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Research Article

Clinical and Microbiological Investigation of Zoonotic Cryptosporidiosis in two Children by Routine Diagnostic Methods and Quantitative Polymerase Chain Reaction

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Abstract

To investigate the applicability of diagnostic assays for detection of the protozoan parasite *Cryptosporidium parvum* throughout the course of natural zoonotic infections, and to compare oocyst loads with clinical presentation, sequential stool samples from two naturally infected, volunteer siblings were tested by modified Ziehl-Neelsen (mZN), auramine phenol (AP), and immunofluorescence microscopy, enzyme immune assays (EIA) and quantitative PCR (qPCR). *Cryptosporidium* was detected by immunofluorescence microscopy, EIA and qPCR but not by mZN or AP in soft stools passed after acute clinical episodes of cryptosporidiosis. During recuperation, samples were positive only by IFM and qPCR of DNA extracted directly from stools; the latter provided the highest diagnostic index and intermittent detection up to 18 days after recovery from all symptoms. Additionally, quantification by qPCR correlated with symptom severity and clinical presentation in the two patients studied.

ABBREVIATIONS

Qpcr: Quantitative Polymerase Chain Reaction; IFM: Immunofluorescence Microscopy; AP: Auramine Phenol; ELISA: Enzyme-Linked Immunosorbent Assay; Mzn: Modified Ziehl-Neelsen; CT: Cycle Threshold; Opg: Oocysts Per Gram of Stool

INTRODUCTION

The protozoan parasite *Cryptosporidium* is a major cause of gastroenteritis that has been identified as one of the most common aetiological agents of moderate to severe diarrhoea in children, posing a significant risk of death in toddlers, in sub-Saharan Africa and South East Asia [1]. Acute infection outcomes range from asymptomatic carriage to severe diarrhea depending on the age, nutritional status and immunity of the host, and may be influenced by the *Cryptosporidium* species and isolate [2]. Two species predominate in human cryptosporidiosis in most settings, *Cryptosporidium hominis* which is transmitted anthroponotically, and *Cryptosporidium parvum* which is also zoonotic [2]. Studies in the UK, where the mean annual number of reported, laboratory confirmed cases in England and Wales between 2000 and 2012 was 4181 [3], have shown that *Cryptosporidium* species distribution is linked not only to exposures [4] but also to demographic, social, geographical and environmental factors [5,6]. One study estimated that 25% of sporadic *C. parvum* cases were attributable to direct contact with farmed animals [7].

Cryptosporidiosis is under-ascertained even in high-income countries and one contributory factor is the diagnostic assay used [8]. Previously, we have shown that the diagnostic sensitivity and specificity of *Cryptosporidium* assays currently in use in primary diagnostic laboratories in the UK varies [9]. PCR and immunofluorescence microscopy (IFM), provide the highest diagnostic index. When PCR and IFM were used as the

nominated gold standard, the comparative diagnostic sensitivity of auramine phenol (AP) microscopy and three enzyme-linked immunosorbent assays (ELISA) was greater than modified Ziehl-Neelsen (mZN) microscopy and an immunochromatographic test [9]. A similar trend in diagnostic index was reported for PCR, ELISA, AP microscopy and mZN microscopy when stools from high-risk HIV-AIDS patients in India were tested [10]. Although most methods are sufficiently sensitive for diagnosing acute disease, samples requiring diagnosis for infection control purposes or epidemiological investigations may be obtained late in clinical illness or even after patients have recovered or from asymptomatic carriers. To investigate the most appropriate diagnostic assays during the course of natural *Cryptosporidium* infections in children, sequential samples and clinical histories were obtained from two volunteer siblings.

MATERIALS AND METHODS

Clinical history and diagnosis

A seven year old male (child A) who lived on a mixed-livestock family farm was diagnosed with cryptosporidiosis by mZN microscopy of a stool sample collected on 13th May 2013, three days after the onset of gastrointestinal symptoms (Figure 1). The stool sample was subsequently referred to the national *Cryptosporidium* Reference Unit for species identification based on a real-time PCR [11] and genotyping by nested PCR and partial sequencing of the gp60 gene [12,13]. On 14th May, child A's six year old sister (child B) also became symptomatic but a stool sample was not submitted for diagnosis at the time, as it was assumed that she too had cryptosporidiosis.

On 30th April, three-day-old orphan lambs (n=3) had been brought onto the farm for hand-rearing by the children, as in previous years (Figure 1). They bottle-fed the lambs twice daily until child A became ill, when they ceased to do this.

Bottle feeding was done over the gates of the lambs' pen. The children washed their hands afterwards. The lambs had come from a commercial farm, where a four-year-old child who played with the lambs developed diarrhoea, vomiting and dehydration requiring hospitalization, and was diagnosed with cryptosporidiosis by mZN microscopy. However, the sample was not sent for genotyping. Her grandmother developed diarrhoea and vomiting with abdominal cramps, requiring four days off work.

As a public health response to the diagnosis of human cryptosporidiosis, advice was provided by the Local Authority on the prevention of spread [14]. On 21st May, three samples of the orphan lamb faeces were collected from the floor of their pen on the adoptive farm and four (three individual, one pooled) samples were collected at the original farm. Oocysts were sought by IFM (CryptoCel, TCS Biosciences) of faecal smears at the Animal and Plant Health Agency (APHA) laboratory in Weybridge. *Cryptosporidium*-positive faeces were sent to the CRU for detection of *C. parvum* by a real-time PCR [11] and other species by sequencing ~830 bp amplicons from nested PCR of the ssu rRNA gene [15]. Genotyping was undertaken by sequencing part of the gp60 gene [12,13].

Longitudinal study

The children on the adoptive farm were curious about the *Cryptosporidium* diagnosis and likely course of their infection; the family approached the authors at Swansea University Medical School, volunteering to send further samples. This provided a unique opportunity to investigate which diagnostic tests would be best for monitoring natural human infection both clinically and microbiologically. The clinical courses of both children were recorded daily from the onset of symptoms until 16th June. Stool samples were collected up to this date, representing sequential samples from eight to 37 days post-onset of symptoms for child A and from four to 33 days post-onset of symptoms for child B (Figure 1). Stool types according to the Bristol stool chart [16] and timing of clinical symptoms are shown in Figure (1).

Stools were stored at -80 °C before transfer to Swansea for testing by the following diagnostic methods: ELISA in plate and cartridge format performed as per manufacturer's instructions (TechLab *Giardia/Cryptosporidium* Chek and *Giardia/Cryptosporidium* Quik Chek, respectively), mZN and AP microscopy [17] and IFM (Crypto-Cel; TCS Biosciences, UK). ELISA reactions were recorded as positive or negative, mZN and AP microscopy as "oocysts seen" or "oocysts not seen" and IFM scored based on the average number of oocysts seen per field of view (from examination of at least 50 fields of view at x400 magnification): 0, oocysts not seen; 1, one oocyst seen; 3, two to five oocysts; 4, six to ten oocysts; 5, >10 oocysts seen. Scores were converted to estimated numbers of opg [18].

Additionally, DNA was extracted both directly from stools using the QIAamp FastDNA stool kit (Qiagen) with an extended incubation at 95 °C as per manufacturer's instructions, and from a thermally treated, semi-purified, salt floated suspension of oocysts using the QIAamp DNA mini kit (Qiagen) as described previously [19]. DNA extracted by both methods was tested in the same PCR run using the *C. parvum* part of a real-time PCR assay [11] as the infecting species was known from child A's diagnostic sample. The assay was used in quantitative format incorporating a commercial, non-competitive (primer limited) internal control (Primerdesign Ltd., UK) to assess the effect of any PCR inhibitors. To generate a standard curve for quantification, a five-point, 10-fold dilution series of *C. parvum* (Moredun isolate; Creative Science Company, UK) DNA equivalent to 5 x 10^4 to 5 oocysts μl^{-1} was included. The dsDNA was measured (Qubit, Life Technologies, UK) prior to dilution in nuclease-free water. The CT values for the test samples were converted to oocyst-equivalent per g (opg) of stool using the standard curve data analysed with the RotorGene 6000 software programme v1.7 (Corbett Research, UK). Estimates of opg derived from CT values were compared between DNA extraction methods using a Mann-Whitney U-test and with IFM scores over the course of infection and clinical presentation for each child.

Three samples per child (from the beginning, middle and end of positive detection period by qPCR) were also genotyped by sequencing the partial gp60 gene as described above [12,13].

Sequences generated from this study have been placed on GenBank, accession numbers KX495669 – KX495672.

RESULTS AND DISCUSSION

Clinical history and diagnosis

The clinical course and microbiological outcomes of testing

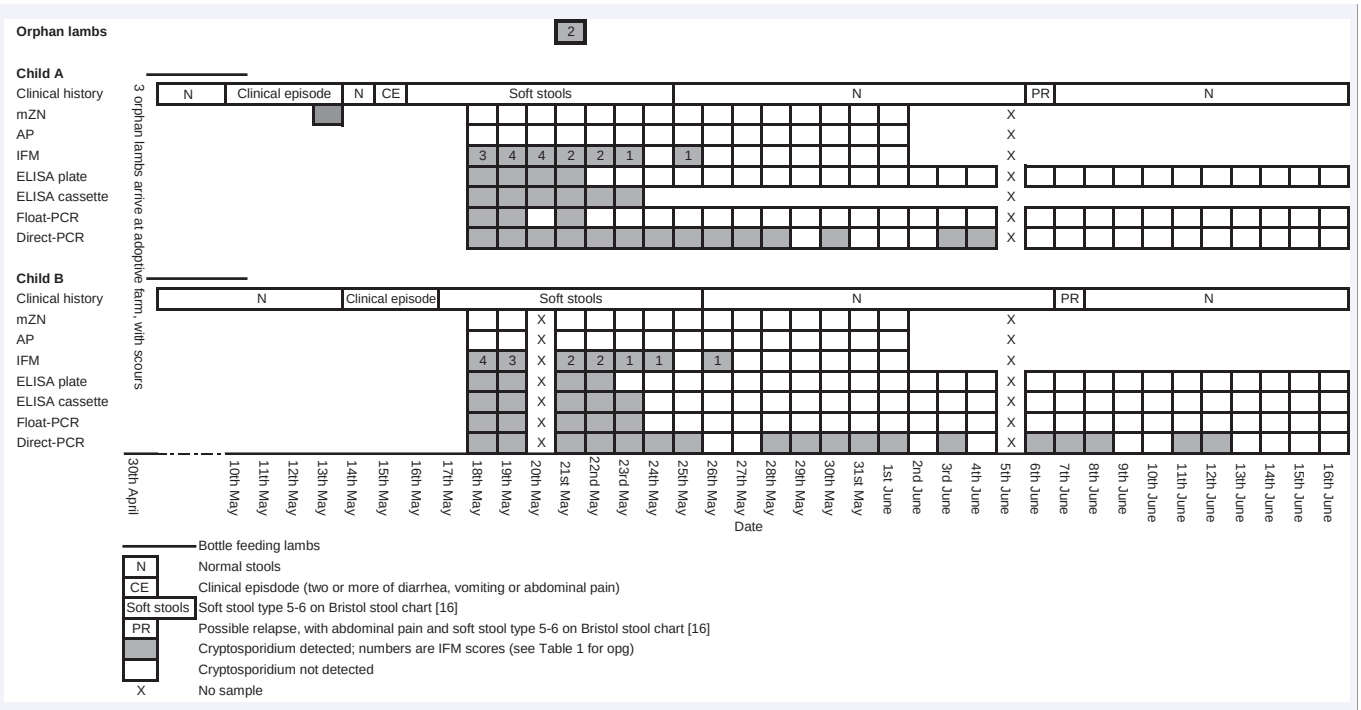

Figure 1 Timeline of exposure to orphan lambs, clinical presentation and detection of Cryptosporidium by stained microscopy, enzyme immunoassays and PCR in two children.

samples from the children are shown in Figure 1. The diagnostic stool and subsequent samples from child A were found to contain *C. parvum*, gp60 allele IIaA13G1R2. The same allele was found in stool samples from child B. Although IIaA13G1R2 has been reported once previously, in a calf in Sweden [20], the sequence was different from those found in our study. According to the standard nomenclature [13] that sequence was gp60 genotype IIaA10G1R1 (Genbank accession number JX183796).

Cryptosporidium oocysts were detected by IFM in one of four samples from lambs on the original farm. The DNA did not amplify with the real-time PCR or gp60 primers for *C. parvum* and was found by sequencing ssu DNA PCR products to contain *Cryptosporidium bovis* (100% match over 761 bp to Genbank accession number AB746197). Oocysts were detected in one of three samples from lambs on the adoptive farm and were identified as *C. parvum* IIaA13G1R2, the same genotype as that found in the children. It is possible that the sampling from the original farm may have missed any remaining *C. parvum* infections in lambs there as only four samples were taken to represent 25 lambs. However, it is likely that other species such as *C. bovis* emerged and *C. parvum* declined in the flock as the lambs became older, a trend which has been reported previously [21].

The finding of *C. parvum* IIaA13G1R2 in human and animal samples indicates zoonotic transmission occurred, at least from the lambs to child A. The time frame for onset of symptoms is also compatible with the possibility that child B may have acquired her infection from her sibling (Figure 1). Transmission of *C. parvum* from lambs to children (and adults) has been reported previously in the UK for *C. parvum* [22-25].

Longitudinal study

The correlation between diagnostic test results and clinical features is shown in Figure (1).

The short interruption in Child A's symptoms on day five after onset is not uncommon in cryptosporidiosis, and has been reported in about one third of cases previously [26]. Overall, child A's symptoms were reported by his mother to be more intense, especially the abdominal pain experienced throughout his clinical episode, and the combination of the clinical episode and abnormal stools lasted longer (15 d) than child B (12 d). Given that the children were only 21 months apart in age it is unlikely that the maturity of their gut mucosa was very different, and both had similar prior exposure to possible *Cryptosporidium* risk factors. It is possible that the infecting doses may have been different, and a larger dose may have produced the more intense symptoms reported by child A. If person-to-person spread occurred from child A to B there could have been some attenuation in virulence. Apart from encouragement of fluid intake, neither of the two children was treated, and neither became dehydrated. Current UK guidance advises that antibiotics should not be given routinely to children with gastroenteritis [27] and there is no licensed therapy for cryptosporidiosis in the European Union [8].

Oocysts were not seen by mZN or AP microscopy in the follow-up stools from either child. Although these stains are used routinely in diagnostic microbiology laboratories in the UK, they lack sensitivity compared with IFM [9] and this study confirms that for samples taken more than a couple of days after acute clinical episodes of cryptosporidiosis, IFM, the ELISA tests used, and PCR-based methods are more suitable for diagnosis. Oocysts were seen by IFM microscopy in all samples from child

Table 1: Estimated quantification of *Cryptosporidium* in daily stools collected from two siblings.

Days post onset of symptoms	Child A			Child B		
	Float-qPCR opg	Direct-qPCR opg	IFM opg	Float-qPCR opg	Direct-qPCR opg	IFM opg
0						
1						
2						
3						
4				$1.0x10^4$	$1.5x10^6$	$>7x10^5$ to $2x10^6$
5				$6.0x10^3$	$3.7x10^5$	$>1.5x10^5$ to $7x10^5$
6	No sample					
7				$2.5x10^3$	$2.5x10^5$	$>1x10^4$ to $1.5x10^5$
8	$7.9x10^3$	$5.7x10^5$	$>1.5x10^5$ to $7x10^5$	$2.6x10^2$	$7.2x10^4$	$>1x10^4$ to $1.5x10^5$
9	$2.9x10^3$	$3.1x10^5$	$>7x10^5$ to $2x10^6$	$1.2x10^2$	$7.0x10^4$	$1x10^3$ to $1x10^4$
10		$1.6x10^5$	$>7x10^5$ to $2x10^6$		$6.1x10^3$	$1x10^3$ to $1x10^4$
11	$8.2x10^2$	$1.8x10^5$	$>1x10^4$ to $1.5x10^5$		$3.7x10^3$	0
12		$2.9x10^4$	$>1x10^4$ to $1.5x10^5$			$1x10^3$ to $1x10^4$
13		$4.7x10^3$	$1x10^3$ to $1x10^4$			0
14		$5.3x10^3$	0		$1.0x10^4$	0
15		$1.2x10^4$	$1x10^3$ to $1x10^4$		$1.6x10^2$	0
16		$4.6x10^3$	0		$2.7x10^4$	0
17		$2.5x10^3$	0		$1.5x10^4$	0
18		$2.0x10^3$	0		$4.3x10^3$	0
19			0			
20		$2.4x10^3$	0		$4.5x10^3$	
21			0			
22	No sample					
23					$4.4x10^3$	
24		$1.3x10^3$			$2.1x10^3$	
25		$2.0x10^3$			$4.0x10^3$	
26				69		
27						
28					$1.7x10^3$	
29					$4.1x10^3$	
30						

Abbreviations: qPCR: Quantitative Polymerase Chain Reaction; IFM: Immunofluorescence microscopy; opg: oocysts per gram of stool

A between days 9 and 14 post-onset of symptoms and in Child B between days 5-11 post- onset (Figure 1). The oocyst scores had declined over time (Figure 1; Table 1), but it is likely that the IFM scores would have been highest during the clinical events. When compared with stool consistency, oocysts were detected by IFM in stools of both children the day after stools returned to normal (Figure 1). However, it is likely that oocysts were present for some days after that but below the threshold of detection of IFM (reported to be $>10^3$ opg with $<5 \times 10^3$ opg unlikely to be seen [28].

The ELISA in cassette format (*Giardia/Cryptosporidium* Quik Chek) provided more positive reactions for *Cryptosporidium* than the plate format (*Giardia/Cryptosporidium* Chek) and was as good as or better than float-PCR. There were no *Giardia* positive reactions, and *Giardia* was not suspected. High performance of the Quik Chek assay has been reported previously [29], providing a rapid test requiring minimal training and equipment that can be used at the point of care. It is recommended that positive ELISA reactions are confirmed by another method [9] and our study demonstrates that IFM or PCR are suitable for this.

PCR for *C. parvum* performed on DNA extracted directly from stools provided positive results in more samples than the other tests (Figure 1). Although improved diagnosis by PCR has been reported previously [30], we have provided a timeframe of recuperation and detection following symptomatic cryptosporidiosis. Sample preparation affected the results: the calculated number of opg was at least 2 $\log_{10}$ greater for direct extraction compared with salt flotation prior to thermal treatment and DNA extraction, and were closer to those estimated from IFM scores (Table 1). Three samples from Child A and five samples from Child B were positive by PCR from both of the DNA extraction methods (Table 1) and the differences in opg between extraction methods were statistically significant for both sets of samples ($p=0.049$ and $p=0.009$ respectively). The difference was probably due to the efficiency of the salt flotation compared with the more predictable performance of the kit-based direct DNA extraction process. There was no evidence for inhibition of the *Cryptosporidium* PCR from either extraction method as the CT values of the IC were within the normal expected range of 31 ± 3 [31]. The opg derived from the qPCR of DNA extracted directly from stools showed that child A probably had a greater

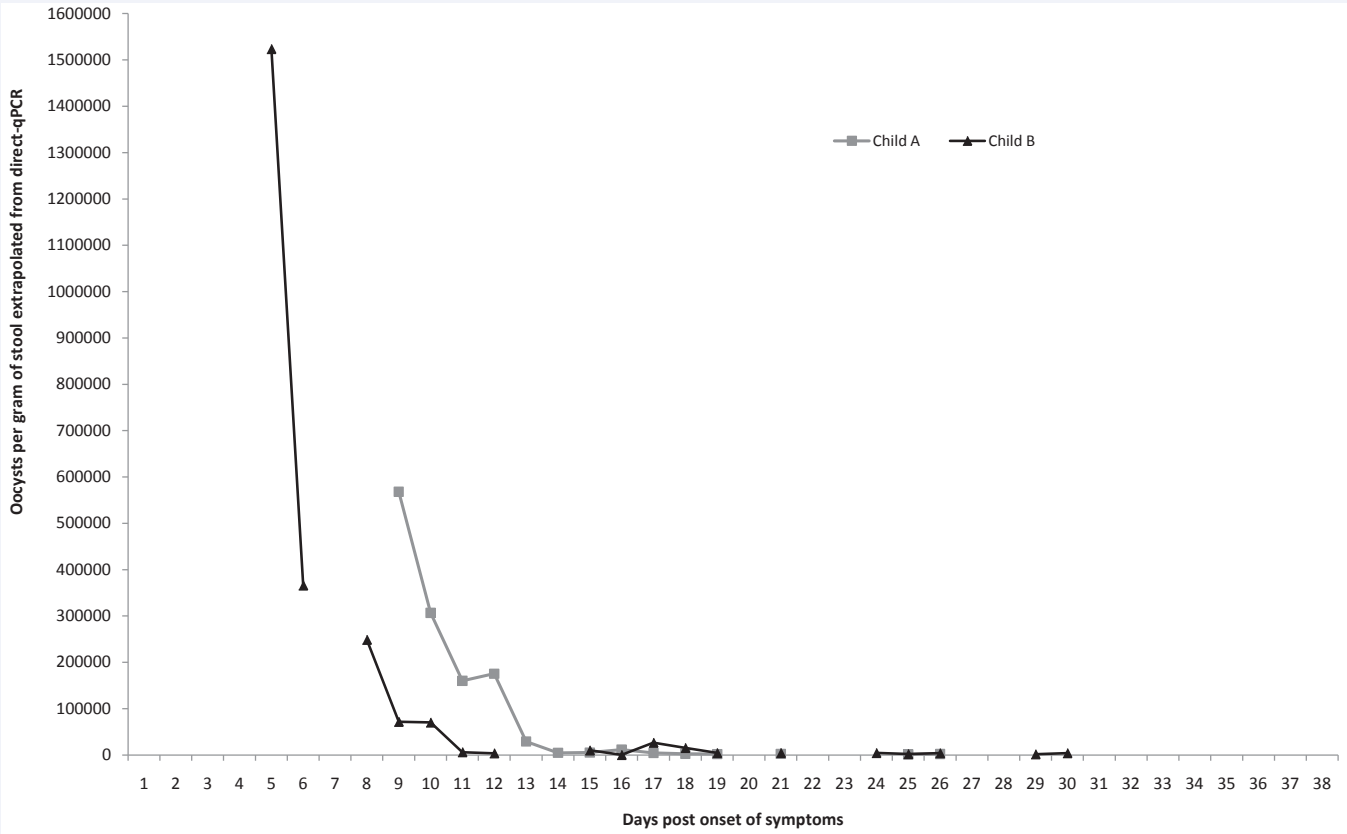

Figure 2 Real-time polymerase chain reaction quantification of Cryptosporidium parvum in stools collected from two children during the course of clinical infection.
Footnote: All data points shown were above the limit of detection

intensity of infection, reflected by his clinical history (see above), although DNA was detected in child B's stools for longer (Figure 2). In order to compare different methods, our calculations assume the *Cryptosporidium* DNA detected by PCR is derived from oocysts. The flotation method would select for oocysts but DNA extracted directly from stool may contain that derived from other *Cryptosporidium* life cycle stages, the proportion of which we were unable to tell. Despite this limitation, extracting DNA directly from stools for PCR provided detection in these recuperating immunocompetent patients where other methods did not. It is likely that these children had an effective immune response to *Cryptosporidium* infection which down-regulated oocyst production rapidly. In some patients, oocyst detection has been reported to continue for weeks following cessation of symptoms [32] and immunodeficient patients may have variable immunity and ability to interrupt oocyst production [33]. Simultaneous increase in *Cryptosporidium* DNA and worsening clinical presentation has been reported previously in two immunocompromised patients, and following treatment of one patient with intravenous immunoglobulins to treat a hypogammaglobulinemia, correlation of clinical improvement and decrease in DNA was observed [34]. However, in another study of diarrhoea patients attending a clinic in Bangladesh, no difference was reported in CT values of *Cryptosporidium* qPCR on cryptosporidiosis patients compared with asymptomatic controls [35], although it was not stated at which point in their infection samples were taken. In the two patients we tested in our study, we have shown DNA extraction directly from stools and qPCR to be a useful means of monitoring *Cryptosporidium* infection and clearance.

CONCLUSION

This study demonstrates that lack of detection by routine diagnostic methods does not indicate lack of infection and provides a timeline for detection during symptomatic phase and recuperation. Oocysts can be detected in stool after symptoms cease and may present a risk of transmission. Where clinical suspicion is high, but samples are collected after the acute clinical episode, PCR of DNA extracted directly from stools provides a higher diagnostic index than the other methods tested and qPCR may provide accurate monitoring of *Cryptosporidium* infection and clearance.

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CONFLICT OF INTEREST

Alere supplied the TechLab *Giardia*/*Cryptosporidium* Chek and Quik Chek kits for this study free of charge.

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