The E2 glycoprotein is necessary but not sufficient for the adaptation of classical swine fever virus lapinized vaccine C-strain to the rabbit

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Abstract

Classical swine fever virus (CSFV) C-strain was developed through hundreds of passages of a highly virulent CSFV in rabbits. To investigate the molecular basis for the adaptation of C-strain to the rabbit (ACR), a panel of chimeric viruses with the exchange of glycoproteins E^{rns}, E1, and/or E2 between C-strain and the highly virulent Shimen strain and a number of mutant viruses with different amino acid substitutions in E2 protein were generated and evaluated in rabbits. Our results demonstrate that Shimen-based chimeras expressing E^{rns}-E1-E2, E^{rns}-E2 or E1-E2 but not E^{rns}-E1, E^{rns}, E1, or E2 of C-strain can replicate in rabbits, indicating that E2 in combination with E^{rns} or E1 confers the ACR. Notably, E2 and the amino acids P108 and T109 in Domain I of E2 are critical in ACR. Collectively, our data prove that E2 is crucial in mediating the ACR, which requires synergistic contribution of E^{rns} or E1.

Keywords: classical swine fever virus; C-strain; E2 protein; adaptation; rabbit

1. Introduction

Viral host range may be expanding through evolutionary adaptation in non-natural hosts (Bitzegeio et al., 2010; Del Prete et al., 2017; Qiu et al., 2005; Terpstra et al., 1988; von Schaewen et al., 2016). For example, a murine tropic hepatitis C virus (HCV) was generated by adapting HCV to use murine orthologues of entry factors (Bitzegeio et al., 2010). The inherent poor ability of Env protein of most human immunodeficiency virus 1 strains to exploit macaque CD4 as a receptor can be improved during adaptation by virus passages in macaques (Del Prete et al., 2017). A significant purpose of the successful adaptation to a non-natural host is to attenuate specific virus strains,

which is of great value for the development of live attenuated vaccines. Species barrier of classical swine fever virus (CSFV), which is classified into the *Pestivirus* genus of the *Flaviviridae* family (Becher et al., 2003), has been overcome through hundreds of passages of a highly virulent CSFV in rabbits, resulting in a highly safe and efficacious vaccine C-strain that is adaptive to the rabbit (Qiu et al., 2005; Terpstra et al., 1988).

CSFV is the causative agent of classical swine fever (CSF), a highly contagious and often fatal disease of pigs. The disease is notifiable to the World Organization for Animal Health (OIE), as it causes significant economic losses to the pork industry in many countries. CSFV has a single-stranded, positive-sense RNA genome of approximately 12.3 kb, which contains a 5′-untranslated region (UTR), a single long open reading frame (ORF) and a 3′-UTR. The ORF encodes a polyprotein of around 3,900 amino acids that is processed into four structural proteins (C, E^{rns}, E1, and E2) and eight nonstructural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Collett et al., 1989; Thiel et al., 1991). Structural proteins are involved in multiple functions (Eblé et al., 2013; Fernandez-Sainz et al., 2008, 2009; König et al., 1995; Li et al., 2007; Liang et al., 2003; Reimann et al., 2004; Riedel et al., 2010; Risatti et al., 2007; Sun et al., 2011; Tamura et al., 2012; van Gennip et al., 2000; Wang et al., 2004; Wang et al., 2015), from virus attachment to entry into target cells (E1 and E2) (Liang et al., 2003; van Gennip et al., 2000; Wang et al., 2004; Wang et al., 2015), induction of protective immune responses (E^{rns} and E2) (Eblé et al., 2013; König et al., 1995; Li et al., 2007; Reimann et al., 2004; Sun et al., 2011), and virulence determinants (C, E^{rns}, E1, and E2) in pigs (Fernandez-Sainz et al., 2008, 2009; Riedel et al., 2010; Risatti

et al., 2007; Tamura et al., 2012).

C-strain, also known as the Chinese hog cholera lapinized virus (HCLV), is an excellent attenuated vaccine that effectively protects pigs against CSF (Terpstra et al., 1988). The vaccine was developed by Chinese scientists through hundreds of passages in rabbits of a highly virulent CSFV in the early 1950s (Qiu et al., 2005). Some other attenuated vaccines have been developed by serial passages in non-susceptible hosts, such as lapinized attenuated rinderpest virus (Walker, 1947) and attenuated equine infectious anemia virus (EIAV) vaccine (Craigo et al., 2010, 2015; Wang et al., 2016), which contribute significantly to the control and eradication of corresponding infectious diseases. Uncovering the molecular basis of viral adaptation to non-susceptible/natural hosts will certainly help to elucidate the mechanisms of the attenuation of these very safe and effective classical vaccines.

Domestic pigs and wild boar are natural hosts of CSFV. Despite the fact that both C-strain and wild-type CSFV can replicate in permissive swine cells, it is the C-strain that is adaptive to the rabbit, characterized by being able to replicate only in the spleen and lymph node in rabbits and causing a fever response. Previously, we have shown that the UTRs of C-strain are essential for its fever induction in rabbits and the coding region is essential for viral replication in the spleens of rabbits (Li et al., 2014). To date, however, which gene(s) is responsible for the adaptation remains elusive. Determination of the molecular basis of viral adaptation facilitates the development of animal models to study virus pathobiology.

For enveloped viruses, the viral envelope proteins can determine the virus tropism (Bitzegeio et al., 2010; Del Prete et al., 2017; Li et al., 2016).

Sequence analysis reveals that the E^{rns}, E1, and E2 glycoproteins of C-strain exhibit dissimilarities to those of the Shimen strain. We hypothesized that the structural proteins may be responsible for the adaptation of C-strain to the rabbit (ACR). In this study, a panel of chimeric and mutant viruses was generated and evaluated in rabbits in terms of fever response and viral replication in the spleens. Our data demonstrate that E2 alone is insufficient for the ACR; or rather, the adaptation results from a synergistic effect of E2 together with E^{rns} or E1, which provides insights for understanding of the adaptation basis of C-strain and developing small animal models for the *Flaviviridae* members, including HCV.

2. Results

2.1. The chimeric or mutant viruses replicate differently in PK-15 or SK6 cells

Seven Shimen-based chimeric viruses expressing the single, double or triple genes of C-strain E^{rns}, E1 or E2 and two C-strain-based chimeric viruses harboring E2 or E^{rns}-E1-E2 (Fig. 1) were rescued from the individual chimeric plasmids constructed with the primers in Table S1 and identified by antigen-capture ELISA, indirect immunofluorescence assay (IFA), and RT-PCR. Viral E^{rns} proteins of progeny viruses were secreted into culture medium (Fig. S1A), and E2 were detected in the infected PK-15 cells (Fig. S1B). Sequence analysis confirmed that the genomic sequences of the rescued chimeras were identical to those of the corresponding chimeric clones.

The growth characteristics of the chimeras were evaluated in swine-derived

PK-15 or SK6 cells relative to their parental viruses (Shimen and C-strain) using a multiple-step growth curve. In comparison with the Shimen strain, the chimeras harboring the E2 protein of C-strain in the Shimen strain backbone exhibited a slightly decreased replication (Fig. 2A). The replication kinetics and virus yields of vSM-HCLVE^{rns} or vSM-HCLVE1 were indistinguishable from those of the Shimen strain (Fig. 2A). In addition, vHCLV-SME^{rns}E1E2 and vHCLV-SME2 had a slightly slower growth rate and to a lower level than that of C-strain (Fig. 2B).

The replication levels of four C-strain-based chimeric viruses harboring different domains or five mutants containing individual or various amino acid(s) replacements with those of the Shimen strain were increased compared with C-strain when three mutants containing two amino acid(s) substitutions with those of the Shimen strain had similar growth characteristics to C-strain, but they were still lower than that of the Shimen strain (Fig. 2C–F, Figs. S2–4).

2.2. The chimeric virus based on the Shimen strain harboring the E^{rns}-E1-E2 of C-strain induces fever response and replicates in rabbits

To firstly verify our speculation that E^{rns}-E1-E2 may play a key role in the ACR, body temperatures of the rabbits inoculated with the chimeric viruses were monitored before inoculation and from 24 to 72 hours post-inoculation (hpi) at 6-h intervals and viral replication in spleens of the rabbits was tested by real-time RT-PCR at 3 days post-inoculation (dpi). A fever response is defined as a 0.5°C higher than body temperature before inoculation at least three consecutive times or 1°C higher for twice. The vSM-HCLVE^{rns}E1E2 but not the vHCLV-SME^{rns}E1E2 induced a fever response (Table 1), suggesting a possible key role of C-strain E^{rns}-E1-E2 in the adaptation. The viral RNA was detected

consistently in spleens of the rabbits inoculated with vSM-HCLVE^{rns}E1E2 (3/3), and the viral replication levels were similar to those of the rabbits inoculated with C-strain (P > 0.05). In contrast to vSM-HCLVE^{rns}E1E2, viral RNA was not detected in spleens of the animals inoculated with vHCLV-SME^{rns}E1E2, Shimen, or Dulbecco's modified Eagle's medium (DMEM) (Table 1). These results demonstrate that E^{rns}-E1-E2 is responsible for the fever response and the replication of C-strain in rabbits.

2.3. The E2 protein in combination with E^{rns} or E1 confers the adaptation of C-strain to the rabbit

Based on the key role of E^{rns}-E1-E2 in the ACR, Shimen-based chimeric viruses expressing the individual and combined structural proteins of C-strain were constructed and evaluated in rabbits to identify which glycoprotein(s) of C-strain is responsible for its adaptation to the rabbit. Viral RNA was detected in the spleens of the rabbits inoculated with vSM-HCLVE^{rns}E2 (2/3) and vSM-HCLVE1E2 (2/3), and the viral replication levels were similar to those of the rabbits inoculated with C-strain (P > 0.05). In contrast, viral RNA was not detected in the spleens of the animals inoculated with vSM-HCLVE^{rns}E1, vSM-HCLVE^{rns}, vSM-HCLVE1, vSM-HCLVE2, Shimen, or DMEM (Table 2).

The animal experiment was repeated once again to verify the adaptability of vSM-HCLVE^{rns}E2 and vSM-HCLVE1E2 in rabbits with consistent results, which demonstrate the presence of viral RNA of vSM-HCLVE^{rns}E2 (4/4) or vSM-HCLVE1E2 (3/4) in spleens with a similar replication level to that of the rabbits inoculated with C-strain (P > 0.05) (Table 3). Furthermore, the two chimeras were recovered from the spleens of the inoculated rabbits (Fig. 3A), and *E^{rns}*, *E1*, and *E2* genes were detected (Fig. 3B). E2 protein of

vSM-HCLVE^{rns}E2 or vSM-HCLVE1E2, but not vSM-HCLVE^{rns}E1 or DMEM, was detected in the rabbit spleens by immunohistochemistry (Fig. 3C). Importantly, the substitutions of E^{rns}-E2 or E1-E2 but not E^{rns}-E1, E^{rns}, E1 or E2 alone of the Shimen strain with the counterparts of C-strain confer the ability of inducing the fever response for the Shimen strain (Fig. 3D, Table 2). The full-length sequence analysis showed no mutations in the genomes of the two chimeras. Remarkably, re-isolated vSM-HCLVE^{rns}E2 (vSM-HCLVE^{rns}E2-R) and vSM-HCLVE1E2 (vSM-HCLVE1E2-R) remained infectious in rabbits (Table S2).

Collectively, our data provide strong evidence that E2 together with E^{rns} or E1 confers the ACR, while E2 itself is insufficient for the adaptation.

2.4. The C-strain-based chimeric virus expressing the E2 of the Shimen strain is unable to induce fever response and do not replicate in rabbits

Since E2 together with E^{rns} or E1 confers the ACR as demonstrated above, we tried to clarify whether E2 is necessary for the ACR using the C-strain-based chimeric virus vHCLV-SME2 harboring the E2-coding region from the Shimen strain. Intriguingly, the replacement of the Shimen E2 protein completely abolished the viral RNA replication and fever response induced by C-strain in rabbits (Table 3), indicating that the C-strain E2 is essential for the ACR.

2.5. Domain I in the C-strain E2 is critical to the ACR

There are 21 different amino acids in the E2 protein between C-strain and the Shimen strain (Fig. 4A), which are located in four different domains in E2 (Fig. 4B). To determine the key residues of E2 involved in the ACR, four

chimeric viruses harboring the different domains of the Shimen E2 in the context of C-strain were rescued and evaluated in rabbits (Fig. S2A and S2B), and vHCLV-SME2 was used as a control. We found that in comparison with the other three chimeras (vHCLV-SME2DomainII, vHCLV-SME2DomainIII, and vHCLV-SME2DomainIV), vHCLV-SME2DomainI was unable to induce fever response in all the rabbits and the viral replication was not detected in three out of four rabbits infected with this virus (Fig. 4C and D, Table 4), demonstrating that Domain I was necessary for the ACR. Surprisingly, a revertant mutation L108P in Domain I was observed in E2 of the vHCLV-SME2DomainI-R isolated from the rabbit inoculated with the vHCLV-SME2DomainI (Fig. 4E). Additionally, one of the three rabbits inoculated with vHCLV-SME2 showed a fever response and the viral replication was detected in the spleen (Table 4). Further sequence analysis demonstrated that a rabbit-adapted mutation I109T occurred in Domain I, which is highly conserved among various lapinized vaccines (data not shown).

2.6. Amino acids P108 and T109 in the C-strain E2 are critical to the ACR

Based on the above findings of the revertant mutations at positions 108 and 109, two mutants harboring various amino acid substitutions in the background of C-strain were generated and evaluated in rabbits in order to determine the key amino acid(s) in Domain I associated with the adaptation (Fig. 5A, Fig. S3A and S3B). The mutant vHCLV-SME2DomainI-1 harboring the substitutions I4T, V23I, D49N, N67S, and S80I was adaptive to the rabbit (Fig. 5B-E, Table 5). However, the infection of the mutant vHCLV-SME2DomainI-2 containing K105G, P108L, and T109I did not induce fever response in all the inoculated rabbits (Fig. 5C and D, Table 5),

suggesting that substitutions K105G, P108L, and T109I abolished the fever response induced by C-strain. Meanwhile, lower level of viral replication of vHCLV-SME2DomainI-2 was detected in spleens of three inoculated rabbits and no replication in one rabbit (Fig. 5E, Table 5). Similar results were obtained in a repeated experiment (Table 6). Remarkably, while viral replication of vHCLV-SME2DomainI-2 was detected in the spleens of 3/4 (Table 5) or 2/4 rabbits (Table 6), a revertant mutation at site 108 (L108P) in the E2 protein of vHCLV-SME2DomainI-2-R recovered from the inoculated rabbits was observed in two independent experiments (Fig. 5SA). Collectively, our data suggest that the amino acids K105, P108, and T109 in E2 play a critical role in the ACR.

To identify the exact contribution of K105, P108, or T109 to the ACR, a panel of mutant viruses containing individual and combined mutations of K105G, P108L, or T109I was generated and evaluated in rabbits (Fig. S4A and S4B). Viral replication or fever was observed in the rabbits infected with all the mutants but not the one harboring P108L and T109I (Fig. 6A–D, Table 6). Although the mutant harboring P108L or mutant containing P108L and T109I were isolated from the inoculated rabbit (Fig. 6B), sequence analysis reveals a revertant mutation occurred at position 108, which was not present in the inoculated viruses (Fig. 5SB). These indicate that amino acids P108 and T109 are essential for the ACR.

To examine whether the viruses were successfully inoculated into the animals, CSFV-specific antibodies were tested (Li et al., 2014). The results demonstrated that the anti-CSFV antibodies were detected in the rabbits inoculated with the chimeric viruses at 7 or 10 dpi (Tables 1–6), demonstrating

the successful inoculation with the viruses.

3. Discussion

In this study, we constructed a series of chimeric viruses with the exchange of viral glycoproteins between C-strain and the Shimen strain and a number of mutants harboring various amino acid substitutions to investigate the contribution of the viral glycoprotein(s) or amino acids to the ACR. Our data demonstrate that the E2 protein in combination with either E^{rns} or E1 confers the ACR and the residues P108 and T109 in Domain I of E2 are essential for the adaptation.

Our data demonstrate the CSFV Shimen strain could be adapted to the rabbit through exchanging the C-strain envelope glycoproteins for its counterparts. Viruses can be adapted to cell cultures or animal models by continuous *in vitro* or *in vivo* passages in order to obtain high-titer viruses, attenuated vaccine strains or adapted viral mutants to new hosts (Chan et al., 2012; Mathiesen et al., 2015; Qiu et al., 2005; Scheel et al., 2008, 2011; Tamura et al., 2012). For example, an adapted HCV with 1,000-fold more infectious titers than the parental virus was generated by serial passages in cell cultures (Chan et al., 2012). Notably, intergenotypic recombinants encoding the structural proteins, p7, and NS2 of different HCV genotypes were adapted to the cell cultures, indicating that chimeric viruses generated by reverse genetics system can effectively acquire the adaptation to cell tropism (Scheel et al., 2011). In our study, without hundreds of passages in rabbits, the Shimen strain acquired the adaptation to the rabbit by reverse genetics system of CSFV, which provides a strategy for developing rabbit-adapted CSFV vaccine candidates in the future.

In the current study, we demonstrated for the first time that the E2 protein in combination with either E^{rns} or E1 defines the ACR. A previous study has indicated that higher-titer adapted HCV possesses 13-amino acid changes in C, E1, E2, p7, NS2, NS5A, and NS5B (Mathiesen et al., 2015). Similarly, readaptation of the live attenuated CSFV vaccine strain GPE^{-} to pigs requires synergistic effects of E2 and NS4B (Tamura et al., 2012). These studies indicate that multiple viral proteins confer the adaptation of the viruses to cells or non-natural hosts. Intriguingly, E2-E^{rns} or E2-E1 of C-strain can confer the adaptation of the Shimen strain to the rabbit, which expands our understanding of the complex molecular basis of the ACR.

The rabbit experiments were repeated two times with consistent results, indicating that vSM-HCLVE1E2 and vSM-HCLVE^{rns}E2 were adaptive to the rabbit. However, the replication or fever response induced by the two chimeras was not detected in all the rabbits (Tables 2 and 3), possibly due to the nature of the rescued viruses or the absence of other elements. We have demonstrated that the UTR substitution with the counterpart of the Shimen strain abolishes the fever response induced by C-strain (Li et al., 2014). In this study, E2 replacement with the counterpart of the Shimen strain also abolished the fever response, suggesting that various genes may be responsible for the C-strain's ability to induce the fever response. Reasonably, fever response was not induced in all the rabbits inoculated with vSM-HCLVE1E2 or vSM-HCLVE^{rns}E2 due to the absence of the UTRs of C-strain in the two chimeras. Therefore, additional factors may be needed to increase the adaptation robustness. Both E2 glycoprotein and UTR are CSFV virulence determinants in pigs (Liang et al., 2003; Wang et al., 2008). In our previous

and current studies, we verified that the two genes affect the pathogenesis of C-strain in rabbits. This implies the correlation of virulent determinants of different CSFV strains in pigs and rabbits.

It has been shown that the binding of viral envelope and its receptor(s) usually determines viral tropism at the entry level (Del Prete et al., 2017; Li et al., 2016). Previously, our data confirmed that the antibodies against the E2 can prevent the fever response induced by C-strain infection and viral replication (Sun et al., 2011). To date, only heparan sulfate (HS) and the laminin receptor (LamR) have been identified as crucial E^{rns}-binding cellular attachment receptors for CSFV (Chen et al., 2015; Hulst et al., 2000, 2001). Recently, it has been demonstrated that a human and chimpanzee-specific HCV could gain entry into mouse cells without the need of human entry factors by accommodating mutations in the E1/E2 complexes of the virus (Bitzegeio et al., 2010). An unknown E2-binding entry receptor(s) may contribute to the unique adaptability of C-strain to the rabbit, which needs further definition.

In this study, we further confirmed that Domain I and more exactly the amino acids P108 and T109 in the domain are essential for the ACR. Notably, the revertant mutation at position 108 of C-strain-based mutant genome occurred with more frequency after inoculation (Fig. 4E, Fig. S5), suggesting that the genome of C-strain remains genetically stable and evolutionarily advantageous in rabbits. Crystal structure analysis of the glycoprotein E2 from bovine viral diarrhea virus (BVDV) demonstrated that Domain I plays the key role in the viral entry step (El Omari et al., 2013). The residues P108 and T109 may contribute to the different structure characteristics and functionalities of the C-strain E2 from that of the Shimen strain, thus determining the using of

specific entry receptor(s) of rabbit cells, which needs to be investigated in future.

HCV and BVDV are both members of the *Flaviviridae* family. To date, though HCV cell culture models have been developed (Chan et al., 2012; Mathiesen et al., 2015; Saeed et al., 2015), a small animal model for HCV infection is needed for evaluation of vaccines or antivirals. BVDV has also the same requirements for a small animal model. To this end, the replication of chimeric viruses harboring the E2 and E1 or E^{rns} of CSFV in the context of BVDV or HCV in rabbits will be evaluated in the future, which possibly provides insights for the development of a rabbit model for BVDV or HCV infection.

In summary, we demonstrate that E2 itself is necessary but insufficient to confer the ACR and synergistic contribution of E^{rns} or E1 is required for the adaptation of the virus in rabbits, which gives an insight into the adaptation basis of C-strain and provides a clue to the development of a rabbit model for the *Flaviviridae* family members, such as BVDV and HCV.

4. Materials and methods

4.1. Cells and viruses

PK-15 (a porcine kidney cell line) (ATCC; CCL-33), or SK6 (a swine kidney cell line) (A gift from the Department of Microbiology, National Veterinary Institute, Sweden) cells were cultured in DMEM (Gibco) supplemented with 5% fetal bovine serum (FBS) (Gibco) at 37°C in a humidified 5% CO_2 incubator. The FBS is free of antigen of BVDV and antibodies against BVDV. Primary lapine spleen lymphocytes isolated from the healthy rabbits were cultured in RPMI-1640 medium containing 10% FBS. The CSFV Shimen strain (GenBank

accession number AF092448.2), HCLV strain (C-strain) (AY805221), the HCLV (vHCLV) rescued from the infectious clone pCSFV-HCLV of C-strain (Zhang et al., 2017), and rescued chimeras or mutants were propagated in SK6 cells in DMEM supplemented with 2% FBS.

4.2. Construction of chimeric or mutant full-length cDNA clones

The E^{rns}-, E1-, E2-, E^{rns}-E1-, E1-E2-, or E^{rns}-E1-E2-coding sequences of C-strain were amplified from a full-length infectious cDNA clone pCSFV-HCLV (Li et al., 2014) and the flanking segments of corresponding genes were amplified from the plasmid pBRCISM, a full-length cDNA clone of the Shimen strain (Li et al., 2013a), by PCR using high fidelity polymerase PrimeSTAR (TaKaRa) with the primers listed in Table S1. Then, the fragments which contained the E^{rns}-, E1-, E2-, E^{rns}-E1-, E1-E2-, or E^{rns}-E1-E2-coding sequences of C-strain were generated by fusion PCR. Subsequently, the PCR products were cloned into the backbone of pBRCISM-5'h (Li et al., 2013a) via the restriction enzymes *Xho*I and *Kpn*I (New England BioLabs), creating the pSM5'h-HCLVE^{rns}, pSM5'h-HCLVE1, pSM5'h-HCLVE2, pSM5'h-HCLVE^{rns}E1, pSM5'h-HCLVE1E2, and pSM5'h-HCLVE^{rns}E1E2, respectively. Finally, the *Xho*I-*Bam*HI fragments from the plasmids above were each linked with pBRCISM-3'h (Li et al., 2013a), giving rise to pSM-HCLVE^{rns}, pSM-HCLVE1, pSM-HCLVE2, pSM-HCLVE^{rns}E1, pSM-HCLVE1E2, and pSM-HCLVE^{rns}E1E2, respectively.

The strategy described above was used to exchange the E^{rns}- and E2-encoding sequences. The E^{rns}-coding sequences of C-strain were fused with the flanking fragments of the corresponding E^{rns} from pSM5'h-HCLVE2 using fusion PCR with the primers (Table S1). Subsequently, the PCR products

were inserted into the plasmid pBRCISM-5'h, resulting in pSM5'h-HCLVE^{rns}E2. Finally, the *Xho*I-*Bam*HI fragment from pSM5'h-HCLVE^{rns}E2 was cloned into pBRCISM-3'h, creating the chimeric full-length cDNA clone containing the E^{rns}- and E2-coding sequences of C-strain, designated as pSM-HCLVE^{rns}E2.

The E^{rns}-E1-E2 or E2 encoding sequence of the Shimen strain was fused with the flanking fragments of the corresponding E^{rns}-E1-E2 or E2 sequence from pCSFV-HCLV using fusion PCR. Subsequently, the PCR products were cloned into the backbone of pCSFV-HCLV via the restriction sites *Xho*I and *Bam*HI (New England BioLabs), resulting in pHCLV-SME^{rns}E1E2 and pHCLV-SME2, respectively.

Based on the crystal structure of the BVDV-1 E2 protein (El Omari et al., 2013, Li et al., 2013b), four chimeric infectious clones harboring different domains of the Shimen E2 protein in the background of C-strain were constructed as described above (Fig. 4D). In addition, the amino acid substitutions were introduced into the C-strain infectious clone pCSFV-HCLV to construct a panel of mutants by QuikChange® site-directed mutagenesis kit (Stratagene) according to its instructions.

All of the chimeric or mutant infectious cDNA clones were confirmed by sequencing and multiple restriction digestion.

4.3. Recovery of chimeric or mutant viruses

The chimeric or mutant viruses were generated as described previously with a modification (Li et al., 2013a). Briefly, PK-15 or SK6 cells were individually transfected with 4 μg of each plasmid in 4-μl X-tremeGENE HP DNA transfection reagent (catalog no. 06366236001; Roche) and passaged ten times (P1 to 10). The rescued viruses were harvested by three freeze-thaw

cycles. The E^{rns} protein of the chimeric or mutant viruses was examined by a CSFV antigen test kit (catalog no. G871; IDEXX) according to the manufacturer's protocols. The E2 expression protein from chimeric or mutant viruses was tested by IFA using an anti-E2 MAb (Peng et al., 2008).

4.4. The growth curves of the rescued chimeric or mutant viruses in PK-15 or SK6 cells

PK-15 or SK6 cells in a 24-well plate were infected with the rescued chimeras or mutants, vHCLV, C-strain, or Shimen strain at a multiplicity of infection (MOI) of 0.1. After adsorption for 2 h at 37°C, the inocula were replaced with fresh medium and the cells were incubated at 37°C and 5% CO_2. The supernatants were harvested at 12-h intervals, and the viral titers were determined as described previously (Li et al., 2013a) and calculated using the Reed-Muench method (Reed and Muench 1938) and expressed as 50% tissue culture infective dose ($TCID_{50}$) per milliliter (ml). Average values and standard deviations for three independent experiments were determined.

4.5. Inoculation experiments in rabbits

New Zealand white rabbits of 14-week-old were randomly assigned to different groups of 3 to 6 each, and were inoculated intravenously (i.v.) via the marginal ear vein with the indicated viruses, parental viruses, or DMEM according to Tables 1-6 and S2.

To monitor the fever response, the rectal temperatures of all the rabbits were recorded every 6 h from 24 to 72 hpi. Three or four rabbits were selected from each group and euthanized at 3 dpi and the rabbits showing the fever response were chosen preferentially to be euthanized. Since the production of

the antibodies against the CSFV E2 is an important indicator of successful inoculation of Shimen strain, C-strain or their mutants in rabbits (Li et al., 2014), at 3, 7, or 10 dpi, the serum samples of the remaining three or two rabbits were collected to determine the anti-E2 antibodies using a CSFV antibody test kit (catalog no. G311; IDEXX) according to the manufacturer's protocols. All of the rabbits were euthanized at 10 dpi.

4.6. RNA extraction, reverse transcription, and real-time RT-PCR

The total RNA was harvested using the TRIzol reagent (Invitrogen). The cDNA synthesis was performed in a total volume of 20 μl containing 200 ng of total RNA, 20 U of Moloney murine leukemia virus reverse transcriptase (TaKaRa), 200 μM deoxynucleoside triphosphates (TaKaRa), and 4 μl of 5 × reverse transcriptase buffer. The mixture was incubated at 42°C for 1 h and then at 75°C for 15 min.

The RNA copies of the inoculated viruses or C-strain in spleens of the rabbits were quantified by a real-time RT-PCR assay (Zhao et al., 2008). Real-time RT-PCR was performed in a total volume of 25 μl containing 3 μl of cDNA, 2.5 μl of 10 × Ex Taq buffer, 2 μl of dNTPs (2.5 mM each), 1 μl of each CSFV-F/CSFV-R (10 μM), 0.5 μl of the probe CSFV-FAM (10 μM), and 2 U of Ex Taq hot start polymerase (catalog no. RR006A; TaKaRa). Cycling conditions included pre-denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, and annealing/extension at 60°C for 45 s. Experiments on each sample were performed in triplicate. The viral RNA copy numbers were calculated based on standard curve.

4.7. Viral isolation

In the animal experiments, three or four rabbits of each group inoculated with different viruses were euthanized at 3 dpi and spleens were collected and subjected to viral isolation. Briefly, the spleen tissues were grinded, diluted with phosphate-buffered saline (PBS) containing appropriate penicillin, and filtered through a 0.45-μm filter. Subsequently, SK6 cells were inoculated with the supernatants and continuously passaged for three times. Re-isolated viruses were tested by ELISA, RT-PCR, and sequencing.

4.8. Immunohistochemistry

The spleens of the inoculated rabbits were subjected to immunohistochemistry examinations as described previously (Ferrari et al., 1998).

4.9. Statistical analysis

Differences between groups were examined for statistical significance using Student's *t*-test by SPSS 14.0 software. An unadjusted *P*-value of less than 0.05 was considered significant.

4.10. Ethics statement

The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Heilongjiang Province of the People's Republic of China. The protocols were approved by the Committee on the Ethics of Animal Experiments of Harbin Veterinary Research Institute (HVRI) of the Chinese Academy of Agricultural Sciences (CAAS). The animal experiments were approved by the Committee on the Ethics of HVRI of CAAS with the license SYXK(Heilongjiang)2011022 (Approved numbers: 20152006, 20152058, 20162067, 20162095, 20162111, SY-2017-Ra-002, and

SY-2017-Ra-003). The rabbits were housed under controlled conditions of humidity (40-70%), temperature (22-28°C) and light (100-200 lx) in accordance with the National Standards of Laboratory Animal Environment and Facilities (GB14925-2010) at HVRI. Animals were observed at least twice daily by trained personnel.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant numbers 31772774, 31400146, and 31630080) and the Chinese Academy of Agricultural Sciences Special Basic Scientific Research Foundation (grant number Y2017CG24).

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Supporting information

Fig. S1. Generation of the chimeric viruses with exchange of envelope glycoproteins E^{rns}, E1, or/and E2 between C-strain and the highly virulent CSFV Shimen strain. A. E^{rns} protein of the progeny virus was expressed. The supernatants from the chimeras of passage 4 (P4) were tested by an antigen-capture ELISA (IDEXX) according to the manufacturer's introductions. **B.** E2 protein of the CSFV chimeras was tested in infected cells. PK-15 cells infected with CSFV chimeras of P4 were examined by an indirect immunofluorescence assay using an anti-E2 monoclonal antibody. Scale bar, 400 μm.

Fig. S2. Identification of the C-strain-based chimeric viruses harboring the different domains of Shimen strain E2 protein. The expression of the E^{rns} **(A)** or E2 protein **(B)** of the rescued chimeric viruses was tested by antigen-capture ELISA (IDEXX) according to the manufacturer's introductions or indirect immunofluorescence assay using an anti-E2 monoclonal antibody. Scale bar, 200 μm.

Fig. S3. Identification of the mutant vHCLV-SME2Domain I-1 harboring

the substitutions I4T, V23I, D49N, N67S, and S80I, and the mutant vHCLV-SME2Domain I-2 containing K105G, P108L, and T109I. The expression of E^{rns} protein **(A)** or E2 protein **(B)** of the rescued mutant viruses was tested by antigen-capture ELISA or indirect immunofluorescence assay using an anti-E2 monoclonal antibody. Bar = 200 μm. Scale bar, 200 μm.

Fig. S4. Identification of the C-strain-based mutants harboring individual or combined replacements K105G, P108L, and T109I. The rescued various mutants were identified by antigen-capture ELISA **(A)** or indirect immunofluorescence assay using an anti-E2 monoclonal antibody **(B)**. Scale bar, 200 μm.

Fig. S5. Sequence analysis of the revertant mutation in the recovered viruses. A. The revertant mutation L108P occurred in the E2 protein of the virus re-isolated from three rabbits infected with vHCLV-SME2DomainI-2. The chromatogram harboring the mutation is present. **B.** The revertant mutation L108P occurred in the E2 protein of the virus re-isolated from the rabbit infected with vHCLV-E2P108L/T109I. The chromatogram harboring the mutation is present.

Figure legends

Fig. 1. Schematic representation of infectious cDNA clones of the chimeric CSFV. The genomes of the chimeric viruses derived from the highly virulent CSFV Shimen strain and lapinized attenuated vaccine C-strain are illustrated. White boxes indicate proteins from the Shimen strain while purple

indicates proteins derived from C-strain. Noncoding regions derived from the respective parental viruses are shown in black.

Fig. 2. Chimeric or mutant viruses replicate differently in PK-15 (A) or SK6 cells (B–F). A. PK-15 cells were infected with vSM-HCLVE^{rns}E1E2, vHCLV-SME^{rns}E1E2, vSM-HCLVE^{rns}, vSM-HCLVE1, vSM-HCLVE2, vSM-HCLVE^{rns}E1, vSM-HCLVE^{rns}E2, vSM-HCLVE1E2, or Shimen at a multiplicity of infection (MOI) of 0.1. **B.** SK6 cells were infected with vHCLV-SME^{rns}E1E2, vHCLV-SME2, vHCLV, or C-strain at MOI of 0.1. **C.** SK6 cells were infected with vHCLV-SME2DomainI, vHCLV-SME2DomainII, vHCLV-SME2DomainIII, vHCLV-SME2DomainIV, or parental viruses at MOI of 0.1. **D.** SK6 cells were infected with vHCLV-SME2DomainI-1, vHCLV-SME2DomainI-2, or parental viruses at MOI of 0.1. **E.** SK6 cells were infected with vHCLV-E2K105G, vHCLV-E2P108L, vHCLV-E2T109I, or parental viruses at MOI of 0.1. **F.** SK6 cells were infected with vHCLV-E2K105G/P108L, vHCLV-E2K105G/T109I, vHCLV-E2P108L/T109I, or parental viruses at MOI of 0.1. The viral titers were determined and expressed as 50% tissue culture infective dose ($TCID_{50}$) per milliliter. The error bars represent the standard deviations for three replicates.

Fig. 3. The E2 glycoprotein in combination with E^{rns} or with E1 confers the adaptation of C-strain to the rabbit. A. Chimeric viruses vSM-HCLVE^{rns}E2 and vSM-HCLVE1E2 but not vSM-HCLVE^{rns}E1 were re-isolated from the spleens of the inoculated rabbits. The supernatants of isolated vSM-HCLVE^{rns}E2, vSM-HCLVE1E2 or vSM-HCLVE^{rns}E1 from the

rabbit spleens were examined by CSFV antigen-capture ELISA (IDEXX). **B.** The corresponding genes of isolated vSM-HCLVE^{rns}E2 or vSM-HCLVE1E2 from the spleens of the rabbits were amplified by RT-PCR from the supernatants of isolated chimeras infected cells. Lane 1, DNA marker; Lane 2, mock control; Lanes 3 to 5, the *E^{rns}*, *E1*, and *E2* genes of vSM-HCLVE^{rns}E2; Lanes 6 to 8, the *E^{rns}*, *E1*, and *E2* genes of vSM-HCLVE1E2. **C.** Evaluation of viral replication in the spleens by immunohistochemistry. The presence of viral antigens in the spleens from the inoculated animals was tested by immunohistochemical staining using an anti-CSFV E2 antibody. Scale bar, 50 μm. **D.** Substitutions of E^{rns}-E2 or E1-E2 but not E^{rns}-E1 of the Shimen strain with the counterparts of C-strain enable the Shimen strain to induce fever response in rabbits. The rectal temperatures of the rabbits inoculated intravenously with vSM-HCLVE^{rns}E1, vSM-HCLVE^{rns}E2, vSM-HCLVE1E2, C-strain, or Shimen were monitored from 24 h post-inoculation (hpi) to the 72 hpi at 6-h intervals. Temperature results of representative viruses are shown.

Fig. 4. Domain I in E2 is critical to the adaptation of C-strain to the rabbit. **A.** E2 amino acids alignments between C-strain (GenBank accession number: AY805221) and the Shimen strain (GenBank accession number: AF092448.2). **B.** The predicted 3D structure of CSFV E2. Domain I is shown in light green, Domain II in dark green, Domain III in yellow, and Domain IV in red. **C.** The E^{rns} protein of the re-isolated chimeric viruses from the inoculated rabbits was detected by antigen-capture ELISA. **D.** The genomes of the chimeric viruses based on C-strain harboring the different domains of the CSFV Shimen strain E2 are illustrated. Light green indicates Domain I from the Shimen strain E2,

Domain II in dark green, Domain III in yellow, and domain IV in red. "√" represents that the virus is able to replicate and induce fever response in rabbits, while "×" indicates negative results. **E.** The revertant mutation L108P occurred in the E2 protein of the virus re-isolated from the rabbit infected with vHCLV-SME2DomainI. The chromatogram harboring the mutation is present.

Fig. 5. The amino acids K105, P108, and T109 in Domain I of E2 play a key role in the adaptation of C-strain to the rabbit. A. Amino acid differences in Domain I of E2 between C-strain and the Shimen strain are present. **B.** The expression of E^{rns} protein of the re-isolated mutant viruses from the inoculated rabbits was determined by antigen-capture ELISA. **C.** The replication and induced fever response characteristics of the mutants vHCLV-SME2DomainI-1 and vHCLV-SME2DomainI-2 in rabbits are shown. "√" represents the virus is able to replicate and induce fever response in rabbits, while "×" indicates negative results. **D.** Detection of viral antigens in the spleen samples by immunohistochemistry. Viral antigens in the spleens from the animals inoculated with the mutant viruses vHCLV-SME2DomainI-1, or vHCLV-SME2DomainI-2 were tested by immunohistochemical staining using an anti-CSFV E2 antibody. Staining results of representative spleen samples are shown. Scale bar, 50 μm. **E.** Substitutions of K105G, P108L, and T109I completely abolish the fever response induced by C-strain. The rectal temperatures of the rabbits inoculated intravenously with vHCLV-SME2DomainI-1, vHCLV-SME2DomainI-2, C-strain, or Shimen were monitored every 6 h from 24 to 72 h post-inoculation (hpi). Temperature results of representative viruses are shown.

Fig. 6. The amino acids P108 and T109 in E2 are critical to the adaptation of C-strain to the rabbit. A. The genomes of the C-strain-based mutants harboring different amino acid substitutions with those in the E2 Domain I of the highly virulent CSFV Shimen strain are shown. The mutation at position 105 is indicated in light red, 108 in yellow, and 109 in blue. The replication and induced fever response characteristics of the mutants in rabbits are shown. "√" represents the virus is able to replicate and induce fever response in rabbits, while "×" indicates negative results. **B.** The expression of E^{rns} protein of the re-isolated mutant viruses from the inoculated rabbits was determined by antigen-capture ELISA. **C.** Detection of viral antigens in the spleen samples by immunohistochemistry. Viral antigens in the spleens from the animals inoculated with the mutant viruses based on C-strain harboring different amino acids replacements in Domain I of the Shimen strain E2 were tested by immunohistochemical staining using an anti-CSFV E2 antibody. Staining results of representative spleen samples are shown. Scale bar, 50 μm. **D.** Substitutions of the amino acids at sites 108 and 109 abolished the fever response induced by C-strain in rabbits. The rectal temperatures of the rabbits inoculated intravenously with vHCLV-E2P108L/T109I, C-strain, or Shimen were monitored from 24 to the 72 h post-inoculation at 6-h intervals. Temperature results of representative viruses are shown.

Figure1

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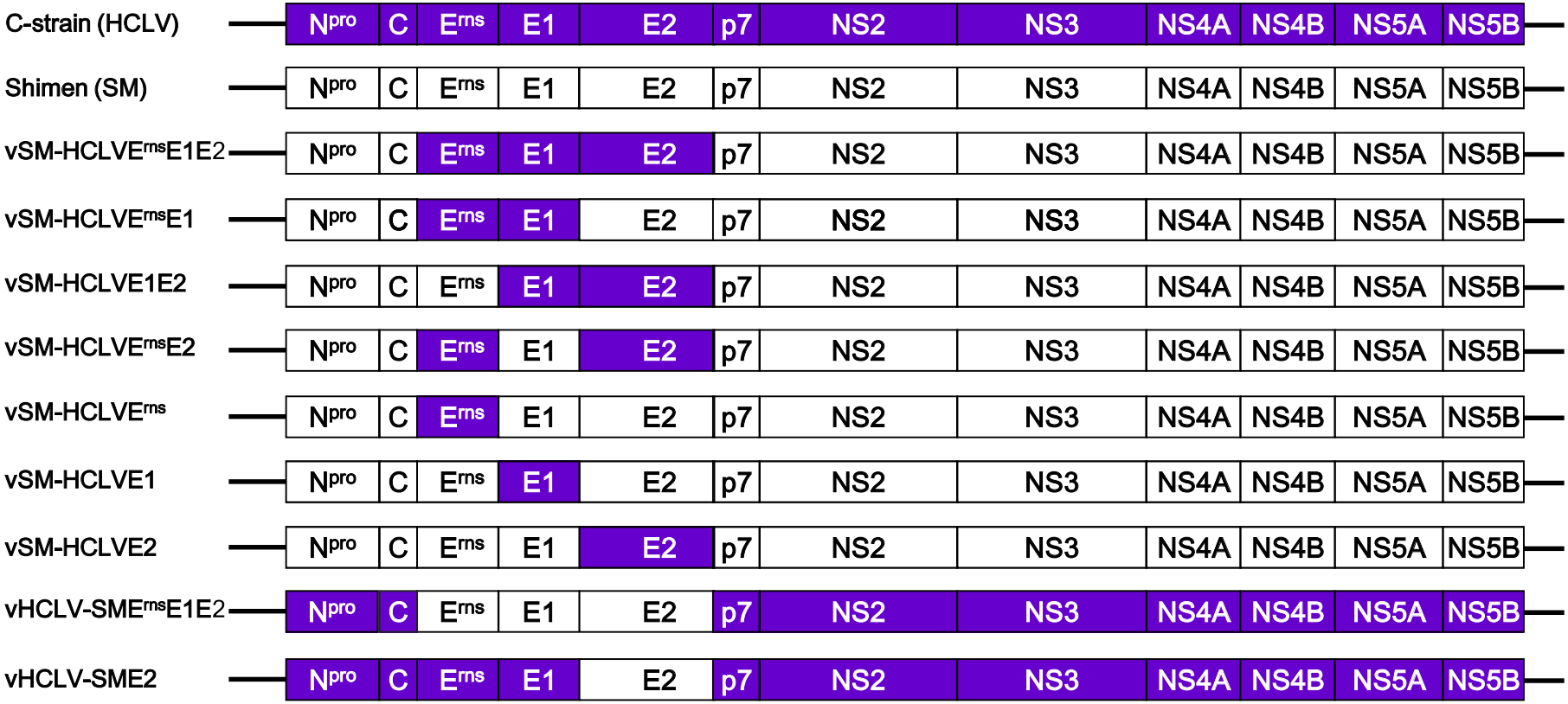

Figure 2

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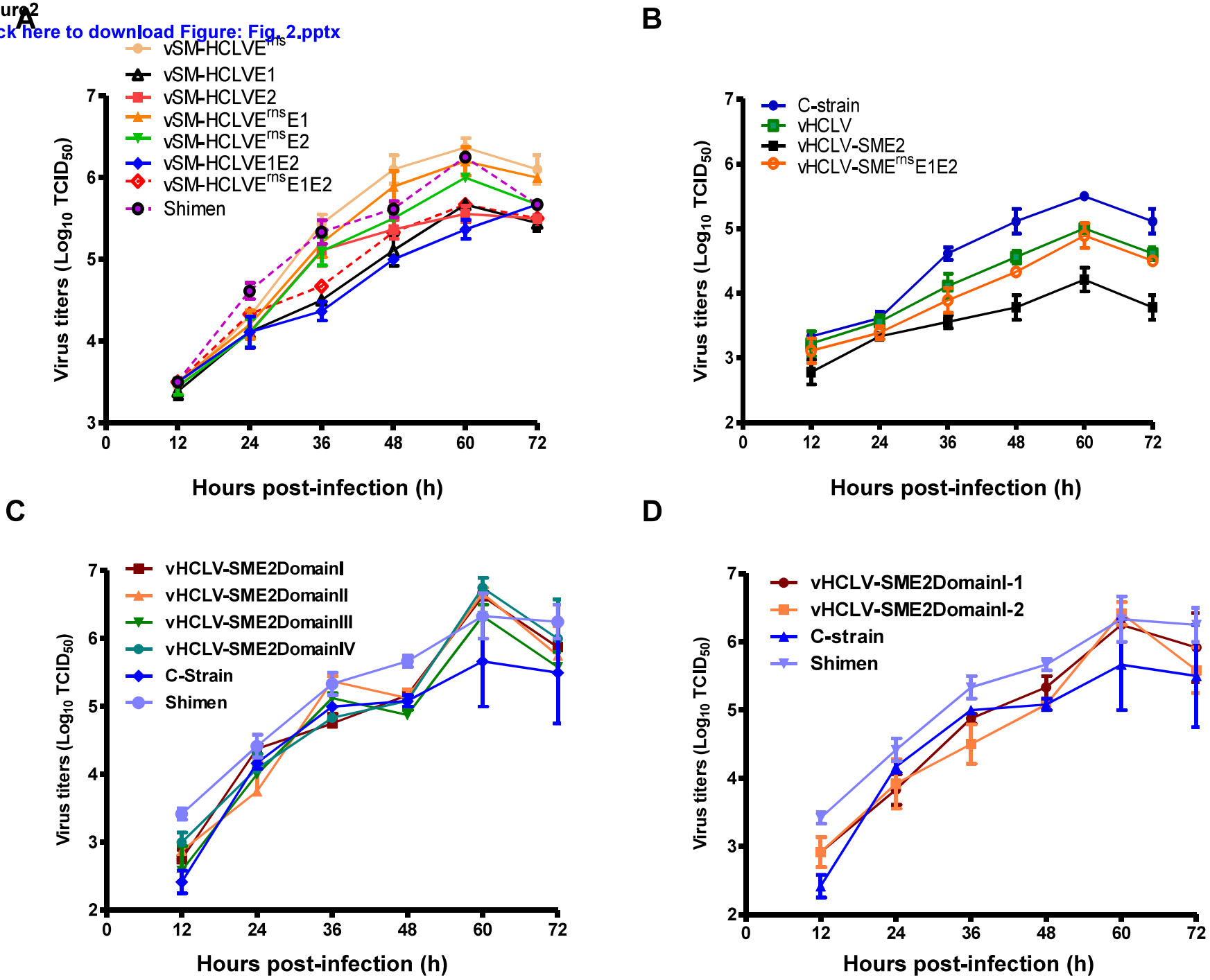

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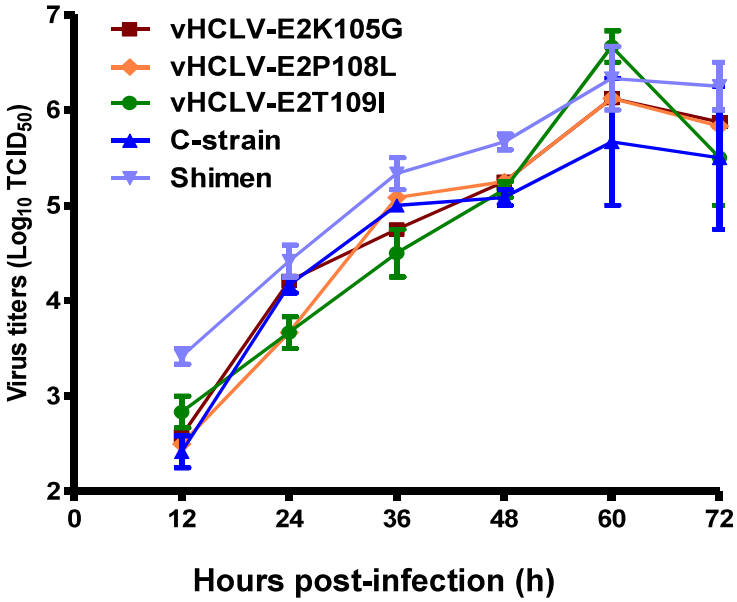
vHCLV-E2K105G
vHCLV-E2P108L
vHCLV-E2T109I
C-strain
Shimen
Virus titers (Log10 TCID50)
Hours post-infection (h)
0
12
24
36
48
60
72
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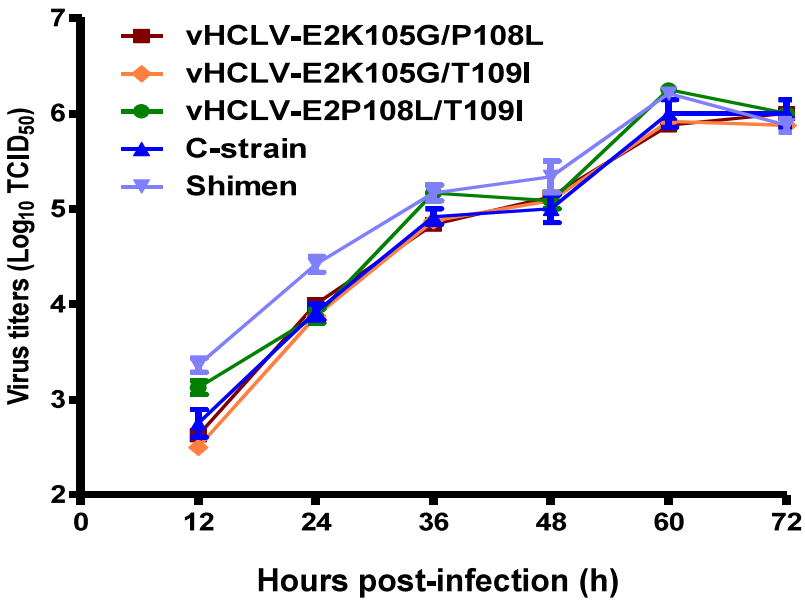
vHCLV-E2K105G/P108L
vHCLV-E2K105G/T109I
vHCLV-E2P108L/T109I
C-strain
Shimen
Virus titers (Log10 TCID50)
Hours post-infection (h)
0
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24
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48
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72
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Figure3

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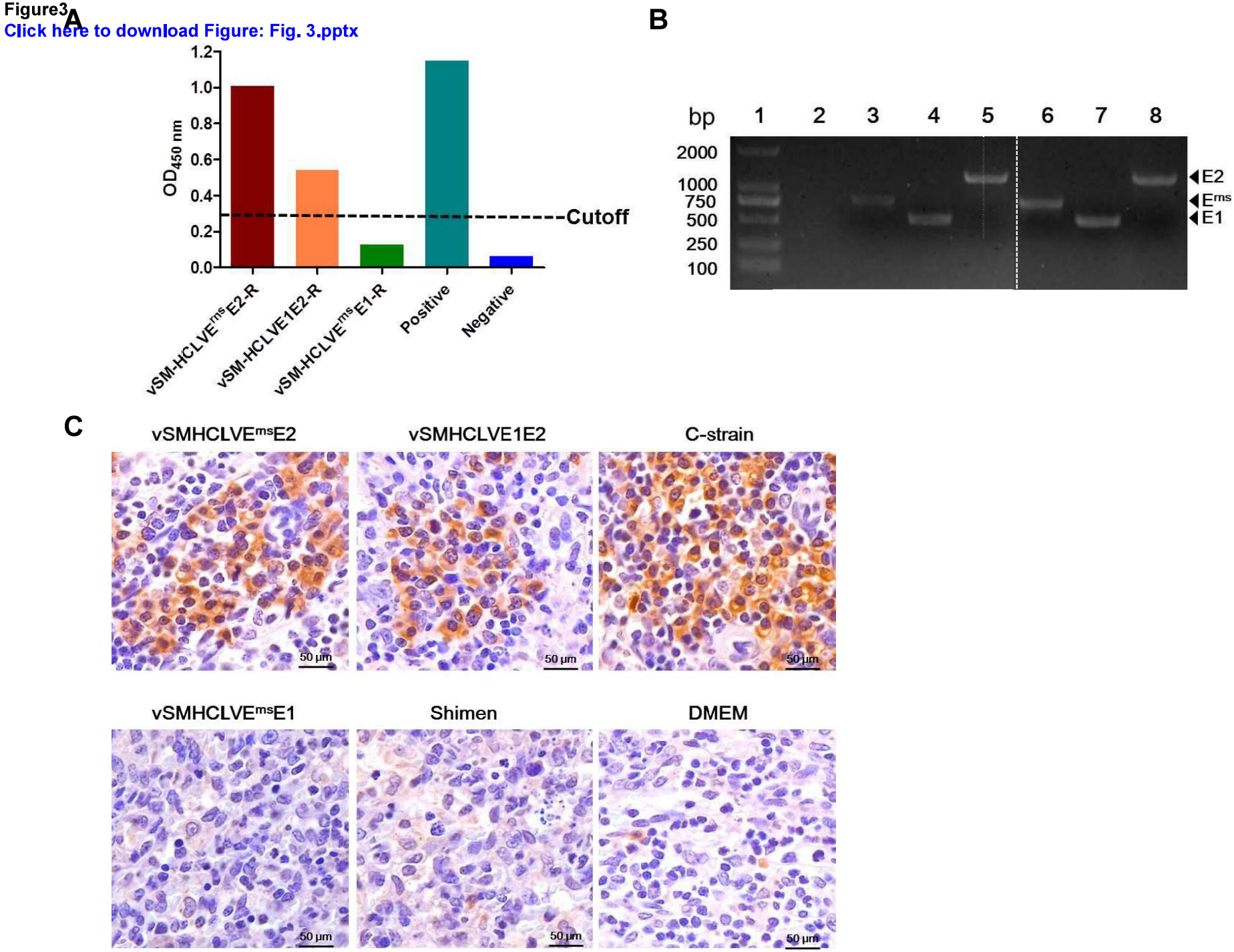


D

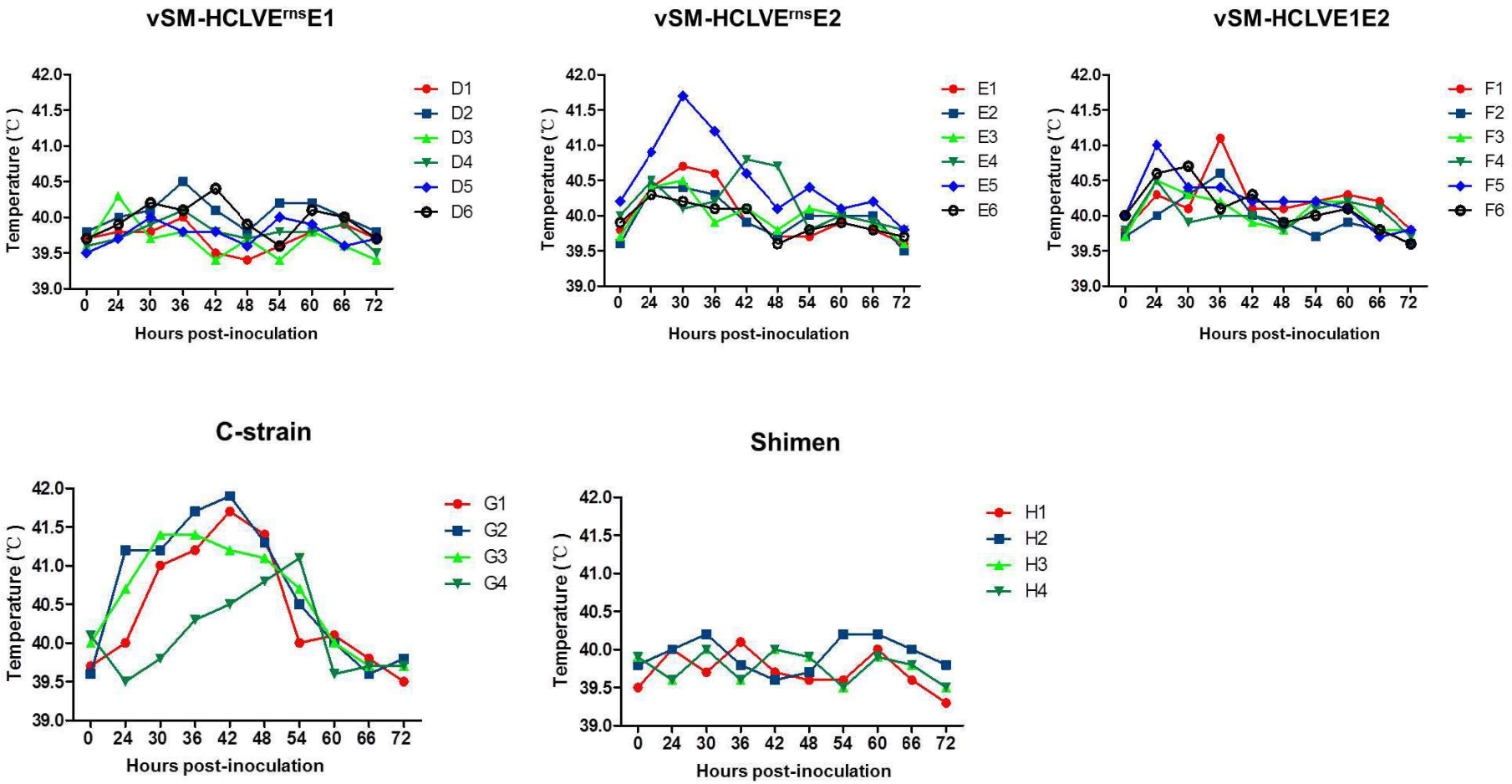

vSM-HCLVE^rnsE1
Temperature (℃)
42.0
41.5
41.0
40.5
40.0
39.5
39.0
0 24 30 36 42 48 54 60 66 72
Hours post-inoculation
D1
D2
D3
D4
D5
D6
vSM-HCLVE^rnsE2
Temperature (℃)
Hours post-inoculation
E1
E2
E3
E4
E5
E6
vSM-HCLVE1E2
Temperature (℃)
Hours post-inoculation
F1
F2
F3
F4
F5
F6
C-strain
Temperature (℃)
Hours post-inoculation
G1
G2
G3
G4
Shimen
Temperature (℃)
Hours post-inoculation
H1
H2
H3
H4

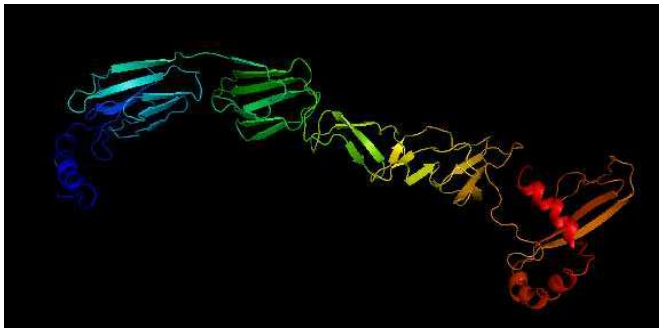
Figure4

Click here to download Figure: Fig. 4.pptx

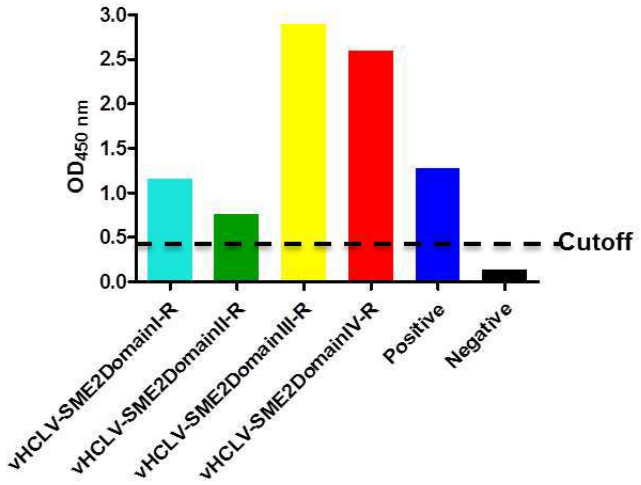
A

```
Shimen-E2.seq ASTTAFLICLIKVLRGQIVQGVIWLLLVTGAQGRLACKEDYRYAISSTNEIGLLGAGGLTTTWKEYSHDLQLNDGTVKAICVAGSFKVTALNVVSRRYLASLHKGALLISVTFELLFDGTNPSTEEMGDDFGFGLCPFDTSPVVKGKYNT
HCLV-E2.seq   ...I.................V.......................D.................N............S.......................K..PT.....................RS.................

Shimen-E2.seq TLLNGSAFYLVCPIGWTGVIECTAVSPTTLRTEVVKTFRREKPFPHRMDCVTTTVENEDLFYCKLGGNWTCVKGEPVVYTGGQVKQCKWCGFDFNEPDGLPHYPIGKCILANETGYRIVDSTDCNRDGVVISAEGSHECLIGNTTVKVHA
HCLV-E2.seq   .....................................D.....................................V....R......DG.....................................T.................

Shimen-E2.seq SDERLGPMPCRPKEIVSSAGPVRKTSCTFNYAKTLKNKYYEPRDSYFQQYMLKGEYQYWFDLDVTDRHSDYFAEFVVLVVVALLGGRYVLWLIVTYIVLTEQLAAG
HCLV-E2.seq   ...................M........T.....R.....................A.............................V.........
```

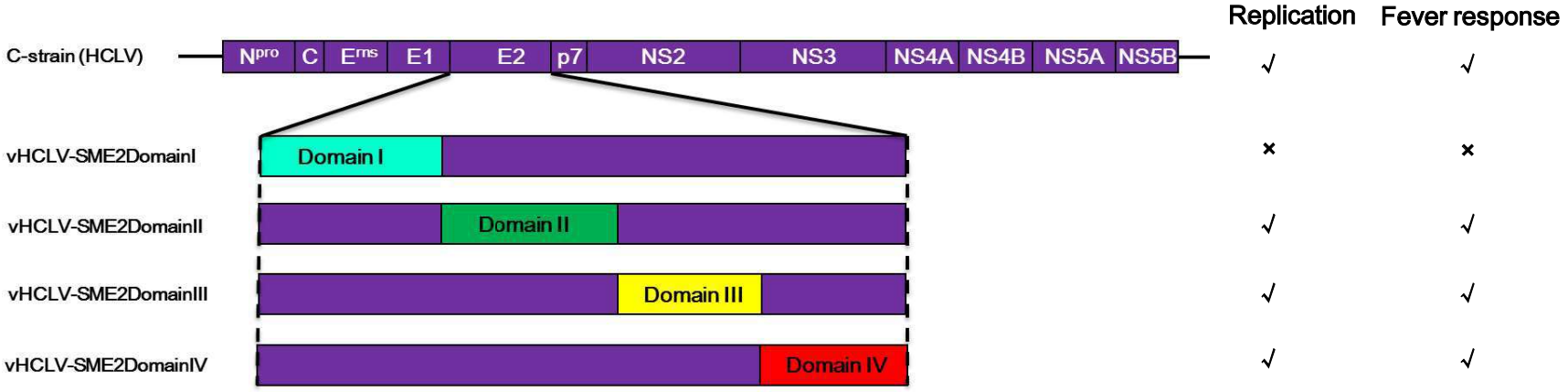
B



C



D



E

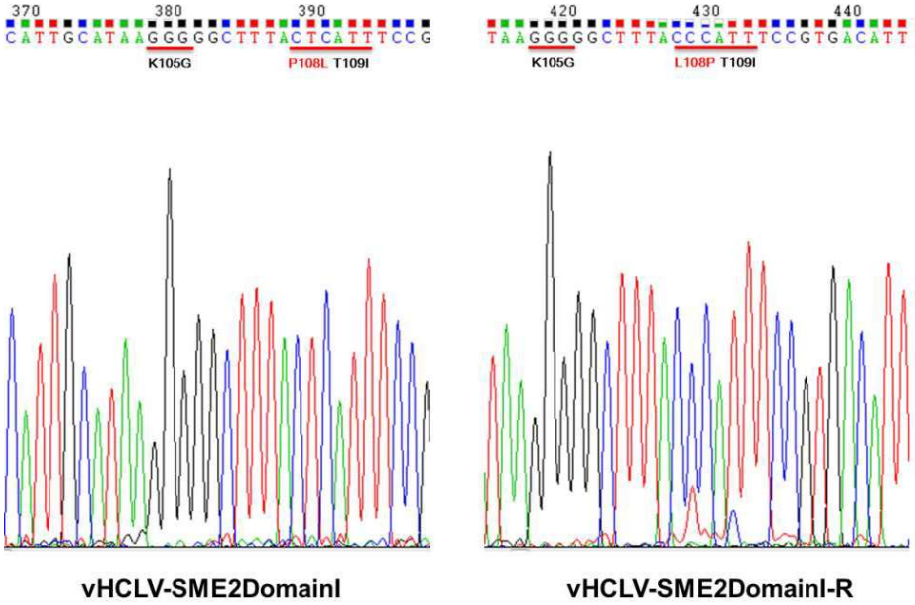

370
380
390
CATTGCATAAGGGGGCTTTACTCATTTCCG
K105G
P108L T109I
420
430
440
TAAGGGGGCTTTACCCATTTCCGTGACATT
K105G
L108P T109I
vHCLV-SME2DomainI
vHCLV-SME2DomainI-R

Figure5

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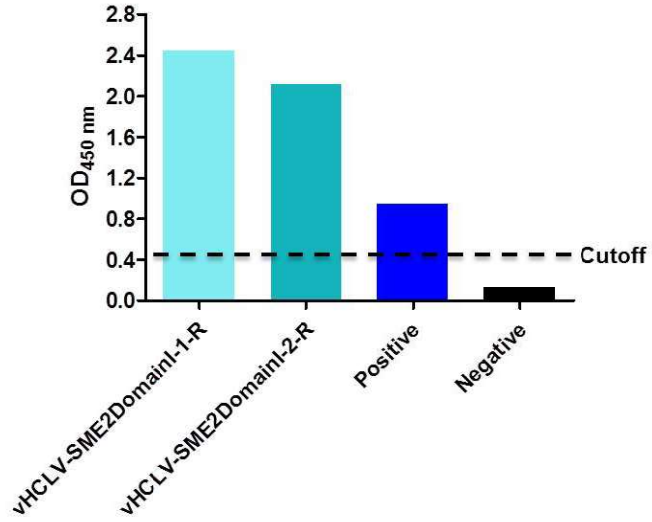
A

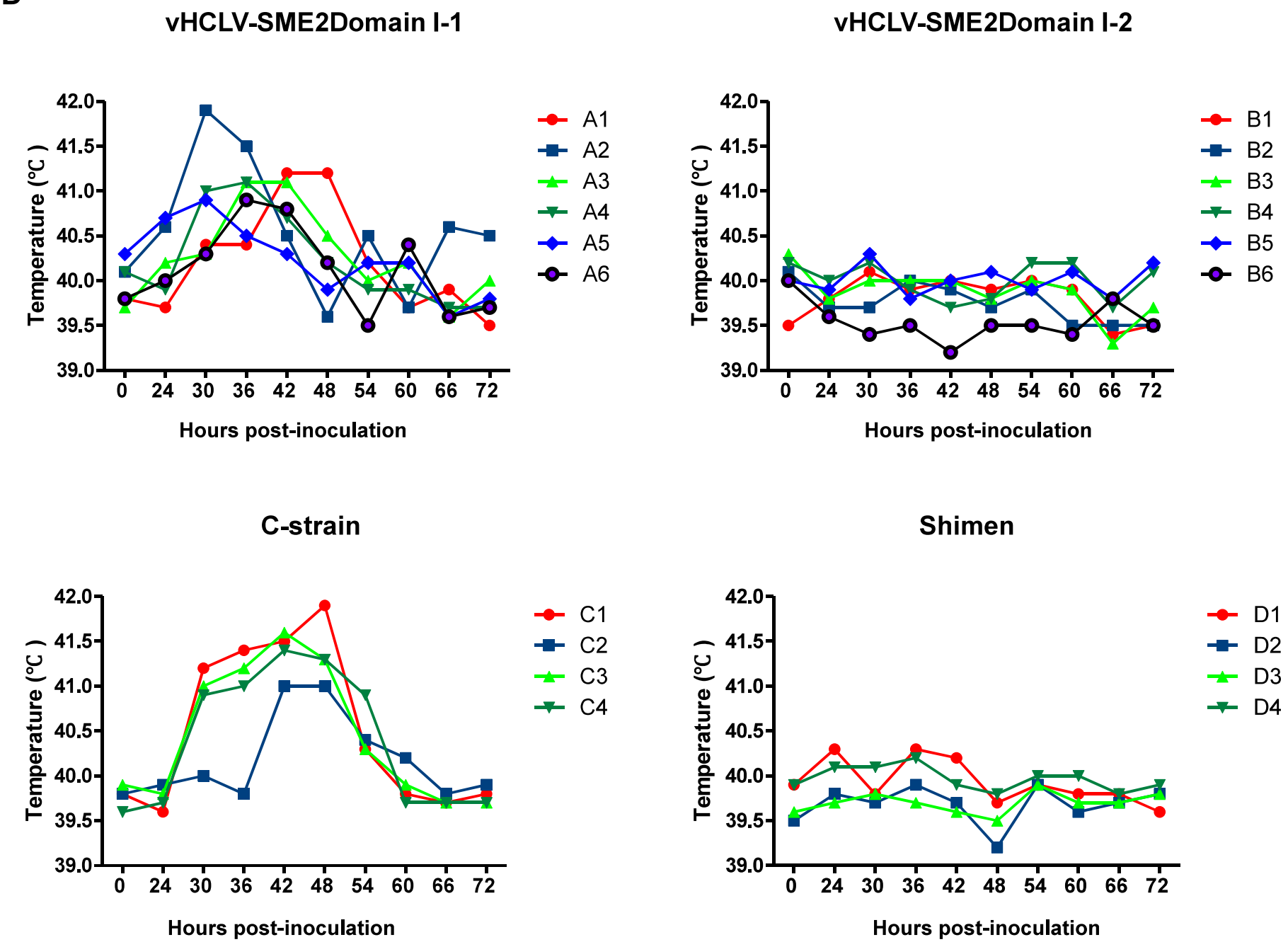
	Domain I-1					Domain I-2		
Amino acid position	4	23	49	67	80	105	108	109
Shimen	T	I	N	S	I	G	L	I
C-strain	I	V	D	N	S	K	P	T

B



C

D


vHCLV-SME2Domain I-1
Temperature (°C)
42.0
41.5
41.0
40.5
40.0
39.5
39.0
0 24 30 36 42 48 54 60 66 72
Hours post-inoculation
A1
A2
A3
A4
A5
A6
vHCLV-SME2Domain I-2
Temperature (°C)
42.0
41.5
41.0
40.5
40.0
39.5
39.0
0 24 30 36 42 48 54 60 66 72
Hours post-inoculation
B1
B2
B3
B4
B5
B6
C-strain
Temperature (°C)
42.0
41.5
41.0
40.5
40.0
39.5
39.0
0 24 30 36 42 48 54 60 66 72
Hours post-inoculation
C1
C2
C3
C4
Shimen
Temperature (°C)
42.0
41.5
41.0
40.5
40.0
39.5
39.0
0 24 30 36 42 48 54 60 66 72
Hours post-inoculation
D1
D2
D3
D4

E

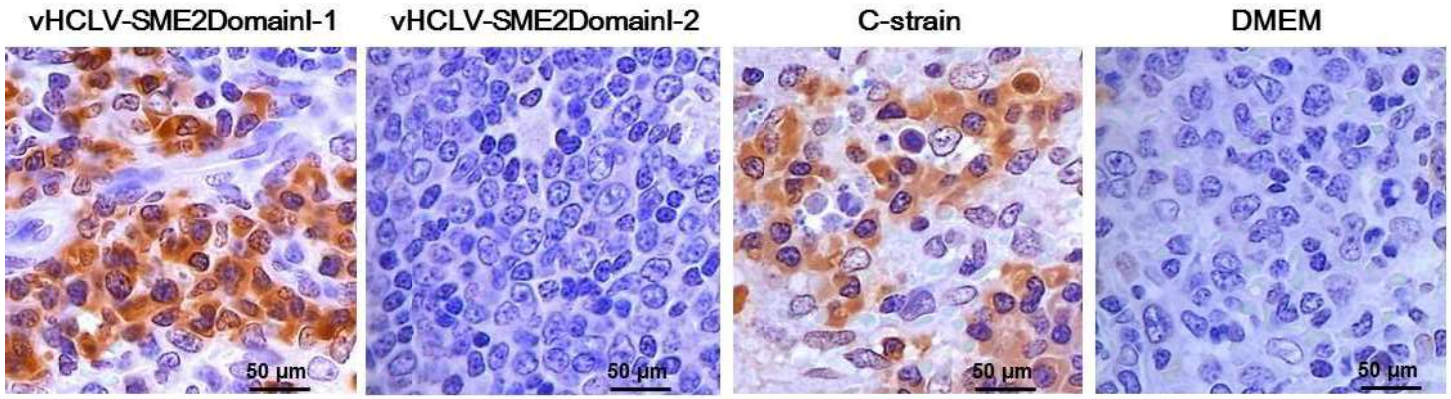
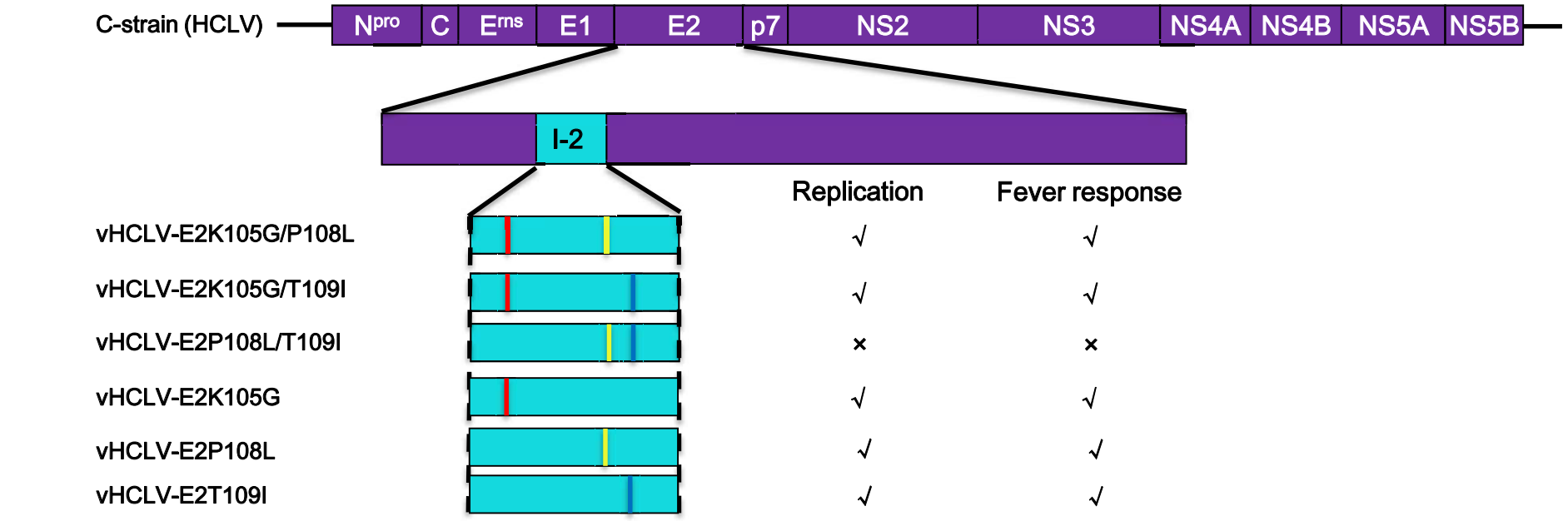
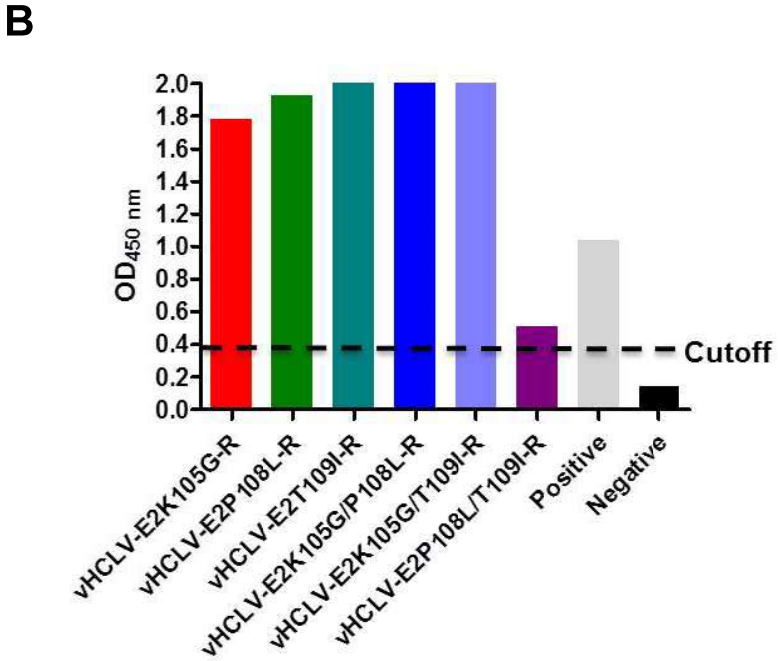

vHCLV-SME2DomainI-1
vHCLV-SME2DomainI-2
C-strain
DMEM
50 μm
50 μm
50 μm
50 μm

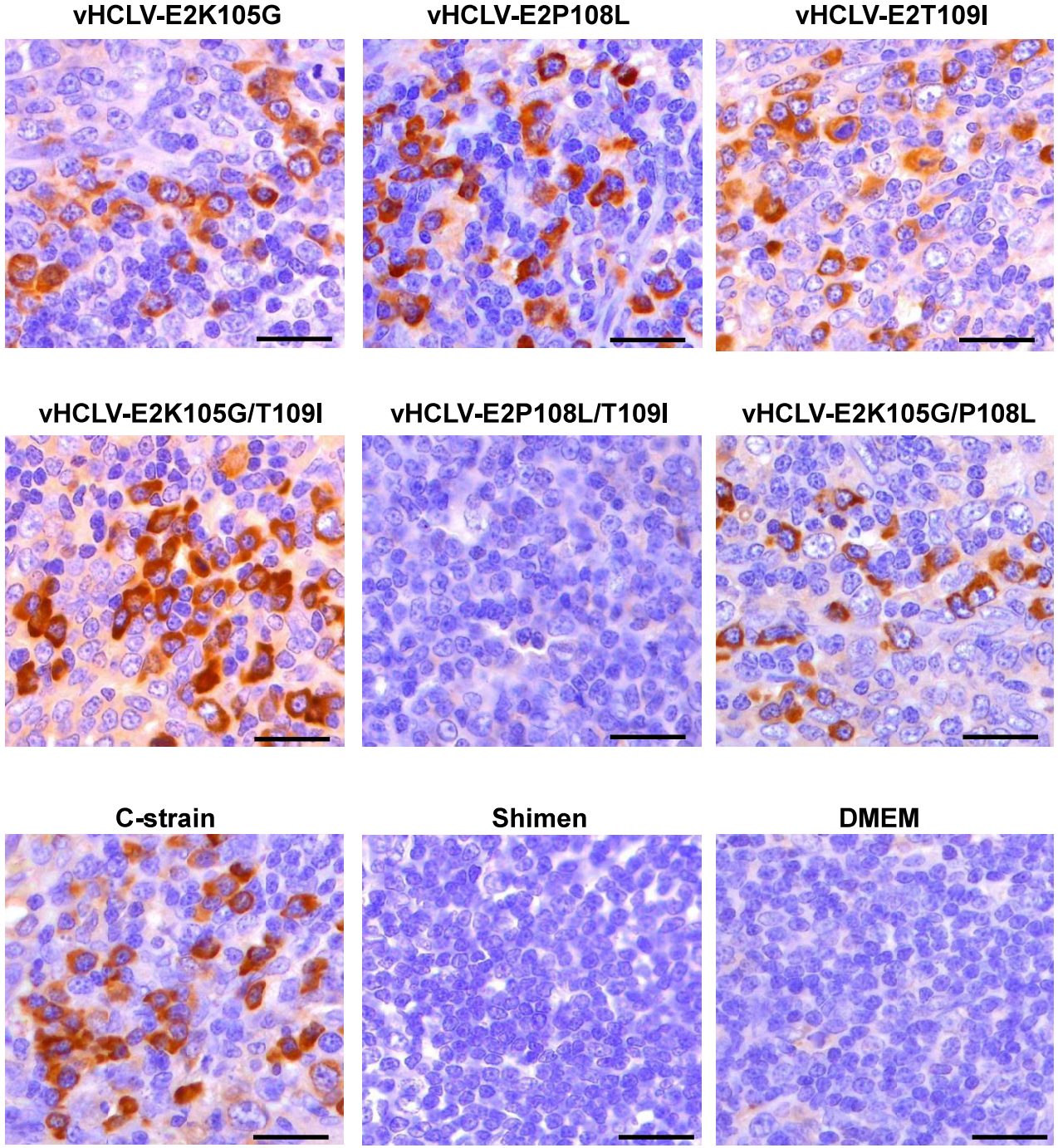
Figure6

Click here to download Figure: Fig. 6.pptx





C


vHCLV-E2K105G
vHCLV-E2P108L
vHCLV-E2T109I
vHCLV-E2K105G/T109I
vHCLV-E2P108L/T109I
vHCLV-E2K105G/P108L
C-strain
Shimen
DMEM

D

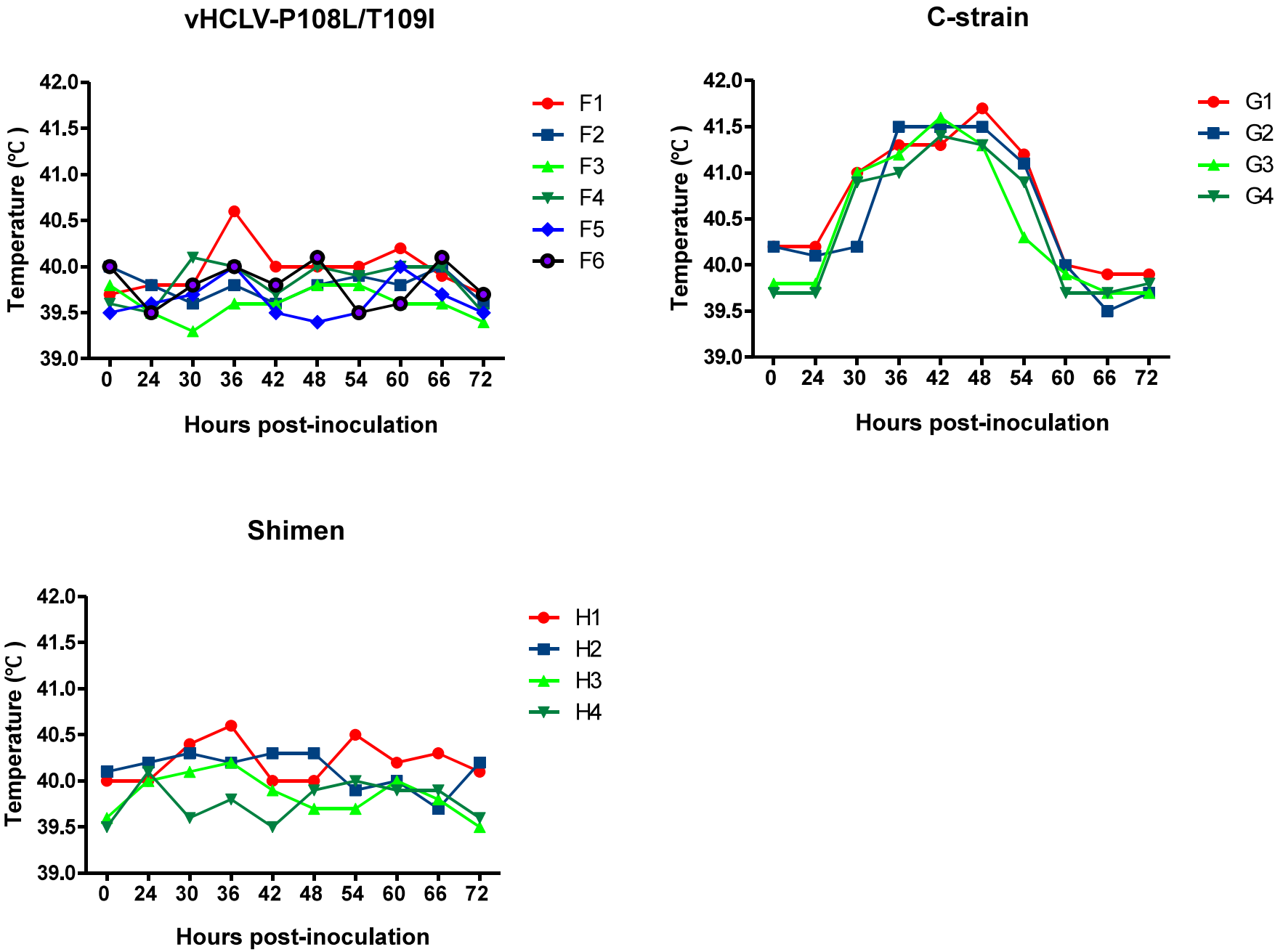

vHCLV-P108L/T109I
Temperature (°C)
42.0
41.5
41.0
40.5
40.0
39.5
39.0
0 24 30 36 42 48 54 60 66 72
Hours post-inoculation
F1
F2
F3
F4
F5
F6
C-strain
Temperature (°C)
42.0
41.5
41.0
40.5
40.0
39.5
39.0
0 24 30 36 42 48 54 60 66 72
Hours post-inoculation
G1
G2
G3
G4
Shimen
Temperature (°C)
42.0
41.5
41.0
40.5
40.0
39.5
39.0
0 24 30 36 42 48 54 60 66 72
Hours post-inoculation
H1
H2
H3
H4

Table 1 The infectivity of the chimeric viruses exchanging E^{rns}-E1-E2 between C-strain and the highly virulent CSFV Shimen in rabbits.

Groups	Inocula	Dose ($TCID_{50}$)	Fever induced	No. of viral replication /total	Mean viral RNA copies in the spleens (copies/μl)	Seroconversion at 10 DPI
1-1	vHCLV-SME^{rns}E1E2	10^4	0/6	0/3	No Ct	3/3
1-2	vSM-HCLVE^{rns}E1E2	10^4	2/6	3/3	2.63×10^3	3/3
1-3	C-strain	10^4	6/6	3/3	3.18×10^3	3/3
1-4	Shimen	10^4	0/6	0/3	No Ct	3/3
1-5	DMEM	1 ml	0/6	0/3	No Ct	0/3

Note: Ct, cycle threshold; DPI, days post-inoculation.

Table 2 The infectivity of the Shimen-based chimeric viruses harboring E^{rns}, E1, E2, E^{rns}-E1, E^{rns}-E2, or E1-E2 protein of C-strain in the genetic background of the Shimen strain in rabbits.

Groups	Inocula	Dose ($TCID_{50}$)	Fever induced	No. of viral replication/ total	Mean viral RNA copies in the spleens (copies/μl)	Seroconversion at 10 DPI
2-1	vSM-HCLVE^{rns}	10^4	0/6	0/3	No Ct	3/3
2-2	vSM-HCLVE1	10^4	0/6	0/3	No Ct	3/3
2-3	vSM-HCLVE2	10^4	0/6	0/3	No Ct	3/3
2-4	vSM-HCLVE^{rns}E1	10^4	0/6	0/3	No Ct	3/3
2-5	vSM-HCLVE^{rns}E2	10^4	3/6	2/3	1.64×10^3	3/3
2-6	vSM-HCLVE1E2	10^4	3/6	2/3	1.65×10^3	3/3
2-7	C-strain	10^4	5/6	3/3	1.55×10^3	3/3
2-8	vHCLV	10^4	5/6	3/3	6.85×10^3	3/3
2-9	Shimen	10^4	0/6	0/3	No Ct	3/3
2-10	DMEM	1 ml	0/6	0/3	No Ct	0/3

Note: Ct, cycle threshold.

Table 3 The infectivity of the chimeric viruses carrying E2 protein of the Shimen strain in the genetic background of C-strain or E^{rns}-E1, E^{rns}-E2, or E1-E2 protein of C-strain in the context of the Shimen strain in rabbits.

Groups	Inocula	Dose ($TCID_{50}$)	Fever induced	No. of viral replication/ total	Mean viral RNA copies in the spleens (copies/μl)	Seroconversion at 10 DPI
3-1	vHCLV-SME2	10^4	0/6	0/4	No Ct	2/2
3-2	vSM-HCLVE^{rns}E1	10^4	0/6	0/4	No Ct	2/2
3-3	vSM-HCLVE^{rns}E2	10^4	2/6	4/4	1.21×10^3	2/2
3-4	vSM-HCLVE1E2	10^4	2/6	3/4	1.17×10^3	2/2
3-5	C-strain	10^4	6/6	4/4	2.63×10^3	2/2
3-6	vHCLV	10^4	6/6	4/4	1.80×10^3	2/2
3-7	Shimen	10^4	0/4	0/4	No Ct	2/2
3-8	DMEM	1 ml	0/4	0/4	No Ct	0/2

Note: Ct, cycle threshold.

Table4

Table 4 The infectivity of the C-strain-based chimeric viruses carrying the different domains of E2 protein of the Shimen strain in rabbits.

Groups	Inocula	Dose ($TCID_{50}$)	Fever induced	No. of viral replication/ total	Mean viral RNA copies in the spleen (copies/μl)	Seroconversion at 10 DPI
4-1	vHCLV-SME2DomainI	10^4	0/6	1#/4	7.80×10^2	2/2
4-2	vHCLV-SME2DomainII	10^4	4/6	4/4	7.49×10^3	2/2
4-3	vHCLV-SME2DomainIII	10^4	5/6	4/4	4.83×10^4	2/2
4-4	vHCLV-SME2DomainIV	10^4	5/6	4/4	2.52×10^4	2/2
4-5	vHCLV-SME2	10^4	1/3	1*/2	4.10×10^2	1/1
4-6	C-strain	10^4	4/4	2/2	1.49×10^3	2/2
4-7	Shimen	10^4	0/4	0/2	No Ct	2/2
4-8	DMEM	1 ml	0/4	0/2	No Ct	0/2

Note: "**#**" represents a revertant mutation L108P occurred in the genome of re-isolated virus. "*" represents a revertant mutation I109T occurred in the genome of re-isolated virus. Ct, cycle threshold.

Table 5 The infectivity of the mutant vHCLV-SME2DomainI-1 harboring the substitutions I4T, V23I, D49N, N67S, and S80I or the mutant vHCLV-SME2DomainI-2 containing the replacements K105G, P108L, and T109I in rabbits.

Groups	Inocula	Dose ($TCID_{50}$)	Fever induced	No. of viral replication/ total	Mean viral RNA copies in the spleen (copies/μl)	Seroconversion at 10 DPI
5-1	vHCLV-SME2DomainI-1	10^4	5/6	4/4	3.01×10^3	2/2
5-2	vHCLV-SME2DomainI-2	10^4	0/6	3#/4	5.91×10^2	2/2
5-3	C-strain	10^4	4/4	2/2	1.75×10^3	2/2
5-4	Shimen	10^4	0/4	0/2	No Ct	2/2
5-5	DMEM	1 ml	0/4	0/2	No Ct	0/2

Note: "**#**" represents a revertant mutation L108P occurred in the genome of re-isolated virus from the three rabbits. Ct, cycle threshold.

Table 6 The infectivity of the C-strain-based mutants with individual and various combined mutations of K105G, P108L, or T109I in rabbits.

Groups	Inocula	Dose ($TCID_{50}$)	Fever induced	No. of viral replication/ total	Mean viral RNA copies in the spleen (copies/μl)	Seroconversion at 10 DPI
6-1	vHCLV-E2K105G	10^4	4/6	4/4	2.07×10^3	2/2
6-2	vHCLV-E2P108L	10^4	3/6	4/4	5.51×10^2	2/2
6-3	vHCLV-E2T109I	10^4	4/6	4/4	6.69×10^3	2/2
6-4	vHCLV-E2K105G/P108L	10^4	3/6	4/4	9.98×10^2	2/2
6-5	vHCLV-E2K105G/T109I	10^4	4/6	3/4	2.85×10^3	2/2
6-6	vHCLV-E2P108L/T109I	10^4	0/6	1#/4	1.36×10^2	2/2
6-7	vHCLV-SME2domainI-2	10^4	0/4	2/4	4.78×10^2	-
6-8	C-strain	10^4	4/4	2/2	8.60×10^3	2/2
6-9	Shimen	10^4	0/4	0/2	No Ct	2/2
6-10	DMEM	1 ml	0/4	0/2	No Ct	0/2

Note: "**#**" represents a revertant mutation L108P occurred in the genome of re-isolated virus from the one rabbit. Ct, cycle threshold.

Fig. S1

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Fig. S2

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Fig. S3

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Fig. S4

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Fig. S5

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TableS1

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TableS2

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