Establishment and Characterization of a Cell Line from the Mosquito *Anopheles albimanus* (Diptera: Culicidae)

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A new cell line designated LSB-AA695BB, was established from embryos of the mosquito Anopheles albimanus. *The primary culture was initiated in April, 1995, and the first passage was made 48 days later. Serial subcultures of the cells have been carried through 90 passages from Abril 1995 to February 1996. The cells were grown at 28°C in MK/VP12 medium, supplemented with 20% fetal bovine serum; the pH tolerance ranged between 6.8 to 7.0. The cells have also been adapted to MM/VP12 medium under the same pH, temperature and serum concentration. The majority of the cells were a fibroblast-type. Isozyme characterization showed a pattern similar to that of* An. albimanus *pupae and adults but distinct from* Ae. taeniorhynchus *and* Ae. albopictus *(C6/36) mosquito cell lines. The culture was shown to be free of mycoplasma, bacteria and fungi. Microsporidia contamination of transovarial transmission was controlled with 6.0 mg/ml of albendazole.*

Key words: cell line - karyotype - isozyme patterns - microsporidia

The mosquito *Anopheles albimanus* Wiedemann is a major vector of human malaria in tropical America. Cell line are currently available for two *Anopheles* species: *An. stephensi* liston (Schneider 1969, Pudney & Varma 1971) and *An. gambiae* Giles (Marhoul & Pudney 1972). Insect cell cultures are useful for fundamental studies of cell physiology, genetics and biochemistry; they are also useful as a substrate to isolate and to identify arboviruses and to study parasites (Vaughn 1976, Buckley 1976, Hink 1980, Maramorosch 1980, 1987, Mitsuahashi 1981, 1983, Grace 1982). In 1995, Bello et al. reported primary cell cultures of *An. albimanus* and described their most significant characteristics, but these cells could not be serially subcultured. This report describes the establishment of a new continuous line of *An. albimanus* cells and some of its characteristics.

MATERIALS AND METHODS

Primary cell cultures - Embryonated eggs were collected using the procedure described before by Bello et al. (1995). The incubation time for these eggs was 28 hr. Sterilization of the eggs was done with a 1.6% sodium hypochlorite solution and with 70% ethanol; during inmersion of the eggs, each of the substances was stirred continuously for 10 min. After washing the eggs three times with destilled sterile water, they were rinsed with the MK/VP12 medium (Pudney & Varma 1971). Finally, 1 ml of egg mass was placed into a 2 ml Ten Broek homogenizer where the eggs were broken mechanically. The resultant suspension was placed in a 25 cm² plastic tissue culture flask containing 5 ml of the growth medium. The cells were maintained in an incubator at 28°C.

Medium - The cell culture was initiated and grown in the MK/VP12 medium, supplemented with 20% fetal bovine serum (FBS) and a mixture of penicillin (100 units/ml) and streptomicin (100 units/ml). Albendazole was used to control microsporidian infections in the embryonic cell explants and also to eliminate the parasite with prolonged treatment through several subcultures. Albendazole (Sigma) was dissolved in dimethyl sulfoxide (DMSO, 2.5 mg/100 ml) and added to the medium, three different concentrations were made: 9.0 mg/ml, 6.0 mg/ml and 3.0 mg/ml. The medium was sterilized by passage through a 0.22-mm millipore filter. After the initial cell growth, the culture medium containing albendazole was changed every two days. Infected cell cultures were exposed to the drug for 60 days. After albendazole treatment, medium containing the drug was replaced by drug-free medium, containing all other supplements (FBS, antibiotics) (Haque et al. 1993)

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Subcultures - The first successful subculture was obtained in April of 1995; since that time, the cells have been maintained for over 90 passages. When confluency was reached, the cell monolayer was scraped with a rubber policeman and the cells were dispersed by gentle pipetting. The first subcultures were developed at a split ratio of 1:2 at an interval of 7 days, however the split ratio was increased gradually up to 1:10 and subcultures were made every 5-6 days. The cells were transferred to 25 cm² flasks, which contained 5 ml of fresh culture medium. The incubation temperature was 28°C and daily observations were performed using an inverted Olympus CK-2 microscope.

Morphological characteristics - Cell morphology was observed and photographed using an inverted microscope with phase contrast and a "Leitz" microphotographic system in increments of 100 to 400X magnification.

Cytogenetic characteristics - Chromosomes were prepared according to the Schneider (1987) technique, with some modifications. Cell exposition time to colchicine solution was 3 hr. For the C-banding softening, four day maturation blank sheets were used; these sheets were submerged in 0.2 N hydrocloric acid for 50 min. After washing with tap water the sample were placed in 5% $Ba(OH)_2$ for 1 min at 53°C, and finally incubated at 54 °C in a double citrate solution (2XSSC) for 1 hr. Staining was done with a 2% Giemsa solution (Motara & Rai 1977, Marchi et al. 1980).

Analyses of isozyme patterns - Isozymatic phenotypes from four systems were examined; phosphoglucose isomerase (PGI-5.3.1.9), phosphoglucomutase (PGM-2.7.5.1), mannose phosphate isomerase (MPI-5.3.1.8) and phosphogluconate dehydrogenase (6-PGDH-1.1.1.4.4). Isozymes were resolved by electrophoresis technique on cellulose acetate, following the procedures described by Brown and Knudson (1980). Cell samples were simultaneously electrophoresed with adult and pupae extracts from *An. albimanus*. The isozyme patterns of our cell line were compared with that of the C6/36 clone of *Ae. albopictus* (Igarashi 1978) and with a culture of *Ae. taeniorhynchus* (Bello et al. 1995). All samples were treated with their own cell buffer, that corresponded to each system. Later the cells were frozen and thawed three consecutive times (liquid nitrogen and room temperature). The cell suspension was centrifuged at 1000g for 10 min and then the supernatant was placed in sample wells with a Zip-Zone applicator (Helena Laboratories B, Texas) and transferred to a cellulose acetate membrane for electrophoresing.

Sterility tests - Cell cultures were periodically checked for sterility, taking two drops of concentrated cells in culture medium and transferring them to tubes containing heart-brain broth infusion or Sabouraud medium. The broth was incubated at 36°C for one week and the medium at room temperature for 15 days. Testing for mycoplasma was carried out by the cytologic method, staining with the fluorescent dye Hoeschst-33258 (Chen 1977, Cahoon et al. 1978, Oro 1984).

Cryopreservation - For freezing and cryopreservation, monolayers 80% confluent were detached and the cells were adjusted to $5X10^6$/ml with fresh medium containing 20% fetal bovine serum and 10% DMSO. The suspension was dispersed into sterile cryotubes and refrigerated at 5°C, frozen overnight at -70°C, and the placed in liquid nitrogen for permanent storage (Hsu et al. 1972, Braude et al. 1986, Léry & Fédière 1990).

RESULTS

Cell line - A record of passage for 90 times is shown in Fig. 1. The cell line was designated LSB-AA695BB. The primary and early subcultures showed slow growth. The estimated population doubling time was about 24 hr. The growth rate gradually increased and became constant after 15 passages. The culture developed a monolayer of firmly attached cells to the flask surface. The initial cells have remained from the embryonic tissue explants were maintained in MK/VP12 medium, with variations in the FBS concentration; beginning from the 10th passage, the FBS was reduced gradually from 20 to 15% and finally to 10% after the 40th passage. Cell cultures were also adapted to the MM/VP12 medium (Varma & Pudney 1969) from passage 25 level onward. The pH range of the medium was 6.8 to 7.0 and the optimum incubation temperature for cells was 28°C. A CO_2 atmosphere was not required. The viability of frozen cells was shown after the second week and also after six months of storage in liquid nitrogen. There was no evidence of cell contamination with mycoplasma, bacteria or fungi. Microsporidia contamination of transovarial transmission was controlled with 6.0 mg/ml of albendazole. When the cell cultures were exposed to the drug for up to 17 subcultures during a two-month period, the parasite was eradicated completely. After drug removal the cell cultures were not reinfected and they have remained microsporidia free. Little control of the parasite was observed at 3.0 mg/ml albendazole, while at 9.0 mg/ml, an infection decrease was observed together with a cell growth reduction, and marked citophatic effect, at four weeks of treatment. Embryonic tissues explanted in albendazole-free medium, showed a high parasite infection by the second week after initiation, together with a high spore population density, and a low cell growth, preventing the formation of a cell monolayer.

Characteristics - The majority of cells had a fibroblastic-type morphology (Fig. 2). The number of diploid chromosomes in metaphase was predominantly 6 (2n=6)(Fig. 3A); tetraploidia were registered in a smaller proportion. Using the C-banding technique, it was possible to precisely identify centromeric regions (Fig. 4).

Isozymatic patterns - The isozymatic phenotypes were one band each for the four systems analyzed (PGI, PGM, MPI, 6-PGDH). These results coincided with those obtained from *An. albimanus* pupae and adult samples simultaneously analyzed with the cell cultures. The average values of relative mobilities of each of the isozymatic systems is shown in Table. They correspond to the present cell line in relation with C6/36 clone and the *Ae. taeniorhynchus* cell line. Diagrams of the isozymatic patterns for each of the different systems of cell line can be observed in Fig. 5.

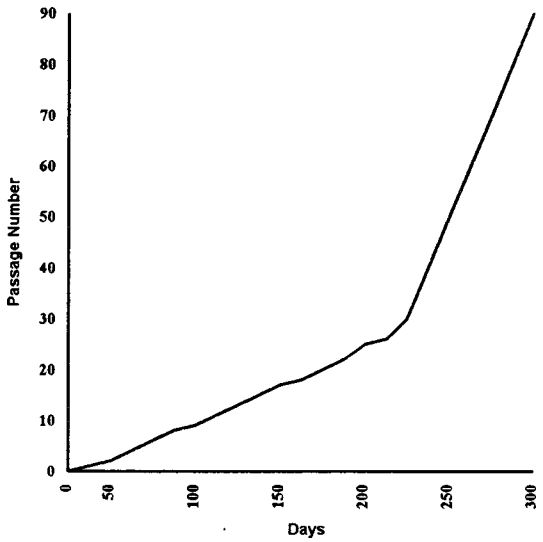

Fig. 1: number of developed passages of the cultured cells within time intervals.

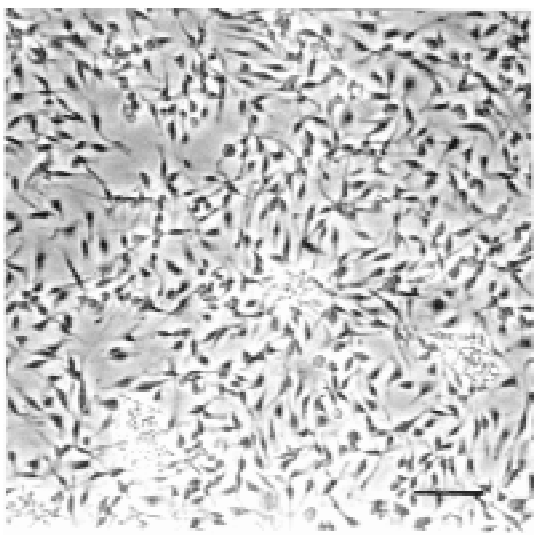

Fig. 2: predominant cell type in the LSB-AA695BB cell line, with fibroblastic cells. Bar=200 mm.

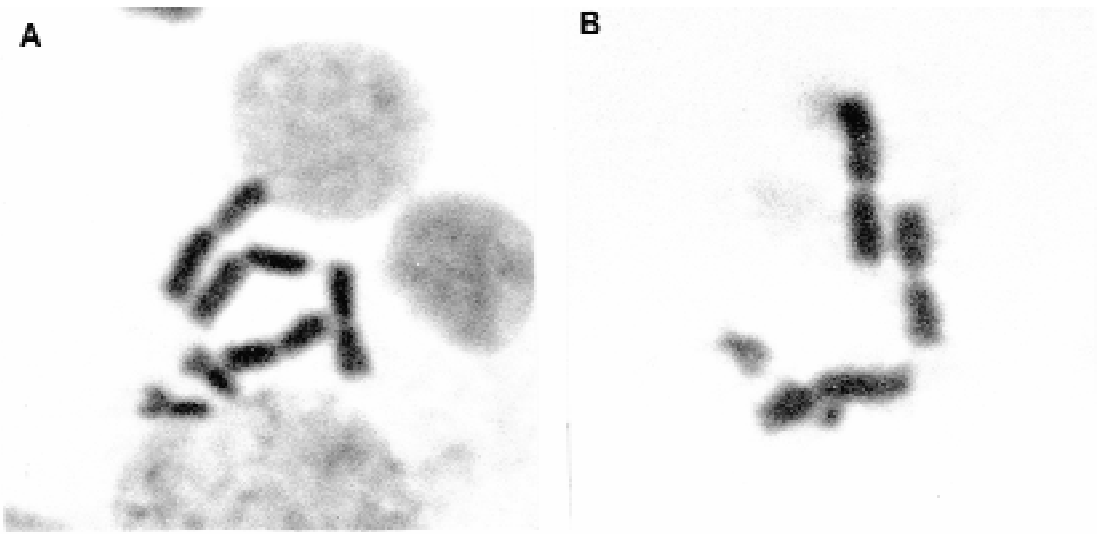

Fig. 3: giemsa-staining metaphase chromosome preparations from LSB-AA695BB cell line showing: A. diploid chromosome complement; B. aneuploid chromosome complement.

Fig. 4: metaphase karyotype from LSB-AA695BB cell line showing centromeric bands.

TABLE

Comparison of the relative isozymatic mobilities of three mosquito cell line

	Isozymes			
Cell lines	PGI	PGM	MPI	6-PGDH
LSB-AA695BB	115.92	87.1	63.23	120.05
Aedes taeniorhynchus	104.6	74.65	51.66	94.11
			86.4	
C6/36[a]		79.77	46.37	
	100	100	100	100

a: isozymatic mobilities were recorded relative to the C6/36 isozyme patterns.

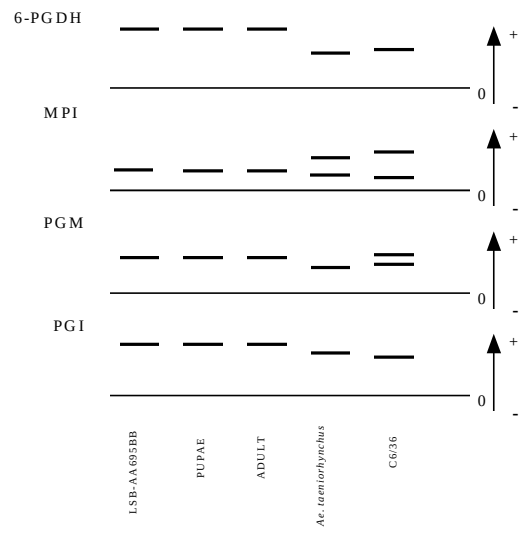

Fig. 5: diagrammatic representation and comparison of the isozymatic patterns from four systems.

DISCUSSION

The effective antimicrosporidial activity of albendazole, here reported, coincided with previous observations (Haque et al. 1993, Ditrich et al. 1994, Weiss et al. 1994). However, in those studies albendazole was used to test the *in vitro* antimicrosporidial activity on induced infections, while in the present report, the drug was used to control and eliminate natural infections against microsporidia of transovarial transmission, which were explanted from infected embryos. This antimicrosporidial treatment was necessary to develop a continuous cell line. It was also possible to isolate and maintain *in vitro* microsporidia of transovarial transmission, with the present technique of cell cultures, without the drug.

The *An. stephensis* and *An. gambiae* cell culture were initiated with recently eclosionated larvae; these were cut into fragments and submitted to enzymatic treatments. In the present cell line, primary cell cultures were obtained from embryonated eggs in a relatively short time; the first successful subculture was made after six weeks.

Primary cell growth pattern and morphology coincided with the description by Bello et al. (1995) for their species; except that the first successful subculture of the former was obtained after only two weeks. The slower growth of the present culture may have been due to the presence of parasites, the effect of the antimicrosporidial drug, and/or because of the frequent medium changes necessary to dilute the free spores.

The cell morphology of the present line was mostly of the fibroblast-type, with characteristics similar to those described for the *An. stephensis* var. *mysorensis* cell culture (Pudney & Varma 1971) but different from that reported for *An. gambiae* (Marhoul & Pudney 1972) and *An. stephensis* (Schneider 1969), which were predominantly epithelioid.

The diploid number of chromosomes for the cell line is 2n=6; this coincided with the number established for most *Anopheles* with the exception of the *Chagasia* genus, whose chromosomatic complement was of 4 pairs (2n=8) (Clements 1992).

In cultures exposed to albendazole for a relatively prolonged time (3 months), it was noticed that, many of metaphases showed dispersed chromosomes with a tendency toward aneuploids (Fig. 3B). Structural type chromosomic aberrations were also noted, most frequently the duplication of short arms in one of the chromosomes of pair 2. However, after removal of the drug and upon subculturing up to 30 passages, the cytogenetic analysis showed normal cells, mostly diploid metaphases.

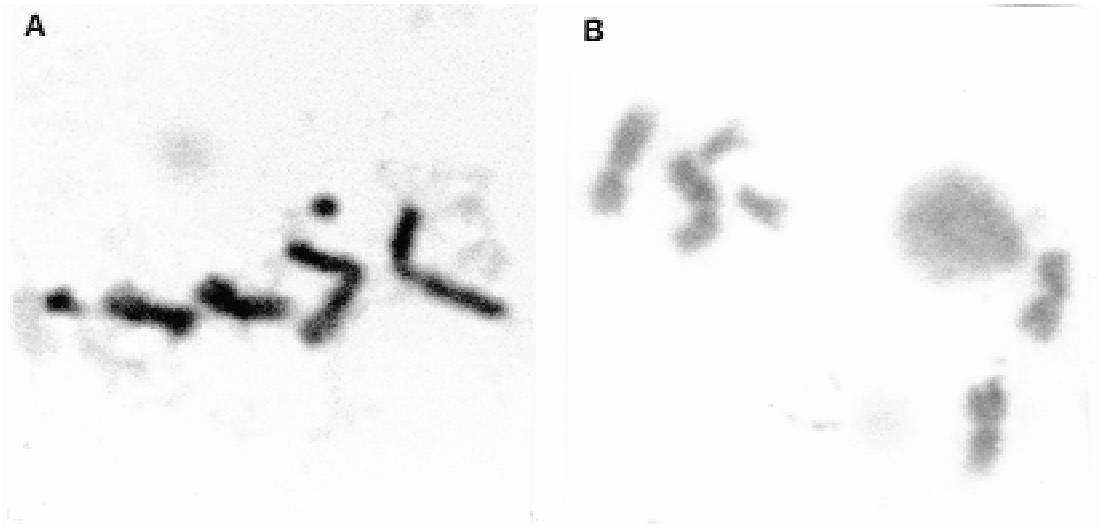

Fig. 6: metaphase karyotypes from LSB-AA695BB cell line A: male; B: female.

In the karyotype, pair 1 is the smaller, heteromorphic sexual chromosome, this description is similar to that reported for somatic chromosomes obtained from brain and gonad cells of *An. albimanus* as reported by Hobbs (1962). In the female XX pair and the male XY (Fig. 6), the X chromosome is acrocentric whereas the Y is smaller and of irregular shape; as a general feature, At metaphase, the majority (90%) of the cells were of the female XX karyotype; this distribution is probably a random one and not related to adaptive changes. When hundreds of embryonic eggs are observed, the sexual ratio is expected to be in similar proportions, however, it is possible that this ratio may deviate in some years. Braude-Zolotarjova et al. (1986), observed that in eggs cultures of *Drosophila virilis* cells observed for six years. Female XX karyotype cells were predominant the first year; later, the male karyotype increased and by passages 120, five years later, female cells were completely replaced by cells with male XY karyotype.

The isozymatic phenotypes of this cell line were identical with those obtained from *An. albimanus* pupae and adult samples, which indicates that cells in the cultures were representative of the original species. However, when isozymatic patterns of this cell line were compared with zymograms of *Ae. taeniorhynchus* cells and the C6/36 clone, a characteristic and different isozymatic profiles were observed for each of above mentioned lines.

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