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# GROWTH AND MAINTENANCE OF *AEDES ALBOPICTUS* CELL LINE, CLONE C6/36, IN DIFFERENT MEDIA.*

Maria Luisa, BARBOSA**
Iray Maria ROCCO**
Julia M. M. Souza FELIPPE**
Aurea Silveira CRUZ**

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SUMMARY: The sensitivity of *Aedes albopicts* cell line, clone C6/36, to arbovirus isolation and growth has been being shown by several authors. The present work has verified which, among several media under the same conditions, would be the most efficient one for C6/36 cultivation and viral isolation. The L-15 medium has proved to be the best among others (MEM, DMEM and 199) with the quickest viral isolation (DEN-1). Also, in this medium, the samples could be observed until the $14^{th}$ day, without significative cellular death. The results reccommend L-15 medium as the most efficient and economic one for the purposes.

## INTRODUCTION

Among the arboviruses that infect man, dengue flavivirus is one of the most important. Paul, *et al.* (1969) shoud, for the first time, that *Aedes albopictus* mosquito cells were susceptible for the dengue virus isolation. Since then, different mosquito cell lines have been tested with the same purpose (Tesh, 1979; White, 1987; Kuno *et al.*, 1971; Kuno *et al.*, 1985). Based on the advantages offered by this methodology such as: they are more sensitive than the vertebrate cell culture system, are easier to manipulate, to maintain and to grow at ambient temperature (Chappell *et al.*, 1971) and remain as stable cultures for 14 days without medium change (Race *et al.*, 1979).

Despite the existence of other mosquito cellular cultures for dengue isolation, the *Aedes albopictus* cell line, clone C6/36, has proved to be one of the most sensitive (Igarashi, 1978). In these cells the dengue virus infection can be easily observed by the characteristic cytopathic effect (CPE) (Kuno, 1983; Kuno *et al.*, 1985; Gubler *et al.*, 1984).

In this work, studies with the dengue-1 virus (DEN-1) in C6/36 cells were made, in different media, to verify the suitability between cells and medium that provides advantages in the isolation and propagation of dengue virus.

* Key Words: C6/36 cells. Cellular culture medium. Viral isolation. Cytopathic effect. Dengue virus.

** Instituto Adolfo Lutz, Av. Dr. Arnaldo, 355 – São Paulo – SP – CEP: 01246-900 – Brazil.

## MATERIALS AND METHODS

*a) Growth and Maintenance Media*

The following media were tested:

–. Leibowitz nº. 15 (L-15 – Sigma) modified with TPB (Tryptose Phosphate Broth), 2.95% of non essential aminoacids solution and 2% of L-glutamin.

– 199 medium (Gibco).

– Minimum Eagle Medium (MEM – Adolfo Lutz Institute, Cellular Culture Laboratory) according to Rechcigl (1977) supplemented with 0.2mM of non-essential aminoacids solution.

– Dulbecco Minimum Eagle Essential Medium (DMEM – Sigma).

All media were supplemented with 10% heat-inactivated at 56°C for 30 minutes of foetal bovine serum and contained 10 I.U/ml of penicillin and 100 uL/mL of streptomycin; for cell maintenance, 2% of FBS was added.

*b) Cellular Adaptation to Media*

*Aedes albopictus* cell line (clone C6/36) cultures, with $5x10^5$ cells/ml, kindly supplied by Dr. Igarashi, Department of Preventive Medicine, Research Institute of Microbial Diseases, Osaka University (Osaka, Japan), were used. The C6/36 cells were adapted to L-15, 199, MEM and DMEM media, through ten serial passages. For each passage, the cells were propagated in four 250 mL glass flasks (500,000 cells/mL) with different medium each and observed to verify their integrity.

After the $10^{th}$ passage, the culture tubes were prepared as follows: one million cells with 2 mL of growth media (L-15, MEM, DMEM or 199) were propagated and incubated at 28°C for 24 hours. The grouth media was then substituted by their respective maintenance media and then the cells were infected with 30 μL. of different inocules. The tubes were incubated at 28°C for at least ten days.

*c) Viral Growth Test in Different Media*

For the viral growth test in cells cultivated in different media, one sample of a DEN-1 patient isolation from serum, identified and typified by immunofluorescence (Gubler *et al.*, 1984) using monoclonal antibodies supplied by the Division of Vector Borne Virus, Center for Infectious Diseases, Centers for Disease Control, Fort Collins, CO, was used.

The $2^{nd}$ and $14^{th}$ passages of this sample were made and inoculated in the C6/36 cells grown in different media. The inoculum consisted of 30 μL of viral suspension which titre was $10^{7.37}$/100 mL or diluted from $10^{-1}$ to $10^{-6}$. Three tubes of each media per sample dilution and three control tubes were used for each media. All tubes were observed daily and immunofluorescence tests were performed.

*d) Human Sera*

To verify the sera toxicity on the clone C6/36 in the different media tested, 40 patients sera were picked up at random, from a universe of 2,117 dengue suspected sera to viral isolation. Later, other 40 samples of 142 DEN-1 positive sera (isolation and immunofluorescence) were used for the re-isolation in the clone C6/36 kept in L-15 medium. All samples tested came from sera harvested during the 1990/1991 epidemy in the State of São Paulo. The inoculation volume of the sera was defined to be 30 uL after tests with volumes ranging from 10 to 50 uL. The toxic effect was intense with volumes above to 40 uL.

*e) Isolation in L-15 Media and Immunofluorescence*

After 24 hours of the cells' growth, tubes containing L-15 media modified were innoculated with 30 uL of each serum sample. Ten test tubes were used for each one of the 40 tested sera. They were observed with inverted microscope (Zeiss) from the first to the tenth day, accompanied by immunofluorescence daily tests (Gubler *et al.*, 1984).

TABLE 1

C6/36 cells' Adaptation to the Different Media

| Passage | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Media | | | | | | | | | | |
| L - 15 | + | + | + | + | + | + | + | + | + | + |
| MEM | + | + | + | + | + | + | + | + | (*) | (*) |
| DMEM | + | + | + | + | + | + | + | (*) | (*) | (*) |
| 199 | + | + | + | + | (*) | * | # | @ | @ | @ |

+ – Interger cellular monolayer, few cells on the supernatant.

(*) – Cellular monolayer showing first degeneration signs.

* – Cellular monolayer with 50% of fallen cells.

# – Cellular monolayer completely degenerated, 100% of fallen cells.

@ – Not possible.

TABLE 2

Cytopathic effect observed in infected C6/36 cells, maintened in different media. Second passage virus.

| Day | Medium | C | $10^{0}$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | L-15 | - | - | - | - | - | - | - | - |
| | DMEM | - | - | - | - | - | - | - | - |
| | MEM | - | - | - | - | - | - | - | - |
| | 199 | - | - | - | - | - | - | - | - |
| 2 | L-15 | - | - | - | - | - | - | - | - |
| | DMEM | - | - | - | - | - | - | - | - |
| | MEM | - | - | - | - | - | - | - | - |
| | 199 | - | - | - | - | - | - | - | - |
| 3 | L-15 | - | (+) | - | - | - | - | - | - |
| | DMEM | - | - | - | - | - | - | - | - |
| | MEM | - | - | - | - | - | - | - | - |
| | 199 | - | - | - | - | - | - | - | - |
| 4 | L-15 | - | + | (+) | - | - | - | - | - |
| | DMEM | - | - | - | - | - | - | - | - |
| | MEM | - | - | - | - | - | - | - | - |
| | 199 | - | - | - | - | - | - | - | - |
| 5 | L-15 | - | + | + | (+) | - | - | - | - |
| | DMEM | - | (+) | - | - | - | - | - | - |
| | MEM | - | (+) | - | - | - | - | - | - |
| | 199 | - | - | - | - | - | - | - | - |
| 6 | L-15 | - | + | + | + | (+) | - | - | - |
| | DMEM | - | + | (+) | - | - | - | - | - |
| | MEM | - | + | (+) | - | - | - | - | - |
| | 199 | (*) | (+) | - | (*) | (*) | - | - | - |
| 7 | L-15 | - | + | + | + | + | (+) | - | - |
| | DMEM | - | (*) | (*) | + | (+) | - | - | - |
| | MEM | - | (*) | (*) | (+) | (+) | - | - | - |
| | 199 | * | * | (*) | * | * | (*) | (*) | - |
| 8 | L-15 | - | + | + | + | + | (+) | - | - |
| | DMEM | (*) | * | * | + | (+) | - | - | - |
| | MEM | (*) | * | * | * | * | - | - | - |
| | 199 | * | * | * | * | * | * | * | (*) |

- : No CPE

(+): Less than 25% of cells with CPE

+ : Plus than 75% of cells with CPE

(*) : 25% or less of dead cells

* : 75% or more of dead cells

C -: Not inoculated control cells.

TABLE 3

Cytopathic effect observed in infected C6/36 cells, maintened in different media. Fourteenth passage virus.

| Day | Medium | C | $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | L-15 | - | (+) | - | - | - | - | - | - |
| | DMEM | - | - | - | - | - | - | - | - |
| | MEM | - | - | - | - | - | - | - | - |
| | 199 | - | - | - | - | - | - | - | - |
| 2 | L-15 | - | + | (+) | - | - | - | - | - |
| | DMEM | - | - | - | - | - | - | - | - |
| | MEM | - | - | - | - | - | - | - | - |
| | 199 | - | - | - | - | - | - | - | - |
| 3 | L-15 | - | + | + | (+) | (+) | - | - | - |
| | DMEM | - | - | - | - | - | - | - | - |
| | MEM | - | - | - | - | - | - | - | - |
| | 199 | - | - | - | - | - | - | - | - |
| 4 | L-15 | - | + | + | + | (+) | (+) | - | - |
| | DMEM | - | (+) | - | - | - | - | - | - |
| | MEM | - | (+) | - | - | - | - | - | - |
| | 199 | - | (+) | - | - | - | - | - | - |
| 5 | L-15 | - | + | + | + | + | (+) | - | - |
| | DMEM | - | (+) | (+) | - | - | - | - | - |
| | MEM | - | (+) | (+) | - | - | - | - | - |
| | 199 | (*) | (+) | (+) | (+) | - | (*) | - | - |
| 6 | L-15 | - | + | + | + | + | + | - | - |
| | DMEM | - | * | + | (+) | (*) | - | - | - |
| | MEM | - | * | (*) | (*) | (*) | (*) | - | - |
| | 199 | * | (*) | (*) | (*) | - | * | - | - |
| 7 | L-15 | - | + | + | + | + | + | (+) | - |
| | DMEM | - | * | * | * | (*) | (*) | - | - |
| | MEM | (*) | * | * | * | (*) | - | - | - |
| | 199 | * | * | * | * | * | * | - | (*) |
| 8 | L-15 | - | + | + | + | + | + | (+) | - |
| | DMEM | - | * | * | * | * | (*) | - | - |
| | MEM | - | * | * | * | * | * | - | - |
| | 199 | * | * | * | * | * | * | * | * |

- : No CPE

(+): Less than 25% of cells with CPE

\+ : Plus than 75% of cells with CPE

(*) : 25% pr less of dead cells

* : 75% or more of dead cells

C - : Not inoculated control cells.

TABLE 4

Toxicity Evaluation of Inoculated Sera on the Different Media Over C6/36 Cells.

| DAY | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|
| MEDIA | | | | | | | | | |
| | C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| L-15 | C+ | (+) | (+) | + | + | + | + | + | + |
| | S | 2.5* | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| | C | 0 | 0 | * | 0 | 0 | 0 | 0 | 25 |
| DMEM | C+- | - | - | - | (+) | + | + | 50 | 100 |
| | S | 12.5 | 12.5 | 12.5 | 25 | 25 | 32.5 | 32.5 | 32.5 |
| | C- | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 25 |
| MEM | C+ | - | - | - | (+) | + | 50 | 50 | 100 |
| | S | 37.5 | 37.5 | 37.5 | 50 | 50 | 55 | 55 | 55 |

C- : Not inoculated control cells.
C+ : Control cells inoculated with DEN- 1 in the 14th passage.
S : Inoculated sera samples.
- : Interger cells.
(+) : Less than 25% of cells with CPE.
+ : More than 75% of cells with CPE.
* : Percentage of tubes with fallen cells.

All tests were made in glass containers incubated in common bacteriologic incubator. No $CO_2$ incubator was used in any step of this work.

## RESULTS

### *1 - Adaptation of C6/36 Cells to Different Media*

The C6/36 cell the showed best adaptation to L-15 media (table 1), not showing any significative signs of degenation until the 10$^{th}$ day.

In the MEM and DMEM media, dead cells were observed in the supernatant, after the 8$^{th}$ day and the monolayer showed signs of loss of confluency.

The 199 medium was the less adequate, once in the 5$^{th}$ passage more than 50% of the cells were dead, the cellular monolayer was not confluent in several points and the monolayer was unfastened.

The cells were observed until the 50$^{th}$ day only in the L-15 media and they had good aspect until the 14$^{th}$ day. After the 15$^{th}$ day, the tubes showed a progressive rise in the number of unfastened cells on the supernatant. Nevertheless, transfers made with bottles maintained for 50 days without medium change showed recuperation of the monolayer and sensitive cells for the dengue isolation.

### *2 - Test of viral Growth and Demonstration of CPE in Different Media*

The cell cultures were maintained in different media and inoculated with DEN-1 virus at different passages (2$^{nd}$ and 14$^{th}$) and at the dilutions previously cited, showed CPE from the 3$^{rd}$ day on with virus from the 2$^{nd}$ passage and from the 1$^{st}$ day with virus from the 14$^{th}$ passage in cells with L-15 media. This CPE was observed only in cells with MEM and DMEM from the 5$^{th}$ and 4$^{th}$ days on, with the passages 2 and 14 respectively, and this CPE was significantly less intense. With 199 medium this effect was seldom observed. Figure 1 shows the DEN-1 effect 48 hours after inoculation in C6/36 cell line with the different media used and their controls.

### *3 - Test of Toxic Effect of Sera Used for Isolation in Clone C6/36 Cells, in Different Media*

Obtained results showed the effectiveness of L-15 media in relation to the toxic effect of the sera over the cells (table 4).

From 40 tubes inoculated with different sera, only 2.5% of cellular unfasting after 24 hours occurred in cells with L-15 media and 12.5% and 37.5% in cells with DMEM and MEM media, respectively. These proportions increased in subsequent days. The observation of the tubes was impossible from the 9$^{th}$ day on because the tubes of the negative controls

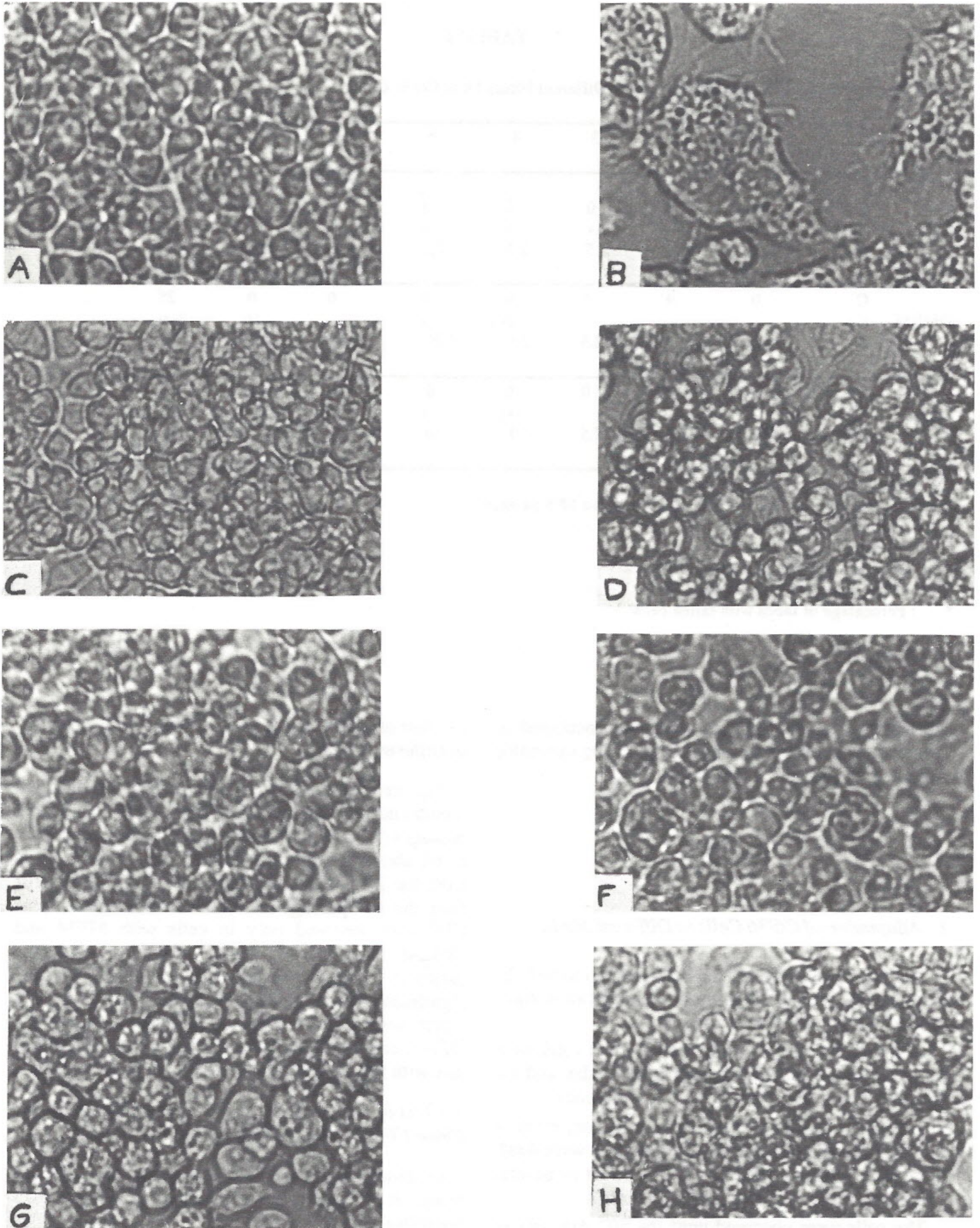


FIGURE 1

Morphology of C6/36 *Aedes albopictus* cell line grown in different media. Carl Zeiss optic microscope JE Naval, X125.

A, C, E and G: control cells in L-15, MEM, DMEM and 199 media respectively; B, D, F, and H: same media but inoculated with 30 ul of a $10^{-7}$. 37/100 ul dilution of DEN-1.

showed a large number of unfastened cells in media other than L-15, were cells presented no alteration until the last day of observation. 199 medium was not used in this test as it was already verified the cells did not maintain their integrity after the 5th passage.

*4 – Isolation and Immunofluorescence*

It was verified that C6/36 cells, maintained in L-15 medium and inoculated with patients sera showed CPE in 25% of them after 72 hours. The immunofluorescence revealed that 20% of the samples tested resulted positive after 24 hours of inoculation (table 5).

TABLE 5

TABLE 5 – Chronological Order of Positivity by CPE Occurrence and Immunofluorescences in 40 Sera Samples Inoculated in C6/36 Cells in L-15 Medium.

| Days after Inoculation | CPE | Immunofluorescence | |
|---|---|---|---|
| 1 | 0 (0.0%) | 80 | (20.0%) |
| 2 | 0 (0.0%) | 100 | (25.8%) |
| 3 | 100 (25.0%) | 90 | (22.5%) |
| 4 | 150 (37.5%) | 10 | (2.5%) |
| 5 | 110 (27.5%) | 10 | (2.5%) |
| 6 | 30 (7.5%) | 10 | (2.5%) |
| 7 | 10 (2.5%) | 0 | (0.0%) |
| Total | 400 (100%) | 300 | (80%) |

For each serum sample 10 tubes were made and observed for both CPE and immunofluorescence.

## DISCUSSION

The *Aedes albopictus* cell line clone C6/36's high grade of susceptibility has been shown in the dengue virus isolation (Igarashi *et al.*, 1978). The identification of the isolated virus was at first made by the hemmaglutination inhibition test (Clarke *et al.*, 1958) and afterwards by complement fixation (Kuberski *et al.*, 1977). However, the sensibility was higher when the immunofluorescence technique with anti-Dengue monoclonal antibodies specific type sera, was used (Gubler *et al.*, 1984).

Kuno *et al.* (1985), showed that the detection of dengue virus in C6/36 cells is directly linked to the viral titre and incubation period, as well as the fusion of infected cells with formation of syncitia is influenced by the infecting viral strains, the pH and composition of media, the culture age and also by the kind of container surface were the cells are being cultivated (plastic or glass). (Randolph et al, 1990).

Several authors (White, 1987; Gubler *et al.*, 1984; Tesh, 1979; Igarashi, 1978; Kuno *et al.*, 1985) tested the growth and better culture conditions for C6/36 cell line and its utilization in viral isolation, using different growth media and cell line maintenance. However, no one tested the several media under the same conditions, what makes impossible to determine which is the most adequated medium for cellular growth and viral isolation. To know which is the best medium is of extreme importance during the dengue epidemics once it is when an efficient virus isolation method is essential.

This work's universe is 142 patient sera, with positive isolation of DEN-1, proceeding from the epidemic area in São Paulo state.

In the first isolated samples, when C6/36 cells in L-15 were used, a decrease on the time of CPE occurrence was observed. Afterwards, 40 samples were taken randomly from the previous 142 so that a more accurate analysis of the results to verify the initial observation (tables 2, 3 and 5) could be made. A real decrease of 72 hours between the inoculation and the CPE occurrence was found (figure 5). Data showed that the L-15 media is the most efficient in the present conditions, being a more rapid method of viral isolation.

Another advantage of L-15 media is that the samples can be observed until the 14th day without significative cellular death. This is not the case with MEM, DMEM and 199 media, particularly with the last one which had a high number of dead cells in the supernatant from the 5th day on (tables 2 and 3).

The results are intriguing, due to the composition of the several media. It is known that the 199 medium is extremely rich of vitamins, which is not the case with L-15 medium, as well as in the non-essential aminoacids composition. As far as MEM is concerned, it is the poorer in aminoacids and salts. All the tested media have bicarbonate as buffer agent except the L-15 which buffering are base-free aminoacids. The acid metabolites percentage is reduced by the substitution of glicosis for galactosis and sodium piruvirate.

Therefore it can be inferred that one or more L-15 factors or components may interfere positively in the production of the cytopathic effect in the cell-virus interation. Since these results were obtained with incubation in common bacteriologic incubators, they may be different if $CO_2$ incubator is used.

Data presented are extremely important due to the necessity of this laboratory of producing viral antigens, as well as viral isolation and using for them the most efficient and economic media.

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BARBOSA, M.L.; ROCCO, I.M.; FELIPPE, J.M.M.S. & CRUZ, A.S. – Crescimento e manutenção de linhagem celular *Aedes albopictus* clone C6/36 em diferentes meios. *Rev. Inst. Adolfo Lutz, 53*(1/2):63-70, 1993.

RESUMO: A sensibilidade da linhagem de células *Aedes albopictus,* clone C6/36, para isolamento e crescimento de arbovírus, tem sido demonstrada por vários autores. O presente trabalho verificou, qual entre vários meios, nas mesmas condições, seria mais eficiente para cultivo de C6/36 e isolamento viral. O meio L-15 mostrou ser o melhor entre outros (MEM, DMEM e 199), com mais rapidez no isolamento viral (DEN-1). Também, nesse meio, as amostras puderam ser observadas até o 14º dia, sem morte celular significativa. Os resultados recomendam o meio L-15 como o mais eficiente e econômico para os propósitos apresentados.

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