluenza type 3 (PI3) virus have been isolated from milk. Bovine herpesvirus 1, FMD, and PI3 viral infections caused clinical mastitis, whereas BHV4 infection caused subclinical mastitis. Viruses such as bovine herpesvirus 2 (BHV2), cowpox, pseudocowpox, FMD, vesicular stomatitis virus, and papillomavirus cause teat lesions. These viruses may damage the integrity of the udder and indirectly contribute to mastitis. It is probable that virus-induced immunosuppression underlies mastitis but experimental evidence to support this assumption is lacking. Very few epidemiological studies support a

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causal relationship between viral infections and bovine mastitis in field conditions (Wellenberg et al., 2002b).

The aim of this study was to determine if such an association between the presence of select viral etiological agents, including BHV1, BHV2, BHV4, and bovine viral diarrhea virus (**BVDV**) and elevated somatic cells ($>200,000$ cells/mL of milk) in composite quarter milk samples in the absence of bacterial IMI.

MATERIALS AND METHODS

Study Population

A large dairy herd in central Pennsylvania consented to participate in the study. The DHIA computerized data set for milk SCC for 1,964 cows in this herd was analyzed. A total of 1,798 lactating cows had SCC data, of which 1,479 lactating cows (101, 605, 552, and 221 cows in lactations 1, 2, 3, and ≥ 4 , respectively) had SCC information for at least 3 sampling test periods. Using a random number generation technique, 10% of the cows ($n = 148$, including 10, 61, 55, and 22 cows in lactations 1, 2, 3, and ≥ 4 , respectively) were selected for the study. During the course of sample collection, 9 cows left the study (5, 2, 1, and 1 cows in lactations 1, 2, 3, and ≥ 4 , respectively) as they were either segregated from the milking herd, culled, or dried off, resulting in complete set of samples from 139 lactating cows (5, 59, 54, and 21 lactating cows in lactations 1, 2, 3, and ≥ 4 , respectively) for analysis. Milk samples from 139 cows were examined for SCC, bacteriological analysis, and detection of BHV1, BHV2, BHV4, and BVDV by real-time PCR. The 139 cows at the time of sampling were between 90 and 120 DIM. In this herd, all adult cows are vaccinated at the time of drying off using killed vaccine against BHV-1 virus (infectious bovine rhinotracheitis, **IBR**), BVDV, PI3, and bovine respiratory syncytial virus (**BRSV**); at the time of breeding, modified live IBR, BVD, PI3, and BRSV were used.

Collection of Milk Samples

Composite quarter milk samples were collected aseptically according to National Mastitis Council guidelines (National Mastitis Council, 1990). Just before milking, teats were carefully cleaned using cotton wool dipped in 70% ethanol. The first few streaks of milk were discarded and approximately 3 mL of milk was collected from each mammary gland quarter into a sterile 15-mL screw-cap centrifuge tube. Milk was collected before morning milking in the milking parlor. Composite quarter milk samples were collected from each of the 139 cows in the study on d 1, 3, and 5. All milk

samples were shipped on ice to the laboratory within 2 to 3 h of collection. Milk samples were processed on the same day of collection for determination of SCC and bacteriological analysis. The remaining milk samples were stored in 2-mL Eppendorf tubes at -20°C for subsequent analysis.

Determination of SCC in Composite Quarter Milk Samples

Each milk sample was mixed thoroughly by inverting the tube several times. The milk sample (60 μL) was drawn into a milk cassette and SCC was estimated using a DeLaval somatic cell counter (DeLaval Inc., Kansas City, MO).

Bacteriological Culture Analyses of Composite Quarter Milk Samples

The composite quarter milk samples were examined for the presence of contagious and environmental mastitis pathogens as described previously (Jayarao et al., 2004). Partitioned Petri dishes (quad plates) were used for bacterial isolation. Each quadrant of the Petri dish contained the following media, respectively: (1) Edwards modified medium (Oxoid, Basingstoke, UK) with colistin sulfate and oxolinic acid medium (**EMCO**; Sawant et al., 2002) for isolation and presumptive identification of *Streptococcus agalactiae* and environmental streptococci; (2) MacConkey's agar (Oxoid) for isolation of coliform and non-coliform gram-negative bacteria; (3) Baird-Parker agar (**BPA**; Difco, Becton Dickinson, Sparks, MD) for isolation of *Staphylococcus aureus* and CNS; and (4) 5% sheep blood agar for detection of hemolytic bacteria. The milk samples were mixed thoroughly by gently inverting the tubes several times. A loopful (~ 10 μL) of raw milk was streaked onto each medium and the quad plates were incubated at 37°C for 48 h. Colonies on EMCO medium were selected and streaked on 5% sheep blood agar and incubated for 48 h at 37°C . All isolates were examined for Gram reaction and catalase production, and were identified using API 20 Strep (BioMerieux, Hazelwood, MO).

Colonies suggestive of *Staph. aureus* and CNS on BPA were selected for speciation. Selected colonies were streaked on 5% sheep blood agar and incubated for 48 h at 37°C . The isolates were examined for hemolysis, coagulase test, and catalase production and identified using API-Staph (BioMerieux; National Mastitis Council, 1999).

Based on colony morphology and lactose fermentation on MacConkey's agar (Oxoid), isolates were selected for bacterial identification. Gram stain and biochemical tests such oxidation-fermentation test, oxidase test,

methyl red test, production of indole and acetyl methyl carbinol, and citrate utilization were performed. Coliforms and non-coliform gram-negative bacteria were identified to species level with API 20E or API 20 NE bacterial rapid identification kits (BioMerieux).

Composite quarter milk samples (~500 µL) were pre-enriched in *Mycoplasma* enrichment broth (University of California Davis Biological Media Services; www.vmbms.ucdavis.edu) and incubated for 48 h at 37°C in a moist 10% CO₂ incubator. The pre-enriched broth (100 µL) was absorbed onto a sterile swab and streaked onto a *Mycoplasma* agar plate (University of California Davis Biological Media Services). These plates were incubated for 7 d at 37°C in a moist 10% CO₂ incubator. *Mycoplasma* colonies were viewed under a low-power microscope.

Extraction of DNA from Cellular Fraction of Composite Quarter Milk Sample

The DNA was extracted from the composite quarter milk samples as described by Wellenberg et al. (2001) with minor modifications. An aliquot of 1.5 mL of the composite quarter milk sample was thoroughly vortexed and defatted by centrifugation at $1,500 \times g$ for 10 min. The defatted supernatant (1 mL) was transferred to another Eppendorf tube and centrifuged at $16,873 \times g$ for 5 min. About 800 µL of supernatant was discarded and the cell pellet was suspended in the remaining 200 µL of supernatant. Cell lysis, digestion with protease, DNA precipitation, and elution were performed using QIAamp Blood and Tissue kit (Qiagen, Valencia, CA). The DNA was stored at -20°C and examined by real-time PCR assay.

Real-Time PCR Assay

Viral DNA/RNA of Reference Strains. Viral DNA of reference strains for BHV1 (IBR strain), BHV2 (bovine herpes mammillitis strain), and BHV4 (DN599

strain) were obtained from S. M. Goyal (University of Minnesota, St. Paul). Viral RNA for BVDV (NADL strain) was obtained from the Animal Diagnostic Laboratory at Penn State (University Park). The concentration of viral DNA and RNA was determined by measuring the optical density at 260 nm using a spectrophotometer (DNA Quant II, Pharmacia Biotech, Cambridge, UK) and then stored at -20°C until further use for the standardization of real-time PCR.

Selection of Primers. Primers were selected to amplify the glycoprotein H gene for BHV1, glycoprotein B gene for BHV2, glycoprotein L gene for BHV4, and a highly conserved region in the 5' untranslated region (UTR) for BVDV. The primers for BHV were designed using Beacon Designer (Premier Biosoft International, Palo Alto, CA). The primers for BVDV were obtained from the Animal Diagnostic Laboratory at Penn State (Bhudevi and Weinstock, 2001). Primers were synthesized by Operon Biotechnologies (Huntsville, AL). The primer sequences are shown in Table 1.

Specificity of Primers. The specificity of the primers was judged by 2 methods. In the first method, the primer sequences were analyzed for specificity using the BLAST software (www.ncbi.nlm.nih.gov/BLAST). In the second method, primers and the standard DNA templates were interchanged and assayed to determine whether the gene product was amplified.

Optimization of Real-Time PCR Assay. A SYBR Green-based real-time PCR assay was developed to detect BHV1, BHV2, BHV4, and BVDV from the cellular fraction of composite quarter milk samples. The optimal PCR condition was determined to be the lowest concentration of the standard template required to amplify the correct gene fragment corresponding to each virus. Optimization was carried out for various concentrations of the primers and standard templates. For the DNA viruses (BHV1, BHV2, BHV4), the reaction mixture in each tube contained 1 µL of DNA standard template, 12.5 µL of Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA), and a

Table 1. Primers (forward, F, and reverse, R) used for detection of bovine herpesvirus (BHV) 1, 2, and 4, and bovine viral diarrhea virus (BVDV) in milk by real-time PCR

Virus	Primer sequence	GenBank Accession number/reference
BHV1	F: 5'-GTA AGG GTA TAT TAT TGA TTG C-3' R: 5'-GAC AGT GAG TAT GAG GAC-3'	X58867
BHV2	F: 5'-TTC GCA CAA CCG CCT AAC-3' R: 5'-CAA TCA GAG TCC GCC TTA CC-3'	M21628
BHV4	F: 5'-ATG CCC ATT CGG AAT AAC TTT AG-3' R: 5'-CAT TGA CTT GTT GAT GCT TTC TC-3'	Z79634
BVDV	F: 5'-TAG CCA TGC CCT TAG TAG GAC-3' R: 5'-GAC GAC TAC CCT GTA CTC AGG-3'	Bhudevi and Weinstock (2001)

final concentration of 0.8 μ M (2 μ L) each of forward and reverse primers for respective viral DNA, with the total volume of 25 μ L made up with sterile PCR-grade water. The reaction was subjected to real-time PCR in the Stratagene MX 3000P instrument. The PCR conditions were as follows: 1 cycle at 95°C for 10 min followed by 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s for 40 cycles. The standard DNA template for each virus served as the positive control. The no-template control served as the negative control, which contained PCR-grade water instead of the DNA template.

The reaction mixture for BVDV (an RNA virus) contained 2 μ L of RNA, 12.5 μ L of Brilliant SYBR Green QRT-PCR Master Mix (Stratagene) along with 0.07 μ L of reverse transcriptase, 0.8 μ M (1 μ L) each of the primer set, and sterile PCR water to make up the total reaction volume of 25 μ L. The PCR program for detection of BVDV was 50°C for 30 min for 1 cycle, 95°C for 10 min for 1 cycle, followed by 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s for 40 cycles. A positive control was maintained using the BVDV standard template. No-template control was maintained using PCR-grade water instead of the standard template. An additional control without reverse transcriptase was maintained for the BVDV PCR assay. The PCR yielded the expected products for BHV1 (128 bp), BHV2 (112 bp), BHV4 (175 bp), and BVDV (96 bp; Figure 1).

Interpretation of Results. Results were interpreted by assessing the cycle threshold (Ct) for each amplified product and its melting temperature (Tm). The Ct is the number of cycles required to produce the first detectable fluorescence above background. The Ct value and the amplification of the products were evalu-

ated using the amplification plot for each set of PCR reactions. A positive reaction was assigned a Ct value. The test was considered as negative when no amplification occurred or the Ct value was >35 cycles. The Tm of each amplified product was compared with the Tm of the standard template. The test was considered positive when the Tm of the sample matched the Tm of the standard template and had a Ct value <35 cycles.

Sensitivity and Specificity of Real-Time PCR Assay. The sensitivity of the real-time PCR assay was evaluated by establishing standard curves using 10-fold serial dilution of the standard templates in PCR-grade water. Standard curves were established by plotting fluorescence values (dR) versus Ct produced for standard templates. This was used to assess the linear dynamic range (amplification step) of the real-time PCR assay. Standard curves were obtained for BHV1, BHV2, BHV4, and BVDV using the respective standard templates. The correlations (R^2 values) of 10-fold serial dilutions of standard template for BHV1, BHV2, BHV4, and BVDV were 0.993, 0.990, 0.979, and 0.904, respectively. The Ct value for the BHV and BVDV viruses ranged between 10 and 33 cycles. The slopes of the standard curve were -4.405, -3.099, -3.315, and -4.253 for BHV1, BHV2, BHV4, and BVDV, respectively. The real-time PCR assay was able to detect 8.2, 8.4, 10.3, and 213.6 pg of BHV1, BHV2, and BHV4 DNA and BVDV RNA templates, respectively. Figure 2 shows the amplification plots for 10-fold serial dilutions of BHV2 DNA.

The specificity of the real-time PCR assay using the standard templates was evaluated by product size and by sequencing. The PCR products were separated by gel electrophoresis using a 2% agarose gel and sequenced. The nucleotide sequences were then compared with the GenBank sequences using the BLAST software.

Data Analysis. The data from all cows were transferred to an Excel spreadsheet (Microsoft Corp., Redmond, WA). The mean SCC for each cow was log-transformed ($\log_{10}$) for statistical analysis. All statistical analyses were performed using JMP software (version 4.0; SAS Institute Inc., Cary, NC). Single-factor ANOVA and Student's *t*-test were used to determine the differences in the mean SCC between the following groups. The cows were categorized into 2 groups based on presence or absence of BHV in composite quarter milk samples (BHV+ or BHV-). Post hoc pairwise comparisons and single-factor ANOVA were also done to determine the differences between the groups. These groups were further stratified into 2 subgroups based upon bacterial infection: bacteria-positive (Bact+) or bacteria-negative (Bact-), resulting in 4 groups total: BHV1+/Bact+, BHV1+/Bact-, BHV1-/Bact+, and

Figure 1. Real-time PCR products using bovine herpesvirus (BHV) 1, 2, and 4 and bovine viral diarrhea virus (BVDV) DNA PCR products on 2% agarose gel. Lanes: S = 100-bp molecular weight standard; C = control (no template); 1 = BHV1; 2 = BHV2; 3 = BHV4; 4 = BVDV.

Figure 2. Amplification plots for 10-fold serial dilutions of bovine herpesvirus (BHV) 2 DNA. Plot 1 = 8.4 ng of DNA/ μ L; 2 = 0.84 ng of DNA/ μ L; 3 = 0.084 ng of DNA/ μ L; 4 = 0.0084 ng of DNA/ μ L; 5 = control (no template).

BHV1–/Bact–. Similar groups were formed for BHV2 and BHV4 viruses. A *P*-value of < 0.05 was considered a significant association between the mean SCC of the different groups.

RESULTS

Prevalence of BHV and BVDV in Dairy Herd

A cow was considered positive for presence of BHV or BVDV in milk if the composite quarter milk sample was positive on at least 1 of the 3 composite quarter milk samples. Real-time PCR revealed that 69 of 139 (49.7%) of the composite quarter milk samples from cows were positive for BHV in milk. Composite quarter milk samples from 10.8, 28.7, and 0.7% of cows were positive for BHV1, BHV2, and BHV4, respectively. A few cows were positive for 2 types of BHV in the same sample. None of the 139 cows was positive for BVDV in composite quarter milk samples (Table 2).

Three first-lactation cows were positive for BHV1 and BHV2 + BHV4, respectively. Compared with cows in second lactation, cows in first, third, and fourth lactations had a higher percentage of milk samples positive for BHV1. Cows in the second and third lactations had a higher percentage of milk samples positive for BHV2 compared with cows in the fourth lactation. Milk samples positive for both BHV1 and BHV2 were the highest in the fourth lactation cows. We detected BHV4 in only one third-lactation cow, whereas simultaneous isolation of BHV2 and BHV4 was observed in 1 cow each in first, second, and fourth lactations (Table 2).

Frequency of Detection of BHV by Real-Time PCR Assay

Composite quarter milk samples were collected from all 139 cows on 3 alternate days: 1, 3, and 5. The frequency of detection of BHV1, BHV2, and BHV4 was

Table 2. Effect of lactation on prevalence of bovine herpesvirus (BHV) 1, 2, and 4 infection (%) in composite quarter milk samples

BHV type ¹	Lactation				Total (n = 139)
	1 (n = 5)	2 (n = 59)	3 (n = 54)	4 (n = 21)	
BHV+	40.0	45.8	55.6	47.7	49.7
BHV1+	20.0	6.7	12.9	14.2	10.8
BHV2+	0.0	28.8	37.0	14.2	28.7
BHV1 and BHV2+	0.0	8.4	3.7	14.2	7.2
BHV4+	0.0	0.0	1.8	0.0	0.7
BHV2 and BHV4+	20.0	1.6	0.0	4.7	2.2
BHV–	60.0	54.2	44.4	52.3	50.3

¹Positive on at least 1 of 3 composite quarter milk samples for BHV on real-time PCR assay.

Table 3. Frequency of detection of bovine herpesvirus (BHV) 1, 2, and 4 in by real-time PCR assay from 3 composite quarter milk samples from 139 lactating cows

Frequency of positive test (positive /total)	Total no. (%)		
	BHV1	BHV2	BHV4
1/3	24 (17.2)	46 (33.0)	2 (1.4)
2/3	1 (0.7)	6 (4.3)	1 (0.7)
3/3	0	1 (0.7)	1 (0.7)

the highest on d 1 compared with d 3 and 5 (Table 3). On d 1 of sampling, BHV1, BHV2, and BHV4 were detected in 17.2, 33, and 1.4% of composite quarter milk samples respectively, whereas on d 3 of sampling, only 0.7, 4.3, and 0.7% of the composite quarter milk samples from the cows were positive, and on d 5 of sampling, BHV1 and BHV4 were detected in 1 cow each (Table 3).

Association of BHV and SCC in Cows With and Without BHV and Bacterial IMI

To assess if BHV alone was associated with increase in SCC in the absence of bacterial IMI, we compared the mean SCC in composite quarter milk of cows in the BHV+/Bact+ group (448,000 cells/mL) with that of cows in BHV+/Bact− group (95,000 cells/mL; Table 4). A significant difference ($P < 0.0001$) in the mean SCC was observed between the 2 groups. Similarly, a significant difference was observed between BHV+/Bact+ group (448,000 cells/mL) and the BHV−/Bact− (55,000 cells/mL). We observed no difference ($P = 0.49$) in the mean SCC in composite quarter milk samples from cows that were BHV−/Bact+ (445,000 cells/mL) compared with cows that were BHV+/Bact+ (448,000 cells/mL). The same was found for cows that were BHV+/Bact− (95,000 cells/mL) and BHV−/

Bact− (55,000 cells/mL). We observed a significant difference when comparing SCC counts in composite quarter milk samples of cows that were BHV+/Bact− (95,000 cells/mL) and BHV−/Bact+ (445,000 cells/mL). A significant difference ($P < 0.0001$) was found in the mean SCC of composite quarter milk samples between cows that were BHV−/Bact+ (445,000 cells/mL) compared with cows that were BHV−/Bact− (55,000 cells/mL). A single-factor ANOVA showed a significant difference in SCC in composite quarter milk samples between 4 groups of cows examined (Table 4).

Association of BHV1, BHV2, BHV4, and Bacteria on SCC in Composite Quarter Milk Samples

Cows were placed in different groups based upon presence or absence of specific BHV and bacteria as indicated in Table 5. A group of 15 cows positive for BHV1 was further classified into cows that were BHV1+/Bact+ ($n = 9$), with a mean SCC of 128,000 cells/mL of milk and cows that were BHV1+/Bact− ($n = 6$), with a mean SCC of 202,000 cells/mL. No significant difference ($P = 0.29$) was observed in the mean SCC between the 2 groups. A total of 40 cows had BHV2 in their composite quarter milk samples, 31 of which also had bacteria (BHV2+/Bact+) with an SCC of 468,000 cells/mL, whereas bacteria (BHV2+/Bact−) were not detected in composite quarter milk samples from 9 cows with an SCC count of 72,000 cells/mL. A significant difference ($P < 0.0001$) was observed in the mean SCC between the 2 groups. A group of 10 cows positive for BHV1 and BHV2 were further classified into cows that were BHV1,2+/Bact+ ($n = 6$), with a mean SCC of 1,389,000 cells/mL of milk, and cows that were BHV1,2+/Bact− ($n = 4$), with a mean SCC of 57,000 cells/mL. A significant difference ($P < 0.0001$) was observed in the mean SCC between the 2

Table 4. Association of bovine herpesvirus (BHV) and bacteria (Bact) on SCC in composite quarter milk samples¹

Item	Status			
	BHV+/Bact+	BHV+/Bact−	BHV−/Bact−	BHV−/Bact+
No. of samples	50	19	17	53
SCC (×1,000 cells/mL)				
Mean	448	95	55	445
Range	128–1,483	57–202	12–465	23–4,452
Variance	2.848	2.342	2.06	2.658
Student's <i>t</i> -test (<i>P</i> -value)				
BHV+/Bact−	3.798 (<0.0001)	—	—	—
BHV−/Bact−	4.863 (<0.0001)	1.084 (0.14)	—	—
BHV−/Bact+	0.182 (0.49)	3.912 (<0.0001)	5.015 (<0.0001)	—

¹Single-factor ANOVA: *F* (12.7); *F* critical (2.672); *P* (0.0001).

Table 5. Influence of bovine herpesvirus (BHV) 1, 2, and 4 and bacteria (Bact) on SCC in composite quarter milk samples

Organism	No. of samples	SCC ($\times 1,000$ cells/mL)			Student's <i>t</i> -test (<i>P</i> -value)
		Mean	Range	Variance	
BHV1+/Bact+	9	128	28–1,278	2.846	0.556 (0.29)
BHV1+/Bact–	6	202	26–1,225	2.919	
BHV2+/Bact+	31	468	18–3,987	2.52	3.443 (<0.0001)
BHV2+/Bact–	9	72	19–1,392	2.174	
BHV1,2+/Bact+	6	1,389	460–4,220	1.44	5.043 (<0.0001)
BHV1,2+/Bact–	4	57	30–286	1.655	
BHV4+/Bact+	1	281	—	—	
BHV2,4+/Bact+	3	1,483	263–3,822	2.655	

groups. A single composite quarter milk sample with a SCC of 281,000 cells/mL from a cow with BHV4+/Bact+ was observed, whereas composite quarter milk samples from 3 cows that were positive for BHV2,4+/Bact+ ($n = 3$) had a mean SCC of 1,483,000 cells/mL (Table 5).

Bacterial Species Isolated from Composite Quarter Milk Samples

The bacterial species isolated from composite quarter milk samples are listed in Table 6. *Staphylococcus aureus* was isolated from 23 of 139 (16.5%) composite quarter milk samples, including 11 and 12 from BHV+ and BHV– composite quarter milk samples, respectively. A total of 13 species of CNS, including *Staph. capitis*, *Staph. chromogenes*, *Staph. cohnii*, *Staph. epidermidis*, *Staph. hyicus*, *Staph. simulans*, *Staph. lentus*, *Staph. maltophilia*, *Staphylococcus* spp. (unidentified CNS), *Staph. warneri*, and *Staph. xylosus*, were isolated from composite quarter milk samples. With the exception of *Staph. chromogenes* (7 isolates from BHV+ vs. 2 isolates from BHV– composite quarter milk samples), the predominance of a CNS species in BHV+ or BHV– composite quarter milk was not observed. A total of 10 streptococci and streptococci-like organisms were isolated from composite quarter milk samples (Table 6). *Aerococcus viridans* was the most frequently isolated organism in this group followed by *Streptococcus uberis*. As observed with CNS, the predominance of streptococci or streptococci-like organisms in BHV+ or BHV– composite quarter milk was not observed.

A total of 12 gram-negative bacterial organisms were isolated from composite quarter milk samples. *Escherichia coli* and *Klebsiella pneumoniae* were the most frequently isolated organisms. As observed with CNS, streptococci, and streptococci-like organisms, no single species predominated in BHV+ or BHV– composite quarter milk. Gram-positive rods were isolated from 24 out of 139 (17.2%) composite quarter milk samples. As

observed with other bacterial species, the predominance of any particular organism was not observed in either BHV– or BHV + composite quarter milk samples.

DISCUSSION

The role of viruses in bovine mastitis is not completely understood. The isolation of viruses or detection of viral DNA in the mammary gland tissue and milk from mastitis cows is suggestive of viruses being one of the etiological agents of bovine mastitis. Polymerase chain reaction-based assays to detect viral infectious agents are now widely used. These assays are more sensitive and accurate in detecting viral infection compared with virus isolation (Vilcek et al., 1994; Alegre et al., 2001; Wellenberg et al., 2001). In recent years, conventional PCR assays have been replaced by real-time PCR assays to identify infectious agents. In our study, we developed a real-time PCR assay to detect bovine herpesviruses, including BHV1, BHV2, and BHV4, as well as BVDV, from the cellular fraction of composite quarter milk samples collected from lactating dairy cows.

The study was conducted on a large dairy herd in central Pennsylvania. In this herd, all adult cows are vaccinated at the time of drying off using killed vaccine against IBR, BVD, PI3, and BSRV, and at the time of breeding using modified live IBR, BVD, PI3, and BRSV. Milk samples from 139 cows were collected from 90 to 120 d in lactation. The purpose of collecting milk samples during the mid-lactation period was to provide an interval of least 60 d following administration of live modified vaccine at the time of breeding before collection of milk samples for analysis. The time interval of 60 d would result in elimination of the vaccine strain of the virus from the cow and thereby significantly reduce the possibility of false-positive results using the real-time PCR assay.

Application of the real-time PCR assay revealed that 49.7% of the 139 cows surveyed had BHV in composite quarter milk samples, of which 10.8, 28.7, and 0.7%

Table 6. Bacterial species (no. of isolates) isolated from composite quarter milk samples infected or not with bovine herpesvirus (BHV)

Species	BHV+ (n = 69)	BHV– (n = 70)	Total (n = 139)
Staphylococci			
<i>Staphylococcus aureus</i>	11	12	23
<i>Staphylococcus capitis</i>	4	6	10
<i>Staphylococcus chromogenes</i>	7	2	9
<i>Staphylococcus cohnii</i>	4	4	8
<i>Staphylococcus epidermidis</i>	5	6	11
<i>Staphylococcus hyicus</i>	4	8	12
<i>Staphylococcus simulans</i>	1	0	1
<i>Staphylococcus lentus</i>	2	4	6
<i>Staphylococcus maltophilia</i>	1	1	2
<i>Staphylococcus sciuri</i>	0	1	1
<i>Staphylococcus simulans</i>	1	0	1
<i>Staphylococcus</i> spp.	4	5	9
<i>Staphylococcus warneri</i>	1	3	4
<i>Staphylococcus xylosus</i>	7	6	13
Streptococci and streptococci-like organisms			
<i>Aerococcus viridans</i>	31	32	63
<i>Streptococcus acidominimus</i>	1	1	2
<i>Streptococcus bovis</i>	2	1	3
<i>Streptococcus mutans</i>	0	1	1
<i>Streptococcus pneumoniae</i>	0	1	1
<i>Streptococcus uberis</i>	8	10	18
<i>Enterococcus avium</i>	0	1	1
<i>Enterococcus faecium</i>	0	1	1
<i>Enterococcus faecalis</i>	1	0	1
Gram-negative bacteria			
<i>Enterobacter asburiae</i>	1	0	1
<i>Enterobacter cloacae</i>	1	1	2
<i>Escherichia coli</i>	5	7	12
<i>Enterobacter</i> spp.	1	0	1
<i>Klebsiella ornitholytica</i>	1	0	1
<i>Klebsiella oxytoca</i>	1	2	3
<i>Klebsiella pneumoniae</i>	2	6	8
<i>Pantoea</i> spp.	1	1	2
<i>Proteus mirabilis</i>	0	1	1
<i>Pseudomonas aeruginosa</i>	0	1	1
<i>Proteus vulgaris</i>	0	2	2
<i>Serratia</i> spp.	1	0	1
Miscellaneous organisms			
<i>Micrococcus</i> spp.	1	0	1
Unidentified bacteria	1	0	1
Gram-positive rods	11	13	24
<i>Kocuria varians</i>	1	0	1
<i>Mycoplasma</i>	0	1	1

of composite quarter milk samples had BHV1, BHV2, and BHV4, respectively. The prevalence of BHV in composite quarter milk samples was not influenced by lactation age. With the exception of BHV1, cows positive for BHV were mostly in their second or higher lactation. Prevalence of BHV2 was higher than that of BHV1 or BHV4, and more so in second-lactation cows. The prevalence of BHV in a herd over a period of time can vary considerably because of the latency of BHV in the host and reactivation due to changes in lactation physiology or immune status (Nylin et al., 2000).

Cows can shed these viruses intermittently or establish latency that can then be reactivated on exposure of the animal to stress or immunosuppressive drugs

(Donofrio et al., 2000). The viruses can be re-excreted without clinical signs and transmitted to other susceptible hosts (Alegre et al., 2001). In our study, composite quarter milk samples were collected from lactating cows on 3 alternate days (d 1, 3, and 5); BHV1 was detected in 17% cows in 1 out of 3 milk samples, BHV2 was detected in 33% cows, and BHV4 was detected in 1.4% cows in 1 out of 3 samples. The difference in the frequency of detection of viral DNA in milk on 3 different occasions raises the possibility of intermittent shedding of the virus in milk by persistently infected animals.

Bovine herpesvirus 2 often infects young heifers and young cows at first parity or in first lactation (Wellenberg et al., 2002b). In our study, only 5 cows

were in the first-lactation age group, which made it difficult to draw any inferences on BHV2 presence in composite quarter milk samples from primiparous cows. Experimental studies with a European strain of endometriotropic BHV4 virus showed that the virus is shed intermittently in milk, saliva, and semen and may account for prolonged latent infections (Frazier et al., 2002).

Bovine viral diarrhea virus was not detected in composite quarter milk samples from cows surveyed during the study using reverse transcription real-time PCR. All of the cows in this herd were vaccinated against BVDV. The virus may be cleared from vaccinated herds, and vaccination can be an important factor for the zero or low prevalence of infection in the herd (Niza-Ribeiro et al., 2005).

In our study, we attempted to determine the influence of BHV in composite quarter milk samples on SCC in the presence or absence of bacterial IMI. Although our inferences are based on a small number of samples, the findings of the study suggest that composite quarter milk samples from BHV+/Bact+ cows had significantly higher SCC than the composite quarter milk samples from BHV+/Bact- cows. Similarly, composite quarter milk samples from cows with BHV+/Bact+ showed significantly higher SCC compared with composite quarter milk samples from BHV-/Bact- cows. The same was observed in cows with (1) BHV+/Bact- and BHV-/Bact+, and (2) BHV-/Bact- and BHV-/Bact+ in their composite quarter milk samples. These observations strongly suggest that BHV is less likely to be associated in increase in SCC in milk, and that an increase in SCC in milk is more likely due to bacterial IMI than to BHV infection. A similar study involving a larger sample size and different herds would be valuable to validate the findings of our study.

On further examination of specific BHV, the mean SCC between Bact+ cows (128,000 cells/mL) and Bact- cows (202,000 cells/mL) did not differ significantly ($P = 0.29$) for BHV1+ cows. The mean SCC of BHV1+/Bact- cows were higher than those of BHV1+/Bact+ cows. Bovine herpesvirus 1 is shown to transiently depress cell-mediated immunity, resulting in secondary bacterial infections (Meyer et al., 2007). The bovine udder has been reported to be susceptible to BHV1, especially in nonimmune animals (Corner et al., 1967; Wellenberg et al., 2002a). Bovine herpesvirus 1 has been isolated from natural cases of mastitis. It can propagate and produce mastitis in the lactating bovine mammary gland. It is not, however, considered to be a major primary udder pathogen (Corner et al., 1967).

Somatic cell counts in composite quarter milk samples from cows with BHV2+/Bact- were significantly lower than those from BHV2+/Bact+ cows. A similar obser-

vation was made with cows with BHV1,2+/Bact- in their composite quarter milk samples compared with cows with BHV1,2+/Bact+ in their milk. The role of BHV2 on the immune system or immune stimulation has not been extensively reported and needs to be further investigated.

Using a nested PCR assay, Kalman et al. (2004) detected BHV4 in milk samples from lactating cows and found that 90% of the cows shed BHV4 in milk, regardless of bacterial status of the udder. In our study, we found only 1 cow that was positive for BHV4, and 3 cows with BHV2, 4+/Bact+ in composite quarter milk samples; therefore, no statistical conclusions could be drawn regarding differences in mean SCC.

The bacterial species isolated in composite quarter milk samples from cows surveyed in our study have been widely reported in literature as etiological agents of clinical and subclinical mastitis in dairy cattle (Wilson et al., 1997; Fox et al., 2005; Olde Riekerink et al., 2006). Organisms including *A. viridians*, *Strep. uberis*, *E. coli*, and *K. pneumoniae* were frequently isolated from composite quarter milk samples. None of these organisms were found to be predominant in BHV+ cows or BHV- cows. A probable confounder in our study could be the fact that culture methods used may have overlooked at the possibility of slow-growing pathogens (e.g., *Prototheca* spp. or small cell variants of *Staph. aureus*), which may take an additional 3 to 5 d to grow on 5% sheep blood agar plates. Despite 3 negative cultures, the possibility of pooling quarter milk samples may have affected the frequency of isolation, when the pathogens are in low numbers especially in the samples were collected over 5 d as opposed to 3 wk.

Sampimon et al. (2009) evaluated the API Staph ID 32 (BioMerieux) and Staph Zym (Rosco, Tåstrup, Denmark) systems for identification of CNS isolated from bovine milk. The tests were compared with an *rpoB* DNA sequencing method. Their study showed that API Staph ID 32 was able to correctly identify 41% of the CNS compared with the DNA sequencing method. In our study, we used API-Staph to identify staphylococci, and based on the work of Sampimon et al. (2009), a misclassification error of CNS is a possibility. Although the likelihood of species misclassification does not affect the overall findings of the study, it does indicate the need to use a highly discriminatory identification method when conducting a prevalence or epidemiologic study on mastitis pathogens.

Fridgut and Stram (2006) isolated *Porphyromonas levii* along with BHV4 from clinically affected animals, whereas Miyano et al. (2004) reported the absence of bacterial organisms from mammary tissue preparations from a cow that had died of suspected BHV4 clinical mastitis. Wellenberg et al. (2001), in their study us-

ing a PCR-based method, were able to identify BHV4 DNA in milk samples with *Strep. uberis* and high SCC (>1,000,000 cells/mL). In our study, however, only 1 composite quarter milk sample was positive for BHV4 DNA in milk.

Wellenberg et al. (2002b) reported that the effect of BHV1 on bovine mastitis was not clear because the number of reported cases of mastitis in which BHV1 played a role was low. They also stated that BHV1 and BHV4 were not primary udder pathogens, although BHV4 could play an indirect role in subclinical mastitis. The results of our study with respect to the association of SCC and BHV support the findings of Wellenberg et al. (2002b)

CONCLUSIONS

Real-time PCR can be used to detect BHV1, BHV2, and BHV4 DNA in composite quarter milk samples from lactating cows. In our study, BHV1 and BHV2 were more prevalent than BHV4 in the study population. However, BHV were not widespread among cows of a particular lactation age group. Similarly, the presence of BHV in milk was not associated with a particular genus or bacterial species. Observations made with respect to the frequency of detection of BHV in composite quarter milk samples raises the possibility of intermittent shedding of the BHV in milk by persistently infected cows and may strongly influence the detection of BHV in milk. A statistical difference was observed in the mean SCC between cows with BHV alone (absence of concurrent bacterial infection) and cows without BHV and bacterial IMI. The same was observed in cows with BHV2 and BHV1 and BHV2 DNA in composite quarter milk samples. The findings of our study suggest that, in the herd we examined, BHV1, BHV2, and BHV4 are probably not major udder pathogens. The major influence on milk SCC was bacterial IMI, suggesting that the tested viral etiologies have little influence on mastitis.

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