Tropical Medicine and Health Vol. 32 No. 1, 2004, pp. 5-14

FURTHER EVIDENCE THAT GENOTYPE I AND GENOTYPE II OF *CRYPTOSPORIDIUM PARVUM* ARE DISTINCT

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Accepted 3, February, 2004

Abstract: Three new genes of *Cryptosporidium parvum* were cloned, including a gene encoding methionine aminopeptidase, one encoding chaperonin containing T-complex protein 1 delta (TCP-1 delta) and one with unknown function. DNA sequence analysis indicated that these genes are quite conserved, but there were some base pair differences between genotype I and genotype II isolates. These differences were confirmed by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the 3 genes from 41 isolates collected from different hosts and geographical origins. In brief, the band patterns generated by endonuclease Hind III or Hinf I restrictions of the gene of methionine aminopeptidase, Sac I restriction of the gene of chaperonin, or Ava II restriction of the unknown gene could differentiate the isolates of *C. parvum* into genotype I and genotype II. PCR primers based on these genes amplified only *C. parvum* genes. Even a single oocyst was detectable with these PCR primers. Thus the results provided further evidence that genotype I and genotype II are distinct, and our three new primers can be used to detect and characterize *C. parvum* isolates with high sensitivity.
Key Word: *Cryptosporidium parvum*, methionine aminopeptidase, chaperonin, genotype, PCR-RFLP

INTRODUCTION

An intestinal protozoan parasite, *Cryptosporidium parvum* is the major causative agent of cryptosporidiosis in humans and livestock. Outbreaks of human cryptosporidiosis are often caused by the contamination of water supplies with this parasite throughout the world. Because of the epidemiological importance, it is desirable to develop a sensitive detection technique for this parasite and accurate genetic classification.

To this end, many efforts have been made to characterize isolates of *C. parvum* in the past decade. The difference in virulence, infectivity, pathogenesis and drug sensitivity has indicated that *C. parvum* is not a uniform species or monophyletic (Morgan *et al.,* 1999a; Okhuysen *et al.,* 1999; Pereira *et al.,* 2002). The present working hypothesis is that *C. parvum* is composed of two main genotypes, genotype I and genotype II. Genotype I (human genotype or anthroponotic genotype) is exclusively found in humans, while genotype II (bovine genotype or zoonotic genotype) was first found in cattle and has also been found in a wide range of mammals, including humans. Some other host-adapted-genotypes have been also found among *C. parvum* isolates, such as the pig, dog and mouse genotype (Morgan *et al.,* 1999b; Sulaiman *et al.,* 2000; Xiao *et al.,* 2000a)

With the enormous development of gene technology, gene diversities between genotype I and genotype II have been reported. The evidence includes sequence and/or PCR-RFLP analysis of several gene loci, such as 18S rRNA (Morgan *et al.,* 1997; Xiao *et al.,* 1999), COWP (Spano *et al.,* 1997; Xiao *et al.,* 2000b), HSP70 (Gobet and Toze, 2001; Sulaiman *et al.,* 2000), TRAP (Spano *et al.,* 1999; Sulaiman *et al.,* 1998), and/Cpgp40/15 (Wu *et al.,* 2003).

In the present study, we cloned 3 new genes of *C. parvum* adapting our previous method to design PCR primers from random amplified polymorphic DNA (RAPD), and further confirmed the distinctness between genotype I and genotype II of *C. parvum* by the sequences and PCR-RFLP analysis of the isolates from different hosts with different geographical origins.

MATERIALS AND METHODS

Parasite isolates

Forty-one isolates of *C. parvum* were used. The details were published in our previous paper (Wu *et al.,* 2003). The host and geographical origins are shown in Table 1.

All fecal samples were preserved in 2% $K_2Cr_2O_7$ and oocysts were isolated using a sucrose flotation method. The samples were refined, contaminated micro-organisms being reduced to a minimum level using an immuno-magnetic separation kit (Dynabeads anti-*Cryptosporidium*, Dynal A. S., Oslo, Norway).

Oocyst DNA was isolated by the method described

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Table 1. Isolates of *C. parvum*.

Code	Host origin	Geographical origin	Genotype
CGJ1	Calf	Gifu, Japan	II
CGJ2	Calf	Gifu, Japan	II
CGJ3	Calf	Gifu, Japan	II
CGJ4	Calf	Gifu, Japan	II
CGJ5	Calf	Gifu, Japan	II
CGJ6	Calf	Gifu, Japan	II
CKJ1	Calf	Kobe, Japan	II
CKJ2	Calf	Kobe, Japan	II
CKJ3	Calf	Kobe, Japan	II
CKJ4	Calf	Kobe, Japan	II
CKJ5	Calf	Kobe, Japan	II
CKJ6	Calf	Kobe, Japan	II
CKJ7	Calf	Kobe, Japan	II
CKJ8	Calf	Kobe, Japan	II
CKJ9	Calf	Kobe, Japan	II
CKJ10	Calf	Kobe, Japan	II
CKJ11	Calf	Kobe, Japan	II
CNJ1	Calf	Nagoya, Japan	II
CI1	Calf	Italy	II
CI2	Calf	Italy	II
CI3	Calf	Italy	II
CI4	Calf	Italy	II
CI5	Calf	Italy	II
CI6	Calf	Italy	II
CI7	Calf	Italy	II
CI8	Calf	Italy	II
GI1	Goat	Italy	II
HJ1	Human	Japan	II
HJ2	Human	Japan	I
HJ3	Human	Japan	I
HI1	Human	Italy	I
HI2	Human	Italy	I
HI3	Human	Italy	I
HI4	Human	Italy	I
HI5	Human	Italy	I
HN1	Human	Nepal	I
HN2	Human	Nepal	I
HN3	Human	Nepal	I
HN4	Human	Nepal	I
HN5	Human	Nepal	I
HN6	Human	Nepal	I

previously (Wu *et al.,* 2000). In brief, oocysts were frozen and thawed repeatedly 5 times, treated at 100℃ for 20 min, and then the samples were digested with proteinase K at a final concentration of 200 μg/ml at 55℃ for 3 hours. The reaction was stopped by heating at 95℃ for 5 min. The treated samples were then directly used as a template for PCR.

Development of PCR primers

PCR primers were produced according to our previously described methods (Nagano *et al.,* 1996). In brief, RAPD was produced from *C. parvum* DNA (Code# CGJ2) by means of arbitrary primed PCR (AP-PCR) using 10 base pair primers. The three target genes thus obtained were RAPD SB281, SB289 and SB012, which were produced by primers tgatgaccgc, gcgtgctcac and cggcccctgt, respectively. The DNA fragments were extracted and sequenced. Based on these sequences, 3 pairs of primers were developed as shown in Table 2.

Detection sensitivity and specificity of primers

The sensitivity of primers was tested by amplifying serially diluted template DNA. The purified oocyst DNA was diluted at 160, 40, 10, 2.5, 0.625, 0.156, 0.039 and 0.001 pg/μl. Tested primers included the primers SB281, SB289 and SB012, as well as 3 other primers to amplify the genes of COWP (GenBank accession No.: AF266273, Xiao *et al.,* 2000b), HSP70 (GenBank accession No.: AF221535, Sulaiman *et al.,* 2000), and TRAP1 (GenBank accession No.: AF017267, Spano *et al.,* 1998), as shown in Table 2. PCR conditions were as follows: one cycle of initial denaturing at 94℃ for 3 min; 35 cycles at 94℃ for 30 sec, 51-56℃ (see details in Table 2) for 30 sec, and 72℃ for 1 min; and one cycle of final extension at 72℃ for 10 min.

The specificity of the primers SB281, SB289 and SB012 was tested by detecting various kinds of template DNA, including *C. parvum* and control samples of *C. muris*, human, bovine, *Entamoeba histolytica, Giardia lamblia, Blastocystis hominis, Ascaris lumbricoides, Trichomonas vaginalis, Trichinella spiralis and Escherichia coli.*

DNA sequencing and sequence analysis

The RAPD was produced by primer SB281, SB289 or SB012 from 16 isolates, which included 8 calf isolates (Code# CGJ2, CGJ5, CKJ1, CKJ3, CKJ7, CNJ1, CI2 and CI8) from Japan and Italy, one goat isolate (Code# GI1) from Italy and 7 human isolates (Code# HJ1, HJ2, HJ3, HI1, HI2, HN4 and HN6) from Japan, Italy and Nepal. DNA sequence of the RAPD was determined using a Thermo Sequenase cycle sequencing Kit (USB Corporation, Cleveland, USA) and an automatic sequencer (Model LIC-4200, Aloka Co., Ltd., Tokyo, Japan). The sequence data were analyzed using DNASIS Software (Hitachi Software Engineering, Tokyo, Japan). Homology searching on the nucleotide and protein database was carried out using the BLAST program at NCBI (Bethesda, MD, USA). Pairwise sequence alignment and protein identities were performed using CLUSTALW 1.8 software (Jeanmougin *et al.,* 1998) and PHYLIP DNADIST software (distributed by Felsenstein, Department of Genetics, University of Washington, Seattle).

PCR-RFLP

PCR and RFLP were performed according to our previously described methods (Wu *et al.,* 1999). PCR prod-

Table 2. Criteria of PCR Primers.

Primer	Sequence	Annealing Tm	Product size	Target gene
SB281	5'-gatagtgttccatttgagagg-3' 5'-ttagatgcaacaaatacaggat-3'	51.0	553	methionine aminopeptidase
SB289	5'-cccaattcagttctgtctct-3' 5'-aataatgctcctaacaacgc-3'	51.0	505	chaperonin containing TCP-1 delta
SB012	5'-ctccgttcgatgatgcagatg-3' 5'-cggcccctgtagaaataagtca-3'	51.3	434	unknown
COWP	5'-ggttcctcctatgcctttct 5'-gtgggcattcctttgtgac	52.7	869	oocyst wall protein
HSP70	5'-tcagttgccatcagtagagc 5'-tcttcttctcagcctcatca	52.5	841	heat shock protein
TRAP1	5'-ggatgggtatcaggtaataagaa 5'-tagcccagttctgactctctg	52.0	1177	thrombospondin-related adhesive protein

Table 3. Predicted RFLP pattern of RAPD SB281, SB289 and SB012 PCR products.

Isolate	Size of predicated bands				Genotype
	SB281		SB289	SB012	
	Hae III	Hinf I	Sac I	Ava II	
CGJ2	59, 494	75, 478	505	152, 282	II
CI2	59, 494	75, 478	505	152, 282	II
IG1	59, 494	75, 478	505	152, 282	II
HJ2	59, 204, 290	75, 138, 340	232, 273	434	I
HJ3	59, 204, 290	75, 138, 340	232, 273	434	I
HN6	59, 204, 290	75, 138, 340	232, 273	434	I
HI2	59, 204, 290	75, 138, 340	232, 273	434	I

ucts were purified by ethanol precipitation, and then digested with an appropriate restriction endonuclease (Hind III, Hinf I, Sac I and Ava II) according to the manufacturer's instructions. The digests were subjected to electrophoresis.

RESULTS

Sequence analysis

Three dense RAPD bands with an appropriate molecular size of 610 (SB281), 600 (SB289) and 550 bp (SB012) were generated from the template DNA of the isolate CGJ2. The RAPD fragments were isolated from the gel and sequenced (GenBank accession No: AY488139, AY488146, AY488153). Based on the 3 sequences, 3 pairs of primers were developed (Table 2). The RAPD from 16 isolates (including 8 calf isolates from Japan and Italy, one goat isolate from Italy and 7 human isolates from Japan, Italy and Nepal) were produced with the 3 developed primers and sequenced (GenBank accession numbers are shown in Figs 1, 2 and 3).

Homology analysis of the RAPD sequence showed that these genes were quite conserved among different isolates, as shown in Figs 1, 2 and 3. The identities of the sequences of SB281 RAPD, SB289 RAPD, and SB012 RAPD were 98.0-100%, 97.6-100%, and 98.2-100% among the 16 isolates, respectively.

The animal isolates shared nearly the same sequence regardless of their origin. The identities of SB281 RAPD, SB289 RAPD, and SB012 RAPD among the 9 animal isolates were 99.3-100%, 99.6-100% and 99.0-100%, respectively. The same was true for human isolates. The identities of RAPD SB281, RAPD SB289, and RAPD SB012 among the 7 human isolates were 98.4-99.6%, 98.0-100% and 99.3-100%, respectively.

Although DNA sequence was conserved, there were point mutations in human origin isolates (except the isolate HJ1) at identical nucleotide sites. The point mutation pattern was different from the animal origin isolates as shown in Figs 1, 2 and 3.

The DNA sequence of the RAPD fragments were deduced into an amino acid sequence and subjected to GenBank for blast searching. The blast results showed that SB281 was a protein gene encoding methionine aminopeptidase. The deduced amino acid sequence shared 52-57% identities with the sequence of methionine aminopeptidase of other organisms, for example, *Drosophila melanogaster*, *Arabidopsis thaliana*, human, mouse and *Caenorhabditis elegans* (Fig 4). The SB289 was a protein gene encoding

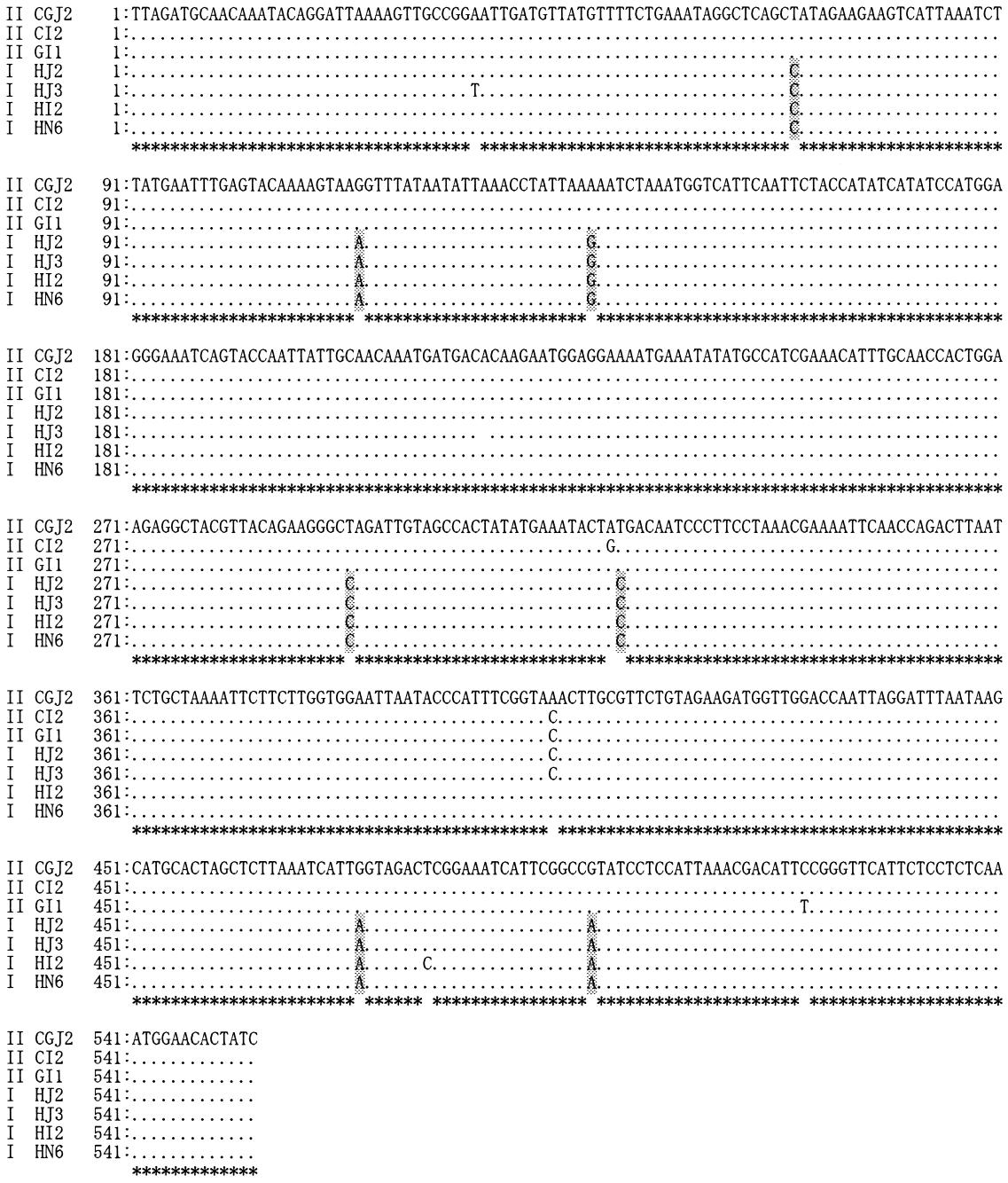

Figure 1. DNA sequence diversities among *Cryptosporidium parvum* isolates in SB281 (methionine aminopeptidase) gene. The genes from 16 isolates were sequenced and typed into genotype I and genotype II, as indicated in the left margin by I and II. Filled circles in the alignment indicate identical base pairs. The differences between genotype I and genotype II are indicated by shading. GenBank accession numbers of CGJ2, CI2, GI1, HJ2, HJ3, HI2 and HN6 are AY488139, AY488140, AY488141, AY488142, AY488143, AY488144 and AY488145, respectively.

chaperonin containing TCP-1 delta subunit. The deduced amino acid sequence shared 54-61% identities with the sequence of chaperonin containing TCP-1 delta subunit of other organisms, such as *D. melanogaster*, *A. thaliana*, human, mouse and *C. elegans* (Fig 5).

Differentiation of C. parvum *isolates by the RFLP method*

After PCR with the primer SB281, SB289 or SB012,

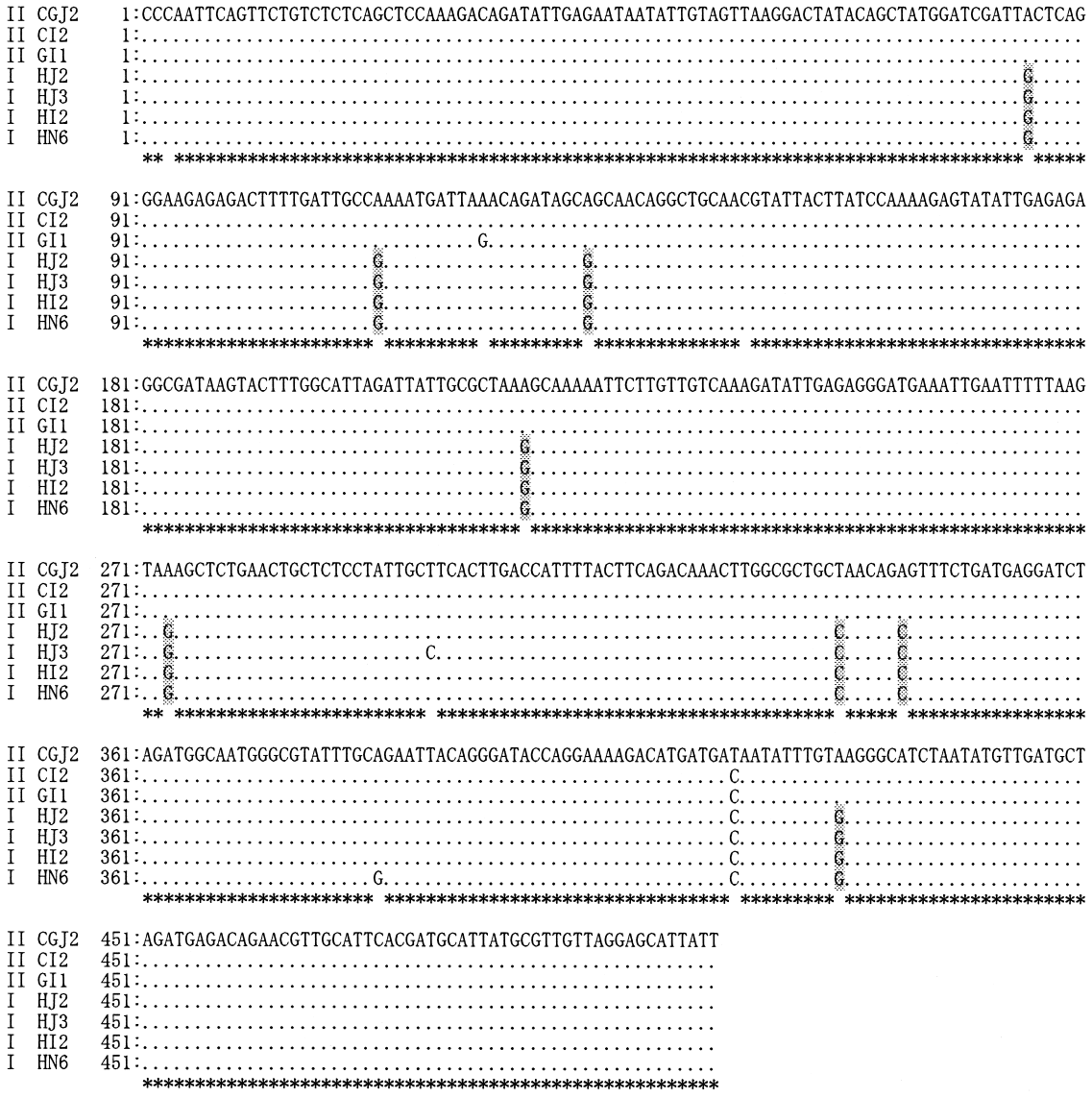

Figure 2. DNA sequence diversities among *Cryptosporidium parvum* isolates in SB289 (chaperonin containing TCP-1 delta subunit) gene. The genes from 16 isolates were sequenced and typed into genotype I and genotype II, as indicated in the left margin by I and II. Filled circles in the alignment indicate identical base pairs. The differences between genotype I and genotype II are indicated by shading. GenBank accession numbers of CGJ2, CI2, GI1, HJ2, HJ3, HI2 and HN6 are AY488146, AY488147, AY488148, AY488149, AY488150, AY488151 and AY488152, respectively.

all 41 DNA samples produced the expected size bands of 553 bp, 505 bp and 434 bp, respectively. Thus 123 (3 times 41) RAPDs were analyzed by PCR-RFLP.

Two kinds of band patterns (Panels A and B in Fig 6) were produced by endonuclease Hind III and Hinf I restriction of the RAPD SB281. All of the calf isolates from both Japan and Italy, the isolate from the goat of Italy and one isolate from a Japanese patient (Code# HJ1) showed the same kind of RFLP pattern of genotype II. All of the human origin isolates, except one Japanese human isolate (Code# HJ1) produced the same kind of RFLP pattern of genotype I, which was different from that of genotype II.

The same results were obtained when the 41 isolates were analyzed by endonuclease Sac I restriction of RAPD SB289 (Panel C in Fig 6) and Ava II restriction of RAPD SB012 (Panel D in Fig 6).

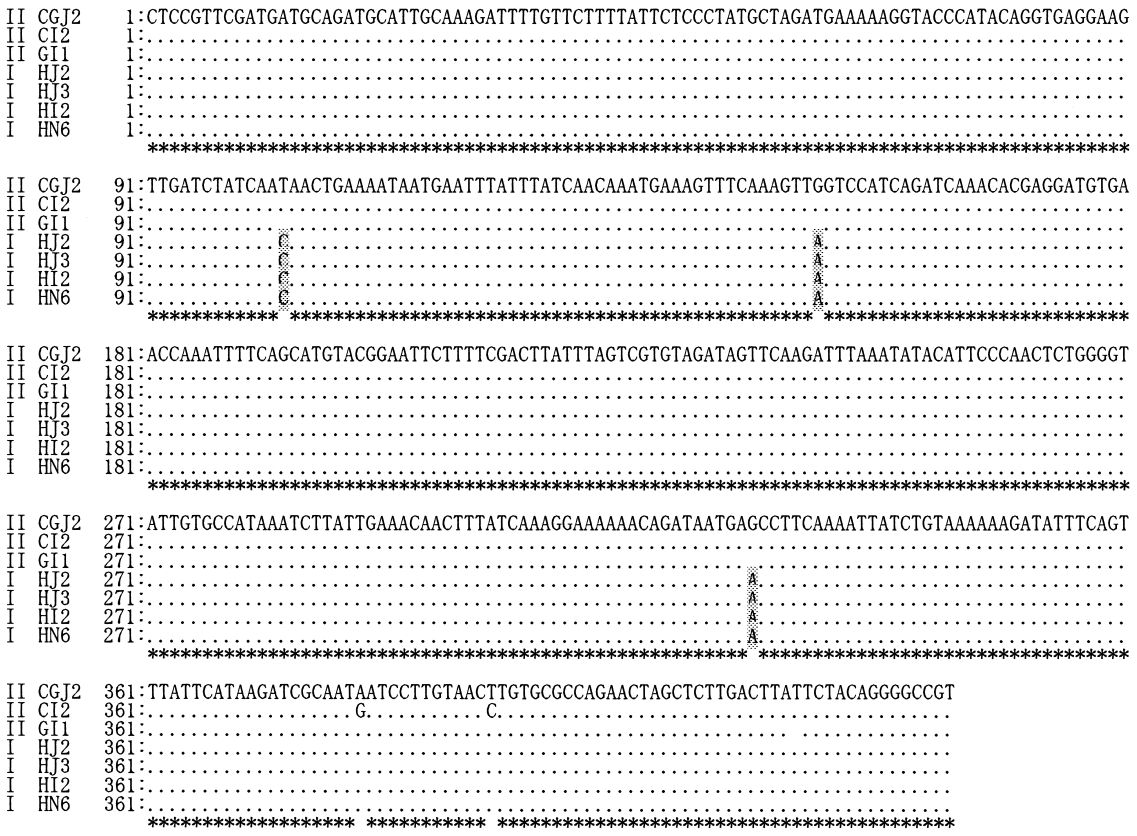

Figure 3. DNA sequence diversities among *Cryptosporidium parvum* isolates in SB012 gene. The genes from 16 isolates were sequenced and typed into genotype I and genotype II, as indicated in the left margin by I and II. Filled circles in the alignment indicate identical base pairs. The differences between genotype I and genotype II are indicated by shading. GenBank accession numbers of CGJ2, CI2, GI1, HJ2, HJ3, HI2 and HN6 are AY488153, AY488154, AY488155, AY488156, AY488157, AY488158, and AY488159, respectively.

Detection sensitivity and specificity of primers

The detection sensitivity of primers was tested by means of PCR with serially diluted template DNA, as described in Materials and Methods. The density of bands tended to be faint when a lower concentration of template DNA was used. The primers SB281, SB289 and SB012 gave the highest sensitivity in detecting template DNA of *C. parvum*. The minimum concentration of template DNA necessary for a positive reaction was 0.156 pg with the primer SB281, 0.039 pg with the primer SB289 and 0.156 pg with the primer SB012 (Panels A, B and C in Fig 7), while the control primers of COWP, HSP 70 and PRAP1 showed a minimum detection concentration of 10 pg, 10 pg and 40 pg, respectively (Panels D, E and F in Fig 7).

All 3 kinds of primers produced bands with the expected size from *C. parvum*, while control samples produced negative results, indicating the species specificity of these constructed primers.

DISCUSSION

Epidemiologically, molecular markers of pathogens are useful in determining the importance of responsible reagents, for example, distinguishing between zoonotic and anthroponotic transmissions. Reportedly, both genotype I and genotype II of *C. parvum* are responsible for the sporadic infection and outbreak of human cryptosporidiosis (McLauchlin *et al.*, 1999; Ong *et al.*, 1999; Sulaiman *et al.*, 1998; Xiao *et al.*, 2000a).

In the present study, 3 new target genes were proposed and assessed for genetic analysis. The sequence analysis of these genes from 41 isolates of *C. parvum* from different hosts and geographical origins indicated that there are identical base pair differences between the isolates from human and animal origins. The PCR-RFLP analysis of the 3 genes confirmed that these 3 new genes are useful for the genotyping of *C. parvum*, because all of the 3 genes showed 2 kinds of RFLP patterns which correspond to genotype I and

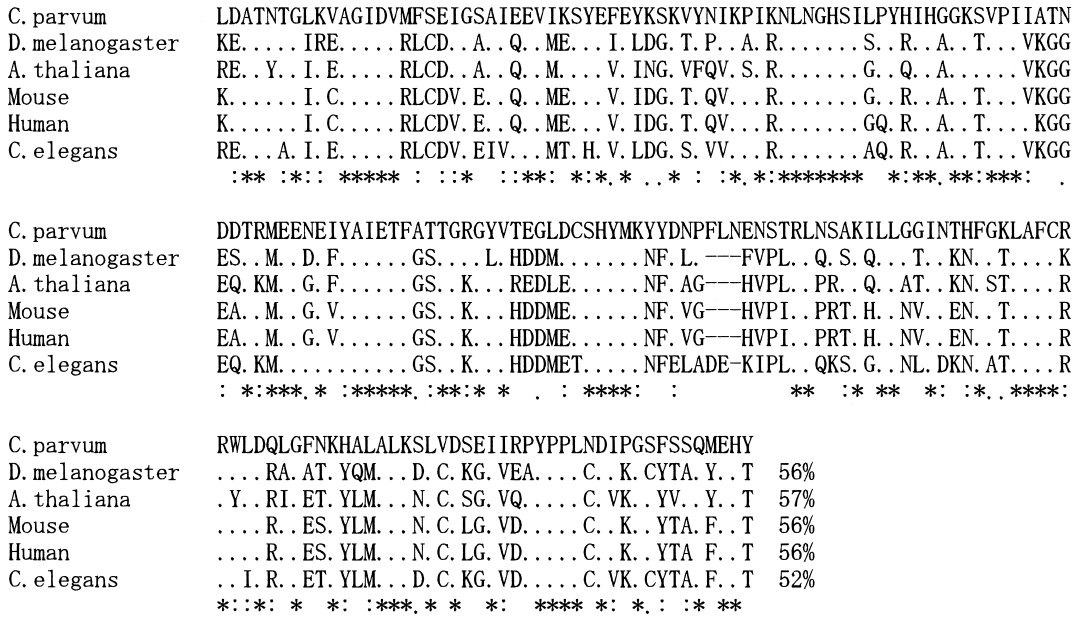

Figure 4. Alignment of the deduced amino acid sequence of methionine aminopeptidase of *C. parvum* and the sequence of *D. melanogaster*, *A. thaliana*, human, mouse and *C. elegans*. Filled circles in the alignment indicate identical base pair and hyphens indicate gaps. The identities of sequence are indicated at the ends of sequences.

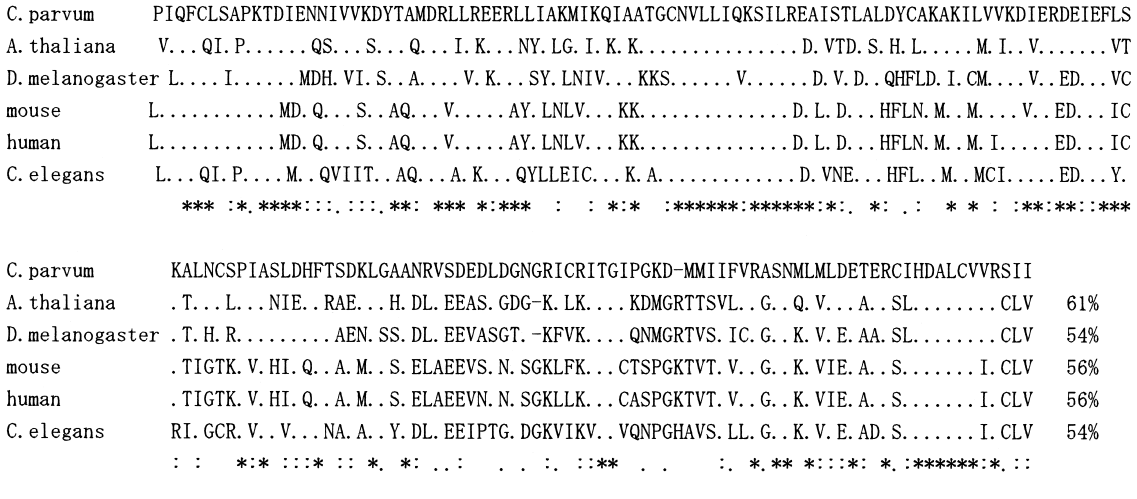

Figure 5. Alignment of the deduced amino acid sequence of chaperonin containing TCP-1 delta subunit of *C. parvum* and the sequence of *D. melanogaster*, *A. thaliana*, human, mouse and *C. elegans*. Filled circles in the alignment indicate identical base pairs and hyphens indicate gaps. The identities of sequence are indicated in the ends of sequences.

genotype II. More important is the fact that the 3 primers amplified only *C. parvum* DNA with high sensitivity, making these candidate primers to detect and genotype *C. parvum* in water contamination at the required quality in terms of sensitivity. Using our method, even low numbers of oocysts in sample water may be amplified with any one of the primers. RFLP analysis with a combination of any one of the three genes can identify animal or human genotypes. Such examples are given by primer SB281/Hind III, SB281/Hinf I, SB289/Sac I or SB012/Ava II.

We have reported that *C. parvum* specific primer SB012 is highly sensitive. The primer SB012 can detect the lowest amount of template DNA necessary for a positive reaction, which was 0.156 pg DNA or even one oocyst, or 50

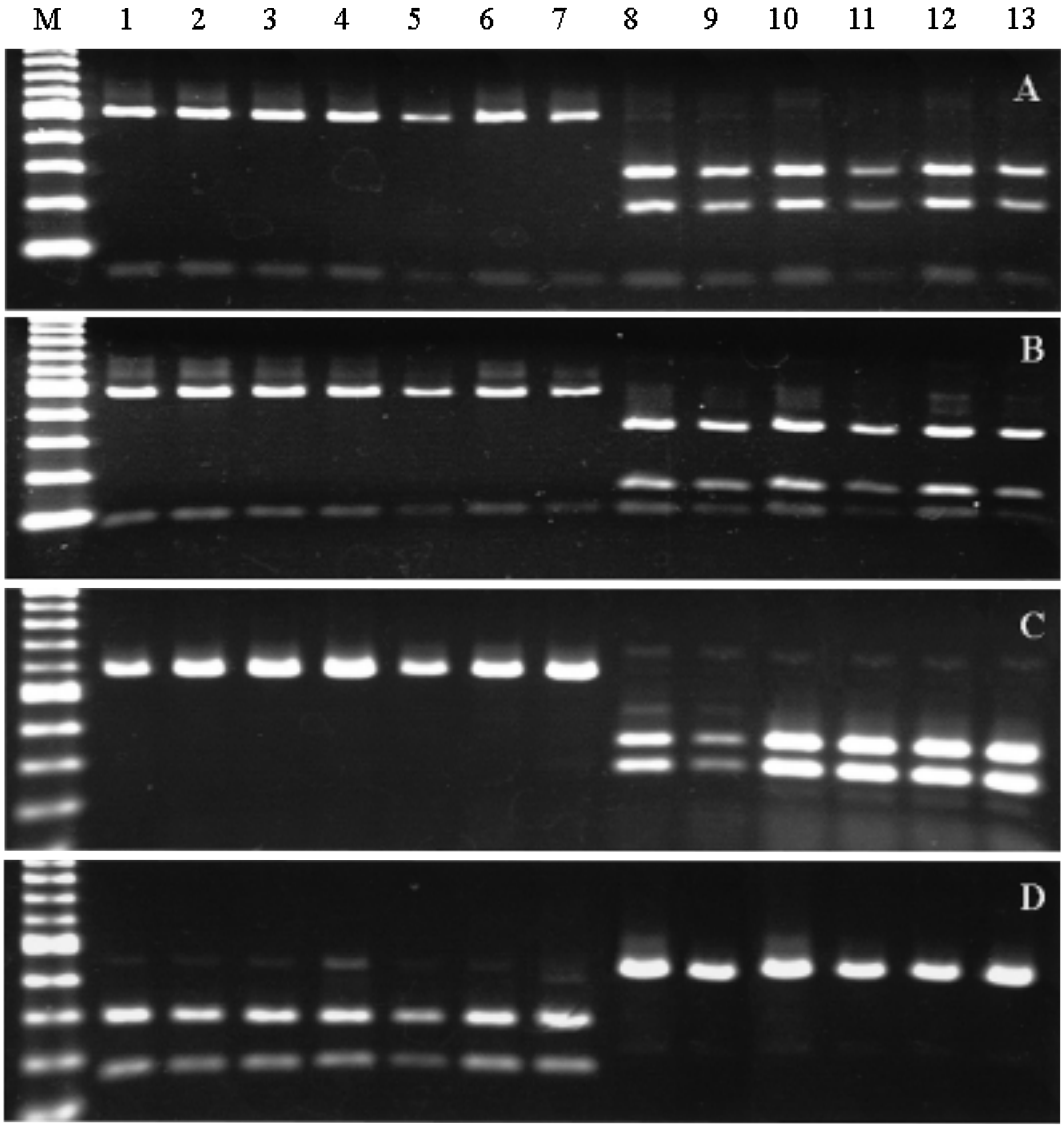

Figure 6. PCR-RFLP analysis of SB281, SB289 and SB012 genes by the restriction of SB281 with Hind III (panel A) and Hinf I (panel B), SB289 with Sac I (panel C), and SB012 with Ava II (panel D). The isolates from Japanese calf (lanes 1-3), Italy calf (lane 4 and 5), Italy goat (lane 6) and one Japanese human (HJ1 in lane 7) give the same kind of band pattern of genotype. The isolates from the other Japanese human (HJ2 and HJ3) in lanes 8 and 9, Italy human (lane 10 and 11) and Nepal human (lanes 12 and 13) show the same kind of band pattern of genotype I. M is Base-Pair Ladder of molecular weight marker.

oocysts in raw water (Wu *et al.*, 2000). The new primers (SB281 and SB289) had equivalent detection sensitivity to primer SB012. PCR detection indicates that these primers have a sensitivity 50 times higher than that of some other primers developed from COWP, HSP70 and TRAP1 and currently being used for genotyping *Cryptosporidium*.

Thus the analysis of the three RAPD genes provided the same genetic characterization to group the isolates to genotype I and genotype II, further evidence for the distinction between the two genotypes of *C. parvum*. Our new primers may also provide selectable methods for reliable elimination of *C. parvum* from tap water and for genotyping of the contaminated oocysts for further epidemiological survey.

ACKNOWLEDGMENTS

We thank Dr. Uga (Department of Medical Technology,

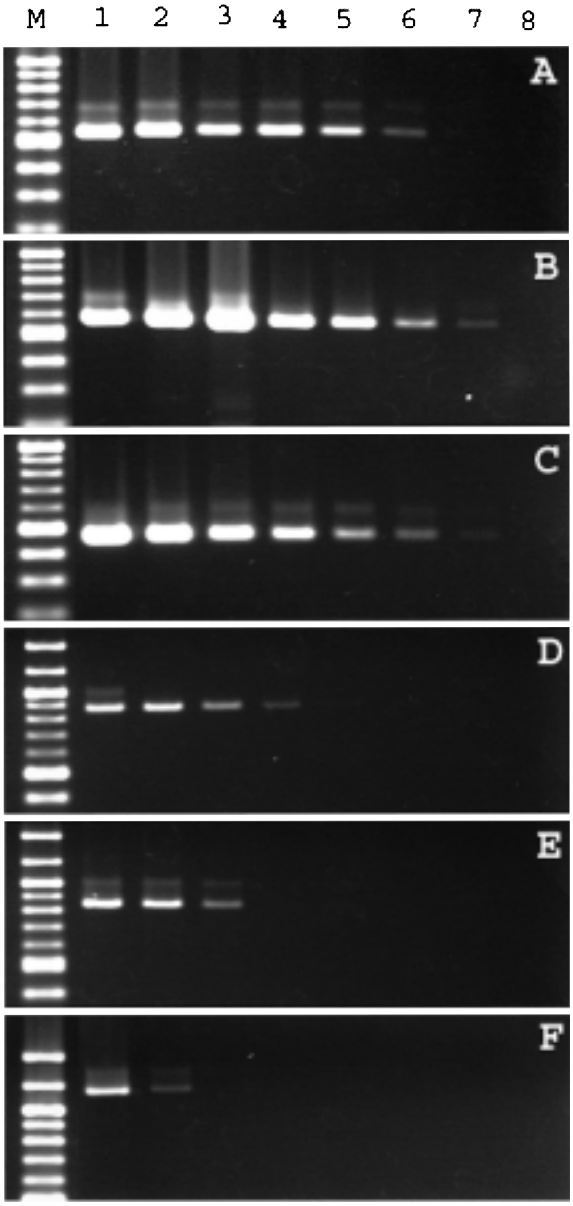

Figure 7. The sensitivity of constructed primer pair SB281, SB289 and SB012 by detecting template DNA and a comparison with other primers. M is 100 Base-Pair Ladder of molecular weight marker; lanes 1 to 8 are DNA amount of 160, 40, 10, 2.5, 0.625, 0.156, 0.039 and 0.001 pg, respectively. Panels A to F are primer SB281, SB289, SB012, COWP, HSP70 and TRAP1, respectively.

Kobe University School of Medicine, Japan) and Dr. Pozio (Laboratorio de Parasitologia, Instituto Superiore de Sanita, Italy) who kindly provided *Cryptosporidium* isolates. This work was supported by a grant-in-aid (no. 13670243) from the Ministry of Education, Sports, Science and Technology, Japan.

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