A single mutation in capsid protein VP1 (Q145E) of a genogroup C4 strain of human enterovirus 71 generates a mouse-virulent phenotype

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We modified the capsid protein of a human enterovirus 71 (HEV71) belonging to subgenogroup C4 (HEV71-C4) to generate a mouse virulent strain, based on the genetic information derived from our previous subgenogroup B3 mouse-adapted virus. Infectious clone-derived mutant virus populations containing the capsid protein mutations VP1-Q145E and VP1-Q145G were generated by site-directed mutagenesis of an infectious clone of a subgenogroup C4 strain. Viruses expressing the VP1-Q145E were virulent in 5-day-old BALB/c mice with 100% mortality rate observed. Skeletal muscle appears to be the primary site of replication of this virus with limb muscle showing severe myositis. Virus was also isolated from spleen, liver, heart and brain of infected mice. This study demonstrates that introducing a key mutation into the HEV71 VP1 capsid protein is able to generate a mouse virulent HEV71 strain from a different genogroup as well as providing an alternative strategy for the generation of mouse virulent HEV71.

In this study, we generated a mouse-virulent HEV71 strain representing subgenogroup C4 by modifying its VP1 capsid protein based on the genetic information derived from our previous mouse-adapted HEV71 (genogroup B3) strain (Chua et al., 2008). HEV71 strain 540v/vNM/05 (subgenogroup C4, HEV71-C4, VP1 gene GenBank accession no. AM490151) was isolated during the 2005 hand, foot and mouth disease outbreak in Southern Vietnam (Tu et al., 2007). We sequenced the whole genome of HEV71-C4 using synthetic oligonucleotide primers designed to amplify overlapping fragments of the genome (Zaini et al., 2012). The complete nucleotide and deduced amino acid sequences of HEV71-C4 (GenBank accession no. JQ965759) was then compared to the available complete genome sequences of HEV71-C4 (GenBank accession no. EU364841), CHO-26M (GenBank accession no. EU376004) and MP-26M (GenBank accession no. EU376005). Importantly, we determined the HEV71-C4 capsid protein amino acid residues to be lysine (K) at position 149 in VP2 and to be glutamine (Q) at position 145 in VP1, respectively (Fig. 1a, b).
We have previously constructed a full-length infectious cDNA clone of HEV71-C4 (strain 540v/vNM/05) by RT-PCR assay, restriction enzyme digestion and cloning in two subgenomic clones using the plasmid pMC18 (Zaini et al., 2012). We have also constructed a full-length infectious cDNA clone of HEV71-C4 containing the CHO cell-adaptation marker, VP2-K149I (C4-VP2I) (Zaini et al., 2012). From previous work, we have established that it is not possible to obtain stable clone-derived virus (CDV) populations that contain only the VP1 mouse-adaptation mutations and that the CHO cell-adaptation mutation VP2-K149I is necessary to increase the stability of the VP1 mouse-adaptation mutations (Chua et al., 2008). Consequently, we decided to use the full-length infectious cDNA clone of C4-VP2I as the parental clone for further genetic manipulation.

Firstly, we showed that residue 145 of HEV71 VP1 capsid protein is highly variable amongst the four strains compared in this study, with residues glycine (G), glutamic acid (E) and glutamine (Q) identified (Fig. 1b). As our parental infectious clone, C4-VP2I, contains a glutamine residue at VP1-145, we decided to construct two variants containing either glutamic acid or glycine at this position in order to investigate the effect of these residues on mouse adaptation and virulence. The VP1-Q145E and VP1-Q145G mutations were introduced into the C4-VP2I full-length clone by site-directed mutagenesis, generating variants C4-VP2I-VP1E and C4-VP2I-VP1G, respectively (Fig. 1c). The QuikChange Lightning Site-Directed Mutagenesis kit (Stratagene) was used with primers 5′-TGACACGCCGAGAGTTGTCGCCACGAG-3′ and 5′-CTGTGGGACACTCTCCGTTGGGTGCGAC-3′ for VP1-Q145E mutagenesis; and primers 5′-GTGCAACCCACGAGAGTTGTCGCCACGAG-3′ and 5′-CTGTGGGACACTCTCCGTTGGGTGCGAC-3′ for VP1-Q145G mutagenesis. The mutagenesis reaction was performed without subcloning and a single clone of each mutant was selected and purified plasmid DNA prepared for sequence analysis. VP1 gene RT-PCR and nucleotide sequencing was undertaken to ensure that the site-specific changes were present in the mutated clones (data not shown).

The infectious cDNA clones of the C4-VP2I-VP1E and C4-VP2I-VP1G variants were transfected into SV40-transformed African green monkey kidney (COS-7) cells (ATCC CRL-1651) using Lipofectamine 2000 (Invitrogen), as described by Zaini et al. (2012). We successfully recovered the CDVs from transfected cells at 2 days post-transfection. The CDVs were passaged once on Vero cells in order to generate high titre working stocks; CDV stocks were found to have viral titres of >10^6 TCID50 ml^-1. VP1 gene RT-PCR and nucleotide sequencing was undertaken to ensure that the site-specific change remained present in the rescued virus populations. Nucleotide sequencing showed that both variants, C4-VP2I-VP1E CDV and C4-VP2I-VP1G CDV, retained the introduced VP1-145 mutation at this passage level with no evidence of partial reversion of the E or G codons to the parental Q codon (data not shown).

We next performed mouse virulence assays to compare the survival of BALB/c mice with the parental or mutant CDVs. Our prior study of the mouse adaptation of HEV71 (subgenogroup B3) showed that mice infected with a lethal dose of mouse-adapted virus at 5–7 days of age survived...
until day 5 post-infection (p.i.) and reached 100% mortality by day 8 p.i. Mice at 14 days of age and older were resistant to mouse-adapted HEV71 infection (Chua et al., 2008). We have also previously investigated oral infection of mice with mouse-adapted HEV71 and found that BALB/c mice are susceptible to infection via the oral route in an age- and dose-dependent manner, although the 50% humane dose (HD\textsubscript{50}) values after oral infection are \(>10^3\)-fold higher than infection via the intraperitoneal (i.p.) inoculation. Thus, for practical reasons, mice were inoculated at 5 days of age via the i.p. route of infection in all of our subsequent experiments. All mouse studies were approved by the University of Sydney Animal Ethics Committee. Groups of six 5-day-old BALB/c mice were infected with approximately \(10^4\) TCID\textsubscript{50} of each of the infectious CDV populations. Mice were observed twice daily for clinical signs of illness for 14 days p.i. As expected, the parental C4-VP2I CDV virus population caused no observable morbidity or mortality in infected mice (Fig. 2a). The C4-VP2I-VP1G CDV population also exhibited a non-virulent phenotype in mice, with no observable clinical signs of infection or mortality up to 14 days p.i., similar to the parental virus (\(P>0.05\)). By contrast, mice infected with the C4-VP2I-VP1E CDV population showed forelimb and/or hindlimb flaccid paralysis, ruffled fur, weight loss and severe lethargy after 3 days p.i. A mortality rate of 100% was observed, with death commencing at 3 days p.i. and reaching 100% by 4 days p.i. (Fig. 2a). The mean survival time of C4-VP2I-VP1E CDV-infected mice was calculated to be 3.3 (±0.5) days.

We next determined the HD\textsubscript{50} values of the parental and mutant viruses. HD\textsubscript{50} values are based on euthanasia of mice as soon as illness is observed rather than waiting until mice die (LD\textsubscript{50}). HD\textsubscript{50} values have been shown not to differ significantly from LD\textsubscript{50} values (Wright & Phillpotts, 1998). Groups of six 5-day-old BALB/c mice were infected by i.p. inoculation with 50 µl of tenfold serial dilutions of the parental or mutant CDVs and observed for 14 days for clinical signs of HEV71 infection. Mice that developed limb paralysis were euthanized. The HD\textsubscript{50} value of C4-VP2I-VP1E CDV in 5-day-old BALB/c mice was determined to be \(2.1 \times 10^2\) TCID\textsubscript{50} following the method of Reed & Muench (1938) (Fig. 2b). This demonstrates that a single amino acid substitution, VP1-Q145E, is able to confer the mouse virulent phenotype upon clone-derived C4-VP2I. By contrast, C4-VP2I-VP1G CDV-infected mice survived beyond 14 days p.i., with no clinically apparent disease observed, indicating that the amino acid substitution VP1-Q145G does not confer the mouse virulent phenotype.

In order to investigate the tissue tropism of C4-VP2I-VP1E, the tissue distribution of mutant virus in infected host tissues was examined. Groups of 12 5-day-old BALB/c mice were inoculated i.p. with the maximum possible dose (\(\sim 10^4\times\) TCID\textsubscript{50}) of the virulent virus, C4-VP2I-VP1E CDV. Three infected mice showing limb paralysis (at 4 days p.i.) were sacrificed, the thoracic cavity opened to expose the heart and whole blood collected by intracardiac puncture. After perfusion with PBS, spleen, liver, heart, skeletal muscle and brain were collected and virus titres in each tissue homogenate were determined by TCID\textsubscript{50} assay on Vero cells (limit of detection=\(\log\textsubscript{10} 2.2\) TCID\textsubscript{50}). Virus infectivity was detected in the spleen, liver, heart, skeletal muscle and brain tissue (Fig. 3a). The virus was detected in high titre in skeletal muscle with a titre \(>10^5\) TCID\textsubscript{50} g\(^{-1}\), indicating that muscle and/or connective tissue is the primary site of C4-VP2I-VP1E CDV infection in mice. The lack of viraemia observed in our study may be due to the lower sensitivity of the TCID\textsubscript{50} assay compared with that of Chua et al. (2008), such that any virus present in blood is below the limit of detection (\(\log\textsubscript{10} 2.2\) TCID\textsubscript{50}) of our assay. Chua et al. (2008) also showed that viral titres in the muscle tissue of mouse-adapted virus-infected mice was \(>10^3\)-fold higher than in any other tissues examined (including viraemia). We also recovered viral genomic RNA from the skeletal muscle homogenate, which was amplified by RT-PCR using primers specific for the VP2 and VP1 genes and the amplicons sequenced. We confirmed that the VP2-149I and VP1-145E mutations persisted after passage in BALB/c mice (data not shown).

We next examined the histopathological changes occurring in the tissues of C4-VP2I-VP1E CDV-infected mice. Groups of 12 5-day-old BALB/c mice were inoculated i.p. with \(\sim 10^4\times\) TCID\textsubscript{50} of the C4-VP2I-VP1E CDV population. Brain, spinal cord and skeletal muscle tissues were
collected from three infected mice showing clinical signs of infection at 4–5 days p.i. For each tissue specimen, several sections (4–5 μm) were cut in a microtome, mounted on glass slides and stained with haematoxylin and eosin. The most notable histopathological changes observed in the infected mice were in skeletal muscle tissue sections (Fig. 3b). Neutrophil infiltration and myocyte necrosis, indicating the presence of severe myositis, was observed in all skeletal muscle sections examined. Although myositis was observed in the muscle tissue surrounding the spinal cord sections, no significant foci of inflammation were observed in the brain and spinal cord sections (cervical and lumbar) of any C4-VP2I-VP1E CDV-infected mice (data not shown). Furthermore, no histological abnormalities were detected in skeletal muscle sections of mock-infected mice (Fig. 3b). The presence of myositis in infected mice and a lack of evidence of central nervous system disease, despite the presence of virus in brain tissue in low titre, suggest that the observed limb paralysis and the cause of death was due primarily to severe inflammatory myositis.

We have shown that residue VP1-145E is a critical determinant of mouse adaptation and virulence of HEV71 in two different subgenogroups, B3 and C4. We demonstrated that the genetic changes that enable mouse adaptation of HEV71 from these two subgenogroups are identical. Mouse virulence was conferred when the amino acid residue at position VP1-145 is the polar acidic glutamic acid (E) residue. Both VP2-149 and VP1-145 are exposed on VP1 external loops, EF and DE, respectively (Chua et al., 2008). These two loops have been shown to
project beyond the virion surface in the mature HEV71 particle (Wang et al., 2012). The CHO-associated VP2 residue I149 appears to stabilize the VP1 residue E145 via an as yet undefined interaction between these two capsid proteins. It has also previously been shown that a synergistic effect of VP2-149 and VP1-145 double mutations in HEV71 enhanced viral infectivity in mouse neuroblastoma (Neuro-2a) cells in vitro and in mouse lethality in vivo (Huang et al., 2012).

Our mouse model provides an excellent and convenient model for the study of vaccine efficacy through passive protection. Wu et al. (2001) showed that immunization of pregnant mice with inactivated HEV71 protected newborn mice against lethal challenge with mouse-adapted HEV71. More recently, Bek et al. (2011) showed that the mouse-passive-protection model was useful in evaluating the protective efficacy of a formalin-inactivated HEV71 vaccine that has since completed a phase I clinical trial (Li et al., 2012), despite the fact that the main cause of death is due to skeletal muscle myositis. We propose that the mouse virulent virus generated from this study to be primarily used in the assessment of the protective efficacy of candidate HEV71 vaccines, especially by providing an additional HEV71 subgenogroup C4, with which to investigate cross-genotype protection.

The subgenogroup C4 mouse-adapted strain described in this study is the first reported mouse virulent HEV71 strain generated by reverse genetic modification of the capsid protein and without serial passage in mice. Other than its use as a passive protection model, this model will also be useful in the evaluation of cross-protective immunity conferred by candidate vaccines produced from strains belonging to different genetic lineages, an issue that has major implications for vaccine design. Even though HEV71 represents a single serotype, large HEV71 epidemics are often associated with genogroup replacement (Huang et al., 2009; Wang et al., 2002) and so concern has been raised that a vaccine based on a single viral genogroup will not provide cross-protective immunity against subsequent infection with viruses belonging to a different genogroup (Arita et al., 2007). Therefore, the development of a subgenogroup C4 mouse-adapted virus model will allow studies of vaccine-induced cross-protective immunity to be extended to include the most recent genogroup C lineage of HEV71 to evolve in Southeast Asia.

This work provides an alternative strategy for the generation of mouse virulent HEV71 without the use of animals during the adaptation process. This approach to the introduction of key mouse-adaptation mutations in the capsid proteins will have a large impact on the HEV71 mouse-adaptation protocol in the future, as mouse-virulent HEV71 virus can now be generated by reverse genetic manipulation of the viral genome in vitro, thus reducing the requirement for animal use. Furthermore, knowledge gained from this study will assist in furthering research on the virulence of this increasingly important human pathogen.

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References


