Type 1 and type 2 immune response profiles of commercial dairy cows in 4 regions across Canada

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Abstract

Diseases of dairy cattle have adverse implications for both the dairy industry and animal welfare. Understanding adaptive immune response profiles of cattle on a national scale will provide insight into the potential for improving health and decreasing disease. The objectives of the present study were to evaluate immune response phenotypes of Holstein cows outside the peripartum period and to determine if antibody isotype bias to putative type 1 and type 2 test antigens is maintained. The cows, housed on commercial farms in 4 key dairy regions across Canada, were immunized with test antigens to measure their ability to mount cell-mediated immune responses (CMIR) and antibody-mediated immune responses (AMIR). Delayed-type hypersensitivity (DTH) was used as an indicator of CMIR and primary and secondary serum antibody of the immunoglobulin (Ig) G1 and IgG2 isotypes was used to determine AMIR to the test antigens. Immune response phenotypes varied significantly among regions, herds, and cows. Cows in Alberta had significantly higher DTH responses and secondary responses to the type 2 test antigen than those in other regions. However, cows in Alberta had significantly lower primary antibody responses. It was found that Alberta had the lowest incidence of mastitis caused by *Escherichia coli* and *Staphylococcus aureus* compared with other regions. The IgG1/IgG2 antibody isotype ratio confirmed the nature of the test antigens. This was the first study to evaluate adaptive immune response profiles and disease incidence of dairy cows on a national scale and it therefore provides a glimpse of the current situation in Canada.

Résumé

(Traduit par Docteur Serge Messier)

Introduction

Infectious diseases, including mastitis, cost the dairy industry billions of dollars a year and contribute to decreased animal health and welfare. In Canada, it has been estimated that a single case of mastitis costs between $110 to $320 and that at any given time, 1 in 5 quarters are infected with mastitis-causing pathogens (1). The immune system largely controls response to pathogenic challenge through innate and adaptive host defences. Significant variation in immune response traits has been observed among dairy cattle (2,3) and this has been correlated with disease (4,5). Breeding primarily for production traits has been associated with a rise in the incidence

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of disease (6) and it has been suggested that including immune response traits in breeding objectives could be a way to increase disease resistance (7–9). Immune response profiles of Canadian dairy cattle have never been evaluated on a national scale. Such an evaluation would provide further insight into the feasibility of breeding cattle for enhanced immunity.

Antibody-mediated immune responses (AMIR) and cell-mediated immune responses (CMIR) have been used as indicator traits of adaptive immune responses of livestock (10–13). The immune system generally responds to extracellular pathogens by mounting type 2 immune responses, which are typically characterized by production of antibody of a particular isotype, immunoglobulin (Ig) G1. The immune system, however, generally responds to intracellular pathogens by a type 1 immune response, typically characterized as CMIR and dominated by production of IgG2. Both CMIR and AMIR are essential for host protection against a variety of infectious diseases. Delayed-type hypersensitivity (DTH) is a commonly used indicator of CMIR or a type 1 response (14,15), whereas antibody to a test antigen is a common indicator of a type 2 response or AMIR. Cytokines also contribute to type 1 and type 2 immune response bias. In cattle, for example, IgG1 has been shown to be a type 2 isotype directed by production of cytokines such as IL-4, whereas IgG2 is a type 1 isotype influenced largely by IFN-γ (16). The isotypic bias as measured by the IgG1/IgG2 ratio has been used to determine the type of immune response elicited to test antigens (17–19), vaccine (20), or infection (21,22). Evaluating CMIR and AMIR, as well as Ig bias, is therefore an effective way to determine the immune response profiles on a cow, herd, and regional level.

A previous study by our group evaluated variation in AMIR to a type 2 antigen in 136 peripartum Holstein cows and heifers from 3 Ontario herds (23). A protocol was subsequently developed to simultaneously test the ability of cattle to mount both AMIR and CMIR, without interfering with commercial diagnostic tests (15). This protocol has been tested in multiple herds in Ontario (11,17,19,24) and on a commercial dairy in Florida (4) and has been proven to be a safe, effective, and reliable method for establishing immune response profiles. Since AMIR and CMIR had only been tested on a limited number of herds in 1 region, the goal of this research was to evaluate adaptive immune response traits on 58 commercial dairy operations for the first time on a national scale. The hypotheses tested were that AMIR and CMIR would vary significantly among cows, herds, and regions across Canada and that antibody isotype bias would be consistent with the type 1 or type 2 nature of the test antigens.

This study was part of a larger study carried out by the Canadian Bovine Mastitis Research Network (CBMRN), a collaborative research network established to decrease the incidence of mastitis, reduce financial losses, and maintain milk quality on Canadian dairy farms. The objectives of the present study were to evaluate immune response phenotypes of Holstein cows outside the peripartum period and housed on commercial farms in 4 key dairy regions across Canada and to determine whether antibody isotype bias to putative type 1 and type 2 test antigens is maintained in herds in each of these 4 regions. Data on clinical mastitis cases were available from the CBMRN and were analyzed for the cows enrolled in this immune response study.

Materials and methods

Animals

Six hundred and eighty Holsteins outside the peripartum period (+/− 4 wk from calving) were enrolled in this study through the CBMRN National Cohort of Dairy Farms (NCDF) (25). Between 10 and 15 cows per herd were enrolled from 58 herds across Canada (26). Cows in each of the 4 regions were evaluated for AMIR and CMIR, with 189 cows from 16 herds in Alberta, 173 cows from 14 herds in Ontario, 117 cows from 12 herds in Quebec, and 201 cows from 16 herds in the Atlantic provinces (New Brunswick, Nova Scotia, and Prince Edward Island). Distribution of stage of lactation was as follows: n = 100 in the first stage [29 to 80 days in milk (DIM)], n = 375 in the second stage (81 to 235 DIM), and n = 205 in the third stage (> 235 DIM).

Immunization protocol

Cows at least 28 DIM were immunized to induce CMIR and AMIR outside the peripartum period. Delayed-type hypersensitivity (DTH) to *Candida albicans*, a putative type 1 test antigen (19), was used as an indicator of CMIR (15). Primary and secondary antibody production to a putative type 2 test antigen, hen egg white lysozyme (HEWL) (19), was used as an indicator of AMIR (23,24). On days 0 and 14, cows received an intramuscular (IM) injection of 0.5 mg of the type 2 test antigen HEWL (Sigma-Aldrich Canada, Oakville, Ontario), 0.5 mg of the type 1 test antigen *C. albicans* (Greer Laboratories, Lenoir, North Carolina, USA), and 0.5 mg of Quil-A adjuvant (Cedarlane Laboratories, Hornby, Ontario) dissolved in 1 mL of phosphate buffer saline (PBS, pH 7.4). Using a 22-ga needle, a 1.0-mL injection was divided and administered intramuscularly on both sides of the neck or rump.

Delayed-type hypersensitivity

To determine the magnitude of DTH, skinfold thickness measurements were taken using spring-loaded callipers (Harpenden Skinfold Calliper; Creative Health Products, Ann Arbor, Michigan, USA) in response to the type 1 test antigen and a PBS control (15,24). Skin thickness measurements were recorded in triplicate at day 21 of the immunization schedule as a baseline measurement (0 h). An intradermal injection of the type 1 test antigen *C. albicans* (0.1 mg dissolved in 0.1 mL of PBS) was then given on the right side of the tail fold and 100 μL of a PBS (control) on the left side using a 28-ga needle. On day 23 (48 h), triplicate skin thickness measurements were repeated at the test and control sites. To determine the increase in skinfold thickness, the log ratio at 48 h of the test site/control site was the response variable, with the log ratio at day 0 fit as a covariate (19).

Serum antibody

Blood was taken at days 0, 14, and 21 to extract sera by centrifugation (700 *g*, 15 min). Sera were stored at −20°C until AMIR to the type 1 and 2 test antigens were evaluated. Serum antibody was determined by a modified enzyme-linked immunosorbent assay (ELISA) (11). The negative control was fetal calf serum (FCS) and the positive control was pooled from day 21 sera after 2 immunizations. Flat-bottomed, 96-well polystyrene plates were coated with 25 μg

Table I. Probabilities of significance for the effects of region, technician, housing, age, and stage of lactation on delayed-type hypersensitivity (DTH) to a type 1 test antigen in Holstein cows from commercial dairy herds across Canada

	Factor						
Time	Region	Technician	Housing	Age	Age * Age	Stage of lactation	0 hours
0 hour	0.3505	0.2021	0.3994	0.4175	0.5428	0.5840	NA
48 hours	< 0.0001	0.5380	0.4242	0.0982	0.1856	0.0018	< 0.0001

NA — factor was not fitted. Significant effects ($P \leq 0.05$) are highlighted.

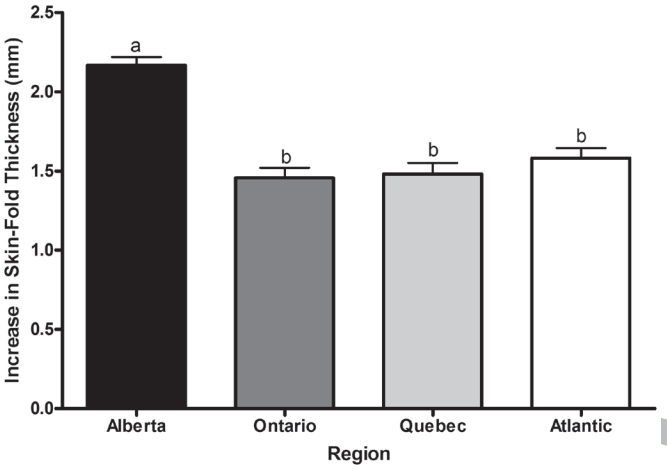

Figure 1. Delayed-type hypersensitivity (DTH) to the type 1 test antigen 48 h after intra-dermal injection in 680 lactating Holstein cows from 4 regions across Canada. Cows were immunized intramuscularly with 0.5 mg of a type 1 and 0.5 mg of a type 2 test antigen emulsified in 0.5 mg of Quil-A adjuvant on days 0 and 14. On day 21, cows received an intradermal injection of 0.1 mg of type 1 test antigen in 0.1 mL of phosphate buffer saline (PBS) on the right tail fold and PBS control on the left tail fold. Double skinfold thickness measurements were taken at 0 h (day 21 before immunization) and 48 h post-intradermal injection using spring-loaded callipers to calculate increase in skinfold thickness as a measure of DTH. Least squares means are reported by region and error bars indicate standard error. Significant differences are reported as $P \leq 0.05$. Different letters indicate significant differences across regions.

of candin (*C. albicans* purified protein extract, Greer Laboratories) or 0.144 mg of HEWL (Sigma-Aldrich Canada) dissolved in 1 mL of carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C for 24 h. Plates were washed with PBS and 0.05% Tween 20 (Sigma-Aldrich Canada) (wash buffer pH 7.4) 3 times and blocked with PBS, 3% Tween 20, 1.5% bovine serum albumin (BSA), and 1.5% FCS for 1 h at room temperature (RT), then washed again 3 times. Control sera were applied in quadruplicate and test sera in duplicate in a quadrant pattern. Sera were diluted in wash buffer at 1/50 and 1/200 for HEWL and 1/200 and 1/400 for candin and incubated for 2 h at RT. Plates were washed 5 times and secondary antibody, sheep anti-bovine IgG1 or IgG2 were conjugated to alkaline phosphatase (Bethyl Laboratories, Montgomery, Texas, USA) was dissolved in Tris-Tween buffer with 0.05% Tween 20 (pH 7.4) and incubated at RT for 1 h. All wash steps were performed with the Elx405 Auto Plate Washer (BioTek Instruments, Winooski, Vermont, USA). p-Nitrophenyl phosphate substrate system (Sigma-Aldrich Canada) was added and incubated for approximately 50 min at RT and optical density (OD) values at 405 nm were obtained using the EL808 Plate Reader (BioTek Instruments). Data were collected with KC Junior Software (BioTek Instruments, Vermont, USA). A correction factor was calculated from the rolling mean of positive control and applied to all data, as described in a previous study (11). The dilutions of the corrected OD values were summed and duplicates were averaged for statistical analysis.

Incidence of mastitis

Milk samples were taken as described previously for the NCDF herds (25). Briefly, farmers sampled cows identified as having abnormal milk or clinical signs of mastitis. A single sample was taken on the day of diagnosis and subsequent samples were taken 2 to 3 wk and 4 to 5 wk later. Samples were frozen for storage at −20°C and submitted to 1 of 4 bacteriology laboratories. A standardized protocol was followed, based on National Mastitis Council guidelines for bacteriology culture and species identification. Data on the number of colonies and species isolated were obtained from the CBMRN database for use in this study. Only the first milk sample for each case was considered for assessment of bacteriology data.

Isolation of *Staphylococcus aureus* and *Streptococcus agalactiae* was considered to cause an intramammary infection if 100 cfu/mL was isolated. If ≥ 200 colony-forming units (cfu)/mL of *E. coli*, *Streptococcus* spp (other than *S. agalactiae*), *Serratia* spp, *Citrobacter* spp, *Proteus* spp, *Salmonella* spp, *Pseudomonas* spp, *Pasteurella multocida* spp, *Klebsiella* spp, *Staphylococcus hyicus*, *Prototheca* spp, *Arcanobacterium pyogenes*, non *S. aureus* coagulase-positive *Staphylococcus*, *Enterobacter* or ≥ 1000 cfu/mL of *Corynebacterium bovis*, coagulase-negative staphylococci (CNS), yeasts, fungi, and *Bacillus* spp were isolated, the cow was considered positive for an intramammary infection (27). Samples were deemed contaminated if more than 3 species were isolated, unless *S. aureus* or *S. agalactiae* were identified, in which case they were enumerated (25).

All cases of mastitis in any quarter were considered, regardless of culture result. A new case in the same quarter was considered if 14 d had passed since the previous case (27). Cows were at risk for the entire 2-year period of NCDF sampling. Days at risk for each cow were calculated as the total number of days in milk from the start to end of the 2-year period based on information available through the CBMRN. Time at risk ended if the cow was either removed from the herd or was not milking. The incidence rate was calculated as the number of cases of mastitis for each cow per 36 500 days at risk (100 cow-years).

Table II. Probabilities of significance for the effects of region, technician, housing, age, and stage of lactation and time on serum antibody responses to a type 1 and type 2 test antigen in Holstein cows from commercial dairy herds across Canada

	Factor							
Response	Region	Technician	Housing	Age	Age * Age	Stage of lactation	Time	Region * Time
IgG1 (type 2 antigen)	0.3344	0.3720	0.8904	0.7850	0.1216	0.3537	< 0.0001	< 0.0001
IgG2 (type 2 antigen)	0.0969	0.0017	0.5562	0.3066	0.4167	0.1391	< 0.0001	< 0.0001
IgG1 (type 1 antigen)	0.0002	0.0901	0.6674	0.8777	0.3084	0.3203	< 0.0001	< 0.0001
IgG2 (type 1 antigen)	0.0004	0.2667	0.0149	0.4203	0.2833	0.0274	< 0.0001	< 0.0001
IgG1/IgG2 (type 2 antigen)	0.0219	0.0025	0.0830	0.8249	0.7421	0.5617	0.0479	0.0787
IgG1/IgG2 (type 1 antigen)	0.0010	0.0005	0.0843	0.012	0.2292	0.1166	< 0.0001	< 0.0001

Significant effects ($P \leq 0.05$) are highlighted.

Statistical analysis

Data for each measure of immune response (DTH, serum antibody, and antibody isotype ratio) were analyzed independently with a linear mixed model using PROC MIXED (SAS Version 9.1.3; SAS Institute, Cary, North Carolina, USA), which included repeated measures. The structure of the variance-covariance matrix of repeated measures was chosen based on the lowest value Akaike information criterion (AICC) (24). Covariance parameters were estimated using restricted maximum likelihood (REML). Residual analysis was conducted to test the assumptions of analysis of variance (ANOVA) using PROC UNIVARIATE (SAS Version 9.1.3). Normality was tested using the Shapiro-Wilk statistic. Residuals were plotted against explanatory variables to determine outliers in the data and the need for data transformation (19). The statistical model used was:

$$y = X\beta + Z\gamma + \varepsilon_{ijk}$$

where: y = response vector of the observation; X = fixed effects of coefficients including region, time, technician, housing, stage of lactation, and age (linear and quadratic) (and their interactions); β = the vector of fixed coefficients to be estimated; Z = model matrix for random effects for the observations including herd; γ = vector of random-effect coefficients; and ε = vector of errors for the observations.

Significant probability values were reported as $P \leq 0.05$. Interactions were tested and nonsignificant ($P > 0.1$) interactions were removed from the model, preserving hierarchy at all times. Data that were not normally distributed were transformed to the natural logarithm and non-transformed data were used for graphs. An unbiased correction factor was added where required for transformation. Least squares means for DTH, antibody, and antibody-isotype ratio were estimated and Tukey's test was used to compare contrasts.

The association of incidence rate of clinical mastitis with region was analyzed by Poisson regression using PROC GLIMMIX (SAS Version 9.1.3). The natural logarithm of the number of days at risk was the offset and herd was fit as a random effect. Least squares means were estimated and Tukey's test was used to test differences among provinces.

Results

Delayed-type hypersensitivity

Region, stage of lactation, and baseline response (0 h) had significant effects on DTH at 48 h, although no variables had an effect on the baseline response, 0 h (Table I). Cows in Alberta had the largest increase in skinfold thickness compared to the other regions (Figure 1). Cows in the first stage of lactation had significantly lower DTH responses than other cows.

Serum IgG1 and IgG2 to the type 2 antigen

Primary (day 14) and secondary (day 21) IgG1 and IgG2 antibody responses to the type 2 antigen were significantly greater than at day 0 ($P < 0.0001$) and secondary responses were significantly higher ($P < 0.0001$) than primary responses in all regions. Region, technician, and age had significant effects on IgG1 and IgG2 responses at certain time points, whereas stage of lactation was found to have no effect (Table II). There were significant differences in baseline (day 0) IgG2 antibody responses among provinces, but no difference in IgG1 antibody (Figure 2). Cows in Alberta had significantly lower primary (day 14) antibody responses, but significantly higher secondary response (day 21) for both IgG1 and IgG2 than cows in other regions (Figure 2).

Serum IgG1 and IgG2 to the type 1 antigen

Primary (day 14) and secondary (day 21) IgG1 and IgG2 antibody responses to the type 1 antigen were significantly greater than at day 0 ($P < 0.0001$) and secondary responses were significantly higher ($P < 0.0001$) than primary responses in all regions. The region had significant effects on IgG1 and IgG2 antibody responses (Table II). Baseline (day 0) antibody (IgG1 and IgG2) was lowest in Alberta and the Atlantic provinces (Figure 3). Cows in Alberta and the Atlantic provinces also had the lowest primary and secondary IgG2 response to the type 1 antigen (Figure 3b), but only cows in Alberta had the lowest primary IgG1 antibody response to the type 1 test antigen (Figure 3a). Quebec had the highest primary IgG2 antibody response (Figure 3b).

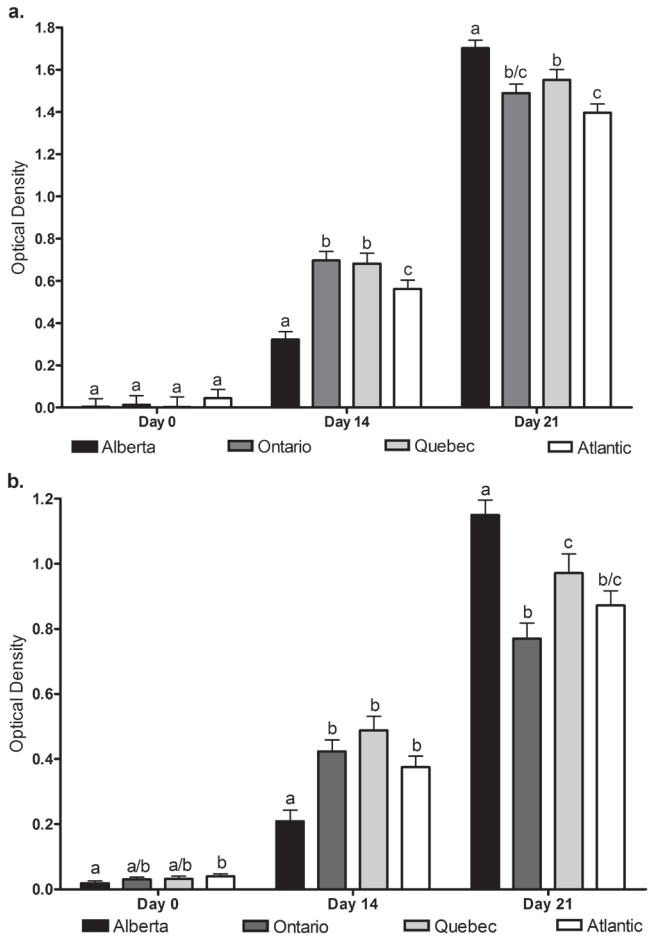

Figure 2 a-b. Serum IgG1 and IgG2 antibody response to the type 2 test antigen at days 0, 14, and 21 in 680 lactating Holstein cows from 4 regions across Canada. Cows were immunized intramuscularly with 0.5 mg of a type 1 and 0.5 mg of a type 2 test antigen emulsified in 0.5 mg of Quil-A adjuvant on days 0 and 14. Sera were collected at days 0, 14, and 21 to measure antibody to the type 2 test antigen by enzyme-linked immunosorbent assay (ELISA). Figure 2a shows IgG1 antibody response and Figure 2b shows IgG2 antibody. Least squares means were reported by region and error bars indicate standard error. Significant differences are reported as $P \leq 0.05$. Different letters indicate significant differences across regions within a time point.

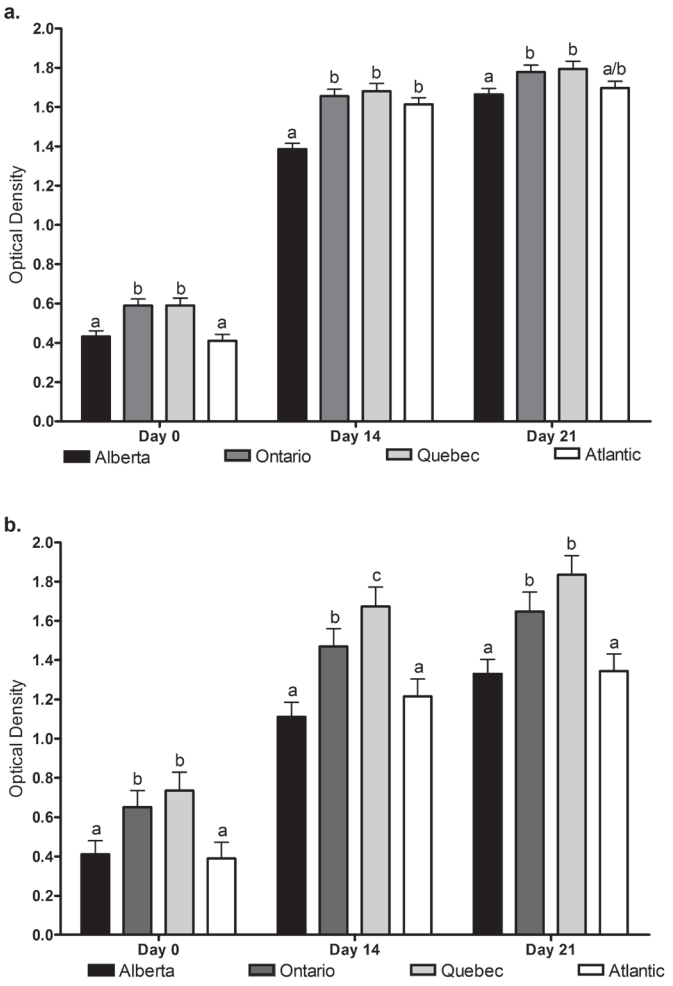

Figure 3 a-b. Serum IgG1 and IgG2 antibody response to the type 1 test antigen at days 0, 14, and 21 in 680 lactating Holstein cows in 4 regions across Canada. Cows were immunized intramuscularly with 0.5 mg of a type 1 and 0.5 mg of a type 2 test antigen emulsified in 0.5 mg of Quil-A adjuvant on days 0 and 14. Serum antibody was measured at days 0, 14, and 21 by enzyme-linked immunosorbent assay (ELISA). Figure 3a shows IgG1 antibody response and Figure 3b shows IgG2 antibody. Least squares means were reported by region and error bars indicate standard error. Significant differences are reported as $P \leq 0.05$. Different letters indicate significant differences across regions within a time point.

IgG1/IgG2 ratio to type 2 and type 1 test antigens

The IgG1/IgG2 ratio of antibody response to the type 2 and type 1 test antigens, respectively, at days 0, 14, and 21 within each region is shown in Figure 4. Region, technician, and time had significant effects on antibody ratio to the type 2 antigen (Table II, Figure 4a). In Ontario, there was a significantly higher ratio of IgG1/IgG2 (indicative of a type 2 response) to the type 2 test antigen at all time points than in other regions. Region, technician, age, time, and the interaction of time by region (time*region), and region by age (region*age) ($P = 0.0093$) significantly influenced the IgG1/IgG2 ratio to the type 1 test antigen (Table II, Figure 4b). In most regions, the ratio of IgG1/IgG2 to the type 1 test antigen decreased significantly over time, indicative of a type 1 immune response (Figure 4b). Cows in the Atlantic provinces had the highest relative ratio to the type 1 test antigen at day 0 (Figure 4b).

Incidence rate of clinical mastitis

Differences in pathogens and the overall incidence rate of clinical mastitis (IRCM) are presented in Table III. Only the incidence rates for *E. coli*, *S. aureus*, and *Streptococcus* spp. are reported due to insufficient data for other pathogens. The IRCM was 24.89 cases per 100 cow-years for all cows across Canada. There were no significant differences in the overall IRCM among regions. The incidence of *E. coli* mastitis was significantly lower in Alberta than in Ontario and the Atlantic provinces, however, and there was significantly less *S. aureus* mastitis in Alberta than in Ontario. Overall, the IRCM associated with environmental pathogens was significantly higher in Ontario than in Alberta and the Atlantic provinces.

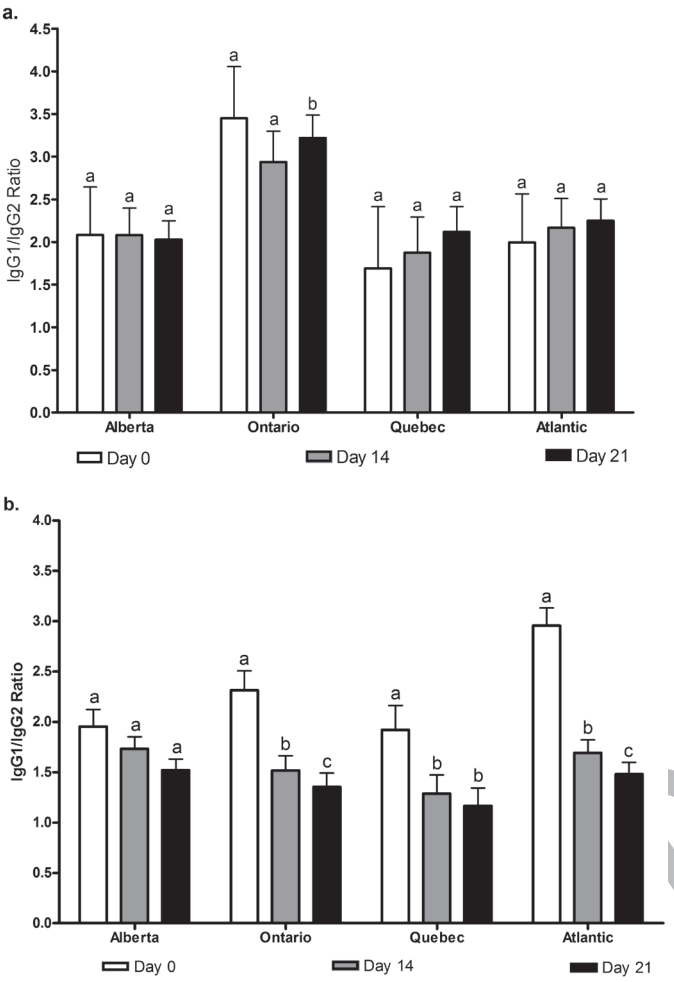

Figure 4 a-b. Serum IgG1/IgG2 antibody ratio to the type 1 and type 2 test antigens at days 0, 14, and 21 in 680 lactating Holstein cows in 4 regions across Canada. Cows were immunized intramuscularly with 0.5 mg of a type 1 and 0.5 mg of a type 2 test antigen emulsified in 0.5 mg of Quil-A adjuvant on days 0 and 14. Serum antibody was measured at days 0, 14, and 21 by enzyme-linked immunosorbent assay (ELISA). Figure 4a shows IgG1/IgG2 ratio to the type 2 test antigen at days 0, 14, and 21 and Figure 4b shows the antibody isotype ratios to the type 1 test antigen. Least squares means are reported by region and error bars indicate standard error. Significant differences are reported as $P \leq 0.05$. Different letters indicate significant differences between time points within a region.

Discussion

This study was the first to evaluate adaptive immune responses to putative type 1 and type 2 test antigens of dairy cows in the 4 main dairy regions across Canada. Type 1 and type 2 immune responses, or more generally CMIR and AMIR respectively, are required to protect dairy cattle from a wide variety of pathogenic challenges. Cell-mediated immune responses (CMIR) and AMIR were measurable in all herds tested across Canada, which indicates that the immunization regimen was efficacious. Importantly, there were significant differences in immune response among cows, herds, and regions. Previously, studies have looked at adaptive immune response traits in several herds, but only in Ontario (17,19,23,24). Other studies have looked at variation in serum antibody to parasites, such as *Dicrocoelium dendriticum* in particular regions of Canada such as Western Canada (28) or *Ostertagia ostertagi* across multiple provinces (29). Regional differences were reported in both cases. Also, seropositivity to bovine leukemia virus, bovine viral-diarrhea virus, *Mycobacterium avium paratuberculosis* (MAP), and *Neospora caninum* have been evaluated both within provinces (30) and across Canada (31). These studies provided an indication of pathogen prevalence, however, rather than immune responsiveness. Specific immune response profiles are important in order to determine the cows' ability to respond to pathogenic challenge and their potential susceptibility or resistance to disease.

In the current study, immune response varied significantly by region. Cows in Alberta had significantly higher CMIR, as measured as DTH, to the type 1 test antigen. This suggests that cows in Alberta are able to mount a stronger type 1 immune response than cows in other regions. Since type 1 immune responses are particularly important in controlling intracellular organisms, this finding may indicate an inherently enhanced ability to respond to intracellular pathogens. Cows in Alberta had the lowest incidence rate of mastitis caused by *S. aureus*, which as reported previously can survive intracellularly as small colony variants (SCVs) in dairy cattle (32). This suggests that cows in Alberta may be better able to control *S. aureus* mastitis, including SCVs, thus minimizing persistent infection with this pathogen. This may contribute to the lower incidence rate of clinical mastitis caused by *S. aureus*. Although cows in Alberta had significantly lower primary antibody responses to the type 2 test antigen, they had significantly higher secondary antibody responses than cows in other regions. Antibody-mediated or type 2 immune responses are important in controlling extracellular pathogens. Since it was found that cows in Alberta had the lowest incidence of mastitis caused by *E. coli* and *S. aureus*, it can be hypothesized that their higher type 2 immune responses translate to an inherently enhanced ability to respond immunologically to control challenge from these pathogens.

Cows in Alberta and the Atlantic provinces had the lowest basal antibody (day 0) to the type 1 test antigen. Even though this antigen tends to be ubiquitous, cows in Alberta and the Atlantic provinces appear to have less environmental exposure to *C. albicans* than cows in Ontario and Quebec. Cows in Alberta and the Atlantic provinces maintained the lowest IgG2 antibody to the type 1 antigen at days 14 and 21. Cows in the Atlantic provinces, however, had similar IgG1 antibody on days 14 and 21 to those in other regions, which indicates that they responded to the immunization in a similar manner regardless of previous exposure.

Cows in Quebec had the highest primary IgG2 antibody responses to the type 1 antigen. In general, IgG2 predominates in response to type 1 antigens (16). Since cows in Quebec had the highest relative IgG2 response to the type 1 antigen, it could be hypothesized that Quebec would also have the highest CMIR (type 1 response), but this was found not to be true. Other studies have shown that bovine IgG2 is a type 1 antibody isotype that has a positive correlation with other type 1 immune responses such as DTH, while IgG1 has a negative correlation with CMIR traits (11,16). At the genetic level, it has been shown that AMIR and CMIR are either genetically independent traits (33) or are negatively correlated (9,12). It is therefore worth noting that cows in Alberta had enhanced DTH but diminished antibody

Table III. Incidence rate of clinical mastitis (IRCM) per 100 cow-years for 680 Holsteins from 58 dairy farms in 4 regions across Canada

	Region				
Pathogen	Alberta (n = 189)	Ontario (n = 173)	Quebec (n = 117)	Atlantic (n = 201)	All Cows (n = 680)
Escherichia coli	1.08[a,b]	5.99[a]	3.64	3.87[b]	3.66
Staphylococcus aureus	1.08[a]	2.82	5.20[a]	2.91	2.82
Streptococcus spp.	1.79	4.93	1.56	1.29	2.44
Culture negative	7.17	4.93	4.16	5.49	5.54
Contagious[d]	2.15	3.52	5.20	5.16	3.94
Environmental[e]	8.97[b]	16.56[b,c]	13.52	9.04[c]	11.83
Overall IRCM	20.80	28.20	27.04	24.21	24.89

[a] IRCM on the same row having a common superscript differ ($P \leq 0.05$).

[b,c] IRCM on the same row having a common superscript differ ($0.05 P \leq 0.10$).

[d] Contagious pathogens were *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus uberis*.

[e] Environmental pathogens were *Escherichia coli*, *Streptococcus* spp. (other than *S. agalactiae*), *Serratia* spp., *Citrobacter* spp, *Proteus* spp, *Salmonella* spp., *Pseudomonas* spp., *Pasteurella multocida*, *Corynebacterium* spp., yeasts, fungi, *Klebsiella* spp.., *Staphylococcus hyicus*, *Prototheca* spp., coagulase-negative *Staphylococcus*, *Arcanobacterium pyogenes*, *Bacillus* spp., non-*S. aureus* coagulase-positive *Staphylococcus*, and *Enterobacter.*

responses to the type 1 antigen at day 14, which supports evidence that these traits may be negatively correlated.

Measuring antibody isotypic bias by evaluating the IgG1/IgG2 ratio over time provides an indication of the type of immune response elicited and can relate to resistance or susceptibility to disease (18). A relative increase in the IgG1/IgG2 antibody ratio would be expected to a type 2 antigen, whereas a decrease would be expected to a type 1 antigen (16). Results of the current study show that the IgG1/IgG2 ratio to the type 1 antigen decreased over time in most regions, which indicates a relative increase in IgG2 production. This further confirms the type 1 nature of this test antigen, *C. albicans*, as previously reported for Holstein cows in University of Guelph research herds (19). Additionally, the IgG1/IgG2 ratio to the type 2 test antigen was maintained over time in most regions, indicating a predominance of IgG1 and therefore confirming HEWL as a putative type 2 antigen. It is noteworthy that these ratios are relative over time, since absolute concentrations of IgG1 and IgG2 antibody are not generally quantified in many livestock species (34).

The variability in the adaptive immune response of the herds across Canada may be correlated with their health status. Of note, regional differences were found in the incidence of clinical mastitis for the cows enrolled in the immune response study, which have been demonstrated previously (27). Cows in Alberta appeared to have the lowest incidence rate of clinical mastitis, although this was not statistically significant. This may be due to the small number of animals used to estimate incidence rates in this study compared to other studies. In Alberta, however, cows had significantly lower incidence of mastitis caused by *E. coli* and *S. aureus* than in other regions. Since cows in Alberta had higher immune responses than cows in other regions, they are likely better immunologically equipped to respond to pathogenic challenge. This demonstrates the potential for cows with higher or inherently enhanced immune responses to get sick less often and save the farmer money. Immune response is a measureable trait that could be used to enhance health through breeding, which may decrease the incidence and impact of disease in the dairy industry (4).

Differences in management practices, such as housing, on dairy farms across Canada may contribute to the variability in immune response found in this study. The CBMRN National Cohort of Dairy Farm herds were enrolled in the current study so that housing would be representative for each region (housing types were to be within 15% of their respective free-stall proportions in Canada). Alberta had the highest proportion of free-stall herds (75.0% or 12/16 herds), followed by the Atlantic provinces (43.8% or 7/16 herds), Ontario (14.3% or 2/14 herds), and then Quebec (8% or 1/12 herds). It has been previously shown that type of housing is associated with the incidence of mastitis pathogens (27), fertility, calving intervals, incidence of teat injuries, ketosis, indigestions, cystic ovaries (35), and stall hygiene (36). Other management factors such as diet and nutritional supplementation may also influence immune response. It has been shown that supplementing the diet with chelated chromium significantly affects the immune function of dairy cattle (37) and selenium deficiency has been correlated with udder health (38).

Environmental factors that cannot be accounted for, including climate and mineral deficiencies in soil and water, could indirectly influence immune response profiles of dairy cattle. These factors directly relate to the survivability of pathogens or other organisms in the environment (30) and infection may cause polarized or suppressed immune responses (16). For example, infection with *Mycobacterium avium paratuberculosis* (MAP) could cause a polarized immune system. As MAP is an intracellular or type 1 pathogen, infection usually persists in the subclinical state for several years. Infection has been shown to influence the number of T and B cells in the lymph nodes (39) and the absolute number of T and B cells would directly impact the ability to respond to challenge. Information on infection status other than mastitis was not available for the cows in this study, but it is suspected that concurrent infections could influence the regional immune response profiles.

A variety of similar factors are likely contributing to differences in regional immune responses in the current study but many of these factors could not be characterized here.

Substantial geographic variation in the distribution of mastitis pathogens across Canada has been demonstrated. In 2008, Olde Riekerink et al (27) found that the incidence rates of clinical mastitis caused by *S. aureus*, *Streptococcus uberis*, and *Streptococcus dysgalactiae (S. dysgalactiae)* were lowest in the western provinces and mastitis caused by *S. aureus* and *S. dysgalactiae* was highest in Quebec. Pathogens cause distinct types of mastitis and stimulate different immune responses, which may explain some of the variability in immune response among regions. A particular pathogen can encourage diverse mechanisms to survive within the host, such as *S. aureus*, which is generally an extracellular bacteria that can persist intracellularly as a small colony variant (SCV) (32). Latent intracellular infection with either *S. aureus* or MAP may influence the host immune system and pathogen prevalence may exert selective pressure on the immune system causing cows to adapt an optimal regional immune response profile. These factors could influence the magnitude of immune response bias to type 1 or type 2 antigens and are worthy of further exploration.

Management factors and pathogen prevalence are merely speculative reasons for the regional differences in the immune response of dairy cattle across Canada. The variations in individual cows seen in this study are probably due to the complex interaction between genetics and the environment. The interplay between these factors is crucial in determining immune response bias, as well as resistance or susceptibility to disease.

In conclusion, this study demonstrates that it is possible to measure adaptive immune responses in multiple commercial dairy herds on a national scale using the University of Guelph patented test system (US Patent #7 258 858). Significant variation was found among cows, herds, and regions and the study provides a glimpse of immune response profiles of Holstein dairy cows in Canada today. Relative immune response bias to putative type 1 and type 2 test antigens was also confirmed. It was found that cows in Alberta have higher type 1 and type 2 immune responses and a lower incidence of mastitis caused by *E. coli* and *S. aureus* than cows in other regions. This may indicate that the cows in this region have an inherently enhanced ability to respond to pathogenic challenge and to control disease compared with cows in other regions. It is likely that cows in each region have adapted optimal immune responses due to individual gene by environment interactions that related to the continually changing antigenic challenges to which they are exposed.

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