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Short communication

Establishment of a rescue system for an autonomous Parvovirus mink enteritis virus

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ABSTRACT

Construction and characterization of a full-length infectious clone (pMEV) of mink enteritis virus are described. Feline kidney cells (F81) were transfected with pMEV containing an engineered *BamH*I site that served as a genetic marker. The rescued virus was indistinguishable from its parental virus. The availability of a MEV infectious clone will facilitate studies of viral replication and pathogenicity and will permit the elucidation of determinants of the host range of the parvovirus.

Mink enteritis virus (MEV), of the genus Parvovirus within the family Parvoviridae, is an autonomous, non-enveloped virus that contains a linear negative single-stranded DNA genome of about 5 kb (Uttenthal et al., 1990). The 3′ and 5′ end of the genome respectively contain a 205 nt and a 62 nt palindromic sequence in Y- and U-shaped configurations. The genome contains two major open reading frames, ORF-L and ORF-R, which express the nonstructural (NS) proteins (NS1 and NS2) and capsid proteins (VP1 and VP2), respectively (Kariatsumari and Horiuchi, 1991). VP2 is the major capsid and antigenic protein which determines host range and pathogenity of MEV (Hueffer and Parrish, 2003). Infection with MEV causes intestinal enteritis, myocarditis and lymphopenia in mink, especially in neonates and young mink.

Within the genus Parvovirus, there are several closely related viruses including feline panleukopenia virus (FPV), mink enteritis virus (MEV) and canine parvovirus (CPV) (Hueffer and Parrish, 2003; Steinel et al., 2000). Although these viruses are highly homologous in capsid protein sequences (>98% homology) and share some antigenic features, they are distinguishable from each other antigenically when tested with monoclonal antibodies (Carlson et al., 1985; Mattyn et al., 1990; Parrish and Carmichael, 1983; Parrish et al., 1988; Parrish, 1994; Reed et al., 1988; Rhode, 1985; Tratschin et al., 1982), and show different host-cell specificities and rates of mutation. FPV replicates in feline cells *in vitro* and in cats and mink *in vivo* whereas CPV replicates in canine and feline cells *in vitro*, but does not infect mink *in vivo*. MEV replicates in feline cells *in vitro*, but does not infect cats and dogs *in vivo* (Truyen and Parrish, 1992). The evolutionary rate of FPV varies slowly by random genetic drift, whereas CPV shows genomic substitution rates similar to those of RNA viruses (about 10^{-4} substitutions per site per year under selective pressure) (Hoelzer et al., 2008; Shackelton et al., 2005). Several variants of CPV have been identified. Soon after the appearance of CPV-2, new antigenic types, CPV-2a and CPV-2b, emerged and replaced the original CPV-2 worldwide (Steinel et al., 1998). These new types differ from the original CPV-2 by their extended host range, now including dogs and cats (Mochizuki et al., 1996). Recently, some research groups have shown evidence of recombination between different CPV antigenic types, CPV and FPV and CPV and MEV under certain conditions (Mochizuki et al., 2008; Ohshima and Mochizuki, 2009; Wang et al., 2012). The origins of CPV and MEV are unclear but it is possible that CPV and MEV are both from FPV or a very closely related carnivore parvovirus (Hueffer et al., 2004).

We have constructed an infectious clone of MEV by applying PCR-based strategies, the inclusion of the synthesized oligonucleotides and the In-Fusion™ assembly system (Zhu et al., 2007). The intention is to provide a means for studying viral replication, transcription and translation functions as well as host-range

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Table 1
Primers used for PCR and Synthesized sequences.

Name[a]	Sequence (5′–3′)	Position[b]
SF1	AGGTGGCGGGCTAATTGTGGG	198–218
SR1	GCTCTCTTTGCCGGAGGTGCC	2327–2347
SF2	ACAAGCGGCAAGCAATCCTC	2063–2082
SR2	TGGTAAGCCCAATGCTCTAT	3671–3690
SF3	TGCTACTCAGCCACCAACTA	3294–3313
SR3	AACATATTCTAAGGGCAAACC	4864–4884
SFInfusionIII	TTGGGCGGGAAAAGGTGGCGGGCTAATTGTGGGCGT	186–221
SRInfusionIII	CTTAACATATTCTAAGGGCAAACCAACCAACCACCCACAC	4848–4887
SFInfusionIV	TTAGAATATGTTAAGGACCAAAAAA	4873–4897
SRInfusionIV	TCCCCGGGAGTCAGGGAGCGAGGAAGCGGAAGA	Sequence in vector
SF4	AACATGGCTTTAGATGACATT	3107–3127
SR4	TTCCCATTTGAGTTACACCAC	3765–3785
I	CGCCACCTTTTCCCGCCCAAGTTTAAACACACAAACC GCCTATCATTCTTTAGAACCAACTGACCAAGGAGCGT ACGTATGACGTGTGACTGCCGTAGGCAGCGCGCGC	1–109
II	GGCAGCGCGCGCAGCGCTCATCACGTCATACGTACGT GAACTTGGTCAGTTGGTTCTAAAGAATGATAGGCGG TTTGTGTGTTTAAACTTGGGCGGGAAAAGGCCTGAC TGACTCCCGGGGG	98–208
5′ end	TTAGAATATGTTAAGGACCAAAAAAATCAATAAAAGA CATTTAAAACTTAATGGTCTCGTATACTGTCTATAAG GTGAACTAACCTTACCATAAGTATCAATCTGTCTTTA AGGGGGGGGTGGGTGGGAGATGCACAATATCAGTAGA CTGACTGGCCTGGTTGGTTGCGCTTAATCAACCAGAC CGCTACGCGGTCTGGTTGATTAAGCAGAGCAACCAAC	4930–5151

[a] Primer names are organized in groups. Prefixes: SF, forward PCR primer; SR, reverse PCR primer.
[b] The nucleotide position within the genome is based on GenBank accession number AF184212.

determinants. The strain of MEV-L used in this study, was isolated from Liaoning Province of China. Synthesized oligonucleotides and specific primers (Table 1) were based on genomic sequences of MEV ABASHIRI strain (GenBank accession number: D00765.1). About 10 μl of the MEV-L infected cell culture medium was adopted to boil as PCR template. The strategy we adopted is shown in Fig. 1. Briefly, synthesized and PCR-amplified fragments were cloned and assembled together to construct a full-length clone pMEV. The final sequence of this clone was determined. Compared with reference sequence (MEV ABASHIRI strain), there were a total of 7 nucleotide substitutions within the coding regions in MEV-L strain. Among these, three substitution T101I, I219V and A300P were within VP2 sequence except for H135Y in NS1 region and D616V in NS1/NS2 region. Although in all these variations only VP2 residue 300 is in the vicinity of CPV host rang determinates including VP2 residues 93, 103, 300 and 323 (Chang et al., 1992; Parker and Parrish, 1997), it is not critical amino acids of FPV host range determinates (VP2 residues 80, 564, 568) (Agbandje et al., 1993). Phylogenetic tree showed that FPV and MEV are from one clade (Cotmore et al., 2006) and VP2 residue 300 of MEV is not conserved amino acid in different MEV strain from NCBI. It is possible that variation of VP2 residue 300 of MEV-L strain does not affect its host range. There were also some changes in the non-coding regions in MEV-L strain, mainly at the 5′ end.

Five μg pMEV was mixed with lipofectamine 2000 (Invitrogen) and used to transfect a subconfluent monolayer of F81 cells. Typical MEV cytopathic effect (CPE) developed by day 3 post-transfection in F81 cells, while mock-transfected cells remained normal. The rescued virions (rMEV) produced by *in vitro* transfection were infectious, as the transfected cell lysates (P0 rMEV) were successfully used to infect F81 cells, with typical MEV CPE developing by day 2 post-infection (Fig. 2a). The infectious titer of rMEV stock prepared from transfected cells was determined to be $10^{5.75}$ $TCID_{50}$/ml.

To differentiate rMEV from the parental virus (MEV-L), and to exclude the possibility of a contaminant, pMEV had been constructed containing a translationally silent substitution, A3520G, to create a unique *BamH*I restriction site. To show that rMEV was indeed derived from pMEV, a pair of primers, SF4 and SR4 (Table 1), was designed using MEV-L and rMEV as templates to amplify a 679 bp fragment. *BamH*I cleaved the fragment from rMEV into two fragments (253 bp and 426 bp), while the corresponding fragment from MEV-L was not digested (Fig. 2b).

To characterize rMEV, immunofluorescence assays (IFA) were conducted using rMEV polyclonal antibody. Briefly, F81 cells were infected with passage 6 (P6) material of rMEV and MEV-L at an m.o.i. of 0.1, then fixed and stained at 40 h by IFA. Both rMEV and MEV-L-infected cells were stained positive, indicating that rMEV displayed infection kinetics similar to those of MEV-L (Fig. 2c).

The growth kinetics of rMEV was evaluated by infecting F81 cells with P6 rMEV at an m.o.i. of 0.1. Multiple-step growth curves of rMEV and MEV-L were constructed by determining the virus titers ($TCID_{50}$/ml) at indicated time points (Pizzi, 1950). As shown in Fig. 2d, the growth curve of rMEV was similar to MEV-L, although between 10 and 36 h, rMEV titers were consistently slightly lower than those of MEV-L.

To investigate if rMEV remained stable during further passaging, rMEV was serially passaged in F81 cells at an m.o.i. of 0.1. PCR was performed with primers SF3 and SR3 (Table 1) to amplify a fragment including the introduced *BamH*I site using infected cell culture medium of the twentieth rMEV passage as template. A fragment of about 1500 bp was amplified and sequenced. Results showed that the engineered *BamH*I site and its flanking sequences had remained unchanged (data not shown), demonstrating that rMEV maintained genetic stability.

In this study, we have shown that a full-length MEV clone (pMEV) was constructed and that was infectious when transfected into F81 cells. A novel *BamH*I restriction site was introduced into pMEV to differentiate rMEV from MEV-L. rMEV retained the biological properties of MEV-L. The genetic manipulations described here have several advantages over methods previously reported (Maria Söderlund-Venermo et al., 2001; Parrish, 1991). Only a small amount of virus was needed to construct the full-length infectious clone and use of the In-Fusion™ assembly system avoided difficulty of endonuclease site selection. The reverse genetics platform