

**ZOONOTIC *CRYPTOSPORIDIUM PARVUM* IN DIARRHEA PATIENTS IN CHANGCHUN, CHINA**

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**ABSTRACT**

Genotyping/subtyping of *Cryptosporidium* parasite in diarrhea patients with different genders, ages and seasons in Changchun, China were conducted in a study aiming to assess the genetic diversity extent of *Cryptosporium* spp. with their phylogenetic relationships among the analyzed samples. *Cryptosporidium parvum* was detected in two (0.32 %) of 617 diarrhea patients. The infection rate was detected in female children in summer season. The presence of *Cryptosporidium parvum* family IIa implied zoonotic transmission.

**KEYWORDS:** cryptosporidiosis, *Cryptosporidium*, diarrhea, zoonotic diseases.

**INTRODUCTION**

Human cryptosporidiosis caused by *Cryptosporidium* parasite; has been recognized worldwide as the most common cause of protozoal diarrhea leading to significant morbidity and mortality in industrialized nations and developing countries (Clark, 1999). Although person-to-person transmission has been considered the major route of *Cryptosporidium* transmission, zoonotic transmission of this protozoan may also occur (Marshall *et al.*, 1997). *Cryptosporidium* oocysts may remain viable in water for over 140 days (Hooda *et al.*, 2000). The oocysts are very resistant to the most common disinfectants (Campbell *et al.*, 1982) making them difficult to be destroyed by conventional chlorination treatment. *Cryptosporidium* is a morphologically identical but genetically different multiple genotypes parasite (Xiao and Ryan, 2004; Abe *et al.*, 2006). In humans, *Cryptosporidium hominis* (*C. hominis*) and *Cryptosporidium parvum* (*C. parvum*) are the most common causes for the majority of infections (Xiao, 2010). *C. hominis* genotype is exclusively found in humans, whereas *C. parvum* is found in humans, domestic, and wild animals (Xiao and Ryan, 2004; Abe *et al.*, 2006).

The small subunit (SSU) rRNA gene multi-copy nature and hypervariable semiconserved regions, gave it the widespread usability in *Cryptosporidium* genotyping (Xiao, 2010). Subtyping tools have been used widely in studies concerning the transmission of *C. hominis* in humans and *C. parvum* in humans and ruminants. The 60-KDa glycoprotein (gp60) gene is considered to be one of the popular subtyping tools for *C. parvum* DNA sequence analysis. Variations in the number of (gp60) trinucleotide repeats and its extensive sequence differences in the non-repeat regions, categorize *C. parvum* and *C. hominis* each to several subtype families (*C. parvum*, IIa, IIb, IIc, IId, IIe, IIe, IIe, IIe; *C. hominis*, Ia, Ib, Id, Ie) (Abe *et al.*, 2006). In China, infection rate of *Cryptosporidium* is 42%-58% in children less than 16 years of age (Zu *et al.*, 1994; USEPA, 2001). Therefore, research on *Cryptosporidium* on species and genotype level is considered an important issue for accurately identifying the organism and assessing its genetic characterization.

**MATERIALS AND METHODS**

During two years investigational period (2010-2012), a total of 615 human diarrhea fecal samples (different ages, different gender and different seasons) (Table 1) were collected from the biggest two hospitals in Changchun, Jilin province (Jilin hospital No. 1 and 2), China. The study aimed to assess the genetic diversity extent of *Cryptosporium* spp. with its relationship among the analyzed samples. Written consent from each participant or their parent/kin prior to participation was taken. The study was approved by Norman Bethune College of Medicine Research Ethics Committee.

The analyzed samples were stained using modified Ziehl Neelsen stain for *Cryptosporidium* parasite detection (Garcia and Bruckner, 1997). Positive samples were kept freshly at -40°C for PCR investigation. The microscopic positive fecal samples were processed for DNA extraction by passing the stool-PBS (phosphate-buffered saline) suspension (Pieniazek *et al.*, 1999) through QIAamp DNA stool Mini Kit (Qiagen sciences, Maryland, USA) following the manufacturer's handbook instructions.

**Polymerase Chain Reaction (PCR)** amplification was performed to identify a fragment of *Cryptosporidium* parasite on species subtype level using previously described nested PCR protocols (Xiao et al., 1999; Feltus et al., 2006). *Cryptosporidium* parasite were identified at the species level targeting SSU rRNA gene using PCR analysis giving an expected band of 830 bp (Xiao et al., 1999), followed by identification at the subtype level targeting (gp60) gene giving an expected band of 900 bp (Feltus et al., 2006). Two tubes were used to represent each sample during the PCR. The PCR products were sent to Sangon Biotech (Shanghai) company for sequencing. To confirm the sequence accuracy, two directional sequencing were requested using secondary PCR product.

The nucleotide sequences received from the company were assembled using ChromasPro Program ([http://technelysium.com.au/?page\\_id=27](http://technelysium.com.au/?page_id=27)). Multiple alignments using Clustal X 2.1 Package (<http://www.clustal.org/download/current/>) were carried out for the DNA sequences obtained from the analyzed samples together with published sequences of *Cryptosporidium* subtypes. *C. parvum* (gp60) nucleotide sequences reported previously (Xiao, 2010; Wang and Cui, 2005) were used in the alignment of the obtained DNA sequences. Nucleic sequences editing, manipulation and analysis were performed using BioEdit program version 7.0.5 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). A neighbor-joining tree using Mega 4.1 software program (<http://www.megasoftware.net/mega4/mega.html>) was constructed for *Cryptosporidium* subtypes sequences to estimate its phylogenetic relationships based on the evolutionary distances calculated by Kimura-2-parameter analysis. Bootstrapping method of 1,000 pseudo replicates used to assess the tree reliability (Figure 1).

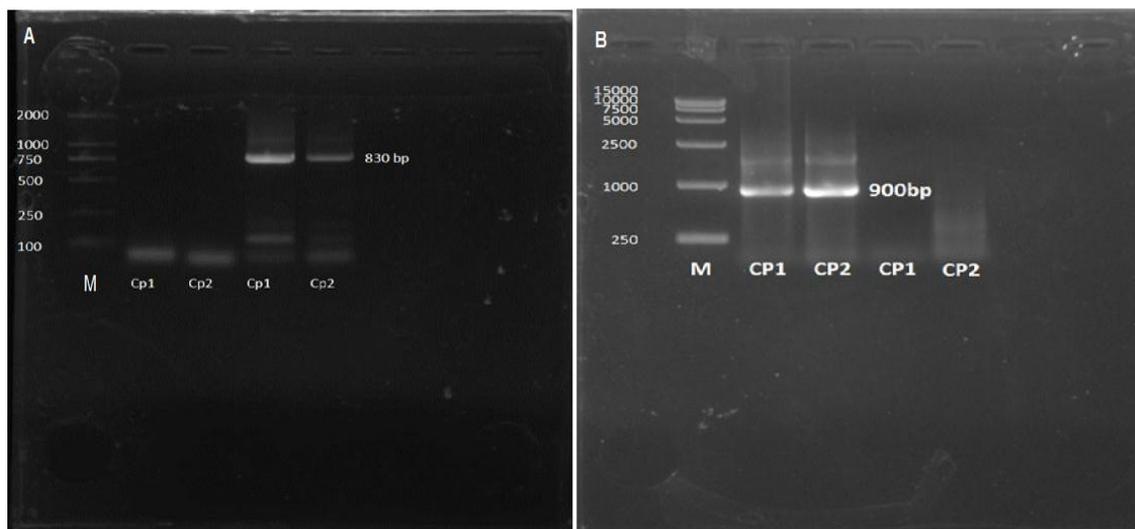
**Statistical analysis** was performed using the SPSS 17.0 computer software statistical package and the Pearson chi-square test was used to test the significance of differences between two qualitatively expressed relations, P values of <0.05 were considered significant.

## RESULTS AND DISCUSSION

In order to understand the epidemiology and establish baseline genetic data for *Cryptosporidium* parasite in Changchun city, we examined 617 human fecal samples microscopically. Molecular, sequencing, genotyping and subtyping analysis of the suspected positive samples gave the following results:



**Picture 1: *Cryptosporidium* oocyst with modified Ziehl Neelsen stain x100**



Picture 2: Panel (A): Genotyping molecular nested PCR of (SSU rRNA) of *Cryptosporidium parvum* (Cp) positive samples, two tubes representing each sample, Panel (B): Subtyping molecular nested PCR of (gp60) gene of *Cryptosporidium parvum* (Cp), two tubes representing each sample.

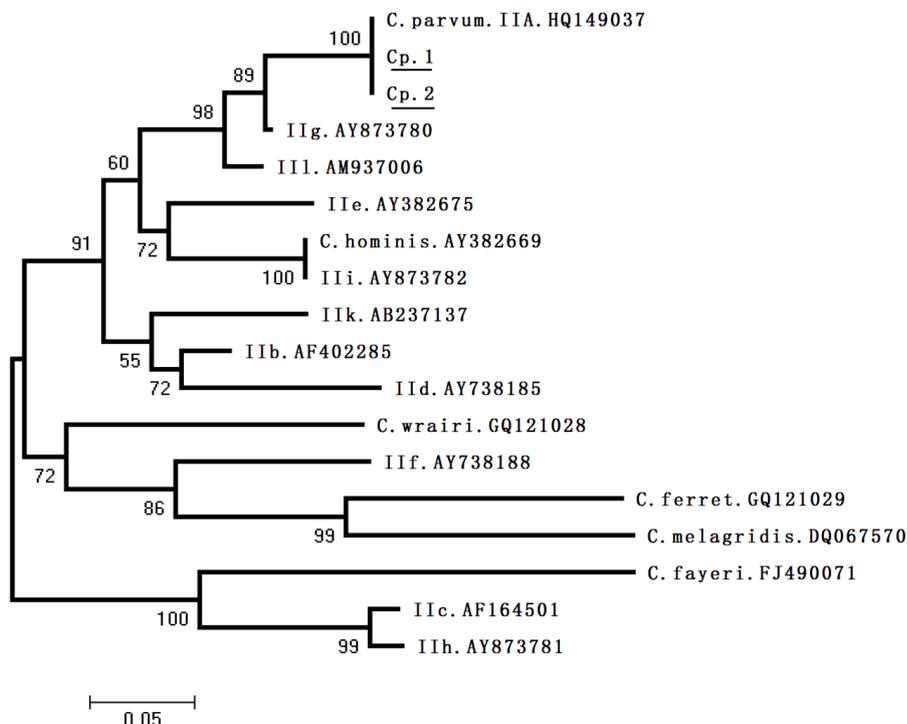


Figure 1: Phylogenetic tree representing the relationship between *C. parvum* in human samples (underlined samples) and published *C. parvum* subtypes associated with other *Cryptosporidium* species using neighbour-joining analysis of (gp60) nucleotide sequences. Formation of a phylogenetic group with reference *C. parvum* subtype family IIa (accession no. HQ149037). Values of >50% are shown using 1,000 pseudo replicates bootstrapping method.

### Distribution of *Cryptosporidium* according to age, gender and season.

Microscopically identified two positive samples out of 617 using modified Ziehl Neelsen stain (Picture 1), giving very low infection rate of (0.32%) compared to other studies in China (Anhui, Changsha, Guangdong, Jiangsu and Yunnan provinces) which gave a prevalence rate of 1.33%, 4.90%, 4.25%, 1.6% and 5.29% respectively (Steve and Stephen, 2010). A report by Chalmers *et al.* (2009) revealed that there has been a decline in *Cryptosporidium* cases since 2001 (Chalmers *et al.*, 2008; Chalmers *et al.*, 2009). Changchun people are keen to boil water before drinking which can be reasonable explanation for the low infection rate in Changchun, as *Cryptosporidium* pathogen can be killed by boiling water for 1 minute. A case control study revealed that the consumption of unboiled tap water at home is significantly and positively associated with illness (Willocks *et al.*, 1998). In this study, the patients were divided to 3 groups according to their ages (1day-10 years, >10-40 years and >40 years old). The two infected patients were female children belonging to the age group between 1 day to 10 years. Children between 2-10 years old were recorded to be mostly infected with *Cryptosporidium* and the infection is more prevalent in female patients than males for all age group (Sherchand and Shrestha, 1996).

Exposure to contaminated drinking water with *Cryptosporidium* is an important risk factor leading to the *Cryptosporidium* infection. In Changchun, China, availability of standard purified water to the public is not present. Most of Changchun people depend on using bottled mineral and boiled water. In daycare centers and kindergartens, there is an absence of bottled and boiled water for children which put them in a high risk to be exposed with *Cryptosporidium* infection. The samples were collected in different seasons of the year. Both infections appeared in summer season which might return to warm temperature and rains associated with this season (Jiantang *et al.*, 2010), that are preferable for *Cryptosporidium* to continue its lifecycle (Bern *et al.*, 2002).

### Genotyping and sub-typing of *Cryptosporidium* parasite in Changchun patients

Genotyping and subtyping of the microscopically positive two samples (Picture 2) indicated that *Cryptosporidium parvum* was the apparent genotype in our samples in accordance with a Kuwaiti study which showed that children were almost exclusively infected with *C. parvum* (Sulaima *et al.*, 2005). However, dominance of *C. hominis* in children was mentioned in other studies (Thailand and Malawi) (Tiangtip and Jongwutiwes, 2002- Peng *et al.*, 2003). Distribution of *Cryptosporidium* genotypes in humans shows a great difference indicating differences in infection sources (Learmonth *et al.*, 2004).

Many species of wild animals (dogs, cats, goats and mice) can be infected with *Cryptosporidium parvum*, which is the same species that infects humans. *Cryptosporidium parvum* is also has been found in dairy and beef cattle. After heavy rains, water runoff from pastures into rivers being an important source of *Cryptosporidium* in surface waters. (Graczyk *et al.*, 1998). *C. parvum* subtype family IIa was the present subtype in this study samples. Family IIa is commonly seen in both humans and ruminants (Sulaima *et al.*, 2005). Most of the studies have shown that calves are commonly infected with IIa subtype family (Xiao, 2010). Thus, the presence of *C. parvum* and subtype family IIa in a population can be considered a result of zoonotic transmission. Our samples were identical to a published isolate (IIaA19G2R1) of (accession no. HQ149037) (Hadfield *et al.*, 2011). Many studies in North America, Europe and Australia, mentioned that the common bovine IIa subtypes were also dominant *C. parvum* subtypes in humans in these areas (Xiao, 2010). Constructed phylogenetic tree for the present study sequences combined with the published sequences of *Cryptosporidium* subtypes, showed that this study sequences formed a major phylogenetic cluster with *Cryptosporidium parvum* IIa family (Figure 1). It is speculated that detailed collection of data concerning water and food sources and history of animal contact, may prove to be more helpful in identifying source of infection and risk factors in cryptosporidiosis.

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