RESEARCH NOTE

DEVELOPMENT OF PCR-RFLP METHOD TO DISTINGUISH BETWEEN *CRYPTOSPORIDIUM PARVUM* AND *C. HOMINIS* IN TAIWAN WATER SAMPLES

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Abstract. *Cryptosporidium*, a protozoan pathogen that causes cryptosporidiosis has emerged as an important source of diarrheal illness among humans and animals. The current routine laboratory technique used for *Cryptosporidium* diagnosis is light microscopy with acid-fast staining but the technique has low efficiency and sensitivity for species-specific identification. Single PCR to amplify a 220 bp fragment of 18 S ribosomal DNA of *C. parvum* and *C. hominis* was developed. The restriction enzymes, *Taq*I and *Vsp*I, were used to distinguish between amplicons of human and bovine *C. parvum* genotype. Water samples, collected from Lo-Na, Ton-Pu, Ho-Ping, and Jen-Ai, Taiwan contained only bovine *C. parvum* genotype whereas in the Ton-Pu and Jen-Ai samples *C. hominis* was also present. Thus, the used of PCR-RFLP allowed successful identification of *Cryptosporidium* in water samples and differentiation between human and bovine species.

Keywords: *Cryptosporidium*, PCR, RFLP, genotype, water sample, Taiwan

INTRODUCTION

Cryptosporidium spp are common pathogenic protozoa that cause cryptosporidiosis, a diarrheal illness caused by waterborne transmission worldwide. This parasite has a global distribution and is of veterinary and public health concern because of its ability to cause gastrointestinal diseases, its ubiquitous presence in the environment, and its propensity to cause waterborne and foodborne outbreaks. High prevalence of waterborne transmission of cryptosporidiosis has been reported (Marshall *et al*, 1997; Barwick *et al*, 2000). For example, ten instances of cryptosporidiosis outbreaks were noticed from 1984 to 1994 (the most serious outbreak being in Milwaukee in 1993 with 419,914 cases).

Cryptosporidium infects a large number of vertebrate species, including humans, cats (*Felis catus*), and dogs (*Canis familiaris*). The *Cryptosporidium* genus currently contains at least 24 valid species and over 40 genotypes, most of which are host adapted and have a narrow host

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range (*eg*, *C. canis* mainly in dogs, *C. felis* mainly in cats, and *C. hominis* in humans) (Thompson *et al*, 2008; Xiao and Fayer, 2008). Some species or genotypes, most notably *C. parvum* and *C. cervine*, have a broader host range, which includes ruminants and humans (Xiao and Fayer, 2008; Xiao and Feng, 2008). Within *C. parvum*, 2 genotypes have been distinguished: type 1 genotype, "human genotype" (H type) and type 2 genotype, "cattle genotype" (C type) (Peng *et al*, 1997). These two genotypes are now recognized as two different species, *C. hominis* (formerly type 1) and *C. parvum* (formerly type 2) (Xiao and Fayer, 2008; Xiao and Feng, 2008).

C. parvum has recently been implicated in waterborne outbreaks in developed and developing countries (Robertson and Gjerde, 2001; Nishi *et al*, 2009; Moulin *et al*, 2010). In southern and central Taiwan, *Cryptosporidium* spp were found in raw water samples from rivers (Hsu *et al*, 1999b; Hu, 2002). However, in these studies, *Cryptosporidium* spp were detected using Ziehl-Neelsen acid-fast stain and fluorescent antibody staining, methods with low sensitivity as a minimum number of 10^3-10^5 oocysts in 1 gram of fecal matter is required for detection (Weber *et al*, 1991). In addition, microscopic examination cannot distinguish between different *Cryptosporidium* spp.

The purpose of this study was to establish a PCR method to detect the 18S rRNA gene of *Cryptosporidium* spp and restriction fragment length polymorphism (RFLP) to identify the presence of specific species in water samples.

MATERIALS AND METHODS

Water samples

Four samples of mountain water from Sinyi (Lo-Na and Ton-Pu village) and Jen-Ai Township, Nantou County, Heping District, and Ho-Ping District, Taichung City of central Taiwan were used in this study (Fig 1). From each household 50 liters of water were collected and pumped through a 3 m membrane filter (Millipore, New South Wales, Australia). The membrane, together with the retained sediment, was dissolved in 100% acetone and washed with 95% ethanol, followed by 70% ethanol (Aldom and Chagla, 1995). The residue of the membrane was washed

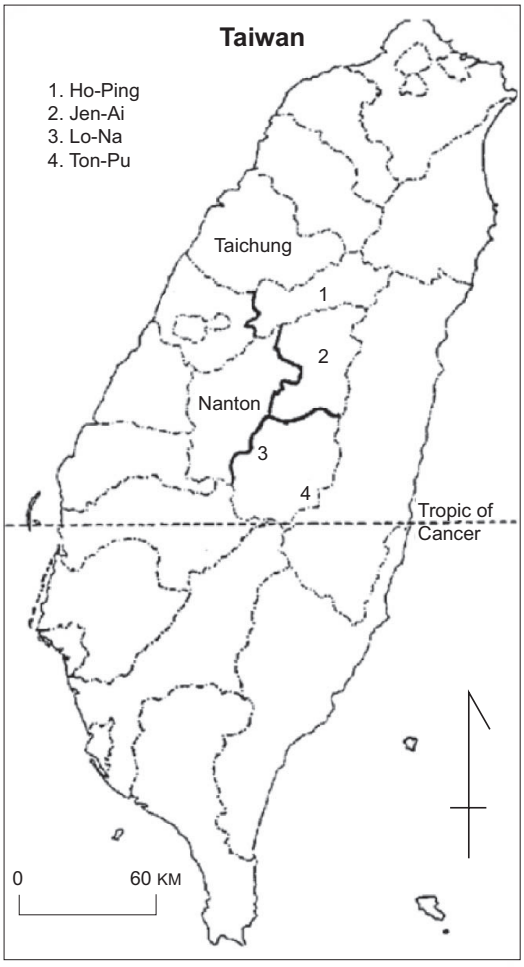

Fig 1–Map of Taiwan showing the sites where water samples were taken: Nantou and Taichung County 1. Ho-Ping, 2. Jen-Ai, 3. Lo-Na, 4. Ton-Pu.

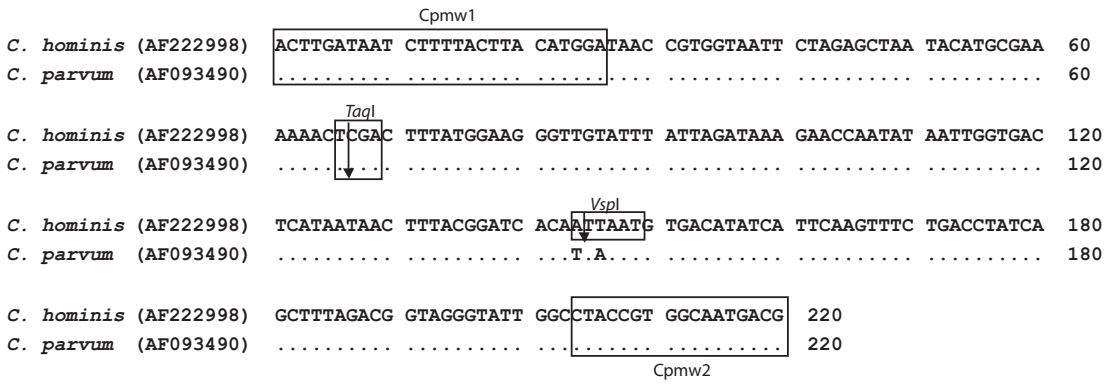

Fig 2–18S rDNA sequence of *C. parvum* and *C. hominis*. Location of PCR primers (Cpmw1, Cpmw2) and sites of restriction enzymes *Taq*I and *Vsp*I are shown.

with phosphate-buffered saline (PBS), and the sediment was stored in a sterile Eppendorf tube at 4ºC until further use.

PCR

Nucleic acids in the sediment from the water samples were extracted using an UltraClean® Soil DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA) and stored at -20ºC before being analyzed. A new pair of primers to detect and discriminate between *C. parvum* and *C. hominis* was designed: forward primer, Cpmw-1 (5'-ACTTGATAATCTTTACTTA-CATGGA-3'), and reverse primer, Cpmw-2 (5'-CGTCATTGCCACGGTAG-3'). A total reaction volume of 50 l, including 2.5 l of *Cryptosporidium* genomic DNA and 10xPCR buffer, containing 1.5 mM $MgCl_2$, 0.5 M of each primer, 200 M of each dNTP, and 1 U GeNei Taq DNA polymerase (Bangalore Genei, India), was used for PCR. The thermocycling was as follows: 45 cycles of 94ºC for 1 minute, 55ºC for 2 minutes, 72ºC for 1 minute, and a final step at 72ºC for 10 minutes.

PCR-RFLP analysis

A 10 l aliquot of the PCR amplicon was digested for 2 hours at 37ºC with 10 U of both *Taq*I and *Vsp*I enzymes (Gibco/Life Technologie, Grand Island, NY) in 35 l of 1x React-2 buffer (Gibco/Life Technologies/Invitrogen). Undigested controls were analyzed together with digested fragments in 2% agarose gel-electrophoresis at 100 V for 0.5 hour containing ethidium bromide (0.5 g/ml) in both the gel and running buffer.

DNA sequence analysis

The PCR amplicons were sequenced by Tri-I Biotech, Taiwan and sequences were analyzed using BioEdit software (Hitachi Software Engineering, Tokyo, Japan, http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

RESULTS

PCR of DNA isolated from the water samples generated 220 bp fragments. The sequence of 220 bp fragment was 100% identical to the sequence of *C. parvum* 18S ribosomal RNA gene (AF222998) and *C. parvum* bovine genotype (AF093490) (Fig 2). Therefore, the DNA samples were identified as being specific fragments of *Cryptosporidium* 18S rDNA.

The 220 bp fragment produced by *C. parvum* and *C. hominis* has specific *Taq*I site that can be digested into 2 fragments of

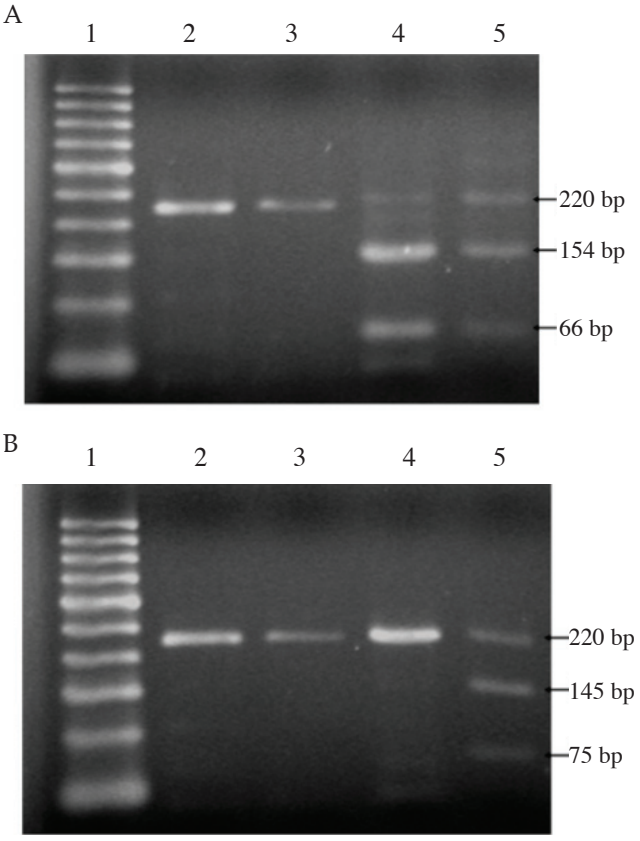

Fig 3–Gel electrophoresis of PCR amplicons from 18S rDNA of *Cryptosporidium* spp. Lane 1: 50 bp marker, lane 2, 4: *C. parvum* bovine genotype; lane 3, 5: *C. parvum* human genotype. (A) *Taq*I digestion, (B) *Vsp*I digestion.

66 bp and 154 bp (Fig 3A). Additionally, the 220 bp fragment of *C. hominis* has a specific *Vsp*I site that can be digested into 2 fragments of 75 bp and 145 bp (Fig 3B). Restriction enzyme analysis of 220 bp amplicons from water samples from all 4 locations were digested by *Taq*I into 66 bp and 154 bp fragments. Moreover, the 220 bp amplicons from Ton-Pu and Jen-Ai were digested by *Vsp*I into 2 smaller fragments of 75 bp and 145 bp, but some 220 bp amplicons remained intact after enzyme treatment. The 220 bp amplicons from Lo-Na and Ho-Ping remained intact following *Vsp*I digestion. From these results, it is apparent that *Cryptosporidium* spp in water samples from Lo-Na and Ho-Ping belonged to *C. parvum*, and those in samples from Ton-Pu and Jen-Ai belonged to *C. hominis*.

DISCUSSION

Currently, identification of *Cryptosporidium* spp is typically performed by Ziehl-Neelsen acid-tast or fluorescent staining of samples followed by microscopy (Newman *et al*, 1993; Alves *et al*, 2000). However, identification accuracy may be affected by the presence of pseudo-positive substances, interference from other spontaneous fluorescent materials (Rosenblatt and Sloan, 1993), or changes in sample preservation methods (*eg*, cryopreservation) leading to alterations in oocyst appearance (Ward and Wang, 2001). Among the various *Cryptosporidium* spp, only *C. parvum*, *C. hominis*, *C. felis*, *C. canis*, *C. meleagridis*, and *C. muris* have infected humans (Xiao *et al*, 2000; Gatei *et al*, 2002; Dalle *et al*, 2003). When testing environmental samples, microscopy is not suitable for species-specific identification. Therefore, because of its high specificity and sensitivity, as well as the ability to distinguish among species, molecular biology methods (such as PCR and PCR-RFLP) are being used in detection of *Cryptosporidium* spp (Spano *et al*, 1997).

Several studies have illustrated that PCR amplification of 18S rRNA gene can produce *C. parvum*-specific amplicons (Morgan *et al*, 1997; Patel *et al*, 1999). However, for distinguishing between

Cryptosporidium spp using 18S rRNA gene as the target, restriction enzymes are needed in order to identify the specific DNA sequences (Xiao *et al*, 1999; Sturbaum *et al*, 2001). In this study, we used newly designed primers to conduct the PCR test and found that amplicons of 220 bp were produced from genomic DNA of *C. parvum*, *C. wrairi,* and *C. meleagridis* (data not shown). We also used restriction enzyme *Taq*I to distinguish *C. parvum* from *C. meleagridis* and *C. wrairi*, whose 220 bp amplicon cannot be digested by this enzyme. In addition, *C. parvum* were treated with *Vsp*I which digests *C. parvum* human genotype (*C. hominis*) into 2 fragments but not *C. parvum* bovine genotype. Analysis of the PCR amplicons from water samples obtained from 4 locations identified *C. parvum* rather than *C. meleagridis* or *C. wrairi*. Furthermore, *C. parvum* from Lo-Na and Ho-Ping were of the *C. parvum* bovine genotype, whereas those from Ton-Pu and Jen-Ai samples were mixed genotypes, *C. hominis* and *C. parvum* bovine genotype. These two areas in Taiwan are popular tourist attractions and have higher population densities than the other two regions. This may be a potential factor for the presence of of *C. hominis* oocysts in water samples.

Previous studies investigating *Cryptosporidium* in Taiwan's water sources have mostly used specific fluorescence microscopy (Hsu *et al*, 1999a,b; Hsu *et al*, 2001) and have detected an average of 22.1 oocysts in 100 liters of water samples. The present study confirmed the existence of *Cryptosporidium* spp using single PCR assay method. In addition, restriction enzyme analysis showed that the water samples from the 4 locations contained *C. parvum* (both human and bovine genotypes) capable of infecting humans. However, the susceptibility of humans to *Cryptosporidium* spp varies greatly between individuals, but it has been reported that even a single oocyst can cause infection (Wu *et al*, 2000). Therefore, constant monitoring of *Cryptosporidium* spp may help prevent waterborne outbreaks of cryptosporidiosis in Taiwan.

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