The Korean Journal of Parasitology
Vol. 34, No. 1, 15-20, March 1996

Differentiation of Korean isolates of *Entamoeba histolytica* from *Entamoeba dispar*

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Abstract: Cysts of *Entamoeba histolytica* are still found from humans in Korea, but not all of the cysts are known as pathogenic. The non-pathogenic strain is regarded as a different species, *E. dispar*. In this study, Korean isolates of conventional *E. histolytica* were subjected for the differentiation by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. Human stools were screened by routine microscopic examination, and cyst or trophozoite positive stools were inoculated into Robinson media. The cultivated trophozoites were prepared for DNA extraction, and the DNAs were used for PCR with common primers of P1 gene. The PCR products were digested with 3 restriction enzymes and RFLP was observed. Also anti-sense primers containing the cleavage site of each restriction enzyme were designed for differentiation only by PCR. The PCR products of Korean isolates S9, S12, YS-6, and YS-27 were spliced by *Taq* I and *Xmn* I but not by *Acc* I, and the isolates S1, S3, S11, S15, S16, S17, S20, YS-17, and YS-44 were spliced by *Acc* I but not by *Taq* I and *Xmn* I. These RFLP pattern correlated well with PCR products by the species specific primers. The findings confirm that the Korean isolates S9, S12, YS-6, and YS-27 are *E. histolytica* and others are *E. dispar*. In Korea, most of the asymptomatic cyst carriers are infected by *E. dispar*, not by *E. histolytica*.

Key words: *Entamoeba histolytica*, *Entamoeba dispar*, Korean isolates, polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP)

INTRODUCTION

Entamoeba histolytica Schaudinn, 1903 is a pathogenic amoeba which grows primarily in the intestine of human. There had been evidences that pathogenic and non-pathogenic strains are present in *E. histolytica* (Mirelman, 1987; Edman *et al.*, 1990; Blanc, 1992). The two strains were proved different in genetic structures (Tannich *et al.*, 1989; Tannich and Burchard, 1991; Abd-Alla *et al.*, 1993), and it is finally concluded that pathogenic and non-pathogenic strains are different species which have evolved for several million years. Therefore, the nomenclature was rearranged as that the pathogenic amoeba is *Entamoeba histolytica* and non-pathogenic amoeba is *Entamoeba dispar* Brumpt, 1925 (Diamond and Clark, 1993). However clear-cut differentiation of them still remains problematic because the two species are same

• Received Jan. 15 1996, accepted Feb. 1 1996.
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in their morphologic characteristics, and thus differentiation is tricky.

The amoebas are differentiated through various molecular biologic methods including the analysis of the amplified P1 gene by PCR and RFLP (Tannich and Burchard 1991), zymodeme analysis (Blanc, 1992), antigen specificity (Gonzalez-Ruiz *et al.*, 1992; Lotter *et al.*, 1992), and determination of the specific coproantigen (Haque *et al.*, 1993; Gonzalez-Ruiz *et al.*, 1994).

Infection of *E. histolytica* has taken a high toll in the population all across the world and it is estimated that more than 50 million people throughout the world are infected with *E. histolytica*. It is assumed that 10% out of the infected people develop clinical enteritis or extraintestinal abscess. In the United States, the number of clinical patients with amebiasis runs up to 70,000 annually. A recent paper recorded the cyst positive rate of *E. histolytica* was alarming as 0.9% in U.S.A. (Kappus *et al.*, 1994). However, the overall prevalence of *E. histolytica* infection may be overestimated due to same morphology of *E. histolytica* and *E. dispar*. Most of the reports have mixed the two.

Strictly speaking, the same situation is true in Korea. Prevalence of *E. histolytica* in Korea has been overestimated definitely since the rate usually included *E. histolytica*, *E. dispar* and *E. hartmanni* altogether. Based on the report by Kessel (1925), the first report in Korea on prevalence of amoeba cysts, 41% out of the total 208 examinees passed cysts of *E. histolytica*. Since then, the cyst positive rates maintained 5~10% until early 1970's and Lee (1973) showed a cyst positive rate of 7.7% out of 4,447 examinees. Afterwards, according to rapid industrialization throughout the country, improvement in water-supply facilities, decreases in the use of human manure and fertilizer, and amelioration in individual hygiene, the positive rate of cysts fell off below 1% in the late 1970's and decreased to 0.5% in the late 1980's and 1990's (Hong, 1994). But differentiation of the three *Entamoeba* species has never been tried in Korea.

At present the differentiation of pathogenic *E. histolytica* from non-pathogenic *E. dispar* is desperately needed in Korea. The objective of this study was to differentiate *E. histolytica* from *E. dispar*, isolated from humans in Korea, through DNA analysis of P1 gene by PCR and RFLP.

MATERIALS AND METHODS

1. Amoebas

The isolates of amoebas used in the experiment were 13 altogether. These were S1, S3, S9, S11, S12, S15, S16, S17, S20, YS-6, YS-17, YS-27, and YS-44, isolated from 13 patients or cyst carriers in Korea. These specimens were collected at the Seoul National University Hospital, Seoul Paik Hospital, and Yonsei Medical Center. The strain of HM-1: IMSS was the standard of *E. histolytica*. The human stools which were cyst positive by formalin-ether concentration technique were cultivated in Robinson medium, and the subcultivated trophozoites in Robinson medium or TY5GM-9 were used for this analysis. The axenized isolates, YS-27 (Chang *et al.*, 1995) and HM-1:IMSS, were maintained in the medium TYI-S-33.

2. Preparation of DNA

The cultivated trophozoites were washed in cold saline and resuspended in 400 μl of water. The bursted trophozoites were successively treated with phenol, chloropane and chloroform. The supernatant was stored frozen and used as template DNA for PCR.

3. Polymerase chain reaction (PCR)

The oligonucleotides of P1 gene synthetised in the Korea Biotech Co. were used as primers. The sense primer was P1-S17 (5'-GCAACTA-GTGTTAGTTA-3'), and the common antisense primer was P1-AS20 (5'-CCTCCAAGATATGT-TTTAAC-3') for both species (Tannich and Burchard, 1991). The species specific antisense primers were pathogenic P1-AS16 including *Xmn* I restriction site (5'-AAGCCCTTCCTGTATC-3') for *E. histolytica* and nonpathogenic NP1-AS16 including *Acc* I restriction site (5'-TAGACGTACCAGTATA-3') for *E. dispar*.

The reaction mixture for PCR was 50 μl which was composed of the primers 100 pmole, dNTP mix 10 mM each, tDNA 50~100 μg, and *Taq* DNA polymerase 2.5 unit. After

keeping for five minutes at 94°C, 3 step thermocycling was repeated 35 times; 30 seconds at 94°C for denaturation, 30 seconds at 57°C for annealing, and 45 seconds at 72°C for extension. After completion of the cycles, the reaction was held for 10 minutes at 72°C. The PCR products were electrophoresed in 1% agarose mini-gel containing ethidium bromide, and visualized through UV illumination. The photopicture was taken with a polaroid camera. The PCR product was precipitated by mixing with ethanol and sodium acetate salt. The DNA precipitate was spun down and dried, and resuspended in 30-50 μl water.

4. Restriction fragment length polymorphism (RFLP)

The redissolved PCR products were aliquated into 8 μl, and 10 × buffer solution 1 μl and 1 μl of each endonuclease were added. The enzymes reacted for 2 hours at 37°C for *Xmn* I and *Acc* I, and at 65°C for *Taq* I. The results were observed through running on 1.5% agarose gels as mentioned above.

RESULTS

The DNA bands shown in Fig. 1 are the 482 bp product extended with the common primers and 292 bp product with species specific primers. The 482 bp product was digested by restriction enzymes, *Taq* I, *Xmn* I, and *Acc* I as presented in Fig. 2. The PCR products of 4 Korean isolates; S9, S12, YS-6, and YS-27, were cut by *Taq* I and *Xmn* I into 2 bands but not by *Acc* I. They also made PCR product with the primer pairs of *Xmn* I sequence, P1-AS16 (Fig. 1). Therefore these four isolates are *E. histolytica*. The PCR products with common primers of the isolates, S1, S3, S11, S15, S16, S17, S20, YS-17, and YS-44, were cut by *Acc* I but not by *Taq* I and *Xmn* I. Of course they showed PCR products with the primers of the *Acc* I sequence, NP1-AS16 (Fig. 1). The 9 isolates are confirmed as *E. dispar*. The present findings and clinical records are summarized in Table 1.

DISCUSSIONS

The present findings confirm that most of the asymptomatic cyst passers of *E. histolytica* in Korea are not infected by *E. histolytica* but infected by *E. dispar*. Out of 13 Korean isolates, S9, S12, YS-6 and YS-27 were proved as *E. histolytica*. The S9 was isolated from a 10-year old girl who suffered from bloody diarrhea at Inchon in 1994 and S12 was isolated in 1994 from a Korean man who had been to Indonesia where diarrheal colitis appeared first. YS-6 and YS-27 were isolated in 1960s at Yonsei Medical Center from patients of colitis and liver abscess respectively. YS-27 is an axenized isolate (Chang *et al.*, 1995).

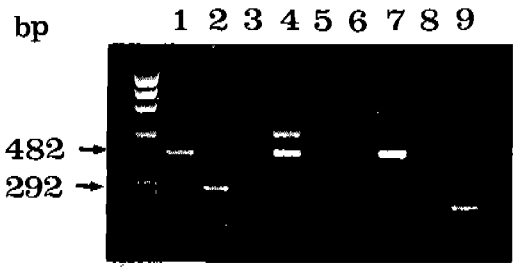

Fig. 1. PCR products of HM-1 & S12 (*E. histolytica*) and S15 (*E. dispar*) with the primer pairs of P1-S17 and 3 antisense primers. **1-3,** HM-1 amoeba template DNA with primers of comnon P1-AS20, pathogenic P1-AS16, and non-pothogenic NP1-AS16; **4-6,** S12 amoeba template DNA with primers of common P1-AS20, pathogenic P1-AS16, and non-pathogenic NP1-AS16; **7-9,** S15 amoeba template DNA with primers of common P1-AS20, pathogenic P1-AS16, and non-pathogenic P1-NAS16.

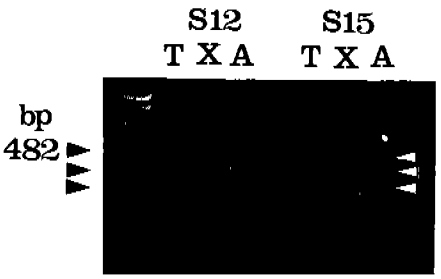

Fig. 2. Restriction patterns of 482 bp PCR products of S12 (*E. histolytica*) and S15 (*E. dispar*) template DNAs with common primers of P1-S17 and P1-AS20. S12 product was digested into two fragments; 321 and 161 bp by T, and 291 and 191 bp by X. S15 product was digested into two fragments, 292 and 190 bp by A. Restriction enzymes: T, *Taq* I; X, *Xmn* I; A, *Acc* I.

Table 1. Differentiation of *E. histolytica* and *E. dispar* among 13 Korean *Entamoeba* isolates

Isolates (year)	PCR with primers			Restriction of 482 bp			Symptoms (travel history)	Species
	AS20	AS16	NAS16	*Taq* I	*Xmn* I	*Acc* I		
S1 (1993)	0	—	0	—	—	0	diarrhea	*E. dispar*
S3 (1993)	0	—	0	—	—	0	symptomless	*E. dispar*
S9 (1994)	0	0	—	0	0	—	diarrhea	*E. histolytica*
S11 (1994)	0	—	0	—	—	0	symptomless	*E. dispar*
S12 (1994)	0	0	—	0	0	—	diarrhea (Indonesia)	*E. histolytica*
S15 (1994)	0	—	0	—	—	0	symptomless	*E. dispar*
S16 (1994)	0	—	0	—	—	0	symptomless	*E. dispar*
S17 (1994)	0	—	0	—	—	0	symptomless	*E. dispar*
S20 (1995)	0	—	0	—	—	0	symptomless	*E. dispar*
YS-6 (1960s)	0	0	—	0	0	—	diarrhea	*E. histolytica*
YS-17 (1960s)	0	—	0	—	—	0	symptomless	*E. dispar*
YS-27 (1969)	0	0	—	0	0	—	liver abscess	*E. histolytica*
YS-44 (1970s)	0	—	0	—	—	0	symptomless	*E. dispar*
HM-1:IMSS	0	0	—	0	0	—		*E. histolytica*

S1, S3, S11, S15, S16, S17, YS-17, and YS-44 were found as *E. dispar*. The S1 was isolated from a patient of diarrhea but others were from cases who were found cyst positive by routine stool examination without any related symptoms of amoebiasis. All of the symptomless carriers and one (S1) of the 4 diarrheal patients (S1, S9, S12, and YS-6) were proved to be infected by the non-pathogenic *Entamoeba*. Since the amoeba, *E. dispar*, is not invading the intestinal mucosa, the carrier of S1 isolate had diarrhea which must have been caused by other undetermined reasons.

Cysts of *Entamoeba* species recovered in human stools are classified into 3 species by microscopic morphology, *E. coli*, *E. hartmanni*, and *E. histolytica* (including *E. dispar*). *E. coli* cyst is larger than 10 μm and has 1~8 nuclei. The cyst of *E. hartmanni* is smaller than 10 μm in diameter. The cyst sized 10~20 μm with 1~4 nuclei is *E. histolytica* or *E. dispar*. However, *E. dispar* cyst is indistinguishable from that of *E. histolytica* on the basis of morphological traits. Furthermore, some papers reported that one isolate of non-pathogenic *E. histolytica* was changed into pathogenic one during long-term cultivation (Mirelman *et al.*, 1986a & b). Some groups asserted that the pathogenic and non-pathogenic characteristics were changeable. After confusing debates, it is now accepted that the confusing reports were the outcome of contamination during cultivation (Clark and Diamond, 1993).

Differentiation of *E. histolytica* from *E. dispar* is practically crucial for definite diagnosis and proper management of the patients. Although the cyst-passers have symptoms of colitis, further study should find out the real etiology if the cysts are confirmed as *E. dispar*. The cyst-passers of *E. dispar*, *E. hartmanni* and *E. coli* should not be essentially treated by anti-amoebic medication.

In Korea, cyst-passers of *E. dispar* are much more than those of *E. histolytica*. Only less than one quarter of the cyst positives are the real targets of medical cares for amebiasis. This is the common feature throughout the world except for hyperendemic areas (Tannich and Burchard, 1991).

Development of an easier and handier method to differentiate *E. histolytica* from *E. dispar* is essential because PCR and RFLP analysis used in the present study is rather complicated, tricky, and costful. Furthermore it takes several days for cultivation. Therefore, simplification of PCR and RFLP remains necessary. Diagnosis by PCR only is much easier than PCR and RFLP as revealed through the present study. The antisense primers which include the sequences of *Xmn* I and *Acc* I restriction sites produce different amplification between the two species. The primer pair with sequences of *Xmn* I site amplifies the DNA fragment of only *E. histolytica* while the primer with sequences of *Acc* I site amplifies the DNA of only *E. dispar*.

These primer pairs can differentiate the two species by PCR only.

To adopt the PCR technique in mass screening, direct extraction of amoeba DNA from feces without cultivation is more effective than DNA extraction from cultivated amoebas. The method described by Katzwinkel-Wladarsch *et al.* (1994) requires no cultivation of amoebas and provides more sensitive and selective differentiation. This method is simple because all extraction steps can be performed at room temperature and there are no steps of precipitation and hybridization. The whole procedure can be performed in one day. We should screen reproducibility and accuracy of the method.

Immunodiagnosis by screening the coproantigen which reacts with the monoclonal antibody is the best candidate at present for differentiating these amoebas (Haque *et al.*, 1993; Gonzalez-Ruiz *et al.*, 1994). It must be compared that which one is more feasible in definite diagnosis between the rapid one step PCR and the coproantigen screening.

In conclusion, the present study proves that non-pathogenic *E. dispar* is more prevalent than *E. histolytica* in Korea. All of asymptomatic cyst carriers and even one patient with diarrhea were infected by *E. dispar*. PCR only with selective primers of P1 gene can be successfully applied to differentiate *E. histolytica* from *E. dispar*.

ACKNOWLEDGEMENT

The authors would highly appreciate technical assistance of Mr. Jae-Lip Kim in Department of Parasitology and Institute of Endemic Diseases, Seoul National University College of Medicine.

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=초록=

우리 나라에서 분리한 이질아메바(*Entamoeba histolytica*)와 동형아메바(*Entamoeba dispar*)의 감별

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이질아메바(*Entamoeba histolytica*)와 동형아메바(*Entamoeba dispar*, 同形아메바)의 포낭은 형태학적으로 구분이 안되어 종 감별에 관하여 논란이 있어 왔다. 최근에 이 둘이 별종이며 특히 이질아메바는 병원성이고, 동형아메바는 비병원성임이 확인되어 그 감별이 중요한 의미를 갖게 되었다. 이 연구에서는 우리 나라의 아메바 분리체를 중합효소 반응과 제한효소 반응을 이용하여 두 종으로 감별하였다. 1994-1995년에 대변을 통상의 방법으로 검색하여 포낭이나 영양형이 발견된 검체를 로빈슨 배지에서 배양하고 배양된 영양형에서 DNA를 분리하였다. P1 유전자 염기서열 중에서 시발체(primer)를 만들어 중합효소 반응으로 482 bp 크기의 산물을 얻고 이를 제한효소(*Taq* I, *Xmn* I, *Acc* I)로 처리하였다. 또한 *Xmn* I과 *Acc* I 제한효소의 특이 염기서열을 포함하는 시발체를 제작하여 따로 중합효소 반응을 시행하였다. 그 결과 13개 분리체 중에서 S9, S12, YS-6, YS-27의 482 bp 산물은 *Taq* I과 *Xmn* I에 의하여, 그 외의 분리체 산물은 *Acc* I에 의하여 절단되었다. 이 결과는 특이 염기서열 시발체의 중합효소 반응에서 얻은 산물과 일치하였다. 이 결과에 의하여 분리체 S9, S12, YS-6는 대장염 환자에서, YS-27은 간농양 환자에서 분리한 병원성의 이질아메바(*E. histolytica*)이고, 분리체 S1, S3, S11, S15, S16, S17, S20, YS-17, YS-44는 무증상의 포낭배출자에서 얻은 비병원성의 동형아메바(*E. dispar*)로 구별할 수 있었다. S1은 설사 환자에서 얻은 분리체이지만 동형아메바임을 확인하였고, 따라서 이 환자의 설사는 다른 원인에 의한 것으로 판단된다. 이로써 비병원성인 동형아메바가 우리 나라에서도 병원성 이질아메바보다 더 흔하게 존재한다는 것을 처음으로 기록하며 *E. dispar*의 우리 말 이름을 동형아메바로 제안한다.

〔기생충학잡지, 33(4): 15-20, 1996년 3월〕