

Seasonality of *Cryptosporidium* oocyst detection in surface waters of Meru, Kenya as determined by two isolation methods followed by PCR

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ABSTRACT

Meru, Kenya has watersheds which are shared by wildlife, humans and domesticated animals. These surface waters can be contaminated by the waterborne pathogen *Cryptosporidium*. To quantify the seasonality and prevalence of *Cryptosporidium* in Meru regional surface waters, we used a calcium carbonate flocculation (CCF) and sucrose floatation method, and a filtration and immunomagnetic bead separation method, each of which used PCR for *Cryptosporidium* detection and genotyping. Monthly water samples were collected from January through June in 2003 and 2004, bracketing two April-May rainy seasons. We detected significant seasonality with 8 of 9 positive samples from May and June ($p < 0.0014$), which followed peak rainy season precipitation and includes some of the subsequent dry season. Six of 9 positive samples revealed *C. parvum*, and 3 contained *C. andersoni*. None contained *C. hominis*. Our results indicate that Meru surface waters are *Cryptosporidium*-contaminated at the end of rainy seasons, consistent with the timing of human infections reported by others from East Africa and contrasting with the onset of rainy season peak incidence reported from West Africa.

Key words | calcium carbonate flocculation, *Cryptosporidium*, Kenya, PCR detection, seasonality, US EPA Method 1623

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INTRODUCTION

Cryptosporidium is a protozoan parasite which usually causes asymptomatic or mild human clinical disease, although severe dehydrating disease and death can occur in immunocompromised populations, such as HIV-infected people. No clearly effective therapy for cryptosporidiosis in immunocompromised people exists (Griffiths 1998; Tzipori & Ward 2002; Abubakar *et al.* 2007), and thus the avoidance of *Cryptosporidium*-contaminated water is important. Zoonotic and human-to-human transmission occurs, often via surface and recreational waters, and the disease is underdiagnosed except in those with persistent diarrhea (Bagley

et al. 1998; Griffiths 1998; Hunter *et al.* 2001; Rose *et al.* 2002). Prevalence rates of ~ 10% to 33% in those with AIDS have been reported from developing countries (Kaplan *et al.* 1996; reviewed in Griffiths 1998). Malnutrition, which impairs cellular immunity, is another recognized risk factor for cryptosporidiosis (Gendrel *et al.* 2003).

We report initial data on *Cryptosporidium* in surface waters of Meru, Kenya, which lies north of Nairobi. Watersheds in Meru arise on Mt. Kenya in wildlife preserves, and water flows east through intensively farmed, densely populated areas before reaching arid, pastoral areas. Thus, Meru

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surface waters could be contaminated by parasite oocysts from wildlife, human, or pastoral animals, and infect people with AIDS or malnutrition as well as others in the general population. In this pilot study, we repetitively collected monthly samples from predetermined sites during January to June, which is before, during, and after the intensively rainy period of April and May. We also field tested two methods for parasite isolation and detection from surface waters during 2003 and 2004. Our approach included *Cryptosporidium* molecular genotyping of the positive water samples so as to better understand the potential sources of the parasites.

MATERIALS AND METHODS

Water collection sites

Meru water supplies are untreated surface waters taken directly from rivers or transient, seasonally flooded areas, which may be piped to households or common urban water distribution points. Water collection sites are often common watering points for domestic and wild animals. Ten sites were sampled. Five sites were on the Kathita River, which supplies Meru Town and surrounding villages. The highest Kathita River site (site A, [Figure 1](#)) is where the river exits a protected wildlife forest preserve. Downstream sites, coded B, C, E, and F, reflect a graded transition of increasing human and animal influence. Site C is the intake for the Meru Town municipal water system, which is chlorinated but unfiltered. Site D is the sewage outflow from Meru Town. Four other sites were chosen because of mixed human and animal use. These included a forest pool where elephants drink (Luuria, site G), and two sites near Kiina Town northeast of Meru Town. Kiina Tap (H) and Kiina River (I) are used by pastoralists for their herded animals, and by wildlife such as elephants, as they border Meru National Park. Miriti Well (site J), one of a group known as the Kiina Wells, is further northeast and is used by both pastoralists and wildlife. Sites H - J are in Isiolo District, and are in the far eastern, lower parts of the Meru ecosystem. Sites were mapped using a global positioning satellite (GPS) monitor ([Figure 1](#)). Under ideal conditions, all sites could be visited in a 3 day period excepting Miriti Well which required a fourth day.

Sampling framework and precipitation data

Samples were obtained during the first six months of 2003 and 2004, bracketing a major precipitation period which occurs in March and April. This period was chosen so that we could analyze 2003 samples and revise our 2004 sampling strategy if required. Precipitation data was obtained from the Meteorology Department, Ministry of Transport and Communication, Meru Weather Station, housed at Kenya Methodist University (KEMU) in Meru.

Sampling methods

We used a calcium carbonate flocculation (CCF) and sucrose gradient floatation technique in 2003, and a filtration method in 2004, each followed by immunomagnetic isolation of parasite oocysts and PCR analysis. Prior work ([Kato *et al.* 2003](#)) documented that the CCF method could successfully isolate oocysts from Meru waters.

Calcium carbonate flocculation (CCF) followed by sucrose gradient floatation

Ten litres of water were collected into plastic containers certified as free from contaminants (Fisher Scientific, Pittsburgh, USA). Samples were brought to KEMU, saturated with CaCl_2 and NaHCO_3 , and adjusted to pH 10 with NaOH ([Vesey *et al.* 1993](#)). Insoluble CaCO_3 crystals were allowed to settle and the supernatant (~9 L) discarded. The precipitate was dissolved in sulfamic acid. $\text{K}_2\text{Cr}_2\text{O}_7$ (25 g per L) was added to kill pathogens until processing, which was typically within a week. Samples were centrifuged at $5,000 \times g$ for 10 minutes, and the supernatant was discarded leaving ~20 ml of concentrated suspension. The suspension was thrice washed with distilled water at $5,000 \times g$ for 10 min at 4°C with supernatant removal via vacuum leaving 3 ml of suspension and sediment. The washed sediment then had distilled water added to a total volume of 7 ml and was thoroughly mixed. Seven ml of sucrose solution (specific gravity 1.18) was layered below the sediment solution via syringe, and centrifuged at $3,000 \times g$ for 20 minutes at 4°C in a 15 ml polystyrene tube. Two separate layers were obtained, with soil sediments at the bottom. The top 2 ml of the sucrose layer was removed using a Pasteur pipette, diluted with water in

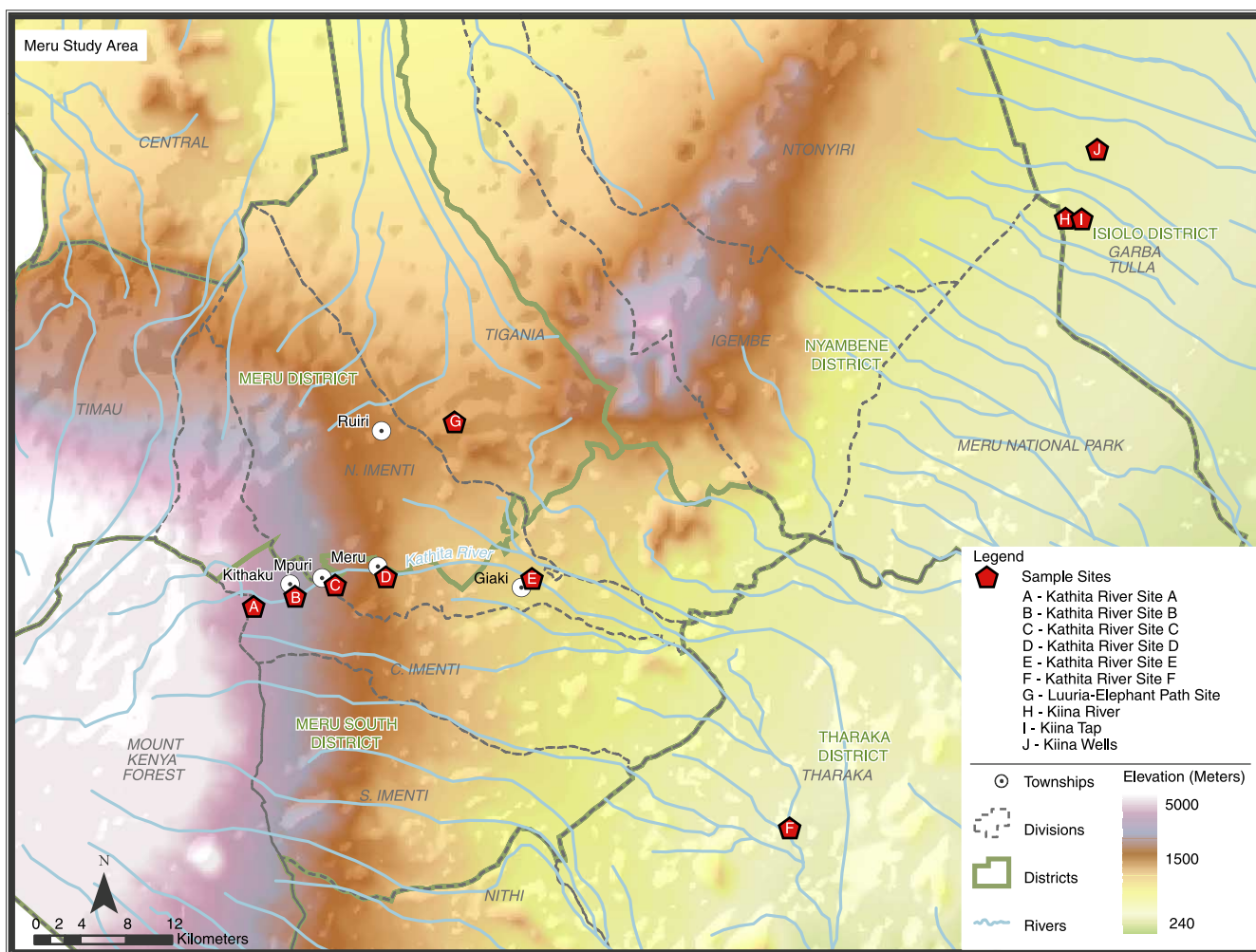


Figure 1 | Map of the Meru, Kenya Region.

another 15 ml tube, and centrifuged at $1,800 \times g$ for 3 min at 4°C . The supernatant was aspirated leaving $500 \mu\text{l}$. This was repeated 3 times leaving a final volume of $500 \mu\text{l}$ which was subjected to immunomagnetic separation (IMS).

Filtration

For consistency between Boston and Meru, we used US Environmental Protection Agency (EPA) Method 1623, as our Boston laboratory is required by the EPA to consistently only use Method 1623, given its certification for *Cryptosporidium* and *Giardia* testing in US ambient surface waters (US EPA 2005). In Kenya, water samples were pumped through a Filta-MaxTM (IDEXX, Westbrook, ME)

filter in a polycarbonate housing, using a car-battery powered pump. Samples ranged from 30 to 40L, with the exact volume measured by an in-line meter. The filter was stored in a glass jar with 300 ml of phosphate-buffered saline (pH 7.2, 0.01% Tween 20) and transported to KEMU. Oocysts were washed out of the filter according to the manufacturer's instructions (IDEXX 2002). In brief, a bolt is released allowing the compressed filter to expand, allowing retained oocysts to be eluted from the sponge matrix. The wash solution is filtered, and oocysts trapped on a membrane are eluted into ~ 10 ml of fluid. The sample is centrifuged to a pellet of < 0.5 ml and then purified by IMS (see below). If the pellet was > 0.5 ml the sample was split per Method 1623.

Immunomagnetic Separation (IMS) and DNA extraction

IMS was conducted using the Dynabeads GC-Combo (DynaL Biotech, Oslo, Norway) protocol. In brief, anti-*Cryptosporidium* antibodies attached to magnetic beads were mixed with samples prepared as described above to a volume of 1 ml. The oocysts were removed through magnetic removal of the beads, then dissociated from the beads using an acid wash step. The acid wash supernatant was aspirated and used for DNA extraction. Oocyst extracts underwent five freeze thaw cycles after the addition of 180 μ l of tissue lysis buffer (50 Mm Tris-HCl [pH 8.5], 1Mm EDTA [pH 8], 0.5% sodium dodecyl sulfate) in cryovials which were incubated for 1 minute in liquid nitrogen and 1 minute in a 56°C heating block per cycle. Samples were centrifuged at 14,000 \times g for 30 s and then incubated with 20 μ l proteinase K (Sigma, St Louis, MO.) for 2 hours at 55°C while being rotated, incubated at 90°C to denature the proteinase K, chilled on ice for 1 minute, and centrifuged at 14,000 \times g for 3 minutes. Supernatants were transferred to a new 1.5 ml tube, 200 μ l of ethanol was added, then vortexed for 15 seconds. The mixture was loaded into a QIAmp spin column (Qiagen Inc, Valencia, California) and centrifuged at 6,000 \times g for 1 minute. This was repeated using wash buffers described by the manufacturer. The final supernatant was stored at -20°C for use in PCR.

PCR and RFLP Protocols

Nested PCR followed by restriction enzyme digestion was carried out using SSU primers, and VSP1 and SSPI enzymes respectively (Xiao et al. 1999). Reaction volumes of 100 μ l consisted of premixed reagents containing 10 mM of each of the four deoxynucleoside triphosphates, 10 \times PCR buffer, 25 mM MgCl₂, PCR grade water and Taq polymerase (Invitrogen). 2 μ l of template DNA was used. The primary run was done using 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCATTTCCTTCGAAACAGGA-3' primers. The samples were incubated in the thermocycler at 94°C for 3 minutes to denature template DNA. This was followed by 35 cycles, each consisting of 94°C for 45 s, 55°C for 45 s and 72°C for 1 minute and a final extension at 72°C for 10 minutes. A secondary amplification using the primary products the primers

5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA-3' was performed using cycling conditions identical to those in the primary PCR. Amplicons were fractionated on a 2% agarose gel and visualized by ethidium bromide staining to confirm the presence of characteristic *Cryptosporidium* products (Figure 2). To differentiate genotypes, 10 μ l of positive secondary PCR products were digested in a 40 μ l (total volume) reaction mixture containing 20 U of SspI (New England Biolabs, Beverly, Mass.) or 20 U of VspI (GIBCO BRL, Grand Island, N.Y) and 4 μ l of the appropriate restriction buffer at 37°C for 1 hr (Xiao et al. 1999). As *C. andersoni* and *C. muris* have identical SspI and VspI restriction patterns, they were differentiated by digesting secondary products with 20 U of DdeI (New England Biolabs) at 37°C for 1h under conditions recommended by the supplier. Digested products were fractionated on 2% agarose gel and visualized by ethidium bromide staining. All positive detections were confirmed in duplicate by RFLP analysis of additional, independent PCR products from the same water sample. Positive controls included

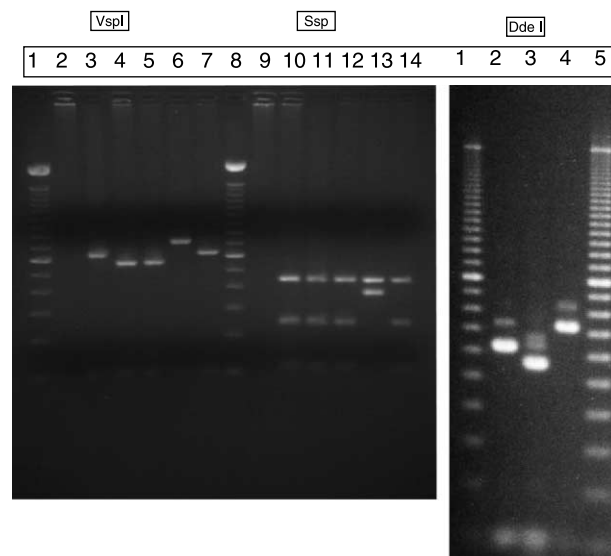


Figure 2 | The results of restriction fragment length polymorphism (RFLP) analysis using Ssp1, Vsp1, and Dde1 for species and genotype determination respectively. Panel A: Lane 1: 100 bp ladder; 2: negative control; 3: TU502 (*C. hominis*); 4: IOWA (*C. parvum*); 5: Water sample (Karinda); 6: Water sample (Kiina river); 7: *C. hominis* spiked stool sample; 8: 100 bp ladder; 9: negative control; 10: TU502 (*C. hominis*); 11: IOWA (*C. parvum*); 12: Water sample (Karinda); 13: Water sample (Kiina river); 14: *C. parvum* spiked stool sample. Panel B: Lane 1: 100 bp ladder; 2: *C. parvum*; 3: *C. hominis*; 4: *C. andersoni*; 5: 100 bp ladder.

TU502 *C. hominis* (Akiyoshi *et al.* 2002), obtained from Dr. Saul Tzipori, Tufts University Cummings School of Veterinary Medicine, North Grafton, MA and the Iowa *C. parvum*, obtained from Pleasant Farms, Troy, ID (Abrahamsen *et al.* 2004).

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 15. Specific comparisons (*t*-tests and chi-square analyses) are denoted in the text.

RESULTS AND DISCUSSION

Oocyst detection: by method and by season

Monthly samples were collected and analyzed for January through June 2003, and January through June of 2004. In 2003 we used the CCF method, and in 2004 the filtration method. All sample concentrates underwent PCR for the detection of oocysts. We thus sampled 6 months of both low and high rainfall in two sequential years. In 2003, all 10 sites were sampled monthly. In 2004, sampling at Kiina Wells became dangerous and was discontinued, as drought had increased the chance of interactions with wild elephants. In January and June of 2004, adverse road conditions made it impossible to visit several other sites.

108 samples were acquired and 9 (8.3%) were PCR positive for oocysts (Table 1). Two of 60 (3.3%) samples obtained in 2003, versus 7 of 48 (14.6%) samples from 2004,

were positive. The detection rate in 2004 was 4.375 fold higher than in 2003, which did not reach statistical significance ($p = 0.075$, 2-tailed Fisher's exact test). Oocyst detection rates varied significantly by month ($\chi^2 = 13.53$, 5 df, $p < 0.019$). Table 2 displays detection rates before, during, and after the peak in precipitation which occurred in March and April of each year, together with monthly cumulative precipitation for each time period. The apparent seasonal period peak of detection at the end of the rainy season is significant ($\chi^2 = 13.17$, $p < 0.0014$). Figure 3 displays precipitation data recorded in Meru. Two peaks of precipitation usually occur each year. The years of 2003 and 2004 were atypical in that there was rainfall in the months separating the late 2003 and early 2004 precipitation peaks. The months when we detected oocysts corresponded to the nadirs immediately following seasonal peaks of precipitation (June 2003, March 2004, June 2004) or the descending right-hand slope of a period of decreasing precipitation after a peak (May 2004).

Genotypes of *Cryptosporidium* detected in water samples

C. parvum was detected six times. It was isolated three times from the sewage waste stream from Meru Town, from Meru Town's water intake site, from a site downstream of Meru Town, and from a pool of water frequently used by elephants (Table 3). *C. hominis* was not detected from any site. *C. andersoni* was detected 3 times (Table 3). This species has been linked to cattle and does not appear to be

Table 1 | Temporal pattern of *Cryptosporidium* detection in Meru, Kenya waters, using a calcium chloride precipitation technique (CCF) or filtration, followed by immunomagnetic bead separation and PCR

Month	2003 testing using CCF			2004 testing using filtration			Totals based on both years		
	Positive	Negative	Total 2003	Positive	Negative	Total 2004	Positive	Negative	Total
January	0	10	10	0	4	4	0	14	14
February	0	10	10	0	9	9	0	19	19
March	0	10	10	1	8	9	1	18	19
April	0	10	10	0	9	9	0	19	19
May	0	10	10	4	5	9	4	15	19
June	2	8	10	2	6	8	4	14	18
Total	2	58	60	7	41	48	9	99	108

Table 2 | *Cryptosporidium* water sample testing by seasonal period

Water sample testing by seasonal period			
Seasonal period, cumulative precipitation in mm water (Mean \pm SD)	Positive	Negative	Total
Before peak rainfall (January–February), 54 \pm 44 mm	0	33	33
Peak rainfall (March–April), 207 \pm 204 mm	1	37	38
After peak rainfall and start of traditional dry season (May–June), 41 \pm 48 mm	8	29	37
Totals	9	99	108

pathogenic to humans. All 3 *C. andersoni* isolates were detected from the Kiina River, where pastoralists water their herds of cattle.

DISCUSSION

In this pilot study, we detected *Cryptosporidium* surface water contamination in the Meru, Kenya region over the first six months of two sequential years (2003 and 2004). The period at the end of the sampled rainy season, and the beginning of the following drier, cool season (May and June) was significantly associated with oocyst detection in surface waters. This corresponded to the nadirs of precipitation between precipitation peaks, or to periods of tapering rainfall (Figure 3).

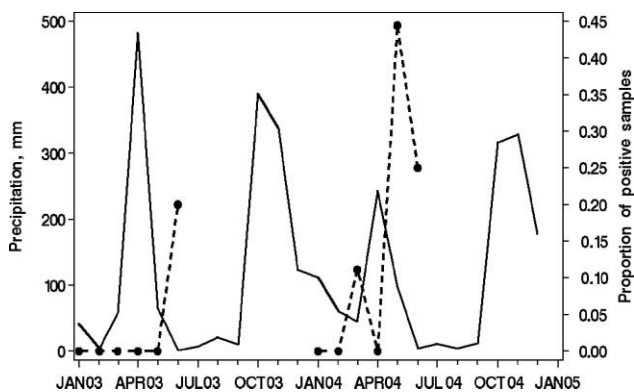


Figure 3 | Actual monthly precipitation in millimetres in Meru, Kenya by month of 2003 and 2004 and proportion of positive Meru Region surface water samples by month of 2003 and 2004. The solid black line represents precipitation in mm (left axis scale), and the dotted black line represents the proportion of positive surface water samples (right axis scale), by month (x axis, horizontal).

Cryptosporidium oocysts are believed to be flushed by rain into surface waters, and so one might have expected peak detection during the onset of the rainy months. During March and April, the volume of water which falls is so great that it is possible that the concentration of oocysts in surface waters is relatively low, because of dilution. Alternatively, there may be little *Cryptosporidium* transmission during March and April in Meru, and thus there may be fewer fecally excreted parasites to be swept into water bodies. Our surface water data does not allow us to speculate further on the merits of these and other possible explanations regarding transmission density, as human and animal cryptosporidiosis incidence data is not yet available from Meru.

However, data from other East African sites may be informative. In a recent survey of 4,899 pediatric fecal samples from laboratories near Nairobi, the rainy months of April and May had the lowest infection prevalence (1.5%), suggesting that transmission is less common during this period (Gatei et al. 2006). Infection rates were highest in children during the November to February period, which usually has little precipitation. Studies from Rwanda, Malawi, and Kenya (Bogaerts et al. 1987; Peng et al. 2003; Gatei et al. 2006), respectively, report that the peak incidence of cryptosporidiosis is at the end of rainy seasons and beginning of the drier months, as has a study by Moodley et al. (1991) in Durban, South Africa. In contrast, in Guinea-Bissau, West Africa, the peak prevalence of clinical cryptosporidiosis in children is just before, or at the onset of, the rainy season from May to July (Molbak et al. 1990, 1993; Perch et al. 2001). Similar results were reported from Zambia (Nchito et al. 1998). The East African human incidence pattern is congruent with our findings regarding oocysts in surface waters. It is possible that the apparent seasonality of surface water contamination and of human disease, is reflective of different transmission pathways, hosts, and/or *Cryptosporidium* species in East Africa when compared to other locations. We note that as climate change occurs, transmission patterns of many waterborne diseases may shift, and studies in locations with unusual seasonality patterns may help inform our understanding of what climate change may bring (Rose et al. 2002; Hunter 2003; Jagai et al. 2007; Naumova et al. 2007).

Table 3 | Genotypes of detected *Cryptosporidium* by month and year, and by site

Genotypes of Detected <i>Cryptosporidium</i> by time and site			
Year	Month	Genotype	Site
2003	June	<i>C. parvum</i> (formerly called bovine genotype Type 2)	Site D. Karinda (sewage waste stream from Meru Town) on the Kathita River
2003	June	<i>C. andersoni</i>	Site G. Kiina River
2004	March	<i>C. andersoni</i>	Site G. Kiina River
2004	May	<i>C. parvum</i>	Site F. Luuria (standing pool in elephant path in forest)
2004	May	<i>C. parvum</i>	Site C. (Intake for Meru Town water supply), Kathita River
2004	May	<i>C. parvum</i>	Site D. Karinda
2004	May	<i>C. andersoni</i>	Site G. Kiina River
2004	June	<i>C. parvum</i>	Site E. Tharaka (Marimanti) on the Kathita River
2004	June	<i>C. parvum</i>	Site D. Karinda

A limitation of our study is that we did not directly compare our two oocyst isolation methods in a side-by-side evaluation during the same year. We substituted the filtration method for the CCF method after unexpectedly low rates of isolation in 2003. While the direct field filtration of 30–40 L of water led to higher PCR detection rates than did the CCF method with 10 L samples, this did not reach statistical significance. The differences in detection that we encountered might be due to the natural variation between the years tested; inhibition of PCR by environmental contaminants (Sluter *et al.* 1997; Loge *et al.* 2002); or additional losses encumbered in the “double purification” via CCF and then IMS. Egorov *et al.* (2002) and Karanis *et al.* (2006) found CCF useful when oocysts were detected via immunofluorescent identification. Although Monis & Saint (2001) reported that the precipitation method is inhibitory for PCR detection, preliminary data (Kato *et al.* 2003) had suggested that our initial approach would be successful. In order to address this issue properly, side by side comparisons of the two methods would be needed which would quantify overall recovery efficiency, as we may easily have suffered from falsely negative results in 2003. We used PCR methods for detection, rather than visual ones, because of our interest in the genotypes of the *Cryptosporidium* that might be found in this ecologically unusual region.

C. parvum, but not *C. hominis*, was thrice detected from human sewage waste waters from Meru Town, as well as from the intake site for the town water supply and

downstream of Meru Town. Studies from Kenya and Uganda (Tumwine *et al.* 2005; Gatei *et al.* 2006) show that *C. hominis* is the dominant species infecting children in the urban centers of Nairobi and Kampala. *C. parvum* is adapted to multiple host species, including wildlife, humans and cattle (Fayer 2004). *C. hominis* has only been reliably detected in primate hosts, although two studies report its detection in dugongs (Morgan *et al.* 2000) and cattle (Smith *et al.* 2005). Cattle herding is uncommon in the intensely farmed Meru Town region, while pastoralist herding is more common northeast of Meru Town. We detected *C. andersoni* thrice from the Kiina River, where pastoralists water their cattle. *C. andersoni* is not thought to be pathogenic for humans, and is dominantly a parasite of older cattle. *C. parvum* infects HIV infected and uninfected children, while *C. meleagridis* and *C. muris* are more frequently found in HIV-infected persons (Caccio 2005). In the rural regions of Kenya such as Meru, the spectrum of *Cryptosporidium* species infecting humans may differ from that in urban areas given different land use patterns (Naumova *et al.* 2005) and the use of the same water by wildlife as well as by people and herded animals. Our data is consistent with, but not proof of, endemic waterborne *Cryptosporidium* transmission in Meru, including potential transmission between wildlife, domestic animals, and humans. Further environmental, epidemiological, and molecular investigations are needed to confirm or refute this thesis. In particular, molecular analysis of *Cryptosporidium* isolates at the sub-type level from

potential animal reservoirs, surface waters and humans may be helpful in this regard (Ajjampur *et al.* 2007). We find it intriguing that *C. parvum* was detected from a pool after use by migrating elephants at Luuria, as according to the local population these waters cause diarrhea in people after the elephants have passed through.

CONCLUSION

The Meru region of Kenya is an appropriate ecological zone for studying potential transmission through water between wildlife, humans and domesticated animals. We document *C. parvum* in the Kathita River, which serves all these groups, as well as *Cryptosporidium* from waters which serve humans, cattle and wildlife, such as elephants. Oocysts were significantly more commonly detected in surface waters at the end of rainy seasons and at the beginning of dry seasons, congruent with studies which have shown peak human disease during the same period in East Africa. This seasonality differs from that noted in West Africa and northern temperature regions. Continued environmental, epidemiological, and molecular studies will be needed to address questions regarding the actors in, and timing of, transmission in this region.

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