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GENETIC ANALYSIS OF *ACANTHAMOEBA* SPP. ISOLATED FROM DIFFERENT GEOGRAPHIC REGIONS OF THE PHILIPPINES

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ABSTRACT

Acanthamoeba spp. have been isolated from water and soil samples collected all over the Philippines. The amoebae were grown on 1.5% NNA lawned with *Escherichia coli*, then cloned using a Skerman™ micromanipulator and axenized prior to mass production in PPYG medium.

Morphological study of *Acanthamoeba* cysts by PATAg r staining method showed three distinct groups: polygonal, rounded and stellate cysts. Phase contrast microscopy of wet mount preparations (Carl Zeiss Axiovert™) and toluidine-stained trophozoites revealed distinguishing features of *Acanthamoeba:* centrally located nucleolus, numerous dense vacuoles and acanthopodia. It is, however, very difficult to distinguish among strains based on trophozoite morphology.

Soluble proteins were extracted by freeze-thawing technique and total protein concentrations were determined by Bradford method. Protein samples (25 µg-100 µg) were loaded on an IEF gel (pH gradient 3.5-9.5) followed by isoelectrofocusing at 400V-1200V for six hours on a Biorad™ horizontal electrophoresis system. Specific substrates for acid phosphatase (Acph), esterase (Est) and alkaline phosphatase (Alph) isozymes coupled with either formazan or tetrazolium stain were reacted with the protein samples.

Analysis of genetic distances (D) calculated from similarity indices (I) of isoenzyme profiles indicates that groupings are not necessarily consistent with cyst morphology but they correlate strongly with geographic distribution. This suggests that further biochemical characterization is necessary to be able to classify Philippine strains. Moreover, pathogenic character of some environmental isolates that show close genetic relationship with pathogenic reference strains needs to be studied.

INTRODUCTION

Acanthamoeba species are among those free-living amoebae that have been recognized to be pathogenic to humans and experimental animals causing a fatal granulomatous amebic encephalitis (GAE) and a debilitating corneal keratitis (Fig. 1-B). They are very ubiquitous and highly resistant to harsh environmental conditions probably owing to their cyst-forming characteristic and their relatively simple nutritional requirements. Although their pathogenic potential has been reputedly limited to a few species, it is believed that the problem lies more deeply on the difficulty of distinguishing among strains which appear so homogenous with respect to their oftentimes unstable morphologic, behavioral and physiological characters (Griffiths et al., 1978). Indeed, conventional criteria for classification schemes in protozoans are so limited. For these reasons, interpretations on the taxonomic position of *Acanthamoeba* have been quite difficult, especially at the species level (Martinez, 1983; Willaert, 1976; and Costas et al., 1983).

In protozoans, isoenzyme pattern analysis has been employed making use of isoelectrofocusing techniques in polyacrylamide and agarose gels (Daggett et al., 1983; De Jonckheere et al., 1984; Pernin et al. 1985; Pernin, 1984; and Adams et al., 1989). This biochemical method may be particularly useful for *Acanthamoeba* in which the availability of stable characters for classification schemes is limited.

More than a hundred strains obtained from soil and water samples from different localities in the Philippines and from clinical specimens have already been cloned (Matias et al., n.d.). In this study, some of these representative *Acanthamoeba* strains were subjected to isoenzyme analysis using acid phosphatase, alkaline phosphatase and esterase in an attempt to show interstrain genetic variability. The resulting zymograms were compared and **similarity indices** (*I*) as well as **genetic distances** (*D*) were calculated. *D* values were subjected to cluster analysis by UPGMA method (Yagita and Endo, 1990), and correlated with geographic distribution and cyst morphology. Moreover, *D* values were used to determine quantitative relationships between known pathogenic *Acanthamoeba* and Philippine isolates whose pathogenic character is yet unknown.

MATERIALS AND METHODS

Isolation and Propagation

Collection of Samples. Soil and water samples as well as clinical specimens from suspected *Acanthamoeba* infections were collected from various sites, namely: Camarines Norte (**Bc**), Cotabato (**Cot**), Davao (**Dav**), Iloilo (**Ilo**), Mindoro Occidental (**Mocc**), Novaliches (**W**), Diliman (**C**) and **Hl** (a clinical isolate). Reference strains include *Acanthamoeba lenticulata, A. quinalugdunensis, A. mauritanensis, A. castellanii* and Renk (a German isolate). Water and soil samples [the latter was resuspended in sterile 0.15M phosphate-buffered saline (PBS), pH 7.2] were vacuum-filtered on glass fiber filters. These glass filters with the trapped particulates were

inverted on petri dishes overlaid with 1.5% non-nutrient agar (NNA) lawned with 24-hour culture of *E. coli* and incubated at 37°C. The bacterized agar plates were observed for amoebal growth after 48-72 hours.

Cloning. Agar plates showing positive growth were harvested by flooding the plates with 10 mL sterile 0.15 M PBS and scraping the agar surface. The cell suspension was pipetted out, pooled together into a centrifuge tube and spun at 1000 rpm for 5 min. The supernatant was discarded and the pellet was washed thrice with PBS by repeated centrifugations. After washing, the pellet was resuspended in 0.5 mL sterile PBS and a small drop was put onto one end of a previously prepared NNA-overlaid slide. The slide was observed under 16X objective. Floating live cysts (indicated by somewhat granular interior) were dragged to the opposite end of the slide using a Skerman™ micromanipulator. Thereupon, the cyst was transferred to a bacterized NNA plate by slicing out the agar overlay from the slide. About 10 cysts were selected per region or clinical sample. The plates were incubated at 37°C and observed daily for amoebal growth.

Axenic Cultivation. Axenization was done by initially growing the cloned cells in *E. coli*-lawned agar plates. Upon reaching confluence and with 90% of the cells in trophozoite stage, the plates were flooded with 10 mL proteose-peptone-yeast extract-glucose (PPYG) liquid medium containing 500 I.U. penicillin and 500 μg/mL streptomycin. The liquid medium was supplemented with 5% calf serum (Gibco™). Cultures that were cleared of bacteria were aseptically transferred to sterile plates without agar overlay. Thereafter, cells reaching exponential growth were subcultured using PPYG to a final cell concentration of 10^6/mL. Aseptic condition was maintained throughout the mass cultivation process. Once the cell concentration reached 10^9/mL (around 3 days of culturing), harvesting was done by pooling the cells into a centrifuge tube and by repeated washing with amoeba saline (AS) (Matias, 1991) at 1,000 rpm for 5 min each washing. Final washing was in 0.01M Tris-HCl (pH 7.2); afterwhich, cells were stored at -20°C until use. The same procedure was followed for the propagation of the reference strains.

Morphological Study of Cysts and Trophozoites

PATAg r Staining of Cysts. Morphological study of cysts was done for each isolate using periodic acid-thicarbohydrazide-silver reduced (PATAg r) staining technique by Matias et al. (1991) with slight modifications. Morphological study of trophozoites was done by washing fresh cultures with PBS and dropping onto slides with and without fixation. For the fixed preparations, 25% glutaraldehyde 0.05 M cacodylate buffer, pH 7.2 was used as fixative followed by toluidine blue staining for 10 min. Cells that were not fixed were immediately observed and photographed.

Preparation of Protein Samples

Extraction of Soluble Proteins. Soluble proteins from *Acanthamoeba* cells were extracted by resuspending the stored cells in one-half volume of extraction buffer (0.15M Tris-HC1, pH 7.2) containing 1 mM protease inhibitors in Eppendorf tubes. The tubes were immersed in acetone containing dry ice for rapid freezing of cells and then transferred to 37°C water bath for immediate thawing. This was done several times until 100% lysis was achieved as monitored under the phase contrast microscope. Lysates were then spun for one hour at 32,000 rpm in a refrigerated Beckman™ ultracentrifuge (4°C) to remove membranous components. Extracts were stored in 100 µl aliquots at –70°C until use.

Determination of Total Protein Concentration. Analysis of total protein concentration according to the method of Bradford (1976) was done for each isolate. Protein solutions of bovine serum albumin containing 1 µg – 10 µg of the protein were pipetted into 12 x 100 mm test tubes in triplicates and adjusted to 0.1 mL with 0.15M PBS, pH 7.2. One mL of Bradford reagent was then added to the test tube and the contents mixed by vortexing. Absorbance at 595 nm was measured after 2 min in 3 mL quartz cuvettes using a Beckman™ DU-60 Spectrophotometer calibrated against a reagent blank prepared from 0.1 mL of the PBS and 1 mL of Bradford reagent. The weight of protein was plotted against the corresponding absorbance resulting in a standard curve which was used for the determination of total protein in the unknown samples. For the unknown samples, 1:100 dilution of extract was made to react with 1 mL Bradford reagent. The resulting absorbance readings were recorded and inputted into a Lotus 1-2-3 regression analysis. Total protein yield was determined by extrapolation of computer-generated regression output of the BSA standards.

Isoenzyme Analysis

Isoelectric Focusing in Polyacrylamide Gel. Gel casting glass plates (10 cm X 12.5 cm X 0.2 cm) pre-treated with commercially available bind saline (LKB™) were used for the preparation of 6% polyacrylamide gel with 0.6 mL Sigma™ ampholine solution (pH 3.5-9.5) (Matias, 1991). The solution was degassed using a vacuum pump for at least 5 min; afterwhich, 0.16 mL riboflavin stock solution was added. The gel mixture was then carefully pipetted in between a plastic gel mould (pre-treated with repel silane, LKB™) and the glass plate, avoiding air bubbles from being trapped. The gel mixture was left to polymerize for one hour under a fluorescent bulb. Electrode strips soaked in respective electrode solutions were then placed on the opposite ends of the gel. Electrode solutions consisted of 1M H_3PO_4 (anode-electrode solution) and 0.1M NaOH (cathode-electrode solution). The gel was prefocused at constant 400 V for 30 min before loading the samples (absorbed in 10 mm x 6 mm filter paper). Differing amounts of protein depending on the isoenzymes to be detected (Table 1) were loaded by putting them 3 cm from cathodic end of

the gel. The time of running was about 2.5 hours at an initial voltage of 400 V increasing the voltage by 200 V after every 30 min up to 1200 V. After electrofocusing, the glass plate (with the gel still anchored on it) was carefully removed from the IEF cooling plate and transferred to ceramic trays.

Detection of Isoenzyme Activity. Gels were incubated with buffered substrate solutions specific for one of the abovementioned isoenzymes (Table 1) at 37°C (Pernin, 1985; Matias, 1991). Substrates (Sigma™) in appropriate buffers with coenzymes and dye complexes include alpha-naphtholphosphate, alpha-naphthylacetate and naphthyl-ASBI-phosphate.

Analysis of Genetic Distances

Construction of Dendrograms. Matrices of genetic distance (*D*) values were calculated from indices of similarity (*I*) among isozyme profiles and then subjected to computer-aided UPGMA cluster analysis for construction of dendrograms where (Nei, 1972):

$$I = \frac{[(Jx)^2 \cdot (Jy)^2]\, Bxy}{\sqrt{(Jx) \cdot (Jy)}} \qquad (1)$$

Jx = band frequency of strain X
Jy = band frequency of strain Y
Bxy = no. of monomorphic bands bet. strains X & Y

and;

$$D = -\log I \qquad (2)$$

RESULTS AND DISCUSSION

Three major groups of *Acanthamoeba* were recognized based on cyst morphology (Figs. 1a-1c). Groupings were made according to the classification scheme by Pussard and Pons (1977). Most C strains exhibited round-shaped cysts including **Ilo1** and **Mocc2** strains and the reference strain, *A. lenticulata*. Polygonal cysts were also observed in some **C** strains (**C**-11, **C**-4) which are the characteristic cystic shape of *A. castellanii* (a pathogenic strain), *A. mauritanensis* and *A. quinalugdunensis*. A clinical isolate, **H**-1, and two **W** strains were also found to be polygonal in shape. The third group consisting of **W**-3, **Bc3**, **Dav4** and **Cot4** showed stellate-shaped cysts.

Geographically isolated strains showed morphological homogeneity based on cyst structure suggesting the wide distribution and adaptability of these organisms. Stellate-shaped cysts appear to be the most widely distributed. Round-shaped cysts seem to be more localized in the central archipelago. An interesting observation

may be noted in the polygonal group to which two pathogenic strains, **H1** and *A. castellanii*, belong.

Figure 2a shows *Acanthamoeba* in trophozoite stage. Numerous dense vacuoles are visible in this wet mount preparation with the diameter varying from 15-45 μm. Stained preparations (Fig. 2b) showed the centrally located, dense karyosome (nucleolus) surrounded by a clear nuclear halo. This has been recognized as one of the distinguishing characteristics of *Acanthamoeba* (Matias et al., 1991).

Soluble protein extracts of *Acanthamoeba* strains showed isoenzyme activities for acid phosphatase, alkaline phosphatase and esterase except for **C**-13 which did not show alkaline phosphatase activity (Figs. 3a-3b). Zymogram patterns of all 20 strains are shown diagrammatically in Figures 4a-4c. Computed Rf values for each band were used to calculate the frequencies of monomorphic or co-migrating bands between any two strains (designated as Bxy) (Table 2). The latter were then used for estimating Table 3 showing similarity index (*I*) and genetic distance using equations (1) and (2) in Materials and Methods. Cluster analysis of genetic distances (*D*) showed two major clusters, clusters A and B, which are 0.615 *D* units apart (Figure 5). Cluster A may be further split into two clusters, A_1 and A_2, with a genetic distance of 0.589. Similarly, cluster B may be subdivided into two clusters, B_1 and B_2, showing a genetic distance of 0.522.

It appears that the distributional patterns of the different *Acanthamoeba* isolates were consistent with the clustering pattern of their respective zymograms. Cluster A comprises the different species collected from the central Philippine archipelago while Cluster B are those collected from the southern part of the Philippine archipelago. However, pathogenic strains *A. castellanii* and **H1** appeared in separate clusters, suggesting that isoenzyme activities for phosphatases and esterase do not reflect pathogenic character. This does not support earlier isoenzyme studies in other closely related groups where common pattern for strains of known pathogenicity has been observed (Pernin, 1984; De Jonckheere, 1982). Isoenzyme analysis of pathogenic and nonpathogenic thermophilic *Naegleria* by Pernin (1984) for seven enzymatic activities including acid phosphatase revealed a common pattern for three pathogenic strains although nonpathogenic strains showed more heterogeneity. Recently, a group of geographically isolated Philippine *Naegleria* strains isolated from a heated swimming pool and a geothermal power plant correlates strongly with a human brain isolate and with *N. fowleri* (a known pathogen) based on isoenzyme pattern and antigenic analyses (Matias, 1991; Yap et al., 1991).

There are seven known pathogenic species of *Acanthamoeba*, namely: *A. astronyxis, A. castellanii, A. polyphaga, A. rhysodes, A culbertsoni, A. palestinensis* and *A. hatchetti* (Warhurst, 1985; Ma et al., 1990). Isoenzyme markers for pathogenicity-related character have not been identified. Since there was only one pathogenic reference strain available for this study, the clinical isolate may belong to any one of the other six species mentioned above. In clusters A_2 and B_2, it would be interesting to test whether the **W** strains, **D4**, **Cot4** and **Mocc2**, are pathogenic or not by means of experimental infection in animals in order to elucidate

more conclusively the possible correlation between pathogenic strains, **H1** and *A. castellanii*, and the abovementioned strains. This, in turn, may give insights on the relationship between isoenzyme activity and pathogenic character.

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Table 1. Staining conditions of enzymes assayed*

Enzyme	Substrate	Dye	Other Reagents	Buffer	Time	Amt. Protein
Acid phosphatase	Alpha-naphthyl phosphate, 100 mg	Fast Garnet BBC 200 mg		Acetate buffer 0.05M, pH 5.0	5 min	25 μg
Esterase	Alpha-naphthyl acetate, 100 mg	Fast Red TR Salt 100 mg	PVP, 500 mg	Phosphate buffer 0.1M, pH 7.4	30 min	100 μg
Alkaline phosphatase	Alpha-naphthyl ASBI-phosphate 300 mg	Fast Red TR Salt 100 mg	PVP, 300 mg NaCl, 1.7 g MgC12.6H20, 20 mg	Tris-HCl buffer 0.2M, pH 9.0	1h	300 μg

*Amounts given are for 100-ml buffer solution.

Table 2. Matrix of Bxy values (number of co-migrating or monomorphic bands)

	C1	C3	C4	C5	C11	C13	W3	W4	W6	Mocc 2	Cot4	H1	Dav4	Ho1	Bc3	*A. lent.*	*A. ql.*	*A. maur.*	Renk	*A. Castel.*
C1		13	10	11	4	4	5	7	5	7	4	5	7	9	11	8	5	7	3	8
C3			10	10	6	4	8	7	5	11	7	6	4	4	7	8	5	8	4	5
C4				7	6	2	6	5	5	11	8	7	4	7	9	10	5	7	2	5
C5					4	3	4	8	6	9	7	8	8	9	11	11	5	7	4	10
C11						9	5	3	6	6	4	4	3	3	6	4	5	3	7	4
C13							5	3	4	6	4	3	4	4	3	3	5	3	7	3
W3								12	10	9	5	3	2	3	3	4	5	8	6	6
W4									13	10	7	4	5	4	4	5	5	9	6	9
W6										7	3	4	4	5	4	3	7	6	8	9
Mocc2											13	12	11	9	9	13	6	12	7	4
Cot4												12	7	6	5	9	3	7	4	3
H1													8	6	7	11	2	6	2	3
Dav4														6	9	9	2	8	4	3
Ho1															9	9	4	6	2	5
Bc3																12	6	5	3	6
A. lent.																	5	9	2	3
A. ql.																		3	8	6
A. maurit.																			5	8
Renk																				4

Table 3. Nei's Genetic Distance (D) calculated from similarity indices of zymogram profiles

	C1	C3	C4	C5	C11	C13	W3	W4	W6	Mocc 2	Cot4	H1	Dav4	Ilo1	Bc3	*A. lent*	*A ql.*	*A maur.*	Renk	*A. Castel.*
C1		0.208	0 352	0 311	0.642	0 626	0 600	0.465	0.600	0 565	0 697	0.623	0.428	0.367	0.301	0 439	0.612	0.516	0.319	0.449
C3			0 331	0 331	0 445	0.605	0.375	0.445	0 579	0.348	0.433	0.523	0.651	0.699	0 477	0.419	0 591	0 438	0.651	0 632
C4				0 517	0.476	0.937	0 530	0.621	0 610	0 378	0 405	0 486	0 681	0 486	0.398	0 352	0.621	0.526	0.982	0 663
C5					0.652	0 761	0.706	0.417	0 530	0.465	0 463	0 428	0.380	0.377	0 311	0 311	0 621	0.526	0.681	0.362
C11						0.176	0.502	0 735	0.423	0.533	0.599	0.622	0.698	0.746	0.466	0.642	0.513	0.786	0.330	0.652
C13							0.486	0.719	0.538	0.517	0.583	0.730	0.557	0.605	0.751	0.751	0 497	0.770	0.314	0.761
W3								0.188	0.255	0 412	0.556	0.801	0 929	0.801	0.822	0 697	0.568	0 415	0 452	0.530
W4									0.153	0.378	0.422	0.688	0.542	0 688	0.709	0 612	0 580	0 375	0.463	0.366
W6										0 521	0.778	0 676	0.628	0 579	0.697	0.822	0.422	0.540	0.327	0.354
Mocc2											0.252	0.310	0.299	0.435	0.456	0 296	0.600	0.349	0.496	0.817
Cot4												0.199	0.385	0.500	0.600	0 345	0.790	0 473	0.628	0.831
H1													0.349	0.523	0 477	0.280	0.989	0.562	0.952	0.854
Dav4														0.474	0.319	0.319	0.940	0.389	0.602	0.806
Ilo1															0.367	0 367	0 688	0 562	0 952	0 632
Bc3																0.263	0.532	0.662	0.796	0.574
A. lent.																	0.612	0 407	0.972	0.875
A. ql.																		0.852	0.338	0.542
A. maurit																			0.593	0 469
Renk																				0.681

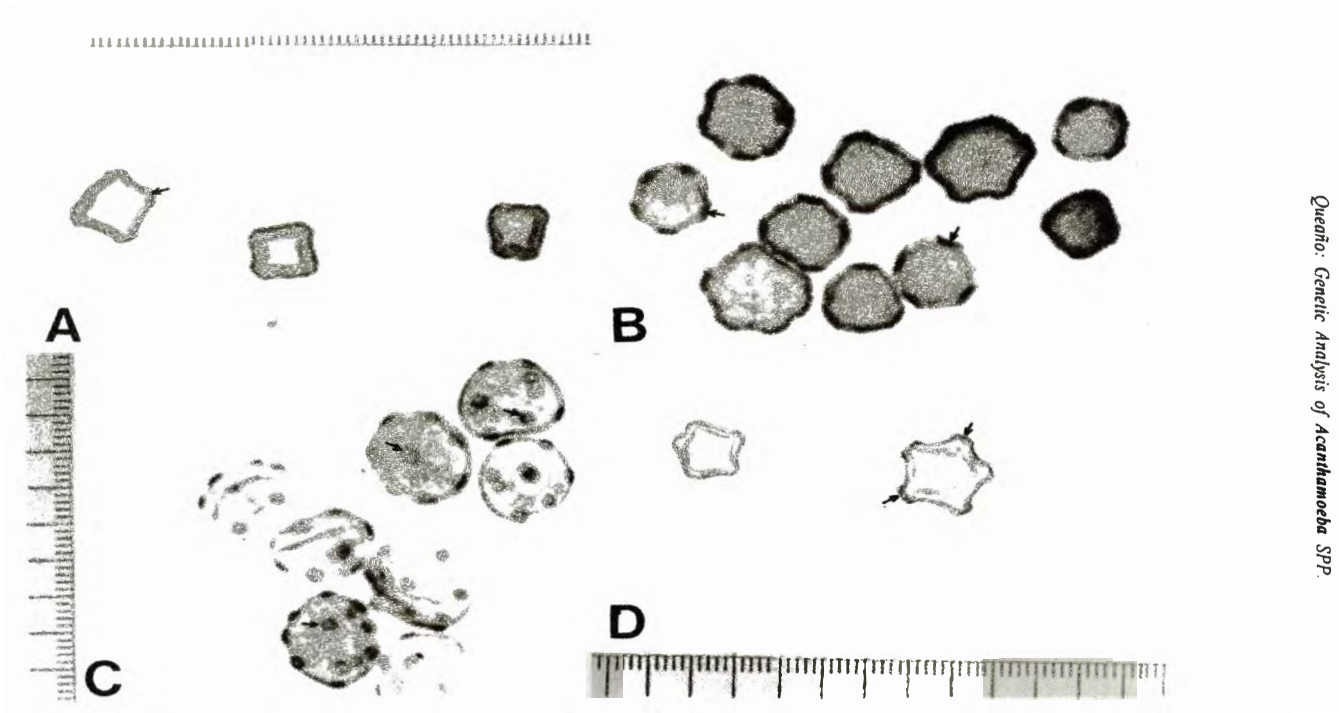

Figure 1. *Acanthamoeba* cysts stained with PATAg r: (a) polygonal cysts with 4-5 sides; (b) polygonal cysts with 6 or more sides; (c) rounded cysts: (d) stellate cysts. Arrows indicate cyst pores. Scale divisions (short bars): 1.19 μm.

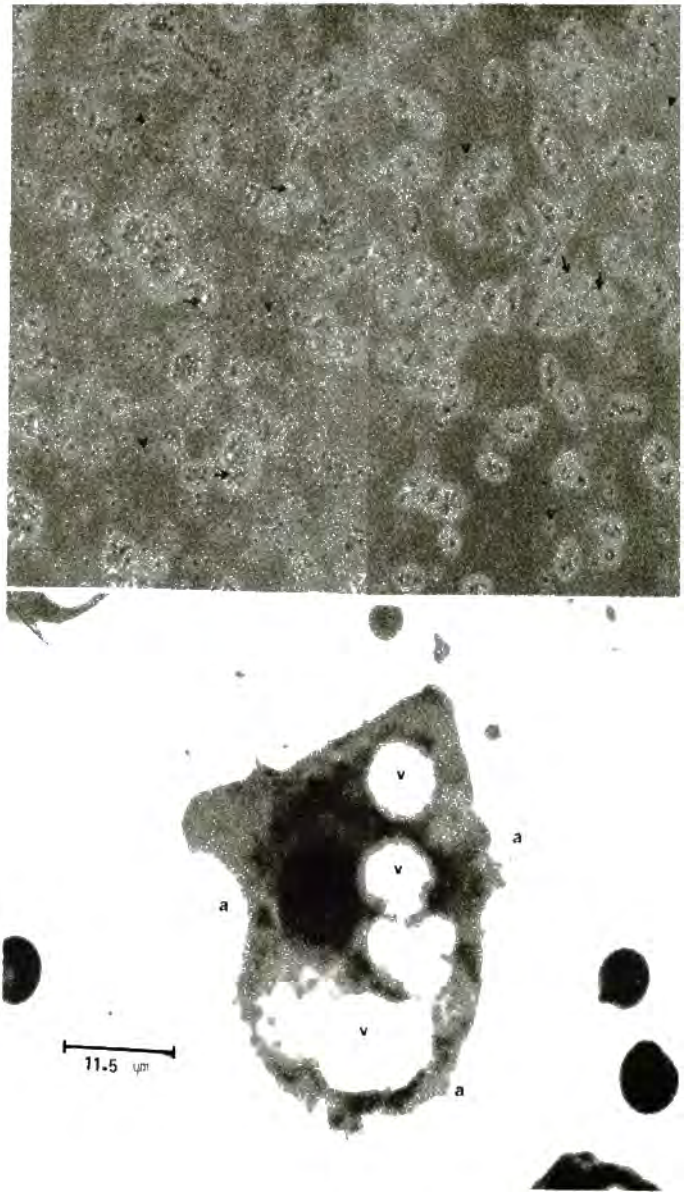

Figure 2. (a) Wet mount preparation of *Acanthamoeba* trophozoites showing dense vacuoles (arrow). Smaller, spherical cells are human RBCs (arrowheads) for size comparisons. Bar: 20 µm. (b) Stained preparation (toluidine blue) of glutaraldehyde-fixed trophozoites. nu – nucleus; nul – nucleolus; ac – acanthopodia; v – vacuoles.

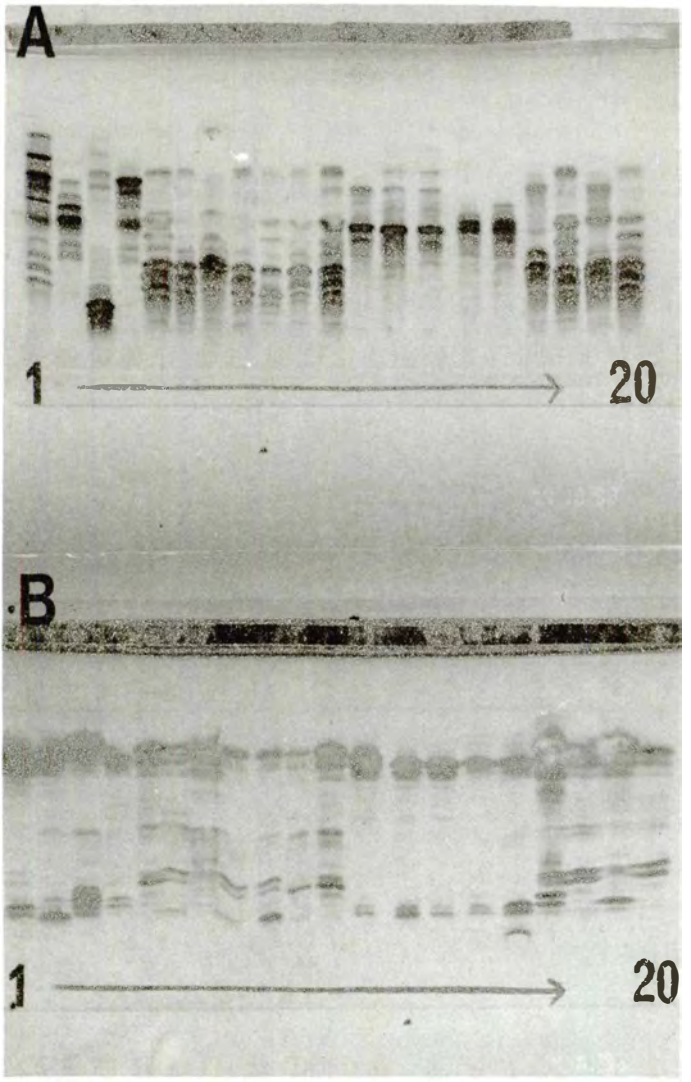

Figure 3. Polyacrylamide gel stained for various isoenzyme activities of soluble protein extracts of *Acanthamoeba* strains isoelectrofocused at 400V-1200V (pH 3.5-9.5). Reference strains: (1) *A. castellanii;* (2) Renk; (3) *A. mauritanensis;* (4) *A. quinalugdunensis;* (6) *A. lenticulata.* Philippine strains: (5) Bc3; (7) Ilo1; (8) Dav4; (9)H1; (10) Cot4; (11) Mocc2; (12) W-6; (13) W-4; (14) W-3; (15) C-13; (16) C-11; (17) C-5; (18) C-4; (19) C-3; (20) C-1. (A) Acid phosphatase activity of soluble protein extracts. (B) Esterase activity of soluble protein extracts. Alkaline phosphatase IEF gel is not shown.

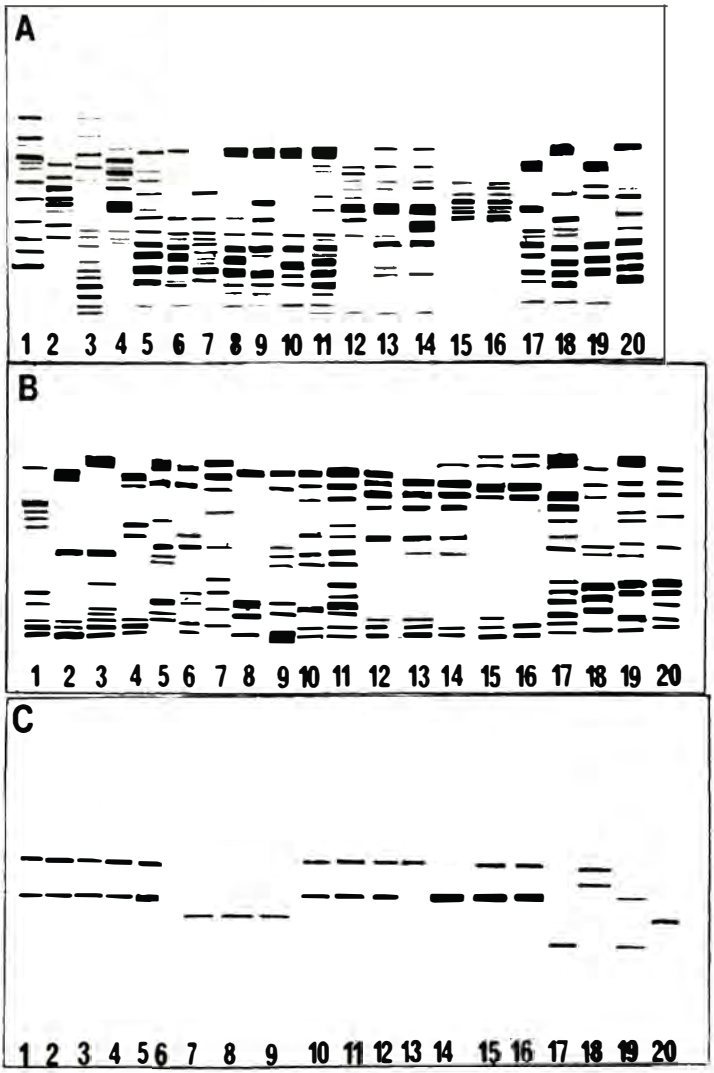

Figure 4. Zymogram patterns of *Acanthamoeba* isolates shown diagrammatically as thin and thick bands. Reference strains: (1) *A. castellanii;* (2) Renk; (3) *A. mauritanensis;* (4) *A. quinalugdunensis;* (5) *A. lenticulata.* Philippine strains: (5) Bc3; (7) Ilo1; (8) Dav4: (9) H1; (10) Cot4: (11) Mocc2: (12) W-6; (13) W-4; (14) W-3; (15) C-13; (16) C-11; (17) C-5; (18) C-4; (19) C-3; (20) C-1. (A) Acid phosphatase zymogram. (B) Esterase zymogram. (C) Alkaline phosphatase zymogram.

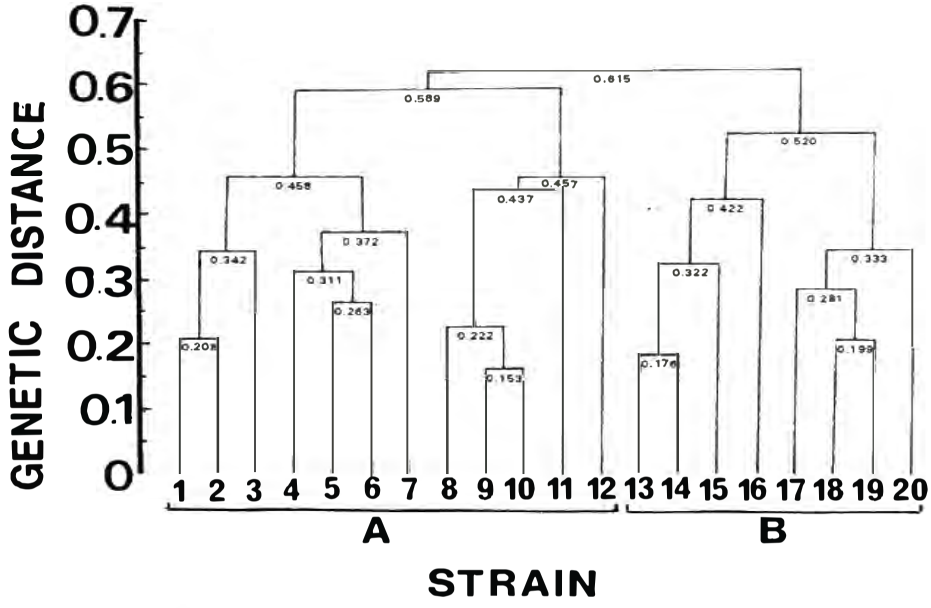

Figure 5. **A dendrogram showing Nei's genetic relationships between major clusters, A and B, and subclusters. Cluster A: (1) C-1; (2) C-3; (3) C-4; (4) C-5; (5) Bc3; (6) *A. lenticulata;* (7) Ilo1; (8) W-3; (9) W-4; (10) W-6; (11) *A. mauritanensis;* (12) *A. castellanii.* Cluster B: (13) C-11; (14) C-13; (15) Renk; (16) *A. quinalugdunensis;* (17) Mocc2; (18) Cot4; (19) H1; (20) Dav4. Cluter A_1: Strains (1) to (7). Cluster A_2: Strains (8) to (12). Cluster B_1: (13) to (16). Cluster B_2: Strains (17) to (20).**