

THE UNIVERSITY OF WARWICK

Original citation:

Stock, Emily, Marriott, Anthony C. and Easton, A. J. (Andrew J.). (2014) The host-range tdCE phenotype of Chandipura virus is determined by mutations in the polymerase gene. *Journal of General Virology*, Volume 95 (Number 1). pp. 38-43. ISSN 0022-1317

Permanent WRAP url:

<http://wrap.warwick.ac.uk/58656>

Copyright and reuse:

The Warwick Research Archive Portal (WRAP) makes this work by researchers of the University of Warwick available open access under the following conditions. Copyright © and all moral rights to the version of the paper presented here belong to the individual author(s) and/or other copyright owners. To the extent reasonable and practicable the material made available in WRAP has been checked for eligibility before being made available.

Copies of full items can be used for personal research or study, educational, or not-for profit purposes without prior permission or charge. Provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way.

A note on versions:

The version presented here may differ from the published version or, version of record, if you wish to cite this item you are advised to consult the publisher's version. Please see the 'permanent WRAP url' above for details on accessing the published version and note that access may require a subscription.

For more information, please contact the WRAP Team at: publications@warwick.ac.uk



<http://wrap.warwick.ac.uk>

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

Emily L. Stock¹, Anthony C. Marriott^{1,2} and Andrew J. Easton^{1*}

¹School of Life Sciences

University of Warwick

Coventry

CV4 7AL

UK

²Public Health England

Porton Down,

Salisbury

SP4 0JG

UK

*Corresponding Author:

Email: a.j.easton@warwick.ac.uk

Abstract: 150 words

Main text (excluding references and figure legends): 2,391

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

2 **Abstract**

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

14

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

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

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

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

50 CV strain 1653514 and temperature sensitive derivatives from it (Gadkari & Pringle, 1980; Rasool &
51 Pringle, 1986) were used. Three *tdCE* mutants were selected on the basis of their previously
52 established high E.O.P. values in CE cells for analysis of the *tdCE* phenotype during this
53 investigation; namely CH112, CH157 and CH256 (Gadkari & Pringle, 1980; Rasool & Pringle, 1986).
54 Wild-type and *tdCE* mutant CV titres were determined by standard plaque assay in permissive (BSC-
55 1 monkey kidney cells) and conditionally permissive (primary cultures of chick embryo (CE)
56 fibroblasts) at 31°C and 39°C. The titres confirmed that the three mutants displayed the *tdCE*
57 phenotype (Supplementary table 1). The E.O.P. scores of the three mutants in CE cells were
58 comparable with those described following the original characterisation, though mutant CH157 had an
59 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
60 <0.7 and in CE cells of >2 (Gadkari & Pringle, 1980; Rasool & Pringle, 1986).

61 To identify the genetic lesions responsible for, and therefore the virus gene(s) associated with, host
62 range requirements, we sequenced the entire genome of each mutant. We also confirmed the
63 nucleotide sequence of the wild type virus. RNA was extracted from wild type CV and *tdCE* mutant
64 stocks using TRIzol LS reagent® (Invitrogen Life technologies), according to the manufacturer's
65 protocol and reverse transcribed to generate cDNA using random hexanucleotide primers. Seven
66 PCR products of approximately 2 kb, which overlapped with each other by 200-400 bp were amplified
67 by PCR from the full length cDNA using *KOD* hot start DNA polymerase (Novagen, Germany) and
68 each PCR product was purified (Qiaquick kit, Qiagen). The primers used to construct PCR products
69 1-7 are shown in Supplementary Table 2. Each PCR product was amplified in duplicate and
70 sequenced three times using different primers (GATC, Germany).

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

73 changes in the RNA polymerase (L) gene and mutants CH112 and CH256 contained additional
74 mutations in the attachment (G) protein gene. Additionally, two of the *tdCE* mutants contained a
75 further silent point mutation(s). Mutant CH112 contained the point mutation U₁₉₈₀→C in the P gene
76 and U₉₉₅₈→C in the L gene at amino acid residues 206 and 1732, respectively. Mutant CH157
77 contained a U₉₀₂₂→C mutation in the L gene at amino acid residue 1420. The presence of the
78 mutations resulting in coding changes in the polymerase gene is consistent with the observation of
79 reduced RNA transcriptase activity *in vitro* at 39°C in 10 of 12 CV *tdCE* mutants relative to wtCV, with
80 CH112, CH157 and CH256 exhibiting efficiencies of transcription at 39°C of 14%, 56% and 37% of
81 the wild-type virus (Rasool & Pringle, 1986).

82 To confirm which of the alterations identified in the mutants were responsible for the *tdCE* phenotype
83 recombinant viruses containing each point mutation, and combinations of the L mutations in the case
84 of mutant CH112, were generated. Quickchange site directed mutagenesis using mutagenic
85 oligonucleotide primers (Supplementary Table 2) was used to produce the C₅₆₉₁→U, C₅₇₆₀→U,
86 C₇₂₁₇→U, G₉₇₃₅→U, G₇₆₉₄→A, U₃₉₄₁→C, U₄₀₃₄→C point mutations in the full length anti-genomic clone
87 of CV (pT7CV). The methylated, non-mutated parental DNA templates were digested with *DpnI*
88 restriction endonuclease and DNA fragments containing the point mutations were re-cloned into
89 pT7CV and the insert sequences verified by DNA sequencing. Combinations of mutations were made
90 by sequential Quickchange reactions with the appropriate mutagenic primers. Rescue of infectious
91 virus was carried out for the wild type virus as control and for each of the nine combinations of point
92 mutations generating coding changes in the virus RNA dependent RNA polymerase gene or one of
93 the two mutations in the attachment (G) protein gene using the reverse genetics system previously
94 described (Marriott & Hornsey, 2011). The genomic plasmid (containing the point mutation(s)) and
95 support plasmids T7N, T7P and T7L were transfected into BSRT-7/5 cells (Buchholz *et al.*, 1999)
96 using *TransIT*®-LT1 transfection reagent (Mirus, USA). After 48 hours, the supernatant was
97 inoculated onto confluent monolayers of BSC-1 cells and virus was harvested when the cytopathic
98 effect was extensive (approximately 24 hours post infection). The titres of the mutant viruses in
99 permissive and non-permissive conditions and the E.O.P. for each are shown in Table 2. The data
100 show that a single amino acid change in the virus RNA polymerase of each mutant was solely
101 responsible for the *tdCE* host range dependent phenotype. All recombinant viruses containing the
102 C₇₁₂₇→U mutation in the L gene of mutant CH112 conferred the *tdCE* phenotype while any

103 combination of the C₅₆₉₁→U and C₅₇₆₀→U point mutations in the L gene alone or together did not
104 (Table 2). Furthermore, the results showed that the mutations identified in the G gene of CH112 and
105 CH256 were not involved in the *tdCE* mutant phenotype. Similarly, only the presence of the G₇₆₉₄→A
106 mutation in the L gene of mutant CH256 and G₉₇₃₅→U in the L gene of mutant CH157 conferred the
107 *tdCE* phenotype.

108 The large protein of rhabdoviruses is multifunctional and has been described as containing 6
109 functional domains of greater amino acid conservation and highly variable intra domains (Poch *et al.*,
110 1990). The molecular interrelationship between the domains in the VSV L protein have also been
111 established with conserved blocks I-IV arranged into a ring structure containing the RNA polymerase
112 activity and an appendage of domains V and VI responsible for capping and methylation respectively
113 (Rahmeh *et al.*, 2010). The point mutations conferring the host range phenotype of the *tdCE* mutants
114 are located between domains III and IV (CH112 C₇₂₁₇→U) or within domain IV (CH256 G₇₆₉₄→A) or VI
115 (CH157 G₉₇₃₅→U) of the L gene (Figure 1A). The G₉₇₃₅→U change within domain VI lies in a stretch
116 of highly conserved amino acids and immediately precedes a GXGXG motif (GDGSG sequence)
117 located 19 amino acids downstream of a lysine residue, which have been implicated in
118 polyadenylation or protein kinase functions (Cherian *et al.*, 2012). While the methyltransferase
119 activity of the L protein has been proposed to also lie within domain VI the CH157 mutant polymerase
120 displays a normal mRNA capping function (Rasool & Pringle, 1986).

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

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

139 To determine whether the *td*CE point mutations disrupt the intracellular localisation of the P protein in
140 the replication complexes the L gene of the P/RFP virus was mutated to produce three *td*CE mutants,
141 r112P/RFP (C₇₂₁₇→U), r157P/RFP (G₉₇₃₅→U) and r256P/RFP (G₇₆₉₄→A) corresponding to *td*CE
142 mutants CH112, CH157 and CH256, respectively. DNA fragments containing one of the three critical
143 *td*CE point mutations were cloned into pT7CV-PRFP and the inserted portions sequenced.

144 Recombinant P/RFP viruses were then recovered as described previously and used to infect cells at
145 an M.O.I of 1 pfu per cell. Cells were fixed with 10% formaldehyde in PBS when fluorescence was
146 clearly visible and mounted onto glass slides using aqueous mounting medium containing DAPI stain
147 (VectorLabs). Fluorescence was visualised using a Leica SP5 confocal fluorescence microscope.

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

161 release (Das *et al.*, 2006). It is likely that the images in Figure 1 demonstrate the late stages of CV
162 infection. There was no visible RFP expression in CE cells infected with P/RFP mutant L viruses at
163 39°C until 24 hours post-infection, whereas RFP was clearly visible after just 8 hours when the cells
164 were incubated at 31°C (the latter was also true for CE cells inoculated with the wild type L virus at
165 either temperature and BSC-1 cells under any condition). The lack of P/RFP-containing foci in CE
166 cells at the restrictive temperature, taken together with previous data showing defects in *in vitro*
167 transcriptase activity and polypeptide synthesis, supports the hypothesis that the defects in the
168 mutant L proteins lead to disruption of replication complexes, reduced viral RNA synthesis and hence
169 reduced viral protein synthesis. The three point mutations examined lead to similar phenotypes,
170 despite the amino acid changes being located in separate domains of the CV L protein.

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

176 References

- 177 **Bhatt, P. N. & Rodrigues, F. M. (1967).** Chandipura: a new Arbovirus isolated in India from patients
178 with febrile illness. *Indian J Med Res* **55**, 1295-1305.
- 179 **Buchholz, U. J., Finke, S. & Conzelmann, K. K. (1999).** Generation of bovine respiratory syncytial
180 virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and
181 the human RSV leader region acts as a functional BRSV genome promoter. *J Virol* **73**, 251-
182 259.
- 183 **Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A. & Tsien, R. Y.**
184 **(2002).** A monomeric red fluorescent protein. *Proc Natl Acad Sci U S A* **99**, 7877-7882.
- 185 **Chadha, M. S., Arankalle, V. A., Jadia, R. S., Joshi, M. V., Thakare, J. P., Mahadev, P. V. & Mishra, A.**
186 **C. (2005).** An outbreak of Chandipura virus encephalitis in the eastern districts of Gujarat
187 state, India. *Am J Trop Med Hyg* **73**, 566-570.
- 188 **Cherian, S. S., Gunjekar, R. S., Banerjee, A., Kumar, S. & Arankalle, V. A. (2012).** Whole genomes of
189 Chandipura virus isolates and comparative analysis with other rhabdoviruses. *PLoS One* **7**,
190 e30315.
- 191 **Das, S. C., Nayak, D., Zhou, Y. & Pattnaik, A. K. (2006).** Visualization of intracellular transport of
192 vesicular stomatitis virus nucleocapsids in living cells. *J Virol* **80**, 6368-6377.
- 193 **Fontenille, D., Traore-Lamizana, M., Trouillet, J., Leclerc, A., Mondo, M., Ba, Y., Digoutte, J. P. &**
194 **Zeller, H. G. (1994).** First isolations of arboviruses from phlebotomine sand flies in West
195 Africa. *Am J Trop Med Hyg* **50**, 570-574.
- 196 **Gadkari, D. A. & Pringle, C. R. (1980).** Temperature-sensitive mutants of Chandipura virus. II.
197 Phenotypic characteristics of the six complementation groups. *J Virol* **33**, 107-114.

198 **Gurav, Y. K., Tandale, B. V., Jadi, R. S., Gunjekar, R. S., Tikute, S. S., Jamgaonkar, A. V., Khadse, R. K.,**
199 **Jalgaonkar, S. V., Arankalle, V. A. & Mishra, A. C. (2010).** Chandipura virus encephalitis
200 outbreak among children in Nagpur division, Maharashtra, 2007. *Indian J Med Res* **132**, 395-
201 399.

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

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

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

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

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

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

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

218 **Rahmeh, A. A., Schenk, A. D., Danek, E. I., Kranzusch, P. J., Liang, B., Walz, T. & Whelan, S. P.**
219 **(2010).** Molecular architecture of the vesicular stomatitis virus RNA polymerase. *Proc Natl*
220 *Acad Sci U S A* **107**, 20075-20080.

221 **Rao, B. L., Basu, A., Wairagkar, N. S., Gore, M. M., Arankalle, V. A., Thakare, J. P., Jadi, R. S., Rao, K.**
222 **A. & Mishra, A. C. (2004).** A large outbreak of acute encephalitis with high fatality rate in
223 children in Andhra Pradesh, India, in 2003, associated with Chandipura virus. *Lancet* **364**,
224 869-874.

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

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

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

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

237

238

239 **Table and Figure legends**

240 **Table 1** Nucleotide positions and coding changes identified in recombinant CV *tdCE* mutants.

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

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

250 **B.** Intracellular localisation of mutated rCV-P/RFP viruses in BSC-1 and CE cells.
251 BSC-1 (a-h) and CE (i-p) cells infected with rCV (a, e, i, m); rCV CH112 (C₇₂₁₇ to U) (b, f, j,
252 n); rCV CH157 (c, g, k, o); rCV CH256 (d, h, l, p) P/RFP viruses at an M.O.I. of 1. Cells
253 were incubated at 31°C (a-d, i-l) or 39°C (e-h, m-p) and fixed in 4% formaldehyde when
254 RFP fluorescence was clearly visible. This was 8 hours post-infection for BSC-1 cells, CE
255 cells incubated at 31°C and CE cells infected with rCV and incubated at 39°C. CE cells
256 incubated with the mutant viruses at 39°C were fixed 48 hours post-infection. Nuclei were
257 stained with DAPI.

Table 1

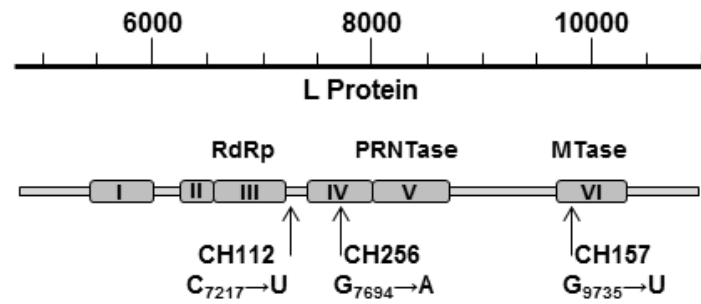
tdCE mutant	E.O.P. In CE cells	Point mutation	Gene	Amino acid change
CH112	4.30	U ₃₉₄₁ → C	G	Ser ₂₉₇ to Pro
		C ₅₆₉₁ → U	L	Ser ₃₁₀ to Leu
		C ₅₇₆₀ → U	L	Pro ₃₃₃ to Leu
		C ₇₂₁₇ → U	L	Pro ₈₁₉ to Ser
		U ₉₉₅₈ → C	L	Non coding
		U ₁₉₈₀ → C	P	Non coding
CH157	2.61	G ₉₇₃₅ → U	L	Gly ₁₆₅₈ to Val
		U ₉₀₂₂ → C	L	Non coding
CH256	3.84	U ₄₀₃₄ → C	G	Tyr ₃₂₈ to His
		G ₇₆₉₄ → 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 → 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 → 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 → 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} → 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} → 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} → 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} → 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 → 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 → 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 → 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 → C)	3.5×10^8	1.2×10^8	3.1×10^7	1.8×10^7	0.46	0.24

Figure 1

A



B

