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Herd characteristics and cow-level factors associated with *Prototheca* mastitis on dairy farms in Ontario, Canada

L. Pieper,*[1] A. Godkin,† U. Roesler,‡ A. Polleichtner,‡ D. Slavic,§ K. E. Leslie,* and D. F. Kelton*
*Department of Population Medicine, University of Guelph, Ontario, Canada, N1G 2W1
†Veterinary Science and Policy Group, Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA), Guelph, Ontario, Canada, N1G 4Y2
‡Institute of Animal Hygiene and Environmental Health, Free University of Berlin, 10115 Berlin, Germany
§Animal Health Laboratory, University of Guelph, Ontario, Canada, N1G 6R8

ABSTRACT

Prototheca spp. are algae that cause incurable acute or chronic mastitis in dairy cows. The aim of this case-control study was the identification of cow- and herd-level risk factors for this unusual mastitis pathogen. Aseptically collected composite milk samples from 2,428 milking cows in 23 case and 23 control herds were collected between January and May 2011. A questionnaire was administered to the producers, and cow-level production and demographic data were gathered. In 58 of 64 isolates, *Prototheca* spp. and *Prototheca zopfii* genotypes were differentiated using PCR and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. All isolates were identified as *Prototheca zopfii* genotype 2. The mean within-herd prevalence for *Prototheca* spp. was 5.1% (range 0.0–12.5%). Case herds had a significantly lower herd-level prevalence of *Staphylococcus aureus* and a higher prevalence of yeasts than did control herds. The final logistic regression model for herd-level risk factors included use of intramammary injections of a non-intramammary drug [odds ratio (OR) = 136.8], the number of different injectable antibiotic products being used (OR = 2.82), the use of any dry cow teat sealant (external OR = 80.0; internal OR = 34.2), and having treated 3 or more displaced abomasums in the last 12 mo OR = 44.7). The final logistic regression model for cow-level risk factors included second or greater lactation (OR = 4.40) and the logarithm of the lactation-average somatic cell count (OR = 2.99). Unsanitary or repeated intramammary infusions, antibiotic treatment, and off-label use of injectable drugs in the udder might promote *Prototheca* udder infection.

Key words: dairy cow, mastitis, *Prototheca*, case-control study

INTRODUCTION

Prototheca is a colorless, unicellular algae that can cause granulomatous mastitis in dairy cows. *Prototheca* spp. have been isolated from cases of clinical and subclinical mastitis in several countries, including Canada (Dion, 1982), the United States (Pore et al., 1987; Anderson and Walker, 1988; Jordan et al., 2003), Brazil (Costa et al., 1996; Corbellini et al., 2001; Bueno et al., 2006), Germany (Baumgärtner, 1997; Rösler and Hensel, 2003; Tenhagen et al., 2005; Möller et al., 2007), and Japan (Osumi et al., 2008).

Prototheca spp. consists of *Prototheca wickerhamii*, *Prototheca stagnora*, *Prototheca ulmea*, *Prototheca zopfii*, *Prototheca blaschkeae* (Rösler et al., 2006), and *Prototheca cutis* sp. nov. (Satoh et al., 2010). *Prototheca zopfii* genotype 2 (Möller et al., 2007; Aouay et al., 2008; Marques et al., 2008) and *P. blaschkeae* (Aouay et al., 2008; Marques et al., 2008) have both been identified as causing clinical and subclinical mastitis. *Prototheca zopfii* genotype 1 has induced subclinical mastitis in challenge trials (Roth, 2009; Ito et al., 2011) but is generally not associated with naturally occurring mastitis. Of the rarely occurring cases of human protothecosis, a few have been associated with *P. zopfii* and *P. blaschkeae* (Rösler et al., 2006; Lass-Flörl and Mayr, 2007; Zhang et al., 2010), suggesting a zoonotic potential of bovine disease due to those *Prototheca* species.

Different *Prototheca* species have been found in the dairy farm environment, usually in moist areas containing organic matter, such as mud (Anderson and Walker, 1988), spoiled feed stored in silos (Baumgärtner, 1997), leftover feed in mangers (Anderson and Walker, 1988), and dirty water troughs (Anderson and Walker, 1988; Costa et al., 1997). Additionally, they have been identified in feces of cows (Anderson and Walker, 1988; Enders and Weber, 1993; Costa et al., 1997), calves (Costa et al., 1997), sows (Pore and Shahan, 1988), sheep (Pore and Shahan, 1988), and rodents (Pore and Shahan, 1988). However, the isolation of *Prototheca* from the dairy herd environment seems to be independent from previous *Prototheca* isolation from mastitis

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[1]Corresponding author: pieperl@uoguelph.ca

samples in the same herd (Blaschke-Hellmessen and Schuster, 1984; Anderson and Walker, 1988).

The prevalence of *Prototheca* spp. in routine milk samples is very low (0.1% of submitted samples) (Wilson et al., 1997). However, the prevalence of cows with *Prototheca* mastitis could reach more than 30% of the lactating herd during a *Prototheca* outbreak (Costa et al., 1997; Dubravka et al., 2006).

Because no treatment has yet been found to cure *Prototheca* mastitis in vivo (Anderson and Walker, 1988; Lagneau, 1996; Jánosi et al., 2001), emphasis should be placed on identifying risk factors and implementing prevention strategies on affected dairy farms. The aim of this case-control study was to identify risk factors associated with *Prototheca* mastitis on dairy farms in Ontario, Canada.

MATERIALS AND METHODS

Data Collection

For this case-control study, 23 herds with repeated (2 or more) *Prototheca*-positive milk culture results from mastitis samples within the last 2 yr were selected by Ontario veterinarians. For selection of the control herds, the veterinarians named 4 suitable herds that could serve as controls. These farms needed to be similar to the respective case herds in the number of cows and stall type for milking cows, but with no history of *Prototheca* mastitis within the last 2 yr. Out of these 4 herds, the investigators randomly picked a control herd by drawing a herd name from a hat. The farms were included in the study if they were enrolled in milk recording through CanWest DHI (Guelph, ON, Canada). For 1 case-control pair, 2 additional control herds had to be sampled because the first and second herds originally enrolled as controls were found to have *Prototheca*-positive milk samples and a history of *Prototheca* mastitis from more than 2 yr ago. All statistical analyses were performed without these 2 "false" control herds.

Between January 2011 and May 2011, the selected case and control herds were visited once during milking time by the researchers, the herd veterinarian, or a veterinary technician for aseptic collection of composite milk samples from each lactating cow. Dry cows were not sampled. Milk samples were taken after the usual premilking udder preparation by the farmer or milking staff, and before attachment of the milking unit. The teat ends were cleaned with an alcohol swab, the first few strips of milk were discarded, and a sample from all milking quarters was collected into one vial per cow. A clinical examination of the cows was not performed. Milk samples were submitted to the Animal Health Laboratory (University of Guelph, Ontario, Canada) for identification of *Prototheca* spp. and other mastitis pathogens. Ten microliters of each milk sample was plated on sheep blood agar plates (Oxoid Ltd., Basingstoke, UK) using a calibrated loop (Thermo Fisher Scientific Inc., Waltham, MA) and incubated at 35°C ± 2°C for 48 h. *Prototheca* spp. were identified using colony morphology and Gram stain. Other mastitis pathogens were identified using colony morphology, Gram stain, and biochemical pattern according to National Mastitis Council guidelines (Hogan et al., 1999). The laboratory personnel were blinded to case or control status of the sampled herds. A random subset of 58 *Prototheca* isolates was shipped in slant agar tubes (Bio-Media Unlimited Ltd., Woodbridge, ON, Canada) to the Institute of Animal Hygiene and Environmental Health, Free University of Berlin (Berlin, Germany), for further species and genotype differentiation using PCR and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (**MALDI-TOF MS**; Bruker Daltonics, Bremen, Germany) with methods described previously (Rösler et al., 2006; von Bergen et al., 2009; Murugaiyan et al., 2012).

A questionnaire developed and pretested by the research team was administered via interview. The questionnaire contained 54 short questions about herd characteristics, management, and medication practices (Table 1). Further herd- and cow-level information was gathered using DHI records (Tables 1 and 2).

Statistical Analysis

Statistical analyses were conducted using the statistical software package STATA 10.1 (StataCorp, College Station, TX). For each herd and each mastitis pathogen, the within-herd prevalence was calculated by adding up the number of isolates divided by the number of milk samples. The within-herd prevalence in case and control farms was compared using the Mann-Whitney U test. The following variables were created with information from the original questionnaire responses: changed dry cow treatment within the last 3 yr (yes/no), number of different intramammary antibiotic mastitis treatment products (**NrIntramam**), number of different injectable antibiotic treatment products (**NrInjectable**; both continuous), and use of injectable sulfonamides (yes/no). The questionnaire data were screened for differences between case and control farms using descriptive statistics, univariable logistic regression for categorical variables, and the Mann-Whitney U test for continuous variables. Variables identified as risk factors ($P < 0.1$) were tested for collinearity using the Spearman rank correlation (ρ). High correlations ($\rho > 0.6$) were found between use of Spectramast LC

Table 1. Descriptions and levels of risk factors for herd-level analyses (information gathered from questionnaire unless otherwise specified)

Risk factor	Description and levels
Herd size	Count of milking and dry cows
Stall type	For dry and milking cows separately: tiestall, freestall, bedded pack, other
Housing at calving	Single cow in a tiestall, single cow in pen with pack, multiple cows in freestalls, multiple cows in a pen with pack, other
Bedding type[1]	For milking and dry cows separately: long straw, chopped straw, sawdust, sand, rubber mats, pasture mats, none, other
Source of drinking water for cows	Dug well; drilled well; surface pond, stream, or river; municipal; other
Source of water for cleaning the milking equipment	Dug well; drilled well; surface pond, stream, or river; municipal; other
Access to pond stream or river	Any adult cow, at any time of the year: yes, no
Other animals on farm[1]	Dogs, cats, horses, pigs, sheep, goats, other, none
Frequency of cleaning feed bunk or manger	For lactating and dry cows separately: more than once per day, once per day, every other day, once per week, less frequently than once per week
Access to pasture or yard	At any time of the year, for pasture (>3 acres) and yard (≤3 acres) separately: dry cows, milking cows, dry and milking cows, none
Attended at shows or fairs	During last 2 yr: yes, no
Purchased cattle[1]	Milking cows, heifers, calves (<6 mo), bulls
Flooring type	For milking cows: slatted, solid, other
Type of milking system	Pipeline, parlor, robotic
Automatic take-off	Yes, no
Technical details of milking equipment	Height of vacuum, pulsation rate, length of premilking stimulation time, yield at automatic take-off
Milking order	None, independent from disease status; healthy cows before mastitis cows
Disinfection of milking unit between cows	Yes, no
Premilking teat disinfection	Premilking teat dip, wash with disinfectant only, wash with disinfectant and dry, none
Tool used to wipe teats before milking	Reusable dry cloth towel, reusable damp cloth towel, single-use dry paper towel, single-use moist paper towel, single-use "baby wipe" type product, nothing, other
Postmilking teat dip	Yes, no (if yes, application technique and product)
Wearing of milking gloves	Farmer and milking staff: all do; no one does; some do, but not all
Disinfection of milking gloves or hands between cows	Farmer and milking staff: all do; all do, at some cows; no one does; some do, but not all
Prestripping before milking unit attached	On hand, shoe, or floor; on a strip cup or plate; not done
Antibiotic products for dry cow treatment	Product name and duration of usage
Intramammary antibiotic mastitis treatment, injectable mastitis treatment, teat channel dilators, udder creams or balms	During the last year, names of products used
Teat sealant	Internal, external, none (if used, product and duration of usage)
Teat surgeries	During the last 2 yr: yes, no
Off-label drug use in the udder (ImINID)	Yes, no (if yes, product name)
Number of cows with treatments	During last year, for ketosis, displaced abomasum, mastitis, and lameness
Bacterial count,[2] ×1,000 cfu/mL	Over 12 mo, average monthly bulk tank bacterial count
Milk yield,[3] kg	Over 12 mo, average monthly milk yield per day and 305-d lactation
Milk components,[3] kg	Over 12 mo, average monthly milk fat and milk protein per day and 305-d lactation
SCC,[2,3] ×1,000 cells/mL	Over 12 mo, average monthly bulk tank SCC and weighted cow SCC

[1]Multiple answers possible.

[2]From Dairy Farmers of Ontario (DFO), Mississauga, ON, Canada

[3]From CanWest DHI (Guelph, ON, Canada) records.

(Pfizer Animal Health, Pfizer Canada Inc., Kirkland, QC, Canada) as a lactating cow mastitis treatment and NrIntramam ($\rho = 0.65$) and between use of sulfonamides and NrInjectable ($\rho = 0.61$). The more general and biologically plausible variables NrIntramam and NrInjectable were tested first in the model.

Table 2. Description of risk factors for cow-level analyses from DHI records

Risk factor	Description
Lactation number	Current lactation number
Milk yield, kg	Milk production from last herd test-day and estimated over 305 d
Milk components, kg	Milk fat and protein production from last herd test-day and estimated over 305 d
SCC, ×1,000 cells/mL	SCC from last herd test-day and estimated over 305 d
Previous lactation number	Lactation number in previous lactation
Previous lactation-average SCC, ×1,000 cells/mL	SCC in previous lactation
Pedigree	Cow's dam and sire identification number

Manual forward selection using a *P*-value of 0.05 was chosen for logistic regression model building. Only biologically meaningful interactions were tested in the model. An interaction between NrInjectable and the intramammary injection of a non-intramammary drug (**ImINID**) was not significant ($P = 0.64$). Linearity was assessed for continuous variables and in the case of a nonlinear relationship between the predictor and outcome variable, the predictor variable was either transformed or categorized. The Hosmer-Lemeshow goodness-of-fit test was used to assess the model fit (5 groups, $P = 0.36$). By plotting raw residuals, standardized residuals, leverage, and delta-beta (influence of the specific covariate pattern on model)against the predicted outcome, covariate patterns that fit poorly in the model or that had a great influence on the model were visually identified. One covariate pattern was identified as very influential because of a high number of observations.

The model-building process described previously was repeated for the cow-level risk factors in case farms. Control farms were not included in these analyses, as the cows, by default, do not have a probability of having a *Prototheca*-positive laboratory result. Logarithmic transformation was performed on all SCC variables before the analyses. Risk factors were compared among cows with *Prototheca*-positive, culture-negative, and other mastitis pathogen-positive laboratory results using the Kruskal-Wallis rank sum test, and for post hoc comparisons, the Mann-Whitney U test. High correlations were found between lactation number and cow age in days ($\rho = 0.93$), and test-day SCC and lactation-average SCC ($\rho = 0.80$). Lactation number and lactation average SCC were, as the more informative variables, tested in the model. Cows with mastitis pathogens other than *Prototheca* spp. were excluded from the final model for cow-level risk factors. An interaction term between age category and lactation-average SCC was not significant in the model ($P = 0.71$). The model fit was assessed using the Hosmer-Lemeshow goodness-of-fit test (5 groups, $P = 0.21$). Five covariate patterns showed high Pearson residuals and high standardized residuals. Four were single observations of *Prototheca*-positive, first-lactation heifers with lactation-average SCC of 240,000 to 962,000 cells/mL. One covariate pattern consisted of 7 observations of first-lactation heifers with a lactation-average SCC of <50,000 cells/mL, whereas 1 of them had a *Prototheca*-positive laboratory result. Only those 5 first-lactation heifers had *Prototheca*-positive laboratory results.

A random effects model accounting for clustering by herd was tested. It was not significantly different from the more parsimonious logistic regression model without random effects (likelihood ratio test; $P = 0.11$). Therefore, the ordinary logistic regression model is shown in the results. A probability of $P < 0.05$ was considered significant in all analyses.

RESULTS

All cows in this study were Holstein-Friesian dairy cows. The mean number of cows in case and control herds was 66.9 (SD: 33.3) and 61.5 (SD: 20.3), respectively. According to the CanWest DHI Progress Report for Ontario (CanWest, 2009), 82.3% of Ontario dairy herds keep from 1 to 99 cows. However, the mean number of cows in the study herds was lower than the average number of cows per herd based on that report. The 305-d milk production per cow in the study farms was lower than the average 305-d milk production of Holstein-Friesian cows in Ontario. Furthermore, the smaller size of the study herds was represented in the higher percentage of tiestalls compared with the average percentage of tiestalls in DHI dairy herds (Table 3). Case and control herds did not differ significantly in 305-d or test-day milk yield, fat yield, or protein yield per cow over the last 12 mo before sampling. Over the same period, case herds tended to have higher SCC ($P = 0.06$) and bacterial counts ($P = 0.06$) in the bulk tank milk compared with control herds. We found no difference in the number of times the producer exceeded the limits of 500,000 cells/mL ($P = 0.23$) or 50,000 cfu/mL ($P = 0.64$) in bulk tank samples. The weighted average SCC over the last 12 mo before herd sampling were higher ($P = 0.04$) in case than in control herds.

Overall, 2,428 milk samples were cultured: 1,229 from case farms and 1,199 from control farms. Culture-negative results were obtained from 83.0% of all milk samples, and 0.62% of samples were contaminated. Significant differences in within-herd prevalence between case and control herds were found for *Prototheca* spp., *Staphylococcus aureus*, and yeasts (Table 4). *Prototheca* spp. were isolated from 64 milk samples from 18 different herds. The within-herd prevalence of *Prototheca* spp. in case herds ranged from 0.0% to 12.5% of sampled cows, with an average of 5.1%. In 5 case farms, no *Prototheca* spp. were isolated. The average within-herd prevalence for *Staph. aureus* in case herds was less than half that in control herds. Yeasts, *Corynebacterium bovis*, *Enterobacter cloacae*, *Klebsiella* spp., *Escherichia coli*, *Proteus* spp., and *Enterococcus* spp. were only isolated from case herd samples, whereas *Streptococcus agalactiae* were only isolated from samples from 1 control herd. *Prototheca* spp. were isolated together with *Staph. aureus* in 4 samples. Other combinations of pathogens were not observed. Using PCR and MALDI-TOF MS, all analyzed isolates were identified as *Prototheca zopfii* genotype 2. The isolates were similar to

Table 3. Demographic characteristics of case and control farms

Parameter	Case	Control	DHI[1]
Cows/herd, no. (SD)	66.9 (33.32)	61.5 (20.32)	75
305-d Milk production per cow, kg (SD)	9,304 (1,806.9)	9,271 (1,607.8)	9,650
Stall type for milking cows, no. (%)			
Tiestall	20 (87.0)	21 (91.2)	72.3
Freestall	3 (13.0)	2 (8.7)	26.5

[1]CanWest DHI (2009).

the type and reference strains SAG 2021^T, RZ II-2, and RZ II-3 (Culture Collection of the Institute of Animal Hygiene and Environmental Health, Free University of Berlin, Germany) that were previously isolated from clinical bovine mastitis in Germany.

Significant differences between case and control farms, based on univariable analysis of the questionnaire sections, are presented in Table 5. The use of an internal dry cow teat sealant was identified as a risk factor for being a case herd [odds ratio (**OR**) = 5.00, $P = 0.033$]. OrbeSeal (bismuth subnitrate, Pfizer Animal Health) was the only internal teat sealant used on the study farms. Most commonly, antibiotic dry cow treatment was administered to all cows at the end of lactation. On 1 case and 1 control farm, only multiparous cows were treated, and on 4 control farms, cows only received dry treatment based on herd-specific conditions (e.g., high SCC). Interestingly, ImINID was identified as the strongest risk factor (OR = 9.6, $P = 0.003$). For this procedure, the producers most commonly used Predef 2x (isuflupredone acetate, Pfizer Animal Health; 5 producers), penicillin (Durvet Inc., Blue Springs, MO; 5 producers), or Excenel RTU (ceftiofur hydrochloride, Pfizer Animal Health; 3 producers) as off-label intramammary infusions.

Table 6 shows the mean number of different antibiotics used, as well as the mean frequency of disease treatments in case and control farms. Case farms used a significantly higher NrIntramam. Even though not statistically significant, the data suggest that case farms may also use a higher NrInjectable ($P = 0.06$). The frequencies of treatments were similar between case and control farms for the most common diseases, with exception of abomasal displacement. Case farms tended to have treated more abomasal displacements during the past 12-mo period ($P = 0.051$) than control farms.

We observed no differences in other analyzed risk factors between case and control farms. The final model for herd-level risk factors included ImINID (OR = 136.8), NrInjectable (OR = 2.82), the use of any dry cow teat sealant (external OR = 80.0; internal OR = 34.2), and having treated 3 or more displaced abomasums in the last 12 mo (**≥3DA**; OR = 44.7; Table 7).

For case farms, DHI data were available for 1,174 of 1,229 sampled cows (95.5%). In case farms, cows with *Prototheca*-positive laboratory results were significantly

Table 4. Mean, SD, minimum, and maximum within-herd prevalence (%) of isolated pathogens (number of isolates/number of cows sampled) in case and control herds

	Case (n = 23)				Control (n = 23)				
Pathogen	Mean	SD	Minimum	Maximum	Mean	SD	Minimum	Maximum	*P*-value
Prototheca spp.	5.14	3.97	0.00	12.50	0.00	0.00	0.00	0.00	<0.001
Staphylococcus aureus	5.59	4.95	0.00	14.29	12.12	10.14	0.00	34.00	0.03
CNS	1.70	2.56	0.00	8.70	2.59	3.43	0.00	12.07	0.31
Streptococcus dysgalactiae	0.16	0.74	0.00	3.57	0.24	0.83	0.00	3.57	0.57
Streptococcus agalactiae	0.00	0.00	0.00	0.00	0.45	2.14	0.00	10.26	0.32
Streptococcus uberis	0.31	0.76	0.00	2.78	0.53	1.78	0.00	7.14	0.48
Streptococcus spp.	0.17	0.82	0.00	3.92	0.21	0.70	0.00	2.50	0.59
Arcanobacterium pyogenes	0.56	1.25	0.00	4.55	0.97	1.56	0.00	5.00	0.30
Serratia mercescens	0.65	1.64	0.00	7.50	0.34	0.93	0.00	3.57	0.33
Pasteurella multocida	0.09	0.44	0.00	2.13	0.25	0.67	0.00	2.17	0.31
Yeasts	0.70	1.21	0.00	3.70	0.00	0.00	0.00	0.00	0.005
Corynebacterium bovis	0.25	1.03	0.00	4.88	0.00	0.00	0.00	0.00	0.15
Enterobacter cloacae	0.16	0.57	0.00	2.50	0.00	0.00	0.00	0.00	0.15
Klebsiella spp.	0.11	0.52	0.00	2.50	0.00	0.00	0.00	0.00	0.32
Escherichia coli	0.11	0.55	0.00	2.63	0.00	0.00	0.00	0.00	0.32
Proteus spp.	0.10	0.50	0.00	2.38	0.00	0.00	0.00	0.00	0.32
Enterococcus spp.	0.04	0.18	0.00	0.88	0.00	0.00	0.00	0.00	0.32
No bacterial pathogen	83.16	6.10	71.43	97.22	80.45	10.28	58.14	98.33	0.44
Overgrowth with contaminants	0.96	1.40	0.00	4.17	0.47	1.02	0.00	3.57	0.18

Table 5. Univariable analysis of significant farm-level risk factors for being a case farm

Parameter/question	Case (n = 23)		Control (n = 23)		Odds ratio	*P*-value
	No.	%	No.	%		
Access to pond, stream, or river at any time						
Yes	0	0.0	4	17.4	0.0	0.036
Other animals on the farm[1]						
Dogs	20	87.0	13	56.5	5.13	0.022
Cats	21	91.3	21	91.3	1.00	1.000
Horses	5	21.7	5	21.7	1.00	1.000
Pigs	0	0.0	1	4.4	—	
Goats	3	13.0	1	4.4	3.30	0.295
Other	5	21.7	1	4.4	6.11	0.080
None	0	0.0	1	4.4	—	
Wear milking gloves						
All do	15	65.2	11	47.8	Referent	
Some do, but not all	1	4.3	7	30.4	0.10	0.048
No one does	7	30.4	5	21.7	1.02	0.970
Changed dry cow treatment within the last 3 yr						
Yes	11	47.8	3	13.0	6.11	0.010
Teat sealant[1]						
None	3	13.0	10	43.5	Referent	
External	2	8.7	1	4.4	6.67	0.172
Internal	18	78.3	12	52.2	5.0	0.033
Antibiotic intramammary mastitis treatment during lactation[2,3]						
Special Formula 17900-Forte Suspension[5]	19	82.6	19	82.6	1.00	1.000
Cefa-Lak[6]	12	52.2	10	43.5	1.42	0.555
Pirsue Sterile Solution[5]	12	52.2	8	34.8	2.05	0.234
Spectramast LC[5]	19	82.6	10	43.5	6.18	0.006
Sulfonamides (Borgal,[7] Trivetrin,[7] Trimidox[8])[3,4]						
Yes	21	91.3	15	65.2	5.60	0.032
Nonantibiotic mastitis treatment[2,3]						
Banamine Sterile Solution Injectable[7]	9	39.1	5	21.7	2.31	0.200
Anafen Injection 100 mg/mL[9]	11	47.8	7	30.4	2.10	0.227
Aspirin[10]	11	47.8	5	21.7	3.30	0.063
Predef 2x Sterile Aqueous Suspension[5]	9	39.1	3	13.0	4.29	0.044
Oxitocin Injection[11]	5	21.7	5	21.7	1.0	1.000
J-Vac[9]	1	4.4	1	4.4	1.0	1.000
Other	7	30.4	4	17.4	2.08	0.300
Intramammary injection of a nonintramammary drug[3]						
Yes	11	47.8	2	8.7	9.63	0.003

[1]Use of any teat sealant.
[2]Multiple answers possible.
[3]During the last 24 mo.
[4]Sulfonamides = use of any injectable sulfonamide.
[5]Pfizer Animal Health, Pfizer Canada Inc. (Kirkland, QC, Canada).
[6]Boehringer Ingelheim (Canada) Ltd. (Burlington, ON, Canada).
[7]Intervet/Schering-Plough Animal Health, Intervet Canada Corp. (Kirkland, QC, Canada).
[8]Vétoquinol Canada Inc. (Lavaltrie, QC, Canada).
[9]Merial Canada, Inc. (Baie d'Urfé, QC, Canada).
[10]Different manufacturers.
[11]Bimeda-MTC Animal Health Inc. (Lavaltrie, QC, Canada).

Table 6. Mean (SD) of number of different antibiotics used during the last 24 mo and of number of disease treatments in the last 12 mo in case and control farms

Parameter	Case (n = 23)	Control (n = 23)	*P*-value
Number of different intramammary antibiotics[1]	2.70 (0.93)	2.04 (0.88)	0.018
Number of different injectable antibiotics[1]	1.96 (1.19)	1.35 (1.07)	0.060
Ketosis[2]	9.00 (6.65)	8.04 (6.54)	0.565
Abomasal displacements[2]	4.17 (4.26)	2.96 (1.52)	0.051
Mastitis[2]	15.41 (14.92)	13.91 (16.38)	0.239
Lameness[2]	7.83 (9.17)	7.91 (6.84)	0.493

[1]During the last 24 mo.
[2]During the last 12 mo.

Table 7. Odds ratio (OR), 95% CI, and significance level of the final logistic regression model for herd-level risk factors for being a case herd

Parameter[1]	OR	95% CI	*P*-value
ImINID	136.8	5.40; 3,463.74	0.003
NrInjectable	2.82	1.043; 7.653	0.041
Teat sealant			
None	Referent	—	—
External	80.0	1.11; 5,765.89	0.045
Internal	34.2	2.22; 526.75	0.011
≥3 DA	41.1	2.69; 628.34	0.008

[1]ImINID = intramammary injection of a nonintramammary drug; NrInjectable = number of different injectable antibiotic products; Teat sealant = use of any teat sealant; ≥3 DA = having treated 3 or more displaced abomasums in the last 12 mo.

older than cows with culture-negative results or cows with other mastitis pathogens (Table 8). Cows that tested positive or negative for *Prototheca* did not differ in stage of lactation or test-day milk, fat, or protein yields. Cows that were culture positive for *Prototheca* spp. or other mastitis pathogens had higher test-day SCC and lactation-average SCC than culture-negative cows. Four *Prototheca*-positive cows had test-day SCC <100,000 cells/mL, and 1 *Prototheca*-positive cow had a lactation-average SCC <100,000 cells/mL. Using the pedigree information from DHI, no mother-daughter pair could be detected among the *Prototheca*-positive cows in this study, and cows with *Prototheca*-positive laboratory results were offspring of many different sires. The final model for the cow-level risk factors (Table 9) indicates higher odds of having a laboratory result positive for *Prototheca* in multiparous cows and in those with high lactation-average SCC.

DISCUSSION

This case-control study was conducted to identify herd- and cow-level risk factors for *Prototheca* mastitis. Laboratory, production, and questionnaire data were compared for 23 case-control pairs of herds. Multivariable logistic regression models were utilized to predict the probability of being either a case farm or a *Prototheca*-positive cow in a case farm.

Case and control farms were similar to each other in basic demographic parameters. Furthermore, because 82.3% of Ontario dairy farms have a herd size of <100 cows, the studied farms can be considered representative of the majority of Ontario dairy farms in terms of herd size. Both case and control farms had a lower milk production per cow than average DHI farms, which might be associated with the smaller average herd size of the study farms.

A *Prototheca* spp. herd-level prevalence of 5.1% from our study is similar to that described in the literature for

Table 8. Description (median; 25% and 75% quartiles in parentheses) of cow-level risk factors for a *Prototheca*-positive laboratory result in case farms

Parameter	*Prototheca*-positive cows (n = 61)	Other mastitis pathogen-positive cows (n = 131)	Culture-negative cows (n = 982)	*P*-value
Lactation number	3 (2–4)[a]	2 (1–4)[b]	2 (1–3)[c]	<0.001
Age of animal on test date, d	1,957 (1,375–2,418)[a]	1,642 (1,274–2,257)[a]	1,357 (1,027–1,835)[b]	<0.001
DIM	184 (108–236)	194 (95–277)	163 (89–258)	0.249
Test-day milk yield, kg	29 (23.8–35.5)	28.9 (23.1–28.7)	30.5 (24.8–37.0)	0.256
Test-day fat yield, kg	1.095 (0.931–1.326)	1.185 (0.956–1.423)	1.187 (0.976–1.395)	0.219
Test-day protein yield, kg	0.949 (0.802–1.098)	0.98 (0.848–1.181)	0.988 (0.836–1.157)	0.406
Test-day SCC, ×1,000 cells/mL	436 (244–747)[a]	259 (71–870)[a]	68.5 (33–158)[b]	<0.001
Lactation estimate of 305-d milk yield, kg	9,200 (8,529–10,561)[ab]	9,359 (8,274–10,618)[a]	9,064 (7,994–10,300)[b]	0.037
Lactation estimate of 305-d fat yield, kg	348 (307–393)	355 (313–399)	342 (302–389)	0.094
Lactation estimate of 305-d protein yield, kg	292 (260–329)[ab]	292 (264–333)[a]	283 (254–319)[b]	0.044
Lactation-average SCC, ×1,000 cells/mL	552 (318–944)[a]	336 (108–798)[b]	87 (44–212)[c]	<0.001
	(n = 56)	(n = 97)	(n = 669)	
Previous lactation number	2 (1–3.5)[a]	2 (1–3)[ab]	2 (1–3)[b]	0.009
Previous lactation-average SCC, ×1,000 cells/mL	260 (122–621)[a]	214 (82–530)[a]	98 (58–244)[b]	<0.001

[a–c]Values within a row with different superscript letters are different at $P < 0.05$.

Table 9. Odds ratio (OR), 95% CI, and significance level of the final logistic regression model for cow-level risk factors for *Prototheca*-positive laboratory results versus a culture-negative result (other mastitis pathogens excluded) in case farms

Parameter	OR	95% CI	*P*-value
Second lactation or greater	4.40	1.697; 11.399	0.002
Log-transformed lactation-average SCC	2.99	2.295; 3.896	<0.001

herds with endemic *Prototheca* mastitis (Baumgärtner, 1997; Bueno et al., 2006). Among those isolates that were further investigated, only *Prototheca zopfii* genotype 2 was identified. This supports the predominant role of *Prototheca zopfii* genotype 2 in the pathogenesis of bovine mastitis caused by *Prototheca* spp. (Ahrholdt and Roesler, 2011).

Five case farms did not yield *Prototheca*-positive milk culture results. However, we decided to include those farms as cases because they met the predefined case definition, based on a history of repeated *Prototheca* spp. mastitis cases within the last 2 yr. In this study, where the herds were only cultured once using composite milk samples, it is very likely to see one or more false-negative culture results based on the imperfect sensitivity of the test and the intermittent shedding of the organism. Furthermore, in small herds, such as those in Ontario, where very few cows are positive for *Prototheca* spp. at a given time, it is also likely that a *Prototheca*-infected cow had been culled for mastitis or other reasons in the period preceding the sampling day, and would therefore not appear in the culture results. Finally, because only lactating cows were sampled, *Prototheca*-positive cows that were dry at the time of the herd visit would not have been tested. In combination, all of these factors might have decreased the herd- and cow-level sensitivity of the culture results, and it is likely that the true prevalence of positive herds and cows was higher than detected in this study.

The mean within-herd prevalence of *Staphylococcus aureus* was considerably lower in case herds than in control herds. It might be that the veterinarians who selected the farms were biased in their choice and selected controls with poor performance in terms of udder health. This might have influenced the risk factor analyses and biased the estimates toward the null.

Several case reports about herds with outbreaks of *Prototheca* mastitis have described poor milking hygiene (lack of one or more of the following: prestripping, cleaning the udder prior to milking, disinfection of milking unit between cows, or postmilking teat dip) and wet, dirty, and muddy outdoor areas on those farms (Costa et al., 1996; Baumgärtner, 1997; Corbellini et al., 2001; Bueno et al., 2006). This case-control study is the first large-scale study that assessed farm-level risk factors objectively. Surprisingly, we did not find that specific farm characteristics, specific farm management, or milking hygiene deficiencies were risk factors for *Prototheca* mastitis on the case farms.

Baumgärtner (1997) and Hodges et al. (1985) reported problems with the milking equipment in farms where *Prototheca* outbreaks occurred. Most of the producers in this study were unable to provide the precise technical data for milking equipment function on their farm. Therefore, an association between milking equipment performance and *Prototheca* mastitis, if present, could not be detected in this study because of lack of appropriate data.

Other risk factors that have been mentioned in the literature were the extensive use of antibiotics, change of dry cow treatment, and unsanitary udder injections (Spalton, 1985; Corbellini et al., 2001; Jánosi et al., 2001). In the current study, we found that variables associated with intramammary treatment (internal teat sealant, ImINID) and NrInjectable were farm-level risk factors for *Prototheca* mastitis. As in any case-control study, this association alone does not conclusively prove a cause-effect relationship. Producers might have implemented these practices in an effort to control *Prototheca* mastitis in their herds. It remains unclear whether *Prototheca*-positive cows were treated with these drugs, because treatment records for individual cows were not available. However, considering that yeasts were only found on case farms, this likely suggests that *Prototheca* is, like yeast, an opportunistic pathogen. Furthermore, unsanitary udder injections might have introduced *Prototheca* spp. into the udder. Baumgärtner (1997) presumed that *Prototheca* infections are promoted by high infection pressure on affected farms together with antibiotic treatments that inhibited the competitive natural udder flora.

The final logistic regression model for farm-level risk factors included the variable $\geq$3 DA. To our knowledge, an association between displaced abomasum and mastitis caused by *Prototheca* has not been previously reported. A high number of displaced abomasums can be caused by factors such as poor ration formulation, poor feed hygiene and quality, or inadequate feed bunk management, all of which might be indicators of overall farm management and cleanliness.

Similar to the present study, Tenhagen et al. (1999) found that higher lactation number was a risk factor for

Prototheca mastitis. On the other hand, 25% of their *Prototheca*-positive cows were primiparous, whereas in our study, only 8.2% of affected cows were primiparous. The same study by Tenhagen et al. (1999) reported higher SCC during lactation in cows that were later affected by *Prototheca* mastitis. Those authors suspected that the disrupted integrity of the udder tissue acted as a *Prototheca* mastitis-promoting factor.

The logistic regression model for cow-level risk factors suggested that culturing milk samples for *Prototheca* spp. from multiparous cows and those with high SCC might be an efficient way to screen for infected cows in farms where *Prototheca* spp. has been confirmed previously. Nevertheless, we found several culture-positive cows with test-day SCC <100,000 cells/mL and some primiparous cows with *Prototheca*-positive laboratory results. It might be that those cows with low SCC became infected shortly after the DHI test day, or that a high SCC from the affected quarter was diluted in the composite DHI test sample due to agalactia caused by the *Prototheca* mastitis. On the other hand, SCC <100,000 cells/mL in cows with *Prototheca zopfii* infection have been reported in quarter and composite milk samples (Tenhagen et al., 2005; Bueno et al., 2006). As suggested (Rösler and Hensel, 2003; Bueno et al., 2006), eradication attempts should use repeated testing of the whole herd rather than testing only high-risk cows. Testing only the high-risk animals for *Prototheca* spp. might miss infected animals that could later perpetuate the infection in the herd. Furthermore, in the present study, composite milk samples, instead of quarter milk samples, were used for milk culture. This might have decreased the sensitivity of the laboratory culture results (Baumgärtner, 1997; Tenhagen et al., 2005) and biased the estimates of the cow-level parameters toward the null. It may be that *Prototheca* mastitis eradication attempts should use quarter milk samples.

The current work was a preliminary investigation. The sample size might have been too small and the data collection methods too general to detect differences in milking hygiene and farm management practices. The wide confidence intervals in the final herd-level model indicate uncertainty in the estimates because of the small sample size. Further research is needed to confirm the results, to investigate intramammary application hygiene, to objectively assess environmental hygiene, and to assess associations with milking equipment function. Moreover, in this study, prevalent instead of incident cases at the farm and cow levels were used. As such, a temporal pattern in support of a cause-effect relationship could not be established. Last, the questionnaire administration was by interview, and the individual asking the questions was not blinded with respect to the status of the herd. This approach might have introduced bias and increased the magnitude of the observed associations. Special training for questionnaire administration was not provided. Nevertheless, mainly multiple-choice questions asking for farm practices during a specific time with discrete answer categories were used, leaving little room for misclassification. Because the literature review that was used to develop the questionnaire revealed mainly anecdotal evidence of many different risk factors, the researchers did not know which of these risk factors would play a major role in the development of *Prototheca* mastitis on farm. Furthermore, a conscious effort was made to aim for neutral administration of questions during the interview.

CONCLUSIONS

Prototheca mastitis is an emerging disease that should be considered in the differential diagnosis of cases of mastitis that are nonresponsive to antibiotic treatment. Determination of mastitis-causing agents is necessary for effective therapy, timely culling decisions, and specific preventive interventions. A producer–veterinarian relationship should be established and treatment options discussed to avoid excessive, unsuccessful, and extra-label antibiotic use for mastitis. *Prototheca* spp. might act as an opportunistic pathogen and may be promoted by antibiotic-induced suppression of the natural udder flora.

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