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A riboprinting scheme for identification of unknown *Acanthamoeba* isolates at species level

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Abstract: We describe a riboprinting scheme for identification of unknown *Acanthamoeba* isolates at the species level. It involved the use of PCR-RFLP of small subunit ribosomal RNA gene (riboprint) of 24 reference strains by 4 kinds of restriction enzymes. Seven strains in morphological group I and III were identified at species level with their unique sizes of PCR product and riboprint type by *Rsa* I. Unique RFCP of 17 strains in group II by *Dde* I, *Taq* I and *Hae* III were classified into: (1) four taxa that were identifiable at the species level, (2) a subgroup of 4 taxa and a pair of 2 taxa that were identical with each other, and (3) a species complex of 7 taxa assigned to *A. castellanii* complex that were closely related. These results were consistent with those obtained by 18s rDNA sequence analysis. This approach provides an alternative to the rDNA sequencing for rapid identification of a new clinical isolate or a large number of environmental isolates of *Acanthamoeba*.

Key words: *Acanthamoeba*, riboprinting scheme, rapid identification

INTRODUCTION

The taxonomy of *Acanthamoeba* has been revised several times (Pussard and Pons, 1977; De Jonckheere, 1983; Visvesvara, 1991; Stothard et al., 1998; Chung et al., 1998). Pussard and Pons (1977) classified the genus *Acanthamoeba* into three morphological groups on the basis of cyst size and shape. Group I consists of *Acanthamoeba* spp. of which cysts are relatively large, with distinctly stellate endocysts and smooth spherical ectocyst. Group II and group III *Acanthamoeba* spp. have smaller cyst (diameters less than 18 μm): Group II species has polygonal to stellate endocyst with irregular or winkled ectocysts, whereas the cyst of group III species has rounded or slightly angular endocyst with thinner and smooth or slightly wrinkled ectocyst. Classification by Pussard and Pons (1977) has been recognized being practical, but taxonomy of *Acanthamoeba* at the species level remains unclear. However, De Jonckheere (1983) suggested that some species designated by Pussard and Pons (1977) should be invalid synonyms of the other species on the basis of alloenzyme analysis. Jacobson and Band (1987) reported alloenzyme heterogeneity among strains assigned to *A. polyphaga*. Therefore, results reported by De Jonckheere (1983) and Jacobson and Band (1987) suggest that a different alloenzyme pattern should not be used as the sole criterion to establish a new species nor a morphological analysis be used alone.

Recently, Stothard et al. (1998) classified 53 strains of *Acanthamoeba* into 12 sequence types based on 18S rDNA sequence analysis.

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The results were inconsistent with some species designations. Chung et al. (1998) applied a simpler technique, riboprinting, to subgenus classification of *Acanthamoeba* and the results coincided well with those of Stothard at al. (1998). Nevertheless, when a number of unidentified *Acanthamoeba* isolates were collected from clinical samples or environments, identification of the isolates by analysis of 18S rDNA complete sequences would be too labor-intensive, time-consuming, and expensive for most laboratories. Riboprinting can be a substitute of 18S rDNA sequencing as suggested by Chung et al. (1998). In this paper, we provide a new strategy based on the riboprinting for rapid identification of unknown *Acanthamoeba* isolates.

MATERIALS AND METHODS

Acanthamoeba

Twenty-four strains including 19 (neo) type strains of the genus *Acanthamoeba* which were previously assigned to 19 species, were obtained from ATCC (Table 1). According to the morphological grouping of Pussard and Pons (1977), two strains belong to group I, 5 strains to group III and the other 17 strains to group II. They were cultured axenically in Proteose peptone-Yeast extract-Glucose (PYG) medium or Proteose peptone-Yeast extract-Glucose-Cysteine (PYGC) medium at 25°C or 37°C.

Extraction of genomic DNA, PCR amplification of 18S rDNA, and restriction enzyme digestion

Genomic DNA of *Acanthamoeba* was obtained by the method described by Kong and

Table 1. List of *Acanthamoeba* 24 reference strains obtained from ATCC

No.	Strain	ATCC No.	Virulence	Environmental source	Geographic source	Reference	Former species designation
1	Castellani	30011	+	yeast culture	England	Douglas (1930)	*A. castellanii*
2	L3a	50240	+	swimming pool	France	Pussard & Pons (1977)	*A. lugdunensis*
3	Vil3	50241	nd[a]	swimming pool	France	Pussard & Pons (1977)	*A. quina*
4	Jones	30461	+	keratitis	U.S.A.	Jones et al. (1975)	*A. polyphaga*
5	SH621	50254	nd	human feces	France	Pussard & Pons (1977)	*A. triangularis*
6	Nagington	30873	+	keratitis	England	Nagington et al. (1974)	*A. polyphaga*
7	Ma	50370	+	keratitis	U.S.A.	Ma et al. (1981)	*A. castellanii*
8	Singh	30973	-	soil	England	Singh (1952)	*A. rhysodes*
9	1652	50253	-	soil	England	Pussard & Pons (1977)	*A. mauritaniensis*
10	AA2	50238	-	soil	Morocco	Pussard & Pons (1977)	*A. divionensis*
11	AA1	50251	-	soil	France	Pussard & Pons (1977)	*A. paradivionensis*
12	Neff	30010	-	soil	France	Neff (1957)	*A. castellanii*
13	P23	30871	-	fresh water	U.S.A.	Page (1967)	*A. polyphaga*
14	Chang	30898	+	fresh water	U.S.A.	Byers et al. (1990)	*A. castellanii*
15	BH-2	30730	+	ocean sediment	U.S.A.	Sawyer et al. (1977)	*A. hatchetti*
16	RB-F-1	50388	nd	ocean sediment	U.S.A.	Sawyer et al. (1993)	*A. stevensoni*
17	S-7	30731	+	beach bottom	U.S.A.	Sawyer (1971)	*A. griffini*
18	Ray & Hayes	30137	nd	soil	U.S.A.	Ray & Hayes (1954)	*A. astronyxis*
19	OC-15C	30867	nd	river	U.S.A.	Lewis & Sawyer (1979)	*A. tubiashi*
20	A-1	30171	+	tissue culture	U.S.A.	Singh & Das (1970)	*A. culbertsoni*
21	OC-3A	30866	+	GAE[b]	U.S.A.	Moura et al. (1992)	*A. healyi*
22	GE-3a	50252	-	swimming pool	France	Pussard & Pons (1977)	*A. pustulosa*
23	Reich	30870	-	soil	Israel	Reich (1933)	*A. palestinensis*
24	PD_2S	30841	nd	swimming pool	France	Molet & Ermolieff-Braum (1976)	*A. lenticulata*

[a]nd; not determined.

[b]GAE; granulomatous amebic encephalitis.

Chung (1996). The primers and method for PCR amplification of 18S rDNA were the same as described by Chung et al. (1998). In order to check the size of the PCR products, the amplified DNA of 24 strains were electrophoresed on 2.5% agarose gel with DNA size standards (*Hind* III digested λ phage DNA, Poscochem, Korea; Amplisize, Biorad, U.S.A.). Four kinds of restriction endonucleases (*Hae* III, *Dde* I, *Rsa* I, and *Taq* I; Poscochem, Korea) which have recognition sequences of four nucleotides were used to generate comparative riboprints. The amplified DNA of each strain was digested with 5-10 units of each restriction enzyme for 2 hr in recommended buffers at 37°C, except for *Taq* I (67°C). The digested DNA was electrophoresed on 2.5% agarose gel for 1.5 hr with *Hae* III digested ΦX174 DNA as DNA size marker. The gels were stained with ethidium bromide and photographed under an UV transilluminator.

Development of the riboprinting scheme

The strains were divided into three groups by morphological characteristics of amoeba and cysts. In each group, restriction phenotypes of strains were determined for each restriction enzyme used. Therefore, differences of PCR product sizes and restriction phenotypes were used to build a scheme for distinguishing and identifying unknown *Acanthamoeba* isolates.

RESULTS

Two strains in morphological group I, *Acanthamoeba astronyxis* and *A. tubiashi*, had bigger PCR product size of 18s rDNA than that of other strains in group II or group III. The size of their PCR products was approximately 2.7 kb for *A. astronyxis* and 2.6 kb for *A. tubiashi*. In addition to the difference of PCR product size, the riboprint of each strain by restriction enzyme *Rsa* I was unique (Fig. 1).

Morphological group II, to which most isolates from clinical and environmental sources belong, contains 17 strains including 12 type or neotype strains. The PCR product size of *A. griffini* is larger than that of other strains, because of the intron in its 18s rDNA (Gast et al, 1996; Ledee et al., 1996). Sixteen strains whose PCR products were approximately 2.3 kb, were subjected to riboprinting. Table 2 and Fig. 2 show the riboprint types of 16 *Acanthamoeba* strains by *Dde* I and *Taq* I. The ribodemes by *Dde* I divided 16 strains into two types. Seven strains including Castellani, the type strain of *A. castellanii*, which showed the same riboprint by *Dde* I were assigned as *A. castellanii* complex. Riboprinting by *Taq* I was applied to differentiate 9 strains with B type by *Dde* I. By *Taq* I, nine strains were divided into four subgroups. *A. castellanii* Neff strain was unique from the other strains. Four strains, including *A. rhysodes* Singh, *A. mauritaniensis* 1652, *A. divionensis* AA2, and *A. paradivionensis* AA1, were in a subgroup, because of their similar pattern by *Taq* I. Chang strain fomerly assigned to *A. canstellanii* and *A. hatchetti* BH-2 strain was classified to an another subgroup with the same pattern. Riboprint by *Hae* III or *Hha* I differenciated *A. polyphaga P23* from *A. stevensoni* RB-F-1, which belonged to the same subgroup by *Taq* I (Fig. 2).

Five strains in morphological group III showed differences in PCR product sizes and their riboprints by *Rsa* I (Fig. 1). *Acanthamoeba lenticulata* had the largest PCR product. Although the other four strains had

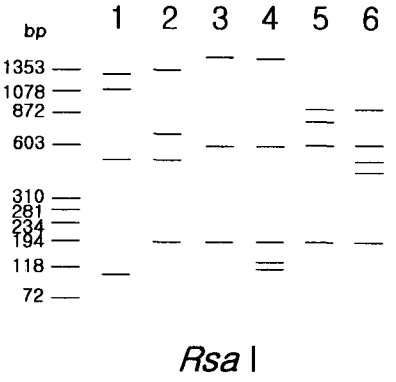

Fig. 1. Schematic representation of riboprinting patterns of *Acanthamoeba* strains, belonging to morphoolgical group I and III by *Rsa* I restriction enzyme. Lane 1, *A. astronyxis* Ray & Hayes; 2, *A. tubiashi* OC-15C; 3, *A. culbertsoni* A-1; 4, *A. healyi* OC-3A; 5, *A. pustulosa* GE-3a; 6, *A. palestinensis* Reich.

Table 2. Riboprint types of *Acanthamoeba* strains belonging to morphological group II

Strain	Riboprint types		Species or species complex
	Dde I	*Taq* I	
Castellani	A	A	*A. castellanii* complex
L3a	A	A	*A. castellanii* complex
Vil3	A	A	*A. castellanii* complex
Jones	A	A	*A. castellanii* complex
SH621	A	A	*A. castellanii* complex
Nagington	A	A	*A. castellanii* complex
Ma	A	A	*A. castellanii* complex
Singh	B	B	*A. rhysodes*
1652	B	B	*A. rhysodes*
AA2	B	B	*A. rhysodes*
AA1	B	B	*A. rhysodes*
Neff	B	A	*A. polyphaga*
P23	B	C	*A. polyphaga*
Chang	B	D	*A. hatchetti*
BH-2	B	D	*A. hatchetti*
RB-F-1	B	C	*A. stevensoni*

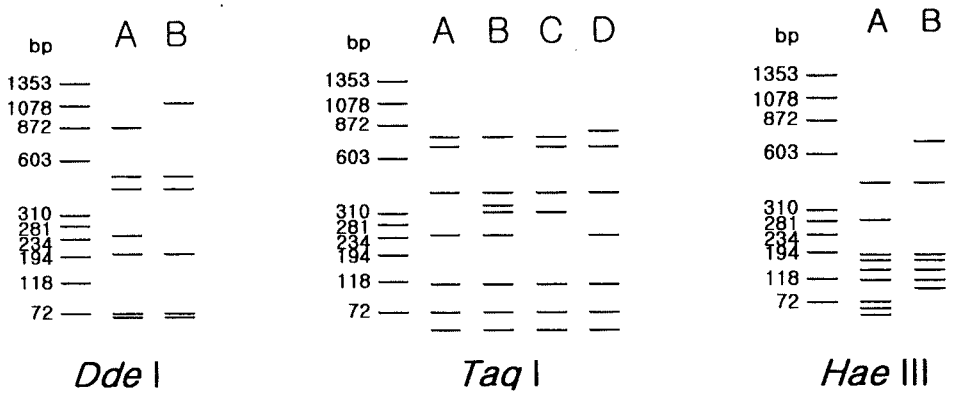

Fig. 2. Schematic representation of riboprint types of *Acanthamoeba* strains, belonging to morphological group II by 3 kinds of restriction enzymes.

similar PCR product size, they were distinguishable from each other by *Rsa* I riboprints.

Fig. 3 represents the riboprinting scheme of 24 strains analyzed in the present study by their morphological grouping, 18s rDNA PCR product sizes, and its riboprints by restriction enzymes. By this scheme, eleven strains of *Acanthamoeba* were identified at the species level.

DISCUSSION

This study presents a riboprinting scheme for identification of unknown *Acanthamoeba* isolates by using PCR/RFLP of the small subunit ribosomal DNA. Considering genetic diversity among strains of genus *Acanthamoeba*, limited number of strains were applied to build a scheme, however, the present study provides a simple guideline for identification, because 24 strains included 19 type or neotype strains of *Acanthamoeba* spp. previously assigned.

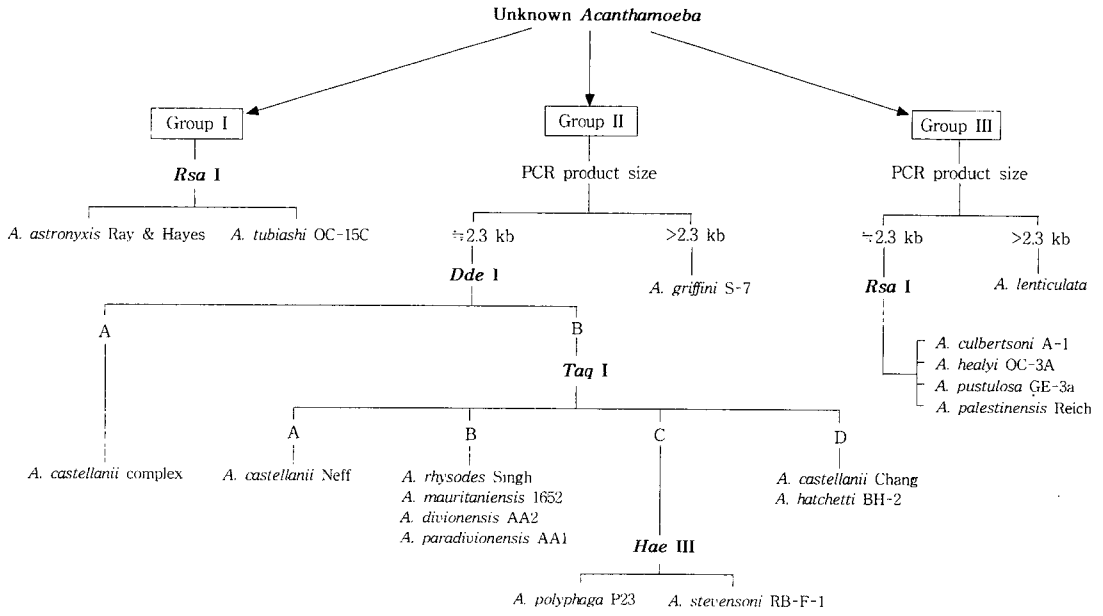

Fig. 3. Riboprinting scheme with 24 reference strains of *Acanthamoeba*.

Pussard and Pons (1977) classified strains in genus *Acanthamoeba* into three groups, according to their morphological characteristics, and the morphology has recognized to be practical for intrageneric grouping (Stothard et al., 1998; Chung et al., 1998). However, because of variability of cyst morphology by culture conditions (Stratford and Griffiths, 1978), species identification by morphology alone can hardly be possible (Visvesvara, 1991).

This study provides a time-saving alternative to sequence analysis for identification of unknown *Acanthamoeba* isolates. For mitochondrial (Mt) DNA RFLP and alloenzyme analysis, it takes time to get enough number of trophozoites by culture. Furthermore, the results of MtDNA RFLP and alloenzyme analyses in *Acanthamoeba* are too heterogeneous to be applied for identifying unknown isolates at the species level (Chung et al., 1996; Kong et al., 1995). Nuclear 18S rDNA sequence analysis is recognized as an objective and reliable method for species identification (Gast et al., 1996; Ledee et al., 1996; Stothard et al., 1998). However, it takes time to sequence approximately 2,300 bp or longer 18S rDNA especially when a large number of isolates are to be tested.

In addition, our study serves a guideline for future studies that can resolve some taxonomic problems in the genus *Acanthamoeba*. For example, type strains (1652, AA2, and AA1) of *A. mauritaniensis*, *A. divionensis*, and *A. parasivionensis* showed riboprints identical to that of *A. rhysodes* Singh (Chung et al., 1998). Three former type strains were isolated and recorded as new species by Pussard and Pons (1977). Chung et al. (1998) suggested the *A. mauritaniensis*, *A. divionensis*, and *A. parasivionensis* should be renamed as *A. rhysodes* under the law of priority. Chang strain previously assigned to *A. castellanii* was found to be related most closely with BH-2, the type strain of *A. hatchetti*, by nuclear and Mt riboprinting (Chung et al., 1998; Yu et al., 1999). Furthermore, the Chang and BH-2 had many comigrating DNA fragments on MtDNA RFLP analysis (unpublished data). To confirm these taxonomic revisions, 18S rDNA sequence analysis should be further pursued.

Acanthamoeba castellanii complex corresponds to T4 sequence type of Byers group. Stothard et al. (1998) suggested that various species in T4 might be reclassified as

A. castellanii because the sequence type includes the type strain for that species. However, the molecular characteristics of strains in T4 were quite heterogeneous and many species which belonged to this clad have already been assigned. We would like to assign this subgroup (T4 sequence type) as species complex as Costas and Griffith suggested (1986).

Although the scheme proposed here is useful, some caution should be taken in when applying the scheme to identify environmental or clinical isolates of *Acanthamoeba*, when the isolate contains intron in 18S rDNA. Among 17 strains in group II examined in this study, only *A. griffini* had intron in its 18S rDNA. However, we found two clinical isolates containing intron in 18S rDNA and both isolates showed the highest sequence homology of 18S rDNA with the type strain of *A. castellanii* (unpublished data). In such cases, riboprinting of 16S-like Mt rDNA which has no intron can be helpful, for confirmation of assignment by sequence analysis of nuclear 18S rDNA. Additionally, investigators should determine the morphological grouping of the isolates before applying the scheme for identification.

In conclusion, the riboprinting scheme supplemented with morphological grouping could provide rapid identification of unknown *Acanthamoeba* isolates. This scheme would provide an alternative to rDNA sequence analysis especially when many isolates are to be identified.

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