Letter to the Editor

Identification of a Newly Isolated Getah Virus in the China-Laos Border, China*

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In this study, we isolated a virus strain (YN12031) from specimens of *Armigeres subalbatus* collected in the China-Laos border. BHK-21 cells infected with YN12031 exhibited an evident cytopathic effect (CPE) 32 h post-infection. The virus particles were spherical, 70 nm in diameter, and enveloped; they also featured surface fibers. Molecular genetic analysis revealed that YN12031 was closely related to alpha viruses such as Chikungunya virus and Sindbis virus, and located in the same clade as MM2021, the prototype of Getahvirus (GETV) isolated in Malaysia in 1955. Phylogenetic analysis of the E2 and capsid genes further revealed that YN12031 was located in the same clade as the Russian isolate LEIV/16275/Mag. Analysis of the homology of nucleotides and amino acids in the coding area and E2 gene demonstrated that the YN12031 isolated from the China-Laos border (tropical region) was related closest to the LEIV/16275/Mag isolate obtained in Russia (North frigid zone area) among other isolates studied. These results suggest that GETV can adapt to different geographical environments to propagate and evolve. Thus, strengthening the detection and monitoring of GETV and its related diseases is very crucial.

Key words: Getah virus; China-Laos border; Phenotypic characteristics; Molecular evolution

Getah virus (GETV) was first isolated from *Culex* samples collected in Malaysia in 1955. The prototype virus strain was MM2021[1]. GETV belongs to the genus *Alphavirus* of the family Togaviridae and is a mosquito-transmitted arbovirus[2]. To date, GETV has been identified in about 10 countries or regions, including Australia[2], Malaysia[1], Japan[3], China[4], Mongolia[5], and Russia[5]. GETV can cause fever, body rashes, and leg edema in horses[3], as well as fetal death and reproduction disorders in pigs[6]. Thus, GETV is an important animal pathogen. Although antibodies neutralizing GETV have been identified in human serum samples in Malaysia, northern Australia, and Hainan Province in China[4,7], GETV has not been reported to cause human diseases.

An arbovirus investigation was conducted in the China-Laos border region (longitude 100°5′E, latitude 21°69′N) in August 2012. Specimens of *Armigeres subalbatus* collected during this investigation were ground and centrifuged. The obtained supernatant was utilized to inoculate BHK cells and the resulting cytopathic effect (CPE) was monitored[8]. After 32 h, BHK-21 cells infected with YN12031 exhibited an obvious CPE, including rounding up, aggregation, and exfoliation (Figure 1A). This CPE progressed rapidly to the '+++' level (i.e., 75% of the cells became cytopathic) about 48 h post-infection.

BHK cells inoculated with YN12031 were visualized by transmission electron microscopy (TEM)[8]. YN12031 exhibited a typical alphavirus morphology; the virus particles were spherical, 0-70 nm in diameter, enveloped, and featured surface fibers (Figure 1B1). After infection, the BHK cells were centrifuged, sectioned, and visualized by TEM. Virus particles were evident, and the majority were located in cytoplasmic vesicles. Virus particles contained a core with a high electron density (Figure 1B2).

doi: 10.3967/bes2017.028

*This work was supported by grants from National Natural Science Foundation of China (81290342 and 81501757); and Development Grant of State Key Laboratory of Infectious Disease Prevention and Control (2014SKLID103); and the Special National Project on Research and Development of Key Biosafety Technologies (2016YFC1201900), China. The founders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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To understand the plaque morphology and proliferation of YN12031 in tissue culture cells, we first observed the formation of virus plaques in BHK-21 cells and then detected dynamic changes in virus multiplication by plaque assay[9]. The plaques were 1.09 mm (1.15 ± 0.35 mm, *n* = 10, 2d) in diameter and regular in shape with distinct edges (Figure 1C). Following infection of BHK-21 cells at multiplicity of infeetion (MOIs) of 1 and 2, YN12031 proliferated rapidly about 8-24 h post-infection and reached peak titers of $1 \times 10^{7.69}$ and $1 \times 10^{7.55}$ pfu/mL, respectively. These titers decreased thereafter, reaching minima of $1 \times 10^{6.77}$ and $1 \times 10^{5.95}$ pfu/mL, respectively, at 72 h. Following infection at an MOI of 0.5, the YN12031 titer increased rapidly from 8 h to 16 h. The titer increased slowly from 16 h to 32 h, at which point it peaked ($1 \times 10^{7.86}$ pfu/mL). The YN12031 titer decreased slowly afterward, reaching a level comparable with that following infection at an MOI of 1 ($1 \times 10^{6.76}$ pfu/mL) at 72 h. These results are shown in Figure 1D.

BHK-21 cells infected with GETV exhibited an obvious CPE 32 h post-infection, and the CPE level reached '+++' after 48 h. The virus titer peaked 32 h post-infection [$1 \times 10^{7.86}$ pfu/mL (MOI = 1/2)] and then decreased gradually (Figure 1D). Previous studies have reported that BHK-21 cells infected with Sindbis virus (YN87448), the model virus of alphavirus, can exhibit an obvious CPE at 24 h and that the virus titer peaks ($1 \times 10^{9.5}$ pfu/mL) at 36 h[10]. By comparison, the CPE caused by infection with Japanese encephalitis virus, a flavivirus with linear positive-sense single-stranded RNA, appears at 48-72 h, and the highest viral titer could reach $1 \times 10^{7.1}$ pfu/mL[9]. These findings reveal that alphaviruses can cause CPEs in tissue culture cells faster than other virus types can.

Whole-genome amplification of YN12031 was performed using the GETV gene amplification primers described in Table 1. The amplified products were examined by agarose gel electrophoresis, purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA USA), and then sequenced directly. The sequences obtained were assembled, edited, and corrected using SeqMan in DNASTAR[8]. The coding region of YN12031 is 11,166 nt in length and encodes 3,720 amino acids. The length of the non-structural gene is 7,404 nt and located in the region between 79 and 7,482 nt. The non-structural gene codes four non-structural proteins (NSP1-NSP4) with a total of 2,467 amino acids. The structural gene is 3,762 nt in length and located in the region between 7,527 and 11,288 nt. This gene encodes a variety of structural proteins (E1, E2, E3, 6K, and capsid protein) with a total of 1,253 amino acids.

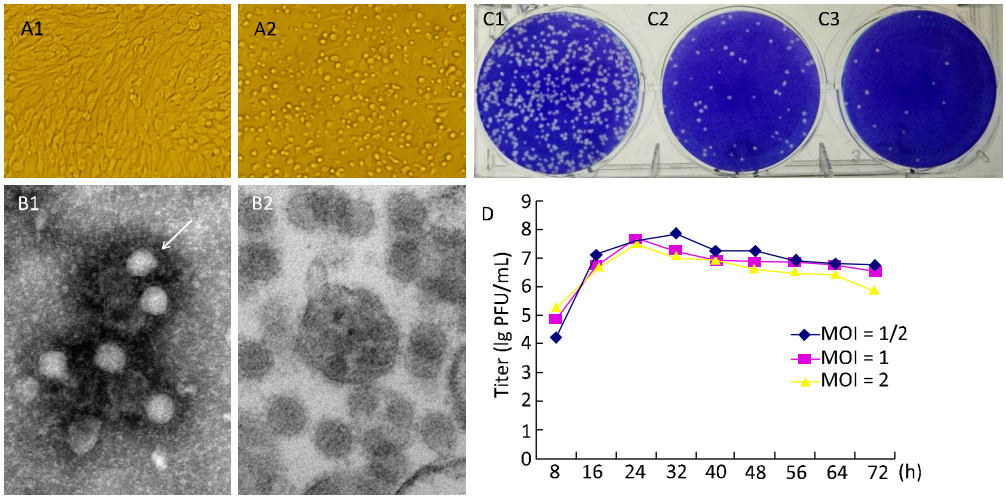

Figure 1. Biological characteristics of YN12031. (A) Cytopathic effect of YN12031 in BHK-21 cells (200 × magnification). (A1) Control (uninfected BHK-21 cells; 48 h). (A2) Infected BHK-21 cells 48 h post-infection showing rounding and exfoliation. (B) Electron micrographs of YN12031 particles negatively stained with 2% potassium phosphotungstate. (B1) Black arrow indicates an intact particle. (B2) Morphology of virus particles. (C) Plaques formed after inoculation of (C1) 10^{-4}, (C2) 10^{-5}, and (C3) 10^{-6} dilutions of YN12031. (D) Growth curve of YN12031 in BHK-21 cells.