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Cryptosporidiosis among Children with Diarrhea Admitted to Hospital Selayang and Hospital Sungai Buloh, Selangor, Malaysia

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Abstract

This study was conducted to verify the prevalence of cryptosporidiosis among children in Selangor. Consenting children aged 12 years and below, admitted with diarrhea to Hospital Selayang and Hospital Sungai Buloh, were included as subjects. Each stool sample was divided into five aliquots and tested for cryptosporidiosis using direct wet mount, Sheather's sugar flotation, formalin-ether sedimentation, modified Ziehl-Neelsen staining, and direct monoclonal fluorescent antibody test. Of 130 stool samples, 6 (4.62%) were positive for *Cryptosporidium* spp. All children positive for cryptosporidiosis were aged < 4 years. Direct wet mount, formalin-ether sedimentation concentration, modified Ziehl-Neelsen staining, and direct monoclonal fluorescent antibody testing showed the same number of positive samples (4.62%); Sheather's sugar flotation detected one less positive sample (3.85%).

Keywords: cryptosporidiosis, children, diarrhea, hospital, Malaysia

Introduction

The *Cryptosporidium* genus consists of zoonotic apicomplexan obligate intracellular parasites. These invade the microvillus border of the gastrointestinal epithelium and the lining of respiratory organs in a wide range of vertebrate hosts, including humans [1,2]. They are a common cause of diarrhea for both humans and animals [3]. The prevalence of *Cryptosporidium* ranges from 1-4% in Europe and North America, to 1-37% in Africa, Asia, Australia, and South and Central America [4-7].

Cryptosporidiosis has a higher incidence rate in developing countries, particularly among children, malnourished individuals, institutionalized patients, and immunocompromised individuals (*eg*, AIDS cases) [8]. In developing countries, *Cryptosporidium* mostly infects children aged < 5 years, and peaks in children aged < 2 years [9,10]. In industrialized countries, however, cryptosporidiosis also occurs in adults because of food- or water-borne outbreaks [11,12]. *Cryptosporidium* causes gastrointestinal illness in both immunocompetent [13-16] and immunocompromised individuals [15,17]. In

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immunocompetent individuals, cryptosporidiosis is a self-limiting illness, but in those who are immunocompromised, infection can be unrelenting and fatal [4,13].

In Malaysia, documented prevalence rates of *Cryptosporidium* infection are between 0.9% and 23% [5,18-23]. The highest rate (23%) was found in HIV-positive intravenous drug users; the lowest rate (0.9%) was reported among children. However, very few of these documented cases have focused solely on pediatric cases.

Using both microscopy and PCR techniques, Menon *et al* [5] showed that 0.9% of children hospitalized with acute diarrhea in Kota Bharu were positive for *Cryptosporidium parvum*. Ludin *et al* [18] reported a cryptosporidiosis prevalence of 4.3% in Penang. Although the reported figures in Malaysia are relatively low when compared to prevalence rates of 37.3% in Jordan [6], 35.7% in Nicaragua [7] and 12.1% in Ethiopia [24], it is necessary to document the incidence of cryptosporidiosis among children in this country accurately, to improve the management of the disease.

The aim of this study was to document the prevalence of cryptosporidiosis among children with diarrhea admitted to Hospital Selayang and Hospital Sungai Buloh, in Selangor, and to evaluate the accuracy and reliability of the 5 different laboratory methods used in the diagnosis of *Cryptosporidium* infection.

Materials and methods

Samples collection

The study was conducted in two tertiary hospitals in the state of Selangor, Malaysia between September 2010 and March 2012. Hospital Selayang is 10 km northwest of Kuala Lumpur, while Hospital Sungai Buloh is 20 km northwest of the capital. All consenting children aged 12 years and below, who had been admitted to these two hospitals with diarrhea over the duration of study, were included in the study.

Data were collected via structured questionnaire. The questionnaire included personal details, demographic data, dietary information, sources of water, contact with animals, and clinical signs and symptoms. Parents or guardians were interviewed directly on behalf of their children by trained research personnel. Ethical clearance was obtained from the National Institute of Health, a subdivision of the Malaysian Ministry of Health.

Processing of samples

One hundred and thirty (130) stool samples were collected over the period of the study. Immediately upon collection, a few drops of 10% formalin were added to aid preservation. All samples were then transferred in sealed containers to the Parasitology Laboratory in Sungai Buloh Campus, Universiti Teknologi MARA (UiTM). Each sample was divided into 5 aliquots for testing using the methods described below.

Direct wet mount

A drop of 0.9% normal saline and a drop of 1% Lugol's iodine solution were placed side by side on a glass slide. The stool sample was then mixed into the drops using an applicator stick. The slide was then covered with a cover slip and observed under a digital compound microscope (Olympus BX53, Japan) at magnifications of x40 and x100.

Concentration method

i) Sheather's sugar flotation
As described by Blagburn & Butler [25], a suspension of stool was first prepared in a test tube using 2 g of stool, then combined with Sheather's sugar solution (specific gravity ~1.27) for microscopic examination at magnifications of x40 and x100.

ii) Formalin-ether sedimentation
The formalin-ether sedimentation method was used as described by Allen & Ridley [26] and observed under a microscope at magnifications of x40 and x100.

Modified Ziehl-Neelsen acid-fast staining (Cold Kinyoun)

The Cold Kinyoun technique was performed as described by Henriksen & Pohlenz [27]. It was

observed under a microscope at magnifications of x40 and x100.

Direct immunofluorescence assay

PARA-TECT™ *Cryptosporidium*/*Giardia* Direct Fluorescent Assay (Medical Chemical Corporation, USA) is an *in-vitro* immunoassay that uses antibodies against the organism's cell wall. An aliquot of 0.1 ml stool suspension was spread over the slide well and left to air dry; 0.1 ml of conjugate was added, followed by the addition of 0.1 ml of counterstain. The reagents were then mixed and spread over the entire well. The slide was incubated in a light-protected humidity chamber for 30 min. After this, it was rinsed with wash buffer to remove excess reagents. A drop of mounting medium was added to the well and a cover slip applied. The slide was observed under a fluorescence microscope (Olympus BX61, Japan) using an FITC filter (excitation 490 nm, emission 525 nm) at x40 magnification.

Results

By direct wet mount, *Cryptosporidium* oocysts appeared spherical in shape with a thick cell wall. The diameter range of the observed oocysts was 4-6 µm (Fig 1A, 1B). Lugol's iodine solution was primarily used in wet-mount preparations to differentiate oocysts from yeasts. *Cryptosporidium* oocysts do not stain with this solution, so they remain colourless, while yeasts stain yellowish-brown.

Using Cold Kinyoun, *Cryptosporidium* spp oocysts stain pinkish-red on a blue or green background, depending on the counterstain used (Fig 1C). By immunofluorescence assay, the oocysts fluoresce an apple-green colour against a dark background. Their microscopic appearances are shown in Fig 1D.

Of 130 stool samples, 6 (4.62%) were positive for *Cryptosporidium* spp. All the children with positive results were aged < 4 years.

Of the 5 tests used, 4 – direct wet mount, formalin-ether sedimentation concentration, modified Ziehl-Neelsen staining, and direct monoclonal fluorescent antibody test – gave the same results (4.62%). Sheather's sugar flotation, however, was only positive in 5 children (3.85%). The comparative results of the 5 methods are shown in Table 1.

The correlation of infection rate to gender, age, locality, source of drinking water, and presence of pets or livestock, is shown in Table 2. There was no difference in the distribution of cases between males and females. All infected individuals lived in urban areas. These areas are supplied with tap water from water treatment plants and have structured plumbing. Four of the 6 positive cases (66.67%) were Malays, while the rest were Chinese and Indians. None of the families owned any animals.

Discussion

The prevalence rate of cryptosporidiosis (4.62%) among the hospitalized children in this study is similar to that in previous reports from Malaysia, where infection rates were between 0.9-11% [5,18-20,23]. A majority of infected cases were children aged < 4 years. This agrees with other Malaysian studies [5,19]. Several other countries, meanwhile, have reported the highest frequency of cryptosporidiosis in children aged < 3 years, *eg* Ireland [28], China [29], and Bangladesh [30].

The youngest child with cryptosporidiosis was 6 months old; the oldest was 3 years and 4 months. Although *Cryptosporidium* spp infection has been reported in various age groups, records suggest that children are most susceptible to infection [31-33]. The most likely explanation for this age-dependent pattern is probably underdeveloped innate immunity among infants and toddlers. In addition, it could also be associated with children's habit of crawling on all surfaces and putting objects freely into their mouths, especially in early infancy.

The low prevalence of infection in this study could be attributed to the demographic distribution of the subjects, the use of treated water, and the absence of household pets. Treated water supplies are available to about 99% of urban and 91% of rural populations in Malaysia [34]. Earlier studies in these areas revealed no

oocysts in treated water [35,36], although they were found in filter backwash water samples taken from treatment plants. Studies in other countries that have reported a high prevalence of cryptosporidiosis cite consumption of untreated drinking water from wells and springs [6], poor levels of sanitation, and contamination of the water supply [7] as key factors in their high prevalence.

As all infected subjects lived in urban areas with proper amenities and without domestic pets, it is logical to conclude that the source of infection is probably anthroponotic. However, nothing concrete can be proven without further testing, by genotyping or molecular identification of species.

Regarding the 5 different methods used to detect *Cryptosporidium* spp oocysts in stool samples, all shared similar results, except for

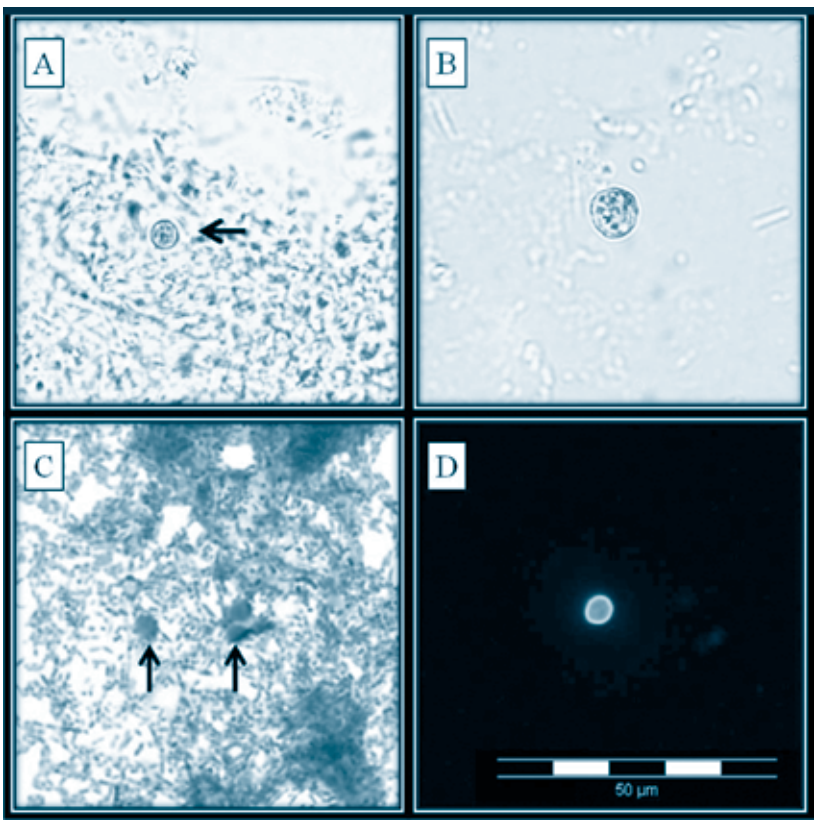

Fig 1 ***Cryptosporidium*** **spp oocysts: (A) wet mount x40; (B) wet mount x100; (C) Cold Kinyoun x40; and (D) direct immunofluorescence antibody x40.**

Table 1 Five laboratory methods for identification of *Cryptosporidium* spp oocysts.

Method	No. of positive samples	Percentage of positive samples (%)
Direct wet mount	6	4.62
Sheather's sugar flotation	5	3.85
Formalin-ether sedimentation	6	4.62
Modified Ziehl-Neelsen staining	6	4.62
Direct monoclonal fluorescent antibody	6	4.62

Table 2 Demographic data of *Cryptosporidium*-positive children.

Age	Gender	Race	Locality	Source of drinking water	Presence of animals
6m	Female	Malay	Urban	Tap water	Nil
1y 4m	Male	Malay	Urban	Tap water	Nil
1y 7m	Male	Indian	Urban	Tap water	Nil
1y 9m	Female	Malay	Urban	Tap water	Nil
2y	Female	Chinese	Urban	Tap water	Nil
3y 4m	Male	Malay	Urban	Tap water	Nil

m = months; y = years

Sheather's sugar-flotation method. This may be due to distortion of oocysts over time as a result of osmotic effects, so making identification more difficult. Iodine staining and size measurement help greatly in identifying *Cryptosporidium* spp oocysts in wet mount preparations. Modified Ziehl-Neelsen and direct monoclonal fluorescent antibody techniques are both categorized as specific stains, as they are both readily able to differentiate *Cryptosporidium* spp from other matter. With acid-fast staining, oocysts retain a carbolfuchsin pinkish-red colour, while the background matter takes the counterstain, appearing as methylene blue or malachite green. On the other hand, the direct immunofluorescence method works by attaching *Cryptosporidium*-specific fluorescent-labelled antibodies to the oocyst wall, which emits an apple-green color when viewed under a fluorescence microscope. This provides for an excellent screening and confirmation method, provided the lab is equipped with a fluorescent microscope and a supply of anti-*Cryptosporidium* monoclonal antibodies. We recommend modified Ziehl-Neelsen staining to screen for *Cryptosporidium* spp in hospitals, since it is cheap, rapid, and easy to interpret. Although direct wet mount can detect the same amount of positives as acid-fast stain, it needs experts in the field to differentiate the parasite from other matter. Direct immunofluorescent antibody could be used to confirm acid-fast positive samples, or to diagnose cases of persistent diarrhea with unknown cause.

In relation to the detection of oocysts, while some studies have reported discrepancies in what is picked up between direct microscopy, concentration technique, acid-fast staining, and direct immunofluorescence antibody [6,37,38], it is not uncommon for results to be similar [39]. Menon *et al* [5] found the prevalence of cryptosporidiosis-positive by modified Ziehl-Neelsen sample was similar to PCR assay results, reputedly more sensitive than conventional microscopy [40].

While there is currently no specific treatment for cryptosporidiosis, nitazoxanide or paromomycin combined with azithromycin has been the preferred medication of choice. It is regrettable many clinicians do not feel it necessary to test for cryptosporidiosis in immunocompetent hosts, when infection is largely self-limiting; testing allows for the education of patients, facilitates recognition of an outbreak, and may lead to implementation of measures to prevent the spread of infection [41-43].

Based on the findings of this study, several control measures can be taken to help prevent the spread of infection. It is normal in urban settings nowadays for both parents to be working, and day-care centers are in increasing demand. Hygiene standards for these places should be improved and monitored regularly, as children are highly susceptible to infection. Moreover, all drinking water should be boiled before consumption to help eliminate pathogens. Parents should not bring diaper-wearing or non-potty trained children to

swim in public recreational waters, because there is a high possibility of them contaminating the water through inadvertent shedding of oocysts, or accidentally ingesting oocysts in bathing areas contaminated by other users.

Conclusion

This study confirms the prevalence rate of cryptosporidiosis in children who were admitted with diarrhea to two urban hospitals, in parallel with results of investigations in other regions of the country. In addition, this study confirms that most laboratory methods used to detect *Cryptosporidium* spp are diagnostically reliable, although Sheather's sugar flotation test did fail to detect one sample.

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