The host-range *td*CE phenotype of Chandipura virus is determined by mutations in the polymerase gene

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Chandipura virus strain I653514 GenBank accession number: KF468775
Chandipura virus mutant *td*CE CH112 GenBank accession number: KF468772
Chandipura virus mutant *td*CE CH157 GenBank accession number: KF468773
Chandipura virus mutant *td*CE CH256 GenBank accession number: KF468774

Abstract

The emerging arbovirus Chandipura virus (CV) has been implicated in epidemics of acute encephalitis in India with high mortality rates. The isolation of temperature-dependent host range (*td*CE) mutants, which are impaired in growth at 39°C in chick embryo (CE) cells but not in monkey cells, highlights a dependence on undetermined host factors. We have characterised three *td*CE mutants, each containing one or more coding mutations in the RNA polymerase gene and two containing additional mutations in the attachment protein gene. Using reverse genetics we show that a single amino acid change in the virus polymerase of each mutant is responsible for the host range specificity. In CE cells at the non-permissive temperature the discrete cytoplasmic replication complexes seen in mammalian cells or at the permissive temperature in CE cells were absent with the *td*CE mutants, consistent with the *td*CE lesions causing disruption of the replication complexes in a host-dependent manner.

Chandipura virus (CV) is a member of the Vesiculovirus genus of the family *Rhabdoviridae* containing a genome of 11,119 nucleotides of non-segmented, negative sense RNA (Marriott, 2005). CV is an emerging virus that has been associated with epidemics of acute encephalitis in several Indian states with high fatality rates in children. CV was first isolated in 1965 from two adults presenting with febrile illness in Nagpur district, Maharashtra state, India (Bhatt & Rodrigues, 1967) and was later isolated from a patient suffering from encephalopathy syndrome in 1980 (Rodrigues *et al.*, 1983). CV epidemics occurred in Andhra Pradesh and Maharashtra in 2003, Gujarat state in 2004 and Maharashtra in 2005 and 2007 (Chadha *et al.*, 2005; Gurav *et al.*, 2010; Tandale *et al.*, 2008). The epidemic case fatality rates were high: 41% in Maharashtra, 55.6% (183 out of 329 cases) in Andhra Pradesh in 2003, 78.3% (18 out of 23 cases) in Gujarat in 2004 and 44% (34 out of 78 cases) in Maharashtra in 2007 (Chadha *et al.*, 2005; Gurav *et al.*, 2010), giving an average of 54.8%.

Serological studies have shown that exposure to CV is widespread in India with 94-97% of adults in Andhra Pradesh during the 2003 outbreak being seropositive (John, 2010). The only known natural route of transmission of CV is by phlebotomine sand flies (Mavale *et al.*, 2007; Rao *et al.*, 2004), although transmission in the laboratory of the virus by *Aedes aegypti* mosquitos has also been demonstrated (Mavale *et al.*, 2005). CV has also been isolated in West Africa (Fontenille *et al.*, 1994; Traore-Lamizana *et al.*, 2001), indicating that it may be widespread in tropical regions.

The critical involvement of host cell factors in the replication of vesiculoviruses was highlighted by the isolation of VSV Indiana, VSV New Jersey and CV temperature-dependent host range (*td*CE) mutants by mutagenesis using 5-fluorouracil (Gadkari & Pringle, 1980; Pringle, 1978; Rasool & Pringle, 1986). These mutants are characterised by growth impairment at 39°C in primary avian cells in culture but not in monkey cells. The *td*CE mutant phenotype has been arbitrarily defined as viruses with an efficiency of plating (E.O.P.) in BSC-1 (monkey) cells of <0.7 and in chick embryo (CE) cells of >2 to reflect significant growth impairment at 39°C compared to 31°C in CE cells while showing normal growth in BSC-1 cells at both temperatures. This phenotype demonstrates a dependence of these *vesiculoviruses* on undetermined host factors. Analysis of *in vitro* transcription assays showed reduced RNA transcriptase activity resulting in significantly lower amounts of viral RNA synthesised in the *td*CE mutants prepared in avian cells at the restrictive temperature and viral protein synthesis was also found to be either considerably depressed or non-existent in all *td*CE mutants grown in avian

cells at the restrictive temperature (Rasool & Pringle, 1986). This led to the suggestion that the growth restriction is caused by diminished RNA synthesis which in turn results in decreased production of viral polypeptides. *In vitro* RNA methylation activity of the virus RNA dependent RNA polymerase was found to be normal in all *td*CE mutants (Rasool & Pringle, 1986). We have investigated the underlying molecular basis of host range specificity, the CV *td*CE phenomenon using a reverse genetics approach.

CV strain 1653514 and temperature sensitive derivatives from it (Gadkari & Pringle, 1980; Rasool & Pringle, 1986) were used. Three *td*CE mutants were selected on the basis of their previously established high E.O.P. values in CE cells for analysis of the *td*CE phenotype during this investigation; namely CH112, CH157 and CH256 (Gadkari & Pringle, 1980; Rasool & Pringle, 1986). Wild-type and *td*CE mutant CV titres were determined by standard plaque assay in permissive (BSC-1 monkey kidney cells) and conditionally permissive (primary cultures of chick embryo (CE) fibroblasts) at 31°C and 39°C. The titres confirmed that the three mutants displayed the *td*CE phenotype (Supplementary table 1). The E.O.P. scores of the three mutants in CE cells were comparable with those described following the original characterisation, though mutant CH157 had an E.O.P. of 0.83 in BSC-1 cells which was outside of the desired range of an E.O.P in BSC-1 cells of <0.7 and in CE cells of >2 (Gadkari & Pringle, 1980; Rasool & Pringle, 1986).

To identify the genetic lesions responsible for, and therefore the virus gene(s) associated with, host range requirements, we sequenced the entire genome of each mutant. We also confirmed the nucleotide sequence of the wild type virus. RNA was extracted from wild type CV and *td*CE mutant stocks using TRIzol LS reagent® (Invitrogen Life technologies), according to the manufacturer's protocol and reverse transcribed to generate cDNA using random hexanucleotide primers. Seven PCR products of approximately 2 kb, which overlapped with each other by 200-400 bp were amplified by PCR from the full length cDNA using *KOD* hot start DNA polymerase (Novagen, Germany) and each PCR product was purified (Qiaquick kit, Qiagen). The primers used to construct PCR products 1-7 are shown in Supplementary Table 2. Each PCR product was amplified in duplicate and sequenced three times using different primers (GATC, Germany).

The nucleotide sequences were aligned and the mutations identified. Each mutant contained only a very small number of mutations, summarised in Table 1. All of the *td*CE mutants contained coding

changes in the RNA polymerase (L) gene and mutants CH112 and CH256 contained additional mutations in the attachment (G) protein gene. Additionally, two of the *td*CE mutants contained a further silent point mutation(s). Mutant CH112 contained the point mutation U_{1980}→C in the P gene and U_{9958}→C in the L gene at amino acid residues 206 and 1732, respectively. Mutant CH157 contained a U_{9022}→C mutation in the L gene at amino acid residue 1420. The presence of the mutations resulting in coding changes in the polymerase gene is consistent with the observation of reduced RNA transcriptase activity *in vitro* at 39°C in 10 of 12 CV *td*CE mutants relative to wtCV, with CH112, CH157 and CH256 exhibiting efficiencies of transcription at 39°C of 14%, 56% and 37% of the wild-type virus (Rasool & Pringle, 1986).

To confirm which of the alterations identified in the mutants were responsible for the *td*CE phenotype recombinant viruses containing each point mutation, and combinations of the L mutations in the case of mutant CH112, were generated. Quickchange site directed mutagenesis using mutagenic oligonucleotide primers (Supplementary Table 2) was used to produce the C_{5691}→U, C_{5760}→U, C_{7217}→U, G_{9735}→U, G_{7694}→A, U_{3941}→C, U_{4034}→C point mutations in the full length anti-genomic clone of CV (pT7CV). The methylated, non-mutated parental DNA templates were digested with *DpnI* restriction endonuclease and DNA fragments containing the point mutations were re-cloned into pT7CV and the insert sequences verified by DNA sequencing. Combinations of mutations were made by sequential Quickchange reactions with the appropriate mutagenic primers. Rescue of infectious virus was carried out for the wild type virus as control and for each of the nine combinations of point mutations generating coding changes in the virus RNA dependent RNA polymerase gene or one of the two mutations in the attachment (G) protein gene using the reverse genetics system previously described (Marriott & Hornsey, 2011). The genomic plasmid (containing the point mutation(s)) and support plasmids T7N, T7P and T7L were transfected into BSRT-7/5 cells (Buchholz *et al.*, 1999) using *Trans*IT®-LT1 transfection reagent (Mirus, USA). After 48 hours, the supernatant was inoculated onto confluent monolayers of BSC-1 cells and virus was harvested when the cytopathic effect was extensive (approximately 24 hours post infection). The titres of the mutant viruses in permissive and non-permissive conditions and the E.O.P. for each are shown in Table 2. The data show that a single amino acid change in the virus RNA polymerase of each mutant was solely responsible for the *td*CE host range dependent phenotype. All recombinant viruses containing the C_{7127}→U mutation in the L gene of mutant CH112 conferred the *td*CE phenotype while any

combination of the C_{5691}→U and C_{5760}→U point mutations in the L gene alone or together did not (Table 2). Furthermore, the results showed that the mutations identified in the G gene of CH112 and CH256 were not involved in the *td*CE mutant phenotype. Similarly, only the presence of the G_{7694}→A mutation in the L gene of mutant CH256 and G_{9735}→U in the L gene of mutant CH157 conferred the *td*CE phenotype.

The large protein of rhabdoviruses is multifunctional and has been described as containing 6 functional domains of greater amino acid conservation and highly variable intra domains (Poch *et al.*, 1990). The molecular interrelationship between the domains in the VSV L protein have also been established with conserved blocks I-IV arranged into a ring structure containing the RNA polymerase activity and an appendage of domains V and VI responsible for capping and methylation respectively (Rahmeh *et al.*, 2010). The point mutations conferring the host range phenotype of the *td*CE mutants are located between domains III and IV (CH112 C_{7217}→U) or within domain IV (CH256 G_{7694}→A) or VI (CH157 G_{9735}→U) of the L gene (Figure 1A). The G_{9735}→U change within domain VI lies in a stretch of highly conserved amino acids and immediately precedes a GXGXG motif (GDGSG sequence) located 19 amino acids downstream of a lysine residue, which have been implicated in polyadenylation or protein kinase functions (Cherian *et al.*, 2012). While the methyltransferase activity of the L protein has been proposed to also lie within domain VI the CH157 mutant polymerase displays a normal mRNA capping function (Rasool & Pringle, 1986).

The location of the *td*CE lesions within the polymerase protein gene coupled with the defect in virus RNA synthesis in the mutants suggest that in CE cells the formation of functional replication complexes may be affected. Attempts to insert marker sequences within the CV L gene in a recombinant virus were unsuccessful and the resulting polymerase proteins were non-functional. As an alternative approach to study the replication complexes we generated a recombinant CV in which red fluorescent protein was fused in-frame within a putative hinge region of the CV phosphoprotein (P/RFP). This was identified by homology with the hinge region in the P protein of VSV which allows insertion of green fluorescent protein (GFP) while retaining function (Das *et al.*, 2006). We inserted the open reading frame for the red fluorescent protein, mRFP1 (Campbell *et al.*, 2002) between residues 213 and 214 of the CV P protein. Sites for *Hin*dIII and *Sac*I were introduced into the P gene, the mRFP1 gene was amplified and the product was digested with *Hin*dIII and *Sac*I and ligated in-

frame into the corresponding sites of the modified P gene. Finally the *Afl*II to *Pfl*23II fragment containing the P-RFP fusion was inserted into the full-length antigenomic CV plasmid, pT7CV (Marriott & Hornsey, 2011), to give plasmid pT7CV-PRFP. Primer details are given in Supplementary Table 2. Single-step growth of the resulting virus rCV-P/RFP in BSC-1 cells showed a very similar virus yield (8.09 ± 0.07 $\log_{10}$ pfu/ml) to the recombinant wild-type virus (8.22 ± 0.02 $\log_{10}$ pfu/ml) indicating that the RFP insertion had minimal effect on virus growth in cell culture. The fusion protein is stable and the resulting recombinant virus grows well in both mammalian and avian cells.

To determine whether the *td*CE point mutations disrupt the intracellular localisation of the P protein in the replication complexes the L gene of the P/RFP virus was mutated to produce three *td*CE mutants, r112P/RFP (C_{7217}→U), r157P/RFP (G_{9735}→U) and r256P/RFP (G_{7694}→A) corresponding to *td*CE mutants CH112, CH157 and CH256, respectively. DNA fragments containing one of the three critical *td*CE point mutations were cloned into pT7CV-PRFP and the inserted portions sequenced. Recombinant P/RFP viruses were then recovered as described previously and used to infect cells at an M.O.I of 1 pfu per cell. Cells were fixed with 10% formaldehyde in PBS when fluorescence was clearly visible and mounted onto glass slides using aqueous mounting medium containing DAPI stain (VectorLabs). Fluorescence was visualised using a Leica SP5 confocal fluorescence microscope.

The distribution of P/RFP in wild type and mutant L viruses in BSC-1 and CE cells at 31°C and 39°C is shown in figure 1B. In infected BSC-1 cells at either temperature P/RFP was present in large bright cytoplasmic foci located primarily near the cell periphery (figure 1B a-h). Several smaller punctate regions of fluorescence were also visible as well as very faint diffuse staining throughout the cell cytoplasm (figure 1B a-h). A similar pattern of fluorescence was also observed with the RFP/P mutant L viruses in CE cells grown at 31°C and in wild type virus-infected CE cells grown at both temperatures (figure 1B i-m). However, all three mutant L gene viruses showed a significantly different pattern in CE cells infected at 39°C, generating only a very diffuse pattern of RFP-P fluorescence (figure 1B n-p) suggesting that the formation of replication complexes was significantly impaired in non-permissive conditions. Studies in VSV have shown RNP complexes are distributed throughout the cytoplasm but at early stages of infection these are located predominantly adjacent to the nucleus during nucleocapsid synthesis. Subsequently the complexes are seen to traffic in a microtubule-dependent manner towards the cell membrane for virion assembly prior to progeny

release (Das *et al.*, 2006). It is likely that the images in Figure 1 demonstrate the late stages of CV infection. There was no visible RFP expression in CE cells infected with P/RFP mutant L viruses at 39°C until 24 hours post-infection, whereas RFP was clearly visible after just 8 hours when the cells were incubated at 31°C (the latter was also true for CE cells inoculated with the wild type L virus at either temperature and BSC-1 cells under any condition). The lack of P/RFP-containing foci in CE cells at the restrictive temperature, taken together with previous data showing defects in *in vitro* transcriptase activity and polypeptide synthesis, supports the hypothesis that the defects in the mutant L proteins lead to disruption of replication complexes, reduced viral RNA synthesis and hence reduced viral protein synthesis. The three point mutations examined lead to similar phenotypes, despite the amino acid changes being located in separate domains of the CV L protein.

In conclusion, the data presented here demonstrate that the conditional growth defect for each of three CV *td*CE mutants, CH112, CH157 and CH256 is caused by single amino acid changes in the virus RNA polymerase protein. The lesions responsible for the severe growth defect severely affect the ability to assemble large replication complexes in the cytoplasm of cells at the non-permissive conditions.

References

Bhatt, P. N. & Rodrigues, F. M. (1967). Chandipura: a new Arbovirus isolated in India from patients with febrile illness. *Indian J Med Res* **55**, 1295-1305.

Buchholz, U. J., Finke, S. & Conzelmann, K. K. (1999). Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *J Virol* **73**, 251-259.

Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A. & Tsien, R. Y. (2002). A monomeric red fluorescent protein. *Proc Natl Acad Sci U S A* **99**, 7877-7882.

Chadha, M. S., Arankalle, V. A., Jadi, R. S., Joshi, M. V., Thakare, J. P., Mahadev, P. V. & Mishra, A. C. (2005). An outbreak of Chandipura virus encephalitis in the eastern districts of Gujarat state, India. *Am J Trop Med Hyg* **73**, 566-570.

Cherian, S. S., Gunjikar, R. S., Banerjee, A., Kumar, S. & Arankalle, V. A. (2012). Whole genomes of Chandipura virus isolates and comparative analysis with other rhabdoviruses. *PLoS One* **7**, e30315.

Das, S. C., Nayak, D., Zhou, Y. & Pattnaik, A. K. (2006). Visualization of intracellular transport of vesicular stomatitis virus nucleocapsids in living cells. *J Virol* **80**, 6368-6377.

Fontenille, D., Traore-Lamizana, M., Trouillet, J., Leclerc, A., Mondo, M., Ba, Y., Digoutte, J. P. & Zeller, H. G. (1994). First isolations of arboviruses from phlebotomine sand flies in West Africa. *Am J Trop Med Hyg* **50**, 570-574.

Gadkari, D. A. & Pringle, C. R. (1980). Temperature-sensitive mutants of Chandipura virus. II. Phenotypic characteristics of the six complementation groups. *J Virol* **33**, 107-114.

Gurav, Y. K., Tandale, B. V., Jadi, R. S., Gunjikar, R. S., Tikute, S. S., Jamgaonkar, A. V., Khadse, R. K., Jalgaonkar, S. V., Arankalle, V. A. & Mishra, A. C. (2010). Chandipura virus encephalitis outbreak among children in Nagpur division, Maharashtra, 2007. *Indian J Med Res* **132**, 395-399.

John, T. (2010). Chandipura virus - what we know & do not know. *Indian J Med Res* **132**, 125-127.

Marriott, A. C. (2005). Complete genome sequences of Chandipura and Isfahan vesiculoviruses. *Arch Virol* **150**, 671-680.

Marriott, A. C. & Hornsey, C. A. (2011). Reverse genetics system for Chandipura virus: tagging the viral matrix protein with green fluorescent protein. *Virus Res* **160**, 166-172.

Mavale, M. S., Fulmali, P. V., Ghodke, Y. S., Mishra, A. C., Kanojia, P. & Geevarghese, G. (2007). Experimental transmission of Chandipura virus by Phlebotomus argentipes (diptera: psychodidae). *Am J Trop Med Hyg* **76**, 307-309.

Mavale, M. S., Geevarghese, G., Ghodke, Y. S., Fulmali, P. V., Singh, A. & Mishra, A. C. (2005). Vertical and venereal transmission of Chandipura virus (Rhabdoviridae) by Aedes aegypti (Diptera: Culicidae). *J Med Entomol* **42**, 909-911.

Poch, O., Blumberg, B. M., Bougueleret, L. & Tordo, N. (1990). Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. *J Gen Virol* **71 (Pt 5)**, 1153-1162.

Pringle, C. R. (1978). The tdCE and hrCE phenotypes: host range mutants of vesicular stomatitis virus in which polymerase function is affected. *Cell* **15**, 597-606.

Rahmeh, A. A., Schenk, A. D., Danek, E. I., Kranzusch, P. J., Liang, B., Walz, T. & Whelan, S. P. (2010). Molecular architecture of the vesicular stomatitis virus RNA polymerase. *Proc Natl Acad Sci U S A* **107**, 20075-20080.

Rao, B. L., Basu, A., Wairagkar, N. S., Gore, M. M., Arankalle, V. A., Thakare, J. P., Jadi, R. S., Rao, K. A. & Mishra, A. C. (2004). A large outbreak of acute encephalitis with high fatality rate in children in Andhra Pradesh, India, in 2003, associated with Chandipura virus. *Lancet* **364**, 869-874.

Rasool, N. & Pringle, C. R. (1986). In vitro transcriptase deficiency of temperature-dependent host range mutants of Chandipura virus. *J Gen Virol* **67 (Pt 5)**, 851-862.

Rodrigues, J. J., Singh, P. B., Dave, D. S., Prasan, R., Ayachit, V., Shaikh, B. H. & Pavri, K. M. (1983). Isolation of Chandipura virus from the blood in acute encephalopathy syndrome. *Indian J Med Res* **77**, 303-307.

Tandale, B. V., Tikute, S. S., Arankalle, V. A., Sathe, P. S., Joshi, M. V., Ranadive, S. N., Kanojia, P. C., Eshwarachary, D., Kumarswamy, M. & Mishra, A. C. (2008). Chandipura virus: a major cause of acute encephalitis in children in North Telangana, Andhra Pradesh, India. *J Med Virol* **80**, 118-124.

Traore-Lamizana, M., Fontenille, D., Diallo, M., Ba, Y., Zeller, H. G., Mondo, M., Adam, F., Thonon, J. & Maiga, A. (2001). Arbovirus surveillance from 1990 to 1995 in the Barkedji area (Ferlo) of Senegal, a possible natural focus of Rift Valley fever virus. *J Med Entomol* **38**, 480-492.

Table and Figure legends

Table 1 Nucleotide positions and coding changes identified in recombinant CV *td*CE mutants.

Table 2 Efficiencies of plating of mutated rCV viruses in BSC-1 and CE cells. Viruses with E.O.P. values >2 in CE cells display the *td*CE phenotype. Virus titres and E.O.P. values are the average from triplicate repeats.

Figure 1 **A.** Diagram of the CV L gene and location of the L protein coding sequences. The nucleotide positions within the 11,119 nt genome are indicated. The location of the 6 domains of the vesiculovirus L protein are indicated (I to VI) and the locations and nature of the *td*CE mutants in the L gene and protein are shown. The locations of the RNA dependent RNA polymerase (RdRp), polyribonucleotidyltransferase, (PRNTase) and methyltransferase (MTase) activities are also indicated.

B. Intracellular localisation of mutated rCV-P/RFP viruses in BSC-1 and CE cells. BSC-1 (a-h) and CE (i-p) cells infected with rCV (a, e, i, m); rCV CH112 (C_{7217} to U) (b, f, j, n); rCV CH157 (c, g, k, o); rCV CH256 (d, h, l, p) P/RFP viruses at an M.O.I. of 1. Cells were incubated at 31°C (a-d, i-l) or 39°C (e-h, m-p) and fixed in 4% formaldehyde when RFP fluorescence was clearly visible. This was 8 hours post-infection for BSC-1 cells, CE cells incubated at 31°C and CE cells infected with rCV and incubated at 39°C. CE cells incubated with the mutant viruses at 39°C were fixed 48 hours post-infection. Nuclei were stained with DAPI.

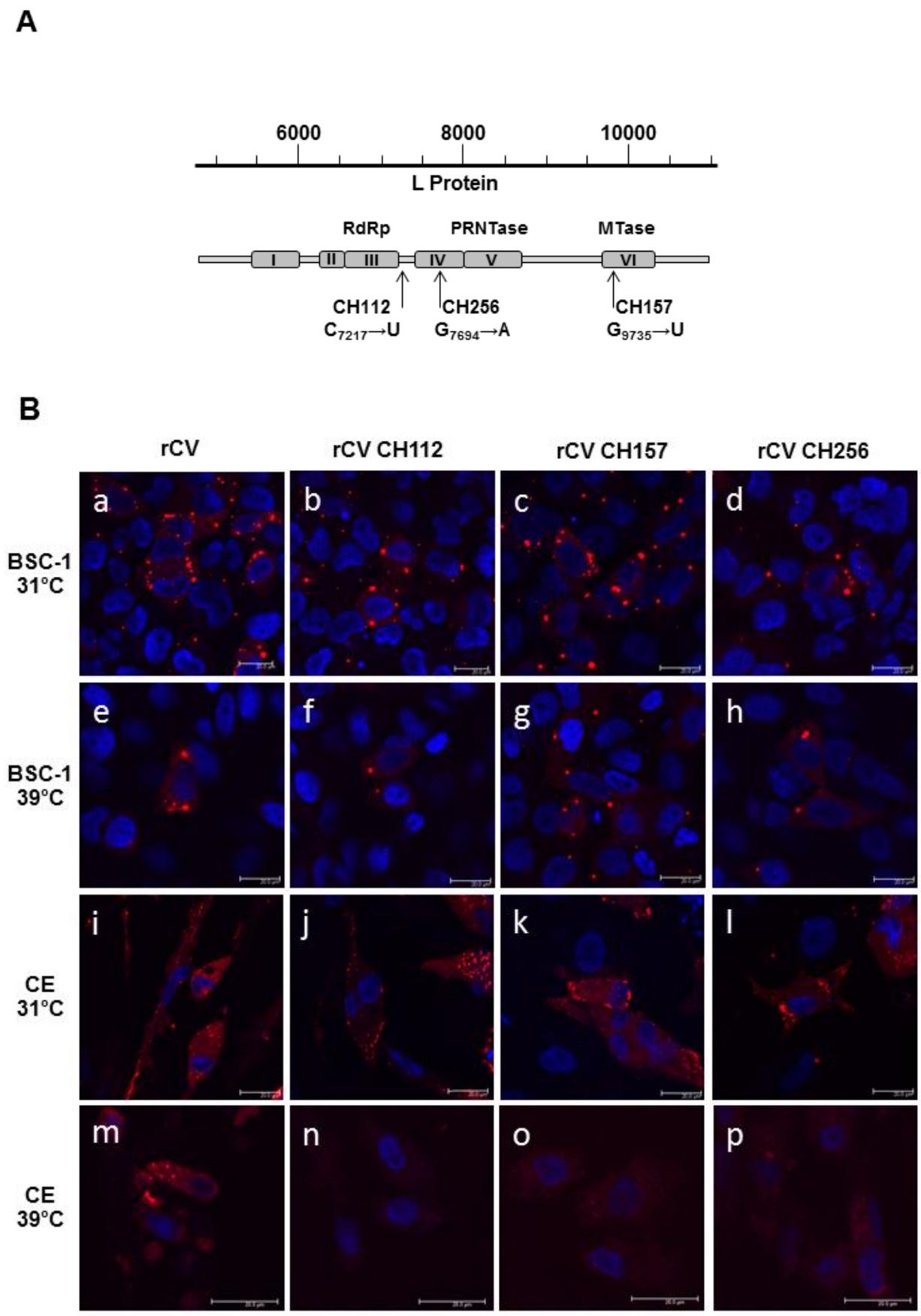
Table 1

***td*CE mutant**	**E.O.P. In CE cells**	**Point mutation**	**Gene**	**Amino acid change**
CH112	4.30	$U_{3941} \rightarrow C$	G	Ser_{297} to Pro
		$C_{5691} \rightarrow U$	L	Ser_{310} to Leu
		$C_{5760} \rightarrow U$	L	Pro_{333} to Leu
		$C_{7217} \rightarrow U$	L	Pro_{819} to Ser
		$U_{9958} \rightarrow C$	L	Non coding
		$U_{1980} \rightarrow C$	P	Non coding
CH157	2.61	$G_{9735} \rightarrow U$	L	Gly_{1658} to Val
		$U_{9022} \rightarrow C$	L	Non coding
CH256	3.84	$U_{4034} \rightarrow C$	G	Tyr_{328} to His
		$G_{7694} \rightarrow A$	L	Ala_{978} to Thr

Table 2

Gene with mutation (s)	Recombinant virus	Titre in BSC-1 cells (pfu/ml)		Titre in CE cells (pfu/ml)		Efficiency of plating	
		31°C	39°C	31°C	39°C	BSC-1	CE
n/a	rCV (wt)	9.9×10^8	3.1×10^8	3.0×10^7	1.3×10^7	0.50	0.36
L gene	rCV 112 ($C_{5691} \rightarrow U$)	9.3×10^8	1.5×10^8	4.9×10^7	1.5×10^7	0.79	0.51
	rCV 112 ($C_{5760} \rightarrow U$)	3.1×10^8	2.8×10^8	2.5×10^7	1.3×10^7	0.04	0.28
	rCV 112 ($C_{7217} \rightarrow U$)	3.8×10^8	8.9×10^7	2.3×10^7	7.5×10^2	0.63	4.49
	rCV 112 ($C_{5691,5760} \rightarrow U$)	4.2×10^8	7.6×10^7	3.9×10^7	9.8×10^6	0.74	0.60
	rCV 112 ($C_{5691,7217} \rightarrow U$)	1.8×10^8	7.6×10^7	1.7×10^7	1.3×10^2	0.37	5.12
	rCV 112 ($C_{5760,7217} \rightarrow U$)	1.1×10^8	2.4×10^7	9.8×10^6	2.8×10^2	0.66	4.54
	rCV 112 ($C_{5691,5760,7217} \rightarrow U$)	1.7×10^8	6.3×10^7	1.2×10^7	3.1×10^2	0.43	4.59
	rCV 157 ($G_{9735} \rightarrow U$)	9.3×10^7	1.4×10^7	2.3×10^7	1.0×10^4	0.82	3.36
	rCV 256 ($G_{7694} \rightarrow A$)	1.1×10^9	2.5×10^8	3.8×10^7	1.8×10^5	0.64	2.32
G gene	rCV 112 ($U_{3941} \rightarrow C$)	7.8×10^8	1.7×10^8	7.8×10^7	1.8×10^7	0.66	0.64
	rCV 256 ($U_{4034} \rightarrow C$)	3.5×10^8	1.2×10^8	3.1×10^7	1.8×10^7	0.46	0.24

Figure 1


A
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L Protein
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II
III
IV
V
VI
CH112
C7217→U
CH256
G7694→A
CH157
G9735→U
B
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rCV CH112
rCV CH157
rCV CH256
BSC-1
31°C
BSC-1
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