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Derivation of cell-adapted Sacbrood virus (SBV) from the native Korean honeybee

Chang-Hee Kweon [1,2], Mi-Sun Yoo [1], Jin-Hyeong Noh, Kondreddy Eswar Reddy, Dong-Kun Yang, Sang-Ho Cha, Seung-Won Kang *

Animal and Plant Quarantine Agency, 480 Anyang-ro, Manan-gu, Anyang-si, Gyeonggi-do, Republic of Korea

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ABSTRACT

Sacbrood virus (SBV), a causative agent of larval death in honeybees, is one of the most devastating diseases in bee industry throughout the world. Lately the Korean Sacbrood virus (KSBV) induced great losses in Korean honeybee (*Apis cerana*) colonies. However, there is no culture system available for honeybee viruses, including SBV, therefore, the research on honeybee viruses is practically limited until present. In this study, we investigated the growth and replication of SBV in cell cultures. The replication signs of KSBV after passages from mammalian cells was identified and confirmed by using combined approaches with nested, quantitative, negative-strand PCR and electron microscopy along with *in vivo* experiment. The results revealed that mammalian cell lines, including Vero cells could support the replication KSBV. Although there were no signs of cytopathic effect (CPE) in cells, it was for the first time demonstrated that SBV could be replicated in cells through the sequential passages linked with cell adaptation. KSBV from the present study would be a valuable source to understand the mechanism of pathogenicity of sacbrood virus in the future.

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1. Introduction

Honeybees are one of the most important pollinators in nature. However, honeybees are susceptible to various pathogens like as bacteria, fungi, protozoa, parasite and viruses. Among them, over 18 honeybee viruses have been identified (Allen and Ball, 1996).

Especially in Asian countries, Sacbrood virus (SBV) is one of the most fatal emerging honeybee virus that cause serious losses both brood and adult in Thailand, India, Vietnam and China (Bailey et al., 1983; Ma et al., 2010, 2011; Nguyen and Le, 2013; Rana et al., 1986). SBV was occurred in Guangdong in 1972 and reemerged in Liaoning China after 36 years. In Vietnam, SBV infection was identified in 2003. These infections caused severe collapse of entire colonies. Since 2010, the entire collapse of apiaries outbroke in Korean honeybees (*Apis cerana*). The causative virus of Korean honeybee colonies was designated as Korean sacbrood virus (KSBV) (Choi et al., 2010; Choe et al., 2010).

SBV is a classified as the order Picornavirales, family Iflaviridae, genus Iflavirus with a positive single-stranded RNA (Christian. et al., 2002; Lanzi et al., 2006; Mayo, 2002). The complete genome of SBV was also sequenced which includes 8832 nucleotides encoding 2858 amino acids (Ghosh et al., 1999).

However, since *in vitro* culture method on bee viruses is not available at present, therefore, the understanding on the biological mechanisms that underlie honeybee viral diseases has been practically limited. For this reason, it is very imperative to develop culture methods that would permit the *in vitro* study of honeybee viruses. Furthermore, the identification of virus replication is an important step toward the understanding of the pathogenic process of viruses in their hosts. In present study, we investigated the growth and replication of SBV in cell cultures. The results revealed that SBV could replicate in mammalian cells through cell adaptation.

2. Materials and methods

2.1. Cells and virus

PK-15, IB-RS-2, Vero and Spodoptera frugiperda (Sf9) cell line were obtained from ATCC (CCL-33, CRL-1835, CCL81 and CRL 1711, respectively. Cloned PK-15 (CPK-15) free from PCV2, IB-RS-2 and Vero cells was regularly maintained in DMEM as described

* Corresponding author. Tel.: +82 31 467 1825; fax: +82 31 467 1828.
E-mail address: kangsw777@korea.kr (S.-W. Kang).
[1] Co-first authors (contributed equally).
[2] Current address: Daesung microbiological LABS, 103, Deogyeong-Daero, Uiwang-si, Gyeonggi-Do, Republic of Korea.

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previously (Kim et al., 2013; Kweon et al., 1999). Sf9 were maintained with Grace's medium (Invitrogen, USA) supplemented with 5% fetal bovine serum (FBS), penicillin (100 unit/ml), streptomycin (100 unit/ml) and amphotericin (0.25 g/ml), respectively. Ten-day-old, embryonated specific pathogen-free (SPF) eggs were also used for the inoculation by the allantoic cavity route. The sample for virus isolation was originated from Korean native honeybee larvae infected with SBV in Korea. For the preparation, SBV-infected larval samples were collected from Korean native honeybees during May 2011, in Jeollanam-do (province), Korea. The larval samples were ground in phosphate-buffered saline (PBS, pH 7.4). The 10% of suspension of homogenized larvae was then filtered through a 0.2 μm membrane (Acrodisc, Gelman). The filtered sample was further diluted to 10-fold with DMEM before inoculation. Initially, the filtrate was blindly passaged in CPK-15 and Sf9 cells at one week interval. The supernatant of each passage was subjected to quantitative reverse transcription polymerase chain reaction (qRT-PCR) and nested RT-PCR (NRT-PCR) analyses.

2.2. Quantitative reverse transcription PCR

qRT-PCR was conducted as described previously (Liu et al., 2010). The RNA extraction and quantitative RT-PCR procedures were conducted according to the manufacturer's protocols using an RNeasy mini kit (Qiagen, Germany) and EXPRESS SYBR® GreenER™ qPCR RT-PCR (Invitrogen, USA), respectively. The internal part of the capsid gene (VP1), one of the major structural proteins of SBV, was also monitored by qRT-PCR in an *in vivo* experiment using cell-adapted SBV.

In the *in vivo* experiment, qRT-PCR was performed using the KVP1 primer set, *i.e.*, forward (F, 1837–1860): 5′GTTTCAAATGCGTTTCACACTGGA3′ and reverse (R, 2193–2169): 5′TCCTCCTCGCATATACACCAAAAC3′, which were designed using sequence information for the capsid protein (VP1) of a Korean SBV isolate (GenBank accession number: HQ322114) (Choe et al., 2010). qRT-PCR was conducted as follows. cDNA was synthesized at 42 °C for 40 min, followed by pre-denaturation at 95 °C for 10 min, before the reaction mixture was subjected to 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s, and elongation at 72 °C for 20 s. After amplification, a melting point analysis was performed from 60 °C to 95 °C using a CFX Connect system (Bio-Rad, USA).

2.3. Nested reverse transcription PCR

The internal part of the capsid gene (VP1) was monitored routinely using NRT-PCR. Primary RT-PCR was conducted using the KVP1 F and KVP1 R primer set to synthesize the complementary DNA. Nested PCR was performed using KVP1 F and the internal primer NKVP1 R (2035–2015): 5′CCGGTAAATAGGCGCTAGCCG3′. The biological assay of SBV-inoculated cells used KVP1 F and the M13 universal forward sequence (5′CCAGTCACGACGTTGTAAAACGGTTTCAAATGCG TTTCACACTGGA3′), with KVP1 R for primary RT-PCR. In this assay, nested PCR was conducted routinely using the M13 primer (5′CCCAGTCACGACGTTGTAAAACG3′) and NKVP1 R. The primary RT-PCR reactions were performed as follows: cDNA was synthesized at 50 °C for 20 min, followed by pre-denaturation at 95 °C for 5 min and 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 1 min, elongation at 72 °C for 1 min, and post-extension at 72 °C for 5 min. Nested PCR was carried out as follows: pre-denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and post-extension at 72 °C for 5 min. The PCR products were analyzed by agarose gel electrophoresis and were visualized under an ultraviolet transilluminator.

2.4. Detection of the replicative form of KSBV by negative strand-specific RT-PCR

In order to develop a negative-strand-specific RT-PCR assay, a negative strand of viral RNA had generated from infected bee larva sample as described (Craggs et al., 2001). Briefly, total RNA was extracted from SBV infected samples. RT was performed with 1 μL (10 pM) primer M13 KVP-F (5′-**GTAAAACGACGGCCAGTG**GTTTCAAATGCGTTTCACA CTGGA-3′). One microliter of the resulting cDNA was amplified in a 20 μL reaction mixture with TOPsimple™ DryMIX-HOT (Enzynomics, Korea). Primers M13-F (5′-GTAAAACGACGGCCAGTG-3′) and KVP1-R (5′-TCCTCCTCGCATATACACCAAAA-3′) were used in the first round PCR and primers M13-F (5′-GTAAAACGACGGCCAGTG-3′) and NKVP1-R (5′-CCGGTAAATAGGCGCT AGCCG-3′) were used in the second round of nested PCR for the detection of negative-strand viral RNA. The 217-bp product obtained from the second round of the nested PCR was confirmed by agarose gel electrophoresis (Fig. 4). We have also performed negative strand PCR in another VP1 position of the SBV. Nearly, 1 μg of total viral RNA was reverse transcribed with primer VP1 (5′-TCC AGT GTG AAA CGC ATT TGA AAC-3′). Primers VP1-F (5′-ACG GTG GGT ATA AGA CCT GGG A-3′) and VP1-R (5′-GCT ATG TCC AAC GTA GTA CGA GG-3′) were used in the PCR reaction for the detection of negative strand viral RNA. KVP1 region of the 443-bp product was obtained and confirmed by agarose gel electrophoresis (Fig. 4). These two PCR reactions were performed same as above nested RT-PCR method.

2.5. Derivation of Cell-adapted SBV

The isolate, designated KSBV, was routinely passaged in 80–90% monolayers of Vero cells in roller culture as described previously (Kweon et al., 1999). For the sequential passage the 10-fold dilution of culture supernatant was inoculated for 1 h in Vero cells and the inoculum was regularly removed before adding medium. Sequential passage of KSBV were normally conducted in VP-SFM (Invitrogen, USA) at one week interval. Since there was no sign of any signs of cytopathogenic effect (CPE) in inoculated cells, the supernatant from each passage was regularly tested for the presence of VP1 by NRT-PCR as described above. Since there was no sign of any signs of cytopathogenic effect (CPE) in inoculated cells, the replication of virus was routinely confirmed by RT-PCR. The supernatant from each passage was regularly tested for the presence of VP1 by NRT-PCR. The assay for the virus titration was conducted through the inoculation of duplicate of cells with 10-fold serial dilution of the supernatant. The supernatant from inoculated cells were subjected to NRT-PCR along with control. TCID50 of virus was indirectly estimated to be the highest dilution of sample that showed the positive signal by NRT-PCR compared to negative cells. In addition, the supernatant was also directly subjected to NRT-PCR by using 10-fold serial dilution. When we compared the positive signals from each assay, it was found that there was at least more than 1-fold difference in positive signal from culture supernatant, partly supporting the infectivity of SBV by NRT-PCR (data not presented here). For morphological characterization, SBV infected larvae were ground in PBS, the mixture was filtered through 0.45 and 0.22 μm. The flow through was centrifuged on a 10–50% (w/v) sucrose gradient at 32,500 rpm for 4 h in a Beckman SW41 rotor (Beckman, USA). The virus fraction was recovered, layered on a 40% (w/v) sucrose gradient and centrifuged at 25,000 rpm for 12 h in a Beckman SW41 rotor. The virus-enriched fraction was resuspended with 200 μL of distilled water. In addition, the culture supernatant from inoculated cells was directly subjected to centrifugation. Briefly, culture debris was removed by centrifugation at 10,000 rpm for 1 min, and then the supernatant was subjected to direct centrifugation at 25,000 rpm for 12 h. The supernatant was removed by decanting

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