An attenuated EMCV-HB10 strain acts as a live viral vector delivering a foreign gene

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We successfully constructed a full-length cDNA infectious clone of the encephalomyocarditis virus (EMCV) HB10 strain and obtained a partially attenuated rEMCV-C9 virus with a shorter poly(C) tract. Our results showed that the length of the EMCV-HB10 poly(C) tract was related to the pathogenicity of the EMCV-HB10 strain in vivo. Using pEMCV-C9 as the backbone, we constructed the novel viral vector pC9-MCS-Δ2A by inserting a cDNA fragment containing a 127 amino acid deletion in the 2A protein, a primary cleavage cassette, a FLAG tag and a multiple cloning site (MCS) at the junction of VP1 and Δ2A. Additionally, the enhanced green fluorescent protein (egfp) gene was cloned into the MCS of pC9-MCS-Δ2A to test its capacity to express foreign proteins. Insertion of the egfp gene did not affect viral replication, and a decrease in EGFP expression was observed within five serial passages. Furthermore, we found that rC9-EGFP-Δ2A was avirulent in vivo, induced neutralizing antibody production and conferred protective immune responses against lethal challenge with EMCV in mice. Taken together, our results demonstrated that we had constructed an attenuated live vector based on an EMCV-HB10 strain with two modified critical virulence factors (the poly(C) tract and 2A protein) that could be used as a candidate live vaccine and a potential live viral vector for foreign antigen delivery.

INTRODUCTION

Encephalomyocarditis virus (EMCV) has a worldwide distribution and a wide animal host range, including rodents, pigs, elephants, non-human primates and humans (Grobler et al., 1995; Kirkland et al., 1989; LaRue et al., 2003; Verlinde & Van Tongeren, 1953). EMCV is a positive-sense, single-stranded, non-enveloped RNA virus that belongs to the genus Cardiovirus, family Picornaviridae. The EMCV genome is approximately 7.8 kb in length and is flanked by two untranslated regions (UTRs). The 5' UTR contains a poly(C) tract and an internal ribosome entry site (IRES), and the 3' UTR terminates with a poly(A) tail (Buenz & Howe, 2006; Cui et al., 1993). The picornavirus genome is translated into a long polypeptide chain, the break in the polypeptide chain at the 2A/2B junction through ribosome skipping resulting in two separate polyproteins (Donnelly et al., 2001). Subsequently, EMCV 3C cleaves the two polyproteins, which yields at least 12 mature proteins: four structural capsid proteins (VP4, VP2, VP3 and VP1) and seven mature proteins, (2A, 2B, 2C, 3A, 3B (VPg), 3C and 3D) (Palmenberg et al., 1984).

Previous studies showed that EMCV infection induces encephalitis, myocarditis, neurovirulence, paralysis and type I diabetes in mice (Cerutis et al., 1989; Psalla et al., 2006; Takeda et al., 1991; Yoon & Jun, 2006). In past decades, several EMCV virulence-related factors were determined and different strategies were adopted to obtain safe and effective live attenuated vaccines. For example, using Mengovirus as a model, researchers found that the length of the poly(C) tract in the 5' UTR determined viral pathogenicity because diminution of the length of the poly(C) tract markedly attenuated viral pathogenicity in mice (Duke et al., 1990; Hahn & Palmenberg, 1995). Additionally, the EMCV 2A protein plays important roles in host protein translation shut-off, apoptosis inhibition and viral pathogenicity (Carocci & Bakkali-Kassimi, 2012). Recently, EMCV 2A was found to be another virulence factor because deletion of the 2A protein by repeated passage of the EMCV virus in vitro also attenuated viral pathogenicity (Carocci et al., 2011).

With the development of infectious clone technology, safer and more effective live attenuated virus vaccines can be obtained as vectors to express foreign antigens (Altmeyer et al., 2011).
et al., 1994; Aubry et al., 2015; Balasuriya et al., 2014). Several factors affect the stability of engineered recombinant viral vectors, including the region of insertion in the viral genome and the size of the inserted cDNA sequence. Previous studies showed that cardioviruses could be used as potential live viral vectors (Altmeyer et al., 1994; Mueller & Wimmer, 1998; Van der Ryst et al., 1998). Foreign cDNA sequences of various length have been inserted into the L-coding region (Altmeyer et al., 1994, 1995) or the junction of the 2A and 2B sequences (Binder et al., 2003). These studies showed that inserted cDNAs in the viral genome were stable when the lengths of the inserted foreign cDNAs were under 500–600 base pairs (bp); larger foreign cDNAs (over 600 bp) inserted into the viral genome were usually unstable when these recombinant viruses were passaged five times in permissive cells (Altmeyer et al., 1994, 1995; Binder et al., 2003).

In this study, we obtained a highly attenuated virus rC9-EGFP-D2A through the artificial deletion of two virulence factors (the poly(C) tract and the 2A gene). The attenuated virus induced neutralizing antibodies and provided protective immune responses against lethal challenge with the EMCV-HB10 strain in mice. Moreover, we found that this live avirulent virus could be used as a candidate viral vector to express EGFP when the foreign gene was inserted at the junction of the VP1 and D2A genes, suggesting its promising application for the delivery of heterologous antigens.

RESULTS

Construction of a full-length infectious EMCV-HB10 cDNA clone

To generate a full-length infectious EMCV-HB10 strain cDNA clone (GenBank accession: JQ864080.1), the 7742-nucleotide-long EMCV-HB10 genome was divided into three overlapping fragments named F1, F2 and F3 (Fig. 1a). F1 was modified with a T7 promoter upstream of the viral 5′ UTR and F3 was modified with a BamH I run-off site downstream of the viral 3′ UTR, resulting in run-off transcription of the authentic genome-length RNA in vitro (Fig. 1b). The fusion PCR product F1-F3 was cloned into the pOK12 vector and named pOK12-F1-F3. Then, the F2 fragment was inserted into pOK12-F1-F3 at two natural restriction sites (Hind III and Afe I) to construct a full-length EMCV-HB10 cDNA designated pEMCV-HB10 (Fig. 1c). Sequence analysis showed that the nucleotide sequence of the cDNA clone (pEMCV-HB10) was identical to the sequence of its parental virus (EMCV-HB10), with the exception of the poly(C) tract. pEMCV-HB10 contained a shorter poly(C) tract (C9) than the poly(C) tract (C7TCTC10) of the EMCV-HB10 genome. Therefore, the pEMCV-HB10 plasmid was designated pEMCV-C9. Then, we successfully rescued the recombinant virus and designated it rEMCV-C9.

Fig. 1. Strategy utilized to construct a full-length infectious cDNA clone of the EMCV-HB10 strain. (a) Structure of the EMCV genomic RNA. (b) Diagram of the three overlapping EMCV-HB10 fragments. The T7 promoter and an artificial run-off site are shown. (c) Schematic diagram of the key steps in the construction of the EMCV-HB10 cDNA clone.
Characterization of the rescued rEMCV-C9 virus in vitro and in vivo

To assess the biological properties of rEMCV-C9, BHK-21 cells were infected with rEMCV-C9 or EMCV-HB10 and then probed with an anti-VP1 monoclonal antibody (mAb). VP1 expression was detectable by immunofluorescence assay (IFA) in both rEMCV-C9- and EMCV-HB10-infected cells (Fig. 2a), suggesting that the rescued rEMCV-C9 could produce progeny viruses. Additionally, viral RNA was extracted from BHK-21 cells infected with either rEMCV-C9 or EMCV-HB10 and RT-PCR (Reverse transcription polymerase chain reaction) was performed to verify the presence of the progeny viruses. The sequence analysis results revealed that the 5′ UTR of rEMCV-C9 contained a C9 poly(C) tract that was different from the 5′ UTR of EMCV-HB10, suggesting that the C9 poly(C) tract could serve as a genetic marker to distinguish the rescued rEMCV-C9 from its parental virus EMCV-HB10 (Fig. 2b). The Western blotting results demonstrated that VP1 expression levels were similar in BHK-21 cells infected with either rEMCV-C9 or its parental virus EMCV-HB10 (Fig. 2c). To assess the growth properties of rEMCV-C9 and EMCV-HB10, the replication efficiencies of the two viruses were tested. We found that rEMCV-C9 replicated as efficiently as EMCV-HB10 in BHK-21 cells (Fig. 2d) and that the plaque morphologies of the two viruses had no marked differences (Fig. 2e).

To investigate whether the length of the poly(C) tract was related to viral pathogenicity in vivo, mice were divided into

Fig. 2. Biological properties of rEMCV-C9 in vitro and in vivo. (a) Immunofluorescence assay (IFA) analysis of BHK-21 cells either mock infected or infected with EMCV-HB10 or rEMCV-C9 at a multiplicity of infection (MOI) of 1 for 12 h. The cells were stained with the anti-VP1 mAb and observed by confocal microscopy. Scale bar: 200 µm. (b) Sequence chromatograms of the poly(C) tracts in the 5′ UTRs of EMCV-HB10 and rEMCV-C9. (c) Western blotting analysis of the whole-cell lysates of BHK-21 cells either mock infected or infected with EMCV-HB10 or rEMCV-C9. The membrane was probed with an anti-VP1 mAb and anti-GAPDH antibody. (d) One-step growth curves of rEMCV-C9 and EMCV-HB10 in BHK-21 cells. BHK-21 cells were infected with rEMCV-C9 or EMCV-HB10 at an MOI of 0.01. At the indicated time points, BHK-21 cell supernatants were harvested to determine the progeny viral titres with the TCID50 assay. (e) Plaque morphology of BHK-21 cells infected with EMCV-HB10 or rEMCV-C9. (f) Survival rates of BALB/c mice infected with different viral doses of EMCV-HB10 or rEMCV-C9.
12 groups and intraperitoneally (i.p.) infected with serial dilutions of rEMCV-C9 (2 × 10^6–2 × 10^2 tissue culture infective doses (TCID_{50}) per mouse), EMCV-HB10 (2 × 10^6–2 × 10^1 TCID_{50} per mouse) or DMEM as a control. We found that mice infected with the parental EMCV-HB10 began to die as early as 3 days post-infection (dpi) and showed hind limb paralysis, which is a typical nervous system symptom. In contrast, mice infected with rEMCV-C9 showed delayed onset of clinical signs. All of the control group mice survived without clinical signs (Fig. 2f). The statistical analysis revealed that the median lethal dose (LD_{50}) of EMCV-HB10 was 2 × 10^{1.67} TCID_{50}, whereas the LD_{50} of rEMCV-C9 was 2 × 10^4 TCID_{50}, which was 213-fold higher than the LD_{50} of EMCV-HB10 (Table 1). Taken together, these results demonstrated that the length of the poly(C) tract in the 5' UTR of EMCV-HB10 did not affect viral replication capability in vitro, but was related to viral pathogenicity in vivo.

### Development of a live recombinant viral vector rC9-MCS-Δ2A

Although the mice infected with rEMCV-C9 still died (Fig. 2f), the lethal dose was much higher and death occurred later; these results indicated that rEMCV-C9 was still virulent. To obtain a safer EMCV-HB10 clone with lower virulence, we adopted a traditional strategy to create an attenuated virus by serial passage in BHK-21 cells. Viral clone C25 was isolated from the 190th passage of EMCV-HB10 by plaque assay. Sequence analysis revealed that C25 had 12 amino acid (aa) replacements and a large deletion of 127 aa in the 2A protein compared with its parental virus EMCV-HB10 (Table 2 and Fig. 3a); thus, C25 was designated EMCV-Δ2A. Previously, the EMCV 2A protein was reported to be a critical virulence factor that played an important role in viral pathogenesis (Carocci et al., 2011). Because the length of the poly(C) tract was related to viral pathogenicity, we speculated that deletion of both the poly(C) tract and 2A protein might be a useful approach to developing a novel, live attenuated virus vector. To obtain a candidate vaccine strain that simultaneously contained a shorter poly(C) tract and a partial deletion of the 2A gene, we amplified the F2 fragment of the EMCV-Δ2A genome to replace the counterpart of plasmid pEMCV-C9. The resultant intermediate plasmid was designated pC9-Δ2A. To test the ability of the plasmid to express heterogeneous antigens, pC9-Δ2A was used as a template. A cDNA fragment containing a duplicated primary cleavage cassette (PCC), a FLAG tag and a multiple cloning site (MCS) was inserted at the junction of the VP1- and Δ2A-encoding sequences. The duplicated artificial PCC was used to ensure normal viral protein packaging and autocatalytic scission of the heterologous protein from the polyprotein (Fig. 3b). The constructed vector was designated pC9-MCS-Δ2A. The recombinant virus was successfully rescued and designated rC9-MCS-Δ2A.

### Table 1. Comparison of the LD_{50} of EMCV-HB10, rEMCV-C9, rC9-MCS-Δ2A and rC9-EGFP-Δ2A in mice

LD_{50} values (in TCID_{50}) were calculated by the Reed–Muench method.

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<th>Death rate (%)</th>
<th>LD_{50} (TCID_{50})</th>
<th>Ratio of LD_{50} EMCV-HB10</th>
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To investigate the pathogenic properties of rC9-MCS-Δ2A in vivo, mice were i.p. infected with rC9-MCS-Δ2A. All mice infected with rC9-MCS-Δ2A at a maximum dose of 2 × 10^6 TCID_{50} per mouse survived without any clinical signs (Table 1), indicating that rC9-MCS-Δ2A is avirulent in mice. To test whether the virus had the ability to express foreign proteins, egfp gene cDNA with two unique restriction enzyme cleavage sites (Aff II and Sal I) was directly cloned into pC9-MCS-Δ2A. Subsequently, we successfully rescued the recombinant virus (named rC9-EGFP-Δ2A). RT-PCR and cDNA sequencing analyses were performed to verify the insertion of the egfp gene. The results indicated that the cDNAs obtained from rC9-MCS-Δ2A and rC9-EGFP-Δ2A presented a difference of 717 bp, corresponding to the
insertion of the egfp gene (Fig. 4a); the sequencing results also supported the insertion (data not shown). To confirm whether the deletion of the 2A gene and the insertion of the egfp gene affected viral replication efficiency, the growth properties of rEMCV-C9, rC9-MCS-Δ2A and rC9-EGFP-Δ2A were assessed. As shown in Fig. 4b, the rC9-MCS-Δ2A and rC9-EGFP-Δ2A viral titres were significantly lower than the rEMCV-C9 titre during the early phase of replication and their growth curves reached a plateau at a similar level 18 h later than rEMCV-C9, suggesting that the viral release of rC9-MCS-Δ2A and rC9-EGFP-Δ2A was substantially delayed in vitro. Notably, the rC9-MCS-Δ2A and rC9-EGFP-Δ2A viral titres exhibited no significant differences, suggesting that the inserted egfp gene did not affect viral replication. These results indicated that the deletion of the 2A gene significantly delayed the release of the EMCV virions and that the insertion of the egfp gene did not affect overall replication efficiency.

To test EGFP expression, rC9-EGFP-Δ2A was serially passaged five times (P1-P5) in BHK-21 cells and the green fluorescence was directly observed. As shown in Fig. S1a (available in the online Supplementary Material), the presence of green fluorescence was directly observed in BHK-21 cells infected with all the different rC9-EGFP-Δ2A passages by confocal microscopy, although the EGFP signal gradually decayed. Next, we tested the stability of the egfp gene in the EMCV genome during different passages. The plaque assay results showed that the percentages of GFP-fluorescent plaques decreased gradually from 97.43 to 33.19% in a passage-dependent manner (Fig. S1b). At present, the mechanism involved in the stability of the egfp gene in the EMCV genome remains unclear.

Western blotting analysis demonstrated that EGFP expression was detectable within five passages, but gradually decreased in a passage-dependent manner. Additionally, we found that two forms of EGFP [free EGFP and VP1-fused EGFP (VP1-FLAG-EGFP)] were simultaneously expressed in the rC9-EGFP-Δ2A-infected BHK-21 cells. Consistent with these findings, rEMCV-C9 only expressed VP1, whereas rC9-MCS-Δ2A expressed VP1 and VP1-FLAG (Fig. 4c). These results suggested that the autocatalytic scission efficiencies of the two PCCs might differ. The first artificial primary cleavage cassette (PCC, 16 aa) was partially cleaved, whereas the natural PCC was completely cleaved when the rC9-EGFP-Δ2A genome was translated (Fig. 3b). Because we detected the expression of a fusion form of EGFP, we investigated the localization of the fusion products and assessed whether VP1-EGFP could be assembled into EMCV viral particles. First, BHK-21 cells were infected with rC9-MCS-Δ2A or rC9-EGFP-Δ2A. Then, the VP1 expression levels in the infected cells were probed with the anti-VP1 mAb and observed under a confocal microscope. EGFP was primarily distributed in the cytoplasm of rC9-EGFP-Δ2A-infected BHK-21 cells and co-localized with VP1 (Fig. 4d). Furthermore, we

Table 2. Comparison of the genome sequences of EMCV-HB10 and EMCV-Δ2A

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isolated and purified virions from the culture supernatants of BHK-21 cells infected with rEMCV-CP, rCP-MCS-Δ2A and rCP-EGFP-Δ2A and then performed Western blotting analysis. As shown in Fig. 4e, VP1-FLAG-EGFP was not detected in any of the different EMCV virions, whereas VP1-FLAG was detected in the rCP-MCS-Δ2A virions. Taken together, our findings reveal that VP1-fused small peptides such as FLAG, but VP1-EGFP, cannot be assembled into EMCV virions and that VP1-FLAG-EGFP is primarily localized in the cytoplasm of rCP-EGFP-Δ2A-infected cells.

rCP-EGFP-Δ2A is an avirulent candidate vaccine that can provide protective immune responses

We investigated the pathogenic properties of rCP-EGFP-Δ2A. Mice were i.p. infected with EMCV-HB10 or rCP-EGFP-Δ2A at a dose of $2 \times 10^5$ TCID$_{50}$. The mice infected with EMCV-HB10 showed typical signs of hind limb paralysis and died at 3–4 dpi, whereas those infected with rCP-EGFP-Δ2A all survived without any clinical signs up to 14 dpi (Fig. 5a). Even when those infected dose was increased up to $2 \times 10^6$ TCID$_{50}$, rCP-EGFP-Δ2A was still avirulent in the infected mice (Table 1). The LD$_{50}$ of rCP-EGFP-Δ2A was greater than $2 \times 10^6$ TCID$_{50}$ and was increased by more than $2 \times 10^4$-fold compared to EMCV-HB10 (Table 1). Next, quantitative real-time PCR (qRT-PCR) was performed to test whether EMCV replication occurred in the brains and hearts of EMCV-HB10- or rCP-EGFP-Δ2A-infected mice. We detected EMCV genomic RNA in the hearts and brains of the mice infected with either the wild-type EMCV-HB10 or rCP-EGFP-Δ2A virus. However, the viral RNA levels in the brains of the rCP-EGFP-Δ2A-infected mice were lower than those in the EMCV-HB10-infected mice (Fig. 5b), suggesting that the replication efficiency of rCP-EGFP-Δ2A is lower in the primary target tissues.

We evaluated the immune protection of rCP-EGFP-Δ2A against lethal challenge with the EMCV-HB10 strain. First, BALB/c mice were inoculated i.p. with a single dose of $2 \times 10^6$ TCID$_{50}$ rCP-EGFP-Δ2A to test the neutralizing antibody levels. We found that mice immunized once with rCP-EGFP-Δ2A produced detectable neutralizing antibodies on day 14 post-vaccination and that the neutralizing antibody levels were higher on day 21. In contrast, the mice immunized with DMEM produced no detectable neutralizing antibodies (Fig. 5c). Next, we challenged the rCP-EGFP-Δ2A-vaccinated mice with $2 \times 10^6$ TCID$_{50}$ of EMCV-HB10 to test the immune protection of rCP-EGFP-Δ2A. As shown in Fig. 5d, 60% of the mice vaccinated with rCP-EGFP-Δ2A survived whereas all mice vaccinated with DMEM died within 4–8 days following challenge with EMCV-HB10. These results indicated that the recombinant attenuated rCP-EGFP-Δ2A provided efficacious protection against EMCV-HB10 in the challenged mice. Interestingly, the sera obtained from the

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**Fig. 3.** Schematic representation of the strategies used in the development of a live vector. (a) Schematic diagrams of the wild-type and deletion mutant EMCV-Δ2A protein. (b) A detailed map of the construction of the recombinant plasmid pCP-MCS-Δ2A from pCP-Δ2A. A polylinker was inserted into the intermediate plasmid pCP-Δ2A, in which the F2 fragment was replaced with F2’ from EMCV-Δ2A. The polylinker contained a duplicated autoproteolytic primary cleavage cassette (PCC, 16 aa), a FLAG tag (27 bp, 9 aa) and a MCS (All II and Sali I, 15 bp, 5 aa). The Gly-Pro residues are autoprotolyic scissio sites.
mice vaccinated with rC6-EGFP-Δ2A recognized exogenously expressed EGFP in the Western blotting analysis and the produced band corresponded to the band recognized by the anti-EGFP mAb (Fig. 5e). Taken together, these results indicated that rC6-EGFP-Δ2A not only induced detectable neutralizing antibodies and conferred partial protection against lethal challenge with EMCV-HB10, but could also be used as a live viral vector to express foreign antigens.

DISCUSSION

The emergence of EMCV infection poses serious potential threats to the health of animals and humans, especially in Chinese swine farms (Zhang et al., 2007b). However, no live attenuated vaccine is available. Recently, the EMCV–HB10 strain was isolated from the organs of dead piglets with acute myocarditis in northern China (Bai et al., 2012; Feng et al., 2015; Lin et al., 2012; Yuan et al., 2014). To date, the virulence and pathogenicity of this virus and the capacity of its genome to tolerate heterologous genes remain unclear. In this study, we successfully constructed a full-length infectious cDNA clone of the EMCV-HB10 strain, which provided us with a useful platform to investigate the biological characteristics of this newly isolated EMCV strain. Then, we developed a highly attenuated live vector by engineering its poly(C) tract and 2A gene. Our findings indicate that the engineered candidate vaccine strain can induce neutralizing antibody production in immunized mice and offer partial protection against lethal challenge with EMCV-HB10 in vivo. Notably, rC6-EGFP-Δ2A could express foreign proteins such as EGFP,

![Fig. 4. rC6-MCS-Δ2A is a potential live virus for foreign gene delivery.](image)

(a) Validation of the egfp gene insertion in the virus vector. The viral RNA isolated from rEMCV-MCS-Δ2A (lane 2) or rC6-EGFP-Δ2A (lane 3) was used as a template to perform RT-PCR with specific primers flanking the MCS sequence. The PCR products were run on 2% agarose gel. (b) BHK-21 cells were infected with rEMCV-C6, rC6-MCS-Δ2A or rC6-EGFP-Δ2A at an MOI of 0.01. At the indicated time points, the cell supernatants were harvested to determine progeny viral titres with the TCID50 assay. (c) BHK-21 cells infected with rC6-MCS-Δ2A, rC6-EGFP-Δ2A or the different passages (from P0 to P5) of rC6-EGFP-Δ2A were harvested at 24 hours post-infection (hpi), and the cell lysates were analysed by Western blotting with the anti-FLAG or anti-VP1 monoclonal antibody. rEMCV-C6 and rC6-MCS-Δ2A-infected cell lysates were used as the controls. (d) BHK-21 cells were either mock infected or infected with rC6-MCS-Δ2A or rC6-EGFP-Δ2A. At 12 hpi, the cells were fixed with the anti-VP1 mAb and observed by confocal microscopy. EGFP expression was directly observed. (e) The rEMCV-C6, rC6-MCS-Δ2A and rC6-EGFP-Δ2A virions were purified from the virus-infected cell supernatants and analysed by Western blotting with the antibodies indicated.
suggesting its possible use as a potential tool to develop bivalent vaccines expressing other viral antigens.

All EMCV strains isolated from pigs contain a poly(C) tract in their 5' UTRs (Feng et al., 2015; Zhang et al., 2007a). An analysis of the EMCV-HB10 genomic sequence revealed that the virus had a poly(C) tract (C_5TCTC_3TC_10) in its 5' UTR. In this study, we rescued a rEMCV-C9 virus that contained a shorter C9 poly(C) tract. We found that rEMCV-C9 replicated as efficiently as its parental virus and exhibited no significant differences in growth kinetics and plaque morphology in BHK-21 cells. However, the statistical analysis revealed that rEMCV-C9 had lower pathogenicity in mice. These data demonstrated that the length of the EMCV-HB10 poly(C) tract played a significant restrictive role in viral pathogenicity in mice, but not in viral replication and infectivity in BHK-21 cells.

Modification of non-coding sequences and deletion of the viral protein-encoding sequences of the viral genome have been successfully applied to generate safer and more effective viral vaccines. A previous study reported that an attenuated EMCV B279/95 strain with a 115 aa deletion in the 2A protein isolated by serial passage showed decreased virus production and a defect in viral particle release compared to its parental virus (Carocci et al., 2011). Our data showed that rC9-MCS-Δ2A, which contained a much longer deletion in the 2A protein (up to 127 aa) and a shorter poly(C) tract, could be rescued. Interestingly, the replication efficiency of rC9-MCS-Δ2A was significantly lower during the early phase of the viral growth curve and reached the plateau later. Our study showed that the 2A deletion decreased the speed of viral particle release, indicating an important role for the 2A protein in the viral release process. We also demonstrated that rC9-MCS-Δ2A was avirulent in mice. Combined with the result shown in Fig. 2f, our results suggested that the length of the poly(C) tract and the 2A protein co-contributed to the attenuation of rC9-MCS-Δ2A.

Several features of Picornaviruses enable them to serve as excellent candidate vaccine vectors (Andino et al., 1994; Miller et al., 2009; Seago et al., 2013). For example, it is easy to obtain several attenuated viral clones of some Picornaviruses by serial passage in permissive cells in vitro and to construct infectious cDNA clones due to their relatively small genome sizes. Previous studies revealed that heterologous cDNAs could be inserted either at the 5' UTR

Fig. 5. Identification of the attenuated recombinant virus rC9-EGFP-Δ2A and its protective efficacy against EMCV-HB10 challenge in vivo. (a) Mortality of BALB/c mice intraperitoneally infected with 2×10⁶ TCID₅₀/mouse of the virulent EMCV-HB10 or rEMCV-C9 virus or the attenuated and recombinant rC9-MCS-Δ2A or rC9-EGFP-Δ2A virus. The survival rates were calculated during the 14-day follow-up period. (b) Real-time RT-PCR was performed to test the viral genomic RNA levels in the brains and hearts of the EMCV-HB10- or rC9-EGFP-Δ2A-infected mice. (c) BALB/c mice were intramuscularly immunized with rC9-EGFP-Δ2A, and the mean titres of neutralizing antibodies against EMCV in the sera from the rC9-EGFP-Δ2A-vaccinated mice were detected at 14 and 21 days post-vaccination (n=6). Error bars indicate the standard deviations. (d) BALB/c mice were intramuscularly immunized with DMEM or rC9-EGFP-Δ2A. The vaccinated mice were intraperitoneally challenged with EMCV-HB10 (2×10⁶ TCID₅₀) at 21 days post-vaccination and the survival rates were recorded daily for 14 days. (e) HEK 293T cells were transfected with the pEGFP-N1 vector. EGFP expression was detected by Western blotting using an anti-EGFP antibody or the pooled serum from mice immunized with rC9-EGFP-Δ2A.

http://jgv.microbiologyresearch.org 2287
or at the junction between the 2A and 2B sequences (Altmeyer et al., 1994; Mattion et al., 1994; Zeng et al., 2013). The maximum size of the heterologous cDNA that could be inserted at the junction of the 2A and 2B sequences of the mengovirus genome was 500–600 bp (Binder et al., 2003). In this study, we successfully constructed and rescued a newly engineered recombinant virus rC9-MCS-Δ2A, which may have the capacity to accept much longer heterologous cDNA sequences. The egfp gene was chosen as the first gene to test the capacity of rC9-MCS-Δ2A for foreign gene expression. Our results showed that insertion of the egfp gene and EGFP expression did not affect the viral growth kinetics (Fig. 4b). The inserted egfp gene was present and its expression was maintained for at least five passages, although EGFP expression showed a gradual decline. The actual mechanism underlying the reduced expression of heterologous genes needs to be investigated. Our findings also revealed that rC9-EGFP-Δ2A expressed two forms of EGFP (VP1-fused EGFP and free EGFP) (Fig. 5d). We proposed that VP1-EGFP might affect the normal assembly of the capsid protein due to its large size; therefore, VP1-fused EGFP was primarily expressed in the cytoplasm and was not assembled into the virions as observed with VP1-FLAG. Moreover, the animal experiments indicated that rC9-EGFP-Δ2A was an avirulent virus in mice; this virus induced neutralizing antibody production in mice and offered partial protection in immunized mice against fatal challenge with virulent EMCV-HB10. Because rC9-EGFP-Δ2A expressed EGFP, infection of mice with this virus induced the production of antibodies specific for heterologous EGFP (Fig. 5e), which provided excellent evidence that the engineered virus could be used as a vector for a bivalent or mixed vaccine. Overall, our results demonstrated that the engineered avirulent virus rC9-MCS-Δ2A could serve as a candidate live attenuated vaccine vector for heterogeneous protein expression.

METHODS

**Cells, viruses and antibodies.** BHK-21 and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO₂. The EMCV-HB10 strain (GenBank accession: JQ864080.1) was propagated in BHK-21 cells. The mouse anti-VP1 mAb was prepared by immunizing mice with a recombinant EMCV VP1 protein. The mouse anti-FLAG mAb, anti-GAPDH polyclonal antibody and the horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). The fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (H+L) and tetramethyl rhodamine isocyanate (TRITC)-conjugated goat anti-mouse antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Construction of the infectious cDNA plasmids.** In this study, four recombinant full-length cDNA plasmids were constructed (pEMCV-C9, pEMCV-HB10, pC9-Δ2A, pC9-MCS-Δ2A and pC9-EGFP-Δ2A). The schematic diagrams are shown in Figs. 1c, 3b. Briefly, the infectious cDNA plasmid pEMCV-C9 was constructed following the steps indicated in Fig. 1c. To construct plasmid pC9-MCS-Δ2A, first we constructed an intermediate plasmid pC9-Δ2A. The F2’ fragment was amplified by PCR from EMCV-A2A using Phusion High-Fidelity DNA Polymerase (NEB, Ipswich, MA, USA) according to the manufacturer’s instructions. The purified fragment was digested with Hind III and Age I and then replaced with the corresponding coding sequences of pEMCV-C9 resulting in plasmid pC9-Δ2A. pC9-MCS-Δ2A was constructed as indicated in Fig. 3(b). Briefly, this plasmid was constructed by overlap PCR amplification from two overlapping fragments (S1 and S2) of pC9-Δ2A. Primers S1-F and S1-R were used to generate fragment S1 with an upstream Adh I site, and primers S2-F and S2-R were used to generate fragment S2 with a downstream Bash I site (Fig. 3b). Overlap extension PCR was performed using the purified S1 and S2 fragments. The PCR products were digested with Adh I and Bash I and cloned into pC9-Δ2A to generate pC9-MCS-Δ2A (Fig. 3b). The egfp gene (GenBank accession no. U55762.1) was amplified from the pEGFP-N1 (Invitrogen, Grand Island, NY, USA) vector and then cloned into the Aff II and Sfl I restriction sites of pC9-MCS-Δ2A, resulting in the plasmid pC9-EGFP-Δ2A. The primers used for the construction of these plasmids are available upon request. All of the constructs were validated by DNA sequencing.

**Production of progeny viruses.** The plasmids pEMCV-C9, pC9-MCS-Δ2A and pC9-EGFP-Δ2A were linearized with BamHI I. The resultant linear plasmids were purified and transcribed in vitro using the Ribomax™ Large Scale RNA Production System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The RNA transcripts were purified and transfected into BHK-21 cells, resulting in the corresponding rescued viruses rEMCV-C9, rC9-MCS-Δ2A and rC9-EGFP-Δ2A 48 hpt (Hours Post-Transfection). When more than 80% of the BHK-21 cells showed cytopathic effects (CPEs), the cell supernatants were collected and titrated in BHK-21 cells using the TCID50 assay.

**One-step growth curve and plaque assay.** To construct the growth curves of EMCV-HB10, rEMCV-C9, rC9-MCS-Δ2A and rC9-EGFP-Δ2A, BHK-21 cells were infected with each of these viruses at a multiplicity of infection (MOI) of 0.01. A one-step growth curve was performed as described in a previous report (Li et al., 2014). For the plaque assay, BHK-21 cells were infected with either EMCV-HB10, rEMCV-C9 or rC9-EGFP-Δ2A at an MOI of 0.001. After incubating for 1 h at 37 °C, the unbound viruses were removed. The BHK-21 cells were washed with 1× PBS and then overlaid with a 2% agarose gel (DMEM supplemented with 2% low-melting point agarose and 2% FBS) and incubated at 37 °C. Visible plaques were imaged at 4 dpi.

**Western blotting analysis and confocal microscopy.** Western blotting analysis was performed as previously described (Huang et al., 2015). For confocal microscopy, BHK-21 cells were infected with EMCV-HB10, rEMCV-C9, rC9-MCS-Δ2A or rC9-EGFP-Δ2A at an MOI of 0.1. At 24 h post-infection (hpi), the IFA was performed as previously described (Huang et al., 2015) and visualized using a Leica SP2 confocal system (Leica Microsystems, Mannheim, Germany).

**Purification of virions.** The supernatants of BHK-21 cells infected with rEMCV-C9, rC9-MCS-Δ2A or rC9-EGFP-Δ2A at an MOI of 0.1 were harvested and clarified by centrifugation at 10 000 × g for 30 min. The clarified virulent supernatants were concentrated and purified at 40 000 rpm. through a 5.5 mL 20% (w/v) sucrose cushion in TNE buffer (50 mM Tris, 100 mM NaCl and 1 mM EDTA, pH 7.4) for 3 h in a 70 Ti rotor (Optima™ L-100XP Preparative Ultracentrifuge, Beckman Coulter, Inc, CA, USA) at 4 °C. The virus pellets were suspended in TNE buffer, loaded on top of a preformed non-linear Optiprep™ density gradient (20, 25, 35 and 45% w/v) in TNE buffer, and subsequently centrifuged at 230 000 × g (SW 41 Ti rotor, Beckman Coulter, Inc, CA, USA) at 4 °C for 5 h. The bands in the TNE buffer corresponding to EMCV were collected and dissolved in 1 mL of TNE buffer.
Mice and inoculation. The animal trials in this study were performed according to the Chinese Regulations of Laboratory Animals and the Guidelines for the Care of Laboratory Animals. BALB/c mice were obtained from Vital River (Beijing, China) and housed in individual ventilated cages inside a biosafety level-2 (BSL-2) laboratory. The license number associated with the research protocol is SY20140203, which was approved by the Laboratory Animal Ethical Committee of Harbin Veterinary Research Institute (LAECHVRI). Five-week-old female BALB/c mice were grouped (n=6) and then infected with serial dilutions of EMCV-HB10 or rEMCV-Cα via the intraperitoneal route. To evaluate the clinical signs and survival rates caused by infection with the genetically engineered virus, the mice were grouped (n=6) and then inoculated with 2×10^5 and 2×10^6 TCID₅₀ per mouse of either rCα-MCS-Δ2A or rCα-EGFP-Δ2A. Mice inoculated with 0.2 ml of DMEM were used as a negative control. All mice were monitored for 14 days for clinical signs, and the survival rates were recorded daily. For vaccination of animals and EMCV challenge, BALB/c mice (six per group) were immunized conventionally with 2×10^6 TCID₅₀ of EMCV-HB10 and then monitored for the appearance of EMCV clinical symptoms for 2 weeks.

Quantitative real-time RT-PCR. Tissue samples were crushed and total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed using Primerscript™ RT Master Mix (Takara Bio Inc., Japan). The products were detected using an Agilent-Stratagene Mx Real-Time qPCR system according to the SYBR Premix DimerEraser™ (Takara Bio Inc., Japan). For quantification, standard curves were generated as previously described (Carocci et al. 2011). Each sample was run in triplicate. Primers and the TaqMan probe used for EMCV detection were located in the conserved sequence of EMCV 1B. The primers used were EMCV-1B-F 5’-GAACCCGACATTCAAGAGAGA-3’ and EMCV-1B-R 5’-CTCAGATTGGAGGGATG-3’. The TaqMan probe used was EMCV-1B-P 5’-(FAM) ACAAGGTCCACTGCCAGAAGT (Eclipse) -3'M. Detection of neutralizing antibodies with the microneutralization test. The sera collected from mock- and rCα-EGFP-Δ2A-immunized mice were assayed for EMCV-HB10 neutralizing antibody titres in BHK-21 cells. All serum samples were inactivated at 56°C for 30 min, twofold serially diluted and then incubated with a viral dose of 200 TCID₅₀ of EMCV-HB10 at 37°C for 60 min. A 96-well tissue culture plate with BHK-21 cells was subsequently inoculated with the mixture and incubated for 96 h. The antibody titres were expressed as the highest dilutions of serum that neutralized 200 TCID₅₀ of EMCV-HB10 in 50% of the wells.

Statistical analysis. Values were expressed as the means±SD. The data were analysed using Student’s t-test in Excel. A P-value of <0.05 was considered significant.

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