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Genetic Characterization and Transmission Cycles of *Cryptosporidium* Species Isolated from Humans in New Zealand

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Little is known about the genetic characteristics, distribution, and transmission cycles of *Cryptosporidium* species that cause human disease in New Zealand. To address these questions, 423 fecal specimens containing *Cryptosporidium* oocysts and obtained from different regions were examined by the PCR-restriction fragment length polymorphism technique. Indeterminant results were resolved by DNA sequence analysis. Two regions supplied the majority of isolates: one rural and one urban. Overall, *Cryptosporidium hominis* accounted for 47% of the isolates, with the remaining 53% being the *C. parvum* bovine genotype. A difference, however, was observed between the *Cryptosporidium* species from rural and urban isolates, with *C. hominis* dominant in the urban region, whereas the *C. parvum* bovine genotype was prevalent in rural New Zealand. A shift in transmission cycles was detected between seasons, with an anthroponotic cycle in autumn and a zoonotic cycle in spring. A novel *Cryptosporidium* sp., which on DNA sequence analysis showed a close relationship with *C. canis*, was detected in two unrelated children from different regions, illustrating the genetic diversity within this genus.

Almost seventy years elapsed between the first observation of *Cryptosporidium* by Edward Tyzzer in 1907 (37) and the realization in 1976 that it was a human pathogen (9). *Cryptosporidium* is an apicomplexan parasite that causes gastrointestinal illness in as many as 152 mammals (10) and numerous other vertebrates. Immunocompetent humans are usually parasitized only by either the *Cryptosporidium hominis* or the *C. parvum* bovine genotype (2, 8, 19, 21, 23, 24, 29, 31, 35, 40, 42); however, an increasing number of *Cryptosporidium* species and genotypes are being implicated as a cause of diarrheal disease in the immunocompetent human population. These include *C. meleagridis* (30), *C. felis* (44), *C. canis* (44), *C. parvum* cervine genotype (28), and a *C. parvum* pig genotype (28). We add to this list a novel *Cryptosporidium* genotype that phylogenetically clustered closest to *C. canis* and was isolated from two unrelated immunocompetent children. The rapid increase in *Cryptosporidium* research since 1976 has seen the number of valid species increase to 20 (27) and decrease to 10 (10), and it is now on the rise again to 13 (32). Clearly, there is great genetic diversity within the genus with more species, genotypes, and variants likely to be uncovered by more-sensitive molecular techniques (6, 12, 13, 15–17, 22, 25, 41).

New Zealand's economy is based on primary food production with dairy, beef, and sheep farming playing the largest role. A preliminary study (unpublished) of the national dairy herd as a potential reservoir for human infection found that 8.8% of 385 calves and 0.7% of 572 cows tested were infected with the *C. parvum* bovine genotype. In the present study we characterize 423 human *Cryptosporidium*-positive fecal specimens from New Zealand by β-tubulin PCR–restriction fragment length polymorphism (PCR-RFLP), 18S ribosomal DNA (rDNA) PCR-RFLP, and 18S rDNA sequencing.

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MATERIALS AND METHODS

Fecal specimens. Between August 2000 and August 2003 several medical diagnostic laboratories in the North Island of New Zealand and one in the South Island forwarded 423 human fecal specimens found to contain *Cryptosporidium* oocysts to the Protozoa Research Unit (PRU) (Table 1). Since it is not a medical diagnostic laboratory, the PRU had to rely on fecal specimens that tested positive for the presence of *Cryptosporidium* oocysts that were referred to it. Two laboratories referred the majority of fecal specimens. One was in Hamilton, a city that services a large intensively dairy farmed region, and the other was in Wellington, a metropolitan district. The fecal specimens did not contain any preservatives and were stored at 4°C until examined.

From time to time the PRU surveys animal populations. *Cryptosporidium* oocysts from a wild rabbit fecal specimen were isolated, characterized, and used for phylogenetic purposes in the present study.

Oocyst isolation. Oocysts were concentrated and removed from the majority of the fecal debris by the formal saline-diethyl ether method (1). A Dynabeads G/C-Combo immunomagnetic bead separation kit (Dynal Biotech ASA, Oslo, Norway) was used to isolate the concentrated oocysts from the remaining heavy fecal debris by following the manufacturer's instructions with minor modifications. Briefly, the oocyst concentrate was made up to 1 ml with water in a microcentrifuge tube, and 100-μl volumes of 10× buffers A and B, plus 25 μl of anti-*Cryptosporidium*-coated paramagnetic beads, were added to the oocysts. The mixture was gently rotated for 1 h at 8 rpm on a Labquake shaker/rotisserie (Barnstead/Thermolyne, Dubuque, Iowa). A magnet, fixed in a housing capable of holding a microcentrifuge tube (made in house), was used to capture the oocyst-bead complex. Debris was removed by washing the oocyst-bead complex twice with 1 ml of 1× buffer A. Oocysts were separated from the beads by the addition of 50 μl of 0.1 M HCl for 10 min with 10 s of vortexing at the start and end of the incubation period. The beads were captured with the magnet before the oocyst suspension was collected and neutralized with 5 μl of 1 M NaOH. Oocysts were stored at 4°C until processed.

Nucleic acid extraction. To each 55 μl of oocyst suspension, 75 μl of TE buffer (1 mM Tris-HCl and 0.5 mM EDTA) containing 1% Nonidet-P 40 and 20 μl of a 20% suspension of Chelex 100 resin (Bio-Rad Laboratories, Hercules, Calif.) were added. The oocysts were subjected to five freeze-thaw cycles of 2 min in liquid air, followed by 2 min in water at 96°C. Cell debris and Chelex 100 resin were deposited by centrifugation at 10,000 × *g* for 1 min. The supernatant containing the nucleic acid was stored at 4°C until processed.

PCR amplification. Initially, all specimens were amplified with the primers for the β-tubulin gene as previously described (5). A PCR product of 592 bp was expected. Any specimens that did not produce a PCR product were reexamined with a nested primer technique targeting the variable region of the 18S rRNA gene. The β-tubulin gene is thought to exist as a single-copy gene per sporozoite (38), and as such it is does not provide as large a DNA template for PCR

TABLE 1. Geographic distribution of *Cryptosporidium* spp. from human infections in New Zealand

Locality	No. of isolates			
	C. hominis	*C. parvum* bovine genotype	Novel genotype	Total[a]
Wellington	109	13		122
Hamilton	40	72	1	113
Hawkes Bay	21	15		36
Southland	1	34		35
Bay of Plenty	6	27		33
Hutt Valley	12	12	1	25
All others	9	50		59

[a] Only localities that referred more than 20 fecal specimens are included.

amplification as did the five-copy 18S rDNA. Each oocyst will thus have 4 copies of the β-tubulin gene available for PCR amplification but 20 copies of the 18S rDNA. The nested primer PCR technique helps to increase the sensitivity of oocyst detection in specimens that contain small numbers of oocysts. Primers for the 18S rDNA were designed by using Primer3 (version 0.9; http://www-genome.wi.mit.edu/genomesoftware/other/primer3.html). The outer primer pair of CRY-L1 (5′-GTT AAA CTG CGA ATG GCT CA) and Cry-L2 (5′-CCA TTT CCT TCG AAA CAG GA) and the inner primer pair of CRY-S1 (5-CTC GAC TTT ATG GAA GGG TTG) and CRY-S2 (5′-CCT CCA ATC TCT AGT TGG CAT A) amplified a 1,397-bp fragment and an 832-bp fragment, respectively. Each 20-μl volume of PCR mixture contained 1× PCR reagent buffer (Invitrogen Life Technologies, Carlsbad, Calif.), 250 μM concentrations of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 100 nM concentrations of each primer, 2.5 U of *Taq* polymerase, and 1 μl of DNA template. Cycling conditions consisted of an initial denaturation step of 96°C for 2 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s, and finally elongation at 72°C for 5 min. PCR amplification was performed in a Perkin-Elmer GeneAmp PCR System 9700 thermal cycler. All PCR products were analyzed by electrophoresis in 1.6% ultrapure DNA-grade agarose (Bio-Rad), stained with ethidium bromide, and visualized on a UV transilluminator.

PCR-RFLP analysis. All β-tubulin PCR products were digested with the restriction enzyme DdeI at 37°C for 3 h (5) and fractionated by electrophoresis in a 3.5% Metaphor agarose gel (BioWhittaker Molecular Applications, Rockland, Maine), stained with ethidium bromide, and visualized on a UV transilluminator. The *C. parvum* bovine genotype PCR product contains a DdeI restriction site that gives two bands of 178 and 414 bp. This site is not present in *C. hominis*, so a single band of 592 bp is observed.

The 18S rRNA gene PCR products were digested with SspI and VspI, as previously described (40), and then analyzed as described above.

18S rDNA PCR sequence and phylogenetic analysis. The 832-bp band containing the 18S rDNA was purified by using a QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany) by following the manufacturer's instructions. An ABI 3730 DNA analyzer (Applied Biosystems) was used to sequence overlapping bidirectional strands of PCR product with at least two PCR products from each fecal specimen sequenced to ensure accuracy. Sequences were assembled with Perkin-Elmer's MT Navigator software and aligned by using CLUSTAL X (36). To aid phylogenetic analysis, 18S rDNA sequences obtained from GenBank were used in a multiple sequence alignment along with human, bovine, dog, and rabbit *Cryptosporidium* sequences from New Zealand isolates. A phylogenetic tree was constructed with PAUP software (34) by using the neighbor-joining method and a general time-relative model to calculate genetic distances. The reliability of branches in the tree were tested by bootstrap values obtained from 1,000 pseudoreplications, with *C. serpentis* as an outgroup.

Nucleotide sequence accession numbers. Published 18S rDNA sequences used for phylogenetic analysis included the following: *C. andersoni*, accession number AB089285; *C. baileyi*, AF093495 (40); *C. canis*, AF112576 (42); *C. felis*, AF112575 (42); *C. meleagridis*, AF112574 (42); *C. muris*, AF093498] (40); *C. hominis*, AF093489 (40); *C. parvum* bovine genotype, AF108864 (26); *C. parvum* pig genotype, AF115377 (42); *C. serpentis*, AF093502 (40); and *C. wrairi*, AF115378 (42). The two novel *Cryptosporidium* sequences and a *C. parvum* rabbit genotype sequence used to interpret results have been deposited with GenBank under the accession numbers AY458612 to AY458614.

RESULTS

β-Tubulin PCR-RFLP. Of the 423 specimens, 405 gave the expected 592-bp PCR product for the β-tubulin gene. PCR-RFLP analysis of this fragment showed that 188 isolates (46%) were *C. hominis* and 217 isolates (54%) were *C. parvum* bovine genotype.

18S rDNA PCR-RFLP and sequencing. The 18 human *Cryptosporidium* isolates that failed to produce a β-tubulin product were amplified by using the 18S rDNA primers. PCR-RFLP analysis was carried out, and the products were sequenced, showing 10 to be *C. hominis*, 6 to be *C. parvum* bovine genotype, and 2 to be a novel genotype. All 18 products were sequenced and aligned over ca. 734 bp, depending on the variation in length of the individual sequences, by using CLUSTAL X (Fig. 1). The two novel isolates were most similar to se-

FIG. 1. DNA sequence alignment through the variable region of the *Cryptosporidium* 18S rDNA, including the novel isolates 2683 and 2893 and the rabbit isolate. Nucleotide position is based on the 734-bp length of aligned sequence and, depending on gaps in the individual sequences, the actual positions are an approximation.

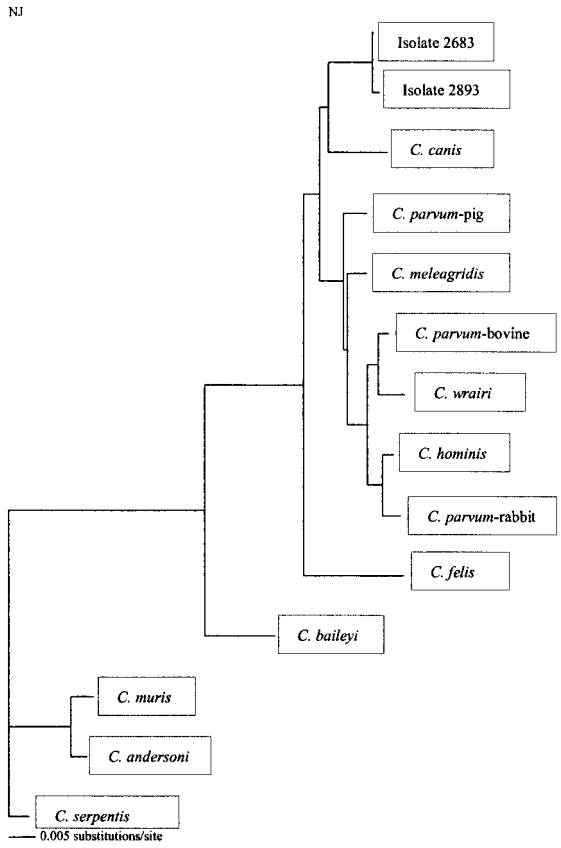

FIG. 2. Neighbor-joining tree analysis of *Cryptosporidium* 18S rDNA showing the phylogenetic relationship of two novel isolates to others in the genus. Analysis was based on genetic distances calculated by the general time-reversible model. *C. serpentis* was used as an outgroup with 1,000 bootstrap pseudoreplications.

quences obtained from dog isolates. A search of the GenBank database did not find an exact match for the two novel *Cryptosporidium* 18S rDNA sequences which differed from each other only by an A-to-G substitution at position 587. Phylogenetic analysis of the *Cryptosporidium* genus by neighbor joining also grouped the novel isolate most closely with *C. canis* (Fig. 2).

Genotype geographic distribution. Geographic distribution of the two main *Cryptosporidium* species causing human infection reflected the land use of the regions from which specimens were referred. The isolates recovered from the Hamilton specimens consisted of 40 (35%) *C. hominis*, 72 (64%) *C. parvum* bovine genotype, and 1 novel *C. canis*-like genotype. Isolates from Wellington were in direct contrast with 109 (89%) *C. hominis* and 13 (11%) *C. parvum* bovine genotypes. The remaining isolates were all from provincial New Zealand: 49 (26%) *C. hominis*, 138 (73%) *C. parvum* bovine genotype, and 1 novel *C. canis*-like genotype (Table 1). All provincial centers referred more *C. parvum* bovine genotype than *C. hominis* with the exception of Hawke's Bay, which referred 21 *C. hominis*-positive specimens during the autumn cryptosporidiosis peak and 15 *C. parvum* bovine genotype-positive specimens during the spring cryptosporidiosis peak. Southland, which is undergoing a boom in dairy farming, referred 35 positive specimens, 34 of which were of the *C. parvum* bovine genotype.

***Cryptosporidium* species seasonality.** There was a marked seasonal shift in transmission route between spring, when almost all human infections were caused by the zoonotic *C. parvum* bovine genotype, and autumn, when the anthroponotic *C. hominis* was dominant (Fig. 3). This pattern has been noted previously (14, 18).

DISCUSSION

We examined the distribution and transmission cycles of *Cryptosporidium* species in New Zealand's human population and evaluated the implications for public health. The results were similar to those of previous studies (18, 29).

One laboratory in Hamilton and another in Wellington forwarded their *Cryptosporidium*-positive fecal samples to the PRU over the period of the study. Other laboratories provided specimens on a more sporadic basis for a total of 423 speci-

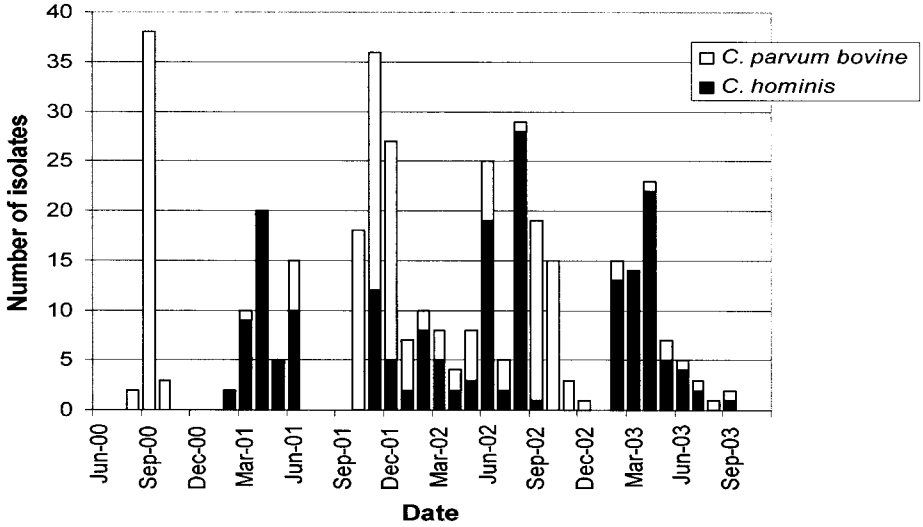

FIG. 3. Seasonal shift in transmission route between *C. parvum* and *C. hominis* human infections.

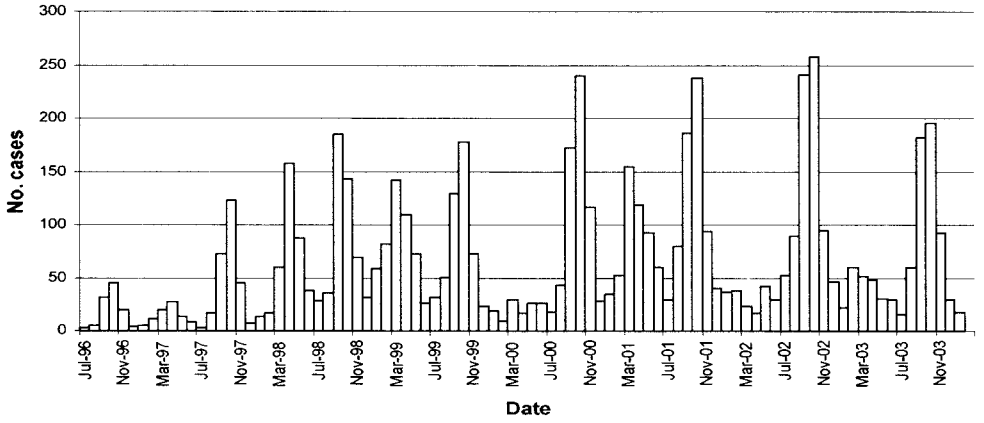

FIG. 4. National monthly number of human cryptosporidiosis cases since the disease became notifiable in July 1996.

mens. Enough specimens were received by the PRU for conclusions to be postulated.

PCR-RFLP of the β-tubulin gene was successful for 405 of the 423 human isolates, differentiating them into *C. hominis* and the *C. parvum* bovine genotype. Early in the study the 18S rRNA region of 20 of the 405 isolates was amplified and sequenced to check for agreement between the two methods. In every case, the results from β-tubulin PCR-RFLP and the sequenced 18S rDNA concurred. Also, there was no variation within the *C. hominis* sequences or the *C. parvum* bovine genotype sequences. PCR-RFLP of the β-tubulin gene is therefore a consistent, reliable, and rapid method that can be used with confidence for speciating isolates from human fecal samples.

In the present study, an isolate from the colon of a shot wild rabbit was characterized. Multilocus PCR-RFLP was performed on 18S rDNA, rDNA internal transcribed spacer region 1 (20), β-tubulin gene (5), *Cryptosporidium* oocyst wall protein gene (35), a polythreonine repeat motif (7), and the ribonucleotide reductase R1 subunit locus (39), with all of the results indicating *C. hominis*. The sequence, however, from the 18S rDNA region of this isolate was not identical to that of *C. hominis* (Fig. 1). This sequence was included in the phylogenetic analysis of strains for the present study (Fig. 2, *C. parvum*-rabbit). Although the sequence is unique and first published by Xiao, it is still most similar to the human genotype (32, 43).

A novel *Cryptosporidium* genotype that clustered closely to *C. canis* was detected in fecal specimens from two children (24 and 14 months of age): one in Hamilton and one in Upper Hutt (close to Wellington). Both strains were detected by nested 18S rDNA PCR, and then only a small amount of PCR product was produced, indicating a light oocyst load with a genotype that may have accidentally infected humans.

The overall numeric distribution of *Cryptosporidium* species was almost equally divided: 198 (46.8%) *C. hominis* specimens and 223 (52.7%) *C. parvum* bovine genotype specimens, along with 2 *C. canis*-like genotype specimens (0.5%). This distribution of *Cryptosporidium* species does not give the full picture, since when the provincial regions are compared to metropolitan Wellington there is a marked difference. Wellington had 109 (89%) *C. hominis* isolates and 13 (11%) *C. parvum* bovine genotype isolates, whereas the provincial regions included 89 (30%) *C. hominis* and 210 (70%) *C. parvum* bovine genotype isolates, with 2 *C. canis*-like genotype specimens. New Zealand's temperate climate allows livestock to remain outdoors on pasture year-round, with the Hamilton region having two calving seasons, resulting in a potential for an increase in the environmental load of oocysts. The spreading of ponded milking shed washings and meat-processing plant effluent as fertilizer onto the land, plus frequent mild rainfall, also disperses the oocysts over pastures and ultimately into rivers and streams. An earlier study found *Cryptosporidium* oocysts to be present in 12.6% of environmental and treated waters tested from throughout New Zealand (4). With today's better sampling, recovery, and detection procedures, this number would be expected to be higher. People living in Wellington have less opportunity for contact with farm animals but would have more chance of being involved in outbreak situations, be it transmitted person to person, water borne, or food borne. The lack of access to farm animals rather than the lack of animals has previously been suggested as an explanation for a decline in cryptosporidiosis caused by *C. parvum* bovine genotype (11).

Cryptosporidiosis has been a notifiable disease in New Zealand since July 1996, with case numbers rising each year. New Zealand's infection rate (i.e., cases per 100,000) for the years 2001 and 2002 were 32.3 and 26.1, respectively; however, the rates for both Hamilton (61.2 and 40) and Wellington (37.4 and 61) were above the national rate (3). Although the national infection rate fell during 2002, Wellington's infection rate increased to 61 as it experienced 2 swimming pool outbreaks resulting in 72 cases of cryptosporidiosis with *C. hominis*. Notified cases have a distinct seasonal pattern, usually showing two clear peaks during spring and autumn (3) (Fig. 4). The autumns of 2000 and 2002, however, did not show peaks, which might be attributed to below average rainfall during autumn for those years (33), and showed a lack of outbreaks. Spring is the main calving and lambing season and, since *Cryptosporidium* is a disease of young animals, this is the time of year when there is a high environmental load with the *C. parvum* bovine genotype. The *C. parvum* bovine genotype was responsible for almost all of the spring cryptosporidiosis cases before a complete change of transmission cycle saw *C. hominis* becoming dominant in the autumn. This seasonality was reflected in the Hamilton specimens, with *C. hominis* prevalent

in the autumn and *C. parvum* bovine genotype prevalent in the spring. Wellington referred fewer positive fecal specimens in the spring, which may reflect the lack of access to young infected animals by its population. Figure 3 shows this seasonal shift in transmission cycles. Since humans are usually infected only by *C. hominis* or *C. parvum* bovine genotype, a more discriminating method, other than PCR-RFLP and 18S rDNA sequencing, may be needed to investigate outbreaks for potential sources.

New Zealand's climate and animal husbandry and farming practices are ideally suited to the spread and survival of *Cryptosporidium* oocysts. Fencing off rivers and streams or planting riparian buffer zones along stream banks could restrict the access of livestock and their excreta to natural waterways. At present, the government is considering submissions on public access across private land to natural waterways, with the fencing of buffer zones being one possibility. Fonterra, New Zealand's largest dairy company, which is a cooperative owned by farmers, is actively encouraging its shareholders to fence off esplanade strips along natural waterways to reduce the concept of "dirty dairying." Effective wastewater management of effluent from farms, meat-processing plants, settlements, towns, and cities could interrupt the transmission cycle, along with keeping the water reticulation of toddlers' public swimming pools separate from the main swimming pool. The tracing of outbreak sources requires molecular techniques capable of discriminating differences within *Cryptosporidium* species and genotypes. Vigilance with public health education programs and strict adherence to the New Zealand Drinking Water Standards would also help to lower the infection rate.

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