Chikungunya virus isolation using simplified cell culture technique in Mauritius

Pyndiah MN, Pursem V, Meetoo G, Daby S, Ramuth V, Bhinkah P, Chuttoo R, Paratian U

Virology Laboratory, Central Health Laboratory, Candos, Mauritius

Med Trop 2012 ; **72** : 63-65

ABSTRACT • During the chikungunya outbreak of 2005 – 2006, the only laboratory facilities available in Mauritius were virus isolation in cell culture tubes and serology. The laboratory was submerged with the large number of blood samples. Comparative isolation was made in human embryonic lung (HEL) and VERO cells grown in 96-well plate. Culture on HEL cells was found to be more sensitive and presence of cytopathic effect (CPE) was observed earlier than in VERO cells. Out of the 18.300 blood samples inoculated on HEL, 11.165 were positive. This virus isolation method was of great help for the surveillance and control of the vectors. In cases of an outbreak a cheap, rapid and simple method of isolating chikungunya virus is described.

KEY WORDS • Chikungunya virus. Human Embryonic Lung. VERO. Cytopathic Effect. Culture.

L'ISOLEMENT DU VIRUS CHIKUNGUNYA PAR LA TECHNIQUE DE CULTURE CELLULAIRE SIMPLIFIÉE À L'ILE MAURICE

RÉSUMÉ • Lors de l'épidémie de chikungunya de 2005-2006, les seules méthodes de diagnostic disponibles à l'Ile Maurice étaient l'isolement viral dans les cultures cellulaires et la sérologie. Le laboratoire a été submergé par un grand nombre d'échantillons sanguins. Des isolements comparatifs ont été pratiqués sur cellules de poumon embryonnaire humain (HEL) et sur cellules VERO cultivées dans des plaques à 96 puits. La culture sur cellules HEL s'est révélée plus sensible avec une présence plus précoce de l'effet cytopathogène que sur les cellules VERO. Parmi les 18 300 échantillons sanguins inoculés sur HEL, 11 165 ont été positifs. Cette méthode d'isolement viral a donc été d'une grande aide pour la surveillance et le contrôle des vecteurs. En cas d'épidémie, une méthode simple, peu onéreuse et rapide de diagnostic est décrite.

MOTS-CLÉS • Virus chikungunya. Poumon embryonnaire humain.VERO. Effet cytopathogène. Culture.

Chikungunya (CHIK) is a mosquito-borne viral disease first described during an outbreak in southern Tanzania in 1952. In the past Chikungunya virus (CHIKV) used to cause epidemics in Africa, Asia and the Indian subcontinent. The virus was endemic in the African continent for many years without causing severe epidemics when in 1999 – 2000 there was a large outbreak in the Democratic Republic of the Congo and in 2007 another outbreak in Gabon. For the first time in 2005-2006, there was an outbreak in the Indian Ocean islands of Reunion and Mauritius, where *Aedes albopictus* was the presumed vector. In India, more than 1 500 000 cases of CHIK were reported in 2006 (1).

CHIK is an acute infection of abrupt onset, heralded by fever and severe arthralgia, followed by other constitutional symptoms and rash lasting for 1 to 7 days. The incubation period is usually 2 to 3 days. Fever rises abruptly, often reaching 39 to 40°C and accompanied by intermittent shaking chills. This acute phase lasts 2-3 days. The temperature may remit for 1-2 days, resulting in a «saddle-back» fever curve. CHIK was initially believed to be mainly an incapacitating disease and nonfatal, but severe forms and deaths have been reported on the Indian Ocean island of La Réunion (2) and in India (3). CHIKV infections without illness do occur. Clinically apparent or silent infection is thought to confer life-long immunity (4).

Human epithelial and endothelial cells, primary fibroblasts and monocyte-derived macrophages are susceptible to infection. Viral entry occurs through pH-dependent endocytosis. Infection is cytopathic and associated with the induction of apoptosis in the infected cell (5).

• *Correspondance : npyndiah@intnet.mu*
• *Article arrivé le 08/02/2012, accepté le 17/02/2012*

The objective of this study was to compare the primary isolation of CHIKV in human embryonic lung (HEL) and VERO cell cultures and to develop a simple, cheap, rapid and efficient method of isolation in 96-well plate.

Materials and Methods

Cell cultures: Diploid human embryonic lung (HEL) and VERO cell cultures were grown in Eagles MEM supplemented with 10% heat inactivated calf serum in 75 x 100 mm borosilicate glass tubes. Once the cell cultures were 100% confluent, the growth medium was replaced by maintenance medium containing 2% inactivated calf serum for HEL and 0% calf serum for VERO cultures. The maintenance medium was then changed at every 3 or 4 days intervals.

HEL and VERO cell cultures were also made in sterile flat bottom 96 well microplates (Greiner) at a concentration of 2 x 10^4cells/150 µL/well. The wells were sealed with an adhesive plastic sheet and incubated in an ordinary 37°C incubator. A confluent cell sheet was usually obtained after 24 hours incubation and the growth medium was then replaced by maintenance medium, as for the tube cultures prior to inoculation.

Blood samples for CHIKV isolation: Medical and nursing staff was instructed to collect 3 to 5 mL of blood from patients, who presented with acute symptoms of CHIK when the body temperature was >38.5°C. The blood samples collected in plain sterile vacuum tubes were shipped immediately to the laboratory, where they were centrifuged at 2500 rpm for 5 minutes. The centrifuged tubes were kept at room temperature in a vertical position until ready for inoculation. From April to August 2005, a

Table 1. Comparison between CHIKV culture and serology, Mauritius 2005.

Isolation of chikungunya virus in cell cultures - 2005		
	Isolation	Serology
Positive	60	46
Negative	46	60
Total	106	106

total of 269 blood samples were received for virus isolation and 18 300 from December 2005 to August 2006.

Virus Isolation in cell culture tubes: 100 µL of patient's serum were inoculated in one HEL and one VERO tube, respectively. The tubes were then incubated at 35°C in a stationary position for 21days. They were examined daily under an ordinary microscope using a 6X objective for any cytopathic effect (CPE).

Virus isolation in microplate: 20 µL of each patient's serum was inoculated into an appropriate well of HEL and VERO microplate respectively. The microplates were sealed as described above, incubated at 35°C for 5 days without fluid change. Up to 95 samples were inoculated per microplate and the last well was used as negative control. Microscopic examination was performed daily under an inverted microscope.

Complement fixation (CF) antigen: The first positive isolate during the outbreak in 2005 was used to infect VERO cells grown in 75 cm^2 tissue culture flasks for the production of CF antigen. Infected cell deposit was glycine extracted as described by K.S. Kim *et al.* (6).

Complement Fixation Test (CFT): Patients sera were screened by the CFT for the presence of antibodies against the CHIKV. The method used was the one supplied by Virion/ Serion GmbH. Friedrich-Bergius-Ring 19. (D-97076 Wursburg, Germany).

Results

Out of the blood samples inoculated at the start of the outbreak nearly all of them was negative because we were dealing with unprecedented cases and had no experience on type of specimen needed. Positive CPE was observed only among samples, which were collected from patients who were in the acute phase of the illness. Focal lesions of rounded, refractile cells spreading rapidly to the whole cell sheet within 24 to 48 hours. Due to apoptosis the refractile cells rapidly changed to granular non refractile ones. The only facilities, which were available at that time for confirmation of Alphavirus was transmission electron microscopy, CFT and seroneutralisation test. Electron microscopy revealed the presence of an Alphavirus.

Most of the CPE appeared within one week of incubation and out of the 269 blood samples received in 2005, 62 were positive, that is 23%. Laboratory results were released within one week after inoculation.

In order to control the specificity of the virus isolation and presence of antibodies out of 106 blood samples obtained from patients with clinical symptoms, 60 were positive for CHIKV and negative for CF antibodies, whereas the 46 which were negative for virus isolation were positive for antibodies as shown in the table 1.

During the outbreak of 2006, more than 18 300 blood samples were received during the period February to July and out of which 11 115 were positive for virus isolation as shown in figure 1. During the peak of the outbreak hundreds of blood samples were received daily for virus isolation. Virus isolation was then performed in 96-well plate, a simple technique which was easily adapted to the routine isolation method.

Table 2. Comparison between CHIKV culture on HEL and VERO cells, Mauritius 2006.

	HEL	VERO
Positive	1143	1005
Negative	567	705
Total	1710	1710

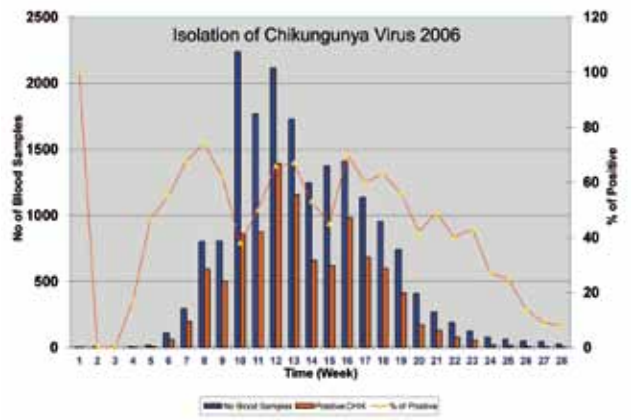

Figure 1. Isolation of CHIKV during year 2006. HEL grown cultures in 96-well plate was used and final reading of the plate was made 5 days after inoculation. In week 10 and 15, isolation rate was less than 50%.

To compare the efficiency of the HEL and VERO cells for CHIKV isolation in microplates,

1710 patient sera were inoculated simultaneously on the two types of culture over a period of 18 days, that is, 95 fresh samples per day. Final examination of the plates was made 5 days after inoculation (table 2).

Comparative primary isolation of CHIKV in HEL and VERO cells

As illustrated in the table 2, HEL was shown to be more sensitive than the VERO cells for primary isolation of CHIKV. In most cases the CPE appeared earlier in the HEL than VERO. In samples collected at the onset of the symptoms CPE was observed between 24 and 36 hours. Otherwise, in most cases, a CPE was observed between the second and third day post inoculation. Very few positives were detected at the end of 5 days incubation. As from then all isolation has been performed in HEL and the results are shown in figure 1. There were only few cases at the beginning of 2006 and it was after week 7 that the number of cases rose and started declining steadily by week 17. On week 10 and 15 there was a drop in the rate of positive isolates.

Discussion

The CHIKV is an Alphavirus with a single strand-positive sense RNA, which acts as mRNA meaning that the replication cycle is short and CPE in cell cultures appears quite early

after infection. As fibroblasts are the main target cells of CHIKV in peripheral tissues (7), the HEL cell was selected for this study. In the present study, HEL cultures have been used successfully and were even much more sensitive than VERO cultures. If VERO cultures were used instead of HEL, more than 10% positive cases would have been missed. In cases of severe outbreaks, it is possible to test large amount of samples for surveillance purposes. It is cost effective to go for vector control in areas where the virus is present in case of spraying and larviciding. During the last outbreak, all new cases of CHIK were reported on a daily basis to the National Communicable Disease Unit, which is responsible for the vector control.

Using the isolation in microplate, it was possible to test nearly every patient with fever >38.5°C seeking treatment at public health institutions. As seen in Figure 1, around week 10 and 15 the rate of virus isolation dropped, because many patients who were already infected with the CHIKV had remittent fever and believed they were infected for a second time (personal information obtained from treating doctors) returned back for treatment.

We have shown that during outbreaks of CHIK, it is cost effective for laboratories possessing cell culture facilities to go for primary isolation in HEL cultures grown in 96-well plate more than to perform RT-PCR.

References

1. WHO. Fact sheet n°327: Chikungunya. WHO Media center, Geneva, 2008. http://www.who.int/mediacentre/factsheets/fs327/en/index.html
2. Lemant J, Boisson V, Winer A, Thibault L, André H, Tixier F *et al.* Serious acute Chikungunya virus infection requiring intensive care during the Reunion Island outbreak in 2005–2006. *Crit Care Med* 2008 ; 36 : 2536–41.
3. Mavalankar D, Shastri P, Raman P. Chikungunya epidemic in India: a major public-health disaster. *Lancet Infect Dis* 2007 ; 7 : 306-7.
4. Lam SK, Chua KB. Alphaviruses. WHO Collaborating Centre for Arbovirus Reference and Research (DF/DHF) ed, Malaya, 1996. http://www.vadscorner.com/alphaviruses.html
5. Sourisseau M, Schilte C, Casartelli N, Trouillet C, Guivel-Benhassine F, Rudnicka D et al. Characterization of reemerging chikungunya virus. *PLoS Pathog* 2007 ; 3 : e89.
6. Kim KS, Moon HM, Sapienza VJ, Carp RI. Complement-fixing antigen of human cytomegalovirus. *J Infect Diseases* 1977 ; 135 : 281-8.
7. Couderc T, Lecuit M. Focus on Chikungunya pathophysiology in human and animal models. *Microbes Infect* 2009 ; 11 : 1197-205.

Bassin des Cormorans à 3 Bassins sur la côte ouest de l'île de la Réunion © Emilie Javelle.