Tropical Biomedicine 28(1): 40–47 (2011)

Prevalence and molecular characterization of *Cryptosporidium* in schoolchildren from department of Rio San Juan (Nicaragua)

Muñoz-Antoli, C.[1], Pavón, A.[2], Marcilla, A.[1], Toledo, R.[1] & Esteban, J.G.[1*]
[1] Departamento de Parasitologia, Facultad de Farmacia, Universidad de Valencia, Av. Vicente Andrés Estellés s/n, 46100 Burjassot-Valencia, Spain
[2] Departamento de Bioanálisis Clínico, Instituto Politécnico de la Salud, Universidad Nacional Autónoma de Nicaragua, Managua, Nicaragua
* Corresponding author email: Jguillermo.Esteban@uv.es
Received 3 July 2010; received in revised form 15 October 2010; accepted 17 October 2010

Abstract. A cross-sectional study of cryptosporidiosis was carried out in Nicaragua. The prevalence of *Cryptosporidium* infection was determined in 272 (110 boys and 162 girls) schoolchildren, aged between 4 to 15 years from department of Rio San Juan. The total percentage obtained for *Cryptosporidium* (35.7%) was one of the highest reported so far. *Cryptosporidium* appeared in 94.8% of multiparasitism cases. No significant statistical differences were detected in the cryptosporidiosis prevalence between loose/watery (22.2%) and soft/formed (36.7%) stool samples. No significant age and sex differences were observed. This is the first report to identify *Cryptosporidium* in Nicaragua at species level, providing a preliminary molecular characterization of all positive samples, such as *Cryptosporidium parvum* (genotype 2). The high prevalence of *C. parvum* suggests that animals may be potential sources of infection for human cryptosporidiosis, although *C. parvum* infections may have originated from humans themselves. The human health problem caused by *Cryptosporidium* in this region may be related to the poor human hygiene/sanitation and contamination of the environment, food, or water supplies. Continuous exposure to the parasite could have been protective against development of symptoms in the children examined.

INTRODUCTION

Intestinal parasitic infections are common in humans around the world. Infections in childhood, pregnancy and those related to AIDS are the most relevant ones. Morbidity and mortality associated with them are high with more than 58 million cases of childhood diarrhoea caused by protozoans per year (Savioli *et al*., 2006).

Cryptosporidium, an apicomplexan parasite, is a major pathogen causing diarrhoea in humans. Knowledge on its taxonomy has increased a great deal in the past decade and, to date, 20 valid species are recognized within the genus reported from mammals, birds, amphibian, reptiles and fish (Ryan *et al*., 2004; Xiao *et al*., 2004; Fayer, 2010). At least eight *Cryptosporidium* species have been reported in humans: *C. hominis* (=*C. parvum* human genotype, genotype 1 and genotype H *sensu* Morgan-Ryan *et al*., 2002), *C. parvum* (=*C. parvum* bovine genotype, genotype 2, genotype B and *C. pestis sensu* Slapeta, 2006), *Cryptosporidium felis*, *Cryptosporidium andersoni*, *Cryptosporidium canis*, *Cryptosporidium meleagridis*, *Cryptosporidium muris*, and *Cryptosporidium suis* (Xiao *et al*., 2004; Cama *et al*., 2008; Chalmers *et al*., 2009; Fayer, 2010).

Information currently available on the prevalence of intestinal parasites in

Nicaragua is rather scarce (Cavuoti & Lancaster, 1992; Tellez *et al.*, 1997; Oberhelman *et al.*, 1998). Related studies have always been biased, referring to only one or a small number of departments and analysing a small number of subjects. To date, cryptosporidiosis in Nicaragua has only been reported in the department of León (Leiva *et al.*, 2006). The aim of the present paper is to report prevalence data on intestinal cryptosporidiosis and to identify the species and genotypes of *Cryptosporidium* that are present in schoolchildren from department of Rio San Juan in order to shed light on the potential ways of cryptosporidiosis transmission in this area.

MATERIALS AND METHODS

Study population

Nicaragua, situated between Honduras (north) and Costa Rica (south), the Atlantic Ocean (east) and the Pacific Ocean (west) is among one of the poorest countries in Latin America. The total population is 5 142 098 inhabitants, 44.7% of whom living in extreme poverty, distributed in 17 departments. The study was carried out in department of Rio San Juan, located in the southeastern part of Nicaragua, 300 km away from the capital, Managua. It has a population of 95 600 inhabitants, a territorial extension of 7 473 km^2 and is made up of five municipalities. The annual mean temperature is 28°C, with annual precipitations of 2 000 to 6 000 ml, making it the zone with the most abundant precipitation in the country. It has a mean altitude of approximately 100 m above sea level. Its economy is based on the production of basic grains, wood exploitation, commercial activities and to a minor extend tourism and fishing.

The study involved a total of 272 schoolchildren (110 boys and 162 girls) between 4 and 15 years of age, attending five primary schools in department of Rio San Juan, concretely three in San Carlos, one in Boca de Sábalos, and one in El Castillo.

The study population originated from urban and rural areas covering various socioeconomic levels, from dwellings in cobbled streets to ruin-like dwellings, streets full of mud and stones, lacking electricity and drinking water and any kind of infrastructure.

Coprological survey

A clean small snap-cap plastic container and a thin applicator stick were given to every child. They were instructed to transfer a small quantity of stool into the container and return it to the research team. One faecal sample per child was collected and the personal data of the student (name, sex, age), as well as faecal consistency, were noted. A total of 18 loose/watery samples and a total of 254 soft/formed samples were analysed.

Each faecal sample was divided into two aliquots. One fixed in formaline 10% for microscopic parasite detection after concentration using the diphasic technique of formol-ether (Knight *et al.*, 1976). The other aliquot was fixed in ethanol 70% to be analysed molecularly. Concretely, 200 µl of the latter were washed in PBS 1X, through centrifugation at 13 000 rpm for 3 min, prior to molecular study.

DNA extraction

Tubes with 200 µl of faecal samples in PBS 1X were processed for DNA extraction. Oocysts were disrupted by subjecting the tubes to six freeze-thaw cycles (2 min in liquid N_2 and then incubated at 100°C for 2 min). DNA was extracted by using QIAamp DNA Stool Mini Kit (QIAGEN), which involved digestion with proteinase K in lysis buffer AL at 56°C for 30 min, purification in a spin column, elution in 50 µl buffer AE, and storage at -20°C until further use.

PCR analysis

The PCR and semi-nested PCR assays targeted the region of the 18S rRNA gene, used to distinguish *Cryptosporidium* species (Hashimoto *et al.*, 2006). Briefly, initial PCR with the primer pair CPB-DIAGF1 and CPB-DIAGR was used to

amplify approximately 435 bp products (Johnson *et al.*, 1995). For semi-nested PCR the primer pair CPB-DIAGF1 and CPB-DIAGR1 resulted in approximately 400 bp DNA products. Semi-nested PCR products were detected on ethidium bromide-stained 1% agarose gels with UV trans-illuminator.

The obtained PCR bands were cut from gel and purified using Ultra Clean 15 DNA Purification kit for gels and solutions (MoBio Laboratoires) before being sequenced.

All sequences were subjected to a BLAST search to determine their homologies using the GenBank database for *C. hominis* (GenBank accession numbers: AF093491 and AF093489), *C. parvum* (GenBank accession numbers: AB513881 and AF093490), *C. felis* (GenBank accession number: DQ836340), *C. andersoni* (GenBank accession number: HQ009808), *C. canis* (GenBank accession number: AY120909), *C. meleagridis* (GenBank accession number: AF112574), *C. muris* (GenBank accession number: X64343), and *C. suis* (GenBank accession number: AF108861). The sequences were aligned with currently available 18S rRNA gene sequences of *Cryptosporidium* using Clustal W.

Phylogenetic analyses were conducted based on evolutionary distance using the software MEGA version 4.0, by the neighbour-joining method, after distance estimation using the Kimura 2-parameter model. In the construction of the genealogical tree, *Eimeria tenella* was utilized as outgroup. The confidence of groups was assessed by bootstrap values using 1 000 replicates.

Statistical analysis

Statistical analyses were carried out using the chi-square test. A P value below 0.05 was considered significant.

RESULTS

The total prevalence for *Cryptosporidium* infection in the 272 children studied was 35.7%. Among all positive cases detected, only five children were parasitized by *Cryptosporidium* only, whilst the other 92 children were cases of multiparasitism. Concretely, *Cryptosporidium* appeared associated with one enteroparasite (14 cases), two (20), three (18), four (15), five (11), six (7), seven (6), and eight (1). This last case was a seven-year old girl, who was parasitized by *Entamoeba histolytica/Entamoeba dispar/Entamoeba moshkovskii*, *Entamoeba hartmanni*, *Endolimax nana*, *Giardia intestinalis*, *Blastocystis hominis*, *Trichuris trichiura*, *Ascaris lumbricoides* and Ancylostomatidae.

No significant statistical differences were detected in the cryptosporidiosis prevalence obtained between loose/watery (4 positives of 18 cases; 22.2%) and soft/formed (93 of 254; 36.7%) stool samples. It is noteworthy that the four loose/watery samples positive for *Cryptosporidium* correspond to the cases of multiparasitism, with a range between three and seven enteroparasites, and in all cases the presence of *G. intestinalis*, species of recognized pathogenicity, was detected.

No statistically significant differences were detected in the *Cryptosporidium* parasitation between boys (36.7%; 95% CI: 27.4%-45.3%) and girls (35.2%; 95% CI: 27.8%-42.5%). Likewise, no statistical differences were obtained in relation to age groups, although the highest percentage of parasitation (53.3%; 95% CI: 28.1%-78.6%) was detected in children between 13 and 15 years of age.

DNA profiles from PCR products gave an identical fragment size of 400 bp (Figure 1). All sequences obtained were aligned to reach a sequence consensus (GenBank accession number HQ331238).

The neighbour-joining tree that was constructed (Figure 2) determined the genetic relationships of the fragment analyzed, compared with the standard sequences from GenBank, and allowed for its identification as *C. parvum* (bovine genotype, genotype 2, genotype B or *C. pestis*) with 100% homology.

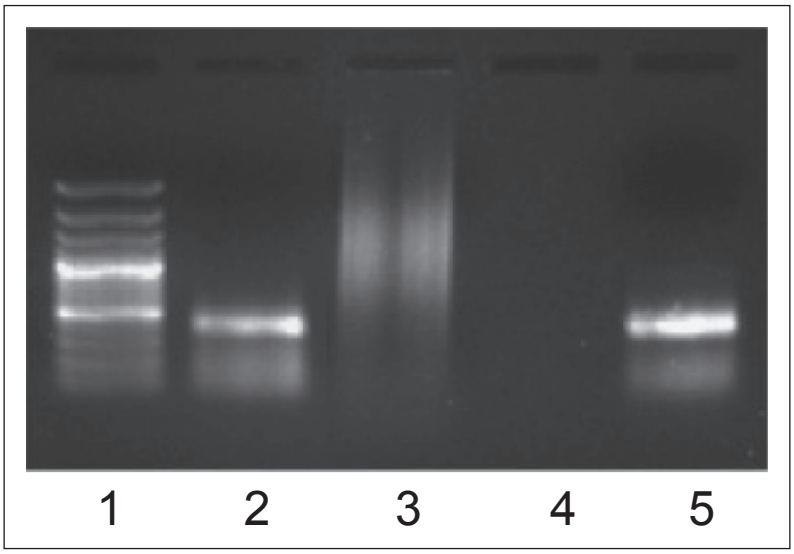

Figure 1. Ethidium-bromide-stained agarose gel of nested-PCR products from faecal samples from Nicaraguan schoolchildren. *Lane 1*: molecular marker; *Lane 2*: positive sample; *Lane 3*: negative sample; *Lane 4*: negative control; *Lane 5*: positive control

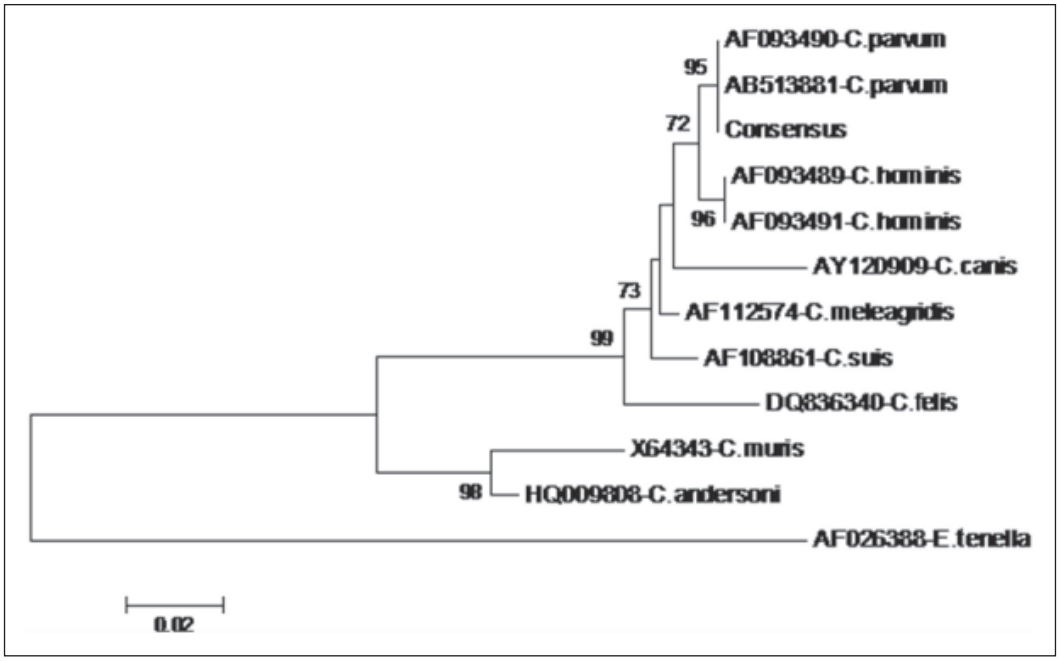

Figure 2. Phylogenetic tree for 18S rRNA gene sequences of *Cryptosporidium* from this experiment (Consensus) and other species published in Genbank. Neighbour-joining consensus tree is shown using *Eimeria tenella* as outgroup. Numbers on the left of the nodes indicate the bootsrap values greater than 70%. Scale bar indicates an evolutionary distance of 0.02 nucleotides per position in the sequence

DISCUSSION

Data available on *Cryptosporidium* species infecting humans in Central America is rather limited, originating from studies involving children, with prevalences of 1.2 to 32% in Guatemala (Cruz *et al.*, 1988; Bern *et al.*, 2000; Laubach *et al.*, 2004), 6.7% in Honduras (Kaminsky, 1991), 4.0% in El Salvador (Reinthaler *et al.*, 1988) and 4.3% in Costa Rica (Mata *et al.*, 1984). In Nicaragua, only one previous study carried out in the department of León demonstrated the presence of the parasite in only 0.7% of 134 subjects above the age of 2 suffering from diarrhoea (Leiva *et al.*, 2006). The prevalence rate obtained in our study (35.7%) is relatively high compared with other studies carried out in other regions of the world.

Cryptosporidiosis is a frequent cause of diarrhoeal disease in humans, with children being more susceptible to *Cryptosporidium* infection than adults (Bhattacharya *et al.*, 1997; Newman *et al.*, 1999; Bern *et al.*, 2000). Nevertheless, appreciable symptoms of diarrhoea were not detected in the schoolchildren at the time of sampling (only 18 out of 272 samples were of loose or watery consistency). The interpretation of these results is complex. Data concerning previous exposures and clinical histories is non-existent. Then, there is the possibility that some students had a recent history of diarrhoea and that many were still shedding oocysts after recovering from this clinical alteration. Consequently, parasitized schoolchildren could have developed diarrhoea after the first *Cryptosporidium* infection months or years before and may have developed some level of immunity due to continual exposure (Esteban *et al.*, 1998). Moreover, frequent cases of multiparasitism among the schoolchildren with *Cryptosporidium* infection must not be neglected, i.e. it is difficult to ascribe diarrhoea to one or another enteroparasite. These facts suggest that these children regularly suffer high intensity exposure to the infective stages of enteroparasites. An immune system that is still in development together with unsanitary living conditions make it easy for the examined pupils to be infected from a very early age, which may be related to posterior protection against parasite-related symptoms (Esteban *et al.*, 1998; Wongstitwilairoong *et al.*, 2007; Siwila *et al.*, 2010).

The non-significant differences in prevalences according to gender and the fact that all age-groups are susceptible to infection could be explained by the lack of gender role differences and by the ingestion of oocysts through several transmission routes (Griffiths, 1998; Clark, 1999).

Among the two genotypes mainly found in human infections, *C. hominis* (genotype 1) appears to be host-specific, and may be expected to reach a higher oocyst density in the human gastrointestinal tract than *C. parvum* (genotype 2), with no host-specificity (McLauchlin *et al.*, 1999). However, to date we have only found *C. parvum* genotype 2 infections, and the high prevalence obtained suggests that animals may be potential sources of infection for human cryptosporidiosis. In rural areas, zoonotic infections of *C. parvum* via direct contact with farm animals have frequently been reported. However, one should be cautions when interpreting the significance of finding parasites traditionally associated with animals in humans (Miron *et al.*, 1991; Peng *et al.*, 1997; Stantic-Pavlinic *et al.*, 2003). It is always tempting to attribute the source of *C. parvum* and other zoonotic *Cryptosporidium* spp. found in humans and the environment solely to animals (Ong *et al.*, 1999; Stantic-Pavlinic *et al.*, 2003). Contradictory to what may be intuitive, many human *C. parvum* infections in certain areas may indeed have originated from humans themselves (Xiao *et al.*, 2004).

The human health problem of this coccidian parasite in the study area may be related to the poor level of hygiene and sanitation of the human population and the contamination of the environment, food, or water supplies. The numerous protozoan and helminth species detected in the area

surveyed and multiple parasite infections detected, including *Cryptosporidium* infection, in the same schoolchildren are illustrative of this situation.

This is the first report that identifies *Cryptosporidium* in Nicaragua at species level and that provides a preliminary molecular characterization of *C. parvum* in all positive samples. Future in-depth studies should be conducted to analyse the diversity of *Cryptosporidium* at species and subtype levels among animals and inhabitants from Nicaragua.

Acknowledgements. We are very grateful to the headmasters and teachers of each school, and parents of schoolchildren participating in this study. We are also grateful to the health authorities the department of San Juan, Dr. F. Canales, A. Rodriguez and W. Ramos for supporting the study. The funds for this study were obtained from the Proyecto de Cooperación al Desarrollo 2007 (s/n) del Vicerrectorado de Relaciones Institucionales y Cooperación from the Universitat de València-Estudi General (Valencia, Spain).

REFERENCES

Bern, C., Hernandez, B., Lopez, M.B., Arrowood, M.J., De Merida, A.M. & Klein, R.E. (2000). The contrasting epidemiology of *Cyclospora* and *Cryptosporidium* among outpatients in Guatemala. *American Journal of Tropical Medicine and Hygiene* **63**: 231-235.

Bhattacharya, M.K., Teka, T., Faruque, A.S. & Fuchs. G.J. (1997). *Cryptosporidium* infection in children in urban Bangladesh. *Journal of Tropical Pediatrics* **43**: 282-286.

Cama, V.A., Bern, C., Roberts, J., Cabrera, L., Sterling, C.R., Ortega, Y., Gilman, R.H. & Xiao, L. (2008). *Cryptosporidium* species and subtypes and clinical manifestations in children, Peru. *Emerging Infectious Diseases* **14**: 1567-1574.

Cavuoti, D. & Lancaster, K.R. (1992). Intestinal parasitism of children on Corn Island, Nicaragua. *The Pediatric Infectious Disease Journal* **11**: 775-776.

Chalmers, R.M., Robinson, G., Elwin, K., Hadfield, S.J., Xiao, L., Ryan, U., Modha, D. & Mallaghan, C. (2009). *Cryptosporidium* sp. rabbit genotype, a newly identified human pathogen. *Emerging Infectious Diseases* **15**: 829-830.

Clark, D.P. (1999). New insights into human cryptosporidiosis. *Clinical Microbiology Reviews* **12**: 554-563.

Cruz, J.R., Cano, F., Caceres, P., Chew, F. & Pareja, G. (1988). Infection and diarrhea caused by *Cryptosporidium* sp. among Guatemalan infants. *Journal of Clinical Microbiology* **26**: 88-91.

Esteban, J.G., Aguirre, C., Flores, A., Strauss, W., Angles, R. & Mas-Coma, S. (1998). High *Cryptosporidium* prevalences in healthy Aymara children from the Northern Bolivian Altiplano. *American Journal of Tropical Medicine and Hygiene* **58**: 50-55.

Fayer, R. (2010). Taxonomy and species delimitation in *Cryptosporidium*. *Experimental Parasitology* **124**: 90-97.

Griffiths, J.K. (1998). Human cryptosporidiosis: epidemiology, transmission, clinical disease, treatment, and diagnosis. *Advances in Parasitology* **40**: 37-85.

Hashimoto, A., Sugimoto, H., Morita, S. & Hirata, T. (2006). Genotyping of single *Cryptosporidium* oocysts in sewage by semi-nested PCR and direct sequencing. *Water Research* **40**: 2527-2532.

Johnson, D.W., Pieniazek, N.J., Griffin, D.W., Misener, L. & Rose, J.B. (1995). Development of a PCR protocol for sensitive detection of *Crytosporidium* oocysts in water samples. *Appied andl Environmental Microbiology* **61**: 3849-3855.

Kaminsky, R.G. (1991). Parasitism and diarrhoea in children from two rural communities and marginal barrio in Honduras. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **85**: 70-73.

Knight, W.B., Hiatt, R.A., Cline, B.L. & Ritchie, L.S. (1976). A modification of the formol-ether concentration technique for increased sensitivity in detecting *Schistosoma mansoni*. *American Journal of Tropical Medicine and Hygiene* **55**: 818-823.

Laubach, H.E., Bentley, C.Z., Ginter, E.L., Spalter, J.S. & Jensen. L.A. (2004). A study of risk factors associated with the prevalence of *Cryptosporidium* in villages around Lake Atitlan, Guatemala. *Brazilian Journal of Infectious Diseases* **8**: 319-323.

Leiva, B., Lebbad, M., Winiecka-Krusnell, J., Altamirano, I., Tellez, A. & Linder, E. (2006). Overdiagnosis of *Entamoeba histolytica* and *Entamoeba dispar* in Nicaragua: a microscopio, triade parasite panel and PCR study. *Archives of Medical Research* **37**: 529-534.

Mata, L., Bolanos, H., Pizarro, D. & Vives, M. (1984). Cryptosporidiosis in children of Costa Rica: a cross section and longitudinal study. *Revista de Biologia Tropical* **32**: 129-135.

McLauchlin, J., Pedraza-Diaz, S., Amar-Hoetzeneder, C. & Nichols, G.L. (1999). Genetic characterization of *Cryptosporidium* strains from 218 patients with diarrhea diagnosed as having sporadic cryptosporidiosis. *Journal of Clinical Microbiology* **37**: 3153-3158.

Miron, D., Kenes, J. & Dagan, R. (1991). Calves as a source of an outbreak of cryptosporidiosis among young children in an agricultural closed community. *Pediatric Infectious Diseases Journal* **10**: 438-441.

Morgan-Ryan, U.M., Fall, A., Ward, L.A., Hijjawi, N., Sulaiman, I., Fayer, R., Thompson, R.C., Olson, M., Lal, A. & Xiao, L. (2002). *Cryptosporidium hominis* n. sp. (Apicomplexa: Cryptosporidiidae) from *Homo sapiens*. *Journal of Eukaryotic Microbiology* **49**: 433-440.

Newman, R.D., Sears, C.L., Moore, S.R., Nataro, J.P., Wuhib, T., Agnew, D.A., Guerrant, R.L. & Lima, A.A. (1999). Longitudinal study of *Cryptosporidium* infection in children in northeastern Brazil. *Journal of Infectious Diseases* **180**: 167-175.

Oberhelman, R.A., Guerrero, E.S., Fernandez, M.L., Silio, M., Mercado, D., Comiskev, N., Ihenacho, G. & Mera, R. (1998). Correlations between intestinal parasitosis, physical growth, and psicomotor development among infants and children fron rural Nicaragua. *American Journal of Tropical Medicine and Hygiene* **58:** 470-475.

Ong, C.S., Eisler, D.L., Goh, S.H., Tomblin, J., Awad-El-Kariem, F.M., Beard, C.B., Xiao, L., Sulaiman, I., Lal, A., Fyfe, M., King, A., Bowie, W.R. & Isaac-Renton, J.L. (1999). Molecular epidemiology of cryptosporidiosis outbreaks and transmission in British Columbia, Canada. *American Journal of Tropical Medicine and Hygiene* **61**: 63-69.

Peng, M.M, Xiao, L., Freeman, A.R., Arrowood, M.J., Escalante, A.A. & Weltman, A.C. (1997). Genetic polymorphism among *Cryptosporidium parvum* isolates: evidence of two distinct human transmission cycles. *Emerging and Infectious Diseases* **3**: 567-573.

Reinthaler, F.F., Linck, G., Klem, G., Mascher, F. & Sixl, W. (1988). Intestinal parasites in children with diarrhea in El Salvador. *Geographia Medica* **18**: 175-180.

Ryan, U.M., Monis, P., Enemark, H.L., Sulaiman, I., Samarasinghe, B., Read, C., Buddle, R., Robertson, I., Zhou, L., Thompson, R.C. & Xiao, L. (2004). *Cryptosporidium suis* n. sp. (Apicomplexa: Cryptosporidiidae) in pigs (*Sus scrofa*). *Journal of Parasitology* **90**: 769-773.

Savioli, L., Smith, H. & Thompson, A. (2006). *Giardia* and *Cryptosporidium* join the "Neglected Diseases Initiative". *Trends in Parasitology* **22**: 203-208.

Siwila, J., Phiri, I.G., Enemark, H.L., Nchito, M. & Olsen, A. (2010). Intestinal helminths and protozoa in children in pre-schools in Kafue district, Zambia. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **104**: 122-128.

Slapeta, J. (2006). *Cryptosporidium* species found in cattle: a proposal for a new species. *Trends in Parasitology* **22**: 469-474.

Stantic-Pavlinic, M., Xiao, L., Glaberman, S., Lal, A.A., Orazen, T., Rataj-Verglez, A., Logar, L. & Berce, I. (2003). Cryptosporidiosis associated with animal contacts. *Wiener Klinische Wochenschrift* **115**: 125-127.

Tellez, A., Morales, W., Rivera, T., Meyer, E., Leiva, B. & Linder, E. (1997). Prevalence of intestinal parasites in the human population of Leon, Nicaragua. *Acta Tropica* **66**: 119-125.

Wongstitwilairoong, B., Srijan, A., Serichantalergs, O., Fukuda, C.D., Mcdaniel, P., Bodhidatta, L. & Mason, C.J. (2007). Intestinal parasitic infections among pre-school children in Sangkhlaburi, Thailand. *American Journal of Tropical Medicine and Hygiene* **76**: 345-350.

Xiao, L., Bern, C., Sulaiman, I.M. & Lal, A.A. (2004). Molecular epidemiology of human cryptosporidiosis. In: *Cryptosporidium: from molecules to disease*, Thompson RCA. (editors) pp. 227-262. Elsevier, Amsterdam, The Netherlands.

Xiao, L., Fayer, R., Ryan, U. & Upton, S.J. (2004). *Cryptosporidium* taxonomy: recent advances and implications for public health. *Clinical Microbiology Reviews* **17**: 72-97.