Restriction enzyme digestion of native and PCR-amplified DNA of an *Acanthamoeba castellanii* isolate

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Abstract

This study attempted to verify the validity of using restricrion fragment length polymorphism (RFLP) patterns of the genomic DNA and polymerase chain reaction (PCR) products of *Acanthamoeba castellanii* to assist in the identification and classification of rhe parasite. Both rhe genomic DNA and the PCR products of the *A. castellanii* using a genus-specific set of primers A3 (TCCCCTAGCAGCTTGTG) and A4 (GTTAAGGTCTCGTTCGTTA) were digested using 27 restriction endonucleases. The patterns of the digested products were analyzed by electrophoresis on agarose gel. The RFLP patterns of the genomic DNA showed different banding patterns with only enzyme *Sma* I, which did nor digest the DNA. The restriction endonuclease-digesrion patterns of the PCR products showed thar 4 restriction endonucleases; *Alu* I, *Bst*N I, *Hinf* I and *Msp* I digested the DNA. Our results showed that the RFLP patterns of rhe genomic DNA and the PCR products may be of use in identifying and classifying rhe *A. castellanii* isolate that we have.

Key Words: *Acanthamoeba castellanii*, genomic DNA; restriction fragment length polymorphism; polymerase chain reaction

Introduction

In recent years medical attenrion has focused on a small, free-living amoeba which causes severe, sight-threatening keratiris. The amoeba, *Acanthamoeba* causes a painful inflammation of the cornea that can lead to blindness if treatment is delayed. Affected persons are commonly soft contact lens users with unhygienic practices (Bacon *et al.*, 1993; Horne *et al.*, 1994). Cases of *Acanthamoeba* keraritis have been reported in Europe, Africa, Israel, Japan, India, Taiwan, Australia, and North and South America. Since the first reported case two decades ago, the number of cases worldwide has increased. As it is nor a reportable disease, the true incidence is not known and may be higher (Visvesvara & Srehr-Green, 1990). In Malaysia, the first reported case was in 1996 involving a woman who had a long history of using contact lenses. She had accidenrly used an old spare lens, which was probably contaminated (Mohamed Kamel & Norazah, 1996). The clinical presentation of rhe infection is often mistakenly diagnosed as herpes simplex infection and in the presence of secondary bacteria infection, the aetiology of rhe keratiris may be missed.

Acanthamoeba keratitis is difficult to treat even with the availability of modern drugs. Among immunocompromised patients, such as diabetics and alcoholics, ir can cause a chronic, subacute encephalitis, often with granuloma formation, termed Granuloma Amoebic Encephalitis (GAE) (Visvesvara & Stehr-Green, 1990). Restriction fragment length polymorphism (RFLP) is a useful method for rhe typing of viruses, bacteria, bacterial plasmids, and eukaryoric pathogens. It has also been used to trace the origin of pathogenic isolates as well as clarifying phylogenic relationships among closely-related organisms. (McLaughlin *et al.*, 1988). McLaughlin and coworkers studied the restriction endonuclease digestion patterns of selected *Naegleria* and *Acanthamoeba* amoebae using *Bgl* II, *Hin*d III, and *Eco*R I. They found that each species displayed a characteristic pattern of repetitive restriction fragments. Digestion of the *Acanthamoeba* species showed fewer fragments than those of the *Naegleria*species (McLaughlin *et al.*, 1988). The mitochondrial DNA's RFLP patterns of eight isolates of *Acanthamoeba* with six restriction endonucleases, namely *Eco*R I, *Bgl* II, *Hin*d III, *Hpa* I, *Sca* I, and *Xba* I, have been studied and 4 distinct mtDNA RFLP phenotypes were discovered for the eight strains (Yagita & Endo, 1990). Three strains morphologically classified as *A. polyphaga* shared a single RFLP phenotype with the Ma strain of *A. castellanii* indicating thar both might be a single species complex (Yagira & Endo, 1990). In another study, 32 morphologically identical strains of *Acanthamoeba* were digested with *Bgl* II, *Eco*R I, and *Hin*d III and the resulting RFLP patterns differentiated the srrains into seven multiple-strain and three single-strain groups (Kilvington *et al.*, 1991). Although the RFLP technique is a useful tool for the differentiation of morphologically-identical *Acanthamoeba* strains, other methods for identifying and classifying *Acanthamoeba* are still needed. The combination of RFLP and polymerase chain reaction (PCR) appears to

be a logical approach. The use of PCR can increase the sensitivity for the detection of parasites in a specimen while the RFLP of the PCR product may be very diagnostic for the parasite being studied. We have therefore, used this approach in our study of an *A. castellanii* isolate, using 27 restriction endonucleases to digest both its genomic DNA and PCR products.

Materials and Methods

The original culture of *A. castellanii* was obtained from the Parasitology Unit of the Department of Microbiology, National University of Singapore from Prof. Mulkit Singh. The parasites were cultured *in vitro* in mycological peptone medium (4%) in 25 mm^3 culture flask (Corning, USA) at room temperature and subculrured every 3 days. Subculture was carried out by placing the culture flask on ice for 10 minutes and then flushing the wall of the culture flask with pasture pipette to detach the adherent parasites (trophozoites) from the walls of the flask and the conrents were transfered into a centrifuge tube. After cenrrifugation at 1,900 g for 10 minutes at 4°C the culture medium was removed and the parasite pellet washed twice with cold sterile phosphate buffered saline (PBS) at 1,900 g for 10 minutes at 4°C.

Extraction of genomic DNA of *A. castellanii* was carried out using a modified method of Riley *et al.* (1991). In this method, the parasite pellet was washed twice with PBS, resuspended in Triton X-100 in reticulocyre standard buffer solution (1ml 1% Triton X-100, 10mM Tris, 10mM NaCl, and 5mM $MgCl_2$), and centrifuged at 1,900 g for 10 minutes at 4°C. The supernatant was then discarded. Five ml of lysis buffer (2% SDS, 10mM Tris HCl pH 8.0, 150mM NaCl, 10mM EDTA) and Proteinase K (500 µg/ml) were added to the pellet. The suspension was incubated at 37°C for 1 hour. Fifty µl of RNAse (100 µg/ml) were added to the suspension which was then incubated at 37°C for 1 hour. The standard phenol/chloroform method for the extraction of DNA was carried out. Precipitation of DNA was then carried out by adding 1/10 volume with 3M sodium acetate (pH 5.2) and 2.5 volume of absolute alcohol to the suspension. The solurion was then incubared overnight at -20°C. Aftet incubation, the solution was centrifuged at 7,000 g for 15 minutes at 4°C. The supernatant was then removed without disturbing the pellet. The pellet was washed with 500 µl of 70% alcohol and centrifuged at 7,000 g for 15 minutes at 4°C. The supernatant was carefully removed using a pipette and the pellet dried under vacuum for 10 minutes. Once dried, the pellet was resuspended in 50 µl of TE buffer pH 8.0 (0.01M Tris-Cl, 0.001M EDTA 8.0). The DNA was then electrophoresed on 2% agarose gel in TBE (Tris-borate EDTA) buffer at 100 volts for 30 minutes. Determination of the content and purity of DNA was determined by spectrophotometry (UV-VIS spectrophotometer, Shimadzu). The size of the genomic DNA was more than 12,216 bp.

Polymerase chain reaction of *A. castellanii* was cartied our using primers based on the study of Vodkin *et al.* (1992). These primers, A3 and A4, were found to be genus-specific for *Acanthamoeba* and have the following nucleotide sequences: Primer A3: TCCCCTAGCAGCTTGTG, Primer A4: GTTAAGGTCTCGTTCGTTA. A 50-µl reaction mixture was set up in a MicroAmp tube (Perkin Elmer, USA) consisting of 0.5 µg DNA, 10x buffer magnesium-free, 2mM of $MgCl_2$, 200µM of deoxynucleotide triphosphate mix (dNTP mix, Boehringer Mannheim, Germany), 20 pmole each of primers A3 and A4, 2.5 unit of Taq DNA polymerase (Promega, USA). The reaction mixture was briefly centrifuged at 500 g for 10 seconds and then amplified by using the GeneAmp PCR sysrem 2400 (Perkin Elmer, USA). The PCR was performed wirh the following temperature cycle: an initial 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 49°C for 1 minute, and primer extension at 72°C for 1 minute. A final elongation step ar 72°C for 5 minutes was also done. The amplified product was then analyzed using 2% agarose gel electrophoresis at 100 volts for 30 minutes and visualized under ultraviolet light. The expected PCR product was 272 basepairs in size.

Restriction enzyme digestion was done on both the genomic DNA and the PCR product of the *A. castellanii.* Twenty seven restriction enzymes (New England Biolabs USA) were used in this study (Table 1). The buffer for each enzyme was provided by the manufacturer and some enzymes were supplemented with bovine serum albumin (BSA). For the digestion reaction of genomic DNA; a reaction mixture consisting of 7.5 µg DNA and 3 units of restriction enzyme per µg of DNA was mixed in an eppendorf microcentrifuge tube together with rhe corteponding 10x buffer, BSA for some enzymes, and enough millipore water to make the final reaction volume of 10µl. The mixture was then incubated overnight at the enzyme's optimal temperature. Electrophoresis was carried out at 30 volts for 5 hours (4 volts/cm of gel) and the gel was stained with ethidium bromide (1 µg/ml for 1 hour). The digestion patterns of the genomic DNA was visualized under ultraviolet light and photographed for permanent record. Digestion reaction for the PCR products was similarly prepared and the electrophoresis was carried out in 2% agarose gel (with 2 µl ethidium bromide) at 100 volts for 30 minutes. 1 Kb Ladder Marker (Pharmacia) was used as DNA marker. The digestion pattern was then visualized under ultraviolet light and photographed.

Results

All enzymes except one digested the genomic DNA. Three enzymes could only partially digest it (Table 1).

Table 1. Restriction digestion of *A. castellanii* using 27 restriction endonucleases.

ENZYMES	Genomic DNA	PCR Product
Alu I	D	D
BamH I	P	N
Bgl I	D	N
BstN I	D	D
Dra I	P	N
EcoR I	D	N
EcoR V	D	N
Hae II	D	N
Hae III	D	N
Hha I	D	N
Hinc II	D	N
Hind III	D	N
Hinf I	D	D
Hpa I	D	N
Hpa II	P	N
Kpn I	D	N
Msp I	D	D
Pst I	D	N
Pvu II	D	N
Sac I	D	N
Sal I	D	N
Sau3a I	D	N
Sca I	D	N
Sma I	N	N
Taq I	D	N
Xba I	D	N
Xho I	D	N

N = Not digested
D = Digested
P = Partially Digested

As seen in Fig. 1, *Msp* I cleaved the genomic DNA in 8 to 9 places starting at 5,090 bp and ending at above 506 bp with DNA smearing towards the end of the lane. *Kpn* I gave two very distinct bands above the 12,216 bp mark, while *Hinc* II gave about 9 to 10 closely-spaced bands. After an initial restriction site at 10,180 bp, the next 8 bands of *Hae* III were evenly-spaced from 1018 to 3,054 bp, with heavy smearing towards the end. *Hpa* I gave 5 indistinct bands, but the 5 bands of *Sal* I were quite distinct. *Eco*R V had 4 bands with still heavy DNA concentration at the top of the lane.

Alu I completely cleaved the genomic DNA with no bands seen (Fig. 2). *Dra* I-digested DNA also showed no distinct banding pattern, but the DNA concentration at the top of the 12,216 bp mark was very sharp. *Xba* I gave 4 bands at 7,126 bp, 4,072 bp, 3,054 bp, and about 1,636 bp. *Hinf* I had no distinct band but showed DNA smearing starting from 2,036 bp downwards. *Xho* I gaves 2 to 3 bands, but DNA could still

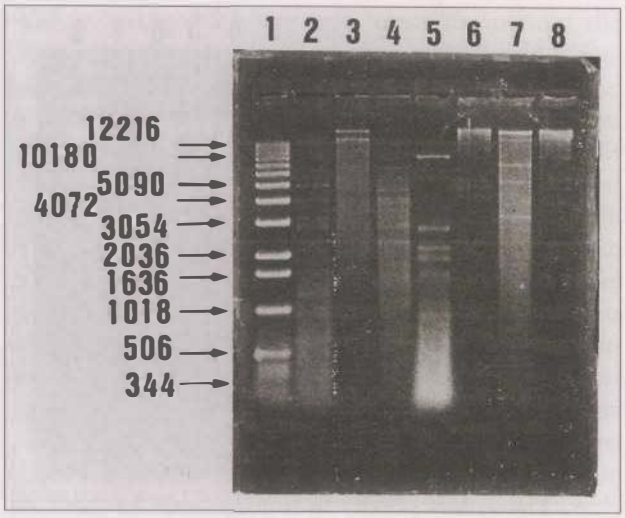

Fig. 1. Electrophoretic patterns of the genomic DNA of *A. castellanii* digested with enzymes: *Msp* I (lane 2), *Kpn* I (lane 3), *Hinc* II (lane 4), *Hae* III (lane 5), *Hpa* I (lane 6), *Sal* I (lane 7) and *Eco*R V (lane 8), 1 Kb DNA laddet matker (lane 1).

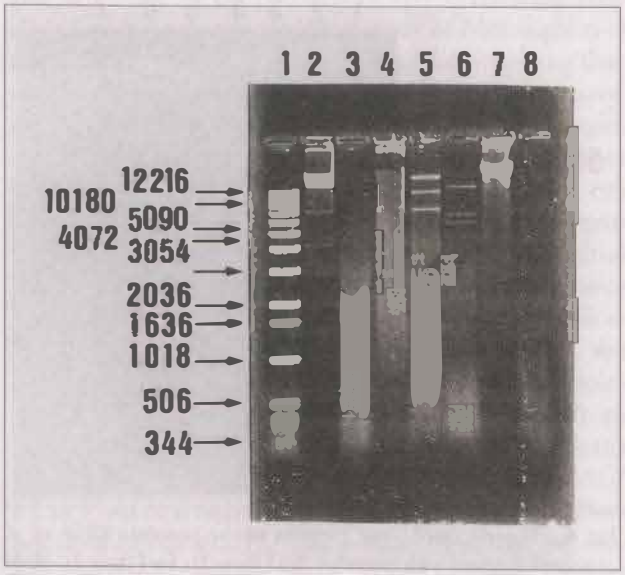

Fig. 2. The electrophoretic patterns of the genomic DNA of *A. castellanii* digested with enzymes: *Xba* I (lane 2), *Hinf* I (lane 3), *Xho* I (lane 4), *Hae* II (lane 5), *Hha* I (lane 6), *Dra* I (lane 7), and *Alu* I (lane 8). 1 Kb DNA ladder marker (lane 1).

be seen near the well. *Hae* II had 8 and *Hha* I had 3 to 6 distinct bands.

Hpa II and *Bam*H I produced only a sharp band with some DNA smearing above the 12,216 bp mark (Fig. 3). *Eco*R I also showed the same band but in addition had 3 bands between 5,090 bp and 10,180 bp. *Hind* III gave 13 distinct bands with heavy DNA smearing at the start of the lane. *Bgl* I gave 5 bands and with some smearing while *Pst* I had 3 bands, with the first 3 bands of both enzymes almost identical in location. *Pvu* II gave 5 hazy bands between 4,072 and 12,216 bp. *Sma* I did not digest the genomic DNA, while *Bst*N I and *Sau3a* I completely digested it (Fig. 4). *Taq* I only

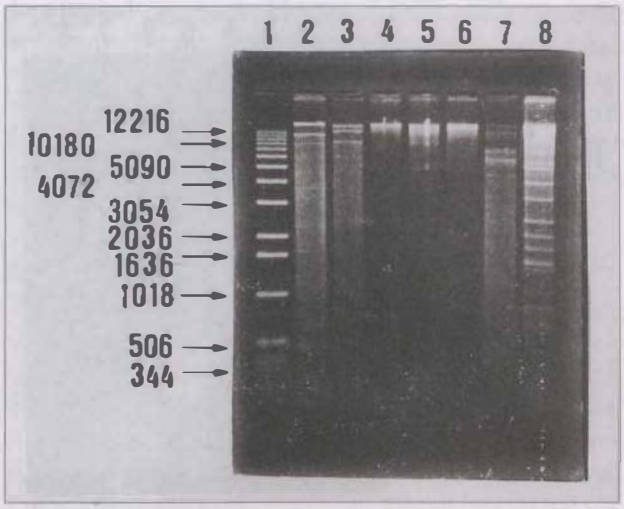

Fig. 3. The electrophoretic patterns of the genomic DNA of *A. castellanii* digested with enzymes; *Bgl* I (lane 2), *Pst* I (lane 3), *Hpa* II (lane 4), *Eco*R I (lane 5), *Bam*H I (lane 6), *Pvu* II (lane 7) and *Hind* III(lane8); 1 Kb DNA ladder marker (lane 1).

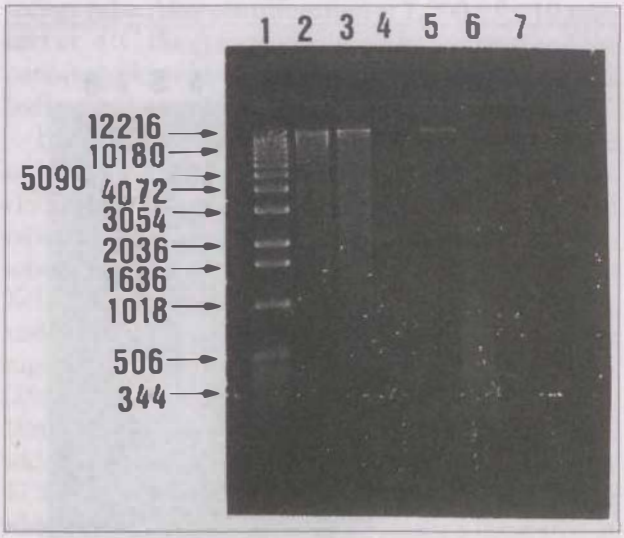

Fig. 4. The electrophoretic patterns of the genomic DNA of *A. castellanii* digested with enzymes; *Sca* I (lane 2), *Sac* I (lane 3), *Bst*N I (lane 4), *Sma* I (lane 5), *Taq* I (lane 6) and *Sau3a* I (lane 7); 1 Kb DNA ladder marker (lane 1).

showed light smearing with no bands throughout the lane. *Sca* I gave about 9 to 10 bands , while *Sac* I had only a single band at 2,036 bp.

Of these enzymes, only 4 could digest the PCR products (Table 1). *Eco*R I, *Pvu* II, and *Pst* I did not digest the PCR product while *Alu* I digested it into 2 distinct bands (Fig.5). *Kpn* I, *Hinc* II, *Hae* III, *Hpa* I, *Sal* I, *Eco*R V, *Xba* I, *Xho* I, *Hae* II (Fig. 6), *Bgl* I, *Hpa* II, *Bam*H I, *Hind* III, *Hha* I, *Dra* I, *Sca* I , *Sac* I, *Sma* I, *Taq* I, and *Sau3a* I (Fig. 7) did not digest the PCR products. *Bst*N I could digest the PCR product into 3 bands at 201 bp, 154 bp, and 134 bp respectively (Fig. 8).

Discussion

In our study, lowering the agarose gel concentration

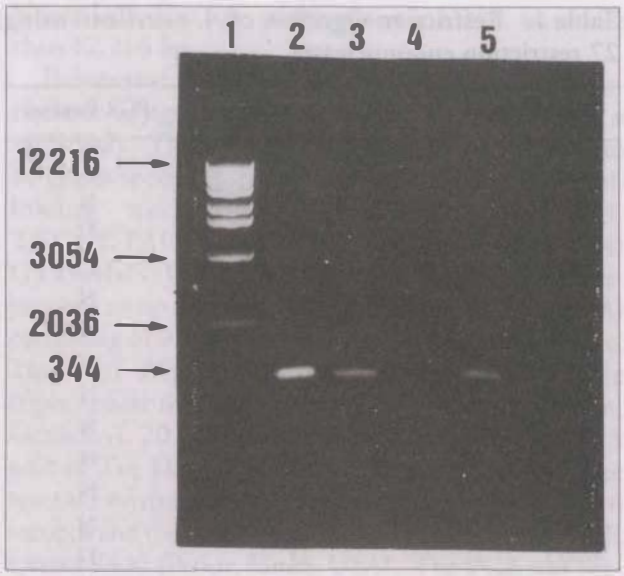

Fig. 5. The electrophoretic patterns of the A3 and A4 PCR product *A. castellanii* digested with enzymes *Eco*R I (lane 2), *Pvu* II (lane 3), *Alu* I (lane 4) and *Pst* I (lane 5); 1 Kb ladder marker (lane 1).

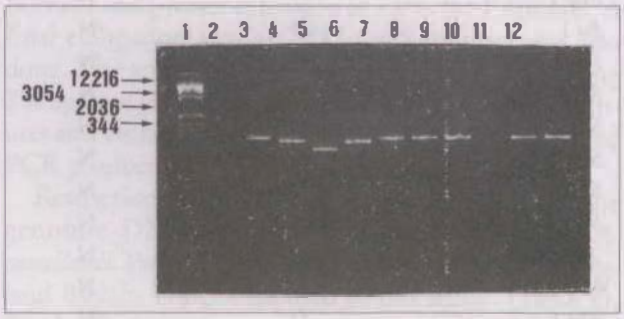

Fig. 6. The electrophoretic patterns of the A3 and A4 PCR product of *A. castellanii* digested with enzymes *Msp* I (lane 2), *Kpn* I (lane 3), *Hinc* II (lane 4), *Hae* III (lane 5), *Hpa* I (lane 6), *Sal* I (lane 7), *Eco*R V (lane 8), *Xba* I (lane 9), *Hinf* I (lane 10), *Xba* I (lane 11), and *Hae* II (lane 12); 1 Kb ladder marker (lane 1).

(0.75%), increasing the amount of restriction enzymes (3 unit of enzyme per μg of DNA), lowering the voltage of the electrophoresis (4 voltage per cm of gel), and omitting the ethidium bromide during the gel run, gave good RFLP patterns for all the enzymes. McLaughlin *et al.* (1988) stated that gentle mixing during DNA isolation, sufficient restriction enzymes, low voltage during electrophoresis, and omission of ethidium bromide from the gel gave better resolution and intensity of bands. For most bacteria and eucaryotes, the banding patterns are too complex for easy analysis. However for some eukaryotes, these sequences, particularly the repetitive ones act as good markers for strain characterization. The discovery of four distinct RFLP phenotypes for the various strains of *A. castellanii* in the study of Yagita & Endo (1990) is proof for DNA sequence diversity within a species . Our results showed that each enzyme gave different banding patterns. This is expected because each enzyme has its own specific recognition

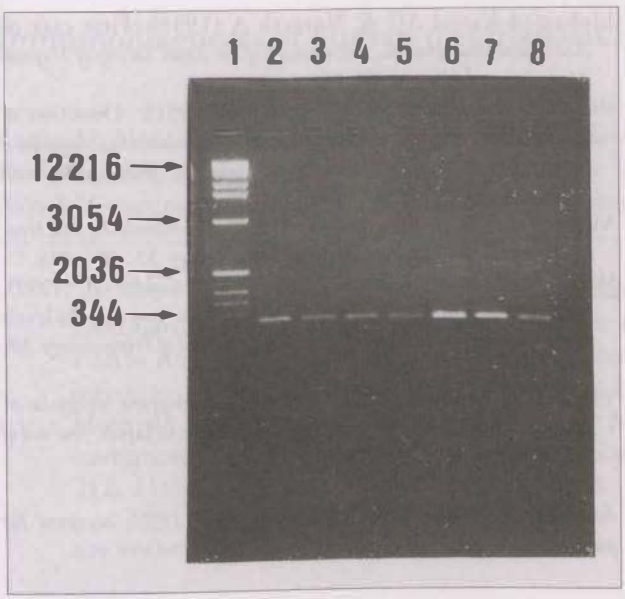

Fig. 7. The electrophoretic patterns of the A3 and A4 PCR product *A. castellanii* digested with enzymes *Bgl* I (lane 2), *Hpa* II (lane 3), *Bam*H I (lane 4), *Hin*d III (lane 5), *Hha* I (lane 6), *Dra* I (lane 7) and *Sca* I (lane 8); 1 Kb ladder marker (lane 1).

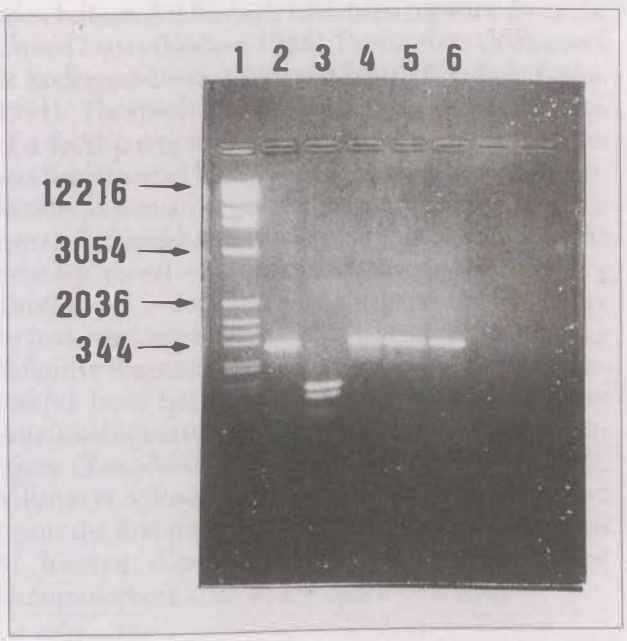

Fig. 8. The electrophoretic patterns of the A3 and A4 PCR product of *A. castellanii* digested with enzymes *Sac* I (lane 2); *Bst*N I (lane 3), *Sma* I (lane 4), *Taq* I (lane 5) and *Sau3a* I (lane 6); 1 Kb ladder marker (lane 1).

sire. However, our findings showed that although both *Msp* I and *Hpa* II had the same recognition sire, their RFLPs were different. The reason for rhis is nor clear. Although both had the same recognition sequence, they were nor isoschizomers. Perhaps, the adjacent nucleotides also determine whether the enzyme can digest a particular sequence. *Alu* I, *Bst*N I, and *Sau3a* I showed complete digestion of the genomic DNA. This suggests rhar repetitive sequences corresponding to rhe enzyme's recognition site are present rhroughour the DNA. On the other hand, the enzymes *Bam*H I, *Dra* I, and *Hpa* II still showed almost-intact genomic band at the beginning of the lane, implying that there was near-toral absence of their recognition sequences in this *Acanthamoeba* isolate or that methylation had occured in these sires. *Sma* I had no recognition sequence at all. *Hin*d III, gave distinct and sharp bands. McLaughlin *et al.* (1988) also noted thar the *Acanthamoeba* species gave fewer, less intensely staining repetitive fragments when compared with those of *Naegleria* species. Comparison of the RFLP patterns of our isolate to patterns demonstrated by several *Acanthamoeba* species in other studies revealed differences. Although rhe bands of *Hin*d III of our isolate appear similar to those of group C in the study of Kilvington *et al.* (1991) and *Bam*H I only gave genomic smearing in both their and our study, we still believe that our isolate was different from theirs when we tested our isolate with other enzymes and showed a different pattern. Comparison of our isolate also with the RFLP patterns of the *Eco*R I-digested *Acanthamoeba* species from the study of McLaughlin *et al.* (1988) revealed no similarities, further implying that our isolate is of a different strain. We also compared our RFLP patterns with those from the study of Yagita & Endo (1990). In their study, the RFLP phenotypes of 8 *Acanthamoeba* isolates obtained from human eye infections, contact lens containers, and soil in Japan were demonstrated by digesting their mitochondrial DNA with 6 restriction enzymes. Although our source of DNA was different, the comparison is still applicable because according to Kilvington *et al.* (1991), the restriction enzyme digestion of *Acanthamoeba* whole-cell DNA gives rise to similar RFLPs originating from the mitochondrial DNA. Surprisingly, our isolate's RFLP patterns appear similar to the phenotype JAC/E1 in 5 out of 6 enzymes used (one enzyme, *Bgl* II, was not used in our study). The possibility is that our isolate has similar genetic make-up and probably belongs to the same phenotype. As anticipated, most enzymes did not digest the small, 272-bp PCR product any further. However, four restriction enzymes, namely *Alu* I, *Bst*N I, *Msp* I, and *Hin*f I, cleaved the products into 2 to 3 distinct bands. For *Alu* I and *Bst*N I, this is not surprising because, as noted above, these 2 enzymes also completely digested the genomic DNA. As the four restriction enzymes gave different banding patterns, the possibility of using the RFLPs of the PCR products for identification appears promising. However, further studies on other isolates of *Acanthamoeba* and closely related organisms must be carried out to determine the validity of using the RFLPs of the PCR product as an aid in classifying and identifying *Acanthamoeba*.

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