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Population-Based Case Control Study of Seroprevalence of *Mycobacterium paratuberculosis* in Patients with Crohn's Disease and Ulcerative Colitis

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There is renewed enthusiasm for exploring the possibility that *Mycobacterium paratuberculosis* may be causative in Crohn's disease (CD). We aimed to determine whether CD subjects are more likely to be *M. paratuberculosis* seropositive than controls. Using our population-based University of Manitoba Inflammatory Bowel Disease Research Registry, we recruited CD and ulcerative colitis (UC) subjects between 18 and 50 years of age for a study involving detailed questionnaires and venipuncture. We accessed the population-based databases of Manitoba Health (single provincial health insurer) to get age-, gender-, and geography-matched controls to our inflammatory bowel disease (IBD) population. We asked enrolling IBD subjects for potential nonaffected sibling controls. We used an enzyme-linked immunosorbent assay (ELISA) for serum antibodies to *M. paratuberculosis* initially developed for cattle but adapted for human use. The rate of positive ELISA results, based on previously published interpretation criteria, was significantly higher for all study groups. There was no difference in *M. paratuberculosis* seropositivity rate among CD patients (37.8%; $n = 283$), UC patients (34.7%; $n = 144$), healthy controls (33.6%; $n = 402$), and nonaffected siblings (34.1%; $n = 138$). For siblings, there was no correlation between *M. paratuberculosis* serological status and that of the corresponding IBD affected sibling. None of the demographic or questionnaire variables studied were predictive of *M. paratuberculosis* status. Subjects with CD and UC were less likely to have ingested unpasteurized milk and less likely to have had a non-tap water source as a primary water source. In conclusion, in this population-based case control study, the *M. paratuberculosis* seropositivity rate was approximately 35% for all groups and there was no difference in rates between CD patients, UC patients, healthy controls, or nonaffected siblings. The much higher rate of seropositivity for subjects from Manitoba, Canada, than for those from Denmark or Wisconsin cannot be obviously explained. While these data seem to refute any association of CD with *M. paratuberculosis*, the high seroprevalence in Manitobans raises the possibility that the high rates of CD in Manitoba could be related to high exposure rates for *M. paratuberculosis*. Hence, the possibility of an association between *M. paratuberculosis* and CD remains inconclusive.

In the early part of this century, the similarities between segmental human intestinal disease and granulomatous disease in cattle were identified (14). In the latter part of this century, with the advent of a modern understanding of the clinical manifestations of Crohn's disease, parallels were drawn between Crohn's disease and mycobacterial diseases of both humans and animals. *Mycobacterium paratuberculosis* causes a disease in cattle known as Johne's disease, a granulomatous inflammatory enteritis associated with diarrhea, wasting, and a predilection for the ileum, sharing many similarities with human Crohn's disease (13, 37, 41).

It is rational to consider that an infectious etiology for Crohn's disease might be acquired through ingestion of foodstuffs. Furthermore, the epidemiology of the disease, which includes rising incidence rates in Western societies concurrent with low rates in developing nations over the second half of the 20th century and high rates among immigrants to Western societies, is consistent with the possibility that a critical infection may be acquired from cattle or other farm animals via milk or meat ingestion, staples of Western diets, and cause Crohn's disease in subjects with the appropriate genetic predisposition (2).

A survey of dairy operations with at least 30 milk cows involving 20 states (representing 79.4% of the dairy population) across the United States found evidence of *M. paratuberculosis* infection in 22% of all dairy herds and 8% of beef herds (13). However, more than half of the infected herds had only one infected cow (30). A survey of 14,932 cows in 304 randomly selected dairy herds in Ontario, Canada, found that 6.1% of tested animals were seropositive for *M. paratuberculosis* by enzyme-linked immunosorbent assay (ELISA) (23). Of all cows presented to the abattoirs for slaughter, 5.5% had *M. paratuberculosis* isolated from their tissue (23).

The search for *M. paratuberculosis* by molecular techniques in colon tissue has produced variable results and about as many positive studies as negative ones (1, 4–7, 9, 15–17, 25, 26, 31, 33, 35, 38, 40, 42). If an organism such as *M. paratuberculosis* is

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ingested at a young age and sets up the immunological machinery to create a chronic intestinal inflammatory disease but cannot consistently be found in the mucosa years after ingestion, then it is plausible that evidence for past infection may reside in deeper layers of the gut or mesenteric lymph nodes, or may simply be measured by serological response. Recently *M. paratuberculosis* has been identified by in situ hybridization to the *M. paratuberculosis*-specific IS*900* gene in tissue specimens of Crohn's disease (36) and in 40% of Crohn's disease granulomas isolated from surgical specimens by laser capture microdissection (34). Others have localized *M. paratuberculosis* by PCR to macrophages and myofibroblasts within the lamina propria (21).

A serological assay has been developed to measure *M. paratuberculosis* in cattle. The sensitivity of the assay is 45%, and the specificity is 99% and is related to *M. paratuberculosis* burdens (39). This assay has been adapted for human testing (11). In view of the variable sensitivity in animals, it is critical to have an appropriate control group when assessing a human population's *M. paratuberculosis* seroprevalence. This assay was chosen for this study, since it has previously been validated with humans (11, 12). While *M. paratuberculosis* positivity with this assay cannot unequivocally prove past infection, it is highly suggestive. The positive control used in this assay was a human inoculated with *M. paratuberculosis* who had a strongly positive reaction at the inoculation site. Furthermore, two other groups have been tested with higher exposure likelihoods than healthy controls, and their ELISA results were significantly higher than those of the healthy controls (12).

In Manitoba, a population-based database of inflammatory bowel disease (IBD) has been developed, and the reported incidence of Crohn's disease (15 patients/10^5 people/year) is among the highest in the published literature (3). The agricultural economy of rural Manitoba and the production of locally consumed beef, dairy, and other animal products enhance the relevance of pursuing *M. paratuberculosis* as a potential etiology for Crohn's disease in Manitoba. Using the population-based University of Manitoba Inflammatory Bowel Disease Research Registry, we conducted a population-based case control study to examine the association between serological evidence of *M. paratuberculosis* infection and Crohn's disease. The following three control groups were used: persons without IBD, persons with ulcerative colitis, and unaffected siblings of persons with Crohn's disease or ulcerative colitis.

MATERIALS AND METHODS

Study design. A population-based case control study was undertaken. Cases were drawn from the University of Manitoba Inflammatory Bowel Disease Research Registry, which has been described previously (3). Briefly, this registry of persons with IBD is based on recruiting subjects through the administrative database of Manitoba Health, which provides comprehensive health care insurance to all residents. Individuals with at least two physician contacts (or at least one if a resident for less than 2 years) were issued, by Manitoba Health, a basic questionnaire and a letter soliciting their willingness to be listed in the registry and possibly contacted for future research studies. Approximately 60% of persons responded to the questionnaires and consented to be listed in the research registry (3). There were no significant socio-demographic or geographical differences between responders and nonresponders. At the beginning of the present study there were 2,890 subjects in the research registry. Persons in this research registry who agreed to participate in future studies furnished a mailing address and a telephone number to facilitate contact. We accessed the registry to enroll subjects under the age of 50 years and mailed them information sheets and questionnaires for the present case control study. The purpose of limiting the study to subjects under 50 years old was to enhance recall for questionnaire completion regarding events of early life as well as to enhance the likelihood of maintenance of antibody responses on serological testing. Furthermore, it has been suggested that Crohn's disease presenting in younger years generally has a different phenotype from that presenting in later years. All subjects completed questionnaires and consent forms for participation and were contacted for venipuncture. The diagnoses of Crohn's disease and ulcerative colitis were verified by chart review for clinical data including, but not limited to, endoscopic, histological, and radiological findings.

Control selection. A population-based set of controls was selected from the Manitoba Health population registry. The Manitoba Health registry contains demographic information on all persons registered with the Manitoba Health public health insurance system. The registry is regularly updated with vital registrations and information from medical and hospital transactions and closely matches population estimates derived from the Canadian census (Statistics Canada) (32). A random sample of registered persons was selected with restriction to the ages of 18 to 50 years, and stratification by gender and 5-year age groups was done to approximate the age and gender distribution of the combined IBD case group in the research registry. Manitoba Health's Information Services generated a mailing list of eligible controls and sent an information package prepared by the investigators explaining the study and requesting participation. The investigators did not know the identity of the controls unless they received a mailed response. For a second control group, we asked patients with IBD to refer us to one or more siblings without IBD. Siblings were not available for all patients; hence, inferences from direct comparisons between average results of IBD patients and sibling controls are limited.

All patients and controls completed a questionnaire and provided a venous blood sample. The questionnaire included demographic and historical questions, including questions about childhood water sources, frequency of consumption of various meats and of unpasteurized milk, and all potential sources of *M. paratuberculosis* (the questionnaire is available upon request from the authors).

ELISA for *M. paratuberculosis*. The ELISA for serum antibodies to *M. paratuberculosis* in cattle (*M. paratuberculosis* antibody test kit; IDEXX Laboratories, Inc., Westbrook, Maine) was adapted for human use. *M. paratuberculosis* antigen-coated 96-well microtiter plates and *Mycobacterium phlei*-containing serum diluent (used to absorb cross-reactivity antibodies) supplied with the kit were used. The kit employs a horseradish peroxidase-labeled protein G conjugate to detect binding of antibodies to the solid-phase *M. paratuberculosis* antigens. On every ELISA plate we ran bovine serum controls supplied with the kit to ensure that each assay was valid by kit criteria. We also ran the same identical human serum controls on every plate and used those optical density (OD) values to calculate sample/positive (S/P) ratios and interpret the assay. All sera were tested in duplicate, including controls.

As a positive-control human serum, we used blood donated by a Wisconsin veterinarian who was accidentally inoculated with the commercial killed vaccine for Johne's disease, resulting in a persistent localized granulomatous tissue reaction at the injection site. A commercial pool of serum from healthy blood donors (Binding Site, Birmingham, United Kingdom) was used as the negative-control human serum. The assay was not validated against proof of *M. paratuberculosis* infection because there are no established (validated, or "gold standard") methods to do so. Instead, we used this large reference population, of healthy Red Cross blood donors, to set the upper limit of normal with 95% confidence. In so doing, we can statistically test when a population of individuals has levels of serum antibodies elevated above those found in Red Cross controls. This assay found differences among two other human populations: differences that make biological sense (12). In 1996 at a meeting of the American Association of Bovine Practitioners in Spokane, Washington, 191 volunteers attending this meeting who represented a group with a high exposure risk to *M. paratuberculosis* were recruited. Another group of 193 farmers from Barron County, Wisconsin, was studied, and this group was considered to have moderate exposure. Both the high-exposure group and the moderate-exposure group had significantly higher ELISA values than the healthy controls (the Red Cross donors) ($P < 0.001$ for each comparison). In fact, 8% of the high-exposure group had ELISA results that were greater than those of the positive control (the veterinarian inoculated with *M. paratuberculosis*).

Sera were diluted 1:20 in a kit serum diluent (identical to the kit protocol for testing bovine sera) and incubated at room temperature for 30 min prior to transfer to the microtiter plate. One hundred milliliters of each diluted serum sample and control serum was dispensed into duplicate microtiter wells and incubated for 30 min at room temperature. Unbound serum components were then removed by washing the wells six times with a phosphate buffer provided with the kit. After removal of residual wash fluid, 100 ml of conjugate was added

TABLE 1. Demographic characteristics of the study groups

Parameter	Value for group[a]			
	Crohn's disease	Ulcerative colitis	Healthy controls	Sibling controls
Age (yr)				
Mean	36.4 ± 7.8^	37.4 ± 7.6^	39.8 ± 5.6	39.3 ± 8.5
range	18–50	18–50	24–49	20–50
Gender (% female)	60.8^	54.9^	72.4	60.1
Urban living (%)	58.4	63.2	59.1	48.5
Born in developing nation (%)	1.1	3.0	2.9	0.6
Childhood consumption of[b]:				
Milk (mean ± SD)	4.65 ± 0.89	4.51 ± 1.09	4.7 ± 0.83	4.63 ± 0.92
Unpasteurized milk	1.07 ± 1.81^	1.23 ± 1.93^^	1.72 ± 2.18	1.57 ± 2.08
Chicken	2.94 ± 0.66^^	3.01 ± 0.65^^	2.82 ± 0.65	2.88 ± 0.64
Beef	3.34 ± 0.73	3.35 ± 0.68	3.37 ± 0.74	3.32 ± 0.72
Pork	2.44 ± 0.87	2.49 ± 0.86	2.55 ± 0.77	2.59 ± 0.79
Childhood primary water source other than tap (%)[c]	29^^	30.9^^	37.4	42.7
Ever lived on a poultry farm	10.6	13.8^^	8.2	14.5
Ever lived on dairy or beef farm	13.1	18.7^^	14.8	23.7
Ever lived on pig farm	7.5	12.3^^	6.7	16.3

[a] *n* values were as follows: for Crohn's disease group, 283; for ulcerative colitis group, 144; for healthy controls, 402; and for sibling controls, 138. ^, $P < 0.001$ versus control; ^^, $P \leq 0.03$ versus control.

[b] Values are means ± standard deviations and indicate frequency of consumption based on the following scale: 0, never; 1, less than once a month; 2, 1 to 3 times a month; 3, 1 to 2 times a week; 4, 3 to 6 times a week; 5, every day. The statistics highlighted in the table are for comparisons of means of scores.

[c] Subjects were given a choice of primary water source during childhood of tap water, well water, or other water sources.

and the plate was incubated for another 30 min. After washing the wells as before, 100 ml of tetramethyl benzidine substrate solution was added to each well and incubated for 15 min. The enzymatic reaction was stopped after 15 min by the addition of 100 ml of stop solution provided with the kit. The OD for each well was measured at 650 nm (EL312; BioTek Instruments, Inc., Winooski, Vt.). Assays were considered valid based on the kit manufacturer's interpretation guidelines.

Mean ELISA OD readings for patient sera were transformed and expressed as the S/P ratio (as done for the cattle assay). However, OD values for the human sera were used as controls in the following calculation: S/P = (sample OD − negative-control OD)/(positive-control OD − negative-control OD). The results of testing 252 serum samples from Red Cross blood donors (Madison, Wis.) with this prototype ELISA for human serum antibodies to *M. paratuberculosis* were used to establish the S/P ratio cutoff for a positive test. A positive test on human sera was defined based on Red Cross donor sera as the mean (0.065) plus 2 standard deviations (2 × 0.123), i.e., S/P = 0.312, which by definition gave the assay a 95% specificity. The relationship between ELISA S/P ratio and likelihood of *M. paratuberculosis* infection in cattle was recently demonstrated (10).

Analysis. Analyses were done with SAS, version 8.2 (SAS Institute Inc., Cary, N.C.). Bivariate analyses were done by chi-square tests, two-sample *t* tests, and one-way analysis of variance. Where respondents were given a choice of questionnaire answers that described a frequency of ingestion (i.e., 0, never; 1, less than once a month; 2, 1 to 3 times a month; 3, 1 to 2 times a week; 4, 3 to 6 times a week; 5, every day), the data were analyzed in two ways. Mean frequencies with standard deviations were calculated (taking a mean of the scores, 0 to 5). Also, the frequency scores were grouped into tertiles (0 + 1, tertile 1; 2 + 3, tertile 2; 4 + 5, tertile 3), and differences between subject groups were compared. Titer data were log transformed to meet the assumptions for analysis of variance. Patients and their siblings were analyzed for *M. paratuberculosis* positivity by McNemar's test for correlated proportions, stratified by Crohn's disease and ulcerative colitis. Variables significantly associated were multivariately analyzed for independence by a backward stepwise discriminant analysis. The significance level was set at alpha = 0.05. Multiple logistic regression analyses were also run for combined groups, and adjusted odds ratios were estimated with 95% confidence intervals (95% CI).

RESULTS

There were 283 Crohn's disease subjects, 144 ulcerative colitis subjects, 402 healthy controls, and 138 sibling controls (Table 1). There were more females among the healthy controls (72.4%) than among the Crohn's disease subjects (60.8%, $P < 0.0001$) and ulcerative colitis subjects (54.9%, $P < 0.0001$). There were no significant gender differences between Crohn's disease patients, ulcerative colitis patients, or sibling controls. Healthy controls were significantly older than Crohn's disease patients (39.8 versus 36.4 years, $P < 0.0001$) and ulcerative colitis subjects (37.4 years, $P < 0.001$), but ages of healthy controls were not significantly different than those of sibling controls (39.3 years). Fifty-eight percent of study subjects were urban dwellers, with no differences in urban versus rural residences between Crohn's disease patients, ulcerative colitis patients, and controls; however, sibling controls were less likely to be urbanites (48.5%, $P = 0.03$). Crohn's disease subjects and ulcerative colitis subjects had similar rates of having first-degree relatives affected with IBD (20.5 and 19.3%, respectively). Only 2% of all subjects were born in a non-Western nation (any nation outside of the United States, Canada, Australia, New Zealand, and western Europe), rendering it difficult to assess the relationship with disease diagnosis versus control status with adequate power; however, Crohn's disease subjects (1.1%) and sibling controls (0.6%) were less likely to have been born in a non-Western nation than ulcerative colitis subjects (3.0%) or controls (2.9%).

Approximately 35% of all subjects were seropositive for *M. paratuberculosis*, and there were no significant differences between groups. The seropositivity rates for each group are as follows (the number of subjects in each group is indicated in parentheses): Crohn's disease patients (283), 37.8%; ulcerative colitis patients (144), 34.7%; healthy controls (402), 33.6%; healthy sibling controls (138), 34.1%. The correlation between the *M. paratuberculosis* serological status of a Crohn's disease or ulcerative colitis subject and an unaffected sibling control was poor (for Crohn's disease, $k = 0.21$, $P = 0.16$; for ulcerative colitis, $k = 0.24$, $P = 0.71$).

In terms of childhood dietary habits, Crohn's disease subjects and controls drank comparable amounts of milk (similar when analyzed by comparing means or tertiles); however, they drank significantly less unpasteurized milk ($P < 0.001$) per

TABLE 2. Percentage of *M. paratuberculosis*-seropositive subjects by demographic variables

Variable	% *M. paratuberculosis* seropositive	Odds ratio (95% CI)
First-degree family member with IBD		
Positive	34.8	1.01 (0.74–1.37)
Negative	34.9	1.00
Birth in non-Western nation		
Positive	33.3	0.93 (0.35–2.50)
Negative	35.0	1.00
Gender		
Female	35.9	1.11 (0.84–1.47)
Male	33.5	1.00
Urban residence		
Positive	37.3	1.27 (0.96–1.68)
Negative	31.9	1.00
Primary non-tap water source as child		
Positive	33.9	0.93 (0.69–1.26)
Negative	35.6	1.00
Lived on poultry farm		
Positive	27.6	0.68 (0.43–1.06)
Negative	36.1	1.00
Lived on dairy/beef farm		
Positive	33.5	0.92 (0.65–1.31)
Negative	35.4	1.00
Lived on pig farm		
Positive	34.4	0.97 (0.62–1.51)
Negative	35.2	1.00

week on average than controls (similar when analyzed by comparing means or tertiles). Ulcerative colitis subjects drank significantly less milk ($P = 0.02$) and less unpasteurized milk ($P = 0.01$) per week on average than controls when comparing mean frequency scores; however, when comparing tertiles of scores, the difference did not reach significance ($P = 0.067$). Crohn's disease and ulcerative colitis subjects drank milk, and specifically unpasteurized milk, with similar frequencies. Crohn's disease subjects drank more unpasteurized milk than sibling controls ($P < 0.015$ comparing means of scores and $P = 0.04$ comparing tertiles of scores). Crohn's disease and ulcerative colitis subjects were less likely to have their childhood primary water supply from sources other than a tap (i.e., well water) (29.0 and 30.9%, respectively) than controls (37.4%) or sibling controls (42.7%) ($P = 0.006$) (Table 1).

While Crohn's disease subjects and ulcerative colitis subjects both ate chicken more often as children than controls, they ate beef and pork in comparable amounts. Subjects with Crohn's disease and controls were significantly less likely to have lived on a poultry, dairy, beef, or pig farm than either ulcerative colitis subjects or sibling controls (Table 1).

M. paratuberculosis serological status was not dependent on gender, age, family history of IBD, birth in a developing nation, urban versus rural living, whether or not time was spent living on a poultry, dairy, beef, or pig farm, or whether subjects used a non-tap water source as a primary water source in childhood. In fact, nothing in our data set was predictive of a positive *M. paratuberculosis* serological status (Table 2).

There were no statistically significant variables on logistic regression analysis that distinguished between Crohn's disease and ulcerative colitis. However, there were independent discriminators that distinguished between a diagnosis of Crohn's disease or ulcerative colitis compared with controls. These included age, gender, having used a non-tap water source as a primary water source in childhood, and frequency of pork ingestion as a child. Combining the Crohn's disease and ulcerative colitis patients as an IBD group for comparison with controls, the adjusted odds ratios of having IBD included the following: age, 0.947 (95% CI, 0.924 to 0.970) for every year older; gender, 0.581 (95% CI, 0.414 to 0.817) less likely if female; having a primary water source in childhood other than tap water, 0.652 (95% CI, 0.481 to 0.923) less likely; ingesting pork as a child, 0.747 (95% CI, 0.807 to 0.919) for every rank increase in use. *M. paratuberculosis* serological status did not discriminate between any groups.

A subset of these subjects participated in a nested case control colonoscopy-plus-biopsy tissue study in pursuit of *M. paratuberculosis* and other zoonoses by PCR (4). Only 8 subjects (6 controls and 2 patients with ulcerative colitis) were *M. paratuberculosis* PCR positive, and of these, 50% were seropositive for *M. paratuberculosis*. Of all IBD patients tested (Crohn's disease plus ulcerative colitis) who were *M. paratuberculosis* PCR negative, 20% were *M. paratuberculosis* seropositive, and of all controls tested who were *M. paratuberculosis* PCR negative, 18% were *M. paratuberculosis* seropositive. Hence, the sample size of *M. paratuberculosis* PCR-positive subjects is not large enough to allow for meaningful comparison with the *M. paratuberculosis* PCR-negative subjects for correlation with seropositivity.

DISCUSSION

M. paratuberculosis has been difficult to culture from humans and has only been isolated from a small number of patients with Crohn's disease (8, 22). Culture can be unreliable, as *M. paratuberculosis* has extremely long incubation times and there has been poor recovery of *M. paratuberculosis* by culture from Crohn's disease tissue. Although ingested organisms would confront the mucosal epithelium first, it is plausible that, for insidious infections, reactions are mainly established in mesenteric nodes. Furthermore, it remains plausible that Crohn's disease is a vasculitis; hence, if antigens circulate, they may not only be more likely to be found in the deeper layers of the bowel but may also initiate a serological response. Hence, the finding of seropositivity might indicate previous (or current) infection. Since most Manitobans ingest meat and drink milk at some time in their lives, they are susceptible to ingesting *M. paratuberculosis*. *M. paratuberculosis* DNA has been identified in pasteurized store-bought milk in both the United Kingdom and Canada (18, 19, 24). The finding of *M. paratuberculosis* in breast milk supports the possibility that that it can be a systemic infection in humans (27). Thus, in attempting to determine whether there is an association with *M. paratuberculosis* seropositivity (and hence *M. paratuberculosis* exposure or infection) with Crohn's disease, it is critical to study appropriate population-based control groups.

We have shown that seropositive rates for *M. paratuberculosis* are statistically similar for patients with Crohn's disease compared to patients with ulcerative colitis, to sibling controls, and to population-based healthy controls in Manitoba. Approximately 35% of Manitobans are seropositive for *M. paratuberculosis*. This is independent of age, gender, dietary habits (as assessed by us for meat, milk, and water source), and whether or not subjects lived in rural areas or at any time on farms.

Considering previous mucosal PCR results, this may seem like a high seroprevalence; however, it has recently been shown that there is discordance between PCR for IS*900* (an *M. paratuberculosis*-specific DNA sequence) in surgically resected bowel tissue and serological response to *M. paratuberculosis* with the same assay employed in our study (11). In that study, 181 subjects underwent both PCR on surgically resected tissue and seroassay, and there was no instance in which a patient was positive by both tests. This supports our results where we assessed seroprevalence among subjects who had undergone colon biopsy for *M. paratuberculosis* PCR and found no obvious association. The other group's study included 272 patients with Crohn's disease, 167 patients with ulcerative colitis, and 275 controls drawn from Wisconsin and Copenhagen County, Denmark (11). The IBD patients were all surgical patients, implying a sicker group of patients than might be expected from a population-based sample. In these surgical Crohn's disease patients, the seropositivity rate was 20.7% in the United States and 6.4% in Denmark. Surgical ulcerative colitis patients were uniformly seronegative. The seropositivity among United States subjects was significantly higher for Crohn's disease patients than for controls ($P = 0.0002$) and ulcerative colitis patients ($P = 0.005$). There was no difference in seropositivity rates among the three groups from Denmark.

To reconcile these results with those from Manitoba, it must be assumed that either Manitobans are simply more exposed to *M. paratuberculosis* than subjects from Wisconsin or Denmark or that they have a different genetic ability to mount a response to the infection. In a serological study of two human groups with higher exposure to *M. paratuberculosis* than healthy blood donors, the group with high exposure (bovine practitioners) and the group with moderate exposure (Wisconsin farmers) both had significantly higher ELISA findings than those of the healthy blood donor group (12). Thus, an important aspect of our study is that, other than direct comparison between Crohn's disease patients and control groups, our data are also reporting on suspected *M. paratuberculosis* exposure rates in Manitoba, which are seemingly high.

Other groups have developed a novel serological assay for *M. paratuberculosis* based on two antigens, p35 and p36, and found higher rates of seropositivity in Crohn's disease patients than has been reported for our assay (28, 29). It is somewhat difficult to reconcile the very high rates of *M. paratuberculosis* seropositivity among healthy nonaffected controls in Manitoba compared to those in Wisconsin. Should *M. paratuberculosis* ultimately be proven to have some association with Crohn's disease, it may be relevant that Manitoba has both high incidence rates of Crohn's disease and high prevalence rates of human contact with *M. paratuberculosis*. It may be that the healthy unaffected controls in Manitoba do not have the genetic machinery to mount the type of inflammatory response to *M. paratuberculosis* infection that leads to Crohn's disease. It is noteworthy that a case control colonoscopy-plus-biopsy study of a nested cohort of this study population with nested PCR for *M. paratuberculosis* revealed a positive rate among the controls of 32%, a rate that is quite comparable to the seropositivity rates in the present study (4). Furthermore, another recent PCR study from the United Kingdom reported a positive rate in control tissues of 26% (5).

The true implications of being *M. paratuberculosis* seropositive by our assay are uncertain. Whereas in cattle seropositivity is related to *M. paratuberculosis* burden (39), it is unknown what seropositivity represents in humans. *M. paratuberculosis* seropositivity in our study correlated poorly with tissue PCR for *M. paratuberculosis* in the nested cohort study we undertook. An antibody response to *M. paratuberculosis* may represent exposure but not necessarily infection. We contend that the high *M. paratuberculosis* seroprevalence in Manitoba does suggest that Manitobans have likely been exposed to this infection in high numbers. Until we can prove that seropositivity in humans confirms exposure, this remains hypothetical. However, as stated above, other groups with potential moderate-to-high exposure to *M. paratuberculosis* also were more likely to be positive than healthy blood donors (12).

Although our questionnaire survey included a variety of items regarding subject demographics and past life experiences, we are herein reporting the results that may have pertained to acquiring *M. paratuberculosis* infection in childhood. Milk is a potential source for acquiring *M. paratuberculosis*. By PCR, *M. paratuberculosis* DNA was found in 11.8% of 567 pasteurized samples from 241 dairy processing establishments in the United Kingdom (24). There were a total of 10 (1.8%) culture-positive samples from the pasteurized samples, suggesting that viable *M. paratuberculosis* is occasionally present at low levels in commercially pasteurized cows' milk in the United Kingdom. Recently, 710 retail milk samples were collected from retail stores and dairy plants in southwest Ontario and tested by PCR for the presence of *M. paratuberculosis*, and 15% of these samples were positive (18). No survivors were isolated from the broth and agar cultures of 44 PCR-positive samples. This suggests that there is either a low number of viable *M. paratuberculosis* organisms in pasteurized milk (and hence undetectable by culture) or simply an absence of live *M. paratuberculosis*. It also raises the possibility that a serological response to *M. paratuberculosis*, as evident in our study, does not conclusively prove that the subject has had an active infection.

Another potential source of *M. paratuberculosis* is water. Environmental *M. paratuberculosis* may be taken up by protozoa, which could potentially implicate water as both a reservoir and a vehicle for transmitting *M. paratuberculosis* to humans (20). Whereas we queried subjects as to their ingestion of non-tap water sources of water as a potential vehicle for *M. paratuberculosis* acquisition, it is also plausible that *M. paratuberculosis* contaminates urban tap lines and water reservoirs. Regardless, primary water sources in childhood did not affect *M. paratuberculosis* serological status.

Our childhood dietary and demographic data may provide some clues to alternate risk factors for IBD. We found that subjects with Crohn's disease were less likely to have ingested unpasteurized milk, less likely to have had non-tap water sources as primary water sources, and ingested pork less frequently and chicken more frequently than controls. We found that subjects with ulcerative colitis were more likely to have lived on a farm and more likely to have ingested chicken than healthy controls, but they also drank less milk and specifically less unpasteurized milk in childhood and were also less likely to have had a non-tap water source of water in childhood. Perhaps there is something protective in ingesting unpasteurized milk or well water against developing either Crohn's dis-

ease or ulcerative colitis. This may support a hypothesis regarding access to various organisms in childhood as being protective against later development of autoimmune-like disease. Finally, the fact that urban dwellers were as likely as rural dwellers to be *M. paratuberculosis* seropositive suggests that if the antibody response represents either infection or exposure to *M. paratuberculosis*, then this can be acquired through accessible sources in urban centers (such as pasteurized milk, commercially available meat, or even urban water sources).

In summary, we found that *M. paratuberculosis* is not serologically specifically associated with Crohn's disease in a community with a relatively high seroprevalence of *M. paratuberculosis* and a high prevalence of Crohn's disease (3). The strength of our study is that the Crohn's disease, ulcerative colitis, and healthy control subjects were population based. Furthermore, for comparison to Crohn's disease subjects, we had three control groups. *M. paratuberculosis* serological status correlated poorly between subjects with Crohn's disease or ulcerative colitis and available sibling controls. Overall, these data do not support an obvious link between *M. paratuberculosis* and Crohn's disease. However, given the high prevalence of Crohn's disease in Manitoba (3) and the high seroprevalence of *M. paratuberculosis* in Manitoba, it is plausible that *M. paratuberculosis* may be necessary but not sufficient to cause some cases of Crohn's disease. Ongoing studies by our group will correlate *M. paratuberculosis* seroprevalence with Crohn's disease-specific genes. Furthermore, it will be important to pursue seroassays with potentially more-specific *M. paratuberculosis* antigens (29). Our data, while not supporting an association of Crohn's disease with *M. paratuberculosis*, do leave open the possibility that populations with high exposure rates to *M. paratuberculosis* are at increased risk of presenting with the disease. Hence, the possibility of an association between *M. paratuberculosis* and Crohn's disease remains inconclusive.

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