

Complete viral protein coding genes NS1 and VP1 sequences and phylogenetic analysis of a novel recombinant Mink Enteritis Virus isolated in China

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Mink enteritis virus (MEV) is a type of virus with an envelope, single-stranded DNA structure that measures about 5 kb long (Xie *et al.* 2023). It belongs to the Parvovirus genus of the Parvoviridae family (Wang *et al.* 2021). MEV is known to cause a highly contagious disease called mink viral enteritis, which is characterized by severe diarrhoea and high rates of morbidity and mortality (Xie *et al.* 2022). This disease has been reported in various countries, and has resulted in significant economic losses in commercial mink production (Jensen *et al.* 2021). MEV is classified as a subspecies of feline parvovirus, along with feline panleukopenia virus (FPLV) and canine parvovirus (CPV), due to their shared DNA sequences of at least 98% and similar antigenic features, as reported by Lin *et al.* (2022). The genome of MEV consists of two open reading frames (ORFs). The left ORF encodes non-structural proteins (NSP), including NS1 and NS2, which are derived from the same gene and contribute to viral replication and regulatory functions. The right ORF encodes two capsid proteins, VP1 and VP2, which are translated from the same ORF but through differential splicing (VP1). These capsid proteins may contribute to viral pathogenicity and virulence. The two ORFs are flanked by inverted terminal repeats, which form self-priming hairpin structures that play an important role in the replication process, as reported by Mao *et al.* (2016).

In mid-October 2017, an outbreak of infectious diarrhoea occurred at a mink farm located at coordinates E123°54' and N47°19' in Qiqihar City, Heilongjiang province, China. This farm housed approximately 700 adult minks, which were primarily raised for fur production. Among these minks, approximately 50, constituting 7.1% of the population, suffered from haemorrhagic diarrhoea. Within 10 days of displaying clinical signs, such as vomiting, anorexia, and diarrhoea, 1.0% (7 individuals) succumbed to the illness. The resulting case fatality rate (CFR) was calculated to be 14.0%, a characteristic rate indicative of a moderately virulent pathogen. Post mortem examinations

revealed lesions consistent with MEV infection, including ulcerative enteritis and intestinal hemorrhage. Five months ago, a single immunization was administered using MEVB attenuated vaccine. The present study aimed to identify MEV in mink samples and analyze the viral gene characteristics of MEV.

In order to obtain virus samples, seven fecal samples were collected from minks displaying symptoms of diarrhoea. Subsequently, all these samples were combined into a single composite sample. The protocol for viral isolation as described by Mao *et al.* 2016 was followed. The confirmation of the presence of viral particles was achieved through transmission electron microscopy (TEM), utilizing Feline kidney (F81) cell culture medium. The spherical virus particles observed had a diameter of approximately 22-25 nm and were envelope-free as depicted in Fig. 1.

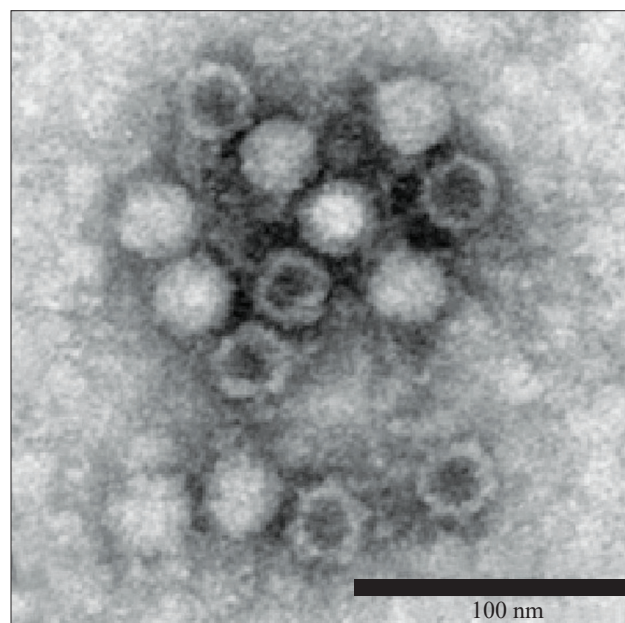


Fig. 1. Electron microscope morphology of the MEV virion (40,000×).

To obtain the complete NS1 and VP1 gene sequences of the MEV-HLJ strain, PCR amplification methods described in a previous study by Wang *et al.* (2012) was followed.

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Table 1. MEV strains cited in present study

Isolate	Accession number	Origin	Collection year	Pathogenicity
MEV-HLJ	MH006576	Heilongjiang, China	2017	Attenuated
MEV-LHV	KT899745	Liaoning, China	2014	Virulent
MEVB	FJ592174	/, China	2013	Attenuated
SD12/01	KC713592	Shandong, China	2012	Virulent
SMPV-11	KP008112	/, China	2011	Virulent
MEV-L	KT899746	Liaoning, China	2010	Attenuated
MEVLN-10	HQ694567	Liaoning, China	2010	Virulent
MEV-SDNH	JX535284	Shandong, China	2009	Virulent
Abashiri	D00765	Japan	/	Attenuated

The resulting sequences were deposited in GenBank and assigned the accession number MH006576. In order to investigate the phylogenetic relationship between the MEV-HLJ strain and other known MEVs, complete NS1 and VP1 gene sequences of nine MEVs, including MEV-HLJ was retrieved from the GenBank (Table 1).

The ClustalX version 2.1 (available at <http://www.clustal.org/clustal2/current/>) was used to align the NS1 and VP1 nucleotide sequences. To assess the accuracy of the phylogenetic tree, the neighbour-joining method-based Kimura 2-parameter model was employed and 1,000 bootstrap replications using MEGA version 6, as recommended by Ohshima and Mochizuk (2013) was conducted. The constructed phylogenetic tree (Fig. 2A) exhibited that the MEV strains were classified into two distinct genotypes: attenuated and virulent groups, as previously described (Wang *et al.* 2012). Surprisingly, the MEV-HLJ strain was grouped into the attenuated group

even though it displayed moderate virulence.

In order to explore the possibility of recombination, the genetic algorithms for recombination detection (GARD) software was utilized to detect the putative parental sequences and locate potential recombination breakpoints in the gene sequences of the MEV-HLJ strain. Present study discovered a possible point of recombination in the MEV-HLJ strain's nucleotide sequence, which was located at nucleotide position 2,075 between the NS1 gene (1-2,007) and VP1 gene (2,101-4,269), as shown in Fig. 2B. No evidence of recombination events was found in the genome sequences of the remaining eight strains that were analyzed using GARD. The gene sequence was separated into two fragments at the potential breakpoint, and distinct phylogenetic trees were constructed for each fragment. The first fragment (1-2,075) was discovered to group with the attenuated category (Fig. 2C), while the second fragment (2,076-4,269) grouped with the virulent

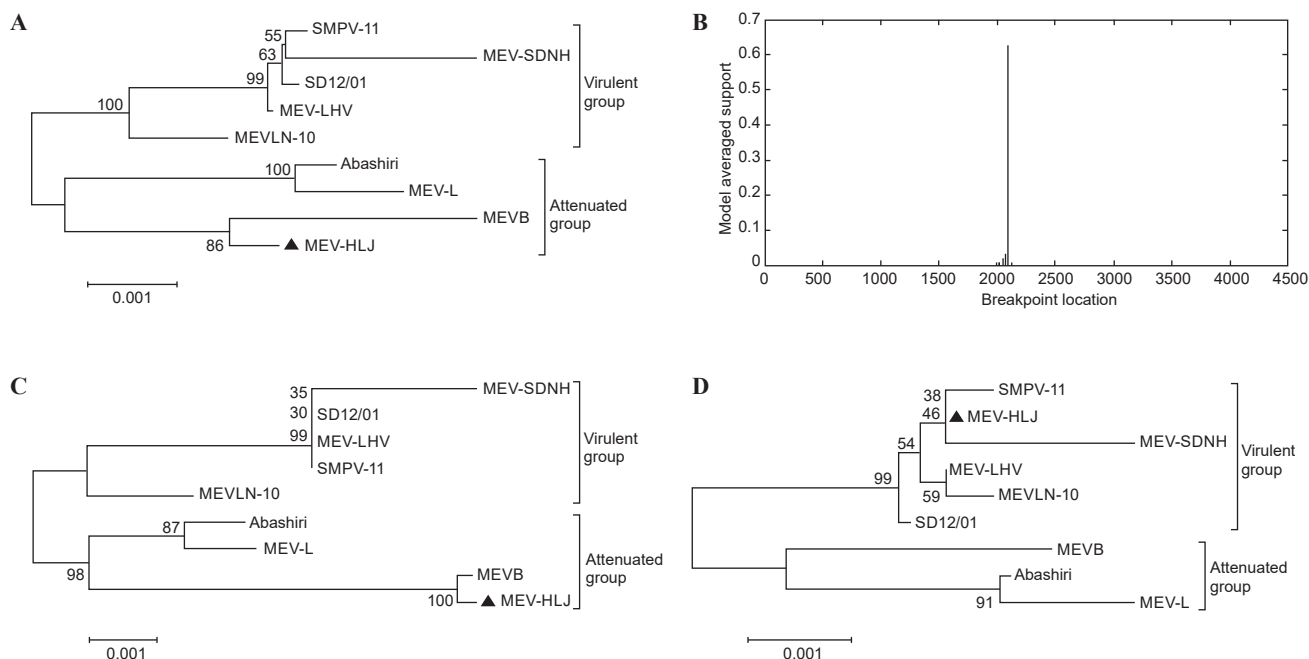


Fig. 2. Phylogenetic and recombination analysis of MEV-HLJ strain: (A) Complete NS1 and VP1 gene sequences were analyzed by utilizing the neighbor-joining method and Kimura 2-parameter model in MEGA6; (B) GARD program detected nucleotide position 2,075 as a probable recombination breakpoint; (C) nucleotide sequences were split into two fragments: 1-2,075 and 2,076-4,269; (D) The fragments were separately analyzed using the neighbor-joining method and Kimura 2-parameter model in MEGA6. The MEV-HLJ strain was marked with solid triangles in the phylogenetic analysis.



Fig. 3. Alignment of homologous amino acid sequences of MEV VP1 protein.

category (Fig. 2D).

Considering that the VP gene of MEV-HLJ is derived from virulent strains, there are specific amino acid differences in the VP protein (Fig. 3). At position 370, both five virulent strains and MEV-HLJ exhibit leucine, whereas three attenuated strains show isoleucine. Similarly, at position 549, five virulent strains and MEV-HLJ feature glutamic acid, while three attenuated strains display proline. It is plausible that mutations in these two amino acids (from isoleucine to leucine and from proline to glutamic acid) might contribute to the heightened virulence observed in MEV-HLJ.

In the nucleotide sequence of the VP1 gene, a notable pattern emerges (Fig. 4). Specifically, at position 1108, both five virulent strains and MEV-HLJ display guanine, whereas three attenuated strains exhibit adenine. Likewise, at position 1646, five virulent strains and MEV-HLJ showcase adenine, while three attenuated strains feature cytosine. It is reasonable to consider that mutations at these two specific nucleotide positions (shifting from adenine to guanine and from cytosine to adenine) may play a role in the heightened virulence observed in MEV-HLJ.

MEV-HLJ is the only recombinant strain of MEV that is discovered so far, with an incidence rate of 7.1% and

a mortality rate of 14.0%. In China, adult minks have generally been exposed to the MEV pathogen or immunized during their juvenile years, which leads to the presence of antibodies against MEV in their bodies. As a result, the morbidity rate for strong virulent strains is relatively low, typically ranging from 10% to 20%, with a mortality rate of approximately 25% to 30%.

Over the years, various researches have noted the occurrence of natural recombination events among distinct CPV antigenic types (Mochizuki *et al.* 2008). In addition, recombination events have been reported between CPV and FPLV (Ohshima *et al.* 2009) and between MEV and CPV (Wang *et al.* 2012). These events have led to the emergence of new genotypes of feline parvoviruses, thereby enhancing the genetic diversity of feline parvoviruses in the natural environment. It has been demonstrated that natural recombination can occur between viral genes of a parvovirus (Martin *et al.* 2011). Given that both MEVs and MEV attenuated vaccines have been extensively utilized in China for several years, the coexistence of attenuated and virulent MEVs on the same farm, or even within a single mink, should not be unexpected. Consequently, in this particular scenario, recombination events could potentially occur under field conditions. This study provides evidence



Fig. 4. Alignment of homologous nucleotide sequences of MEV VP1 gene.

that recombinant MEV strains can also arise within the MEV population. The frequency of such recombinant MEVs may pose challenges in diagnostic testing and in developing effective vaccines against these viral variants. Thus, it is crucial to verify the occurrence of recombination events in attenuated vaccine viruses before considering them as vaccine candidates.

SUMMARY

The primary objective of this study was to detect the presence of MEV in mink samples and investigate the genetic characteristics of the virus. In 2017, a newly identified strain of MEV, named MEV-HLJ, was isolated. The virus's complete NS1 and VP1 gene sequences were amplified using PCR and subjected to phylogenetic analysis. The results indicated that despite moderate virulence, MEV-HLJ belonged to the attenuated group. Recombination analysis using the GARD programme identified a putative recombination breakpoint at nucleotide position 2,075 between the NS1 and VP1 genes. Separate phylogenetic trees were constructed to confirm the recombinant event. This study provides insights into the genetic diversity and evolution of MEV isolates.

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