RESTRICTION ENZYME DIGESTION ANALYSIS OF PCR-AMPLIFIED DNA OF *BLASTOCYSTIS HOMINIS* ISOLATES

I Init[1], AI Foead[2], MY Fong[1], H Yamazaki[3], M Rohela[1], HS Yong[4] and JW Mak[5]

[1]Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia; [2]Faculty of Medicine, Universiti Teknologi MARA, Shah Alam, Malaysia; [3]2895 Aonohara Tsukui Machi, Tsukui Gun, Kanagawa, Japan; [4]Institute of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia; [5]International Medical University, Sesama Center, Plaza Komanwel, Bukit Jalil, Kuala Lumpur, Malaysia

Abstract. Genomic DNA of *Blastocystis* isolates released into 0.1% Triton X-100 was suitable for amplification and yielded similar results as the genomic DNA extracted with standard kit. The specific *B. hominis* primers (BH1: GCT TAT CTG GTT GAT CCT GCC AGT and BH2: TGA TCC TTC CGC AGG TTC ACC TAC A) successfully produced the PCR product of about 1,770 bp with all the 7 *Blastocystis* isolates tested. The restriction fragment length polymorphism (RFLP) patterns yielded by 13 out of 25 restriction endonucleases showed that the 7 isolates could be grouped into 4 subgroups: subgroup-1 consisted of isolate C; subgroup-2 of isolates H4 and H7; subgroup-3 of isolates KP1, Y51 and M12; and subgroup-4 of isolate 27B05. The differences between subgroups manifested as clear-cut RFLP patterns. A common band of 230 bp was revealed by *Eco* R1 in all the *Blastocystis* isolates tested. The band of about 180 bp was revealed by *Alu* I, differentiated symptomatic from asymptomatic isolates of this parasite, and might indicate the pathogenicity of this parasite.

INTRODUCTION

Since the report of *Blastocystis* as an intestinal protozoan, many investigators have attempted to confirm the life-cycle, pathogenicity and taxonomy of this parasite. Medical interest in *B. hominis* has focused on its pathogenicity. Several reports indicated that *B. hominis* could be an important cause of intestinal diarrheal disease in humans and animals (Nguyen and Krech, 1989; O' Gormam *et al*, 1993). There are still divergent views on the etiology of this organism; it may or may not be pathogenic as it is found not only in diarrheal but also in normal feces.

Polymerase chain reaction (PCR) has been utilized for typing and differentiating organisms. PCR can be used to discriminate strain, species and pathogenic potential of organisms. Primers for *B. hominis* have been established by Silberman *et al* (1996) producing an amplicon of 1,770 bp. PCR has successfully shown differences among *Blastocystis* isolates using arbitrary primers (Yoshikawa *et al*, 1996) and also a single set of primers (Init *et al*, 1999). On the other hand, restriction fragment length polymorphism (RLFP) has been used in determining *B. hominis* sub-groups as well as clarifying phylogenic relationship among closely-related organisms (Bohm-Glomin *et al*, 1997). In this study, RFLP PCR was used to detect the variations as well as to study the characteristics of pathogenic *B. hominis* from man and monkey.

Correspondence: Dr Init Ithoi, Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.
Fax: 603-79674754
E-mail: init@um.edu.my

MATERIALS AND METHODS

Blastocystis isolates

Six isolates of *Blastocystis* from human (C, H4, H7, KP1, Y51, 27B05) and one from a

monkey (M12) were used. These isolates were in xenic culture, except isolate C which was in axenic culture. Isolate M12 was obtained from soft stool of a monkey (*Macaca fascicularis*) kept at the Institute for Medical Research animal house, Malaysia. Isolate C was supplied by the National University of Singapore, isolates KP1 and Y51 from patients with chronic diarrhea and crampy abdominal pain, isolates H4 and H7 from healthy persons, and isolate 27B05 from a healthy Malaysian aborigine (Orang Asli). These isolates were selected based on the study using a single set of primers (Init *et al*, 1999). A common intestinal bacterium, *E. coli* (Bac-4), obtained from the culture medium of *Blastocystis* isolate KP1 was used as a control for xenic culture.

Preparation of genomic DNA

Three methods were used to extract genomic DNA. The first method utilized the G-Nome kit (BIO 101) according to the manufacturer's procedures. In the second method, parasites (about 10^8 cells) were quick-frozen, washed and stored at -80ºC, and thawed to produce lysis just before use. The third method employed 0.1% Triton X-100 (1 ml) solution (Sigma) to lyse the newly washed parasites for at least one hour at room temperature before use or samples were kept at -20ºC until use. This latter method was applied to all the *Blastocystis* isolates and bacteria.

Polymerase chain reaction of genomic DNA

Primers used in this study were those developed by Silberman *et al* (1996) from the 26s rDNA of *B. hominis*, namely, primer BH 1 (GCT TAT CTG GTT GAT CCT GCC AGT) and primer BH 2 (TGA TCC TTC CGC AGG TTC ACC TAC A). This reaction mixture contained 0.4 µl of 100 mM stock deoxynucleotide triphosphate, 5 µl of 10X PCR buffer, 2 µl of 50 mM $MgCl_2$, 0.5 µl of Taq DNA polymerase (5 U/µl) and genomic DNA (1 µl of isolate C from G-Nome kit, 10 µl of isolate C quick-frozen and stored at -80ºC, and 10 µl of all isolates from 0.1% Triton X-100 lysis. The mixture was made up to a total volume of 50 µl with sterile Millipore water. PCR was performed at an initial 94ºC for 5 minutes followed by 35 cycles of denaturation at 94ºC for 1 minute, annealing at 55ºC for 1 minute, extension at 72ºC for 1 minute, and a final step at 72ºC for 5 minutes.

Ten µl of PCR products and 1 µl of loading dye (Promega) were loaded in the well of 2% agarose gel. DNA standard markers (100 and 200 bp ladders) were loaded in different wells. Electrophoresis in 1X TBE buffer (AMRESCO) was conducted at 50 volts until the blue dye migrated about 4 cm from the wells. Gel was then stained in 100 ml 1X TBE buffer containing ethidium bromide (0.2 µg/ml) and photographed under short wave length ultraviolet transillumination.

Restriction fragment length polymorphism (RFLP) analysis

A mixture containing PCR product (20 µl), 10X PCR buffer (3 µl), restriction endonuclease (1 unit for 1 µg of PCR product) and sterile Millipore water (to make total volume of 30 µl) was incubated overnight at the appropriate optimal temperature according to manufacturer's procedure. A total of 25 restriction endonucleases (MBI, Fermentas) were used. Digested PCR products were separated by electrophoresis in 3% agarose gel and processed as described above.

RESULTS

PCR products

Genomic DNA of isolate C extracted by using the G-Nome kit and 0.1% Triton X-100 solution produced similar amplicons of about 1,770 bp (Fig 1), while the extract from quick-frozen parasite stored at -80ºC did not produce any band (data not shown). Genomic DNA of all other *Blastocystis* isolates (H4, H7,

Table 1
Restriction fragment sizes of amplicons from *Blastocystis* isolates after digestion with restriction endonucleases.

Enzyme	*Blastocystis* isolates with their restriction fragment sizes in base pair						
	C	H4	H7	KP1	Y51	M12	27B05
Alu I	CWB (230, 270, 320, 400)	CNB	CWB (230, 270, 320, 400)	180, 230, 270, 320, 400	180, 230, 270, 320, 400	180, 230, 270, 320, 400	150, 180, 270, 400, 750
*Bst*O I	500, 650, 700	CWB (600, 1200)	CWB (600, 1200)	600, 1200, 1300	600, 1200, 1300	600, 1200, 1300	250, 350, 1200
Dde I	CWB (280)	180, 280, 380, 400	180, 280, 380, 400	180, 280, 380, 400	CWB (280, 380, 400)	CWB (280, 380, 400)	CWB (380, 400)
Eco RI	230	230	230	230	230	230	230
Hae III	550, 1200	550, 1200	550, 1200	CWB (300, 550, 1000)	CWB (300, 550, 1000)	300, 550, 1000	230, 550, 1200
Hha I	180, 350, 450	350, 450	CNB	350, 450	350, 450	350, 450	350, 450
Hinf I	CNB	280, 350, 550	280, 350, 550	280, 550, 1000	280, 550, 1000	280, 550, 1000	260, 1400
Hpa II	CWB (600, 1200)	CWB (600, 1200)	CWB (600, 1200)	150, 600, 1100, 1300	150, 600, 1100, 1300	150, 600, 1100, 1300	750, 1200
Pvu I	NB	NB	NB	NB	NB	550	550
Pvu II	NB	300	300	NB	NB	NB	NB
Sca I	CWB (200)	NB	NB	NB	NB	NB	NB
Spe I	1100	500, 1000	500, 1000	500, 1000	500, 1000	500, 1000	800, 1770
Taq I	CWB (320, 550, 700, 1000)	320, 550, 700, 1000	320, 550, 700, 1000	320, 550, 700, 900, 1100	320, 550, 700, 900, 1100	320, 550, 900, 1100	320, 550, 900

CWB= Digested and produced weak RFL bands (in parenthesis)
CNB= Digested but did not produce RFL band
NB= Did not show any RFL band

KP1, Y51, M12 and 27B05) using 0.1% Triton X-100 lysis solution also showed a single amplicon of about 1,770 bp (Fig 2). No amplicon was obtained from *E. coli* Bac-4 (Figs 1 and 2).

RFLP patterns

Of the 25 restriction endonucleases (RE) used, 12 (*Bam* HI, *Bgl* I, *Bgl* II, *Dra* I, *Eco* RV, *Hinc* II, *Hind* III, *Pst* I, *Sac* I, *Sal* I, *Sma* I and *Xba* I) failed to digest the amplicons from all the *Blastocystis* isolates tested. However 13 RE (*Alu* I, *Bst*O I, *Dde* I, *Eco* RI, *Hae* III, *Hha* I, *Hinf* I, *Hpa* II, *Pvu* I, *Pvu* II, *Sca* I, *Spe* I and *Taq* I) successfully produced different RFLP patterns (Table 1, Fig 3A-M). A common band of 230 bp was present in all the isolates digested with *Eco* RI (Fig 3D). The differentiation of the 7 *Blastocystis* isolates by these 13 RE are summarized in Table 2.

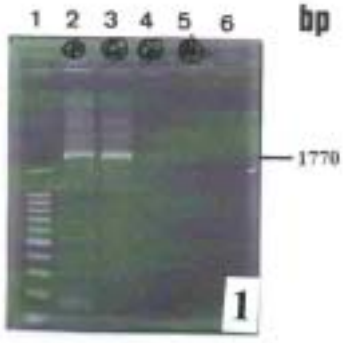

Fig 1–Amplicons from *B. hominis* isolate C and *Escherichia coli*, Bac-4. Lane 1- A 100 bp markers; lane 2 - amplicon from DNA template of isolate C extracted by using G-Nome kit; lane 3 - amplicon from DNA template of isolate C in 0.1% Triton X-100; lane 4 - amplicon from DNA template of Bac-4 extracted by using G-Nome kit; lane 5 – amplicon from DNA template of Bac-4 in 0.1% Triton X-100; lane 6 – water control.

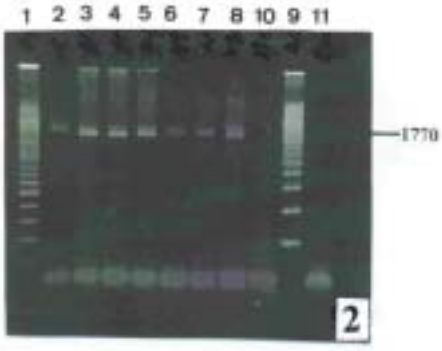

Fig 2–Amplicons from DNA template in 0.1% Triton X-100. Lane 1 - 100 bp markers; lane 2 - *B. hominis* isolate C; lane 3 - H4; lane 4 - H7; lane 5 - KP1; lane 6 - Y51; lane 7 - M12; lane 8 - 27B05; lane 9 - 200 bp marker; lane 10 - *E. coli* Bac-4; lane 11 – water control.

Table 2
Differentiation of 7 *Blastocystis* isolates by 13 restriction endonucleases.

Enzyme	Groups of isolates with similar RFLP patterns			
Alu I	C, H7		KP1, Y51, M12	27B05
*Bst*O I	C	H4, H7	KP1, Y51, M12	27B05
Dde I	C	H4, H7, KP1	Y51, M12	27B05
Eco RI	C, H4, H7, KP1, Y51, M12, 27B05			
Hae III	C, H4, H7		KP1, Y51, M12	27B05
Hha I	C		H4, KP1, Y51, M12, 27B05	
Hinf I		H4, H7	KP1, Y51, M12	27B05
Hpa II	C, H4, H7		KP1, Y51, M12	27B05
Pvu I			M12, 27B05	
Pvu II		H4, H7		
Sca I	C			
Spe I	C	H4, H7, KP1, Y51, M12,		27B05
Taq I	C, H4, H7	KP1, Y51	M12	27B05

DISCUSSION

A combination of PCR and restriction endonuclease digestion has proven to be useful for the identification, and determination of strain and pathogenicity of *Entamoeba histolytica* (Tachibana *et al*, 1991). In this study, a set of *B. hominis* primers developed by Silberman *et al* (1996) was selected for PCR. Besides that, 3 methods were applied to

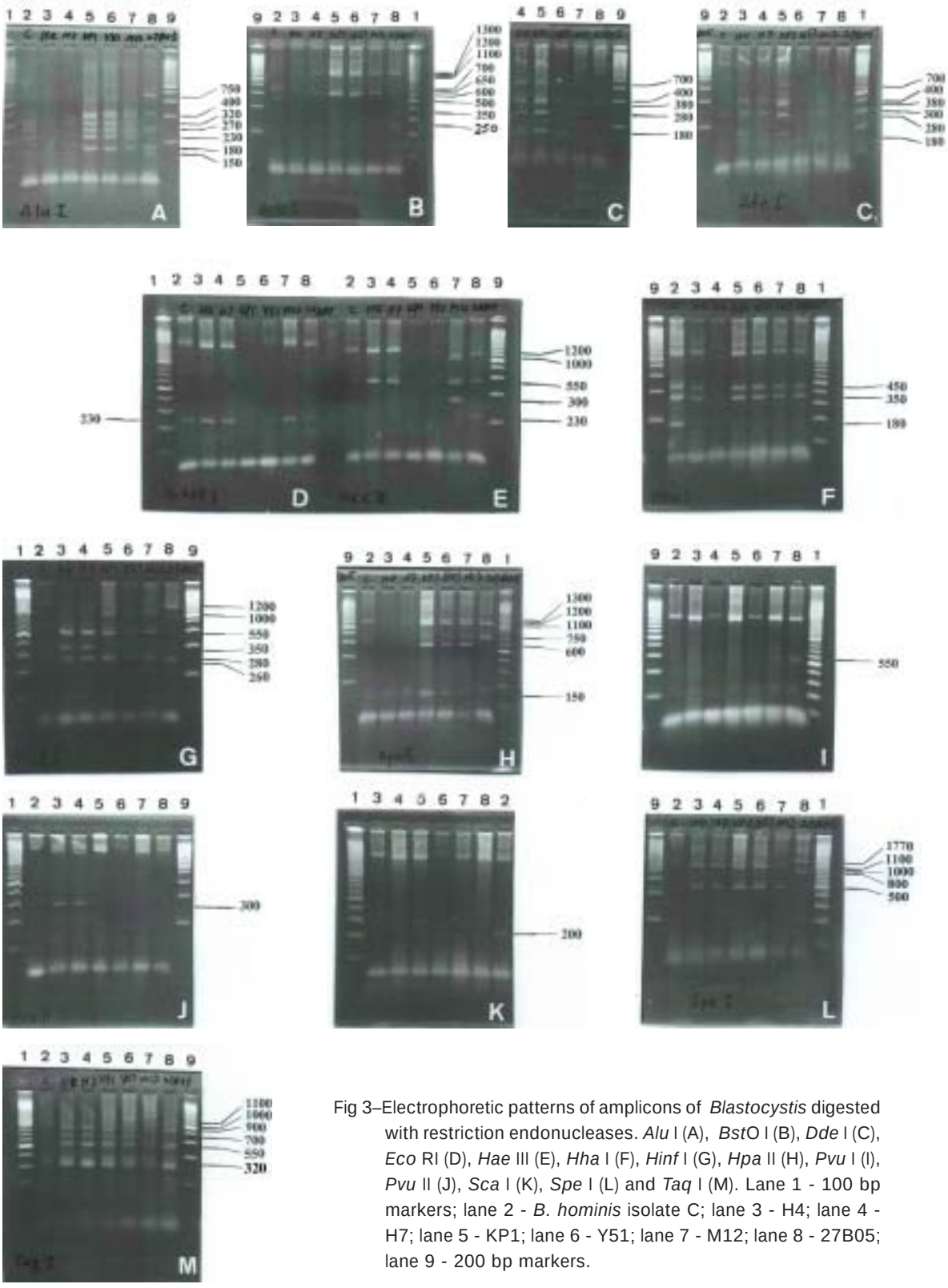

Fig 3–Electrophoretic patterns of amplicons of *Blastocystis* digested with restriction endonucleases. *Alu* I (A), *Bst*O I (B), *Dde* I (C), *Eco* RI (D), *Hae* III (E), *Hha* I (F), *Hinf* I (G), *Hpa* II (H), *Pvu* I (I), *Pvu* II (J), *Sca* I (K), *Spe* I (L) and *Taq* I (M). Lane 1 - 100 bp markers; lane 2 - *B. hominis* isolate C; lane 3 - H4; lane 4 - H7; lane 5 - KP1; lane 6 - Y51; lane 7 - M12; lane 8 - 27B05; lane 9 - 200 bp markers.

extract genomic DNA from *B. hominis,* namely, G-Nome kit, quick-freezing and Triton X-100 extraction.

The amplicons from DNA in 0.1% Triton X-100 solution and from G-Nome kit showed similar purity by electrophoresis. The use of 0.1% Triton X-100 solution to release genomic DNA is simple, fast and cheap and was used in this study. Wilton (1998) had successfully used 0.1% Triton X-100 solution to extract genomic DNA from human buccal cells for amplification in human DNA fingerprinting studies.

As most of the restriction endonucleases used in this study were from old stocks stored at -20ºC, this may have affected their activity. Nevertheless, among the 7 *Blastocystis* isolates studied, isolates C and 27B05 could be distinguished from each other and from the other isolates by *Bst*O I, *Dde* I, *Hinf* I and *Spe* I (Table 2). Isolates H4 and H7 could be distinguished by *Alu* I and *Hha* I, from each other and from the other isolates by the absence of a band. Isolate M12 possessed a unique RFLP pattern with *Taq* I. Isolates KP1 and Y51 could not be differentiated by the 13 restriction endonucleases employed.

The 7 isolates could be classified into four sub-group: isolate C designated as subgroup-1, isolates H4 and H7 as subgroup-2, isolates KP1, Y51 and M12 as subgroup-3, and isolate 27B05 as subgroup-4. Subgroup-1 and -4 concurred with the results obtained by Init *et al* (1999), using a single set of primers (which had the capability of detecting repeated DNA sequence) and designated as Sin-1 and Mal-4 respectively. However subgroup-2 comprised Mal-1 and Mal-2, and subgroup-3 comprised Mal-6 and Mal-7. The similarity of isolate M12 (isolated from the soft stool of a monkey) and isolates KP1 and Y51 (isolated from symptomatic patients) indicates that *B. hominis* not only exist in human host but also in monkey. Abe *et al* (2003) also reported that there are genetic similarity between *B. hominis* isolates from primates and man.

DNA polymorphism of *B. hominis* by amplification of a gene fragment coding for the 16s-like rDNA was reported by Bohm-Gloning *et al* (1997). Amplicons of approximately 850 and 1,100 bp were generated from some of the *B. hominis* isolates. Digestion of these amplicon with *Hinf* I, *Rsa* I and *Alu* I revealed different fragment length profiles, indicating there were 5 *B. hominis* sub-groups, none of which was significantly correlated with the reported disease.

The difference between *B. hominis* isolates from symptomatic and asymptomatic patients was the presence of band of about 180 bp produced by *Alu* I, which only appeared in symptomatic isolates (KP1, Y51 and M12) and isolate 27B05. By using a single set of primers Init *et al* (1999) found a pathogenicity band of 280 bp. These results support the presence of pathogenicity in *B. hominis* and that these two nucleotide sequences may have come from gene(s) governing pathogenicity. Further studies are needed to sequence these nucleotides, determine specific primers, and use them in detecting pathogenic strains of *B. hominis*.

ACKNOWLEDGEMENTS

This study was made possible through financial support from the IRPA Research Grant (06-05-01-0108) of the Ministry of Science, Technology and Environment, Malaysia.

REFERENCES

Abe N, Wu Z, Yoshikawa H. Molecular characterization of *Blastocystis* isolates from primates. *Vet Parasitol* 2003; 113: 321-5.

Bohm-Gloning B, Knobloch J, Walderich B. Five subgroups of *Blastocystis hominis* from symptomatic and asymptomatic patients revealed by restriction site analysis of PCR-am-

plified 16s-like rDNA. *Trop Med Int Health* 1997; 2: 771-8.

Init I, Mak JW, Lokman Hakim S, Yong HS. Strain differences in *Blastocystis* isolates as detected by a single set of polymerase chain reaction primers. *Parasitol Res* 1999; 85: 131-4.

Nguyen XM, Krech T. *Blastocystis hominis*, a parasitic cause of diarrhoea. *Schweizer Med Wochensechrift*. *J Suisse Med* 1989; 119: 457-60.

O'Gorman MA, Orenstein SR, Proujansky R, Wadowsky RM, Putman PE, Kocoshis KA. Prevalence and characteristics of *Blastocystis hominis* infection in children. *Clin Pediatr (Philadelphia)* 1993; 32: 91-6.

Silberman JD, Sogin ML, Leipe DD, Clak CG. Human parasite finds taxonomic home. *Nature* 1996; 380: 398.

Tachibana H, Ihara S, Kobayashi S, Kaneda Y, Takeuchi T, Watanabe T. Differences in genomic DNA sequence between pathogenic nonpathogenic isolates of *Entamoeba histolytica* identified by polymerase chain reaction. *J Clin Microbiol* 1991; 29: 2234-9.

Wilton S. Workshop on advance methods in molecular biology I: DNA typing and mutation detection. Kuala Lumpur: Universiti Kebangsaan Malaysia, 1998.

Yoshikawa H, Nagano I, Yap EH, Singh M, Takahashi Y. DNA polymorphism revealed by arbitrary primer polymerize chain reaction among *Blastocystis* strains isolated from humans, a chicken, and a reptile. *Int J Microbiol* 1996; 43: 127-30.