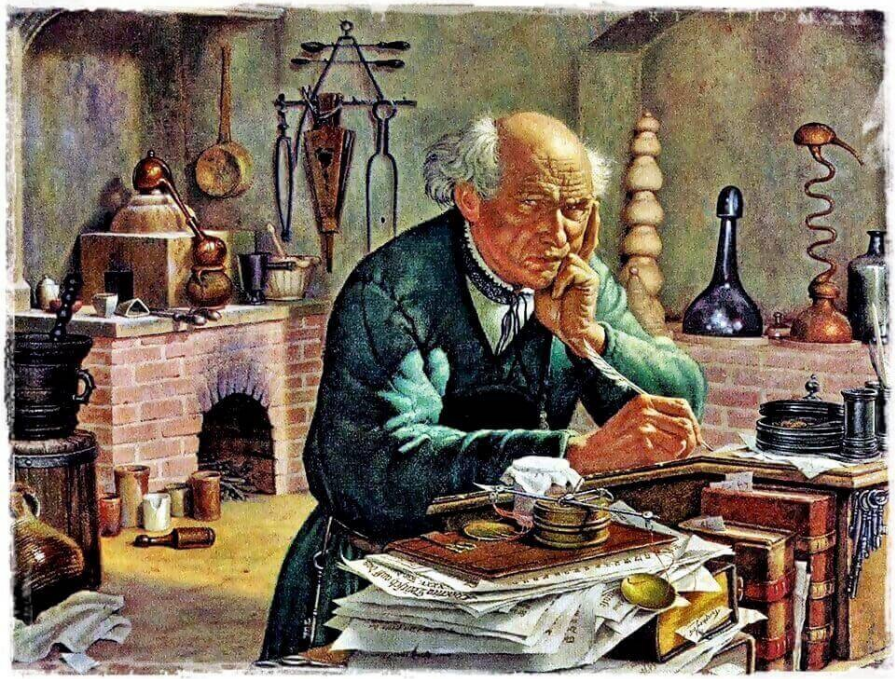
# Toxicologia e outras áreas de investigação e tecnologia – células epiteliais do rim do macaco verde

- Teresa Dias –
mtdias@fc.ul.pt

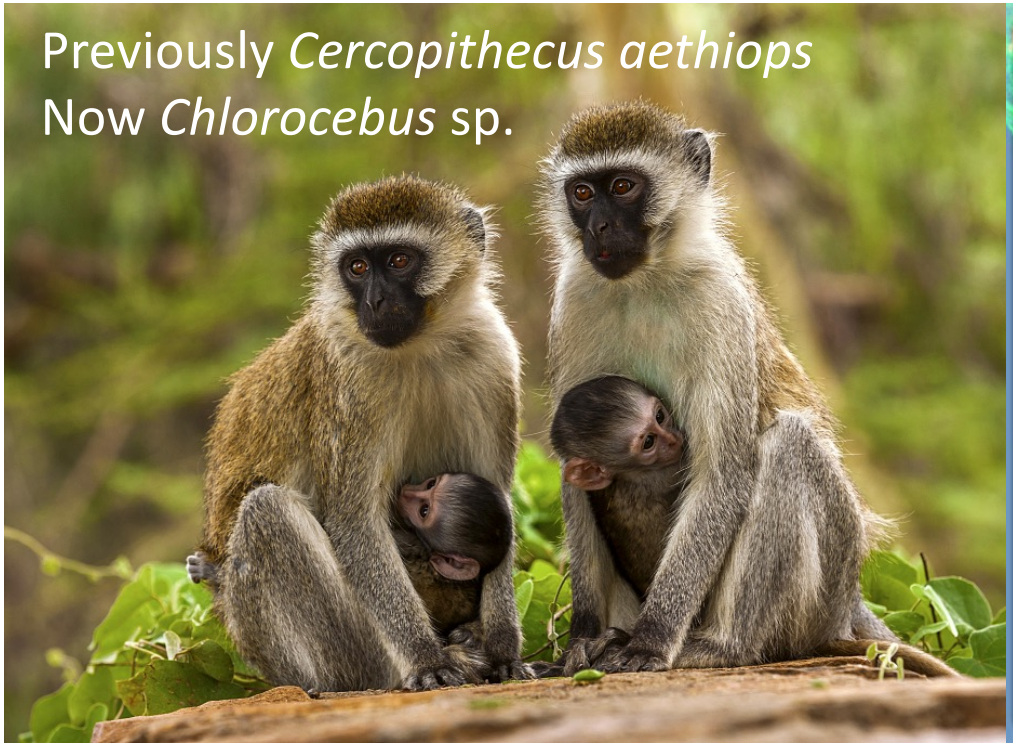


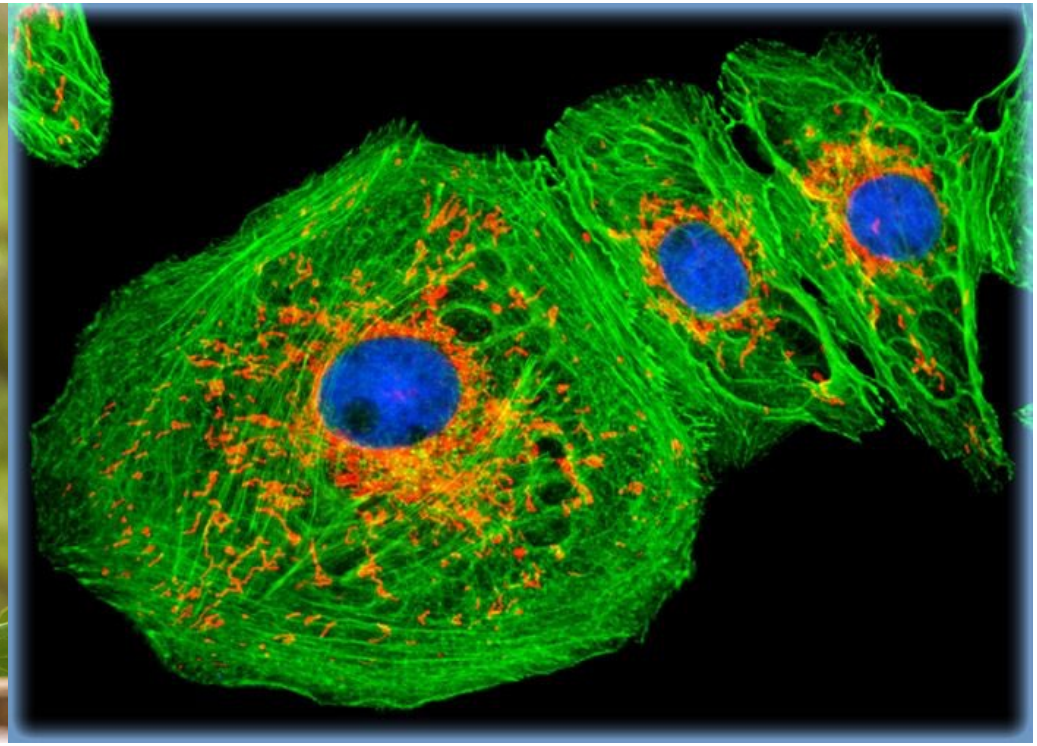
“All substances are poisons. There is none which is not a poison. The right dose differentiates the poison from a remedy”

Philippus Aureolus Theophrastus Bombastus von Hohenheim, mais conhecido por Paracelcius (1493-1541) foi um médico, alquimista, físico, astrólogo e ocultista Suiço-alemão.
Seu pseudónimo significa "superior a Celso (médico romano)".
Também é aclamado pelo seu trabalho na Química e como fundador da Bioquímica e da Toxicologia.

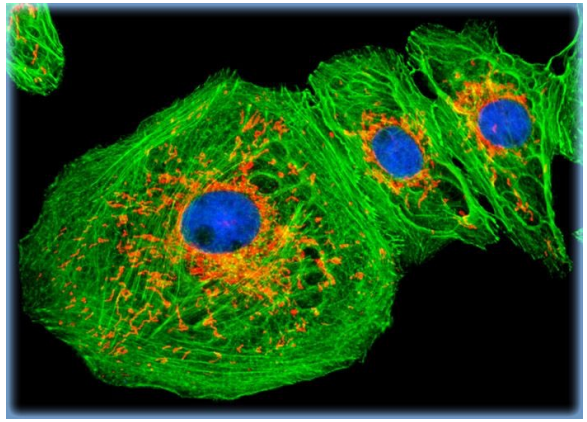
# E assim nasce a… toxicologia

# Células epiteliais do rim do macaco verde africano (”Vero cells”)



# Células epiteliais do rim do macaco verde africano (”Vero cells”)

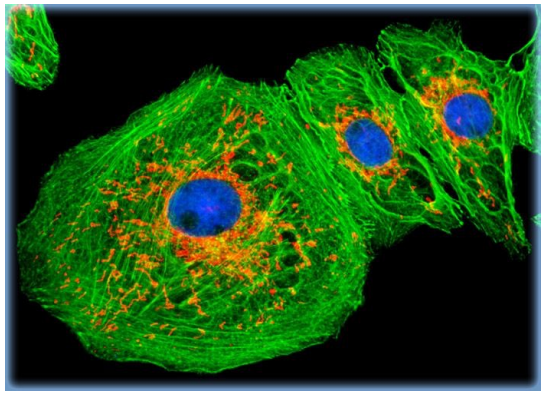


**Vero cells** are a lineage of cells used in cell cultures. The lineage was developed in 1962, by Yasumura and Kawakita (Japan). The original cell line was named ”Vero”: *Verda reno* which means "green kidney" in Esperanto; *vero* means "truth" in Esperanto.

**Vero Cells lineages are**:

- continuous (can be replicated through many cycles of division and not become senescent)
- aneuploid (has an abnormal number of chromosomes).
- unlike normal mammalian cells, Vero cells are interferon-deficient (do not secrete interferon alpha or beta when infected by viruses). However, they still have the interferon alpha or beta receptor, so they respond normally when recombinant interferon is added to their culture media.

# Aplicações das células Vero



**Vero cells** are used for many purposes including:

a) Screening for toxins

e.g., *E. coli* toxins, which were first named "Vero toxin" after this cell line, (now they are called “Shiga-like toxin” due to its similarity to the toxin isolated from *Shigella dysenteriae)*

b) as host cells for eukaryotic parasites

e.g., trypanosomatids [protozoan parasites of the class Kinetoplastida predominately restricted to invertebrate hosts (i.e., monoxenous life-cycle) but several genera are pathogenic to humans, animals and plants, and have an invertebrate vector that facilitates their transmission (i.e., dixenous life-cycle)]

c) As host cells for growing viruses

e.g., to measure replication in the presence or absence of a research pharmaceutical, the testing for the presence of virus (e.g. rabies, influenza, etc.), or the growth of viral stocks for research purposes

JOURNAL OF VIROLOGY, Aug. 1996, p. 5519–5524
0022-538X/96/$04.00+0

Vol. 70, No. 8

# African Green Monkey Kidney (Vero) Cells Provide an Alternative Host Cell System for Influenza A and B Viruses

E. A. GOVORKOVA,[1,2] G. MURTI,[1] B. MEIGNIER,[3] C. DE TAISNE,[3] AND R. G. WEBSTER[1,4]*

*Department of Virology & Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105[1]; D. I. Ivanovsky Institute of Virology, 123098 Moscow, Russia[2]; Pasteur Merieux, 69280 Marcy I'Etoile, France[3]; and Department of Pathology, University of Tennessee, Memphis, Tennessee 38163[4]*

Received 5 March 1996/Accepted 2 May 1996

**The preparation of live, attenuated human influenza virus vaccines and of large quantities of inactivated vaccines after the emergence or reemergence of a pandemic influenza virus will require an alternative host cell system, because embryonated chicken eggs will likely be insufficient and suboptimal. Preliminary studies indicated that an African green monkey kidney cell line (Vero) is a suitable system for the primary isolation and cultivation of influenza A viruses (E. A. Govorkova, N. V. Kaverin, L. V. Gubareva, B. Meignier, and R. G. Webster, J. Infect. Dis. 172:250–253, 1995). We now demonstrate for the first time that Vero cells are suitable for isolation and productive replication of influenza B viruses and determine the biological and genetic properties of both influenza A and B viruses in Vero cells; additionally, we characterize the receptors on Vero cells compared with those on Madin-Darby canine kidney (MDCK) cells. Sequence analysis indicated that the hemagglutinin of Vero cell-derived influenza B viruses was identical to that of MDCK-grown counterparts but differed from that of egg-grown viruses at amino acid positions 196 to 198. Fluorescence-activated cell sorting analysis showed that although Vero cells possess predominantly α2,3 galactose-linked sialic acid, they are fully susceptible to infection with either human influenza A or B viruses. Moreover, all virus-specific polypeptides were synthesized in the same proportions in Vero cells as in MDCK cells. Electron microscopic and immunofluorescence studies confirmed that infected Vero cells undergo the same morphological changes as do other polarized epithelial cells. Taken together, these results indicate that Vero cell lines could serve as an alternative host system for the cultivation of influenza A and B viruses, providing adequate quantities of either virus to meet the vaccine requirements imposed by an emerging pandemic.**

Although used routinely to prepare human influenza virus vaccines and diagnostic reagents, embryonated chicken eggs have potentially serious limitations as a host system, including vaccines, including those against poliomyelitis and rabies (16). Earlier studies indicated that influenza viruses do not replicate productively in Vero cells (2, 15, 18, 29). The repeated addition

JCM Accepted Manuscript Posted Online 11 November 2020
J Clin Microbiol doi:10.1128/JCM.02156-20


# Novel isolation method of porcine epidemic diarrhea virus using suspension vero cells and immunogenicity analysis

Fei-Fei Ge*, De-Quan Yang*, Xin Li, Hou-Bin Ju, Hai-Xiao Shen, Jian Liu, Hong-jin Zhao, Jian Wang
Shanghai Animal Disease Control Center, Shanghai 201103, People's Republic of China

**ABSTRACT** In February and December of 2019 separately, two large-scale outbreaks of diarrhea were observed in the same swine farm with 3000 sows in Shanghai, China. We successfully isolated two PEDV isolates (strain shxx1902 and shxx1912) from clinical samples in this farm using suspension Vero cells in February and December, respectively. The third PEDV strain (SH1302) tested positive in the other farm of Shanghai in 2013 was also isolated using suspension Vero cells. The three isolates were better adapted to growth in adherent Vero cells through serial passages in the suspension Vero cells. The three isolated strains were detected positive by an immune- fluorescence assay and observed through electron microscopy. Phylogenetic analysis of the complete genomic sequence demonstrated that shxx1902(the 5th passage) and shxx1912(the 5th passage) clustered with a new GII subgroup (GII-c), which consisted of SINDEL strains from America (e.g., OH851), and their S gene belonged to GII-a. Both the strains(the 35th passage) have occurred dramatic changes in their genomes compared with the 5th passage. The 5th and 35th SH1302 belonged to GI-b genotype. The anti-N protein antibody titer of the strain shxx1902 was elevated to the same level as the vaccine strain(CV777) in mice. The use of the suspension Vero cells to isolate and propagate PEDV provide an effective approach for studies of the epidemiological characteristics and vaccine development of this virus.

**KEYWORDS:** porcine epidemic diarrhea virus; suspension Vero cells; serial passages; phylogenetic analysis;


Subculture of Adherent Cells
Abnova
Add appropriate volume of fresh medium and mix well

Estudo Orientado em Biologia Molecular e Genética
27.06.2018

# Avaliação da citotoxicidade de produtos com potencialidade antiviral, em diferentes condições de cultura das linhas celulares Vero e Vero E6, utilizando diferentes métodos colorimétricos

Orientado por: Prof. Dra. Maria Filomena Caeiro

Ciências ULisboa

Mafalda Verdial (46359) | Mª. Leonor Santos (47654)
Marta Cardoso (47613) | Mónica Azevedo (47573)

» As plantas e derivados estão ligados a terapêuticas para combater vírus:

## *Solidago virgaurea* L. + Ribavirina

Extractos aquosos desta planta tem capacidade e potencialidade antiviral para alguns vírus (e.g. vírus Influenza A subtipo H5N1 e outros vírus Influenza, HIV, e o vírus herpes simplex tipo 2)


Fármaco antiviral – nucleosídeo sintético que bloqueia a síntese de ácidos nucleicos; eficaz em vírus de DNA e RNA (e.g., sensibilidade *in vitro*: adenovírus, vírus da febre hemorrágica da Crimeia-Congo, hepatite A, hepatite C; vírus herpes simplex tipo 1 e 2, HIV (VIH), influenza vírus tipo A e B, vírus do sarampo, vírus parainfluenza, vírus da febre amarela)

**[Soro Fetal Bovino]** (FBS, Biochrom é o suplemento mais utilizado em cultura de células eucarióticas *in vitro*)

» Em meio suplementado com soro a 10% as células dividem-se rapidamente;

» Poucos dados sobre citotoxicidade em células cultivadas em meio suplementado com soro a 2%;

## Linha Celular

» As linhas celulares apresentam diferentes taxas de crescimento.


## Confluência

» Confluência é a estimativa do número de células que aderiram à placa de cultura, isto é, a proporção de superfície coberta por células.

» As diferentes linhas celulares apresentam taxas de crescimento diferentes e, consequentemente, atingem a confluência em diferentes tempos após a sua subcultura (e.g., as células Vero multiplicam-se mais rapidamente do que as células Vero E6)

# **O**bjetivos

1. Avaliar possíveis diferenças de efeito citotóxico tendo em conta diferenças nas condições experimentais:


2. Determinar qual ou quais o(s) melhor(es) teste(s) colorimétrico(s) se adequa a ensaios de viabilidade celular

Extracto aquoso de flores de *Solidago virgaurea* L. (0-700 μg/ml)

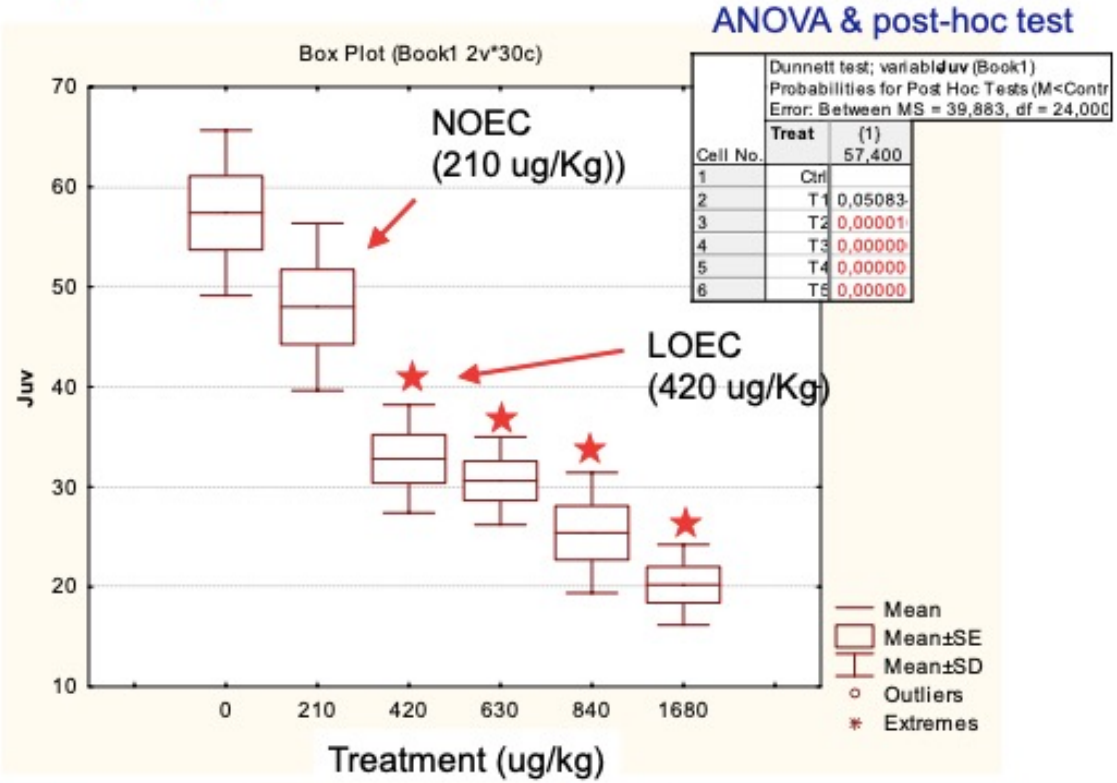
Ribavirina (0-250 μM)

**$CC_{50}$** – [] à qual se observa perda de viabilidade de 50% das células hospedeiras

**CMNC** – [] máxima não citotóxica

(**IS** – índice de selectividade ou terapêutico = $CC_{50}/IC_{50}$)

# Alguns exemplos de testes com organismos do solo




NOEC – No observed effect concentration.
LOEC – Lowest observed effect concentration.
EC50 – median effective concentration. It is the concentration of the substance that causes a specific toxic effect to 50% of the test organism



Fonte: Prof José Paulo Sousa

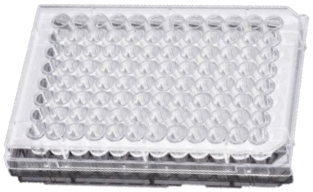
# **T**este do MTT (brometo)

O ensaio de redução do MTT (brometo de 3-4,5-dimetil-tiazol-2-il-2,5-difeniltetrazólio) é um método rápido, frequentemente usado para medir proliferação celular e acitotoxidade.

O teste é baseado na redução do MTT, um sal amarelo solúvel em água, pelo efeito da atividade metabólica celular ligada ao NADH e NADPH, formando cristais insolúveis de formazan, de coloração azul ou roxa. A coloração azul ou roxa é, portanto, um quantificador da viabilidade das células.

Esse mecanismo envolve a atividade da enzima desidrogenase mitochondrial; as células metabolicamente ativas irão converter o MTT, enquanto que células mortas não serão capazes de realizar essa conversão. A redução do MTT está associada às mitcôndrias, citoplasma, e membranas não-mitocondriais (incluindo a membrana plasmática e os lisossomas-endossomas).

# Teste do MTT (brometo)



Descartar
meio da P96

50 µL de MTT a 250 µg/mL

90 min
37°C

100 µL de DMSO

30 min
agitação às escuras
Temperatura ambiente

Leitura a 570 nm

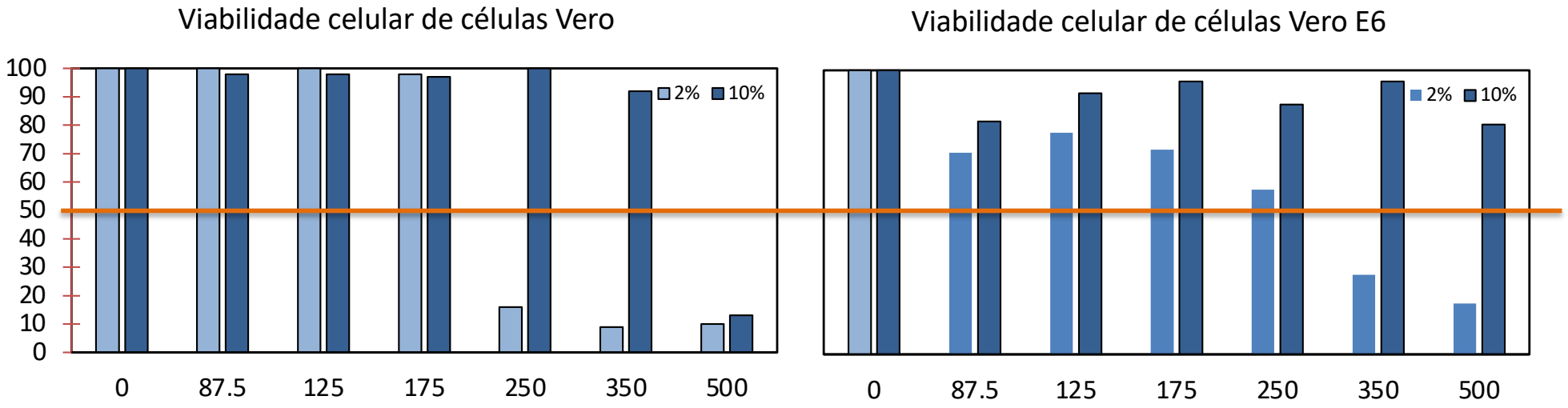
# [FBS] e Linha Celular

| Extracto aquoso de flores de *S. virgaurea* | | | | | |
|---|---|---|---|---|---|
| **Concentração** | **Vero** | **Vero E6** | **Concentrações** | **Vero** | **Vero E6** |
| **500** | 10% | 18% | **700** | 13% | 81% |
| **350** | 9% | 28% | **500** | 92% | 96% |
| **250** | 16% | 58% | **350** | 103% | 88% |
| **175** | 98% | 72% | **250** | 97% | 96% |
| **125** | 102% | 78% | **175** | 98% | 92% |
| **87,5** | 103% | 71% | **125** | 98% | 82% |
| **0** | 100% | 100% | **0** | 100% | 100% |
| **DMEM-FBS2** | | | **DMEM-FBS10** | | |

- Culturas sub-confluentes
- Teste do MTT

**Ribavirina apresenta resposta semelhante**

# [FBS] e Linha Celular




- Culturas sub-confluentes
- Teste do MTT

**Ribavirina apresenta resposta semelhante**

# **C**$C_{50}$ e CMNC

| Extracto aquoso de flores de *S. virgaurea* | | | | |
|---|---|---|---|---|
| | **Vero** | | **Vero E6** | |
| **CMNC** | 161 | 482 | 54 | 549 |
| **$CC_{50}$** | 237 | 624 | 265 | - (*) |
| | **DMEM-FBS2** | **DMEM-FBS10** | **DMEM-FBS2** | **DMEM-FBS10** |

- Culturas sub-confluentes
- Teste do MTT e CV

**Resultados variáveis caso haja alteração do nivel de confluência das culturas**

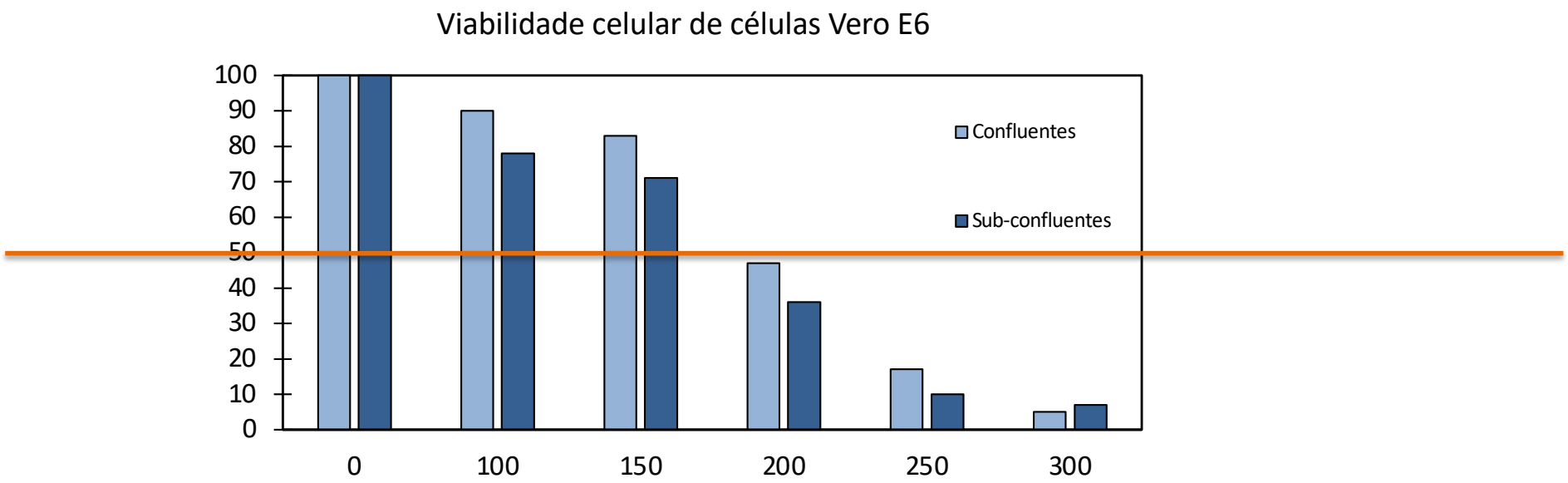
# Níveis de confluência

| Extracto aquoso de flores de *S. virgaurea* - Vero | | | | | | |
|---|---|---|---|---|---|---|
| **Concentração** | Média | D. padrão | **Viabilidade** | Média | D. padrão | **Viabilidade** |
| **300** | 0,1009 | 0,00782 | 5% | 0,0871 | 0,04274 | 7% |
| **250** | 0,3115 | 0,02348 | 17% | 0,1160 | 0,02401 | 10% |
| **200** | 0,8911 | 0,19270 | 47% | 0,4316 | 0,06230 | 36% |
| **150** | 1,5626 | 0,13339 | 83% | 0,8511 | 0,03954 | 71% |
| **100** | 1,6856 | 0,10652 | 90% | 0,9305 | 0,06992 | 78% |
| **0** | 1,8780 | 0,13952 | 100% | 1,1943 | 0,12890 | 100% |
| | **Culturas Confluentes** | | | **Culturas Sub-confluentes** | | |

- Em DMEM-FBS2
- Teste do CV

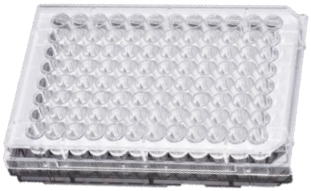
**Ribavirina apresenta resposta semelhante**

# Níveis de confluência




- Teste do MTT

**Ribavirina apresenta resposta semelhante**



Teste do Vermelho Neutro (VN)

Teste de brometo de (3-(4,5-Dimetiltiazol-2-yl)-2,5-diphenyltetrazolio (MTT)

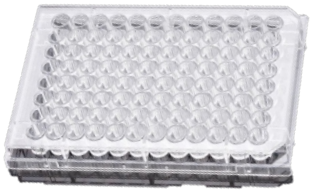
Teste do Cristal Violeta (CV)

$CC_{50}$
CMNC

**$CC_{50}$** – [] à qual se observa perda de viabilidade de 50% das células hospedeiras
**CMNC** – [] máxima não citotóxica

(**IS** – índice de selectividade ou terapêutico = $CC_{50}/IC_{50}$)

# Teste do Cristal Violeta



Descartar
meio da P96

100 µL de formaldeído 1% em PBS-A

30 min
agitação à temperatura ambiente

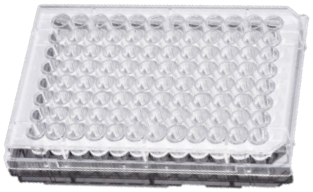
50 µL de CV a 0,2% em 50%
metanol

**0,4% de CV em 100% metanol** ✗

**0,2% de CV em 50% metanol** ✓

**0,1% de CV em 25% metanol** ✗

# Teste do Cristal Violeta



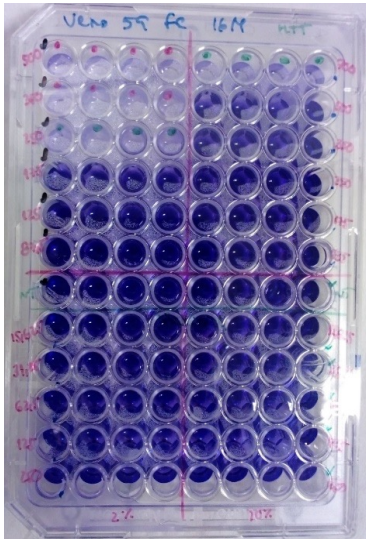
Descartar
meio da P96

100 µL de formaldeído 1% em PBS-A

30 min
agitação à temperatura
ambiente

50 µL de CV a 0,2% em 50%
metanol

20 min
agitação às escuras
Temperatura
ambiente

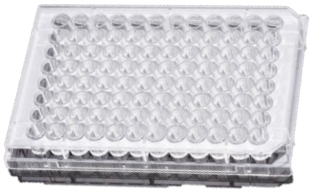


150 µL de SDS 1%
Incubação

Leitura a 570 nm

| | |
|---|---|
| **100 µL de SDS 1%** | ✗ |
| **200 µL de SDS 1%** | ✗ |
| **150 µL de SDS 1%** | ✓ |

# Teste do Vermelho Neutro



Descartar
meio da P96

50 µL de VN

| | |
|---|---|
| 5mg/mL | ✗ |
| 1mg/mL | ✗ |
| 50 µg/mL | ✗ |

90 min
37°C

100 µL de formaldeído 1% em PBS-A

15 min
agitação às escuras
Temperatura
ambiente

100 µL de solução solubilizadora

Incubação

Leitura a 546 nm

# Qual o(s) melhor(es) método(s) para avaliar citotoxicidade?

| E6.2 | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Concentrações** | **MTT** | **VN** | **CV** | **Concentrações** | **MTT** | **VN** | **CV** |
| **500** | 10% | -4% | 6% | **700** | 13% | -3% | 9% |
| **350** | 9% | -2% | 6% | **500** | 92% | 50% | 100% |
| **250** | 16% | 8% | 9% | **350** | 103% | 65% | 99% |
| **175** | 98% | 82% | 101% | **250** | 97% | 68% | 102% |
| **125** | 102% | 84% | 100% | **175** | 98% | 65% | 96% |
| **87,5** | 103% | 78% | 103% | **125** | 100% | 72% | |
| **0** | 100% | 100% | 100% | **0** | 100% | 100% | 100% |
| **DMEM-FBS2** | | | | **DMEM-FBS10** | | | |

- Culturas sub-confluentes

**Ribavirina apresenta resposta semelhante**

# Conclusões

1. **Nivel de confluência e linha celular induz diferenças significativas**
2. **A concentração de soro utilizada é o maior fator de diferença entre os resultados. => necessidade de executar testes de citotoxicidade quando procuramos determinar $CC_{50}$ e CMNC**
3. **Brometo (MTT) e cristal violeta (CV) como melhores testes de avaliação da citotoxicidade**

**Mafalda Verdial (46359) | Mª. Leonor Santos (47654)**
**Marta Cardoso (47613) | Mónica Azevedo (47573)**

# Development of suspension adapted Vero cell culture process technology for production of viral vaccines

Chun Fang Shen [a,*], Claire Guilbault [a], Xiuling Li [b], S. Mehdy Elahi [a], Sven Ansorge [a], Amine Kamen [a,c], Rénald Gilbert [a]

[a] Human Health Therapeutics Research Center, National Research Council of Canada, 6100 Royalmount Ave, Montreal, Quebec H4P 2R2, Canada
[b] China National Biotec Group Company Limited, Beijing, China
[c] Department of Bioengineering, McGill University, Montreal, Canada

ARTICLE INFO

*Article history:*
Available online xxxx

*Keywords:*
Vero cell culture
Viral vaccine
Cell adaptation
Culture media

ABSTRACT

Vero cells are considered as the most widely accepted continuous cell line by the regulatory authorities (such as WHO) for the manufacture of viral vaccines for human use. The growth of Vero cells is anchorage-dependent. Scale-up and manufacturing in adherent cultures are labor intensive and complicated. Adaptation of Vero cells to grow in suspension will simplify subcultivation and process scale-up significantly, and therefore reduce the production cost.

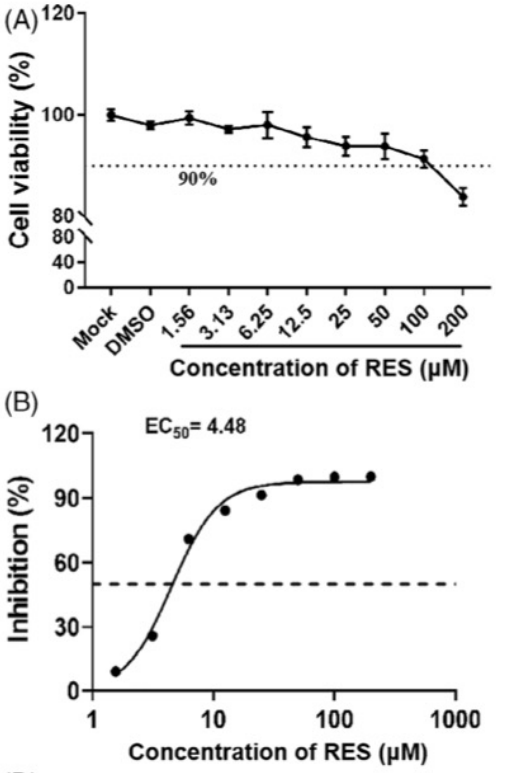
Here we report on a successful adaptation of adherent Vero cells to grow in suspension in a serum-free and animal component-free medium (IHM03) developed in-house. The suspension adapted Vero cell cultures in IHM03 grew to similar or better maximum cell density as what was observed for the adherent Vero cells grown in commercial serum-free media and with a cell doubling time of 40–44 h. Much higher cell density ($8 \times 10^6$ cells/mL) was achieved in a batch culture when three volume of the culture medium was replaced during the batch culture process.

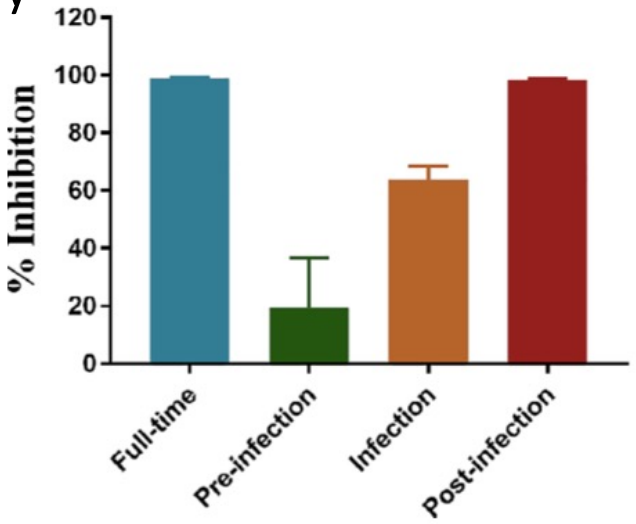
Both adherent and suspension Vero cells from various stages were tested for their authenticity using short tandem repeat analysis. Testing result indicates that all Vero cell samples had 100% concordance with the Vero DNA control sample, indicating the suspension cells maintained their genetic stability. Furthermore, suspension Vero cells at a passage number of 163 were assayed for tumorigenicity, and were not found to be tumorigenic.

The viral productivity of suspension Vero cells was evaluated by using vesicular stomatitis virus (VSV) as a model. The suspension cell culture showed a better productivity of VSV than the adherent Vero cell culture. In addition, the suspension culture could be infected at higher cell densities, thus improving the volumetric virus productivity. More than one log of increase in the VSV productivity was achieved in a 3L bioreactor perfusion culture infected at a cell density of $6.8 \times 10^6$ cells/mL.

Resveratrol inhibits the replication of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in cultured Vero cells






Full-time: Vero cells pre-treated with 50 μM RES for 2 hr, then RES and virus at a multiplicity of infection of 0.01 were simultaneously added into cells for 1 hr. Afterwards, the virus-RES mixture was removed, and the cells were cultured with medium containing 50 μM RES for 48 hr.

Pre-infection treatment: 50 μM RES was added to the cells for 2 hr only, removed and added virus for 1 hr, and then removed the virus and continuously cultured with fresh medium for 48 hr.

Infection experiment: RES and virus were simultaneously added into cells for 1 hr, washed and replaced with fresh medium for 48 hr.

Post-infection treatment: virus was added to allow attachment for 1 hr, washed and replaced with fresh medium contained 50 μM RES for 48 hr.