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LETTER

Identification, Isolation, and Characterization of an Ectromelia Virus New Strain from an Experimental Mouse

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Dear Editor,

Ectromelia virus (ECTV) is a large double-stranded DNA virus belonging to the genus *Orthopoxviruses* in the family *Poxviridae*. It is the causative agent of mousepox, which is a lethal, acute toxic disease with high mortality rate in most strains of laboratory mice. It was first discovered in 1930 when mice were first used as animal models (Marchal 1930). It has long been enzootic in laboratory mouse colonies in Europe, Japan, and China, and later spread to USA (Fenner 1981; Esteban and Buller 2005). To date, there are four ECTV strains with complete genome sequences deposited in GenBank. ECTV strain Hampstead (ECTV-Ham) was the first discovered strain from a laboratory mouse colony in London (Marchal 1930). ECTV strain Moscow (ECTV-Mos) derived from Moscow was considered more virulent than ECTV-Ham based on animal experiments (Andrewes and Elford 1947). ECTV strain Naval (ECTV-Nav) was isolated from an outbreak at the Naval Medical Research Institute in Bethesda, MD (Dick *et al.* 1996). ECTV strain culture-collection ATCC VR-1431 (ECTV-ATCC) was isolated from throat swabs of six individuals who were suffering from erythromelalgia in rural China in 1987. This virus was referred as erythromelalgia-associated poxvirus as indicated by serological tests and viral morphology (Zheng *et al.* 1992). By next generation sequencing in 2012, the virus was finally identified a new ECTV strain closely related to strains ECTV-Mos and ECTV-Nav (Mendez-Rios *et al.* 2012).

Here we report the isolation of a new ECTV strain (ECTV-WH) from an experimental mouse brain in 2013. Pregnant mice (KM strain) were bought from a local company and raised in laboratory until babies were born. In order to isolate virus pathogens from ticks, each of the suckling mice (2-day old) given birth by one mother were inoculated intracerebrally with aliquot homogenates from one tick group as previously described (Zhang *et al.* 2018). Mice which had disease onset within 2 weeks were sacrificed. Brains were harvested and total RNA was purified as described (Zhang *et al.* 2018). RNA was further used for library preparation and Roche 454 sequencing as previously reported (Shu *et al.* 2018). Data analyses and sequence comparison showed that a total of 213,772 reads (GenBank accession number: PRJNA599517) were generated from one of the libraries, including 12 reads related to ECTV and 591 reads related to lactate dehydrogenase-elevating virus (LDV) belonging to the family *Arteriviridae*. We attempted to isolate LDV in the beginning. Clarified brain homogenates (150 μL) were incubated with Marc-145 cells (National Virus Resource Center, IVCAS 9.174) at 37 °C for 2 h, then was replaced with fresh Dulbecco Modified Eagle Medium (DMEM, NZK biotech, Wuhan, China) containing 2% Fetal Bovine Serum (FBS, GIBCO, NY, USA). On 2 days post incubation (d p.i.), several cell foci presented cytopathic effect (CPE) having elongated shapes. Supernatants were harvested on 4 d p.i. and used to infect new healthy Marc-145 cells. CPE was observed from all cells in the third passage (P3) (Fig. 1A), suggesting successful isolation and virus proliferation by cell culture. Detection of LDV RNA was positive from mouse brain but negative from cell culture supernatants (data not shown). EM analyses were performed as described (Shu *et al.* 2018) by using infected Vero cells

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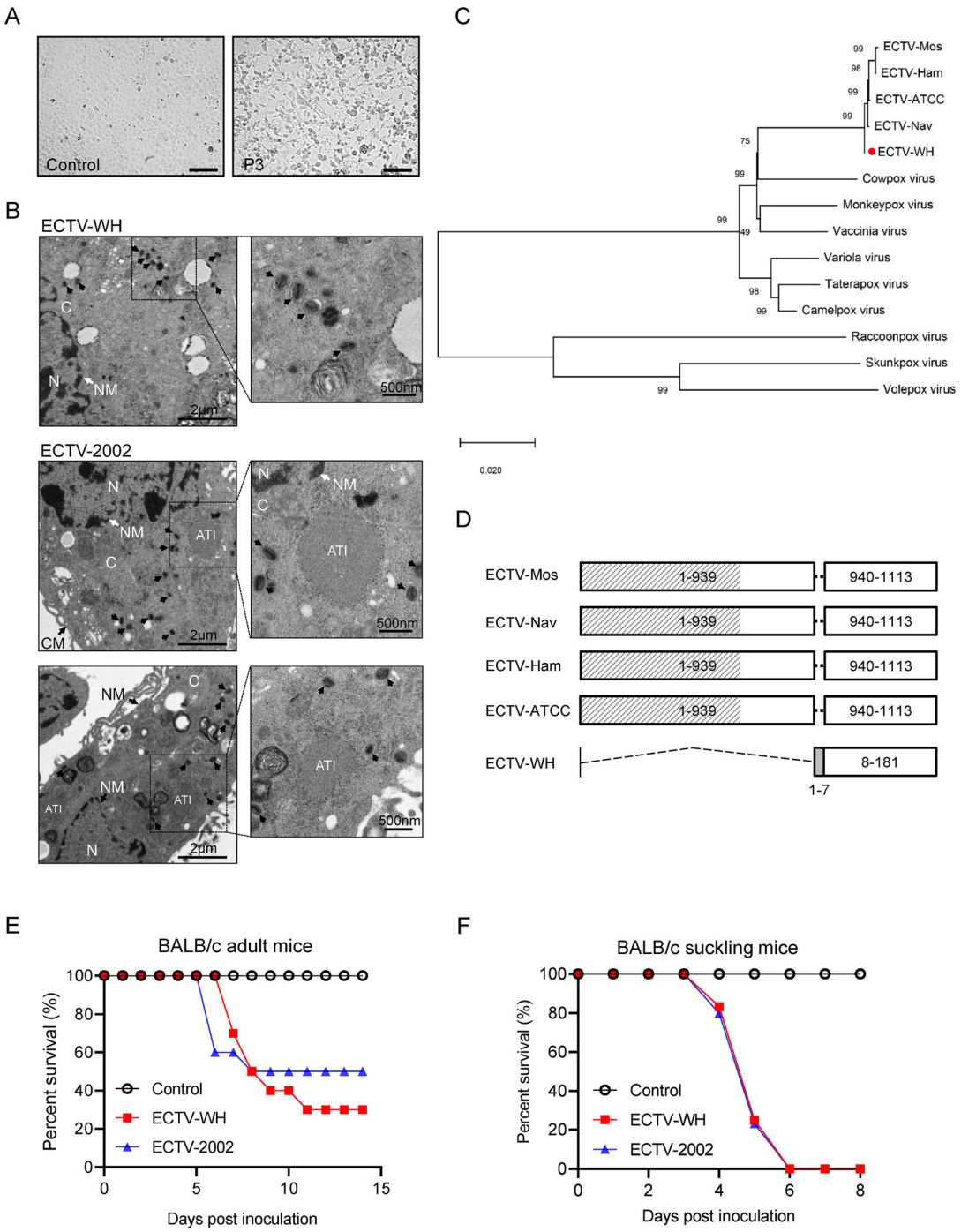

Fig. 1 Isolation of the new ECTV strain ECTV-WH. **A** Images of healthy Marc-145 cells (Control) and ECTV infected Marc-145 cells. Cytopathic effect (CPE) could be observed from the third passage (P3). Bars, 200 μm. **B** Image of ECTV-WH (the upper panel) and ECTV-2002 (the middle and bottom panels) infected cells by transmission electron microscopy. Virons locating in cytoplasm were shown in the enlarged image on the right and were indicated by arrows. ATI, inclusions formed by A-type inclusion protein. Bars, 2 μm and 500 nm, respectively. N, nucleus; NM, nucleus membrane; C, cytoplasm. **C** Phylogenetic tree based on the complete genome sequence of viruses from the genus *Orthopoxvirus*. The isolate ECTV-WH is labeled by red circle. Phylogenetic tree was constructed using the Neighbor-Joining method with bootstrap value of 1000 replicates in MEGA6.0. **D** Schematic presentation of the truncated form of ATI protein encoded by the strain ECTV-WH. Boxes with numbers represent amino acid positions of ATI proteins. The 600 aa conserved region at N-terminus are shaded in bias, and the extra 7 aa of ECTV-WH is shaded in grey. The large deletion in ECTV-WH ATI is indicated by dotted, triangular line. **E** Survival curve of BALB/c adult mice (6–8 week old) inoculated with ECTV-WH, ECTV-2002, and aliquot volume of cell culture medium (Control). **F** Survival curve of BALB/c suckling mice (2-day old) inoculated with ECTV-WH, ECTV-2002, and aliquot volume of cell culture medium (Control).

(ATCC® CCL-81) which are also susceptible to ECTV. Brick-shaped viral particles in cytoplasm were found, which were not LDV particles but presented typical morphology of poxvirus about 250 nm long and 200 nm short (Fig. 1B, the upper panel). Subsequently, ECTV DNA (a fragment of 478 bp in length) was detected from cell culture supernatants in the third passage (data not shown). All these results showed that attempts to isolate LDV failed; however, a new strain of ECTV was isolated.

Complete genome sequence of this new strain (named as ECTV-WH, GenBank number: MN912466) was obtained by Roche 454 sequencing and PCR to fill the gaps (Supplementary methods). The genome is 202,359 bp in length with 97.9% identity to strain ECTV-ATCC including gaps, and contains 205 open reading frames (ORFs) as predicted by softberry FGENESV. As expected, phylogenetic analysis showed that ECTV-WH clusters together with other ECTV strains (Fig. 1C).

Genome organization of ECTV-WH was further characterized. Like other ECTV strains, two long inverted terminal repeats locate at 5′ and 3′-ends of genome sequence respectively. Of the 205 genes, 86 genes were encoded in forward direction, and 105 were encoded reversely (Supplementary Fig. S1). The other 14 genes were pseudo genes, which are fragmented due to mutations, deletions, or insertions that interrupt translation of protein products (Mavian *et al.* 2014). Putative functions of all 205 genes were summarized, including 63 genes related to virus structure and assembly, 51 genes related to host-response modifiers and host-range, 49 genes related to DNA and RNA metabolism, and 42 genes of unknown functions (Supplementary Fig. S1, Table S1). Comparing to ECTV-ATCC, amino acid variations were found from ECTV-WH viral proteins, such as ORF38, ORF78, ORF107, ORF112, ORF146, ORF164, ORF195, and ORF200 (Supplementary Table S2), most of which are related to DNA and RNA metabolism and immunomodulatory. A few frameshifts were found in ECTV-WH ORFs due to nucleotide insertions. For example, comparing to ECTV-Ham and ECTV-Mos, ECTV-WH ORF8 has a 5 bp-insertion (ACCTA) at genome positions 10011–10015. Frameshift mutations were also found in ORF40 and ORF98 due to insertions, which interrupt protein translation and result in protein truncation at C-terminus. Comparing to other ECTV strains, insertion (TTACTA) was found at positions 124418–124423 by adding 2 amino acids (SN) in ORF129. Nucleotide deletions were also found in ECTV-WH ORFs. ORF165 encodes profilin-like (PFL) protein which is highly conserved from all orthopoxviruses and plays role in intracellular transport of viral proteins or intercellular spread of poxvirus (Butler-Cole *et al.* 2007). A guanylic acid (G) deletion was found in the *profilin-like* (*pfl*) gene of ECTV-WH at genome position 152037, making it a pseudo gene that contains two separate fragments (144 bp and 177 bp). Comparing to the full-length ORF147 of other strains encoding *A-type inclusion* (*Ati*) gene (3342 bp), ECTV-WH *Ati* gene is only 546 bp in length. It has a large deletion resulting in 939 aa truncation at N-terminus, and thus would generate truncated form of A-type inclusion (ATI) protein, in contrast to full-length ATI proteins of other strains (1113 aa). ECTV-WH ATI protein (181 aa) is composed of a short fragment (7 aa) insertion at N-terminus and the C-terminus sequence (174 aa) completely identical to other strains (Fig. 1D). Poxvirus ATI protein could form dynamic, mobile inclusions with liquid gel-like properties in cytoplasm, which could embed infectious mature virions (MVs) of poxvirus to provide long-term protection in the environment and promote animal-to-animal transmission (Katsafanas and Moss 2019). Some orthopoxviruses such as variola virus, monkeypox virus and camelpox virus having truncated forms of ATI protein do not form large inclusions for embedding MVs (Meyer and Rziha 1993). ECTV-2002 was isolated from diseased KM mice in 2002 (An *et al.* 2004), which has an intact ATI ORF as confirmed by PCR detection (Supplementary Fig. S2, and Supplementary methods). Inclusions were noted in cytoplasm of ECTV-2002 infected Vero cells but not observed in ECTV-WH infected cells (Fig. 1B). Therefore, ECTV-WH is the first strain encoding truncated ATI protein, which may result in the incompetence to form inclusions. Previous study reported that the absence of ATIs might enhance poxvirus replication in mice and promote spread of free MVs in the route of respiratory transmission (Kastenmayer *et al.* 2014). This indicated that ECTV-WH may have higher replication efficiency *in vivo* and higher transmitting efficiency to cause respiratory infection in mice. Moreover, cowpox virus PFL proteins play a role in intracellular virus spread by interacting with both truncated and full-length forms of ATI proteins (Butler-Cole *et al.* 2007). It may indicate that the fragmented PFL protein and truncated ATI protein of strain ECTV-WH may be an outcome of co-evolution through protein–protein interaction. Pathogenicity of ECTV-WH (10^3 $TCID_{50}$ per mouse) were confirmed using BALB/c adult mice and suckling mice respectively in comparison to ECTV-2002. ECTV-WH and ECTV-2002 infection caused slight body weight loss (data not shown), and resulted in very high mortality in the adult groups (70% and 50%, respectively) (Supplementary Table S4). Fatal cases in BALB/c adult mice occurred during 7–11 days post inoculation (p.i.) for the ECTV-WH group and 6–8 days p.i. for the ECTV-2002 group (Fig. 1E). Survival curve differed between the two groups, but not in the manner of significance (paired *t* test, $P = 0.4168$). For suckling mice, the two groups had similar survival curve (Fig. 1F), and none of them survived (Supplementary Table S4). Generally, those mice died rapidly within hours after having manifestations of asthenia accompanied with tanglesome hair. Moreover, diseased suckling mice had skin lesions with a few of them presenting gangrene of limbs. In addition, a few suckling mice infected

with ECTV-WH had convulsions (Supplementary Table S4). These results suggested that both ECTV-WH and ECTV-2002 are pathogens to cause disease of symptoms to inbred mice, although no significant difference of lethality was found between the two strains. The function and impact of truncated ATI protein on ECTV virulence and pathogenesis needs to be further investigated.

ECTV is closely related to Variola virus, the causative agent of smallpox, and Monkeypox virus, the causative agent of a severe zoonosis. It is an attractive virus model for study of poxvirus pathogenesis, viral immune and inflammatory responses, and could be used for antiviral and vaccine tests. This new ECTV isolate is now preserved in the National Virus Resource Center (Wuhan, China) (IVCAS 6.6094), and could be provided as an important virus resource for study on poxvirus. Unfortunately, the exact origin of this virus was unable to be traced at the moment. Although strain ECTV-WH was isolated from brain of a suckling mouse inoculated with tick homogenates, there was no evidence that ECTV could be vectored by ticks. ECTV could cause asymptomatic infection in some strains of mice, and illness onset could be activated by many factors (An *et al.* 2004). The recorded mousepox outbreak was reported from a local laboratory animal center in 2002, which resulted in a high mortality (62.5%) of 6-week KM mice community (An *et al*. 2004). Typical manifestations of mousepox include ulcers or necrosis on the tail and heel, and toes fall off. However, these were not noted from diseased KM mice in 2002, an outbred strain containing a large gene pool with high heterozygoisity, nor observed from the KM suckling mouse in this report. It was speculated that ECTV caused asymptomatic infection in the previous outbreak (An *et al*. 2004). The KM suckling mouse in this study may also have asymptomatic infection or it was sacrificed before illness onset. Nevertheless, in order to prevent and control ECTV emergence and to reduce ECTV contamination in animal experiments, it is suggested to perform strict management on production and utilization of experimental animals and to detect ECTV asymptomatic infection before animal experiment.

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Compliance with Ethical Standards

Conflict of interest This article does not contain any studies with human performed by any of the authors.

Animal and Human Rights Statement All studies involving animals were conducted according to the animal welfare guidelines of the World Organization for Animal Health.

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