Chikungunya

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- Detection and Quantification of Chikungunya Virus (CHIKV) RNA by real-time RT-PCR
- Evaluation of RNAi (RNA interference) technology in curing Chikungunya Virus infection in-vitro and in-vivo
- Study on pathogenesis of Chikungunya virus (CHIKV) in laboratory animals
- Host immune response/s in Chikungunya cases hospitalized with diverse clinical presentations
- Association of the neurological complications of the Chikungunya virus infection during the current epidemic with specific mutation(s) in the viral genome
- Establishment and characterization of a new *Aedes aegypti* cell line for Chikungunya isolation and propagation
- Growth kinetics of Chikungunya virus in certain cell lines

Detection and Quantification of Chikungunya Virus (CHIKV) RNA by real-time RT-PCR

Deepti Parashar, MD Gokhale, GP Jacob, S Kumar, AC Mishra and VA Arankalle

Introduction

Chikungunya virus (CHIKV), a member of the alphavirus genus, is of considerable public health concern in Southeast Asian and African countries. However, despite serological evidence, the diagnosis of this arthropod-borne human disease is confirmed infrequently and needs to be improved. In fact, illness caused by CHIKV can be confused with diseases such as dengue or yellow fever, based on the similarity of the symptoms, and laboratory confirmation of suspected cases is required to launch control measures during an epidemic.

Objectives

- To develop TaqMan real-time RT-PCR method for detection and quantitation CHIKV RNA
- Demonstration of its utility in documenting CHIKV TOT in field collected and experimentally infected *Aedes* mosquitoes

Work done

CHIKV Primers and probe designing: All the full genome sequences of CHIKV available in GenBank (or our unpublished sequences) were aligned and conserved region were targeted to locate the primers and TaqMan MGB probes using primerexpress software (Applied Biosystems Inc, Foster, CA).

Construction of CHIKV standard: A PCR product encompassing the targeted region was prepared using the confirmed CHIKV strain and cloned into the T7 polymerase expression vector pGEM-T-easy (Promega, Madison, USA) according to manufacturer's instructions. Clone with sense orientation was cut with Spe I and run off transcription was done with T7 polymerase. Transcript was purified, serially diluted and used as RNA standard. Viral RNA was extracted from samples using QIAamp viral RNA mini kit (Qiagen, USA).

The insert sequences for the CHIKV standard were confirmed by using big dye terminator kit on 310 sequence analyzer (Applied Biosystems).

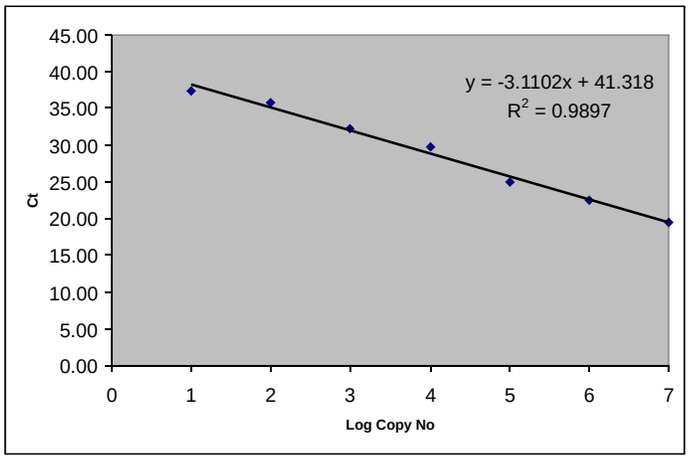


Fig. 1: Standard curve for the CHIKV-specific one step real time RT-PCR assay generated from the amplification plots between the 10-fold serially diluted IVT RNA. Serial 10-fold dilutions of *in vitro* transcribed RNA standard were plotted against the threshold cycle.

Real-time TaqMan RT-PCR assays. One-step real-time RT-PCR assay was performed using an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA) as previously described.

Sensitivity, specificity and reproducibility of the TaqMan assay: The analytical sensitivity and reproducibility of the real time TaqMan RT- PCR was determined using a 10 fold dilutions of in vitro transcribed CHIKV strain in duplicate.

Specificity of CHIKV assay was evaluated by using infected cell extracts with DEN, JE and WN Viruses.

The detection limit of the assay with the CHIK standard template was 10 copies.

No cross reactivity was detected with DEN, JE and WN viruses.

Detection of CHIKV in clinical samples and in *Aedes aegypti* mosquitoes by real time RT-PCR method:

Optimization of RNA isolation procedure: Experimentally infected mosquitoes for detection of CHIKV in a pool of single mosquito as well as one positive mosquito in a pool of 50 were used.

TaqMan real-time RT-PCR method was developed and applied for detection and quantitation of CHIKV RNA in 198 clinical samples collected from Kerala, 36 samples were detected by this system. Out of 198 field collected and pooled mosquitoes (1-50 mosquito(es) / pool), 27 were found to be positive for CHIKV RNA. All the *Aedes aegypti* larvae collected from Andhra Pradesh were found to be negative for CHIKV RNA.

Evaluation of RNAi (RNA interference) technology in curing Chikungunya Virus infection *in-vitro* and *in-vivo*

Deepti Parashar, S Kumar, AB Sudeep, AC Mishra and VA Arankalle

Introduction

Chikungunya is an emerging arboviral infection of immense public health concern in Southeast Asian and African countries. Recently several parts of Indian Ocean islands and India witnessed explosive, unprecedented epidemic. The response to available treatment modalities is not impressive. The advent of RNA Interference--a phenomenon of sequence-specific degradation of RNAs mediated by double-stranded RNA--holds promise as a potential therapy for CHIKV infection.

Objectives

- To evaluate the efficacy of siRNA in inhibiting CHIKV replication in mosquito cells.
- To evaluate the efficacy of siRNA in inhibiting CHIKV replication in animal model.

Work done

siRNA oligos for E 2 gene were used for transfection and 24 hr after transfection cells were infected with CHKV. Cells were observed every 24, 36 and 48-hour PI cells were harvested and real time PCR was done.

Study on pathogenesis of Chikungunya virus (CHIKV) in laboratory animals

DR Patil and VA Arankalle

Introduction

India experienced re-emergence of Chikungunya (CHIK) virus after a quiescence of more than three decades in the form of explosive outbreaks affecting several states and 1.3 million people which may be a gross underestimate This re-emergence was associated with a shift in the genotype of the virus from Asian to African. Clinical complications and deaths were noted during the outbreak, especially in elderly people. Joint pain persisting for months together caused disability and heavy economic losses. Very rapid spread of the virus and increased virulence warrants detailed studies of the virus as well as the disease pathogenesis.

A systematic study in animals yields valuable information and paves way for further in-depth research. Considering this, the study was undertaken with following objectives.

Objectives

- To study tissue tropism and persistence of virus with special reference to joint and associated tissues
- To characterize tissue injury at macro and micro level.
- To understand involvement of different immune cell types in inflammation.

Work Done

Virus stock preparation

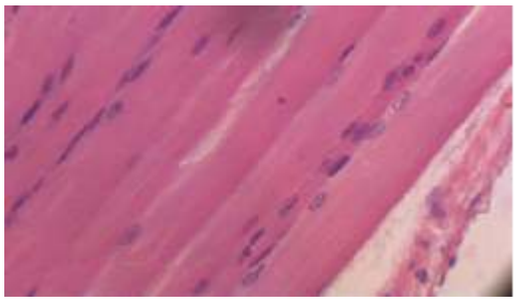
Latest Indian strain of CHIKV (061543): A serum isolate obtained from patient from Andhra Pradesh in 2006 was taken as a representative of 2006 outbreaks. Virus at mouse brain passage level 12 was tested for the susceptibility in mice at different ages by S/C route. As no overt clinical signs ere noted, it was further passaged six times in suckling mice by S/C route. Virus at MB12 level was inoculated by S/C route and the whole carcass was harvested except viscera and skin. It was homogenized and suspension was prepared. This carcass suspension was used to inoculate at next passage. Likewise six passages were made (MC1 to MC6) by S/C route in left inguinal region of suckling mice. Virus stock was prepared in Vero E6 cells using CHIKV at passage level MB12+MC6. Thus the stock virus used for the study was at passage level: MB12+MC6+V-1.

Susceptibility of 8-day-old Swiss Albino and C57BL/6 mice to MB12+MC6+V-1 virus: Swiss Albino and C57BL/6 mice of 8 day old age were inoculated with $10^{4.6}TCID_{50}$ of MB12+MC6+V-1 in the left inguinal region and mice were scored for development of hind limb disfunction and disease Hind limbs stiffness started at 6^{th}PID, which became severe on 8^{th} PID. Hind limb weakness could be observed on 12^{th} PID also though less severe as compared to 8^{th} PID. Necropsy revealed white patches on the hind limb thigh region.

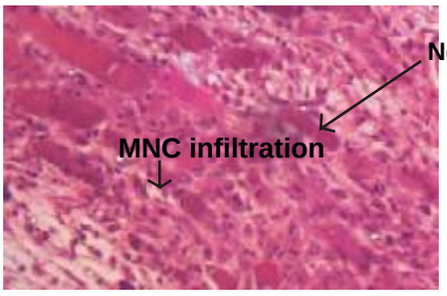
Characterization of tissue injury in 8-day-old Swiss Albino and C57BL/6 mice by histopathology:

Swiss Albino and C57BL/6 mice of 8-day-old age were inoculated with $10^{4.6}$ $TCID_{50}$ of MB12+MC6+V-1 virus by S/C route in the left inguinal region. Different tissues were collected from mock-inoculated control and infected age mached mice at 1, 3, 8 and 12 days post infection (DPI) and fixed in 10% formal saline Tissues were processed for histopathology. Paraffin sections of 5 µ were prepared, stained with Hematoxylin & eosin and evaluated for the microscopic changes

Muscles and joint from ipsilateral as well as contralateral (with respect to injection site) hind limbs of the mock-inoculated control animals were apparently normal without any lesions. Muscles of the hind limbs (ipsilateral as well as contralateral) of the 8 and 12-day post infection animals revealed severe inflammatory lesions such as mononuclear cell infiltration, oedema, moderate to severe muscular degeneration and necrosis with proliferation of fibrous connective tissue. Muscles of left limb (injection site) from 3-day post infection showed mild inflammatory lesion. Joint sections of mouse at 8-day post infection revealed minimal inflammatory cell infiltration (mononuclear) in joint space. Clinical symptoms in the form of hind limb stiffness correlated well with pathological changes observed in hind limbs. Histopathology examination of the liver, spleen, heart, kidney, and brain did not reveal any infection related lesions.



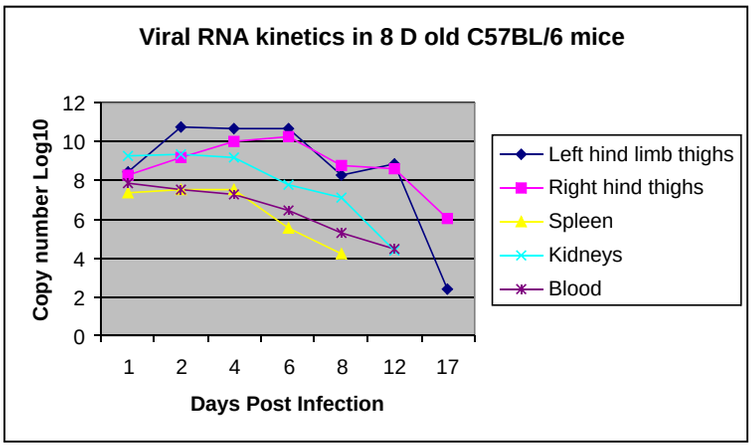
H&E, 40x- 12th PID Control



H&E, 40x- 12th PID infected

Viral RNA kinetics by real time RT PCR in infected 8 day old C57BL/6 mice: Different tissues and whole blood were collected at 1, 2, 4, 6, 8, 12 and 17days post infection (DPI) and stored in RNA letter. At each time point three animals were sacrificed and the tissues and whole blood were collected in RNA letter separately. For each set of tissues one mock-inoculated animal was sacrificed and the tissues and blood were collected in RNA letter similar to infected animals. Tissues and blood were stored at 70°C till assayed.

Viral RNA load was found maximum in the hind limbs (Ipsilateral as well as contralateral). In limbs, viral RNA remained at peak from 2nd to 6th PID (10 11 logs per gram of tissue). Thereafter, declining at 8th PID but still maintaining heavy load through out the observation period (8-9 logs per gram of tissue at 12th PID). Contrary to which, the viral RNA number declined faster in spleen through out the observation period (4 logs per gram of tissue on 8th PID). Decline was comparatively slower in blood (4-5 logs per ML of blood on 12th PID). Ironically the viral load in kidneys was found much higher (8-10 logs per gram of tissue from 2nd to 4th PID). Clearance from kidneys was also slower as compared to spleen and blood Gross and histopathological lesions in the limbs were seen maximum on 8th PID, the time point at which, decline in viral RNA load was observed in most of the limb tissues assayed.



Tissue : Copy number is measured per gram of tissue.

Blood : Copy number is measured as per ml of blood.

Each data point represents arithmetic mean of three mice.

Host immune response/s in Chikungunya cases hospitalized with diverse clinical presentations

Anuradha Tripathy, BV Tandale, SN Ranadhive and VA Arankalle

Introduction

Chikungunya (CHIK) has re-emerged in an explosive epidemic form in Indian Ocean islands and India. An estimated 1.38 million people from 11 states in India were severely affected by Chikungunya virus (CHIKV) infection. During the current epidemic, it was observed that Ahmedabad, the capital of the state of Gujarat experienced clinical complications of CHIKV infection leading to hospitalizations. These included encephalitis and multi-systemic involvements and classical fever cases. A possible role of underlying medical conditions in the progression of the CHIKV infected individuals to a variety of complications was envisaged. Atypical clinical presentations could be attributed to the host factors and/or critical mutations in the viral genome. Such studies have not been reported so far for CHIKV. In fact, even in classical CHIK infection, the dynamics and role of both humoral as well as T cell immune responses have not been established, probably because of the rarity of the disease. No data is available on cellular immune responses as well. Cellular immune responses play important role in the protection against viruses.

Objective

- To understand the cellular immune responses in different clinical manifestations of Chikungunya infection that would help in understanding the pathogenesis of CHIK infection.

Work done

Forty-six Chikungunya cases hospitalized with different clinical presentations (encephalitis cases, n=22, other systemic involvement (OSI) cases, n=12 and classical cases, n=12) were investigated. A control group consisting of seven age and sex matched healthy individuals negative for anti-CHIK IgM and IgG antibodies were also included. Confirmation of Chikungunya infection was assessed by the presence of IgM anti-CHIK antibodies and/or CHIK RNA detection. Seventeen of 46 cases had underlying medical conditions, six of them with more than 2 underlying medical conditions Underlying medical conditions were noted in 10 of 22 encephalitis cases, 5 of 12 OSI cases and 2 of 12 classical CHIK cases. CD4 T cell responses and the levels of pro and anti inflammatory cytokines against the envelope antigens (rE1p and rE2p) were assessed by lymphocyte proliferation assay and Cytometric bead array

Lymphocytes of 0/12 and 6/21 encephalitis cases, 1/6 and 4/12 other systemic involvement cases and 3/10 and 6/12 classical CHIK cases could recognize rE1p and rE2p respectively. Five of the six classical fever cases responding to rE2p were without any underlying medical condition at the time of Chikungunya infection. None of the 7 controls responded to rE1p and rE2p. **(Table 1)**

Table 1: CD4 T cell responses to rE1 and rE2 proteins in CHIKV infected individuals

Responder/Total tested (% Recall response)					
Encephalitis Cases (n=22)		Other Systemic Involvement Cases (n=12)		Classical CHIK Cases (n=12)	
Recall antigen		Recall antigen		Recall antigen	
rE1p	rE2p	rE1p	rE2p	rE1p	rE2p
0/11 (0%)	6/21 (28.5%)	1/6 (16.6%)	4/12 (33.3%)	3/10 (30%)	6/12 (50%)

Due to logistic problems, rE1p induced cytokine assays were carried out only in very few samples. Hence we are considering the results of only rE2p induced cytokines Figure 1 depicts cytokine levels in the PBMC culture supernatants in response to rE2 protein in different patient categories. The cytokine values are expressed in pico-gram as mean ± standard deviation. IL-5 levels from all the three patient categories were significantly higher than those in controls (4297.9 ± 1551.1 pg/ml in encephalitis cases, p<0.01; 4444.4 ± 1666.6 pg/ml in other systemic involvement cases, p< 0.01 and 3867 ± 994 pg/ml in classical fever cases, p=0.02, vs controls 4.9 ± 5.1 pg/ml). Difference in IL-5 levels among different patient categories were not statistically significant (p>0.05).

As compared to the controls (2.6 ±0.92pg/ml), IL-4 was significantly elevated in encephalitis cases (273.9± 166.1 pg/ml, p<0.01) and other systemic involvement (879.1± 1557.3 pg/ml, p<0.01) cases. IL-4 levels in the classical CHIK cases were also higher, though statistically not significant (468± 188 pg/ml, p=0.07**).** IFN-? levels in the encephalitis cases were significantly lower compared to controls (9.6 ± 8.3 pg/ml vs 383.6 ± 798.6pg/ml, p<0.01). No difference was recorded in IFN-? levels of the other two patient categories when compared with controls (p> 0.05). TNF-a**,** IL-10 and IL-2 levels among all cases and controls were comparable **(Fig. 2)**.

Overall, the present study suggests that infection with Chikungunya is associated with a Th2 biased cytokine response irrespective of the outcome and clinical manifestations and emphasizes need for in-depth studies in the pathogenesis of this re-emerging infection.

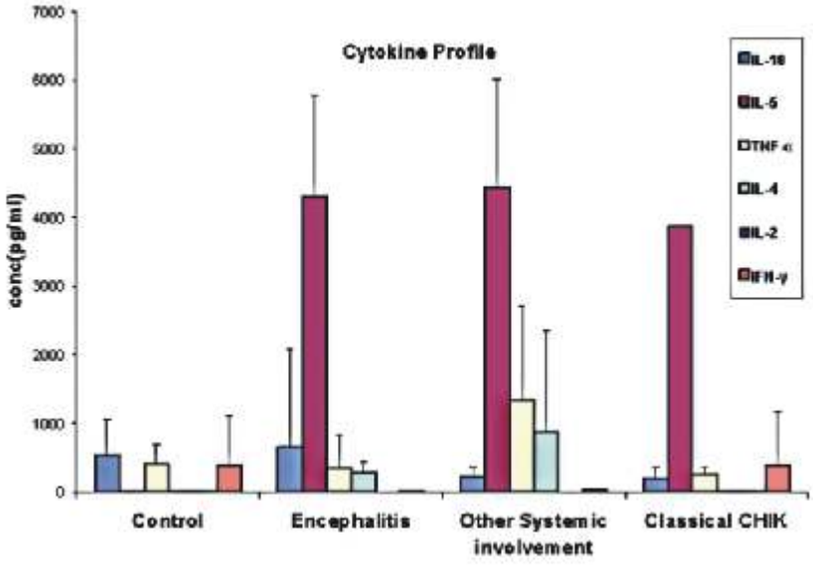


Fig. 2: Cytokine levels in culture supernatants of peripheral blood mononuclear cells after stimulation with CHIKV rE2p in different categories of Chikungunya patients & controls

Association of the neurological complications of the Chikungunya virus infection during the current epidemic with specific mutation(s) in the viral genome

VA Arankalle, SL Hundekar and SS Gandhe

Introduction

Re-emergence of Chikungunya (CHIK) in epidemic form has affected several countries from Indian Ocean islands and India. In addition to the predominant classical presentation, several complications of the infection have been recorded. Both host and viral factors play crucial roles in modulating the course of an infection. This study deals with the viral factors.

Objective

- To examine if the neurological complications of CHIK could be correlated with specific mutations in the viral genome.

Work Done

Full genomes of CHIK viruses from 5 cerebrospinal fluid (CSF)-derived isolates, one CSF and ~8.5 Kb genome from the other CSF were amplified, sequenced and compared with the sequences derived from viruses isolated from the classical cases. These sequences represented the states of Gujrat, Maharashtra and Karnataka. No specific mutations differentiating the two forms of the disease could be identified. Thus, the neurological complications of CHIK infection are not related to the specific mutations in the viral genome and in-depth studies in relation to the host factors need to be undertaken on priority.

Establishment and characterization of a new *Aedes aegypti* cell line for Chikungunya isolation and propagation

AB Sudeep, Deepti Parashar, VA Arankalle and AC Mishra

Introduction

The 2006-07 Chikungunya epidemic in the Indian subcontinent warranted the need for rapid diagnosis and isolation of the virus from field collected serum/arthropod specimens. Since CHIK is vectored by *Aedes aegypti* mosquitoes in India, it was felt necessary to establish a new cell line from the vector species in order to facilitate further studies on the virus at the cellular level.

Objectives

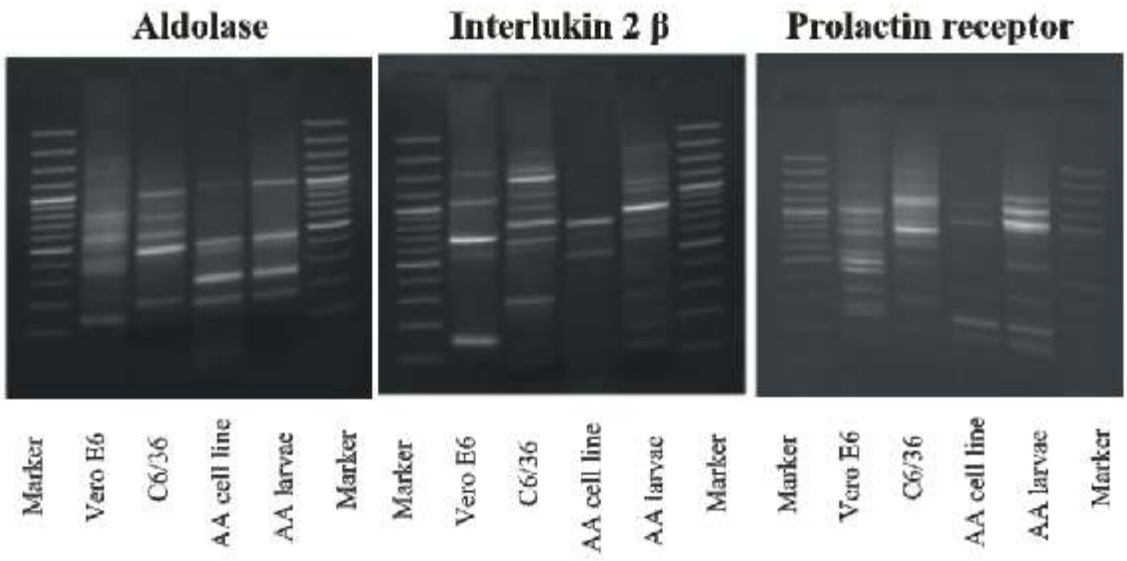
- To establish a new cell line from the vector species for CHIK studies
- To clone the cell line to isolate highly susceptible cell populations to CHIK virus
- Large scale propagation of CHIK virus antigen for diagnostic and vaccine studies.

Work done

A new cell line is established from *Ae. aegypti* mosquito from the neonate larvae and characterized. It is at the 62nd passage level and consists of three different cell types *viz*. epithelial like, fibroblast like and giant cells. The epithelial-like cells formed the majority of the cell population (>90%) and the other two cell types represent rest of

the population. Seventy-six percent cells showed diploid number (2n=6) of chromosomes at the 52nd passage level. The cells showed 10-fold increase in cell number at the 52nd passage level.

Species specificity of the cell line was determined using RAPD primers *viz*. mammalian aldolase, interleukin 2β and Prolactin receptor. The fingerprinting profile clearly demonstrated 100% homology with host insect confirming the origin of the cell line.



Susceptibility of the newly established cell line was studied and found that the cell line replicates (Japanese encephalitis (JE), West Nile (WN), Chikungunya (CHIK), Chittoor and dengue (DEN)) and two sandfly borne arboviruses (Chandipura and vesicular stomatitis virus). However, the cell line did not replicate two tick-borne arboviruses tested *i.e*. Ganjam and Kaisodi viruses. The virus yield of DEN, JE, WN, CHIK and Chandipura viruses in the cell line was very high and the cell line may find application in the detection, isolation and large-scale production of virus antigen.

Growth kinetics of Chikungunya virus in certain cell lines

AB Sudeep, VA Arankalle and AC Mishra

Introduction

The 2006-07 Chikungunya epidemic in the Indian subcontinent warranted the need for rapid diagnosis and isolation of the virus from field collected serum/arthropod specimens. Though many vertebrate and invertebrate cell lines supported the growth of the virus, it was felt necessary to determine the right cell line which is most sensitive and yield high titre for virus isolation and further studies.

Objectives

- To study the sensitivity and virus yield of different cell lines to CHIK virus

Work done

CHIK virus growth kinetics was studied in certain mammalian and insect cell lines to determine the virus yield. C6/36 cell line (a clone of Singh's *Ae. albopictus* cell line) was found the most sensitive and high yielding when a comparative study was carried out **(Fig. 3)**. Among the vertebrate cell lines, Vero E6 cell line yielded high titre of virus. Further studies with other cell lines are in progress.

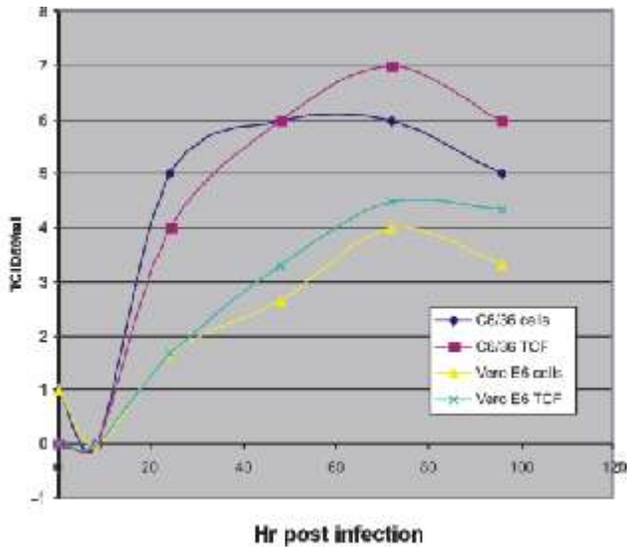


Fig. 3 : Growth of CHIK virus in Vero E6 and C6/36 cell lines

Diagnostic Activity

Despite providing a large number of diagnostic kits to various state laboratories, samples from suspected Chikungunya cases are received at the National Institute of Virology from all over India. These samples were screened for the presence of antiCHIK IgM antibodies in MAC-ELISA test (NIV Kit). Table shows the results of the tests carried out.

Chikungunya testing: 1 April 2007 to 31 March 2008

S. No.	State	Total samples	Chik IgM positive
1	Maharashtra	1682	391
2	Kerala	715	308
3	Gujarat	109	18
4	Pondicherry	02	00
5	West Bengal	14	07
6	Orissa	06	01
7	Tamilnadu	40	22
8	Madhya Pradesh	19	01
9	Delhi	06	02
10	Rajasthan	03	01
11	Karnataka	02	00
Total		**2598**	**751**

Molecular surveillance

Molecular surveillence for CHIK virus was continued. Two genomes from Kerala (2006 and 2007) were sequenced completely. In addition, from over 100 serum samples, small fragments of the genome were amplified and sequenced. These will be used for further analysis.

Publications

- Arankalle VA, Shrivastava S, Cherian S, Gunjikar RS, Walimbe AM, Jadhav SM, et al. Genetic divergence of Chikungunya viruses in India (1963-2006) with special reference to the 2005-2006 explosive epidemic. **J Gen Virol**; 2007 Jul; 88(Pt 7): 1967-76.

Workshops / Conferences / Seminar / Meetings attended

VA Arankalle

- Member of the Indian delegation to France, Presentation on "Chikungunya in India" at France (6-7th Jun 2007).
- WHO meeting of the Experts Group on 'Chikungunya Fever', at Aurangabad, Maharashtra (India) 27-29 September 2007.
- Invited speaker (Topic: Molecular epidemiology of Chikungunya in India.) during an International Workshop on "Molecular Epidemiology and Immunology of Malaria and other Vector Borne Diseases" at RMRC, Jabalpur, October 16-19, 2007.
- Invited speaker (Topic: Re-emergence of Chikungunya in India: Molecular studies.) at the Annual meeting of Indian Academy of Sciences at Trivendrum during 1-4 Nov, 2007.
- Presented multi-centric CHIK project to a special review committee at ICMR on 7th Nov 2007.
- Invited speaker (Topic: HEV Diagnosis" and "Chikungunya in India) at the International Conference on 'Emerging and Re-Emerging Viral Diseases of the Tropics and Sub-Tropics', at New Delhi during December 11-14 2007

Deepti Parashar

- Presented a paper entitled "Development of real-time RT-PCR for detection and quantification of Chikungunya Virus" in the International Conference Emerging and Re - emerging Viral Diseases of the Tropics and Subtropics, organized at IARI, Pusa Road, New Delhi from 11th -14th Dec. 2007.

AB Sudeep

Presented the following papers in the International Conference Emerging and Re - emerging Viral Diseases of the Tropics and Subtropics, organized at IARI, Pusa Road, New Delhi from 11th -14th Dec. 2007.

- Sudeep AB, Jadi RS, Basu A, Arankalle VA and Mishra AC. Growth kinetics of Chikungunya virus in certain cell lines.
- Jadi RS, Sudeep AB, Arankalle VA and Mishra AC. Inactivation kinetics of Chandipura with different agents with reference to immunogenicity.

Supriya Hundekar

- Presented poster titled 'Full Genome Sequence Analysis of Chikungunya Virus isolates From Kerala (India) during 2006-2007' at International Conference on 'Emerging and Re-Emerging Viral Diseases of the Tropics and Sub-Tropics', at New Delhi December 11-14, 2007.

Training programmes / Workshops / Seminars organized

The following persons were trained in animal and insect cell culture.

- Mrs. Rekha Jaiswal, under WHO fellowship undergone one month training in Animal Tissue culture.
- Mr. Chetan Mokashi (M.Sc.), Modern College, Shivajinagar has undergone a project entitled, "Characterization of insect cell lines" as partial fulfillment of M.Sc. degree of University of Pune.
- Ms. Archana Mokashe (M.Sc.), Annasaheb Magar College, Hadapsar has worked under Dr. AB Sudeep on a project entitled; "Growth and yield in *Helicoverpa armigera* NPV in *H. armigera* cell line under different nutritional and temperature conditions".
- Dr. Bharat Bhushan Sharma, from Indore Biotech, Madhya Pradesh has undergone a short-term training course on *in vitro* cultivation of NPVs in certain lepidopteran cell lines.