Short communication

Genetic characterization of a densovirus isolated from great tit (Parus major) in China

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ABSTRACT

During a study of orthophilous viruses in China, a new densovirus (DNV) was isolated from the lung tissue of Parus major (PmDNV-JL). The complete genome of PmDNV-JL was cloned and sequenced. Five open reading frames (ORFs) were identified in the 5166 nt sequence, on the basis of deduced amino acids. It was further shown that this virus caused cytopathic effects (CPE) in Feline kidney cells. The NS1 gene sequence of PmDNV-JL shares 70%–90% nucleotide sequence identity with isolates of the Blattella germanica densovirus (BgDNV) and BgDNV-like virus. Phylogenetic analysis indicated that the predicted amino acid sequences of capsid (VP) and non-structural domain (NS1) of PmDNV-JL clustered with the BgDNV and were similar to BgDNV-HB within the genus Densovirus.

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1. Introduction

Densovirus (DNVs) are non-enveloped, icosahedral, autonomously replicating, single stranded linear DNA viruses that infect arthropods, primarily in insects. Densovirus infection in insects is specific and is found only in invertebrate species. Previous studies have revealed that some orders of insects (Diptera, Lepidoptera, Dictyoptera, Orthoptera, Odonata and Hemiptera), decapod crustaceans and sea stars can be infected with DNVs (Hewson et al., 2014; Shike et al., 2000; Tijssen and Bergoin, 1995; Van Munster et al., 2003; Wang et al., 2005). Densovirus, Ierovirus and Brevidensovirus belong to one subfamily, the Densovirinae, within the family of Parvoviridae. The three genera have been described according to the ultrastructural and histopathological characteristics of the susceptible cells, including nuclear degeneration (Vago et al., 1966). In the majority of cases, the hosts perish from the infection.

Although many researchers have thought that Densovirus can infect only insects, a recent study has shown that a sea star-associated densovirus (SSaDV) causes a disease that can be transmitted from disease-affected sea star to asymptomatic sea star (Hewson et al., 2014). In addition, some studies have also detected Densovirus in the excrement of insectivorous bats (Ge et al., 2012).

The great tit Parus major is a passerine bird with an extremely large range of distribution. It belongs to Family Paridae and is considered Least Concern by IUCN Red List (Birdlife International, 2016). Great tit was split by Päckert et al. into P. major, P. minor and P. cinereus who also transferred P. bokharensis into P. major (Päckert et al., 2005). Thirty-four subspecies are presently recognized. Four large subspecies groups (sectors) are traditionally distinguished: the minor sector from eastern Siberia to the Pacific coast and Japan, southern China and northern Thailand; the major sector in the Western and Northern Palaeartic from North Africa to eastern Siberia; the cinereus sector from India and southern China to the Malayan Archipelago; and the bokharensis sector that bridges the northern and the southern arm of the ring across dry central Asia, except for deserts and the high-altitude steppes of Tibet (Päckert et al., 2005). These birds feed primarily on insects, such as leaf-beetle (Coleoptera: Chrysomelidae), scarab (Coleoptera: Scarabaeoidea), gypsy moth larvae (Lepidoptera: Lymantriidae), geometrid moth larvae (Lepidoptera: Geometridae), mosquitoes (Culex spp.), anthomyiid (Diptera: Anthomyiidae), ant (Hymenoptera: Formicidae), bee (Hymenoptera: Apidae), pine moth (Lepidoptera: Lasiocampidae), leafhopper (Hemiptera: Cicadellidae), stinkbug (Hemiptera: Pentatomidae), ladybug (Coleoptera: Coccinellidae) and...
Tettigonia chinensis (Orthoptera: Tettigoniidae) (Chu, 1988). These insectivorous birds frequently come into contact with various virus-carrying insects, thus potentially increasing the chances of spreading the virus (Davis, 1940; Francy et al., 1989). Therefore, the main aim of this study was to identify whether Densovirus could be found in Parus major that subsist on insects.

2. Materials and methods

2.1. Study site and sample collection

The protocols for animal study were approved by the Animal Care and Ethics Committees (ACEC) of Jilin Agriculture University (approval number JLAU08201407). During December 2014 and February 2015, Parus major (n = 10) were caught in a trap in Changchun (43°05’–45°11’N, 124°18’–127°05’E), Jilin province, China. Parus major (n = 10) were euthanised using intramuscular injection of xylazine (1 mg/kg). After then, each bird was stored in a flask and brought back to the laboratory. The instruments and flasks were treated (moist heat sterilization) at 121 °C for 30 min followed by exposure to ultraviolet (UV) radiation to prevent contamination, and the instruments and DNA extraction with a QIAamp Viral RNA Mini Kit (Qiagen, #52904, Hilden, Germany), according to the manufacturer’s instructions. One pair of PCR amplification primers (BgDNV-HB 1F and 1R) was designed from originally reported BgDNV-like virus genes obtained from GenBank (JQ320376.1) (Table 1), and PCR was used with a TaKaRa Ex Taq PCR kit (TaKaRa, #RR001Q, Dalian, China) (Wang et al., 2012). Positive products were purified with a GenElute™ Gel Extraction Kit (Sigma-Aldrich, #NA1111, Louis, USA) and directly sequenced using an ABI (Applied Biosystems) PRISM 3730XL sequencer.

2.2. Viral particle purification and electron microscopy

Viral particle purification was conducted as described in a previously published protocol (Ge et al., 2012). In brief, each lung sample (n = 10) from Parus major collected in China in 2014, was thoroughly homogenized and resuspended (1:10, wt/vol) in Hank’s balanced salt solution (HBSS, Sigma-Aldrich). Then the viral particles were separated from the supernatant by centrifugation and filtration, followed by digestion with 0.1 mg/ml RNase A and 20 U/ml Turbo Dnase to remove non-particle protected nucleic acids. The viral particles were also examined with an electron microscope (H-7650, Hitachi, Tokyo, Japan).

2.3. Nucleic acid extraction and polymerase chain reaction (PCR)

Supernatant containing viral particles was processed for total RNA and DNA extraction with a QIAamp Viral RNA Mini Kit (Qiagen, #52904, Hilden, Germany), according to the manufacturer’s instructions. One pair of PCR amplification primers (BgDNV-HB 1F and 1R) was designed from originally reported BgDNV-like virus genes obtained from GenBank (JQ320376.1) (Table 1), and PCR was used with an TaKaRa Ex Taq PCR kit (TaKaRa, #RR001Q, Dalian, China) (Wang et al., 2012). Positive products were purified with a GenElute™ Gel Extraction Kit (Sigma-Aldrich, #NA1111, Louis, USA) and directly sequenced using an ABI (Applied Biosystems) PRISM 3730XL sequencer.

2.4. Growth of virus in embryonated chicken eggs

The sterile samples were inoculated in the allantoic cavity of 9-day-old specific pathogen free (SPF) embryonated chicken eggs, the allantoic fluid of SPF embryonated chicken eggs was harvested 72 h after inoculation, and the sample was observed with electron microscope.

2.5. Fluorescence microscopy assay

Feline kidney (F81) cells were cultured with Dulbecco’s Modified Eagle Medium (DMEM, Gibco) containing 1% antibiotic–antimycotic solution, 8% fetal bovine serum (FBS, Gibco) and 2 mM L-glutamine. The F81 cells were plated in 24-well plates and infected with sterile samples containing densovirus that were filtered using 0.22-μm syringe filters (Millipore, Bedford, MA, USA), the cells were incubated with fresh FBS-free medium for 1 h at 37 °C. After washes in PBS, the F81 cells were cultured with DMEM containing 2% FBS at 37 °C and photographed after cytopathic effects (CPE) developed. The F81 cells were fixed with 4% formaldehyde and then stained with 0.1% crystal violet.

Table 1

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence(5′–3′)</th>
<th>Position*</th>
<th>Length of fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BgDNV-HB 1F</td>
<td>ATGAATAATGACGACCACTTTTG</td>
<td>873–895</td>
<td>1602</td>
</tr>
<tr>
<td>BgDNV-HB 1R</td>
<td>TTATCGAATTTGTATATTTTATATA</td>
<td>2452–2474</td>
<td></td>
</tr>
<tr>
<td>BgDNV-HB 2F</td>
<td>CTTGGCCTTATGGGCAAGCACC</td>
<td>1–23</td>
<td>5166</td>
</tr>
<tr>
<td>BgDNV-HB 2R</td>
<td>AAGCCAAGGGCCTATGGGCAAGC</td>
<td>5144–5166</td>
<td>112</td>
</tr>
</tbody>
</table>

*The two pairs of primers were acquired from the sequence of BgDNV-like virus (JQ320376.1).

Fig. 1. Electron-microscopic image of densovirus (Pm-DNV-JL). Micrograph of negatively stained Pm-DNV-JL from the lung (A) and (B) the allantoic fluid of SPF embryonated chicken eggs. Virus particles having an electron-transparent core and an electron-dense wall (thin arrows) or seen as electron-dense spheres (thick arrows). Scale bar, 100 nm. (10,000×).
paraformaldehyde for 30 min. After washes with PBS, the nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI, Sigma Aldrich, St. Louis, Missouri, USA). Coverslips were then analyzed by fluorescence microscopy using an Olympus IX71.

2.6. Virus-genome cloning and sequencing

The full-length genome of a densovirus from *Parus major* lung was amplified, and one pair of degenerate PCR primers (BgDNV-HB 2F and 2R) was designed based on the sequencing results in this study.

### Table 2
Organization of VP and NS coding sequences.

<table>
<thead>
<tr>
<th>ORFs</th>
<th>Proteins</th>
<th>Position (nt)</th>
<th>Numbers of amino acid (aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF1</td>
<td>VP1</td>
<td>4256–2475&lt;sup&gt;a&lt;/sup&gt;</td>
<td>593</td>
</tr>
<tr>
<td>ORF2</td>
<td>VP2</td>
<td>5107–4196&lt;sup&gt;a&lt;/sup&gt;</td>
<td>303</td>
</tr>
<tr>
<td>ORF3</td>
<td>NS1</td>
<td>873–2474</td>
<td>533</td>
</tr>
<tr>
<td>ORF4</td>
<td>NS2</td>
<td>880–1677</td>
<td>265</td>
</tr>
<tr>
<td>ORF5</td>
<td>NS3</td>
<td>327–839</td>
<td>170</td>
</tr>
</tbody>
</table>

<sup>a</sup> The complementary strand.
Table 1), because the sequenced results of this virus isolated from a bird showed high nucleotide similarity (99%) with a BgDNV-like virus isolated from a bat (JQ320376.1). The PCR products of densovirus were then purified and cloned into the pEASY-Blunt vector before sequencing. To analyze the evolutionary characteristics of strain DNV (PmDNV-JL), the complete genome of 5166 nucleotides (nt) in length was sequenced according to a previously published method (Mukha et al., 2006). Five independent clones of each PCR product were used for sequencing and a full-length genome consensus sequence of the densovirus was obtained. The 3′ Rapid Amplification of cDNA Ends (RACE) kit (Takara, #6106, Dalian, China) was performed according to the manufacturer’s instructions to obtain the densovirus of genome terminal sequences of both the sense and antisense strands.

Fig. 3. Maximum-likelihood phylogenetic trees of the entire NS1 (A) and of the entire VP1 (B) aa of densoviruses. Bootstrap proportions are shown for each node. AeDNV, Aedes densonucleosis virus (Moscow, USSR); BgDNV, Blattella germanica densovirus (North Carolina, USA); BmDNV1, Bombyx mori densovirus 1 (Canada); BmDNV5, Bombyx mori densovirus 5 (Nagano, Japan); C6/36DNV, Aedes albopictus C6/36 cell densovirus (Guangdong, China); CeDNV, Caspalia extranea densovirus (Ivory Coast); CpDNV, Culex pipiens densovirus (Guizhou, China); DpDNV, Dendrolimus punctatus densovirus (Hubei, China); DsDNV, Diatraea saccharalis densovirus (Montpellier, France); GmDNV, Galleria mellonella densovirus (Canada); HeDNV, Haemagogus equinus densovirus (Fort Collins, USA); JcDNV, Junonia coenia densovirus (France); MIDNV, Mythimna loreyi densovirus (Canada); PfDNV, Periplaneta fuliginosa densovirus (Hubei, China).
2.7. Phylogenetic reconstruction

A Basic Local Alignment Search Tool (BLAST) search was performed with the deduced amino acid (aa) sequences against those of other viruses from GenBank. Sequence similarity analysis of aa sequences was conducted using the ClustalW method in BioEdit software. Phylogenetic analyses were performed using the maximum-likelihood (ML) method implemented in phyML version 3.1 with 1000 bootstrap replicates. The trees were generated using FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/). The nucleotide sequences of the full-length genome of PmDNV-JL were deposited in GenBank under accession number KU727766. The determination of open reading frames (ORFs) was conducted using a translated BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

3. Results and discussion

In this study, the densovirus was detected in the lung tissue of a Parus major. A positive band from Parus major lung tissue was verified by amplification with identified primers and subsequent sequencing, and was confirmed to contain a densovirus (KU727766). To eliminate the possibility that the Qiagen kit was contaminated with densovirus, the DNA of the densovirus was extracted with the phenol-chloroform method, which confirmed the presence of densovirus (data not shown). In addition, particles of approximately 20 nm in diameter were also observed with electron microscope (Fig. 1 A), the densovirus from the allantoic fluid of SPF embryonated chicken eggs indicated typical densovirus-like particles (about 20 nm in diameter) (Fig. 1 B). The NS1 gene sequence of PmDNV-JL shares 70% and 99% nucleotide sequence identity with isolates of the BgDNV (AY189948.2) and BgDNV-HB (JQ320376.1), respectively. In addition, to evaluate the full-length genome of a densovirus isolated from Parus major lung tissue, the near full-length genomic sequence of PmDNV-JL was amplified, and one pair of degenerate PCR primers was designed according to previously published sequencing results. A 5 kb positive band, which shared sequence similarity with of the genome of a BgDNV-like virus that was isolated from bat feces, was detected by 1% agarose gel electrophoresis, and analyzed by sequencing (KU727766).

The PmDNV-JL was inoculated into F81 cells, and CPE was observed on infection of F81 cells with PmDNV-JL at 48 h (Fig. 2 B). The CPE infected to the all F81 cell within 72 h p.i. (Fig. 2 D). DAPI staining indicated strong blue fluorescence bounding the nuclei of infected F81 cells, and the nuclei of infected cells were markedly dilated compared to those of control cells (Fig. 2 E and F). The DNA of the densovirus was also extracted and the presence of densovirus was confirmed in the infected F81 cells (data not shown). These results indicate that the cells were infected by densovirus that may have been able to effectively reproduce in the nuclei of infected cells.

Sequence analysis of the full-length genome of densovirus (PmDNV-JL) showed that it contained 5166 nt. In addition, the genome of this strain contained five ORFs, whose locations were analogous to those in the known BgDNV, which is transmitted by German cockroach (Blattella germanica) (Mukha et al., 2006). Further analysis showed that NS proteins are encoded by ORF3, ORF4 and ORF5 on one DNA strand, and VP proteins are encoded by ORF1 and ORF2 on the complementary strand, respectively (Table 2). The characteristics of PmDNV-JL indicated that it belonged to the genus Densovirus in the subfamily Densovirinae, family Paroviridae.

The phylogeny analyses showed that the predicted amino acid sequences of the strongly conserved domain of NS1 determined the taxonomic status: PmDNV-JL belonged to the BgDNV cluster including the BgDNV-like virus of the genus Densovirus; nevertheless, Brevidensovirus and Iterovirus grouped separately, and were more distinctly related members of the family (Fig. 3A). In addition, the deduced amino acid sequences of the VP1 showed high similarity (100%) to corresponding amino acids from a BgDNV-like virus isolated from a bat in China (Fig. 3B).

PmDNV-JL was detected from bird lung tissue obtained from the Jilin province of China in 2014. Notably, fecal samples from insectivorous bats from other provinces in China, collected in 2012, had many genetically diverse and novel insect viruses including BgDNV-like virus, which was isolated from the feces of insectivorous bats (Ge et al., 2012). Further study is needed to determine whether these viruses can be transmitted among bats or other species in China. Currently, small DNA viruses have been isolated from insects, but the mixed infection of viruses is a common phenomenon in natural conditions. One study has reported that the mixed infection of densovirus and picornavirus is uniquely found in the larvae of the Egyptian cotton leaf worm (Spodoptera littoralis) (Fédière et al., 1999). However, PmDNV-JL was isolated from the lung tissues of wild birds, whether it could be infected by other pathogens (parasite or other virus) or not is unclear.

4. Conclusion

Until recently, Densovirus was found only in insects and crustaceans, as reported by many publications in the past decade (Li et al., 2001; Molthathong et al., 2013; Valles et al., 2013). One report has recently indicated that SsaDV is associated with tissues from semiotic starfishes (Hewson et al., 2014). We investigated a densovirus isolated from the lung tissue of Parus major (PmDNV-JL) in the Jilin province of China. However, it was not possible to determine whether Parus major was infected by the densovirus or if the densovirus came from insects ingested by the bird without infection of avian cells. Hence, the laboratory experiments are required to confirm densovirus incidence or mortality rates and the potential pathogenic mechanisms in avian species.

Statement

(i) All the authors have agreed to its submission and are responsible for its contents.
(ii) All the authors have agreed that Chun-Feng Wang may act on their behalf regarding any subsequent processing of the paper.

Competing interests

The authors have declared that no competing interests exist.

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