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Short communication

# Isolation, molecular characterization, and phylogenetic analysis of encephalomyocarditis virus from South China tigers in China

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ABSTRACT

Although encephalomyocarditis virus (EMCV) can infect many host species and cause myocarditis and sudden death in many species, little is known about EMCV infection in tigers. A virus was isolated from organs of dead South China tigers with sudden death in southern China. The production of cytopathic effect on BHK cells, and the results of PCR, electron microscopy (EM), and whole genome sequencing indicated that the pathogen was EMCV, the strain was named FJ13. Other pathogenic agents were excluded as possible pathogenic agents. Phylogenetic analyses of the whole genome, ORF (open reading frame) and CCR (capsid coding region) using the neighbour-joining method revealed that EMCV isolates cluster into two groups (group 1 and 2) with two sub-clusters within group 1 (group 1a and 1b), and FJ13 belongs to group 1a. Animal experiment showed that the isolated strain FJ13 could cause clinical symptoms and pathological changes. The results of this study indicated that FJ13 caused myocarditis of tigers and provided new epidemiologic data on EMCV in China.



## 1. Introduction

Encephalomyocarditis virus (EMCV; family *Picornaviridae*, genus *Cardiovirus*) is a group of closely related virus strains; with only two known serotypes (King et al., 2011; Philipps et al., 2012). It seems to infect a wide range of vertebrate hosts, with zoonotic potential for human infection (Canelli et al., 2010; Philipps et al., 2012; Sandwyk et al., 2012). The disease exerts different sequelae in different animals, ranging from asymptomatic persistence in natural reservoirs (rodents) to sudden death in most animal species (Carocci and Bakkali-Kassimi, 2012; Oberste et al., 2009; Philipps et al., 2012). EMCV is a small, non-enveloped, positive sense single-stranded RNA virus, of a genome size of ~7800 bp nucleotides (Palmenberg et al., 1984). The virus genome contains a 5′ and 3′-untranslated regions (UTRs), and a single open reading frame (ORF) encodes a polyprotein that is co- and post-translationally processed into 12 viral proteins (King et al., 2011). Viral dissemination is poorly understood, but transmission is thought to be via fecal-oral route (Carocci and Bakkali-Kassimi, 2012).

EMCV has been reported from wide range of vertebrate hosts, however, pigs are being the most mammalian host impacted with the disease (An et al., 2009; Bai et al., 2012; Koenen et al., 1999; Lin et al., 2012), imposing serious challenge on pig industry. Moreover, occurrence of EMCV in wildlife has been reported in free range wildlife (Billinis, 2009; Grobler et al., 1995), and implicated for acute and highly lethal infections in wildlife in zoos and captivity (Canelli et al., 2010; Jones et al., 2011; Seaman and Finnie, 1987). However, the reported outbreaks remain associated with rodent irruptions (Sandwyk et al., 2012).

The South China tiger (*Panthera tigris amoyensis*) is the most endangered tiger subspecies. Uncontrolled hunting and extensive deforestation since then have resulted in the extinction of all wild South China tigers (Tilson et al., 2004). The present study describes, for the first time, occurrence of EMCV infection in semi-captive, endangered South China tigers. Behavior of the isolated viral strain was studied in cell culture and by animal inoculation test. Full genome sequence of the virus was achieved to determine the molecular identity of the strain.

## 2. Materials and methods

### 2.1. Case description

Three South China tigers were found dead in Meihuashan National Reserve, Fujian province, southern China in Dec, 2012.

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**Table 1**
Clinical and pathological findings of the three dead South China tigers.

| No. | Sex | Age | Death date | Clinical signs | Gross pathology | EMCV Positive (tissue) |
|---|---|---|---|---|---|---|
| 1 | Female | 13 | 17-12-12 | Sudden death | No gross lesions were observed | Heart |
| 2 | Male | 9 | 28-12-12 | Anorexia, depression | Cerebral hemorrhage, pericardial effusion, pleural effusion, jaundice, pulmonary emphysema | Heart<br>Liver<br>Lung |
| 3 | Female | 16 | 31-12-12 | Lethargy, anorexia, depression | Meningeal congestion, cardiomegaly, ascites, pericardic aemorrhages, pulmonary edema, liver hyperaemia | Heart<br>Liver<br>Lung<br>Intestine |

**Table 2**
Oligonucleotide primers used for the amplification of the complete genome of the FJ13 strain of encephalomyocarditis (EMCV).

| Primers | Sequence 5′–3′ | Positions[a] | Amplified fragment | References |
|---|---|---|---|---|
| E-1F | ATTGTATGGGATCTGATCTGGGG | 605–625 | 1064 bp | Juan et al. |
| E-1R | ACNGCDATNACNARNGTCCA | 1771–1790 | | This study |
| E-2F | AARGGNCCNTTYGCNATGG | 1621–1639 | 1281 bp | This study |
| E-2R | NGTYTCYTCRTTNCCRTTNCCC | 2877–2897 | | This study |
| E-3F | MGNYTNACNGARATHTGGGGNAA | 2861–2882 | 1294 bp | This study |
| E-3R | GCATNACNGCDATNGTCATNCC | 4132–4153 | | This study |
| E-4F | CARYTNATHGCNGGNATGACNAT | 4120–4142 | 710 bp | This study |
| E-4R | GGRTTYTGNCCNARRTCRTCC | 4809–4829 | | This study |
| E-5F | TTYGCNGCNATHATGGAYGA | 4795–4814 | 1658 bp | This study |
| E-5R | TTNGCRTAYTCYTTNGCNACC | 6432–6452 | | This study |
| E-6F | GAGGAGGAGTTATTCTTGTCTGAGG | 942–966 | 5′-UTR | Juan et al. |
| E-6R | GGGGCCTAGACGTTTTTTAACCTC | 659–682 | (728) | Juan et al. |
| 5′-RACE outer primer | CATGGCTACATGCTGACAGCCTA | | | |
| 5′-RACE inner primer | CGCGGATCCACAGCCTACTGATCAGTCGATG | | | |
| E-8F | ATGTTGTCATACTATCGTCCAGG | 7430–7452 | 3′-UTR | Juan et al. |
| E-8F | TACCGTCGTTCCACTAGTGATTT | | (132) | |

[a] Nucleotide positions were indicated based on the position of EMCV BJC3 (DQ464062).

The semi-captive South China tigers were housed separately, and were fed freshly culled chickens or beef as their regular feed. For a period of one week, the tigers exhibited illness symptoms, including anorexia, lethargy and general malaise, treatment with antibiotics was unsuccessful. Post-mortem revealed excessive pericardial effusion and cerebral hemorrhage (Table 1). Fresh tissue samples from different organs including heart, liver, spleen, lung, kidney, brain and intestine were collected for bacteriological, virological and histopathological examinations.

### 2.2. Molecular identification of the virus

Viral genomic RNA and DNA were extracted from tissue samples collected by using TIANamp Virus DNA/RNA Kit (TIANGEN, Beijing, China), and subjected to RT-PCR or PCR and screened against a panel of potential pathogens including Encephalomyocarditis virus, Influenza virus, Feline parvovirus, Feline leukemia virus, Feline infectious peritonitis, and Circovirus. The results of PCR indicated that the tested specimens were positive for EMCV but negative for other pathogens tested.

The virus, designated EMCV FJ13, was isolated from heart, liver, lung and intestine tissues, and cultured on BHK-21 cells (Canelli et al., 2010). The cultured cells were harvested and fixed in 2.5% glutaraldehyde for transmission-electronmicroscope (TEM) examination (JEM-1400, Japan).

To elucidate the molecular identity of the isolated virus, the genetic materials were derived from positive tissues by RT-PCR. Both 5′- and 3′-UTRs were amplified by the 5′ and 3′ Full RACE Core sets (TaKaRa Biotechnology Company, Dalian, China). The 5′-phosphorylated primers and the 3′ RACE adapter primer were used as reverse primers to synthesize 1st-strand cDNA, which was used as the template for amplification. RNA region corresponding to the whole ORF of EMCV was amplified and sequenced using a set of specific primers (Table 2). The primers were designed based on the ORF sequence of the BJC3 strain (DQ464062), using Primer Premier 5.0 software. The PCR products were cloned into the pMD19-T vector and sequenced by a commercial corporation (BGI, Beijing, China).

Sequence assembly was carried out using the SeqMan program of the DNASTAR Software. Full genomic sequence of the isolated strain (EMCV FJ13) was deposited in genbank (KF293299). Multiple sequence alignment based on the whole genome, ORF and CCR (capsid coding region, including VP1, VP2, VP3 and VP4) gene sequence of FJ13 and other reference strains in database was conducted using the Clustal W (http://www.clustal.org/). Phylogenetic analysis was performed based on the whole genome, ORF and CCR sequences by the neighbor-joining (NJ) method (Tamura et al., 2011) as implemented in MEGA5.2 software (http://www.megasoftware.net).

### 2.3. Animal inoculation test

Animal inoculation was done to determine the pathogenicity of the EMCV FJ13 in lab animals. Twenty 42-day-old BALB/c mice free of EMCV were divided into 2 equal groups. All mice in the first group were inoculated intraperitonealy with 0.1 mL/mouse containing $10^6$ $TCID_{50}$ of FJ13 strain. Mice in the second group were inoculated with 0.1 mL/mouse of DMEM medium and served as negative controls. At 3 days PI, all inoculated mice exhibited clinical symptoms and hemagglutination inhibition (HI) examination of sera was positive for EMCV. Tissue samples were collected from infected as well as control mice and processed from pathological evaluation.