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Identification of Genotypes of *Cryptosporidium parvum* in Children with Diarrhea by PCR-RFLP

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Abstract

Various *Cryptosporidium* species have been associated with human and animal infections, especially *C. parvum* infects primarily immunocompromized individuals and causes chronic or acute diarrhea. Outbreaks of human cryptosporidiosis are often cause by the contamination of water supplies with this parasite throughout the world. Because the epidemiological importance it is desirable to develop a sensitive detection technique for this parasite and accurate genetic classification. In this study, we collected samples of whole feces from 256 children with diarrhea at Social Security Children's Hospital, Ankara, Türkiye. For detecting the oocysts in feces, we used modified acid-fast staining method and direct immunofluorescent assay. For molecular studies, a fragment of the *Cryptosporidium* COWP and SSU rRNA genes encompassing the hypervariable region was amplified by PCR and then RFLP analysis was performed for distinguishing the *Cryptosporidium* species in the samples. In RFLP analysis *Rsa* I, *Ssp* I and *Vsp* I enzymes were used to identify the species/genotypes of *Cryptosporidium*. In the samples studied, we have determined only *Cryptosporidium parvum* and its two genotypes. The prevalance of *Cryptosporidium* positivity was about 4.7%.

INTRODUCTION

Protozoan parasites of the genus *Cryptosporidium* (Phlyum: Apicomplexa) infect gastrointestinal or respiratory tracts of a wide range of mammals, birds, reptiles and fish. *Cryptosporidium parvum* is the major causitive agent of cryptosporidiosis in humans and livestock. It has recently emerged as a widespread enteric pathogen in humans. The infection is self-limiting in healthy individuals, on the other hand, in malnourished children and immunocompromised patients *C. parvum* may cause a chronic diarrhea that can be life-threatening [1].

Until recently, it was assumed that *C. parvum* was a uniform species, but there is now strong evidence that *C. parvum* is composed of numerous distinct genotypes: a "human" genotype found only in humans, a "cattle" genotype found in many domestic animals and also humans, and a number of other genotypes, some of which appear to be host specific [2].

Recent studies that used PCR-based methods for the analysis of *C. parvum* genetic variation at single loci have coherently demonstrated the existence of two main parasite subpopulations characterized by

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distinct genotypes [3] The vast majority of human infections are caused by one of two genotypes, varyingly designated as type 1/type 2, and recently also assigned to different species, *C. hominis* and *C. parvum.*

The most common genotypic analyses are based on PCR-restriction fragment length polymorphism (PCR-RFLP) analysis and sequencing of the small subunit (SSU) rRNA [4], 70 kDa heat shock protein [5], β-tubulin [6], *Cryptosporidium* oocyst wall protein (COWP) [7], or thrombospondin-related adhesive protein Cryptosporidium-1 (TRAP C1) or TRAP C2 genes [8]. The genes coding for the *Cryptosporidium* oocyst wall protein (COWP) and the small subunit ribosomal RNA (SSU rRNA) are commonly used targets of molecular tool for genotyping *Cryptosporidium* parasites.

Characterization of the COWP gene revealed genetic differences among human and bovine *C. parvum* isolates and *C. wrairi*. Based on the sequence diversity, a simple PCR-RFLP technique was developed to separate three genotypes of *Cryptosporidium* parasites [9]. Since then, this technique has been widely used in the genotyping of *Cryptosporidium* parasites in clinical samples [10].

There are several methods for extracting DNA from *Cryptosporidium* oocysts for PCR analysis. These methods are suitable for extracting DNA directly from fecal samples or from environmental samples containing oocysts. Two sample preparation methods can be used: (1) direct extraction of fecal/environmental DNA; and (2) isolation of oocysts followed by DNA extraction. Direct extraction of DNA is faster, but generally it is preferred to purify the oocysts first, as a portion of these oocysts can subsequently be used for additional studies or animal propagation. Extracting DNA from purified oocysts also reduces the probability of contamination of the DNA with PCR inhibitors. Several density media and gradients have been described for floating or sedimenting oocysts from fecal samples. These methods are used individually or in combination with other purification steps until the required level of the oocyst purification is achieved [11].

The aim of this study was to determine the reliability of molecular techniques and to show that the molecular techniques are more sensitive than conventional microscobic staining and direct immunofluorescent assay techniques in the diagnosis of *Cryptosporidium.* A direct polymerase chain reaction (PCR) method that allowed easy identification of human and animal isolates would greatly assist in the predictive epidemiology of cryptosporidiosis. In our study, we used modified acid-fast staining method to detecting the oocysts in feces. COWP and SSUrRNA genes were chosen for molecular studies. These were amplified by PCR and then RFLP analysis was performed for distinguishing the *Cryptosporidium* species in the samples. In RFLP analysis *Rsa* I, *Ssp* I and *Vsp* I enzymes were used to identify the species/ genotypes of *Cryptosporidium.*

MATERIALS AND METHODS

Samples

Whole feces were collected from enrolled 256 children with diarrhea between 1 July and 31 September 2005 at Ankara Dışkapı Education and Research Hospital of Child Diseases. In molecular studies, Type 1 isolate TU502 [12], originally isolated from a child with diarrhea, and Type 2 isolate Iowa strain were used as control samples.

Oocyst Isolation

Oocycts were purified from the whole faecal samples using ether-lipid extraction and saturated NaCl centrifugation [13].

DNA Isolation

DNA was extracted from purified oocysts by High Pure PCR Template Preparation Kit (Roche, Germany). Cells are lysed during a short incubation with Proteinase K in the presence of a chaotropic salt (guanidine HCl), which immediately inactivates all nucleases. Cellular nucleic acids bind selectively to glass fiber fleece in a special centrifuge tube. The nucleic acids remain bound while a series of rapid "wash and spin" steps remove contaminating small molecules. Finally, low salt elution removes the nucleic acids from the glass fiber fleece. Oocysts were purified according to the manufacturer's recommendations [14].

Modified Acid-fast Staining Method

All stool samples were examined by Kinyoun carbol –fuchsin modified acid-fast stain [15]. A large number of staining techniques have been used to detect Cryptosporidium oocysts. The most widely used have been modified acid-fast procedures, which differentiate red-stained oocysts from similarly sized and shaped green-stained yeast forms.

Cryptosporidium immunochromatographic test

Firstly, we let all the reagents warm up to room temperature before proceeding with the test. Secondly we wrote the patient's name or specimen number on the test tube (foresee one test tube per sample) and then placed all the marked test tubes in a rack. After we completed the preparation of specimen and reagents for this test, we applied all the steps of procedure which are written in the manual of kit. The Crypto-Strip's (Coris BioConcept, Belgique) limit of detection for *C. parvum* is 50-100 oocysts in 100 µL of faeces.

Enzyme Immunoassay

Cryptosporidium Test (Tech Lab, Meridian, Netherlands) is Enzyme Immunoassay (EIA) methods for the detection of *Cryptosporidium* antigens in fecal specimens. This assay utilizes monoclonal and polyclonal antibodies directed against the cell surface antigens of the organism. In the assay, an aliquot of a diluted fecal specimen is transferred to a microassay well. If the *Cryptosporidium* antigen is present, it binds to the immobilized monoclonal antibody. When the *Detecting Antibody* is added, it binds to the antigen-antibody complex. The bound *Antibody* is detected using an anti-rabbit IgG-peroxidase conjugate. Any unbound materials are removed during the washing steps. Following the addition of substrate, a color is detected due to the enzyme-antibody-antigen complexes that form in the presence of *Cryptosporidium* antigen. A positive test result indicates that *Cryptosporidium* oocyst antigen is present in the fecal specimen. A negative result indicates that *Cryptosporidium* oocyst antigen is not present in the fecal specimen. Stool was processed according to the kit manual's recommendations.

COWP PCR-RFLP

We used a PCR-restriction fragment length polymorphism technique for species and genotype-specific diagnosis of *Cryptosporidium* parasites [16]. A PCR product of about 550 bp was amplified first by primary PCR with primers:

Forward 5'- GTAGATAATGGAAGAGATTGTG -3'
Reverse 5'- GGACTGAAATACAGGCATTATCTTG -3'

PCR amplifications were performed in 25 µl; 10x PCR buffer (50 mM Tris-HCl, pH 7.5 at 37°C, 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA) (Fermentas, Canada), 6 mM $MgCl_2$ (Fermentas, Canada), 200 mM (each) deoxynucleoside triphosphate (Fermentas, Canada), 100 nM (each) primer (Metabion, Germany), 2.5 U of *Taq* polymerase (Fermentas, Canada), and 1 to 5 µl of DNA template in a total reaction mixture. DNA amplification was carried out with a Biometra thermocycler for each pair of primers by using the previously described

cycling conditions A total of 36 cycles were carried out, each consisting of 94°C for 50 s, 55°C for 30 s, and 72°C for 50 s, with an initial hot start at 94°C for 2 min and a final extension at 72°C for 10 min.

For restriction fragment analysis, 5 µl of PCR product was digested in a total of 20 µl of reaction mixture, consisting of 20 U of *Rsa*I (Roche, Germany) for species diagnosis and genotyping of *C. parvum* and 1 µl of respective restriction buffer at 37°C for 1 h, under conditions recommended by the supplier. The digested products were fractionated on 3.5% agarose gel and visualized by ethidium bromide staining.

SSU-rRNA PCR-RFLP

We used a PCR-restriction fragment length polymorphism (RFLP) technique for species and genotype-specific diagnosis of *Cryptosporidium* parasites [17]. A PCR product of about 1,325 bp was amplified first by primary PCR with primers:

Forward 5'-TTCTAGAGCTAATACATGCG-3'
Reverse 5'-CCCTAATCCTTCGAAACAGGA-3'

PCR amplifications were performed in 25 µl 10x PCR buffer (50 mM Tris-HCl, pH 7.5 at 37°C, 10 mM $MgCl_2$, 100 mM NaCl, 0.1mg/ml BSA) (Fermentas,Canada), 6 mM $MgCl_2$, 200 mM (each) deoxynucleoside triphosphate, 100 nM (each) primer, 2.5 U of FastStart *Taq* DNA Polymerase (Roche, USA), and 0.25 to 1 ml of DNA template in a total 100-ml reaction mixture.

DNA amplification was carried out with a Biometra thermocycler for each pair of primers by using the previously described cycling conditions (initial hot start, denaturation, annealing, elongation, final extension, and total number of cycles) A total of 35 cycles were carried out, each consisting of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, with an initial hot start at 94°C for 3 min and a final extension at 72°C for 7 min. A secondary PCR product of 826 to 864 bp (depending on isolates) was then amplified from 2 ml of the primary PCR with primers:

Forward 5'-GGAAGGGTTGTATTTATTAGATAAAG-3'
Reverse 5'-AAGGAGTAAGGAACAACCTCCA-3'.

The PCR and cycling conditions were identical to primary PCR, except that 3 mM $MgCl_2$ was used in the PCR. A negative control, consisting of a reaction mixture without the DNA template, was included in each experiment. Each PCR product was analyzed by electrophoresis in a 1.5% agarose gel and was visualized after ethidium bromide staining. The same DNA stock preparation was used as the template in all evaluation experiments.

For restriction fragment analysis, 20 µl of the secondary PCR product was digested in a total of 50 µl of reaction mixture, consisting of 20 U of *Ssp*I (Fermentas, Canada) for species diagnosis or *Vsp*I (Fermentas, Canada) for genotyping of *C. parvum* and 5 µl of respective restriction buffer at 37°C for 1 h, under conditions recommended by the supplier. The digested products were fractionated on 3.5% agarose gel and visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

The results of the experiments belonging to the detection of the positivity and negativity of the samples are given at Table 1, 2 and 3. PCR results of COWP gene region are given at Figure 1. RFLP results COWP gene digested by *Rsa* I is shown at Figure 2. Nested-PCR results of SSU rRNA gene are shown at Figure 3 and 4. RFLP results of *Ssp* I. and *Vsp* I digestions are shown at Figure 5 and Figure 6, respectively. In the samples studied, we have determined only *Cryptosporidium. parvum* and its two genotypes. The prevalance of *Cryptosporidium* positivity was about 4.7%.

Table 1. The number of positive and negative samples detected by Acid-Fast Staining and Enzyme Immunoassay methods.

	Acid-Fast Staining	Enzyme Immunoassay
Positive	11	13
Negative	245	243
Total	256	256

Table 2. The number of positive and negative samples detected by Acid-Fast Staining and Chromotografic Lateral Flow Immunassay methods.

	Acid-Fast Staining	Chromotografic Lateral Flow Immunassay
Positive	11	12
Negative	245	244
Total	256	256

Table 3. The number of positive and negative samples detected by Acid-Fast Staining Method and PCR of 18SrRNA ve COWP genes.

	Acid-Fast Staining	18SrRNA PCR	COWP PCR
Positive	11	12	12
Negative	245	244	244
Total	256	256	256

Current routine diagnostic techniques rely on the recognition of specific oocyst morphologies by light microscopy in stained fecal specimens [4] and there is evidence to suggest that several species of *Cryptosporidium* (including *C. parvum* and *C. wrairi*) produce oocysts indittinguishable by light microscopy [5,6]. However, the taxonomy of the genus *Cryptosporidium* is incompletely defined, and DNA sequence analysis has indicated that this genus is complex and contains additional, as yet, un-named species [7].

Hence, to further understand the epidemiology of this disease, it is important to develop additional methods for indentifying *Cryptosporidium* species in the faeces of infected animals and humans, as well as to able to recognize these organisms in food, water and environmental samples. Although acid fast staining is a reference method for the detection of *C. parvum* oocysts, it is suggested that an alternative test is also needed because microscopic examination is timeconsuming and user-dependent and needs an experienced microscopist, along with difficulty in the detection of oocysts in subclinical infections.

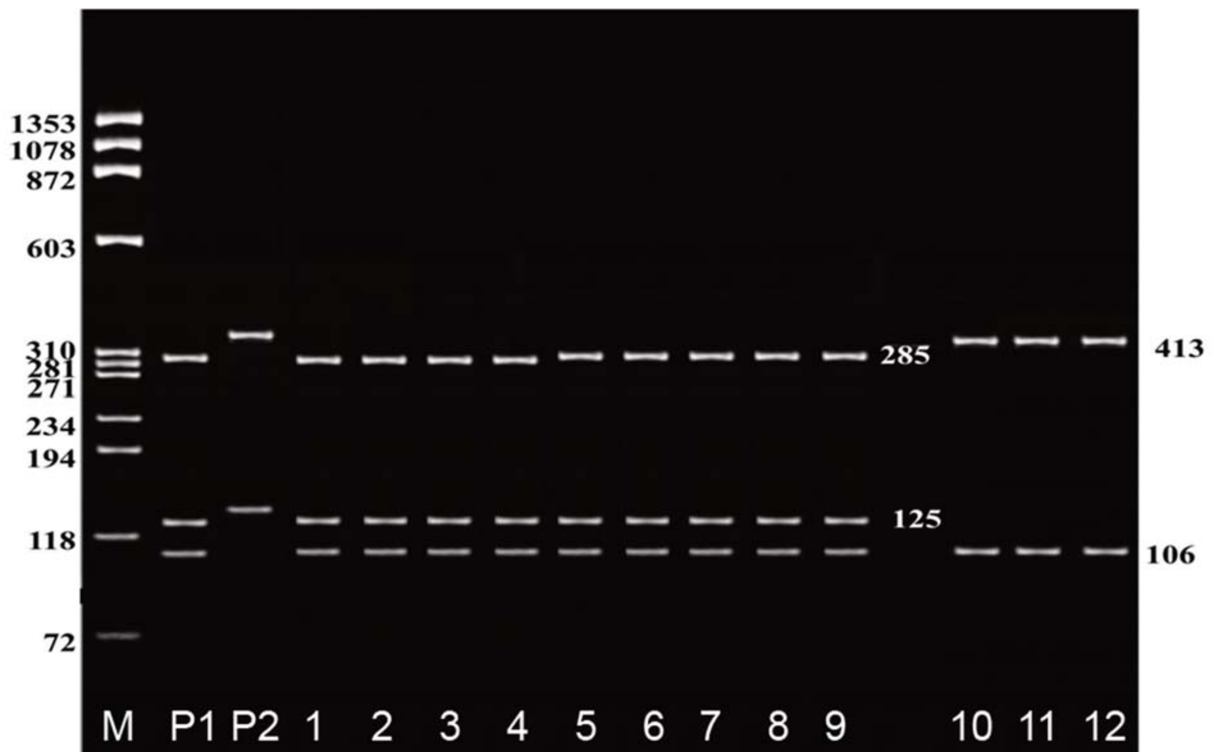

Figure 1. Electrophoretic profiles of the COWP PCR products amplified with the oligonucleotide primers cry-15 and cry-9. M: Marker (DNA ladder ØX174Hae III), 1-12: Sample numbers.

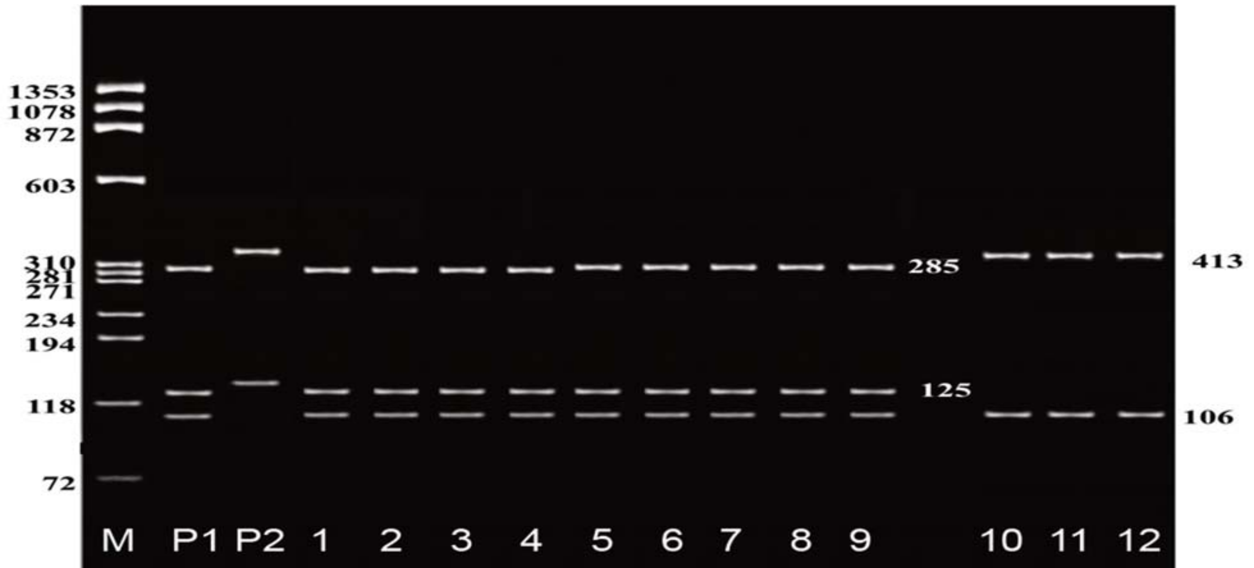

Figure 2. PCR-RFLP analysis of the COWP gene by restriction with Rsa I. M: Marker (DNA ladder ØX174Hae III), 1-12: Sample numbers.

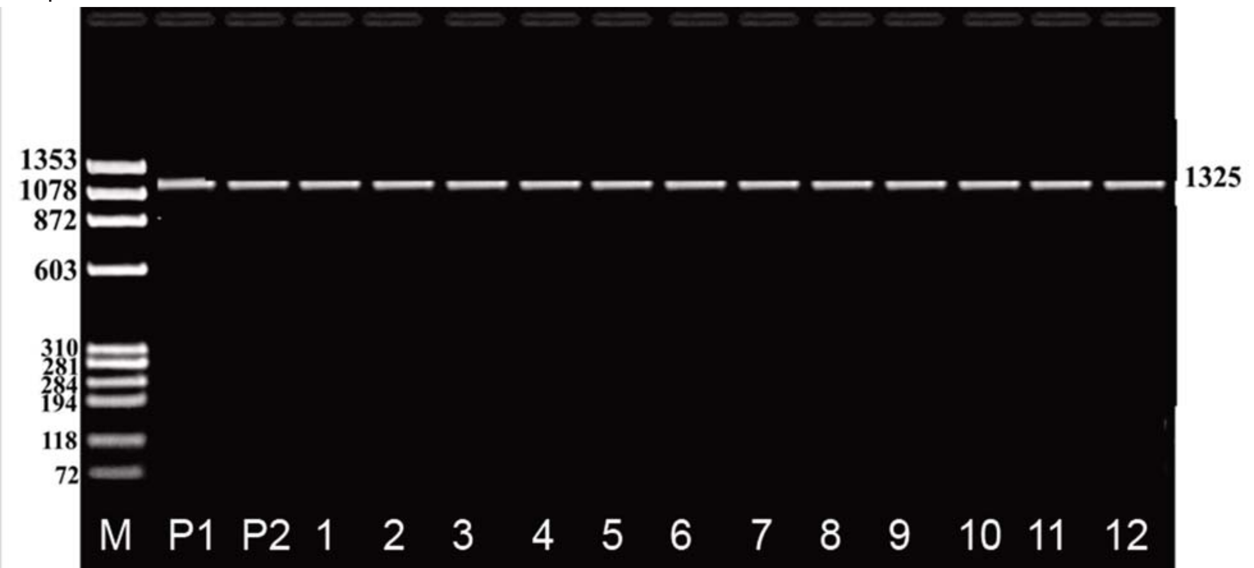

Figure 3. Electrophoretic profiles of the first SSU rRNA nested-PCR products amplified with the 5'-TTCTAGAGC-TAATACATGCG-3' and 5'-CCCTAATCCTTCGAAACAGGA-3'primers. M: Marker (DNA ladder ØX174Hae III), 1-12: Sample numbers.

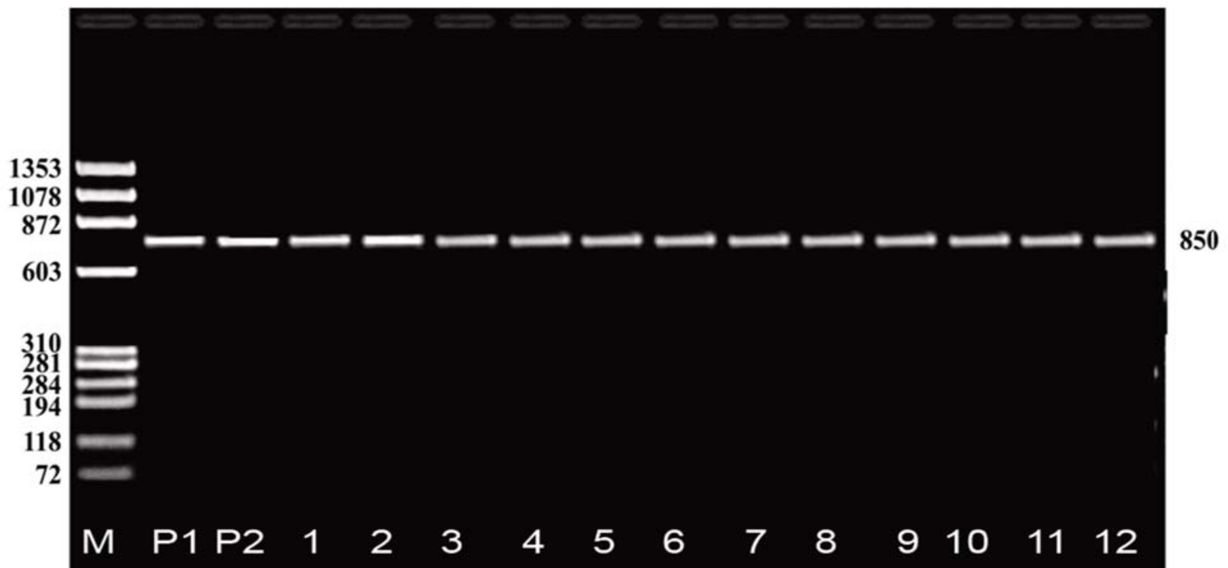

Figure 4. Electrophoretic profiles of the second SSU rRNA nested-PCR products amplified with the 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA-3' primers.M: Marker (DNA ladder ØX174Hae III), 1-12: Sample numbers.

Figure 5. PCR-RFLP analysis of the SSU rRNA gene by restriction with Ssp I. M: Marker (DNA ladder ØX174Hae III), 1-12: Sample numbers.

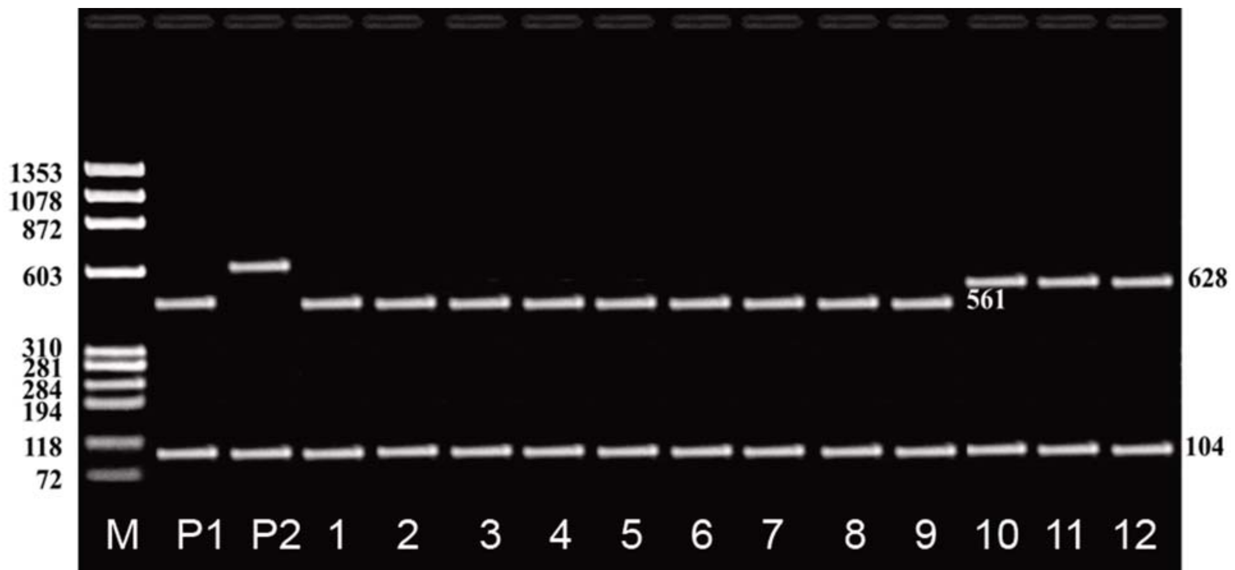

Figure 6. PCR-RFLP analysis of the SSU rRNA gene by restriction with Vsp I. M: Marker (DNA ladder ØX174Hae III), 1-12: Sample numbers.

To our knowledge, no systematic comparison of various oocyst purification methods has been published, and each investigator needs to experiment with different methods before deciding on the one that is most appropriate and meets the requirements of the experiment. Even if a comparative study of different purification protocols were performed, it is likely that the results achieved in one laboratory would not directly apply to another, since the sample matrix has a major impact on oocyst recovery. When we used concentration method for oocysts isolation, we thought that high numbers of Cryptosporidium oocysts were throwed away by the gauze which was used for stool filtration and in the supernatants from both the first and second centrifugation steps. The concentration procedure may be required to prevent such large oocyst losses. So there isn't too many differences between the number of positivity by using PCR amplification and the other methods.

The most common genotypic analyses are based on PCR-restriction fragment length polymorphism (PCR-RFLP) analysis and/or sequencing of the small subunit (SSU) rRNA [4,16,18], 70-kDa heat shock protein [5], β-tubulin [6,19], *Cryptosporidium* oocyst wall protein (COWP) [7,20], or thrombospondin-related adhesive protein

Cryptosporidium-1 (TRAP C1) or TRAP C2 [21] genes.

Cryptosporidium species and genotypes can be distinguished in clinical samples by using PCR based RFLP analysis of COWP and (SSU) rRNA genes [16,17]. RFLP analysis of COWP region amplification product by using *Rsa I* can distinguish *C. parvum* Type 1 and 2 and also *C. wrairi* [22]. In our study, according to *Rsa I* enzyme pattern of COWP (Figure 2), sample number from 1 to 9 showed *C. parvum* Type 2 pattern and sample number from 10 to 12 showed *C. parvum* Type 1 pattern. The PCR-RFLP protocol for 18rRNA gene region was developed by Xiao [17] and shown specific to *Cryptosporidium*. Three *Cryptosporidium* species and two genotypes of *C. parvum* can be distinguished by this protocol. In our study, when the RFLP analyses of 18SrRNA using *SspI* (Figure 5), it couldn't be distinguished any species or genotypes. In the case of *Vsp I* (Figure 6), two genotypes could be identified. According to this pattern, from 1 to 9 showed bovine genotype of *C. parvum* (*C. parvum* Type 2) and from 10 to 12 showed human genotype of *C. parvum* (*C. parvum* Type 1). Both of the results obtained by using *Vsp I* and *Rsa I* enzymes were compatible with each other.

We have demonstrated *C. parvum* oocysts in the stools of one immunocompromised patient with diarrhea whose stools were negative by the Acid Fast Staining Method. This finding may have significant clinical implications and may contribute to a better understanding of the epidemiology of cryptosporidiosis. The degree of sensitivity achieved in this study may be particularly useful in identifying and diagnosing those symptomatic patients with stools negative by the traditional diagnostic methods.

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