

# Prevalence and identification of *Cryptosporidium* species in paediatric patients with diarrhoea

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## Abstract

**Objectives** To determine the prevalence of *Cryptosporidium* infection in children with diarrhoea, identify associated factors and identify the parasite using Polymerase Chain Reaction (PCR).

**Methods** A total of 138 diarrhoeic faecal samples were collected between August 2011 and February 2013, from children under 12 years of age, admitted to paediatric units of Teaching Hospitals, Kandy and Peradeniya, Sirimawo Bandaranayake Childrens' Hospital, Peradeniya and District General Hospital, Matale. One hundred faecal samples collected from healthy children were used as controls. All control and test samples were screened for the presence of *Cryptosporidium* oocysts with Modified Ziehl-Neelsen (MZN) method and PCR.

**Results** Prevalence of *Cryptosporidium* infection among children with diarrhoea was 5.7%. All the cases positive for *Cryptosporidium* were below 3 years of age. The majority (7 out of 8) of the positive cases had watery diarrhoea while none of the healthy children excreted *Cryptosporidium* oocysts in the faeces. Of the 8 positive cases, 6 had a history of animal contact. A large proportion of positive cases used pipe borne municipal water. The majority (66.6%) of positive cases did not consume boiled cooled water. We were able to identify *C. parvum* from one of the eight cases that had diarrhoea.

**Conclusions** The current study shows that *Cryptosporidium* is one of the aetiological agents responsible for childhood watery diarrhoea in Sri Lanka, thus stressing the importance of routine stool examination for *Cryptosporidium* oocysts. This study recommends boiling water as an important measure to prevent the transmission of *Cryptosporidium* oocysts. Further molecular studies are needed to determine the other species of *Cryptosporidium* responsible for cryptosporidiosis in children in Sri Lanka.

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## Introduction

*Cryptosporidium* species are intestinal coccidian parasites that infect a variety of animals including humans.

The two main species of *Cryptosporidium* that infect humans are *Cryptosporidium hominis* and *Cryptosporidium parvum*, the former primarily infects humans [1]. However, *C. parvum* naturally infects several animal species that serve as reservoirs for zoonotic infection, including cattle, sheep and goats. *C. hominis* is mostly associated with outbreaks of cryptosporidiosis in developed countries indicating human to human transmission [2]. In developing countries cryptosporidiosis is a major cause of childhood diarrhoea. This disease is known to cause self-limiting infection in immunocompetent individuals. However, it causes severe and life-threatening diseases in immune-compromised patients [3]. Diagnosis of cryptosporidiosis is mainly based on the detection of *Cryptosporidium* oocysts in faeces. Modified Ziehl Neelsen (MZN) staining method is widely used as a reliable routine technique to detect *Cryptosporidium* oocysts. However, MZN staining is not carried out in routine stool analysis in Sri Lankan laboratories. Thus, cryptosporidiosis remains largely under diagnosed by the current routine laboratory practice in Sri Lanka. Environmentally resistant *Cryptosporidium* oocysts are passed in the faeces of asymptomatic and symptomatic animals and are acquired by ingestion [4,5]. In developing countries, *Cryptosporidium* is responsible for 8% to 19% of cases of diarrhoeal diseases, with significant mortality. Similarly, a few studies done in Sri Lanka have identified *Cryptosporidium* oocysts in diarrhoeic faecal samples of children, and results have shown a prevalence between 6% to 9% [6,7]. In Sri Lanka, *Cryptosporidium* infection has been reported in cattle, buffalo, goats and monkeys indicating the presence of potential zoonotic transmission [8,9].

As all species do not appear to cause infection in humans, and the majority of human infective species cannot be differentiated microscopically because of their identical morphology, both microscopic and molecular methods, which can differentiate *Cryptosporidium* species are required to identify potential sources of infection. To date there have been no studies to identify the *Cryptosporidium* species responsible for human infection in Sri Lanka despite the highlighting of possible zoonotic risk. Thus information about circulating *Cryptosporidium*

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species and genotypes are not available in our country. Hence, the current study was undertaken to determine the prevalence of *Cryptosporidium* infection in children with diarrhoea, identify associated factors and identify *Cryptosporidium* species isolated from infected children with diarrhea.

## Methods

### Data collection

Between August 2011 and February 2013, faecal samples were collected from children with diarrhoea under 12 years of age admitted to paediatric units of Teaching Hospitals, Kandy and Peradeniya, Sirimawo Bandaranayake Childrens' Hospital, Peradeniya and District General Hospital, Matale. WHO criteria were used to determine the nature of the diarrhoeal episodes. An information sheet recorded age, sex, address and clinical symptoms related to the illness for each child. Information regarding the possible mode of acquiring *Cryptosporidium* infection such as source of drinking water, method of water purification, exposure to animals and personal hygiene were collected from parents or the guardian of the child after obtaining informed consent. Faecal samples of the control group were collected from age and sex matched children presenting with illness other than diarrhoea to the same units.

### Detection of *Cryptosporidium* spp. oocysts in faecal samples

Half of each faecal sample was concentrated with modified formalin ethyl acetate technique as this method is more sensitive for the recovery of *Cryptosporidium* oocysts [10]. Subsequently, each concentrated sample was stained with MZN method. If the faecal sample was positive for *Cryptosporidium* oocysts the rest was subjected to Sheather's sugar flotation technique and the concentrate was washed several times with distilled water and stored at -20°C until use.

### DNA extraction

Oocysts stored at -20°C were defrosted at room temperature. Each oocyst sample was suspended in lysis buffer (1M NaCl, 0.5M EDTA, 1M Tris HCl and 10% SDS). Then, the suspension was subjected to 15 cycles of freezing and thawing by immersing in liquid nitrogen and thawing in a water bath at 60°C. Subsequently the debris was removed by centrifugation and the sediment was treated with proteinase K (20mg/ml). DNA was extracted by phenol-chloroform method and the extracted DNA was suspended in sterile water and stored at -70°C until use.

### Polymerase chain reaction (PCR)

*Cryptosporidium*18S rRNA gene fragment was

amplified using the DNA extracts of 8 diarrhoeic faecal samples using Cry2 (5'GCGGGATCCTTGCAAA TGCTTTCG3') and Cry4 (5'GCGAATTCCTGAC ACAGGGAGGTAG 3') primers [8]. *Cryptosporidium* DNA was amplified with 35 cycles of 94°C for 30s, 58°C for 30s and 72°C for 1 minute with final extension at 72°C for 10 minute. PCR products were then analysed using 1.5% agarose gels which were stained with ethidium bromide.

## Results

A total of 138 diarrhoeal faecal samples were collected. Fifty were from males. Of the 138 faecal samples tested for *Cryptosporidium* oocysts, 8 (5.7%) were positive for MZN staining on microscopy. *Cryptosporidium* oocysts were not found in the 130 faecal samples of healthy children without diarrhoea. The majority of the children positive for *Cryptosporidium* oocysts were aged one to three years. Four of the positives were male.

Of the study sample 101 (73.1%) had watery diarrhoea and of them 7 (6.9%) were positive for *Cryptosporidium* oocysts while 37 (26.7%) who had semisolid faeces, only 1 was positive.

In the study group, 67 (48.5%) gave a history of contact with animals. Of the 8 positives 6 gave a history of animal (dogs, cats, and goats, chicken) contact. In those who had animal contact, a large proportion (59.7%) had dogs and of these three cases were *Cryptosporidium* positive. Of the seven children who had contact with goats, two were positive for *Cryptosporidium*. Fever was the predominant symptom in these children (109 out of 138) and six out of eight *Cryptosporidium* positive cases presented with fever.

The study group obtained drinking water from municipal water supply, protected or unprotected wells and tube wells. The majority used piped borne water. Of the *Cryptosporidium* positives, a large proportion used piped borne water (Table 1). Most in the study group used boiled water while three did not drink boiled cooled water. Of these three, two were *Cryptosporidium* positive (Table 2).

**Table 1. Presence of *Cryptosporidium* oocysts in faeces of children in relation to source of drinking water**

| Source of drinking water | Presence of <i>Cryptosporidium</i> oocysts in faeces |             |       |
|--------------------------|--|-------------|-------|
|                          | Positive   | Negative    | Total |
| Pipe borne water         | 5 (8%)   | 58 (92%)    | 63    |
| Protected well           | 2 (5%)   | 42 (95%)    | 44    |
| Unprotected well         | 0  | 4 (100%)    | 4     |
| Tube well                | 1 (7.69%)  | 12 (92.30%) | 13    |

**Table 2. Presence of *Cryptosporidium* oocysts in faeces of children in relation to source of drinking boiled water**

| Water boiling | Presence of <i>Cryptosporidium</i> oocysts in faeces |           |       |
|---------------|--|-----------|-------|
|               | Positive   | Negative  | Total |
| Always        | 4 (4%)   | 107 (96%) | 111   |
| Some times    | 2 (20%)  | 8 (80%)   | 10    |
| Never         | 2 (667%)   | 1 (33%)   | 3     |

All eight samples positive by MZN staining were amplified with *C. parvum* specific primers. *C. parvum* was detected in one sample and seven were negative for PCR. The amplicon was approximately 200-300bp.

## Discussion

The prevalence of *Cryptosporidium* in different countries of the world vary, however, the rate of infection is higher in children in countries [11]. In this study, *Cryptosporidium* oocyst positivity of 5.7% was observed in children with diarrhoea. This rate of positivity is comparable to that reported previously [6,7]. Similarly, the prevalence of *Cryptosporidium* infection in children in the neighboring countries, India and Nepal have been 5.6% and 4.1%, respectively [12,13]. Much higher prevalence of this infection has been reported among children in Nicaragua (35.7%) and Egypt (33.3%) [14,15]. In contrast, lower prevalence rates of 1.5% to 2.7% have been reported from Iran and Bangladesh [16]. These differences could be attributed to standards of living, socioeconomic status and availability of safe drinking water.

In the present study, the majority of the positive cases had watery diarrhoea. Similar findings have been reported in India where the greater proportion of cryptosporidiosis cases had watery diarrhoea [17]. None of the age and sex matched control group in this study were positive for *Cryptosporidium* infection. This is in agreement with the previous studies [18,19]. Contrary to this, asymptomatic carriage rate of 2% to 3% has been reported in India [20].

All the positive cases in this study group were below three years of age. Similar results have been reported in studies done in Ghana and Iran where the *Cryptosporidium* infection rate is high among children under five years of age [21, 22].

*Cryptosporidium* is transmitted through water, person to person contact and from animals to humans. *Cryptosporidium* oocysts are highly resistant to common water disinfection practices including chlorination. The majority of *Cryptosporidium* infected children in the study group used pipe borne municipal water. Similarly, a study done in the USA shows high prevalence of cryptosporidiosis among those who drink municipal water [23].

*Cryptosporidium* oocysts have been recovered from irrigation reservoirs and canals in Sri Lanka [24]. A large proportion of the study group used boiling as a method of water purification. Although boiling inactivates the *Cryptosporidium* oocysts in water, there were four *Cryptosporidium* infected children among this group. This may be due to improper boiling of water or due to consumption of contaminated food. Food such as fresh fruits, vegetables and raw or undercooked shell fish are increasingly recognised as sources of *Cryptosporidium* spp [25]. It is interesting to note that one quarter of those who had *Cryptosporidium* infection did not consume boiled cooled water. This supports the explanation that municipal water purification is not effective in eliminating *Cryptosporidium* oocysts. Zoonotic transmission of cryptosporidiosis after exposure to infected animals has been documented [26]. The majority of positive cases in the current study had a history of contact with animals. In Sri Lanka, calves and goats are considered to be major animal reservoirs [27].

Although cryptosporidiosis is prevalent in Sri Lanka no studies have been carried out to identify the *Cryptosporidium* species. This is the first study that reports of *C. parvum* causing diarrhoea in children in Sri Lanka. The rest of the MZN positive samples were negative with PCR using *C. parvum* specific primers. Those cases could be associated with other *Cryptosporidium* species. The majority of human infections are caused by the species *C. hominis* and *C. parvum*. In European countries, equal percentages of *C. hominis* and *C. parvum* have been documented, however, in developing countries the majority of human infections are due to *C. hominis* [1]. In India too the commonest species isolated from the faecal samples of children with *Cryptosporidium* diarrhea is *C. hominis* [28]. In this study only one isolate was identified as *C. parvum* and the rest was PCR negative using *C. parvum* specific primers. Since we only used *C. parvum* specific primers our species identification was confined to *C. parvum*.

In conclusion, *Cryptosporidium* is one of the possible aetiological factors for childhood watery diarrhoea in Sri Lanka necessitating routine stool examination for *Cryptosporidium* using MZN staining. Boiling water may be useful to prevent the transmission of *Cryptosporidium* oocysts. Molecular studies such as restriction fragment length polymorphism analysis and DNA sequencing are needed to determine the other species responsible for cryptosporidiosis in Sri Lanka.

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