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Construction of Polymerase Chain Reaction Primer to Detect *Cryptosporidium parvum* or *C. muris*

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ABSTRACT

Two sets of polymerase chain reaction primers that amplify DNA fragments of *Cryptosporidium parvum* or *C. muris* were constructed by adapting a simple method, namely cloning and sequencing of randomly amplified polymorphic DNA (RAPD) of corresponding species. The primers successfully amplified genomic DNA of *C. parvum* or *C. muris* as well as sample DNA obtained from feces of a human patient, naturally infected cattle or experimentally infected mice. And we also developed the method to obtain DNA of *Cryptosporidium* from the oocysts in feces.

INTRODUCTION

Cryptosporidium spp. (Apicomplexa: cryptoridiidae) are common enteric protozoan parasites of humans and other animals. There are at least six recognize d species of *Cryptosporidium* (*C. parvum* and *C. muris* which infect mammals, *C. bailevi* and *C. meleagridis* which infect birds, *C. crotalt* infecting reptiles and *C. nasorum* infecting fish). Among them *C. parvum* is considered to be of particular importance to public health because it causes a severe diarrheal disease in man and livestock. Although most infections are probably transmitted directly from person-to-person by fecal-oral route, the transmission by waterborne route is important. When drinking water is contaminated with oocysts, large number of consumers can be infected and cryptosporidiosis outbreaks may occur (Hayes et al.

1989; Smith & Rose 1990; Current et al. 1991; and Mackenzie et al. 1994). Procedures currently used for detection of *Cryptosporidium* in water are complicated, filtering a large volume of water through a cartridge filter, releasing the oocysts from the filter matrix, concentration the elute to a small volume, staining the sample with anti-cryptosporidium antibodies and fluorescence-labeled second antibodies on a membrane filter, and examining by fluorescence microscopy. The recovery rate of oocysts by this method is limited to a low percentage. Therefore critically needed is a means to detect the oocysts with high sensitivity and specificity.

Recent progress in gene technology enabled us to use a DNA probe to assign identity of pathogens. The application of molecular techniques has significantly increased the specificity and sensitivity of diagnostic and research methods (Pershing 1991). PCR may overcome the limitations to detect *Cryptosporidium* (Saiki et al. 1988). In this paper we report primers that allow to amplify DNA fragments of *C. parvum* or *C. muris*.

MATERIALS AND METHODS

Source of oocysts of C. parvum and *C. muris*

Cryptosporidium parvum (Komagome strain): oocysts were originally isolated from an immunocompetent Japanese patient with diarrhea (Masuda et al. 1991), and maintained by subinoculation into immunocompromised adult mice, including athymic nude and severe combined immunodeficiency (SCID) mice. Oocysts were concentrated and purified from homogenates of infected feces by sugar flotation method (Iseki et al . 1989). *Cryptosporidium muris* (RN 66 strain): oocysts were isolated from the house rat, *Rattus norvegicus*, and maintained by subinocuration into adult immunocompetent specific pathogen-free mice (Iseki et al. 1986). Oocysts were concentrated and purified by same method as in *C. parvum*.

Source of test samples

1: Feces from mice infected with the Komagome strain of *C. parvum*. 2: Feces from a Japanese immunocompetent patient with diarrhea in which feces *Cryptosporidium* oocysts were microscopically detected. The patient was suspected to be infected during a two-week travel in India. 3: Feces from mice infected with the RN 66 strain of *C. muris*. 4: Feces from a cattle naturally infected with *Cryptosporidium*.

Preparation of genomic DNA of C. parvum and C. muris

Genomic DNA was prepared from oocysts of *C. parvum* and *C. muris*. In

brief, samples were frozen, mechanically homogenized, and digested with Pronase K at 37°C for 3 hrs. The DNA was precipitated after a phenol/chloroform extraction, and suspended in TE buffer (1 mM EDTA in 10 mM Tris-HCl, pH 7.4).

Cloning and sequencing of target DNA

Randomly amplified polymorphic DNA (RAPD) was produced with 10 bp primer (5'-GCAAGTAGCT-3'). PCR profile was 92°C for 30 sec, 36°C for 60 sec and 72°C for 60 sec, for 45 cycles. The PCR products were electrophoresed in 1.5% agarose gel. As criteria for targets of DNA identification, the DNA fragments should be shared by DNA samples of the intended strains, should be of appropriate length (around 500 bp) and the copy number should be high. DNA fragments that satisfy such criteria were isolated from agarose gel using Ultrafree-C3 HV unit (Nihon Millipore Ltd. Tokyo, Japan), and amplified again with the same AP-PCR condition. Resulting PCR products were purified with Ultrafree-C3 TK unit (Nihon Millipore Ltd. Tokyo, Japan), and ligated to pGEM-T plasmid vector with pGEM-T vector system (Promega Corporation Madison, WI, USA). The recombinant plasmids were introduced into competent cells of *Escherichia coli* JM 109. The plasmid DNA was isolated from *E. coli* by using the alkaline lysis method, and subjected to Taq cycle sequencing reactions using Dye Primer Cycle Sequencing Kit (Perkin Elmer Corporation Norwark, USA). The sequence products were analyzed with an automatic sequencer (model 373AL, Applied Biosystems, USA).

Basing on this DNA sequence data (Fig 1), primers were designed and synthesized. Sequence of primers were as follows: Primer SB48 for *C. parvum*:, Forward 5'-TTTTAGATGGTGAAGTTGGTA-3', Reverse 5'-GGATGTAGTGAA CCTGAAATGTG-3'; Primer SB50 for *C. muris*:, Forward 5'-AGTCTTTGTCTT TGCGTCAG-3', Reverse 5'-TCGTATTTGTATTGGATTCA-3'.

PCR condition

PCR reagents were purchased from Takara Co Ltd (Kyoto, Japan) and regular PCR was carried out according to manufacturer's instruction using the primers SB48 or SB50 with a profile being; step l- 92°C for 3 min, step 2 - 92° C for 30 sec, 51° C for 30sec and 72 °C for 60 sec, with 30 cycles.

Specificity of primers

To check specificity of primers, PCR was carried out. The tested DNA samples included *C. parvum, C. muris, Toxocara canis, Dirofilaria immitis, Taenia saginata, Trichinella spiralis,* and *Blastocystis hominis.*

PCR diagnosis

Template DNA for tests was prepared from feces as listed above. In brief, samples (about 0.1 g) were treated with 390 μl 0.1 M NaOH at 37°C for over night and neutrized by 500 μl of 0.1 M KH_2P0_4, and digested with Pronase K and precipitated according to the method described above.

DNA samples thus prepared were supposed to have very small amount of target DNA. DNA samples of feces contaminated with *Cryptosporidium* were mostly of bacterial origin. In these cases, single PCR was not sensitive enough to produce DNA bands visible after gel electrophoresis and ethidium bromide staining. We adapted double PCR using the same PCR method to amplify the first PCR products.

RESULTS

Sequence of target DNA fragments

Target DNA sequence thus obtained was shown in Fig.1. Other sequence with significant homology could not be detected in searches through the data base. The sequence was registered in GenBank. The accession numbers for the sequence are the fragment of *C. parvum*, U42052, and the fragment of *C. muris*, U42050.

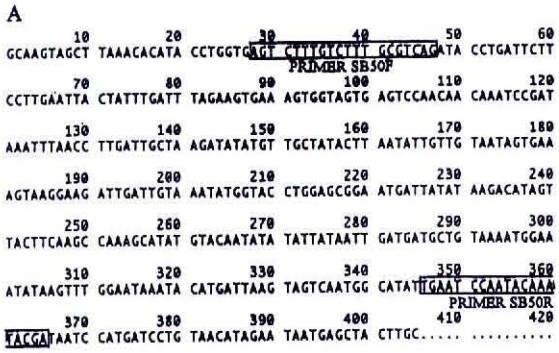

Fig. 1A. The sequence of DNA fragment of *Cryptosporidium parvum*. Designed forward primer is at position 28-47, and reverse primer is at position 346-365. The amplified fragment is 338 bp.

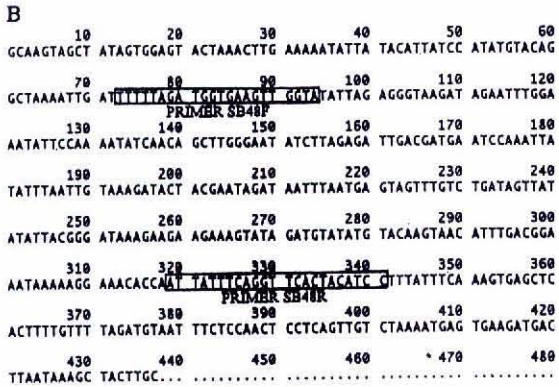

Fig. 1B. The sequence of DNA fragment of *Cryptosporidium muris*. Designed forward primer is at position 73-94, and reverse primer is at position 319-341. The amplified fragment is 269 bp.

PCR results with the primer SB50

As shown in Fig.2A. bands with 338 bp was obtained in the PCR products with the primer SB50 and DNA of *C. parvum*, but not those of the other parasites including *C. muris*. The result suggested that the primer we developed seems to be promising to detect DNA of *C. parvum*.

PCR result with the primer SB48

As shown in Fig.2B. bands with 269 bp was obtained in the PCR products with the primer SB48 and DNA of *C. muris*, but not those of the other parasites including *C.parvum*. The result suggested that the primer we developed seems to be promising to detect DNA of *C. muris*.

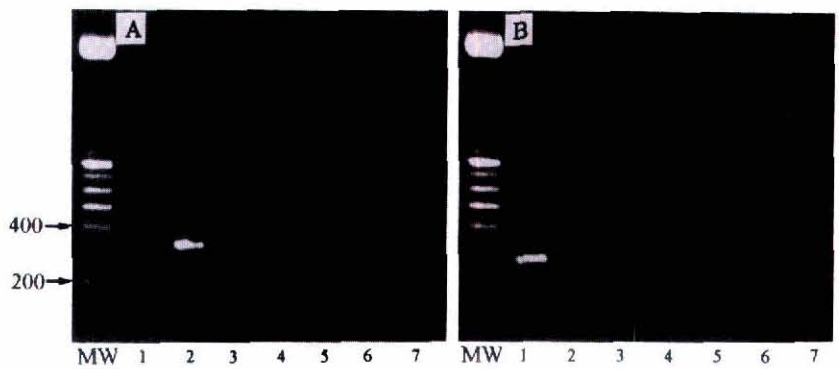

Fig 2. The specificity test of primers for *C. parvum* or *C. muris* with PCR. The DNA samples of *C. muris* (lane 1), *C. parvum* (lane 2), *Toxocara canis* (lane 3), *Dirofilaria immitis* (lane 4), *Taenia saginata* (lane 5), *Trichinella spiralis* (lane 6), and *Blastocystis hominis* (lane7), were used. Panel A: primer for *C. parvum*. Panel B: primer for *C. muris*.

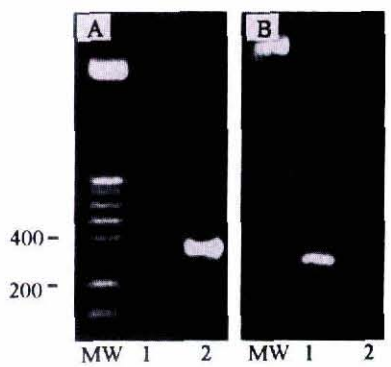

Fig. 3. PCR diagnosis of *C. parvum* and *C. muris*. The DNA obtained from oocysts in the feces of mice infected with *C. muris* (lane 1), or immunocompetent patient with diarrhea (lane 2), were detected using primer for *C. parvum* (Panel A) or primer for *C. muris* (Panel A).

PCR diagnosis of Cryptosporidium

The primers, SB48 and SB50, were tested for a practical use. DNA was isolated from feces of infected hosts, and amplified by the double PCR method. The test samples successfully produced a single band with expected numbers of bases as shown in Fig.3. Although the target DNA fragments are supposed to be very few in test samples of DNA, double PCR with our primers was sensitive enough to detect them.

Same was true for the isolates of *Cryptosporidium* from feces of the human case which was positive by PCR for *C. parvum.* but negative for *C. muris.* Therefore the parasite was identified as *C. parvum.* The isolate of *Cryptosporidium* from cattle feces resulted in the equivalent result that it was positive for *C. muris* but negative for *C. parvum.* Therefore the parasite was identified as *C. muris.*

DISCUSSION

Thus we have developed two sets of primers that can amplify genomic DNA of *C. parvum* or *C. muris.* Most likely application of these probles is DNA diagnosis of *C. parvum* and *C. muris.* Although current techniques of diagnosis of *C. parvum* by identifying oocysts in stool samples with acid-fast or immunofluorescent are generally considered adequate for the routine detection, the threshold of 50,000 oocysts per gram of feces of the methods is considered undesirable sensitivity (Garcia et al. 1987; Rusnak et al. 1989; Weber et al. 1991). Double PCR with our primers was sensitive enough to detect *Cryptosporidium* in feces of patient or infected animal.

The monitoring of water for the presence of cryptosporidial oocysts is necessary, but it is not easy at present because of their small size (4.5-5.5 μm in diameter), small numbers of oocysts in sample, and the difficulty in morphological identification of them amongst other particles and debris.

A variety of protocols are currently available that differentiate *C.parvum* from other strains (Nina et al. 1992; Awad-El-Kariem et al. 1993). However, these methods require a large number of oocysts and are mainly useful for research purpose. PCR with our primers is a suitable technique to differentiate *C. parvum* and *C. muris.*

It is worth noting that DNA of *Cryptosporidium* can be obtained from oocysts in feces by our method, where NaOH treatment is used to destroy oocyst wall of *Cryptosporidium.* After neutralization by KH_2P0_4, the samples are subjected to the standard procedures for DNA extraction.

Although the high discriminatory power of the PCR methods in identification of parasites rely upon DNA polymorphism, nobody can deny the

possibility that the DNA polymorphism may hamper practical use of PCR, because even point mutation of DNA at 3' end of the annealing site may cause failure of PCR, thus likely leading to false negative results.

Same is true for the PCR primer that has been reported previously by Laxer et al. 1991), which was designed to amplify DNA of *C. parvum*. Therefore more extensive test using isolates from different sources are needed to convince usefulness of these primers.

REFERENCES

Awad-El-Kariem, F. M., Robinson, H. A., McDonald, V., Evans, D., & Dyson, D. A. 1993. Is human *cryptosporidiosis* a zoonotic disease? *Lancet 341:* 1535.

Current, W. L. & Garcia, L. S. 1991. *Cryptosporidiosis. Clin. Microbiol. Rev. 4:* 325-358.

Garcia, L. S., Brewer, T. C., & Bruckner, D. A. 1987. Fluorescent detection of *Cryptosporidium* oocysts in human fecal specimens by using monoclonal antibodies. *J. Clin. Microbiol. 25:* 119-121.

Hayes, E. B., Matte, T. D., & O'Brien, T. R. 1989. Contamination of a conventionally treated filtered public water supply by *Cryptosporidium* associated with a large community outbreak of cryptosporidiosis. *N. Engl. J. Med. 320:* 1372-1376.

Iseki, M., Maekawa, T., Moriya, K., Uni S., & Takada, S. 1989. Infectivity of *cryptosporidium muris* (strain RN 66) in various laboratory animals. *Parasitol. Res. 75:* 218-222.

Iseki, M. I 986. Two species of *Cryptosporidium* infecting horse, rat, Rattus norvegicus. *J. Parasitol. 35:* 521-526.

Laxer, M,. A., Timblin, B. K., & Patel, R. J. 1991. DNA sequences for the specific detection of *Cryptosporidium parvum* by the polymerase chain reaction. *Am. J. Trop. Med. Hyg. 45:* 688-694.

MacKenzie, W. R., Hoxie, N. J., Proctor, M. E., Gradus, M. S., Blair, K. A., Peterson, D. E., & Kazimerczak, J. J. 1994. A massive outbreak in Milwaukee of *cryptosporidium* infection transmitted through the public water supply. *N. Engl. J. Med. 331:* 161-167.

Masuda, G., Negishi, M. & Ajisawa, A. 1991. *Cryptosporidium* diarrhea developing in two Japanese adults- one in AIDS and the other in a normal host. *J. J. A. Inf D. 68:* 1614-1619 (In Japanese with English summary).

Nina, J, M. S., McDonald, V., Deer, R. M. A., Wright, S. S., Dyson, D. A., Chiodini, P. L. O, & McAdam, K. P. W. 1992. Comparative study of antigenic composition of oocyst isolates of *Cryptosporidium parvum* from different hosts. *Parasite Immunol. 14:* 227-232.

Pershing, D. H. 1991. Polymerase chain reaction: trenches to benches. *J. Clin. Microbiol. 29:* 1323-1327.

Saiki, R K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., & Erlich, H. A. 1988. Primer directed enzynlatic amplification of DNA with a thermostable DNA polymerase. *Science 239:* 487-491.

Smith, H. V. & Rose, J. B. 1990. Waterborne cryptosporidiosis. *Parasitology Today 6:* 8-12.

Rusnak, J., Hadfield, T. L., Rhodes,M, M., & Gaines, J. K. 1989. Detection of *Cryptosporidium* oocysts in human fecal specimens by an indirect immunofluorescence assay with monoclonal antibodies. *J. Clin. Microbiol. 27:* 1135-1136.

Weber, R, Bryan, R. T., Bishop, H. S., Wahlquist, S. P., Sullivan, J. J. & Juranek, D. D. 1991. Threshold of detection of *Cryptosporidium* oocysts in human stool specimens: evidence of low sensitivity of current diagnostic methods. *J. Clin. Microbiol. 29:* 1323-1327.