Prevalence of contagious mastitis pathogens in bulk tank milk in Prince Edward Island

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Abstract — The purpose of this study was to 1) estimate the herd prevalence of contagious mastitis pathogens in bulk milk from Prince Edward Island (PEI) dairy farms, 2) determine the association between bulk milk culture results and mean bulk milk somatic cell count (BMSCC), and 3) investigate the agreement of repeated bulk milk cultures. Three consecutive bulk milk samples were obtained at weekly intervals from all 258 PEI dairy herds and were cultured using routine laboratory methods. Cumulative prevalence of *Staphylococcus aureus, Streptococcus agalactiae,* and *Mycoplasma* spp. (*M. bovis* and *M. alkalescens*) was 74%, 1.6%, and 1.9%, respectively. Bulk milk somatic cell count of *Staph. aureus*-positive herds was higher than that of negative herds. Agreement for *Staph. aureus* isolation between 3 consecutive tests was moderate (kappa = 0.46). *Mycoplasma bovis* and *M. alkalescens* in bulk milk are being reported for the 1st time in PEI ever and in Canada since 1972.

Résumé — Prévalence des pathogènes contagieux de la mammite dans le lait de citerne en vrac à l'Île-du-Prince-Édouard. La présente étude avait pour objectif 1) d'estimer la prévalence dans le troupeau des pathogènes contagieux de la mammite dans le lait en vrac provenant des fermes laitières de l'Île-du-Prince-Édouard (Î.-P.-É.), 2) de déterminer le lien entre les résultats de culture du lait en vrac et la numération moyenne des cellules somatiques du lait en vrac et 3) d'étudier la concordance entre les cultures répétées du lait en vrac. Trois échantillons consécutifs de lait en vrac ont été obtenus à des intervalles hebdomadaires auprès de l'ensemble des 258 troupeaux laitiers de l'Î.-P.-É. et ont été cultivés en utilisant les méthodes de laboratoire habituelles. La prévalence cumulative de *Staphylococcus aureus,* de *Streptococcus agalactiae* et de *Mycoplasma* spp. (*M. bovis* et *M. alkalescens*) était de 74 %, de 1,6 % et de 1,9 %, respectivement. La numération des cellules somatiques du lait en vrac des troupeaux positifs pour *Staph. Aureus* était supérieure à celle des troupeaux négatifs. La concordance de l'isolement de *Staph. aureus* entre 3 tests consécutifs était modérée (kappa = 0,46). *Mycoplasma bovis* et *M. alkalescens* dans le lait en vrac sont signalés pour la première fois à l'Î.-P.-É. et pour la première fois depuis 1972 au Canada.

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Introduction

Mastitis is the most prevalent and expensive disease on a dairy farm. Knowledge of the prevalence and distribution of mastitis pathogens is critical to the prevention of the disease. Bulk tank milk culture may be used as a monitoring tool in the control and evaluation of clinical and subclinical mastitis (1). This tool may be useful while investigating potential milk quality problems on a dairy farm, such as increased bacterial or somatic cell counts (SCC) are being investigated (1,2). Bulk milk culture is a cheap and convenient method of evaluating milk quality compared with the collection and culturing of individual cow milk samples, and it may be a useful tool for estimating herd level prevalence of contagious mastitis pathogens.

The contagious mastitis pathogens *Staphylococcus aureus, Streptococcus agalactiae,* and *Mycoplasma* spp. reside primarily in the cow's udder; therefore, when they are found in bulk milk, these mastitis causing organisms are strong indicators of the presence of intramammary infections in the herd (3,4). *Staphylococcus aureus* is a gram-positive bacterium, can cause subclinical and clinical mastitis in dairy cows, and is usually associated with elevated SCC (5,6). *Streptococcus agalactiae* is a gram-positive bacterium, is a contagious obligate parasite of the bovine mammary gland, and most often causes subclinical mastitis and elevated cow SCC (5,7). *Mycoplasma* are pleomorphic bacteria that lack a cell wall, are contagious, and can cause high SCC and chronic clinical mastitis (5,8).

Several studies have been performed to estimate the herd prevalence of *Staph. aureus, Str. agalactiae,* and *Mycoplasma* spp. in the United States and Europe (9–14). However, only a few studies have been carried out in Canada to estimate the prevalence of contagious mastitis pathogens in bulk milk. The prevalence of *Str. agalactiae* found in Canadian bulk milk ranged between 6% in Alberta (1993) and 43% in Québec (1992) (15,16). For Prince Edward Island, only Keefe et al (7,17) have studied herd prevalence of *Str. agalactiae* and *Staph. aureus.* They found a herd prevalence of 18% and 70%,

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respectively (7,17). Kelton et al (18,19) found *Staph. aureus* in 58 out of 59 bulk milk samples from Ontario, while 92% of the herds had at least 1 *Staph. aureus* culture-positive cow. In only 1 Canadian study carried out over 30 y ago were *Mycoplasma* spp. found in bulk milk and individual cow milk in Ontario herds: in 33 out of 64 herds and in 182 out of 598 cows (20).

Bulk milk SCC (BMSCC) is used worldwide as a measurement for milk quality. Maintaining a low BMSCC benefits both producers and consumers (21–23). An elevated BMSCC is associated with higher prevalence of subclinical mastitis caused by *Str. agalactiae* and *Staph. aureus.* Herds may experience a high incidence of clinical mastitis even though the BMSCC remains low (24–26). A positive association between herd classification based on BMSCC and the isolation of *Staph. aureus* in bulk milk has been reported (27,28).

Currently, reports of a few studies on culture agreement between individual cow or quarter milk cultures are available (29,30). However, no studies that estimated the agreement between cultures of consecutive bulk milk cultures have been done.

The objectives of this study were to 1) estimate the herd prevalence of contagious mastitis pathogens in bulk milk from Prince Edward Island dairy farms, 2) determine the association between isolation of contagious mastitis pathogens and herd average BMSCC, and 3) investigate the agreement between repeated bulk milk cultures.

Materials and methods

Study population

At the beginning of the study in May 2004, the Prince Edward Island dairy industry consisted of 258 dairy farms. During the sampling period, 1 farm ceased farming. On December 31st, 2003, 193 dairy farms (75%) were enrolled in the milk recording program of the Atlantic Dairy Livestock Improvement Corporation (ADLIC). Among these 193 herds, the average herd size was 59.6 lactating cows, mean annual milk production was 8894 kg/cow (31), and the arithmetic mean BMSCC was 245 000 cells/mL (32).

Sample collection

Three sets of fresh bulk milk samples were collected from all dairy farms on Prince Edward Island at weekly intervals. Samples were collected from the bulk tank by bulk milk haulers who followed a specified sampling protocol. The milk in the tank was agitated for 10 min before a sample was taken from the top of the tank, using a clean, sanitized dipper (33). Samples were then transported on ice to the provincial dairy laboratory and cultured within 24 to 36 h after collection at the farm. After culturing, SCC was determined within 12 h, except for the 1st set of samples. In the 1st set of samples, ethidium bromide tablets were added as a preservative to the milk and SCC was measured 48 h later.

Laboratory analysis

Five different culture media were used to detect *Staph. aureus, Str. agalactiae,* and *Mycoplasma* spp. These were 1) blood agar with the addition of 1 g/L esculin; 2) Vogel Johnson agar, a medium selective for staphylococci; 3) modified Edward's medium with the addition of colistin sulphate (5 mg/L) and oxolinic acid (2.5 mg/L), a medium selective for streptococci (34); 4) modified Hayflick's agar for the culture of *Mycoplasma* spp., and 5) modified Hayflick's broth for *Mycoplasma* spp. enrichment (33–35). After mixing the milk on a vortex shaker for 5 s, 50 μL was dispensed by pipette onto the blood esculin agar, Vogel-Johnson agar, and modified Edward's; 100 μL was dispensed onto Hayflick's agar and into 2 mL of Hayflick's broth. The milk was spread evenly over the plates by a sterile cotton swab and allowed to air dry before incubation.

The Hayflick's broth was mixed on a vortex shaker for a short time and then incubated at 37°C in a moist incubator with 10% CO_2 for 48 h before an aliquot of 100 μL was dispensed onto Hayflick's agar. All Hayflick's agar plates were incubated for 10 d at 37°C in a moist incubator with 10% CO_2. These plates were examined after 48 h and again after 10 d. The blood-esculin agar, Vogel-Johnson agar, and modified Edward's medium were incubated at 37°C aerobically for 48 h. These plates were examined after 24 and 48 h of incubation. *Staphylococcus aureus* was identified by Gram stain, a positive catalase test, α- and β-hemolysis on blood-esculin agar, and a positive tube coagulase test. *Streptococcus agalactiae* was identified by typical appearance on either modified Edward's medium or blood esculin agar, gram-positive staining, a negative catalase test, a positive CAMP test, and a positive latex agglutination test (Remel PathoDx; Remel Europe, Dartford, Kent, United Kingdom). *Mycoplasma* spp. were identified by the typical fried egg appearance on Hayflick's agar. If *Mycoplasma* spp. were cultured, isolates were sent to the Animal Health Laboratory of the University of Guelph for determination of species by an antibody agglutination method (36).

Bulk milk SCC of all samples was determined with an electronic cell counter (Fossomatic Series 400; Foss Electric A/S, Hillerød, Denmark).

Statistical analysis

Geometric mean BMSCC per farm was calculated as the exponent of the average natural logarithm (ln) of the 3 BMSCCs. A Student's *t*-test was used to test if the geometric mean BMSCC was different between pathogen-positive and negative farms. Linear regression was used to determine the strength of association of BMSCC and the frequency of *Staph. aureus* isolation. The agreement of *Staph. aureus* isolation between 2 consecutive samplings was measured by using kappa, which determines the agreement among tests beyond chance. A kappa between 0 and 0.2 is considered a slight, 0.2 to 0.4 fair, 0.4 to 0.6 moderate, 0.6 to 0.8 substantial, and > 0.8 almost perfect agreement (37). Calculation of geometric mean, linear regression, and kappa analysis were performed using a statistical software (Intercooled Stata for Windows, version 8.0; Stata Corporation, College Station, Texas, USA).

True herd prevalence, test sensitivity, and test correlation were determined by using maximum likelihood estimation based on a model described by Evers and Nauta (38), where animal-level prevalence was assumed

Table 1. Proportion of Prince Edward Island bulk milk samples (n = 258) that were culture-positive for contagious pathogens in 3 consecutive weeks

	Week 1 Herds (%)	Week 2 Herds (%)	Week 3 Herds (%)	Cumulative prevalence Herds (%)
Staphylococcus aureus	135 (52.3)	141 (54.8)	142 (55.3)	191 (74.0)
Streptococcus agalactiae	3 (1.2)	3 (1.2)	1 (0.4)	4 (1.6)
Mycoplasma spp.	5 (1.9)	1 (0.4)	1 (0.4)	5 (1.9)[a]

[a]*Mycoplasma bovis* 4 herds, *Mycoplasma alkalescens* 1 herd

Table 2. Frequency of contagious pathogens, isolated 0, 1, 2, or 3 times (out of 3 times) in the successive milk samples in a study of 258 Prince Edward Island dairy herds

	0 out of 3 times Herds (%)	1 out of 3 times Herds (%)	2 out of 3 times Herds (%)	3 out of 3 times Herds (%)
Staphylococcus aureus	67 (26.0)	52 (20.2)	53 (20.5)	86 (33.3)
Streptococcus agalactiae	254 (98.4)	2 (0.8)	1 (0.4)	1 (0.4)
Mycoplasma spp.	253 (98.1)	3 (1.2)	2 (0.8)	0

Table 3. Association of isolation of any pathogen with average bulk milk somatic cell count (BMSCC)

	Mean BMSCC (× 1000 cells/mL)			
Pathogen	Never isolated (# herds)	Isolated 1 or more times (# herds)	Difference (× 1000 cells/mL)	*P*-value
Staphylococcus aureus	129 (67)	169 (191)	39.7	0.001
Streptococcus agalactiae	157 (254)	177 (4)	20.0	0.69
Mycoplasma spp.	158 (253)	137 (5)	-20.5	0.60
Any contagious pathogen	132 (64)	167 (194)	34.7	0.006

Table 4. Mean bulk milk somatic cell count (BMSCC) in relation with frequency of *Staphylococcus aureus* isolation

Frequency of *Staph. aureus* isolation	Number of herds	Geometric mean BMSCC (× 1000 cells/mL)			
		Geometric mean	95% CI	Minimum	Maximum
0 (out of 3)	66	129[a,x]	112 to 148	16	462
1 (out of 3)	51	151[a]	129 to 177	48	537
2 (out of 3)	53	156[y]	134 to 183	56	487
3 (out of 3)	87	188[b,z]	167 to 213	47	607

[ab]Means with different superscripts were different at $P < 0.05$
[xyz]Means with different superscripts were different at $P < 0.10$

to vary between herds. The procedure of maximum likelihood estimation determines a set of parameters that makes the observed data most likely (37). This model was used assuming a perfect test specificity and constant true herd status, but allowing for conditional dependence between test results. For this analysis the SAS procedure for nonlinear mixed models [PROC NLMIXED] (The SAS system for Windows, version 8.02; SAS Institute, Cary, North Carolina, USA) was used.

Results

In total, 773 samples were examined, missing 1 sample in the 3rd week. Reading for *Mycoplasma* spp. could not be done on day 10 for 31 samples due to overgrowth by other organisms. *Staphylococcus aureus* was isolated in bulk milk from 191 (74%) dairy farms (Table 1). In every sampling week, *Staph. aureus* was isolated from at least 135 (52%) samples. Eighty-six (33%) farms tested positive for *Staph. aureus* on every bulk milk sample (Table 2). *Streptococcus agalactiae* was isolated at least once in samples from 4 (1.6%) farms. A *Mycoplasma* sp. was isolated at least once in samples from 5 (1.9%) farms (Table 2). Species determination in cultures from these 5 farms revealed 2 species, *M. bovis* and *M. alkalescens*. *Mycoplasma bovis* was found on 2 farms in 1 sample, on 2 farms in 2 samples. *Mycoplasma alkalescens* was found on 1 farm in 1 sample. *Mycoplasma* spp. were never found in 3 consecutive samples on 1 farm (Table 2).

The model in the maximum likelihood procedure that fitted best the data for *Staph. aureus* consisted of a herd prevalence of 100% (95% CI: 80 to 100), a test sensitivity of 54% (95% CI: 54 to 68) and rho (between test correlation) 0.46 (95% CI: 0.24 to 0.46).

Farms that had at least 1 bulk tank sample positive for any of the contagious pathogens had a geometric mean

BMSCC that was 34 700 cells/mL higher than the counts from farms that had no pathogens isolated (Table 3) ($P = 0.006$). No difference in BMSCC was found between the 5 *Mycoplasma*-positive and the negative herds, or between the 4 *Str. agalactiae*-positive and the negative herds ($P > 0.5$). The BMSCC of *Staph. aureus*-positive herds was 39 700 cells/mL higher than that of negative herds ($P = 0.001$).

The BMSCC increased with increasing frequency of *Staph. aureus* isolation (Table 4). *Streptococcus agalactiae* and *Mycoplasma* spp. were not included, because the number of *Str. agalactiae* and *Mycoplasma*-positive farms were 4 and 5, respectively, and therefore too low from which to draw conclusions.

Kappa for isolation of *Staph. aureus* between weeks 1 and 2, between weeks 2 and 3, and between weeks 1 and 3 was 0.42, 0.49, and 0.46, respectively. The combined agreement between the 3 tests gave a kappa value of 0.46. All kappa values indicated a moderate agreement.

Discussion

The apparent *Staph. aureus* herd level prevalence was in agreement with earlier studies in North America and Europe, where herd level prevalence ranged from 31% to almost 100% (13,14,19,27,39). Prevalence of *Mycoplasma* spp. has been reported for the 1st time on Prince Edward Island, and in Canada, for the 1st time since 1972. However, Canadian laboratories have cultured *Mycoplasma* spp. repeatedly from milk samples.

True herd prevalence, defined as the proportion of herds that have *Staph. aureus*-infected udders, can only be determined if the sensitivity and specificity of testing bulk milk samples is known; therefore, these parameters have to be determined or estimated. For isolates retrieved from bovine mastitis cases, Boerlin et al (40) found a specificity of 100% for the culture method, if *Staph. aureus* was identified by α and β hemolysis on blood agar and a positive coagulase test after 24 h. Therefore, in the statistical approach for the true prevalence, we considered the specificity of our method to be 100%. Another study also reported a high specificity for *Staph. aureus* of 93% (41). Allowing for a lower specificity, the estimated true prevalence would be over-estimated. In the 14-day sampling period, herds could go from a truly negative to a truly positive status for *Staph. aureus* or vice versa. The authors considered it to be unlikely that the infection status of a herd for *Staph. aureus* would have changed in that period.

The *Str. agalactiae* prevalence of 1.6% confirmed a trend of declining prevalence of this pathogen on Prince Edward Island from 18% in 1994 (7). Herd level prevalence of *Str. agalactiae* has decreased considerably over the last years (42,43). Keefe et al (7) reported a herd prevalence of 18% on Prince Edward Island in a study performed in 1994. In the current study, the *Str. agalactiae* herd prevalence appears to be reduced by a factor of 10 since 1994. However, Keefe et al (7) used a more sensitive method than the standard method recommended by NMC (formerly the National Mastitis Council; Verona, Washington, USA; a global organization for mastitis control and milk quality): in addition to modified Edward's medium, they used modified group B streptococcal (GBS) medium. Sawant et al (34) found in a media comparison that modified Edward's medium with the addition of colistin sulphate (5 mg/L) and oxolinic acid (2.5 mg/L) had a sensitivity and specificity of 100%. However, the study was under laboratory conditions, used selected streptococci, and spiked the milk samples. A few authors have estimated *Str. agalactiae* sensitivities from a single bulk milk sample under field conditions: Godkin and Leslie (44) found a bulk milk sensitivity of 21% for *Str. agalactiae* and Bartlett et al (41) found a sensitivity of 35%. Both sensitivities were estimated with single bulk milk samples and compared with individual cow composite and quarter samples, respectively. Sensitivity would have been higher if multiple bulk milk samples had been taken. The true prevalence of *Str. agalactiae* in this study is probably higher than estimated. With a 21% sensitivity and assuming a specificity of 100%, the true prevalence would not be estimated to be higher than 7.5%.

For the last 30 y, no Canadian studies have been performed to determine herd level prevalence of *Mycoplasma* spp. Recent US studies, however, suggested that 1% to 6% of the dairy herds had at least 1 cow with *Mycoplasma*-induced mastitis (10,11,45,46). Sampling bulk tank milk only once may give an underestimation of the prevalence, due to intermittent shedding (46); therefore, multiple sampling should be performed (45). Sensitivity of a single culture of bulk milk samples for *Mycoplasma* spp. ranges from 33% to 59% (47). The *Mycoplasma* spp. that were found in this study are both pathogenic and can cause mastitis (10,47). *Mycoplasma bovis* is considered the most pathogenic *Mycoplasma* sp. (47).

In this study, there was a significant association between the isolation of *Staph. aureus* and the mean BMSCC. This is in agreement with other studies (22,27,28). The frequency of isolation of *Staph. aureus* (amount of times it was isolated from the 3 samples) has been shown to be significantly associated with the BMSCC. Jayarao et al (27) have shown similar associations in a recent study in Pennsylvania. The BMSCC and isolation of *Str. agalactiae* were not significantly associated in their study, but only 4 farms were considered positive. Other studies have shown that isolation of *Str. agalactiae* in bulk milk is highly correlated with high BMSCC (42,49). However, the presence of certain strains of *Str. agalactiae* is not correlated to high BMSCC. A possible explanation is that the bulk tank milk was contaminated with human strains of *Str. agalactiae* (50).

The isolation of *Mycoplasma* spp. and mean BMSCC were not significantly associated in this study. The main reason is most likely that the number of *Mycoplasma*-positive farms was very low. Fox et al (11) have previously reported an association. One explanation could be that the isolation of *Mycoplasma* spp. in bulk tank milk is not related to the number of shedding cows (3). Other explanations could be the low sensitivity of bulk milk culture or that bulk milk was contaminated with *Mycoplasma* spp.

The test agreement of repeated bulk milk cultures was calculated to be moderate. This indicates that the culture of 1 bulk milk sample is not sufficient to correctly classify a herd's *Staph. aureus* infection status.

The apparent herd level prevalence of *Staph. aureus* infection in Prince Edward Island dairy herds is high and similar to that in previous research done elsewhere. As estimated by 3 bulk milk cultures done at weekly intervals, at least 74% of Prince Edward Island herds likely have at least 1 cow with udder infection due to *Staph. aureus*. The prevalence of *Str. agalactiae* has decreased and is low. Two species of *Mycoplasma* were cultured from Prince Edward Island herds for the first time. Reduction of *Staph. aureus* and *Str. agalactiae* infections is a useful tool in the reduction of BMSCC on a dairy farm. The agreement between repeated *Staph. aureus* cultures from bulk milk samples with weekly intervals is moderate; therefore, for reliable determination of the presence of *Staph. aureus,* more than 1 bulk milk sample is needed.

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