[BIO41] Application of PCR-RFLP technique and direct PCR-sequencing analysis to determine the relationships of *Acanthamoeba*

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Introduction

The genus of *Acanthamoeba* comprises of several free-living amoebae and mostly found in environment habitats worldwide. These organisms can infect a variety of mammals, including humans involving brain, eyes, skin, bones and lungs (Armstrong, 2000; Martinez, 1999). The taxonomy of *Acanthamoeba* is has yet to be established and still under review, although species identification of the genus by cyst morphology has been extensively used (Page, 1967). The high variability of the morphology within a clone, limited the availability of the morphology alone as a taxanomic tool (Visvesvara, 1991). New, refined and reproducible methods have been applied to resolve the taxanomy problems.

The RFLP analysis was recently applied in subgenus classification of *Acanthamoeba,* but the result were highly polymorphic among closely related strains (Chung, *et al*., 1998; Kong and Chung, 1996). Comparison of high conserved sequences which have a central function like SSU rDNA gene can reveal phylogenetic relationships of organism. PCR-RFLP analysis of SSU rDNA is a simpler and less expensive substitute for sequence analysis (Yu, *et al*., 1999). Until 1977, determining the sequence of bases in DNA sequencing was a laborious process which could only be applied to small molecules within a cloning (Innis, *et al*., 1990). But nowdays, direct DNA sequencing of PCR products permits the rapid characterization of sequences of interest without the need for subcloning (Newton and Graham,, 1997; Innis, e*t al*., 1990; Biggin *et al*., 1983). The direct PCR Sequencing is the recent systematic studies that provided a new classification of the genus *Acanthamoeba*. The sequence data are very useful for identification and differentiation (Yu, *et al*., 1999; Stothard, *et al.*, 1998). The present study was conducted to evaluate the PCR-DNA based techniques as a tool to distinguish relationships of *Acanthamoeba* spp.

Material and methods

Samples and strains

Three isolates of *Acanthamoeba* and two reference strains from various sources were used in this study. They were labelled as isolate AC, AK, and AP. The reference strains, ACC (*Acanthamoeba castellanii* CCAP 1501/2A) and APC (*Acanthamoeba polyphaga* CCAP 1501/3A) were obtained from Culture Collection of Algae and Protozoa (CCAP, UK). The isolate AC is expected species of *Acanthamoeba castellanii* and a clinical isolate, obtained from Institute of Medical Research, Kuala Lumpur. The isolates were grown in axenic medium, Polypeptone medium at 25^{o}C. Isolate AK is unknown species that isolated from human corneal scrapings of keratitis patients and supplied by Department of Ophthalmology, Hospital of Kuala Lumpur and isolate AP was isolated from the inner side of a water tap. The isolation of the amoebae was done following "cotton swab' technique employed by Devonnshire *et al.* (1990). All the isolate were identified morphologically as *Acanthamoeba* and growth for cultivation (Nakisah and Noriza, 2003; Page, 1988). While strain HV (*Hartmannella vermiformis* CCAP 1534/7B*)* from CCAP, UK was used as a reference negative control.

DNA extraction and PCR Amplification

Samples of *Acanthamoeba* spp. in the logarithmic phase of growth were taken three to four days old after cultivation. The trophozoites phase of the amoebae harvested by centrifugation at 3000 x g for 15 minutes. Pellet of isolates of *Acanthamoeba* (containing 7 x 10^6 to 2 x 10^7) then was washed twice with

Phosphate buffer saline (pH 7.4) before DNA extraction was done using modified Wizard® Genomic Purification System from Promega Corporation (Sambrook, *et al.,* 1989).

A total of five *Acanthamoeba* isolates were amplified using the primers pair FP16-RP16 which is complementary to the 5' and the 3' of the gene, respectively. The SSU rDNA genes of *Acanthamoeba* isolates were identified using standard genus-specific PCR amplification technique. The specific PCR was performed in 25 µl volumes containing 1.5 mM $MgCl_2$, 1X PCR Buffer, 200 µM dNTPs, 0.625 u Taq, 1.0 µM upstream and downstream primers, and 0.5 ng template DNA. A standard amplification program used was amplified the DNA during 35 cycles, each cycle consisting of initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, and chain elongation with *Tag* polymerase at 72°C for 2 min. The primer extension phase was prolonged for 5 minutes at 72°C in the last cycle. The reactions amplification was carried out in a 100-PTC Thermal Cycler (MJ research Inc., USA).

RFLP Digestion and data analysis

Six kinds of Restriction endonuclease enzymes *Dde* I*, Hae* III*, Hind* III*, Hinf* I*, Mbo* I and *Taq* I (Promega corporation, USA), which have recognition sequences of four nucleotides, were used to generate comparative riboprints. Each amplified DNA (1 µg) was digested with 5-10 units of the enzymes in a 20µl reaction mixture containing buffer supplied by the manufacturer (Promega, USA). An analytical scale restriction enzyme digest is usually performed in a volume of 20µl on 0.2 – 1.5µg of DNA. The standard of a typical RE digestion were done followed by manufacturer instructor. The mixture tube then centrifuged for a few seconds and incubated at 37°C (except for *Taq* I - 67°C) for 2 hours.

The digested PCR products then were fractionated on 2.5% agarose gel in TBE electrophoresis buffers. The gel stained with Ethidium Bromide before visual under UV transilluminator and photographed. The sizes of the band presence were determined directly with a standard 100bp DNA ladder (Promega, USA). The gels were analyzed by visual inspection considering all visible bands. Data from the analyses were scored as discrete variables. The unweighted pair group method with arithmetic or UPGMA method was used to represent the similarity relationships among the varieties as a dendrogram according to Nei (1972). The clustering correlations were calculated by unweighted pair group method by SAHN (sequential, agglomerative, hierarchical, and nested) of NTSYS-PC version 1.7 (Applied Biostatics) (Rohlf, 1994).

PCR product purification and sequencing analysis

The amplification of PCR indicated with two primers pairs, FP16-RP16 and CRN5-1137 were done using standard genus-specific PCR amplification technique. The standard program was carried out in 100-PTC Thermal Cycler (MJ research Inc. USA). The PCR products were resolved on 1.2% agarose gel in TBE buffer before stained in Ethidium Bromide and followed by visualized under UV transilluminator to confirm the size of single band. The marker used as a standard for fragment sizing was a 1Kb DNA ladder (Promega, USA). The gene product purified using Wizard® PCR Preps DNA Purification System from Promega Corporation, USA.

The purified *Acanthamoeba* spp. were directly sequenced using an ABI PRISM Dye Terminator cycle sequencing Ready Reaction kit and ABI PRISM Dye Primer cycle sequencing Core kit (Perkin Elmer, California, USA). The primers used were synthesized and supplied by Genosys Biotechnologies Inc., USA. The sequence of SSU rDNA were determined by a DNA Sequencer (Model 373 A, Applied biosystems).

Sequencing data analysis

The SSU rDNA sequences were determined in this study and those from the databases were aligned by the clustal W program version 1.6 (Thompson, *et al*., 1994). Continuous stretches of 1,550 bp and 2,000 bp nucleotide ranges from position 50 bp to 1,500 bp were used for analysis. The aligned sequences were compared with sequences present in GenBank (EMBL, USA).

Results

Specific-PCR amplification

A total of five isolates of *Acanthamoeba* were identified by using genus-specific primers pair, FP16-RP16. The results of this PCR approach are shown in Figure 1(a). All the reference strains, ACC (*Acanthamoeba castellanii* CCAP 1501/2) and APC (*Acanthamoeba polyphaga* CCAP 1501/3A) and a new isolates of AC, AK and AP produced a strong and defined band of approximately 1,550 bp. But for the negative control of strain HV (*Hartmannella vermiformis* CCAP 1534/7B) were obtained no corresponding banding appeared.

PCR-RFLP fragments analysis

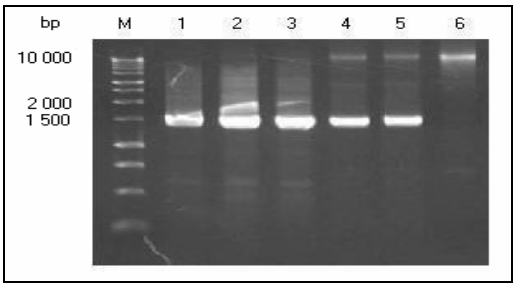
Five isolates of *Acanthamoeba*, including two strains from CCAP, UK were fingerprinted using Restriction Fragment Length Polymorphism (RFLP) technique to determine the genetic relatedness among the isolates tested. In this study, six restriction endonuclease enzymes were used to generate comparative riboprints fragment and estimated the genetic distances. Proportions of co-migrating fragments between *Acanthamoeba* isolates using these enzymes are shown in Figure 2. The fingerprints pattern indicated from restriction enzymes digestion yielded multiple DNA fragments ranging from 50 bp to 1,500 bp.

The fingerprints pattern of enzymes *Hind* III and *Hinf* I shown in Figure 2(a). From the DNA patterns digested with *Hind* III shows extremely similar for three isolates of ACC, APC and AC, which only three bands present in all three isolates (Figure 2(a); Lane 1-3). AK isolate also revealed a presented of three bands but in a different of sizes (Figure 2(a); Lane 4). While for AP isolate, six bands were presented in the DNA pattern (Figure 2(a); Lane 5). Although isolates AK and AP are shown slightly different from the others, there are one band that present in all five isolates tested, the size of the band approximately 350 bp (Figure 2(a); Lane 1-5).

Riboprint patterns of five isolates of *Acanthamoeba* using restriction enzyme *Hinf* I shown in Figure 2(a); Lane 6-10. The DNA fragments revealed the present of three bands in all the isolates tested, accept for AP isolate (Figure 2(a); lane 10). The three sizes of identical bands present in all five isolates are 950 bp, 400 bp and 100 bp approximately (Figure 2(a); Lane 6-9). While there is another one band present in AP isolate at size approximately 50 bp (Figure 2(a); Lane 10). Although the isolate AP patterns slightly differ from the others, the DNA patterns show minor differences.

In the panel of *Dde* I, *Hae* III and *Taq* I fragmentation profile shows an identical DNA patterns revealed in all the isolates tested (Figure 2(b) and Figure 2(c); Lane 1-5). While the RFLP profile digested with *Mbo* I enzyme shows there are four identical DNA pattern bands present in all the isolates tested, accept for AP isolate (Figure 2(c); Lane 10). For AP isolate, there is another one band present at molecular size of 50 bp approximately (Figure 2(c); Lane 10).

(a)



(b)

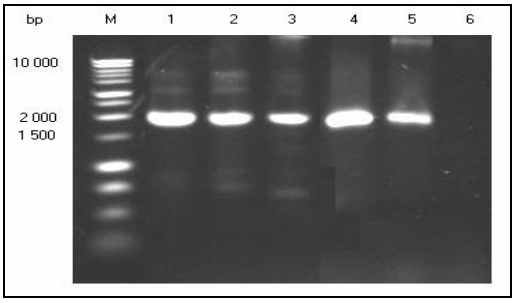
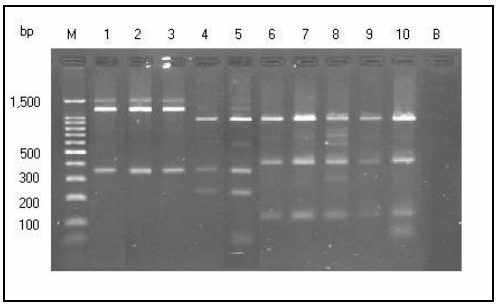
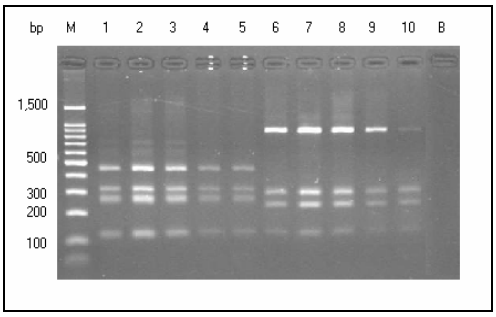


FIGURE 1 Genus specific-PCR amplification, (a) Primer FP16-RP16 (b) Primer CRN5-1137. Lane 1: Strain ACC; Lane 2: Strain APC; Lane 3: Isolate AC; Lane 4: Isolate AK; Lane 5: Isolate AP and Lane 6: Isolate HV. Lane M: 1Kb DNA ladder.

(a)



(b)



(c)

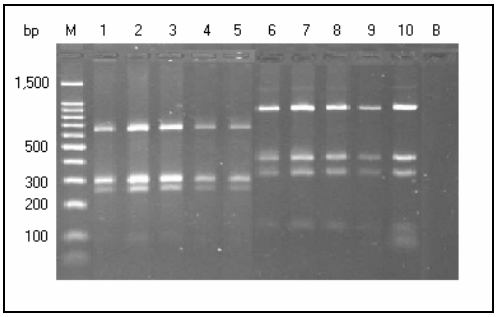


FIGURE 2 RFLP profile of five isolates of *Acanthamoeba* obtained with restriction enzyme, (a) *Hind* III*, Hinf* I (b) *Dde* I*, Hae I*II (c) *Taq* I*, Mbo* I. Lane 1: Strain ACC; Lane 2: Strain APC; Lane 3: Isolate AC; Lane 4: Isolate AK; and Lane 5: Isolate AP digested with *Hind* III*, Dde* I, and *Taq* I. Lane 6: strain ACC; Lane 7: Strain APC; Lane 8: Isolate AC; Lane 9: Isolate AK and Lane 10: Isolate AP digested with *Hinf* I, *Hae* III and *Mbo* I. Lane M: 100bp DNA ladder. Lane B: Blank/Control

Analysis of homology cluster of PCR-RFLP

The estimated genetic distance between *Acanthamoeba* isolates are shown in Figure 3. The cluster tree showed a significant genetic diversity among the isolates tested. The cluster was constructed based on RFLP profile data from six restriction enzymes digestion between five *Acanthamoeba* isolates tested. From the cluster analysis showed the existence of two distinctive groups at level 0.150, with strain ACC, APC and isolate AC forming one group (Figure 3; Cluster 1) and isolate AK and AP falling into another one group (Figure 3; Cluster 2). The phylogenetic tree indicated that isolate AC is clearly more similar to reference strain ACC with genetic distance 0.021, as compared to strain APC with genetic distance 0.51. Although isolates AK and AP were separated from others strain the percentage of the genetic distance was very small, it's about 0.160.

Sequencing Analysis

The PCR amplification obtained from Primers used revealed a strong and clear single band (Figure 1(a) and 1(b)). Two PCR primers Pairs, FP16-RP16 and CRN5-1137 were encoding SSU rDNA gene at molecular weight approximately 1,550 and 2,000 bp respectively. The gene SSU rDNA were aligned by the Clustal W program. The aligned sequences then were compared with the sequences present in GenBank (EMBL) using BLAST.

Sequencing analyses of the gene SSU rDNA obtained from PCR reaction were done to isolate AC, AK and AP. From the results AC isolate showed a high similarity 92% to the gene 16S rDNA of *Acanthamoeba castellanii* strain Ma compared 91% similar to the gene 16S rDNA of *Acanthamoeba* sp. strain CDC V168 (GENBANK). While for isolates AK and AP showed a high similarity: 95% and 92% to the gene 16S rDNA of *Acanthamoeba* sp strain CDC V168, and 94% and 91% similar to the gene 16S rDNA of *Acanthamoeba castellanii* strain Ma (GENBANK) respectively.

Discussion

The Restriction fragments Length Polymorphism (RFLP) analysis was recently applied in subgenus classification of *Acanthamoeba,* but the results were highly polymorphic among to the closely related strain (Chung, *et al.,* 1998; Kong and Chung, 1996). The used of nuclear small-subunit rDNA (SSU rDNA) gene can reveal the phylogenetic relationships among *Acanthamoeba* spp. (Yu, *et al*., 1999; Stothard, *et al*., 1998).

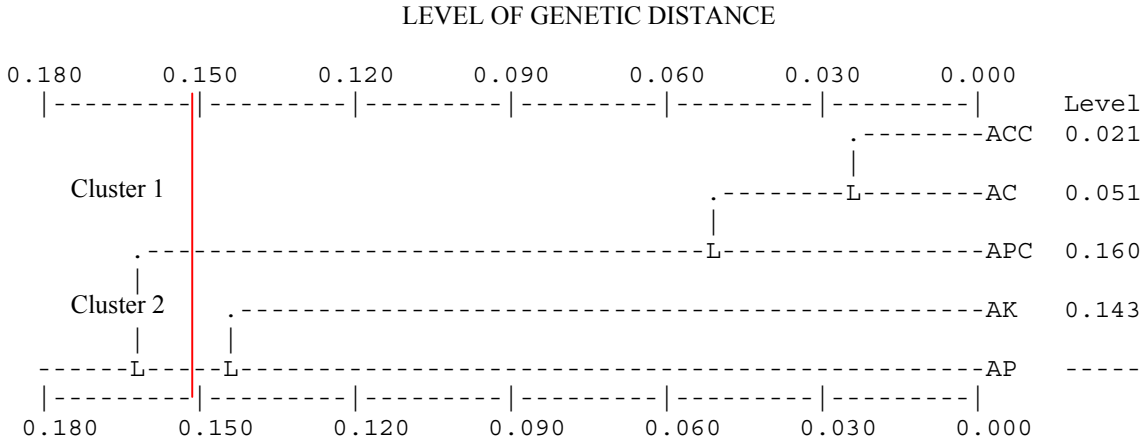


FIGURE 3 Phylogenetic cluster obtained from PCR-RFLP on amplified SSU rDNA of *Acanthamoeba* isolates followed by evaluation using UPGMA clustering method.

Due to the phylogenetic tree obtained in this study showed the existence of two groups at level 0.150, of which one group consists of isolates ACC, AC, and APC and the second group consists of isolate AK and AP. The placement of isolate AC with reference strains ACC (*Acanthamoeba castellanii* CCAP 1501/2A *)*and APC (*Acanthamoeba polyphaga* CCAP 1501/3A) in one cluster are in close agreement with Pussard and Pon (1997) suggestion to group II, since strain ACC and strain APC are assigned in group II. Interestingly, based on the cluster isolate AC clearly more similar to reference strain ACC compared to strain APC (Figure 3; Cluster 1). It can be conclude that isolate AC might belong to *Acanthamoeba castellanii* species. But the second cluster consists of isolates AK and AP cannot be specified and grouped according to pussard and pon's suggestion due to insufficient information available.

However the dendrogram constructed revealed the present of extreme similarities between the isolates. So it can conclude that the genetic distance among the isolates obtained in this study is very minimum to indicate their minimal genetic differences. Although isolates AK and AP cannot be determine their specific species, due to the RFLP results indicated that these isolates might be also in group II according to Pussard and Pon's suggestion.

From the sequencing and GENBANK comparison showed that no significant similarity between the isolates studied to the gene from others genera group of Amoebae. So it proved that these three new isolates of Amoebae studied confirmed as a genera *Acanthamoeba*. However the result can't give a clear identification of the species group for isolates AK and AP, but it showed that the isolates is slightly similar to strain of *Acanthamoeba castellanii* due to high percentage of similarity. For isolate AC the result showed that the isolate clearly closed as *Acanthamoeba castellanii* with the highest percentage of similarity.

The SSU rDNA analysis have recognized particularly well suited for estimating the relationships of *Acanthamoeba*, however due to the labour costing of the technique the variation of the sequence can be missed. Therefore in the future lots of conserved sequence of strains and primers were needed (Kong and Chung, 1996). From the result both of the PCR-based DNA technique indicated the similar finding for the new isolates tested.

In conclusion, the results of this study proven that RFLP and sequencing analysis was a useful tool for rapid characterization of new isolates and for assessment of genetic relatedness among a closely related isolates of *Acanthamoeba*.

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