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Prevalence of *Giardia* Cysts and *Cryptosporidium* Oocysts and Characterization of *Giardia* spp. Isolated from Drinking Water in Canada

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This study was carried out to estimate the prevalence and potential for human infectivity of *Giardia* cysts in Canadian drinking water supplies. The presence of *Cryptosporidium* oocysts was also noted, but isolates were not collected for further study. A total of 1,760 raw water samples, treated water samples, and raw sewage samples were collected from 72 municipalities across Canada for analysis, 58 of which treat their water by chlorination alone. *Giardia* cysts were found in 73% of raw sewage samples, 21% of raw water samples, and 18.2% of treated water samples. There was a trend to higher concentration and more frequent incidence of *Giardia* cysts in the spring and fall, but positive samples were found in all seasons. *Cryptosporidium* oocysts were found in 6.1% of raw sewage samples, 4.5% of raw water samples, and 3.5% of treated water samples. *Giardia* cyst viability was assessed by infecting Mongolian gerbils (*Meriones unguiculatus*) and by use of a modified propidium iodide dye exclusion test, and the results were not always in agreement. No *Cryptosporidium* isolates were recovered from gerbils, but 8 of 276 (3%) water samples and 19 of 113 (17%) sewage samples resulted in positive *Giardia* infections. Most of the water samples contained a low number of cysts, and 12 *Giardia* isolates were successfully recovered from gerbils and cultured. Biotyping of these isolates by isoenzyme analysis and karyotyping by pulsed-field gel electrophoresis separated the isolates into the same three discrete groups. Karyotyping revealed four or five chromosomal bands ranging in size from 0.9 to 2 Mb, and four of the isolates had the same banding pattern as that of the WB strain. Analysis of the nucleotide sequences of the 16S DNA coding for rRNA divided the isolates into two distinct groups corresponding to the Polish and Belgian designations found by other investigators. The occurrence of these biotypes and karyotypes appeared to be random and was not related to geographic or other factors (e.g., different types were found in both drinking water and sewage from the same community). Biotyping and karyotyping showed that isolates from this study were genetically and biochemically similar to those found elsewhere, including well-described human source strains such as WB. We conclude that potentially human-infective *Giardia* cysts are commonly found in raw surface waters and sewage in Canada, although cyst viability is frequently low. *Cryptosporidium* oocysts are less common in Canada. An action level of three to five *Giardia* cysts per 100 liters in treated drinking water is proposed on the basis of the monitoring data from outbreak situations. This action level is lower than that proposed by Haas and Rose (C. N. Haas and J. B. Rose, J. Am. Water Works Assoc. 87(9):81–84, 1995) for *Cryptosporidium* spp. (10 to 30 oocysts per 100 liters).

The probability of contracting giardiasis or cryptosporidiosis from drinking water in Canada is not well understood. There have been several documented outbreaks of giardiasis in British Columbia, Alberta, Ontario, Quebec, New Brunswick, and Newfoundland but only one incidence of waterborne cryptosporidiosis (Ontario) if swimming pools are not counted. Another possible outbreak of waterborne cryptosporidiosis in an agricultural area of Ontario is under way at the time of writing. The population of Canada is low in comparison to its land mass, and the availability of high-quality raw water has slowed the proliferation of multiple-barrier water treatment facilities in some areas. Experience in the United States has clearly shown that even pristine watersheds can be contaminated with pathogenic protozoan cysts, and it is probable that the true incidence of waterborne giardiasis and cryptosporidiosis is greatly underestimated in Canada. Populations may be reinfected at sporadic intervals depending upon varying raw water quality because the ability of both of these parasites to elicit a lasting immune response remains in doubt, although immunization in animals appears to be capable of reducing giardiasis in kittens (11, 13). It is possible that repeated challenge by waterborne pathogens in an area of endemicity might reduce the incidence of infection, but populations are heterogeneous and change continuously. Epidemiological studies have shown that children and the families of infected children are most susceptible to giardiasis (3), especially when hygiene and nutritional status are low. Ultimately, the prevalence of water-

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borne protozoan infections depends upon concentration (dose), viability, and virulence against humans (pathogenicity), assuming that a significant number of the general population is susceptible to infection. The purpose of this study was to measure these properties in *Giardia* isolates collected from Canadian drinking water samples. The presence of *Cryptosporidium* oocysts was also recorded, but isolates were not collected for further study.

The prevalence and concentration of *Giardia* cysts and *Cryptosporidium* oocysts in water supplies can be measured, but many of the cysts in water samples are not recovered by the best available detection methodology. The viability of *Giardia* cysts recovered from water samples is also difficult to measure, primarily because cyst numbers are usually very low. The most commonly accepted methods for *Giardia* cyst viability determination are fluorescein diacetate with propidium iodide (PI) exclusion (17) and in vivo excystation using the gerbil model (1). In vitro excystation is usually impossible with water samples because of the low number of cysts. In this study, portions of certain samples were inoculated into gerbils (*Meriones unguiculatus*), and some samples were treated with PI during the immunofluorescence detection procedure to estimate viability.

The human pathogenicity of *Giardia* cysts in water samples cannot be assessed directly, but it is possible to characterize strains from cultures of trophozoites isolated from gerbils infected with cysts from water supplies. This approach permitted comparisons between *Giardia* isolates from both outbreak and nonoutbreak situations and between waterborne outbreak strains and known human source strains. The major drawback with this strategy is that all isolates recovered by in vitro or in vivo techniques are virtually clones and may not be representative of all the cysts present in potable water. Tibayrenc (19) discussed this problem with respect to *Giardia*, *Entamoeba*, and *Toxoplasma* spp. and stated that genetic differences observed in asexual protozoa may result from clonal populations (as opposed to cryptic species) and not be indicative of real species differences unless they can be correlated with observable biological differences. Recognizing this difficulty, we attempted to evaluate pathogenicity by recovering *Giardia* isolates by use of the gerbil model and comparing them with strains recovered from raw sewage (presumably human in origin) by using pulsed-field gel electrophoresis (PFGE) DNA karyotyping, isoenzyme analysis, and hybridization of DNA isolates with recombinant DNA probes.

Isoenzyme electrophoresis and DNA karyotyping have been used to characterize over 30 *Giardia* strains, including 16 Canadian drinking water isolates associated with the transmission of giardiasis in British Columbia (7). These analyses revealed a diversity of parasites that bore no relationship to either host type or geographic source. Isolate heterogeneity was found in drinking water as well as animal and human source isolates. Parasite heterogeneity was also found within single communities (7). Homan et al. (6) compared 47 *Giardia* isolates from diverse geographic sources with respect to three isoenzymes (malate dehydrogenase, phosphoglucomutase, and glucose phosphate isomerase) and their reaction with three DNA recombinant probes. The isoenzyme approach identified a number of unique zymodemes, but the DNA recombinant probes were able to separate all isolates into two groups that were named Polish and Belgian after the source of the original strains. One of the probes used by Homan et al. (6) reacted equally well with all isolates and was shown to contain a sequence that recognized a large portion of the repeat unit of the DNA coding for rRNA (rDNA). The division of isolates into two main groups was confirmed by two subsequent methods, Southern blot analysis (using probes complementary for genes of *Giardia* tubulin and the cytoskeletal protein giardin) and the use of a single random primer to produce several randomly amplified polymorphic DNA markers. In contrast to several previous studies, Homan et al. (6) concluded that *Giardia duodenalis* can be divided into two different groups, possibly species, by using DNA recombinant probes.

In this study, we used the probes specific for the Polish and Belgian strains to characterize all isolates. The same isolates were also tested for seven different enzyme systems and karyotyped by PFGE. The analyses were undertaken to determine the similarities and differences between waterborne isolates over wide geographic distances in Canada.

MATERIALS AND METHODS

Sample collection. Samples were collected from 72 municipalities across Canada from 1991 to 1995 at approximately monthly intervals. Municipalities were not chosen randomly; rather, they were selected on the basis of their willingness to take and ship samples, their reliance on surface water, and the recommendations of public health authorities. The average number of samples collected per site was 24 (range, 1 to 140). All of the municipalities obtained their raw water from surface waters, and 58 of 72 treated their water by chlorination only, without any filtration. The remaining 14 municipalities employed some kind of filtration, either in pressurized vessels or direct filtration with coagulant. In total, 1,760 samples were collected; these consisted of 1,173 raw water samples, 423 treated drinking water samples, and 164 raw sewage samples. Water samples were collected with samplers constructed in our laboratory as described previously (22). The sampling equipment was assembled in a metal carrying case, and all components in contact with water were made from either brass, plastic, or stainless steel. Influent water (connected by a garden hose) was passed through a pressure-reducing valve calibrated to keep back pressure below 83 kPa (12 lb/in^2). Water leaving the pressure-reducing valve was passed directly through a filter housing containing the filter cartridge or allowed to bypass the filter through a valved bypass loop. Filtered water was directed through a Kent (Ocala, Fla.) C700 water meter and drained through a common effluent with the bypass water. The bypass loop could be closed, causing all of the water to pass through the filter, or opened partially to permit a longer sampling interval with continuous subsampling of the influent water. The filters employed during this study (Delta-Pure Corporation model DW 1-03-9-1) were made of polypropylene string wound around a plastic core and had a nominal porosity of 1 μm. A typical sampling run took place over a 24-h period, with average flow rates of between 1 and 5 liters/min. At least 1,000 liters was filtered, except in some cases of exceptionally high turbidity or high algal loading. All samples were shipped in coolers with blue ice packs by surface transport or by air. Most samples arrived within 48 h of shipping, but some from remote sites did not arrive for 72 h. Samples were picked up immediately whenever they came in and stored at 4°C until analysis (within 2 days on average).

Water sample analysis. Filters were analyzed by the method described by LeChevallier et al. (8), except that filters were entirely hand washed, more of the pellet was stained and examined, and the final pellet (~100 μl) was stained in a 15-ml centrifuge tube and dried on a window slide (Cel-Line Associates, Newfield, N.J.) after washing instead of being stained on a membrane (21). Samples were washed once with phosphate-buffered saline (PBS) between the application of primary antibody and the fluorescein isothiocyanate (FITC) labelling antibody. After the second incubation, the pellet was washed once with PBS and once with deionized water before spotting on a window slide. On some samples, the PI technique of Schupp and Erlandsen (17) was modified by adding PI to the pellet for 5 min after incubation with the primary antibody and before the wash with PBS. Fluorescein diacetate was not used, because cysts were already tagged with monoclonal antibody. After drying, a coverslip was mounted on the window using fluorescence-grade immersion oil and observed with a Zeiss Axioskop microscope equipped with FITC and rhodamine filters for epifluorescence and Plan-Neofluar objectives. Slides were scanned under oil at 400×, and identification was confirmed at 1,000× by shape, size, strength of immunofluorescence, and presence of PI-stained nuclei. This was relatively easy for *Giardia* cysts, but *Cryptosporidium* oocysts are harder to identify. We used the criteria of Rose et al. (16), which include (i) the degree of fluorescence, at least 50% of that seen in controls of fresh oocysts seeded into environmental samples; (ii) a distinct fluorescence around the wall; (iii) a general spherical shape of 4 to 6 μm; and (iv) a folding in the oocyst wall. Control human source *Giardia* cysts were obtained from the Medicine Hat Diagnostic Laboratory and from experimental infections in gerbils during the course of this study. Control *Cryptosporidium* oocysts were obtained from Charles Sterling (University of Arizona) and Eileen Proctor (National Parasitology Reference Centre, Vancouver, Canada). Negative controls were run with each batch. Monoclonal antibodies for *Giardia* and *Cryptosporidium* immunofluorescence detection were obtained from Meridian Diagnostics Inc., Cellab Pty. Ltd., and Waterborne Inc. (New Orleans, La.). All were used routinely at different times, and comparative trials were run frequently to ensure the accuracy of results. Goat anti-mouse FITC conjugates for indirect

tests were obtained from Sigma Chemical Co. (St. Louis, Mo.). The best (i.e., dependable, with bright fluorescence) anti-*Giardia* monoclonal antibody was obtained from Waterborne, and the best anti-*Cryptosporidium* antibody was from Cellab, but all gave dependable reactions. Filters were washed by hand into 1,000 ml of deionized water containing 0.1% sodium dodecyl sulfate and 0.1% Tween 80. All of the wash water was concentrated by centrifugation and clarified by layering over Percoll-sucrose (S.G. 1.09), 1.0 M sucrose (some of the samples that were rich in organic matter were impossible to clean over Percoll-sucrose), or a 50:50 (vol/vol) mixture of both, and the resulting pellet was split between immunofluorescence analysis (10 to 50%) and gerbil infection (50 to 90%).

Sewage sample analysis. Sewage samples were collected in 500-ml containers from a composite sampler whenever possible. Two hundred milliliters of raw sewage was concentrated by centrifugation (650 × *g*) into a pellet, which was clarified by density gradient centrifugation (with 1.0 M sucrose or Percoll-sucrose) if necessary or stained as described above without further processing. The resulting pellet was split equally between immunofluorescence analysis and gerbil infection.

Quality control. Variations in turbidity (particularly clay content), organic matter, and algal concentration all affect cyst recovery efficiency, and all three of these factors varied enormously throughout the study, both temporally at each sampling site and between sampling sites. The large number of samples processed during this study precluded an exhaustive analysis of each. The recovery efficiency of our methodology was evaluated by analyzing a series of control samples made up on the basis of the U.S. Environmental Protection Agency protocol for Information Collection Rule laboratory approval. These samples were provided by Clancy Environmental Consultants (St. Albans, Vt.) as part of a larger Canadian and American survey sponsored by Health Canada and the U.S. Environmental Protection Agency. The results from our laboratory based on the spiked samples did not show any false identifications (unseeded filters were correctly reported as negative), and the recovery efficiency ranged from 2 to 47% (n = 7; $\bar{x}$ = 12%) for *Giardia* cysts and 0 to 5% (n = 7; $\bar{x}$ = 1%) *Cryptosporidium* oocysts. These recovery efficiencies were low but comparable to those obtained by other Canadian and U.S. laboratories (2). The data collected therefore greatly underestimate the true concentrations of *Giardia* cysts and *Cryptosporidium* oocysts present. The major problems with the method occurred during the initial concentration step (10), the density gradient centrifugation, and the identification of cysts and oocysts. The lower limit of detection was not determined directly during this quality control exercise, but on the basis of the lowest recovery efficiency of 1% and the average sample volume in this study of 1,000 liters, at least 100 cysts or oocysts would have to be present in the sample to be detected. This equates to a lower detection limit of 10 cysts or oocysts per 100 liters.

Viability testing for *Giardia* cysts. The viability of *Giardia* cysts was estimated by treating a limited number of water and sewage samples with PI as part of the regular staining procedure and by using a portion of many samples to infect gerbils. The PI dye exclusion method was based on that of Schupp and Erlandsen (17), except that cysts were stained with specific monoclonal antibodies instead of fluorescein diacetate. PI will not penetrate an intact cyst wall, so its absence indicates a living cyst. If the cyst wall is compromised, PI will enter and stain the DNA. The best results were obtained when 100 μl of PI stock solution (0.01% in PBS) was added to approximately 100 μl of pellet at the end of the first incubation period with the primary antibody and the mixture was incubated for 5 min. Empty cyst walls (ghosts) will not take up PI and may be mistaken for healthy cysts but can usually be distinguished because of the uniformity of staining, irregular and swollen shape, and wrinkled appearance (when dried on a slide). Gerbils were infected per os with blunt intubation needles with the portion of the sample reserved for animal infection by the method described by Wallis and Wallis (23). Gerbils were initially obtained from the Animal Sciences Vivarium at the University of Calgary and maintained as a breeding colony in our laboratory. Gerbils were only inoculated between 6 and 12 weeks of age, and all were pretreated with three or five doses of oral metronidazole (6 mg per adult gerbil per day). Despite extensive treatment with metronidazole, gerbils were universally infected with *Trichomonas muris* and *Entamoeba muris*. Gerbils were sacrificed 7 days after inoculation by use of ether and cervical dislocation. Infection was confirmed or rejected by examining biopsy snips of the duodenum and jejunum mounted in PBS for trophozoites by phase microscopy.

Culturing. If trophozoites were present, the entire small intestine was removed and placed in warm RPMI medium (without serum). Trophozoites were stripped from the gut by the procedure described in Wallis and Wallis (23) and transferred to culture in TYI-S-33. The culture medium was supplemented with CLEX (Dextran Products Ltd., Scarborough, Ontario, Canada) instead of fetal bovine serum and piperacillin (2 mg/ml). After isolates had stabilized in culture, they were axenized by adding the following antibiotics sequentially to the culture medium at concentrations of 100 μg/ml: piperacillin (Pipracil; Lederle, Markham, Ontario, Canada), ticarcillin (Ticar; SmithKline Beecham, Oakville, Ontario, Canada), gentamicin (Garamycin; Schering, Point-Claire, Quebec, Canada), imipenem (Primaxin; MSD, Kirkland, Quebec, Canada), cloxacillin (Orbenin; Ayerst, St. Laurentien, Quebec, Canada), and rifampin (Rifidin; Merrell Dow, Richmond Hill, Ontario, Canada). After axenization, isolates were cryopreserved in liquid nitrogen with 7.5% dimethyl sulfoxide.

Isoenzyme analysis. Isolates were recovered from cryopreservation by rapid thawing and washing in TYI-S-33. The isolate was subcultured after retrieval, incubated at 37°C, and examined daily until sufficient growth was achieved to prepare lysates for that isolate. Cultures of trophozoites in the late log phase were harvested and counted in a Neubauer chamber, and pellets were washed three times in sterile PBS. Equal volumes of EDTA, dithiothreitol, and α-aminohexanoic acid were added to the trophozoites, and the pellet was resuspended. The suspension was left overnight at −135°C, thawed on ice to lyse the organisms, and centrifuged at 4°C (Eppendorf Micro-Centrifuge 5415). The supernatant was immediately aliquoted into beads by dropping it into liquid nitrogen and then stored at −135°C until electrophoresis. Electrophoresis was carried out with the Agarose Gel Authentikit System (Innovative Chemistry Inc., Marshfield, Mass.). Lysates (1 to 2 μl) from the different isolates and electrophoresis controls (Innovative Chemistry) were applied to different wells in the gel surface and electrophoresed as described previously (7). Different gels were used for each enzyme system tested. Seven different enzyme systems requiring seven different electrophoresed gels per isolate were used (malic enzyme, nucleoside phosphorylase, phosphoglucose isomerase, phosphogluconate dehydrogenase, malate dehydrogenase, aspartate aminotransferase, and phosphoglucomutase). Following electrophoresis, the gels were incubated with the appropriate substrate for the enzyme tested and then washed, photographed, and air dried. Controls were included on each gel, and replicate testing was carried out whenever possible. Bands were measured (origin to band center), and the migration distance was recorded. Banding patterns were analyzed from the gels, replicates were run wherever possible, and WB (ATCC) was used as a reference strain. Separation into zymodemes was based on band migration differences in a composite of the seven banding patterns obtained from seven systems tested for each isolate. Bands for each enzyme system were measured from the middle of the origin to the middle of the band.

PFGE. Trophozoites from the selected isolates were retrieved from cryopreservation. Fresh TYI-S-33 medium was prepared, and inoculated trophozoites were incubated at 37°C. Cultures were examined daily and subcultured until sufficient numbers of trophozoites from that isolate were available at the late log phase. Trophozoites were harvested by chilling on ice for 15 min followed by centrifuging at 1,050 × *g* for 10 min. Pellets were washed twice in PBS and twice in TSE (10 mM EDTA, 50 mM Tris-HCl [pH 8.0]) and then counted in a Neubauer chamber. Equal volumes of trophozoites and 1% low-melting-point agarose (FMC Bioproducts, Rockland, Maine) were combined to give a final concentration of 2×10^6 cells per ml, and the trophozoite-gel mixture was aliquoted into wells in a gel mold and allowed to solidify. Gel blocks for each isolate were expelled into lysis buffer (EDTA, *n*-lauroyl sarcosine, proteinase K) and incubated at 50°C for 48 h. Lysis buffer was replaced at 24 h. Chromosome blocks were washed and stored at 4°C in EDTA. DNA blocks from selected isolates were loaded into the wells of a 1% agarose gel. Each block was then sealed into the well with molten agarose. Electrophoretic karyotypes were determined with a CHEF-DR11 apparatus. Conditions were set (330-s pulse time for 24 h, 750-s pulse time for 24 h, and 1,800-s pulse time for 48 h) on the pulse switcher as described previously (18), buffer was added (Tris-borate-EDTA buffer), and chromosome blocks were loaded and sealed. After the week-long electrophoresis, the gels were removed, washed in buffer, and stained with ethidium bromide. Photographs were made, and the gel was destained for 48 h with RNase A. The gels were photographed with both Polaroid film and T-Max negatives. Controls (chromosome blocks of *Saccharomyces* spp.) were included on each gel. Replicate testing was carried out as far as possible. Banding patterns were analyzed from photographic records; measurement of migration distances was carried out with T-Max negatives. An estimation of the DNA size of each band was made.

Amplification of isolate DNA using PCR–recombinant-probe hybridization. Isolates were recovered and grown to the late log phase as described above. Parasites were resuspended and incubated in lysis buffer (10 mM EDTA, 50 mM Tris-HCl [pH 8.0], 0.2% Sarkosyl, 0.5 mg of proteinase K per ml) for 1 h at 65°C, and DNA was extracted as described by Homan et al. (6). Characterization of the isolates was performed by PCR amplification of DNA with a Polish and Belgian group-specific oligonucleotide primer for the 16S rDNA and by hybridization of filter-bound total genomic DNA with Polish and Belgian group-specific hybridization probes (20). The PCRs were performed with a 100-μl volume with 5 ng of DNA, Polish or Belgian group-specific forward primers (5′GGTGGATCCT GCCGGAGCG3′ and 5′GGTGGATCCTGCCGGAATC3′, respectively) combined with a universal reverse primer (5′GCTCTCCGGAGTCGAAC3′) using *Taq* polymerase for 35 cycles (95 to 50 to 72°C) under conditions as described by Weiss et al. (24) or with Tth polymerase under the same conditions except with 0.5 mM $MgCl_2$ in the reaction mix. This primer combination amplifies a 296-nucleotide DNA fragment corresponding to the 296 nucleotides 5′ of the 16S rRNA gene. Known Polish (Isr, P1, and MR4) and Belgian (AMC-4 and CM) strains were used as controls. The PCR mixes were extracted once with phenol-chloroform-isoamyl alcohol, precipitated with ethanol, and subsequently analyzed by electrophoresis on 6% polyacrylamide gels. The gels were stained with ethidium bromide and viewed and photographed on a UV lightbox. Total DNA was hybridized with Polish and Belgian group-specific oligonucleotide probes (UCGCGCTCCGGCAGGAT and UCGGATTCCGGCAGGAT, respectively) by loading 7 μg of DNA on Nytran+ filters and hybridization for 16 h with digoxigenin-dUMP-labelled probes at 60°C in 5× SSPE (0.9 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, 0.5% SDS [pH 7.4]). The filters were washed at 37°C in decreasing concentrations of SSPE (5× to 2×), followed by one single wash

TABLE 1. Summary of water and sewage analysis results from microscopy for *Giardia* sp. and *Cryptosporidium* sp.

Sample type	Total no.	*Giardia*-positive samples		*Cryptosporidium*-positive samples	
		No.	%	No.	%
Total	1,760	441	25.1	78	4.4
Raw drinking water	1,173	245	20.9	53	4.5
Treated drinking water	423	77	18.2	15	3.6
Raw sewage	164	119	72.6	10	6.1

for 10 min with 3 M trimethylamine HCl at 50°C. Color development was with anti-digoxigenin-alkaline phosphatase by standard procedures.

RESULTS

Water and sewage samples—*Giardia* cysts. The results of the microscopic analysis of water and sewage samples for *Giardia* cysts are summarized in Table 1. The highest proportion of positive samples was found in raw sewage (72.6%), followed by raw (20.9%) and treated (18.2%) drinking water. Water samples from 53 of the 72 municipalities sampled contained *Giardia* cysts at least once. *Giardia* cysts were most frequently detected in samples from New Brunswick, Nova Scotia, and Newfoundland (Fig. 1), but most of the water samples contained fewer than 2 *Giardia* cysts per 100 liters. *Cryptosporidium* oocysts were found much less frequently. Water samples were found to contain cysts or oocysts at all times of the year but more frequently in late winter-early spring and fall. Seasonal algal blooms in summer made sample analysis difficult, especially for prairie community samples. The highest concentration of *Giardia* cysts (230 cysts per 100 liters) was found in a community in northern Ontario during an outbreak of waterborne giardiasis in 1994 when an attack rate of 30% was experienced in a population of about 1,000. Another site in northern Ontario frequently contained infectious cysts in its drinking water over the 2-month sampling period, but no cases of giardiasis were officially reported. Several isolates were collected from this water supply and raw sewage that were incorporated into the study (isolates SL1, SL2, SL4, SLAS, and SLS3). No significant rise in reported cases of giardiasis was found at any other municipality found to contain *Giardia* cysts in raw or treated water, although raw sewage samples were usually positive (sometimes more than 1,000 cysts per liter). Sewage samples contained as many as 88,000 cysts per liter, but values of under 1,000 cysts per liter were most frequent. The prevalence of *Giardia* cysts and *Cryptosporidium* oocysts in sewage samples is summarized in Fig. 2.

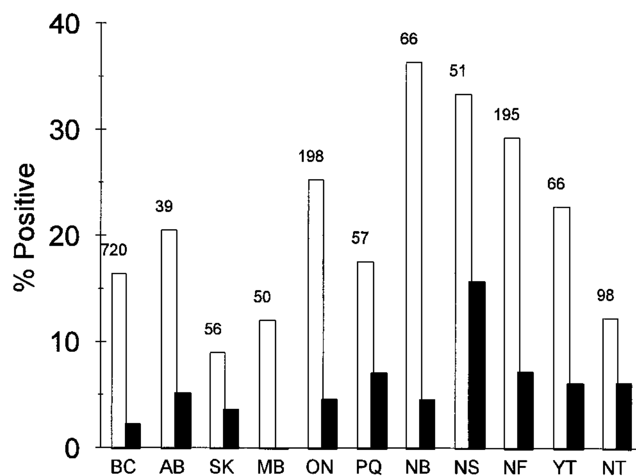

FIG. 1. Prevalence of *Giardia* cysts (□) and *Cryptosporidium* oocysts (■) in raw and treated drinking water (combined) by province and territory. Numbers above bars indicate numbers of samples. Abbreviations: BC, British Columbia; AB, Alberta; SK, Saskatchewan; MB, Manitoba; ON, Ontario; PQ, Quebec; NB, New Brunswick; NS, Nova Scotia; NF, Newfoundland; YT, Yukon Territory; NT, Northwest Territories.

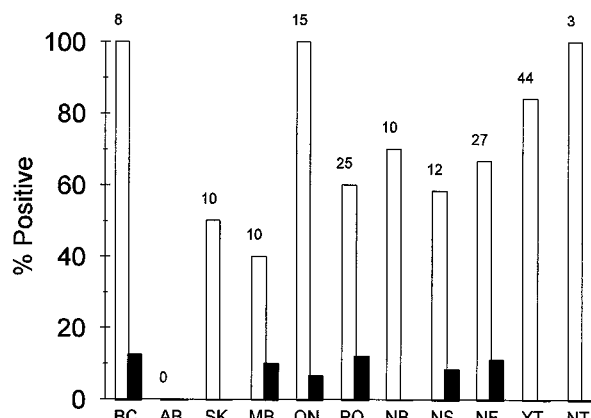

FIG. 2. Prevalence of *Giardia* cysts (□) and *Cryptosporidium* oocysts (■) in raw sewage samples by province and territory. Numbers above bars indicate numbers of samples. Abbreviations are as described in the legend to Fig. 1.

Monitoring data associated with waterborne outbreaks of giardiasis. In several instances, it was possible to obtain water samples during and after outbreaks of giardiasis that appeared to be waterborne on the basis of epidemiological data. These data came from two towns in Newfoundland (Corner Brook and Botwood), two in New Brunswick (St. Quentin and Plaster Rock), and one in Ontario (Temagami). The Newfoundland and Ontario outbreaks were well under way by the time samples were obtained, but the New Brunswick situation was less acute, with an ongoing prevalence of giardiasis that was well above the normal rate reported by health authorities. All of these municipalities treated their water by chlorination alone, with no filtration and minimal contact time before water reached the first customer (5 to 30 min). Results of monitoring are reported in Fig. 3, which shows the range and mean value of *Giardia* cyst concentration in the outbreak communities compared with those of a control community, Whitehorse (Yukon), which did not report any outbreak of giardiasis during the study period. Whitehorse's water is pumped from both the Yukon River and infiltration wells installed close to the river and is treated in the same way as the water of the outbreak communities. The results of monitoring data for 12 months after the outbreaks in the Newfoundland communities and Temagami are included for comparison. Cyst concentrations during the outbreaks were 0.5 to 3 orders of magnitude higher than those found after the outbreaks. Monitoring data from the New Brunswick communities were more sporadic, with high and low concentrations detected in rapid succession.

Water and sewage samples—*Cryptosporidium* oocysts. The results of water and sewage analyses for *Cryptosporidium* spp. are reported in Table 1 and Fig. 1 and 2. The prevalence rates for both raw and treated water were lower than those found for *Giardia* cysts (4.5 and 3.5%, respectively), and most of these positive results were based upon the observation of fewer than 0.5 oocysts per 100 liters. The prevalence rate for raw sewage was approximately double that for water (6.1%), and concen-

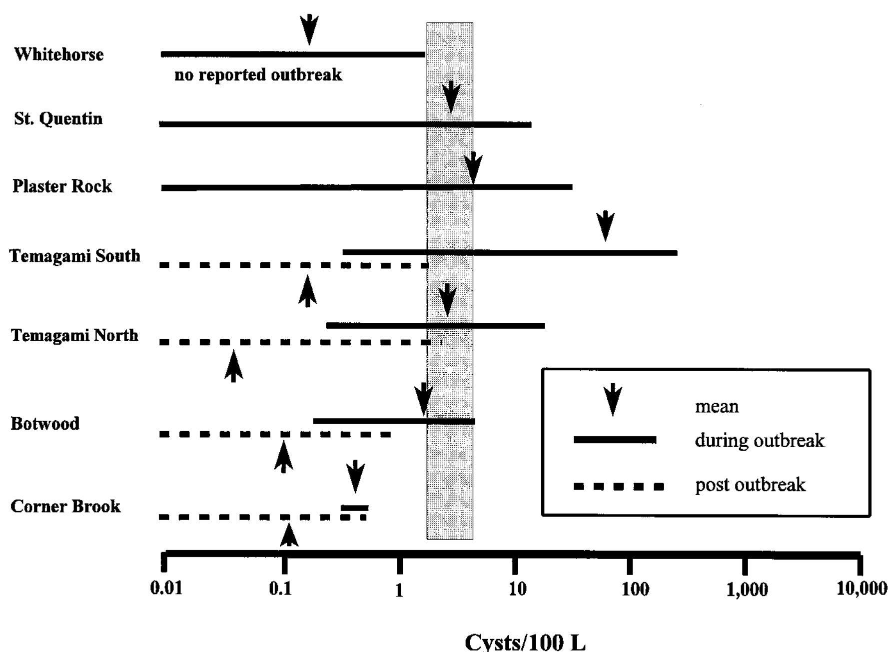

FIG. 3. Proposed action level for *Giardia* cysts in drinking water based on known Canadian waterborne outbreaks of giardiasis.

trations ranged from 1 to 120 oocysts per liter. Positive samples were observed for all provinces (no samples from Prince Edward Island) and territories, and no pattern was observed in their occurrence. The recognition of oocysts was very difficult under the conditions experienced during this study, and it is probable that some mistakes were made in identification.

Determination of viability of *Giardia* cysts by the PI method. Of the 167 positive drinking water and sewage samples that were treated with PI, the average viabilities observed were 24.6% (n = 127) and 38.9% (n = 40), respectively. Of these samples, 33 were used to inoculate gerbils, resulting in eight infections (24%). The number of cysts inoculated was variable but low, ranging from 0 to 272 viable (by PI exclusion) cysts. The actual number of cysts was probably much higher because of the low recovery efficiency of the analytical method, but no clear relationship between dose and infection was observed. The similarity between the percentage viabilities by PI exclusion and gerbil infection is coincidental (many samples containing viable cysts failed to infect gerbils), but the data do indicate that the viability of waterborne cysts is much lower than 100%.

***Giardia* infections in gerbils from water and sewage samples.** A total of 437 samples were inoculated into gerbils, and the results of inoculations of water and sewage samples into gerbils are summarized in Table 2. The rate of *Giardia* infection was 2.2% for raw water (5 of 223 samples), 7.6% for treated water (6 of 79), and 22.2% (30 of 135) for sewage. Four of the six infections from treated water came from the Sioux Lookout (Ontario) water supply over a 4-week sampling period, although no outbreak of waterborne giardiasis was reported from the community. A total of 13 isolates were established in culture (Table 3), and 15 others were lost to contamination (12 of them from sewage sources). The viability of the inoculated cysts was measured in 33 samples, and the rate of infection from these samples was 1 of 12 (8%) for water samples and 7 of 22 (32%) for sewage samples. The difference in infection rates between water and sewage probably only reflects the dose, which was 3.5 times higher in sewage samples on average, but the probability of gerbil infection was higher in samples known to contain viable cysts. Some water samples failed to infect gerbils despite inoculations of more than 500 viable cysts. No infections of gerbils with *Cryptosporidium* oocysts were detected by fecal analysis or duodenal biopsy.

Infectivity of *Giardia* isolates to gerbils. Of the 437 water and sewage samples inoculated into gerbils, it was possible to

TABLE 2. *Giardia* infection results of gerbil inoculation with water and sewage samples

Sample type	Total no. of samples inoculated	No. of positive infections	% Positive infections	No. of isolates cultured
Raw water	223	5	2.2	2
Treated water	79	6	7.6	3
Sewage	135	30	22.2	7

TABLE 3. Summary of *Giardia* strains cultured from gerbils

Strain[a]	Location	Source	Date isolated (mo/day/yr)
BT1*	Newfoundland	Water	9/26/91
CB2*	Newfoundland	Water	3/27/92
CBS1*	Newfoundland	Sewage	9/1/92
CAS9	Yukon	Sewage	2/9/93
CIS	Yukon	Sewage	2/9/93
SLS3	Northern Ontario	Sewage	2/5/93
SLAS	Northern Ontario	Sewage	2/10/93
SL1	Northern Ontario	Water	1/20/93
SL2	Northern Ontario	Water	1/20/93
SL4	Northern Ontario	Water	2/17/93
SM1	Manitoba	Sewage	8/31/93
WES	Yukon	Sewage	4/20/93

[a] Strains associated with documented waterborne outbreaks are indicated by asterisks.

TABLE 4. Dose-response data for infection of gerbils with waterborne *Giardia* cysts

Dose range (no. of cysts)	No. of positive infections	No. of negative infections	Probability of infection
0–25	17	311	0.05
26–50	5	18	0.22
51–75	4	7	0.36
76–100	2	2	0.5
101–125	1	4	0.2
126–150	1	3	0.25
151–175	3	1	0.75
176–200+	5	3	0.63
Total	38	349	

estimate the number of cysts inoculated from 387 samples on the basis of microscopic counts. Recognizing the inherent inaccuracies of the cyst counts, the data were combined into ranges and compared with the probability of infection as shown in Table 4. The relationship between dose and infection is obscured by a small but consistent number of inoculations that failed to infect gerbils despite high numbers of healthy-looking cysts. This was particularly obvious in the samples recovered during an outbreak in northern Ontario where *Giardia* cysts were present in high numbers, at least half of which were morphologically intact and viable on the basis of PI exclusion, but virtually noninfective to gerbils. Only one inoculation produced a weak infection that did not survive repassage, and the same result was obtained with *Giardia* cysts recovered from human and beaver fecal samples from the area.

Isoenzyme analysis. The protein content of lysates was found to be 1 to 10 mg/ml (7). Some enzymes produced diffuse bands, and a double band was observed for one isolate in one system (malate dehydrogenase). Two systems (phosphoglucomutase and nucleoside phosphorylase) were not discriminatory in the testing of these 10 isolates. Three distinct zymodemes were observed (Fig. 4), which divided the isolates into BT1 and CB2 (group I), SLAS, SLS3, SL2, and SM1 (group II), and SL1, SL4, CAS9, and CBS1 (group III). Group I isolates were both waterborne outbreak strains from Newfoundland, but groups II and III included isolates from diverse geographic sources. The sources of all isolates are listed in Table 3. Strain SLAS showed a slight variation in 6-phosphogluconate dehydrogenase but was otherwise closely aligned with other isolates in group II. Water source isolates were found in each group, but zymodeme grouping was not predictive of either geographic source or type of specimen.

PFGE. PFGE banding patterns of chromosomes were reproducible, and four or five chromosomal bands were observed in the set of isolates tested (Fig. 5). Their molecular weights were estimated to be between 1.4 and >2.2 Mbp on the basis of molecular weight standards from *Saccharomyces cerevisiae* (Bio-Rad, Richmond, Calif.). Karyotypes were identified on the basis of estimated size, number, and spatial position. The isolates could be divided into three groups on the basis of karyotype as follows: BT1 and CB2 (group I); SM1, SLS3, SLAS, and SL2 (group II); and SL1, SL4, CAS9, and CBS1 (group III). Group III was similar to but distinguishable from group I and showed the same banding pattern as that of strain WB (ATCC reference strain). Minor bands were sometimes present, and repeat testing did not show any changes in banding patterns.

PCR–recombinant-probe hybridization. The final conclusions reached by using PCR and filter hybridization with Belgian and Polish primers and probes follow. The Polish probe hybridized to DNA extracted from all isolates (CBS1, CAS9, CIS, SL1, SL2, SL4, SMS, WES); however, the Belgian probe hybridized only with those DNAs that were of the Belgian type as suggested by PCR (CB2, SLS3, SLAS, SM1). Therefore, a positive hybridization with the Belgian probe was scored as a positive identification, whereas no hybridization with the Belgian probe combined with a positive hybridization with the

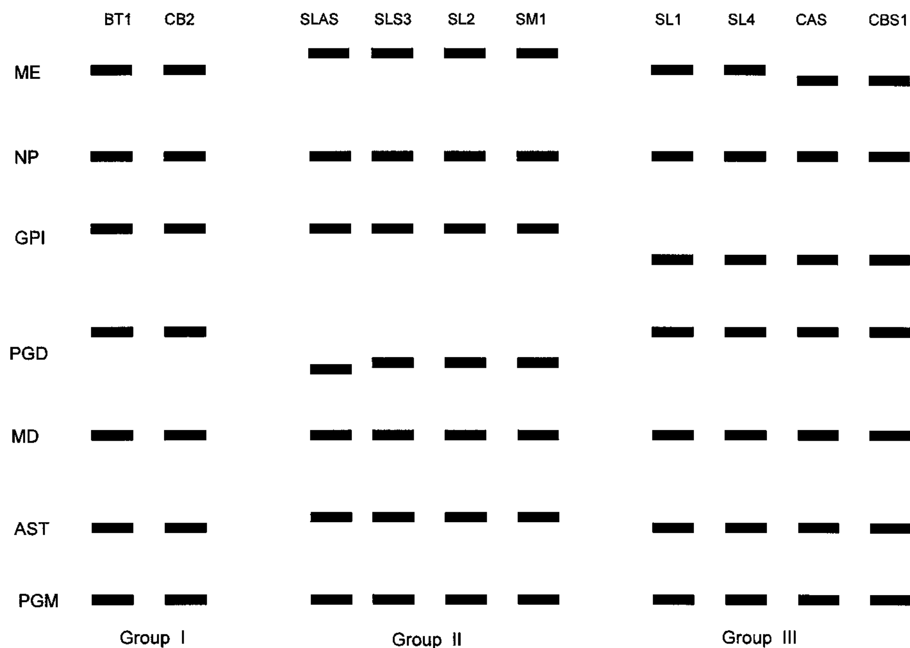

FIG. 4. Results of isoenzyme analysis of *Giardia* isolates showing zymodemes. Abbreviations: ME, malic enzyme; NP, nucleoside phosphorylase; GPI, phosphoglucose isomerase; PGD, 6-phosphogluconate dehydrogenase; MD, malate dehydrogenase; AST, aspartate aminotransferase; PGM, phosphoglucomutase.

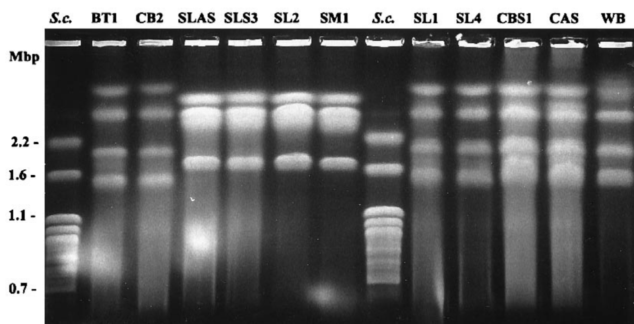

FIG. 5. Pre-RNase PFGE banding patterns of *Giardia* isolates showing group I (BT1, CB2), group II (SLAS, SLS3, SL2, SM1), and group III (SL1, SL4, CBS1, CAS). The WB strain (ATCC) is shown as a reference, and the molecular markers are from *S. cerevisiae* (*S.c.*).

Polish probe was scored as a Polish-type DNA. The positive hybridization with the Polish probe was used only as a control. Sufficient DNA was present on the filters to be detected. The combined results of PCR and hybridization were in agreement, but the PCR of isolate SMS and possibly of SL2, however, suggests them to be a mixture of Polish and Belgian types. Otherwise, the Belgian group of isolates corresponds closely to group II differentiated by isoenzyme analysis (with the exception of SL2 and CB2), and the Polish group includes all of the isolates that fell into group III except SL2, which may be mixed (the WES, SMS, and CIS isolates were not available for isoenzyme biotyping). Unfortunately, BT1 was not available for PCR–recombinant-probe testing.

DISCUSSION

Municipalities with contaminated raw drinking water and minimal water treatment were found in all regions of Canada. Waterborne outbreaks of giardiasis have been reported in British Columbia (7) and the Maritimes, although Ontario, Quebec, and the northern territories have all had problems. One municipality in southern Ontario experienced a suspected waterborne outbreak of cryptosporidiosis during the study. When summed, all of these data resulted in an average prevalence of *Giardia* cysts of approximately 20% in both raw and treated drinking water. These data may be compared with the larger study of Hibler (4a), who found an average of 18% of 4,423 samples to be contaminated with *Giardia* cysts in the United States, and with the smaller study of LeChevallier et al. (8, 9), who found both parasites in most of 85 raw samples and 39% of treated water samples analyzed from water supplies in the northeastern United States. Waterborne *Giardia* contamination in Canadian drinking water samples is not universal but is common enough to require increased awareness on the part of public health authorities and municipal engineers.

The prevalence data reported in this communication do not reflect the results of a predefined, random sampling program but are sufficiently numerous and diverse (Fig. 1) to indicate the prevalence of waterborne *Giardia* cysts and *Cryptosporidium* oocysts in Canadian water supplies and sewage. Data from five known (from Ontario, New Brunswick, and Newfoundland) and six suspected (from Ontario, Quebec, British Columbia, Northwest Territories, and Yukon) waterborne giardiasis outbreaks are included along with routine monitoring data from municipalities that have never experienced an outbreak. Not all of the outbreaks were recognized by health authorities, and many of those infected were treated empirically with metronidazole or were not treated at all.

The comparison of cyst concentrations in Fig. 3 between outbreak and nonoutbreak communities is the basis for a proposed action level for *Giardia* cysts similar to that advanced for *Cryptosporidium* oocysts (4). Haas and Rose (4) proposed an action level of 10 to 30 oocysts per 100 liters, but our data suggest that a lower concentration is more appropriate for *Giardia* cysts. In this study, average *Giardia* cyst concentrations above 3 to 5 cysts per 100 liters in drinking water were always associated with high levels of giardiasis in the communities to which it was supplied. The outbreaks in Botwood and Corner Brook, Newfoundland, suggest that even this action concentration may not be sufficiently conservative to predict the threshold of obvious waterborne giardiasis. In reality, these data illustrate a spectrum of exposure that causes prevalence of giardiasis to range from endemic to epidemic levels in communities.

A single sample containing *Giardia* cysts at concentrations above the action level is cause for immediate concern, but it should be emphasized that Fig. 3 reports average concentrations (the number of samples for each community ranged from 6 to 27) and that repeat sampling is necessary to establish a trend. As many as 10 samples may be required to describe the range of cyst concentrations that a community is experiencing in its water supply. There are presently about 1,000 communities (mostly small) in Canada that treat raw water with chlorination and minimal contact time (less than 30 min [19]), and these municipalities are at the greatest risk. Municipalities that are at risk would benefit from regular monitoring so that the significance of a single sample exceeding the action threshold can be evaluated immediately and appropriate action can be taken without delay.

A total of 164 sewage samples were analyzed by this method, 72.6% of which were found to contain *Giardia* cysts and 6.1% of which contained *Cryptosporidium* oocysts. The prevalence of *Giardia* cysts in sewage samples was much higher than that in drinking water, which confirms that giardiasis is a common enteric parasite in Canada. This was not found to be true for *Cryptosporidium* oocysts. Sewage monitoring has proven to be a useful indicator of the prevalence rate in a community, although concentrations of less than 1,000 cysts per liter probably do not indicate a specific outbreak of giardiasis. High concentrations of cysts in raw sewage in combination with positive results from drinking water are suggestive, however, and should be cause for concern by public health authorities (15). The ubiquity of *Giardia* cysts in sewage is a clear warning against the discharge of untreated sewage to any water that may be used as a potable water source or is inhabited by aquatic mammals such as beaver and muskrat that may become infected with *Giardia* cysts from human sources. Establishment of human-infective *Giardia* spp. in wild animal reservoirs helps to spread the parasite through animal migration and may magnify a low level of contamination to potentially dangerous concentrations. Raw and treated sewage are easy to sample (500-ml grab samples usually contain enough cysts to count and determine viability by the PI method or gerbil infection) and analyze and are a useful monitoring tool for the protection of water resources. At least one municipality in Canada (in the Yukon) will be required to monitor *Giardia* cyst contamination in sewage effluent as a condition of their water license. The analysis of grab samples avoids the cartridge filtration problems inherent in water sampling and is more efficient because of the reduced number of steps. Comparison of *Giardia* and *Cryptosporidium* concentrations in raw and treated sewage also provides a useful indicator of sewage treatment efficiency because the persistence of cysts and oocysts in the environment makes them useful benchmark organisms.

The detection of *Cryptosporidium* oocysts in water and wastewater was infrequent, and no pattern was apparent either temporally or geographically. This may have been because most of the municipalities involved in the study were not situated downstream of agricultural areas or sewage effluents. The accurate determination of oocysts is difficult, and there are several algal species in the right size range that react with antibodies and fluoresce green. It is possible that false-positive identifications were made, but it is equally probable that oocysts were missed in some samples. Oocyst detection was more reliable in sewage, and the prevalence rate was higher, but concentrations were still low. At this time, there does not seem to be a widespread incidence of waterborne cryptosporidiosis in Canada, and *Giardia* cysts are much more common in raw water, treated water, and wastewater.

These results clearly indicate that the number and viability of cysts inoculated are not the only factors determining the probability of infection with some isolates. The inoculum of cysts required to infect humans was found to lie between 10 and 100 cysts by Rendtorff (14), and this is also true of *Giardia muris* in mice (5). Measurements of the 50% infective dose for a *Giardia* isolate from sewage in the Northwest Territories showed that at least 400 PI-negative (viable) cysts were required to infect 50% of gerbils in a controlled experiment (unpublished data). The results of infections from isolates recovered from water samples reported in this study agree with this finding when the factors of viability, host specificity, and counting inaccuracies are ignored. In practice, some isolates (11 of 194) infected gerbils readily even when no cysts were detected in the sample (negative controls were uninfected), and other isolates would not infect at any concentration likely to be found in water supplies. These anomalies in combination with low cyst numbers in many samples resulted in an overall gerbil infection rate of only 9.4%, but this does not mean that the gerbil model is not useful for recovering waterborne isolates. The only alternative is to try and excyst samples into culture, and this approach is unreliable when cyst numbers are low. We speculate that the probability of humans becoming infected with waterborne *Giardia* cysts is similar to that of gerbils in that not all strains will be equally human infective.

Only about half of the samples that infected gerbils were recovered as isolates for further study, and most of the rest were lost because of overgrowth of bacteria in the medium. The isolates available for isoenzyme, PFGE karyotyping, and PCR–recombinant-probe analysis proved to be heterogeneous, with no obvious geographic grouping except for the two waterborne strains from Newfoundland. The same three major groups were recognizable by all three methods of isolate characterization. Group I included strains from Newfoundland (CB2 and BT1), group II consisted of two sewage strains (SLAS and SLS3) and one waterborne strain (SL2) from northern Ontario along with a sewage strain from Manitoba (SM1), and group III included two more waterborne strains (SL1 and SL4) from northern Ontario, a sewage strain from Newfoundland (CBS1), and a sewage strain from the Yukon (CAS9). The fact that both northern Ontario and Newfoundland produced strains that fell into both groups II and III suggests (i) that isolate heterogeneity is present across Canada (as found in British Columbia by Isaac-Renton et al. [7]) and (ii) that these methods cannot distinguish between isolates that are infective to humans and those that are not. The source of the waterborne strains is unknown, but the sewage strains are presumably human in origin and are most probably human infective. The well-known human strain WB falls into group III by DNA karyotyping and has bands in common with all of the isolates. Although these results are inconclusive concerning human pathogenicity, the strains do fall into discrete groups as predicted by the clonal theory of genetic variation advanced by Tibayrenc (19) and Meloni et al. (12). The origin of all of the isolates in this study (either from waterborne outbreaks or sewage) suggests that all of these isolates are potentially infective to humans. In addition, all isolates could be characterized as either Polish or Belgian by their signature sequences in small-subunit rRNA in the same manner as known human isolates.

Finally, the viability of cysts recovered from water and sewage is much less than 100%. The gerbil model is not always an accurate test, because some strains do not appear to be infective to gerbils and false negatives may result. Laboratory experiments employing strains known to be infective to gerbils will still be useful, but unknown isolates produce variable results. PI dye exclusion is a much easier technique to use and can be incorporated into routine detection with monoclonal antibodies. The test works well with the indirect immunofluorescence staining procedure, and it should be added during the last 5 min of incubation with the primary antibody. Experiments with direct kits or the use of PI after the FITC conjugate has been added did not produce satisfactory results.

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