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Chandipura virus growth kinetics in vertebrate cell lines, insect cell lines & embryonated eggs

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***Background & objectives*: Since not much information on Chandipura virus is available, an attempt was made to study the growth kinetics of the virus in certain vertebrate, invertebrate cell lines and embryonated chicken eggs.**

***Methods*: Comparative study of Chandipura virus (CHPV) growth kinetics in three vertebrate cell lines [Vero E6, Rhabdo myosarcoma (RD), Porcine stable kidney (PS) cell lines], two insect cell lines [*Aedes aegypti* (AA) and *Phlebotomus papatasi* (PP-9) cell lines] and embryonated pathogen free chicken eggs was conducted, by tissue culture infective dose 50 per cent ($TCID_{50}$) and indirect immunofluorescence assay (IFA).**

***Results*: All the cell lines and embryonated egg supported the growth of CHPV and yielded high virus titre. The vertebrate cell lines showed distinct cytopathic effect (CPE) within 4-6 h post infection (PI), while no CPE was observed in insect cell lines. PP-9 cell line was the most sensitive system to CHPV as viral antigen could be detected at 1 h PI by IFA.**

***Interpretation & conclusions*: Our results demonstrated that all the systems were susceptible to CHPV and achieved high yield of virus. However, the PP-9 cell line had an edge over the others due to its high sensitivity to the virus which might be useful for detection and isolation of the virus during epidemics.**

Key words *Aedes aegypti* cell line - Chandipura virus - growth kinetics - *Phlebotomus papatasi* cell line - Vero E6 cell line

Chandipura virus (CHPV) belongs to family *Rhabdoviridae*, a hitherto unimportant virus from the standpoint of public health, has been implicated in outbreaks occurred in several districts of Andhra Pradesh (AP), Maharashtra and Gujarat during 2003-2004, killing more than 200 children[1,2]. The outbreak was unique as the disease progressed rapidly and killed children below the age of 15 yr within 48 h of hospitalization with a case fatality rate of >55 and >75 per cent in AP and Gujarat States, respectively. Since little information is available about the virus, vectors, and virus transmission, an attempt was made to study growth kinetics of the virus in certain living systems, *i.e. in vitro* and *in ovo* as part of the characterization of the virus. Comparative studies were also conducted to determine the appropriate system to propagate the virus in large scale and also the most sensitive system for virus detection in case of an outbreak. In the present communication, we report the susceptibility and sensitivity of three vertebrate cell lines, two insect cell

lines and embryonated eggs to the virus using indirect immunofluorescence assay (IFA) and 50 per cent tissue culture infective dose ($TCID_{50}$).

Material & Methods

The study was perfomed at the Microbial Containment Complex, National Institute of Virology, Pune. CHPV (strain number 034627) used in the present study was isolated from Andhra Pradesh, India, from human serum in 2003[1]. The isolate was plaque purified and passaged 4 times in Vero E6 cell line before using for the present study. Vero E6, Rhabdomyosarcoma (RD) and Porcine stable kidney (PS) cell lines were maintained in DMEM (Earle's) medium supplemented with 10 per cent foetal bovine serum (FBS) (Gibco, BRL, USA). *Aedes aegypti* (AA) cell line developed from the neonate larvae (in-house) was maintained in MM (Mitsuhashi Maramorosch) medium[3] supplemented with 10 per cent FBS and used between 25-30 passage levels. The sand fly cell line (PP-9) derived from *Phlebotomus papatasi* provided by Dr Robert Tesh, Department of Pathology, Center for Bio-defense and Emerging Infectious Diseases, University of Texas Medical Branch Galveston, USA, was maintained in Grace's medium (Gibco, BRL, USA) supplemented with 15 per cent FBS. Fertilized special pathogen free white leghorn chicken eggs were procured from Venkateshwara Hatcheries, Pune, candled and incubated for 12 days in an egg incubator at the National Institute of Virology (NIV), Pune.

Growth kinetics in vertebrate cells: Cells were grown to approximately 90 per cent confluency in 24 well plates (Nunc, Denmark) and infected with CHPV at a multiplicity of infection (MOI) of 10. After incubation at 37°C for 1 h with rocking at every 10 min, the virus suspension was discarded and cells were washed thrice with 1x phosphate buffered saline (PBS) to remove unadsorbed virus. Cells were fed with DMEM containing 2 per cent FBS and returned to the incubator. Cells and corresponding supernatants were harvested at an hourly interval as described below. The medium from one well, selected randomly, was harvested using a pipette and stored immediately at -70°C. Attached cells from the well were harvested with a cell scraper (Nunc, Denmark), washed four times with 1x PBS and suspended in 1.0 ml DMEM supplemented with 2 per cent FBS. The cell suspension was freeze-thawed thrice, centrifuged at 2790 x *g* for 10 min, collected supernatant and stored at -70°C. Same procedure was followed for all the subsequent samples. After completion of the experiment, samples were thawed at 4°C, diluted serially and titrated in RD cells. Cells were observed daily, stained after 72 h and 50 per cent tissue culture infective dose ($TCID_{50}$)/ml was determined as described by Reed and Muench[4].

Growth kinetics in insect cells: Confluent monolayers of AA cells and PP-9 cells were grown in Leighton tubes and infected as described above. The cells were incubated for 1 h at 28°C with rocking at every 10 min post infection (PI). After incubation the inoculum was discarded, washed cell sheet thrice with PBS (1x), fed with appropriate maintenance medium and returned back to the incubator. Two tubes each from the two cell lines were removed randomly at an hourly interval upto 10 h. Further collections were made from 24 h post infection onwards for seven days at 24 h interval. All the samples were stored immediately after harvest at -70°C until processed. The samples were freeze-thawed thrice, centrifuged at 2790 x *g* for 10 min at 4°C, titrated the cell lysate in RD cells and determined virus titre as described above.

Indirect immunofluorescence assay (IFA) technique: IFA was carried out with antiserum raised in adult mouse as described earlier[5]. Anti-mouse antibody conjugated with FITC was procured from Sigma Chem. Co., St. Louis, USA and used as per directions of the manufacturer. The stained cells were observed under UV illumination using an Olympus BH2 microscope (Japan).

Growth kinetics in embryonated eggs: Eggs were inoculated with 200 µl virus (2log EID_{50}) through allantoic route and incubated at 35°C. Allantoic fluid (AF) from two eggs selected randomly was harvested at an hourly interval and stored at -70°C. After completion of the experiment, the AF was thawed quickly and titrated in RD cells. Virus titre ($TCID_{50}$/ml) of each sample was determined as described earlier.

Results

Growth of CHPV in different cell lines: Commencement of cytopathic effects (CPE) was observed at 4 h PI in RD cell line and total cell destruction was observed at 16 h PI. The CPE was characterized by rounding of cells followed by foci formation and disintegration of cells leading to total detachment of cells. Fig. a depicts the virus growth pattern in the supernatant and cell pellet. Maximum virus yield obtained in the cell pellet was 5 $\log_{10}$ $TCID_{50}$/ml and supernatant was 7 $\log_{10}$ $TCID_{50}$ at 8 and 10 days PI respectively.

The commencement of CPE in Vero E6 and PS cell lines was observed 6-8 h PI and the pattern of

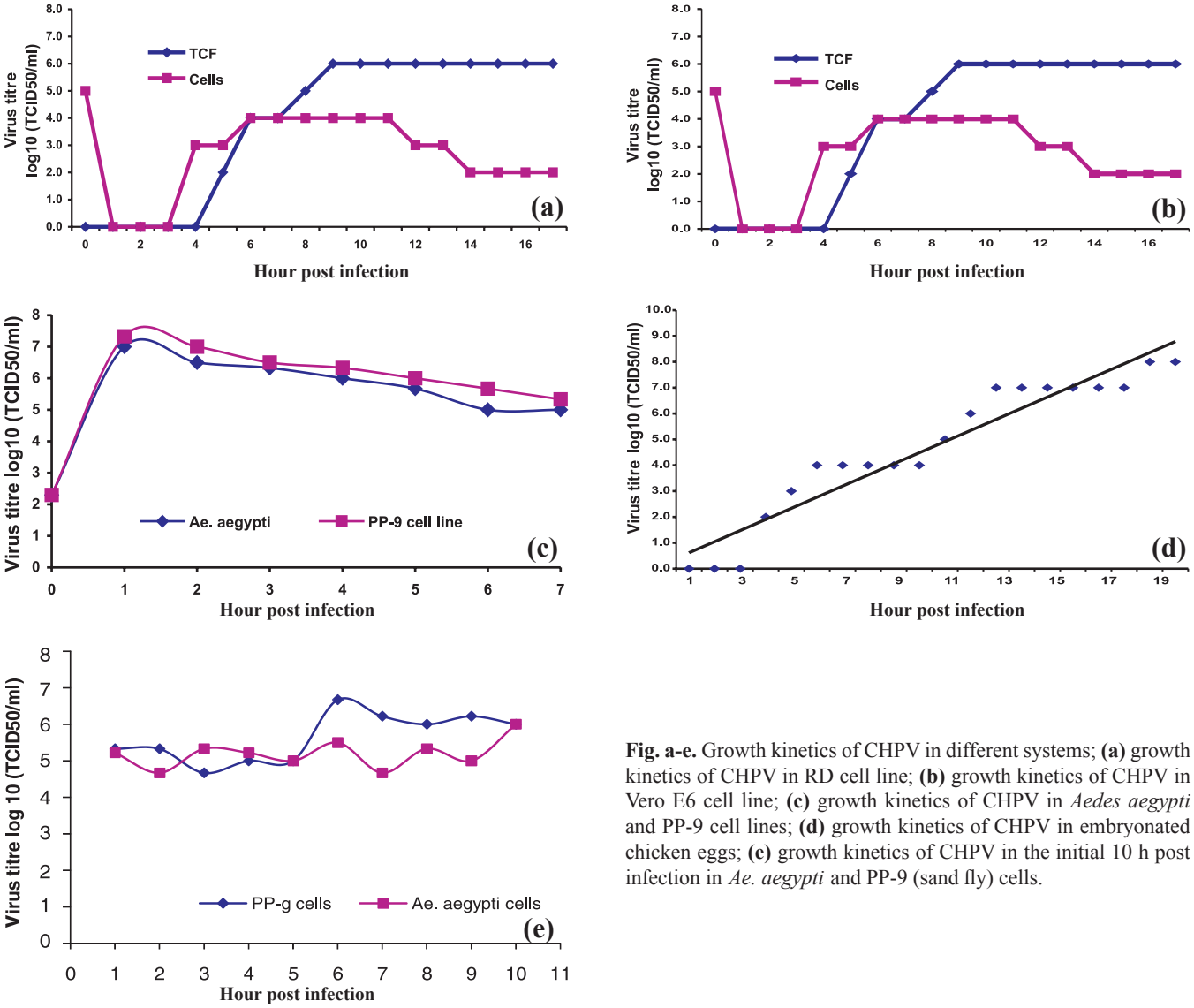

Fig. a-e. Growth kinetics of CHPV in different systems; **(a)** growth kinetics of CHPV in RD cell line; **(b)** growth kinetics of CHPV in Vero E6 cell line; **(c)** growth kinetics of CHPV in *Aedes aegypti* and PP-9 cell lines; **(d)** growth kinetics of CHPV in embryonated chicken eggs; **(e)** growth kinetics of CHPV in the initial 10 h post infection in *Ae. aegypti* and PP-9 (sand fly) cells.

induction of CPE resembled that observed in RD cells. The growth kinetics of CHPV in Vero E6 cell line is given in Fig. b. The virus growth kinetics as well as maximum virus yield in PS and Vero E6 cell lines resembled closely (Data on CHPV growth kinetics in PS cell line not shown). Maximum virus titre observed in the cell pellet was 4 $\log_{10}$ $TCID_{50}$/ml at 6 h PI and in supernatant was 6$\log_{10}$ $TCID_{50}$/ml at 9 h PI.

In both the insect cell lines, *i.e*. *Aedes aegypti* (AA) and PP-9, CPE was not observed at any stage of infection. IFA studies were able to detect the presence of virus antigen from 1 h PI with very distinct intracytoplasmic fluorescence. The maximum virus yields in AA cell line and PP-9 cell line were 7.0 and 7.33 $\log_{10}$ $TCID_{50}$ per ml respectively at 24 h PI (Fig. c). A drop in virus titre was observed on subsequent days in both the cell lines. However, virus titre of 5 $\log_{10}$ $TCID_{50}$/ml was observed upto 7th day PI in the cell lines.

Infected chicken embryos remained healthy upto 6 h PI. Sickness and sluggish movement was observed from 7 h PI onwards followed by death at 26 h PI. A very characteristic pattern of virus growth was observed in embryonated eggs (Fig. d). After a stationary phase of three hours, the first cycle of virus release was observed upto 7 h PI yielding approximately 4$\log_{10}$ $TCID_{50}$/ml virus. Similar cycles were observed after 11 h PI and 18 h PI. Maximum virus yield of 8$\log_{10}$ $TCID_{50}$/ml was achieved at 20 h PI.

Table. Sensitivity of different cell lines to CHPV: a comparative study using IFA

CHPV quantity	Time interval, h PI	Cell lines		
		PP-9	RD	Vero E6
100 pfu/ml	1	+ve	+ve	+ve
	2	+ve	+ve	+ve
	5	+ve	+ve	+ve
10 pfu/ml	1	+ve	-ve	-ve
	2	+ve	-ve	-ve
	5	+ve	-ve	-ve

RD, Rhabdomyosarcoma cell line; Pfu, plaque forming unit; +ve, positive; -ve, negative

Sensitivity of different cell lines to CHPV: PP-9 cell line was observed as the most sensitive cell line to CHPV infection when a different study was carried out to determine the most sensitive cell line among PP-9 cells, Vero E6 and RD cell lines using IFA as the assay system. Very distinct fluorescent virogenic particles were seen inside CHPV infected PP-9 cells at 1 h PI when infected with 10 plaque forming units (pfu). Distinct peri-nuclear fluorescence was detected from 2 h PI onwards. However, both Vero E6 and RD cell lines were unable to pick up infection and replicate with 10 pfu of virus (Table). However, when infected with 100 pfu, all the three cell lines picked up infection and replicated demonstrating that the vertebrate cells needed more inoculum to replicate.

Discussion

The virus induced CPE, characterized by rounding of cells, cell detachment, *etc.*, at 4 h PI in RD cells and at 6-8 h in Vero E6 and PS cell lines suggested the application of these cell lines for diagnostic purpose. Similar results were also reported by earlier workers[5]. The present study also revealed an eclipse phase in vertebrate cell lines and embryonated chicken eggs during which period, no virus replication could be detected. However, a significant observation in the growth kinetics of CHPV in insect cell lines was that the eclipse period was not prominent (Fig. e). It could have been very early and very brief due to rapidity of infection. No significant loss in the titre of adsorbed virus was detected in the insect cells and the logarithmic growth phase of the virus started from 5 h PI. Though the virus did not cause CPE in these cells, virus replication could be monitored by IFA, demonstrating the advantage of the system in combination with IFA for early detection and isolation. IFA has become a very useful tool in the detection of virus antigen and is being used effectively for virus isolation studies[6-8].

In vertebrate cell lines and embryonated eggs, a long latent phase was observed after infection and during the period virus could not be detected by titration in RD cells either in the supernatant or in cells. In embryonated eggs also, CHPV could not be traced in the allantoic fluid. Our earlier data had shown that infected allantoic fluid retained virus without significant loss for 24 h at 37°C (Jadi and others, unpublished data). Therefore, in that study the inoculum adsorbed might have been less and the rate of virus replication in the initial stages would have been low. This could explain for not showing virus in infected allantoic fluid. Henle and Rosenberg[9] also reported similar observations during their studies with influenza virus in chicken embryos. Similar explanation also stands for the vertebrate cells as virus could not be detected in the initial hours after infection in cell pellet or supernatant by virus titration in RD cells. On the contrary, both the insect cells were able to maintain the adsorbed virus without significant loss in virus titre. In both the insect cell lines, the adsorbed virus was maintained up to 5 h PI without significant loss in virus titre and increased gradually thereafter. CPE was never observed in these cell lines and the virus titre was maintained up to 10 days PI without significant change. This indicates that there could be a special mechanism in insect cells, which preserves the viral antigen for replication and transmission as a prerequisite for a vector as both *P. papatasi* and *Ae. aegypti* are capable of transmitting the virus horizontally and vertically[10,11]. The comparative study to determine the sensitivity of different systems has shown that the sand fly cell line was the most sensitive among the systems tested. The test demonstrated that PP-9 cell line could replicate CHPV up to 10 pfu/ml while one of the most sensitive vertebrate cell lines (RD) needed 100 pfu/ml to replicate a cell population of approximately $1x10^6$ cells. Even with IFA, the presence of virus antigen could not be detected when RD and Vero E6 cells were infected with 10 pfu of virus.

As sand flies are the incriminated vector of the virus, as suggested by repeated isolations[2], the high susceptibility of the sand fly cell line was expected. However, it was interesting to note that the *Ae. aegypti* cell line was also equally susceptible to CHPV and produced a comparable yield of virus. Earlier studies conducted in the laboratory also demonstrated the efficiency of *Ae. aegypti* mosquitoes in the transmission of the virus more efficiently than other mosquitoes[12,13].

It has already been documented earlier that embryonated eggs yield high virus titre than the cell lines[14]. Our study also demonstrated high virus yield in embryonated eggs than vertebrate and invertebrate cell lines. However, large-scale production of virus for vaccine in eggs has several disadvantages. One of the major limitations is the high cost involved in the downstream processing, as allantoic fluid contains a large number of proteins in comparison to cell culture medium. Moreover, egg proteins are allergic to some people and hence limit the use of egg-derived vaccines. The mass killing of embryos for virus production is also a matter of concern.

Virus isolation is an important area of research and requires sensitive systems for rapid diagnosis of aetiological agents during epidemics to take corrective measures. A high virus yielding host system is equally important in large-scale propagation of viruses for diagnostic and vaccine development studies.

In conclusion, among the cell lines tested, PP-9 cell line showed highest sensitivity to CHPV, and may be explored further.

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