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Genetic Relatedness Among Isolates of *Acanthamoeba* Based on RAPD Analysis

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Abstract: *Acanthamoeba* is a pathogen frequently infecting brain, eyes, skin and lung of human and animal. Seven *Acanthamoeba* local isolates from various sources, two *Acanthamoeba* type strains and one strain of *Hartmannella vermiformis* were characterized for their genetic variability using randomly amplified polymorphic DNA (RAPD) technique using four different 10-mer oligonucleotides primers. Electrophoresis of the amplification products generated DNA bands ranging from approximately 0.25 to 7.50 kbp in size. A genetic relatedness among the isolates was examined using Dice similarity coefficient as the genetic distance measured between the strains of *Acanthamoeba* and *H. vermiformis*. Three distinct clusters could be separated at genetic distance of approximately 0.330.

Key words : *Acanthamoeba*, DNA polymorphism, Genetic distance, RAPD-PCR

INTRODUCTION

Acanthamoeba is a free-living amoeba and mostly found in environment habitats such as soil, air, dust and water[1]. These organisms can infect a variety of mammals, including humans involving brain, eyes, skin and lungs[2]. The human diseases inflicted by these amoebae are *Acanthamoeba* Keratitis and Granulomatous Amoebic Encephalitis (GAE)[2]. The common species of *Acanthamoeba* that have been reported associated with human diseases are *Acanthamoeba polyphaga*, *Acanthamoeba castellanii* and *Acanthamoeba culbertsoni*[2]. *Acanthamoeba* can be easily isolated from the environment and cultivated on non-nutrient agar culture plates covered with a dense lawn of bacterium *Escherichia coli* (NNA-*E. coli*) or grown in axenic solution such as mycological peptone medium proteose-peptone glucose medium and polypeptone medium[3]. This amoebae species is normally characterized by observing the morphology of its trophozoites and cysts[1]. However, to differentiate between species of *Acanthamoeba* is quite difficult due to very small variations in the morphology of their trophozoite and cysts[4]. The trophozoites have tiny and thin cytoplasmic projection called acanthapodia and the cysts are commonly wrinkled, stellate and sometimes have polygonal shape. Their conventional identification that is mainly based on cysts morphology should be supported by other reliable techniques such as biochemical and molecular approaches[5]. In this regards, DNA analysis is more preferable for species differentiation.

In this study, we attempted to differentiate among the seven isolates of *Acanthamoeba* as well as two type strains of *Acanthamoeba castellanii* CCAP 1501/2A and *Acanthamoeba polyphaga* CCAP 1501/3A and one *H. vermiformis* negative control by means of PCR technique using arbitrary primers to obtain randomly amplified polymorphic DNA (RAPD) fingerprints. This technique is well-known to provide a greater ability to differentiate closely related isolates both of the strain and species level[6]. Moreover, a great advantage of this technique is that the result obtained can be correlated with that obtained by morphological characterisation[7].

MATERIALS AND METHODS

***Acanthamoeba* samples:** Two type strains of *Acanthamoeba* (*Acanthamoeba castellanii* CCAP 1501/2A and *Acanthamoeba polyphaga* CCAP 1501/3A) and one type strain of protozoan *Hartmannella vermiformis* CCAP 1534/7B were purchased from Culture Collection of Algae and Protozoan (CCAP), UK. Seven local isolates of *Acanthamoeba* from various sources were used in this study. They were identified

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morphologically as *Acanthamoeba* and labelled as isolates AC, AK, AP, AR1, AR2, AR3 and AR4[1]. The isolate AC is a brain tumour isolate obtained from Institute of Medical Research, Kuala Lumpur. Isolate AK was obtained from human corneal scrapings of keratitis patients and supplied by Department of Ophthalmology, Hospital Kuala Lumpur and isolate AP was isolated from the inner side of a water tap. The other isolates (AR1, AR2, AR3 and AR4) were isolated from rivers of Kuala Terengganu, Malaysia. Isolation of the amoebae was done by a cotton swab technique employed by Devonnshire *et al.*[8]. All the isolates were grown on a non-nutrient agar supplemented with heat-killed *Escherichia coli* and incubated at 30°C.

Extraction and quantitation of DNA: DNA extraction from PBS-washed *Acanthamoeba* samples was carried out with the Wizard® Genomic DNA purification Kit (Promega, Madison, WI, USA), according to the manufacturers instructions for cells. The concentration and quality of the DNA was estimated by measuring the absorption at 260 and 280 nm in an Ultraspec 300 UV-visible spectrophotometer (Pharmacia Biotech, Cambridge, UK) according to Sambrook *et al.*[9].

DNA amplification by Polymerase Chain Reaction (PCR): A total of 9 decametric 10-mer oligonucleotides of arbitrary primers (synthesized by Genosys Biotechnologies Inc., USA) were used in screening of amplified DNA isolates of *Acanthamoeba*. Based on the number and intensity of the resulting bands, the reproducibility and discriminating potential of the amplified products, four decametric oligonucleotide primers were chosen for RAPD analysis. The primers were GEN 1-60-03 (5'-CTACACAGGC-3'), GEN 1-60-05 (5-GTCCTCAACG-3), GEN1-60-06(5'-CTACTACCGC-3') and GEN 1-60-09(5'-CGTCGTTACC-3). The amplification reactions were performed in 25 µL volumes containing 20-30 ng of template DNA, 1 unit of Taq DNA polymerase, 1.0 mM of dNTPs, 50 pmol of a single primer and 2.5 mM of $MgCl_2$ (Promega, Madison, USA). The reactions were carried out in a 100-PTC Thermal Cycler (MJ Research Inc, Watertown, USA). The amplification program was as follows: an initial denaturation phase at 94°C for 3 min; then 44 cycles of amplification at 94°C for 1 min, 36°C for 1 min and 72°C for 2 min and final extension step at 72°C for 5 min.

Agarose gel electrophoresis: The RAPD-PCR amplification products were fractionated by electrophoresis on 1.2% (w/v) agarose gel and run at 55 V in 1X TBE buffer (pH 8.3). The gels were stained with Ethidium Bromide (0.5 µg mL^{-1}), visualized and photographed under UV light with a Gel Doc 2000 (Bio-Rad, Hercules, USA), which allowed digitalisation of the negative photographs. 1 kb DNA ladder (Promega, Madison, US) was used as a standard marker.

Data analyses: The polymorphisms in PCR amplification products were scored visually for presence and absence of bands at specific molecular weight[10]. Calculations of the genetic distance in the profile of bands were obtained by using the coefficient of Dice[11], followed by cluster analysis of the similarity matrix data using the SAHN (sequential, agglomerative, hierarchical and nested) clustering and UPGMA (unweighted pair group method, arithmetic average) algorithm to group the fingerprints similarity.

RESULTS

The genomic DNA of each strain used in this study was amplified using 4 different primers. A large number of reproducible bands were produced for most of the primers. The results showed that each of the primers produced specific band profiles revealing polymorphisms. The number of bands for each primer varied from 4 to 16. The size of the amplified product ranged from 0.25 to 7.50 kbp.

The RAPD profiles of the nine *Acanthamoeba* strains were compared using primer GEN 1-60-03 which produced bands ranging from 0.50 to 7.50 kbp in size (Fig. 1). Two bands with the molecular weight of approximately 2.00 and 1.70 kbp, were present in almost all the *Acanthamoeba* tested except for strain APC (Fig. 1, lane 2). This primer differentiated the seven *Acanthamoeba* (ACC, APC, AC, AK, AP, AR1 and AR4) as they had different RAPD fragment profiles (Fig. 1, lane 1-6, 9). The isolates of AR2 and AR3 did not show polymorphic profiles, as the present of identical electrophoretic patterns in the gel (Fig. 1, lane 7 and 8).

Among the primers used in this study, primer GEN 1-60-05 generates lesser number of bands. The number of bands in these profiles ranged from 3 to 7 bands per isolate. The amplified products revealed a bands ranging from 0.25 to 7.50 kbp in size (Fig. 2). Differences in intensities and number of bands were observed in all the strains tested. A DNA band with molecular weight of approximately 1.50 and 2.00 kbp in size were present in all isolates of *Acanthamoeba* except for strain of APC (Fig. 2, lane 2). Primer GEN 1-60-05 was effective in differentiating all the isolates of *Acanthamoeba* although this primer gives lesser bands, because almost all the isolates show independent profiles (Fig. 2, lane 1-9).

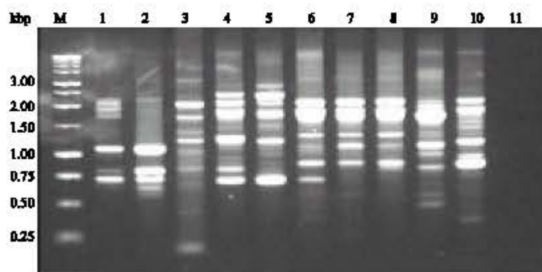

Fig. 1: RAPD profile of *Acanthamoeba* isolates obtained with primer GEN 1-60-03. Lane 1: Strain ACC; Lane 2: Strain APC; Lane 3: Isolate AC; Lane 4: Isolate AK; Lane 5: Isolate AP; Lane 6-9: Isolate AR1-AR4; Lane 10: Strain HV; Lane 11: Control. Lane M: 1 kbp DNA ladder

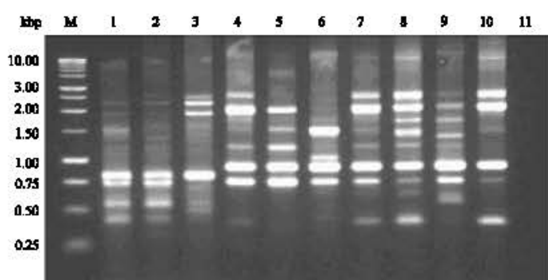

Fig. 2: RAPD profile of *Acanthamoeba* isolates obtained with primer GEN 1-60-05. Lane 1: Strain ACC; Lane 2: Strain APC; Lane 3: Isolate AC; Lane 4: Isolate AK; Lane 5: Isolate AP; Lane 6-9: Isolate AR1-AR4; Lane 10: Strain HV; Lane 11: Control. Lane M: 1 kbp DNA ladder

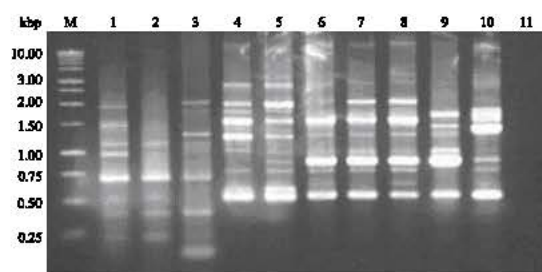

Fig 3: RAPD profile of *Acanthamoeba* isolates obtained with primer GEN 1-60-06. Lane 1: Strain ACC; Lane 2: Strain APC; Lane 3: Isolate AC; Lane 4: Isolate AK; Lane 5: Isolate AP; Lane 6-9: Isolate AR1-AR4; Lane 10: Strain HV; Lane 11: Control. Lane M: 1 kbp DNA ladder

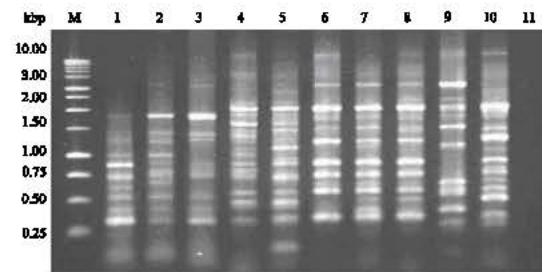

Fig 4: RAPD profile of *Acanthamoeba* isolates obtained with primer GEN 1-60-09. Lane 1: Strain ACC; Lane 2: Strain APC; Lane 3: Isolate AC; Lane 4: Isolate AK; Lane 5: Isolate AP; Lane 6-9: Isolate AR1-AR4; Lane 10: Strain HV; Lane 11: Control. Lane M: 1 kbp DNA ladder

In the panel GEN 1-60-06 of Fig. 3, are showed the amplification profiles obtained with this primer using nine isolates of *Acanthamoeba*. The amplification of this primer was revealed a number of bands in ranged from 5 to 10 bands per isolates and produce a bands ranging from 0.25 to 7.50 kbp in size. The DNA band with the molecular weight 1.00 kbp in size was present in almost all the isolates of *Acanthamoeba* tested (Fig. 3). Isolates of ACC, APC, AC, AK, AP and AR4 showed independent profiles of RAPD fragments (Fig. 3, lane 1-5, 9). While isolates of AR1, AR2 and AR3 had very similar banding pattern (Fig. 3, lane 6-8).

The RAPD analyses also showed that the primer GEN 1-60-09 gave the highest number of bands for almost all the isolates of *Acanthamoeba* tested compared to the other primers. The number of bands in these profiles ranged from 9 to 16 bands per isolate. With this Primer yielded bands ranging from 0.30 to 7.50 kbp in size. It revealed two bands with the sizes of approximately 0.30 and 1.90 kbp in all the isolates tested except for isolate AR4 (Fig. 4). There are another two bands with molecular weight approximately 0.35 kbp in size present in almost all the isolates of *Acanthamoeba* except for isolate AR4 (Fig. 4, lane 9). All the isolates of *Acanthamoeba* tested exhibited different amplification profiles with this primer, showing that this primer is capable of differentiating most of the samples compared to other primers (Fig. 4, lane 1-9). Overall, fingerprints pattern indicated significant diversity among the isolates tested. Gel electrophoresis fragments of strain *H. vermiformis* CCAP 1534/7B generate totally different fragment of bands in all four primers used (Fig. 1-4, lane 10), showing a clear separation from the other isolates of *Acanthamoeba*.

Examination of the DNA fingerprints with cluster analysis showed a significant genetic diversity among the isolates tested. A dendrogram tree in Fig. 5 was constructed based on RAPD profile data from the four primers used (GEN 1-60-03, GEN 1-60-05, GEN 1-60-06 and GEN 1-60-09). The dendrogram showed the existence of

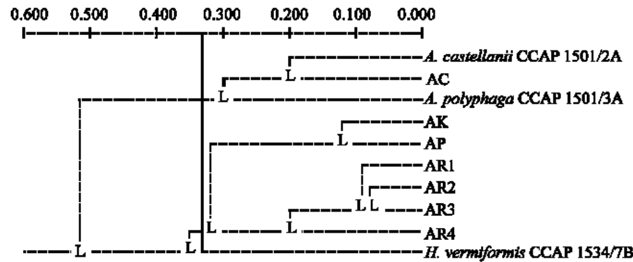

Fig. 5: Dendrogram on the RAPD bands distance of *Acanthamoeba* samples and one strain of *Hartmannella vermiformis* CCAP1534/7B constructed using UPGMA clustering method

three distinct subclusters at cluster cut-off value at the branches (indicated as vertical line) which corresponded to a genetic distance of 0.330, with strain *H. vermiformis* CCAP 1534/7B clearly forming one subcluster and all the *Acanthamoeba* sp. analysed forming another two subclusters.

DISCUSSION

RAPD-PCR is a very powerful DNA fingerprinting technique and has been developed for generating markers to establish genetic linkage maps[12]. This technique that uses arbitrary primers provides a convenient and rapid assessment of the differences in the genetic composition of related individuals[13]. In this study, several primers were initially tested and finally four of them were selected for RAPD-PCR on the basis of the number, intensity and distribution of bands generated that were able to clearly distinguish among isolates. Almost all the primers tested had a unique RAPD patterns, indicating a large diversity among *Acanthamoeba* isolates. The dendrogram generated from RAPD profiles showed the existence of three subclusters at a genetic distance of approximately 0.330, of which one subcluster consists of isolates ACC, AC and APC, the second subcluster consists of isolates AK, AP, AR1, AR2, AR3 and AR4 and the third subcluster consists exclusively of strain HV.

The placement of reference strains ACC and APC in one subcluster by RAPD profiles are in close agreement with the grouping o f these species in group II based on their morphological characteristics as assigned by Pussard and Pon[7]. Interestingly, based on this cluster analysis, a brain tumour isolate AC from Institute of Medical Research, Malaysia clearly more similar to reference strain of *A. castellanii* CCAP 1501/2A with genetic distance only 0.193, as compared to strain APC (*A. polyphaga* CCAP 1501/3A) with genetic distance 0.294. It can be concluded that isolate AC might belongs to *Acanthamoeba castellanii* species (Fig. 5).

In contrast, isolates AK, AP and AR1, AR2, AR3 and AR4 which forming separate cluster from reference strains of *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* could not be identified as either *Acanthamoeba castellanii* or *Acanthamoeba polyphaga*. However, they could be belongs to other species of *Acanthamoeba*. These results are in close agreement with Kong and Chung[14] who recommended that all the new isolates of *Acanthamoeba* sp. should be analysed with various references strains for confirmation of their taxonomic validity. The cluster analysis based on RAPD-PCR fingerprinting obtained was clear enough to allow discrimination between reference strains and local isolates. This cluster analysis technique is in agreement Zhang and Fang[15], that demonstrated the diverse genomic profiles of microbial communities.

REFERENCES

1. Page, F.C., 1988. A new key to freshwater and soil *Gymnamoebae*. Ambleside, Cumbria, UK: Freshwater Biol. Ass., pp: 122.
2. Armstrong, M., 2000. The pathogenesis of human *Acanthamoeba* infection. Infect. Dis. Rev., 2: 65-73.
3. Martinez, A.J., 1985. Free-Living Amebas: Natural History, Prevention, Diagnosis, Pathology and Treatment of Disease. Boca Raton, Florida: CRC Press. ISBN 0849366313.
4. Kilvington, S., J.R. Beeching and D.G. White, 1991. Differentiation of *Acanthamoeb*a strains from infected corneas and the environmental by using Restriction Endonuclease Digestion of whole-cell DNA. J. Clin. Microbiol., 29: 310B314.
5. Byers, T.J., 1986. Molecular Biology of DNA in *Acanthamoeba*, *Amoeba*, *Entamoeba* and *Naegleria*. Intl. Rev. Cytol., 99: 311B341.
6. Maslow, J.N., M.T. Mulligan and R.D. Arbeit, 1993. Molecular epidemiology: Application of contemporary technique to the typing of microorganisms. Clin. Infect. Dis., 17: 153-164.
7. Pussard, M. and R. Pon, 1977. Morphology of the taxonomy of genus *Acanthamoeba* (Protozoa, Amoebida). Protistology, 8: 557-598.
8. Devonnshire, P., F.A. Munro, C. Abernethy and B.J. Clark, 1990. Microbial contamination of contact lens cases in the west of Scotland. Br. J. Opphthalmol., 77: 41-45.
9. Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. 2nd Edn., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

10. Tamplin, M.L., J.K. Jackson, C. Buchrieser, R.L. Murphree and K.M. Portier *et al.*, 1996. Pulsed-field gel electrophoresis and ribotype profiles of clinical and environmental *Vibrio vulnificus* isolates. Applied Environ. Microbiol., 62: 3572-3580.
11. Nei, M., 1972. Genetic distance between populations. Am. Natl., 106: 283-292.
12. Williams, G., A. Kubelik, K. Livak, A. Rafalski and S. Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful genetic markers. Nucleic Acids Res., 18: 6531-6535.
13. Welsh, J. and M. McClelland, 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res., 18: 7213-7218.
14. Kong, H.H. and D.I. Chung, 1996. PCR and RFLP variation of conserved region of small subunit ribosomal DNA among *Acanthamoeba* isolates assigned to either *A. castellanii* or *A. polyphaga*. Kor. J. Parasitol., 34: 127-134.
15. Zang, T. and H.H.P. Fang, 2000. Digitisation of DGGE (denaturing gradient gel electrophoresis) profile and cluster analysis of microbial communities. Biotechnol. Lett., 22: 399-405.