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Research Article

Cryptosporidiosis: Prevalence in Children in Nepal and Bhutan

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Abstract

Objective: To assess prevalence of cryptosporidiosis in children with acute diarrhea and non- diarrhea controls in Nepal and Bhutan.

Methods: A case-control study of acute diarrhea etiology was conducted from 2006-2009 in Nepal and from 2011-2015 in Bhutan. Children aged from 3 months to 5 years with acute diarrhea and asymptomatic controls were enrolled. Commercial ELISA kits were used for detecting *Cryptosporidium*.

Results: In Bhutan, *Cryptosporidium* was detected in 45/1716 (2.6%) of diarrhea cases and 27/1644 (1.6%) of non-diarrhea controls (OR=1.6, 95% CI 1.0-2.6). The overall prevalence of cryptosporidiosis in Bhutan was 2.1% and the prevalence was significantly higher in peri-urban (2.6%) than in rural areas (1.0%) (OR=2.6, 95% CI 1.3-5.3). The highest prevalence detected was observed in children aged 24-47 months old and *Cryptosporidium* was associated with diarrhea in children 24-35 months old (OR=6.2, 95% CI 1.4-27.9). In Nepal, *Cryptosporidium* was significantly detected in 26/1200 (2.2 %) of cases compared to 10/1200 (0.8%) in controls (OR=2.6, 95% CI 1.3-5.5); no significant difference of prevalence between peri-urban and rural areas (1.8% and 1.2%, respectively) was observed. Divided by age groups, the highest prevalence were from children of aged 24-35 and 36-47 months old. Increasing of cases of cryptosporidiosis was observed during the hot and monsoon seasons in both sites.

Conclusion: The results of case-control studies in Nepal and Bhutan suggest that *Cryptosporidium* is prevalent and a significant pathogen. As the prevalence and burden of *Cryptosporidium* may be underestimated, additional surveillance and morbidity studies of cryptosporidiosis in Nepal and Bhutan are warranted.

INTRODUCTION

Cryptosporidiosis is a parasitic infection of medical and veterinary importance affecting humans and many vertebrate species including large and small mammals (cattle, sheep, cats, and dogs), reptiles, fish and poultry [1]. Among 24 recognized species, *C. parvum* and *C. hominis* are two most common species associated with infections in human [1]. *Cryptosporidium* is mainly transmitted to humans through water contaminated with human or animal feces and is of public health concern because of its low infective dose and long survival of the oocysts in the environment [2,3]. Cryptosporidiosis has been well recognized to be highly fatal in immune-compromised hosts and malnourished children [4,5]. Among naïve pediatrics population in developing countries, *Cryptosporidium* was the second most common cause of moderate to severe diarrhea among infants with adjusted attributable fractions ranging from 5.3 - 14.7% in Bangladesh and Mozambique (two of seven international sites) respectively as part of the Global Enteric Multicenter Study (GEMS) [6]. Additionally, *Cryptosporidium* was a significant diarrhea etiologic agent with overall adjusted attributable fractions of 2.0% and 3.8% during the first and second year of life, respectively at

eight sites in developing countries as part of the Interactions of Malnutrition & Enteric Infections: Consequences for Child Health and Development Study (MAL-ED) [7].

In this study, we assessed *Cryptosporidium* infections in children with acute diarrhea (cases) and non-diarrhea (controls) in peri-urban and rural areas in Nepal and Bhutan and describe its association with diarrhea, seasonality, urbanization and age groups.

MATERIALS AND METHODS

Nepal study sites

A case-control study of acute diarrhea etiology was conducted from December 2006 through April 2009 at Kanti Children's Hospital loacted in Kathmandu (peri-urban area) and Bharatpur Hospital in Bharatpur (rural area), a small town in southern Nepal. Peri-urban area is defined as the area that surrounds metropolitan areas and cities. Children aged 3 months to 5 years with acute diarrhea of less than 72 hours duration by history and controls with no history of diarrhea in the previous 2 weeks were enrolled at each hospital.

Bhutan study site

The study sites were Jigme Dorji Wangchuk National Referral Hospital in Thimphu (peri-urban area) and the Regional Referral Hospitals in Mongar, Gelephand Phuntsholing (rural areas). Children aged 3 months to 5 years with acute diarrhea and non-diarrhea controls were enrolled from March 2011 through July 2015.

Enrollment, sample collection and testing

After obtaining informed consent, one stool sample was collected from each subject and demographic and brief clinical data were obtained. At the study sites, a microscopic examination by direct wet smear for ova and parasites was performed. Methanol fixed stool smears were prepared andup to three aliquots of stool (approximately 1 gram each) were preserved in liquid nitrogen or in a –70°C freezer. If sufficient stool was remaining, stool was also preserved in 10% formalin.

Stool samples were kept frozen at -70°C until thawed and tested by ELISA. Commercial ELISA kits (ProSpecT™, Alexon-Trend, MN, USA) were used to screen for the presence of the following parasites specific antigen: *Giardia*, *Cryptosporidium* and *Entamoeba histolytica* in the stool samples following the methods described in the manufacturer's instructions. Acid fast staining was conducted after stool was concentrated using the formalin-ethylacetate sedimentation concentration technique.

Statistical analysis

Associations between age and prevalence of parasite antigens were extrapolated using the Chi-square test. Univariate and multivariate odd ratios (OR) with 95% CI were calculated for each specific parasite. A *P*-value of less than 0.05 was considered statistically significant. All analyses were performed using SPSS version 23 (SPSS Inc; Chicago, Illinois).

RESULTS AND DISCUSSIONS

In Bhutan, a total of 3,360 samples (1,716 cases and 1,644 controls) were examined. The overall prevalence of *Cryptosporidium* infection in Bhutan was 2.1% (72/3360) with *Cryptosporidium* being detected in 2.6% (45/1716) of diarrhea cases and 1.6% (27/1644) of non-diarrhea controls respectively (OR=1.6, 95% CI 1.0-2.6). *Cryptosporidium* was detected significantly more in Thimpu, 2.6% (63/2453), as compared to the three rural sites combined, 1.0% (9/907), (OR=2.6, 95% CI 1.3-5.3). The prevalence by rural sites was 0% (0/290), 2.0% (8/411), and 0.5% (1/206) for Mongar, Gelephu, and Phuntsholing, respectively. *Cryptosporidium* was most frequently observed more in children aged 24-35 and 36-47 months and was associated with the development of acute diarrhea in children aged 24-35 months (OR=6.2, 95% CI 1.4-27.9) (Table 1). In children less than 2 years, detection was relatively similar in both cases and controls with a range of 1-2%. Though *Cryptosporidium* was detected more often in controls than in cases in the older age groups, the difference was not statistically significant.

In Nepal, a total of 2,400 samples (1,200 cases and 1,200 controls) were examined in which 1.5% (36/2400) were positive for *Cryptosporidium. Cryptosporidium* was detected significantly (*P*= 0.007) more frequently in cases, 2.2% (26/1200), when compared to controls, (0.8% (10/1200); (OR=2.6, 95% CI 1.3-5.5). There was no significant difference observed in the frequency of *Cryptosporidium* infection in the peri-urban site, Kathmandu, 1.8% (22/1200) when compared to the rural site in Bharatpur, 1.2% (14/1200). The age groups with the highest prevalence were observed in children aged 24-35 and 36-47 months.

In these case-control studies, the prevalence of *Cryptosporidium* in acute diarrhea cases in children less than 5 years was 2.2% and 2.6% in Nepal and Bhutan, respectively and the prevalence of *Cryptosporidium* in asymptomatic controls was 0.8% and 1.6% in Nepal and Bhutan respectively. Overall, *Cryptosporidium* infection was found to be lower in children less than 5 years than has been previously reported and infection was also highest in older children (24 – 47 months) which differs from other recent studies conducted in similar geographic and socioeconomic settings in the developing world [6,8]. Additionally, *Cryptosporidium* infection was only significantly associated with acute diarrheal cases in children aged 24 – 35 months in Bhutan (*P* = 0.007). However, there are a limited number of studies describing *Cryptosporidium* infection in children in Nepal and we found no studies in the published literature assessing *Cryptosporidium* infection in children in Bhutan.

The prevalence of *Cryptosporidium* was 5.0% and 0% in cases and controls respectively in a limited case-control study assessing enteric pathogens in children aged 6 – 60 months in Kathmandu, Nepal in 1994. Unfortunately, data stratified by age was not provided to show which age range *Cryptosporidium* was most prevalent [9]. Other recent studies described *Cryptosporidium* infection was detected more in children less than 2 years of age as opposed to the older children as reported here. *Cryptosporidium* was detected in 16.3% and 3.1% of acute diarrhea cases and controls respectively in children less than two years old with the highest prevalence reported in children 7 – 12 months of age in Dar es Salaam, Tanzania [10]. In Bangladesh, Korpe et al., reported that *Cryptosporidium* infection was highest in children 10 to 17 months with decreased prevalence as the children aged [11]. In Mozambique, *Cryptosporidium* was significantly

Table 1: Age-specific detection of *Cryptosporidium* among acute diarrhea cases and non-diarrhea controls in Bhutan and Nepal.

Age (months)	Bhutan (N=3,360)			Nepal (N=2,400)		
	Case (N=1,716)	Control (N=1,644)	OR (95%CI)	Case (N=1,200)	Control (N=1,200)	OR (95%CI)
	No. positive/No. tested (%)			No. positive/No. tested (%)		
3-11	9/650 (1.4%)	7/615 (1.1%)	1.2 (0.5-3.3)	11/515 (2.1%)	2/411 (0.5%)	4.5 (1.0-20.3)
12-23	14/545 (2.6%)	11/547 (2.0%)	1.3 (0.6-2.9)	6/374 (1.6%)	3/310 (1.0%)	1.7 (0.4-6.7)
24-35	13/269 (4.8%)	2/247 (0.8%)	6.2 (1.4-27.9)	4/107 (3.7%)	2/179 (1.1%)	3.4 (0.6-19.1)
36-47	6/143 (4.2%)	3/137 (2.2%)	2.0 (0.5-8.0)	4/95 (4.2%)	2/133 (1.5%)	2.9 (0.5-16.1)
48-60	3/109 (2.8%)	4/98 (4.1%)	0.7 (0.2-3.1)	1/109 (0.9%)	1/167 (0.6%)	1.5 (0.1-24.8)
Total	45/1716 (2.6%)	27/1644 (1.6%)	1.6 (1.0-2.6)	26/1200 (2.2%)	10/1200 (0.8%)	2.6 (1.3-5.5)

more associated with diarrhea in cases (19.5%) than in controls (9.5%) in children 0 – 23 months as compared to children aged 23 – 59 months (Cases: 9%; Controls: 8%) [12]. Moreover, both the GEMS and MAL-ED studies highlighted the importance of *Cryptosporidium* infection in children and its significance in morbidity and mortality.

The GEMS study, a case-control study, showed that *Cryptosporidium* infection was highest in children less than two years in both cases and controls (Cases: 6.0 – 19.8%; Controls: 3.3 – 16.5%) as compared to older children (24 – 59 months: Cases: 2.2 – 9.6%; Controls: 2.2 – 12.0%) across all international sites and was significantly associated with moderate to severe diarrhea in this younger age group. This differs from the findings in this study though sites in neither Nepal nor Bhutan were included in the GEMS study [6,8]. In Bhaktapur, Nepal, a town near Kathmandu, which was one of the sites included in the MAL-ED study, *Cryptosporidium* infection was reported in 3.6% of children aged 12 – 24 months with no *Cryptosporidium* detected in children aged 0 – 11 months [7,13,14]. In the present study, between the two sites in Nepal, 1.6% of the cases and 1.0% of the controls in children aged 12 – 23 months were infected with *Cryptosporidium* and 2.1% of the cases and 0.5% of the controls in children aged 3 – 11 months were infected (Table 1). The prevalence of *Cryptosporidium* infection in this report peaked in children aged 36 – 47 months in Nepal at 4.2% and 24 – 35 months in Bhutan at 4.8%. There may be a number of reasons that accounted for this apparent disparity in older children being infected more frequently than younger children unlike the observations in other studies. Reasons attributable to this finding may lie in the difference of detection methods used in the respective studies and the target age population. Other factors may include the *Cryptosporidium* endemicity in Bhutan and Nepal and certain geographic and environmental factors. For a better understanding of transmission dynamics warrants further investigation of *Cryptosporidium* infection and subtypes including the circulating genotypes in Bhutan and Nepal.

A limiting factor of this study is that only one time point stool sample was collected from each case and control. Shedding of *Cryptosporidium* oocysts in stool is sporadic and highly variable from day to day and this may affect *Cryptosporidium* detection in the infected stool [15,16]. The ProSpecT™ *Cryptosporidium* microplate assay has been shown in previous studies to have limited sensitivity in the detection of *Cryptosporidium* in stool with low parasite densities (< 175/10 µl) [17,18]. The *Cryptosporidium* parasite burden in children less than 2 years of age in the present study could have been significantly less compared to the older children resulting in the lower detection rates observed; this may be due to the limitation of the ELISA assay which is the most efficient screening assay available at the time. More recent studies have used PCR assays for the detection of *Cryptosporidium* that are significantly more sensitive and robust when compared to ELISA and microscopy [11,19-21]. Moreover, in our study only those children presenting with acute diarrhea (diarrhea of no more than 72 hours duration) were included as cases. Children with persistent diarrhea (diarrhea greater than 14 days) or prolonged diarrhea (diarrhea greater than 7 days) were excluded from the present study. Several studies have shown that *Cryptosporidium* was significantly associated with persistent and prolonged diarrhea when compared to cases of acute diarrhea [22].

Persistent diarrhea was the most common clinical presentation of *Cryptosporidium* infection in a cohort in Brazil with a prevalence of 6.9% [23]. Furthermore, *Cryptosporidium* oocysts were detected more frequently in persistent diarrhea cases (16.9%) when compared to those with acute diarrhea (8.4%) in this Brazilian cohort. A study in Kenya examining over 4500 samples reported cryptosporidiosis prevalence of 3.8% among acute diarrhea and 7.2% of persistent diarrhea cases [24]. Excluding persistent diarrhea cases in the present study could have impacted the overall prevalence. Speciation and molecular subtyping of the *Cryptosporidium* detected in the study may provide additional data on etiology and severity. Several studies have shown that *C. hominis* is more significantly associated with diarrhea and heavier infections than other species including *C. parvum* [11,25,26].

In Nepal, the highest percent detection of *Cryptosporidium* was found in June (3.1%) and July (4.3%) with another smaller peak in February (3%) while in Bhutan, the highest percent detection rates (4.3 - 5.7 %) were observed from July to September (Figure 1). The months from June to August had the

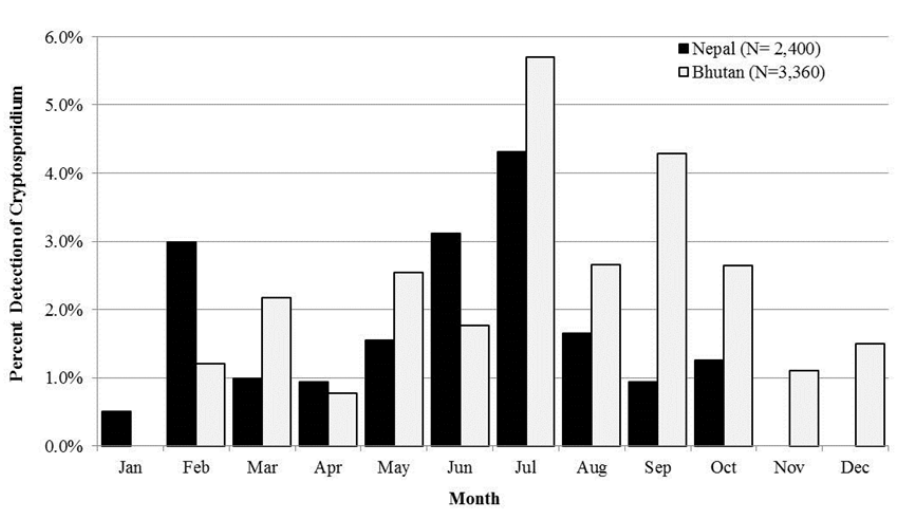

Figure 1 Percent detection of Cryptosporidium by month in all study sites in Bhutan and Nepal.

highest percent detection of *Cryptosporidium* in both Nepal and Bhutan coinciding with the summer/monsoon season in both countries.

A meta- analysis of cryptosporidiosis seasonality reported rising of cases associated with an increase in temperature and precipitation [27]. This is consistent with other reports that an increase incidence was observed during warm, rainy season [23,28]. Cryptosporidiosis is primarily a waterborne diarrheal disease and mainly transmits through contamination of water. Increasing in concentration of *Cryptosporidium* oocysts in surface and drinking water sources was detected after heavy rainfall due to runoff [29]. Additionally, gastrointestinal illness outbreaks attributable to *Cryptosporidium* were associated with recreational water [30]. Increasing of temperature and precipitation may be used for prediction of an increase incidence of cryptosporidiosis [27].

From a total of 108 *Cryptosporidium* positives samples, co-infections with other parasites, a more common association was found with *Giardia lamblia* 8.3% (9/108) where as 0.9% (1/108) was observed with *Entamoeba histolytica*.

CONCLUSION

Despite limitations, the results of these case-control studies in Nepal and Bhutan suggest *Cryptosporidium* is prevalent and a significant pathogen. As the prevalence and burden of *Cryptosporidium* might be underestimated in the current studies, additional surveillance and morbidity studies of cryptosporidiosis in Nepal and Bhutan are warranted.

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DISCLAIMER

The views expressed in this article are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government. Trade names are used for identification purposes only and do not imply endorsement.

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