Isolation and characterization of the full coding sequence of a novel densovirus from the mosquito Culex pipiens pallens

You-gang Zhai,1 Xin-jun Lv,1 Xiao-hong Sun,1 Shi-hong Fu,1 Zheng-da Gong,2 Yun Fen,2 Su-xiang Tong,3 Zhao-xiao Wang,4 Qing Tang,1 Houssam Attoui5 and Guo-dong Liang1

Correspondence
Guo-dong Liang
gdliang@hotmail.com

1State Key Laboratory for Infectious Disease Prevention and Control, Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 100 Ying Xin Street, Beijing 100052, PR China

2Department of Arbovirus and Chlamydia, Yunnan Institute of Endemic Diseases Control and Prevention, 33 Wenhua Road, Dali City 67100, Yunnan Province, PR China

3Xinjiang Center for Disease Control and Prevention, 1 Jianquan Street, Urumqi City 830000, Xinjiang Uygur autonomous, PR China

4Department of Virology, Guizhou Province Center for Disease Control and Prevention, 73 BaGeYan Road, GuiYang City 550004, Guizhou Province, PR China

5Department of Arbovirology, Institute for Animal Health, Pirbright, Woking, Surrey GU24 0NF, UK

Received 7 June 2007
Accepted 19 September 2007

During an investigation of arboviruses in China, a novel densovirus (DNV) was isolated from the adult female Culex pipiens pallens. The virus, designated Culex pipiens pallens densovirus (CppDNV), caused cytopathic effect in C6/36 cells. The virus particles were icosahedral, non-enveloped and had a mean diameter of 24 nm. The complete coding region of CppDNV was found to be 3335 nt and it contained three open reading frames (ORFs). CppDNV shares 82–93 % identical nucleotides with isolates of the Aedes albopictus densovirus [isolates AalDNV-1, AalDNV-2 (C6/36 DNV) and AalDNV-3], Aedes aegypti densovirus (AaeDNV) and Haemagogus equines densovirus (HeDNV). The nucleotide sequence identity among CppDNV isolates exceeds 98 %. Phylogenetic trees based on non-structural (NS1 and NS2) and capsid (VP) genes show that CppDNV clustered with the species AaeDNV and represents a novel variant of this species within the genus Brevidensovirus.

Densoviruses (DNVs) are classified as members of the genera Densovirus, Iteravirus, Brevidensovirus and Pefudensovirus (subfamily Densovirinae) within the family Parvoviridae. They represent a group of non-enveloped viruses, with single-stranded DNA genomes, encapsidated within icosahedrally arranged viral particles. Their host range is limited to a few closely related invertebrates, particularly insects. However, some DNVs also infect and multiply in shrimps. Members of the genera Densovirus and Iteravirus infect lepidopterans (Tijssen & Arella., 1991). Members of the genus Brevidensovirus infect mosquitoes (Ward et al., 2001), while members of the genus Pefudensovirus infect cockroaches. Members of the genera Densovirus and Pefudensovirus have ambisense genomes that are 5.5–6 kb long. Their structural and non-structural proteins are encoded from separate strands, while those of the genera Iteravirus and Brevidensovirus are monosens (encoded from the same strand) and are ~5 and 4 kb long, respectively (Jousset et al., 1993; O’Neill et al., 1995; Fauquet et al., 2005).

The identification of DNVs in various mosquito cells and wild mosquitoes suggests that the brevidensoviruses have a widespread distribution (O’Neill et al., 1995). The type species of the genus Brevidensovirus is Aedes aegypti densovirus (AaeDNV) (Buchatsky., 1989). The genus contains several other isolates including Aedes albopictus densovirus (AalDNV) [this species contains to date three distinct viruses from C6/36 cells all identified as AalDNV, we shall refer to these in this paper as AalDNV-1 (identified by Jousset et al., 1993; Boublik et al., 1994), AalDNV-2 (identified by Chen et al., 2004) and AalDNV-3 (identified by Paterson et al., 2005)]. Culex pipiens densovirus (CpDNV) was isolated from Culex pipiens larvae; however, it is genetically related to the type species.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are EF579756–EF579771.

Supplementary tables are available with the online version of this paper.
of the genus Densovirus, the Junonia coenia densovirus (JcDNV), isolated from a shrimp with a genome of 6 k (Jousset et al., 2000). The Thai-strain densovirus was isolated from the adult Aedes albopictus and Aedes aegypti mosquitoes from Thailand (Kittayapong et al., 1999). The other two mosquito DNV strains [Toxorhynchites amboinensis densovirus (TaDNV) and Haemagogus equines densovirus (HeDNV)] were found in cell lines of Toxorhynchites amboinensis (TRA-284) and Haemagogus equines (GML-HF-12) by using PCR amplification methods (O’Neill et al., 1995).

Here we report the isolation and characterization of a novel brevidensovirus from wild caught adult Culex pipiens pallens mosquitoes in Jinhzhou city (Liaoning province) during an investigation of arboviruses in China. The virus was designated Culex pipiens pallens densovirus (CppDNV) after the vector it was isolated from. The virus was subsequently isolated from other Culex mosquitoes, including Culex pipiens quinquefasciatus, Culex tritaeniorhynchus, Anopheles sinensis and other mosquitoes collected in various provinces of China.

For the purpose of investigating arboviruses in China, we collected mosquitoes from the villages where livestock were bred in the provinces of Liaoning, Yunnan, Xinjiang and Guizhou. Collection locations were within 10 m of hen houses, hog pens and sheep pens. The mosquitoes were captured using an electrical mosquito aspirator. The captured mosquitoes were sorted (blood-fed and male mosquitoes were excluded from our collections) and then stored in liquid nitrogen. Pools of mosquitoes were mixed with 2 ml minimal essential medium (HyClone) supplemented with 2 mM glutamine, 0.12 % NaHCO₃, 100 U penicillin ml⁻¹ and 100 U streptomycin ml⁻¹, followed by grinding in pre-cooled sterile plastic grinding tubes. The ground samples were centrifuged at 12 000 r.p.m. in a microcentrifuge for 20 min at 4 °C, and the supernatant was filtered through a 0.22 μm syringe-driven filter then inoculated into Aedes albopictus C6/36 and BHK-21 hamster cells. The development of cytopathic effects (CPE) was followed up daily.

Sixteen strains in total were isolated from Culex pipiens pallens (two strains), Culex pipiens quinquefasciatus (two strains), Culex tritaeniorhynchus (three strains), Anopheles sinensis (one strain), unclassified Culex mosquitoes (six strains) and other miscellaneous mosquitoes (two strains) from the four provinces of China (see Supplementary Table S1 available in JGV Online). These viral isolates produced the same CPE in C6/36 cell line. Infected cells became round, aggregated and ended up by forming large syncytia by day 4 post-infection. The inoculated BHK-21 cells did not show any CPE and did not support replication of the novel virus isolates. Serological tests by ELISA showed that these isolates did not react with antibodies against any of the members of the genera Flavivirus, Alphavirus and Bunyavirus. Under the electron microscope, the virus isolates appeared as icosahedral and non-enveloped with a mean diameter of 24 nm, which prompted us to verify if these isolates are DNVs. The viral genome was resistant to RNase treatment. To test if the genome is single-stranded DNA (i.e. a densovirus genome), a primer designated DNV3U (‘-TCCATGATAYAKAWAARAAATA-3’’) was designed from the 5’ non-coding region (NCR) of the available sequences of brevidensoviruses. This primer was used in conjunction with the Klenow DNA polymerase. A 4 kb band was observed by agarose gel electrophoresis (AGE, 1 % gel), which is similar in length to those of the genomes of brevidensoviruses.

To sequence the genome of the various CppDNV isolates, DNA was extracted from the supernatants of infected C6/36 cells using a Genome DNA Extraction kit (Tiangen Biotech) and eluted in 60 μl elution buffer according to the manufacturer’s instruction. Four pairs of degenerate PCR primers were designed from previously published densovirus sequences retrieved from the GenBank database (GenBank accession nos: AY095351, X74945, AY310877 and M37899) (see Supplementary Table S2 available in JGV Online). Amplified products were examined by AGE, purified by QiAquick Gel Extraction kit (Qiagen) and directly sequenced. The contig sequences were assembled using ContigExpress software (Vector NTI Advance 10.0.1 package; Invitrogen). Using the designed primers, overlapping PCR products were obtained which generated a 3498 nt long sequence. The sequences of the 16 CppDNV strains were deposited in the GenBank database (GenBank accession nos: EF579756–EF579771). The base composition of the minus strand of isolate JZ-16 was: A, 20.3; T, 42.0; G, 19.6 and C, 18.1 %. Such a sequence with a high T content was similar to that of other members of the genus Brevidensovirus (AaeDNV, 41.3; AalDNV-1, 40.7 and AalDNV-2, 42.4 %) (Afanasiev et al., 1991; Boublík et al., 1994; Chen et al., 2004). Sequence analysis showed that the highest sequence identities (93 % for nucleic acids and 90 % for amino acids) were depicted with AaeDNV (GenBank accession no. M37899), the type species of the genus Brevidensovirus, confirming that CppDNV is a member of this genus.

Analysis of the assembled PCR products from the 16 CppDNV strains showed that they are organized into a partial 3’ NCR, which is 163 nt long, and a coding region, which is 3335 nt long; organized into three ORFs. ORF1 is 2376 nt long and encodes the non-structural protein NS1 (791 aa). ORF2 encoding NS2 (363 aa) overlaps with ORF1 (however, it is encoded from a distinct reading frame). The start codon of ORF2 is located at position 76 downstream of the ATG of ORF1. ORF3 is 1071 nt long and encodes the capsid protein VP (356 aa). ORF3 overlaps with the 3’ end of ORF1 by 112 nt. The sequence identity among 16 strains of CppDNV was fairly high. The identity of the VP gene of the 16 strains is between 99 and 100 % for nucleic acids, and between 98 and 100 % for amino acids. The same identity for both nucleic acids and amino acids was also found in the NS1 and NS2 genes of the 16 strains (see Supplementary Table S3 available in JGV Online).
NS1 of CppDNV contains characteristic signature motifs of proteins involved in transcription and translation found in other brevidensoviruses (Boublik et al., 1994; Chen et al., 2004). Like other members of the family Parvoviridae, NS1 of CppDNV contains the highly conserved replication start motif between amino acids 315 and 375 as well as the NTP-binding sequence and helicase domain between amino acids 550 and 668 (Fig. 1) (Boublik et al., 1994; Shike et al., 2000; Chen et al., 2004). The function of the NS2 protein, the most highly conserved protein in brevidensoviruses, is still unknown. VP of CppDNV contains the highly conserved sequence RGTKRKR (aa 14–20) located within the glycine-rich region, and it is identical to that found in VP of AalDNV-1, AaeDNV, AalDNV-2, AalDNV-3 and HeDNV. In some members of the family Parvoviridae, this glycine-rich conserved region can be cleaved into smaller VPs by proteases (Cotmore & Tattersall, 1987). However, 2 aa deletions were found in VP of CppDNV when compared with AaeDNV and AalDNV-2 (aa 21–22, encoding DE in AaeDNV and ET in AalDNV-2).

To provide additional insight into the relatedness of CppDNV to other brevidensoviruses, nucleic or amino acid sequences were aligned using CLUSTAL_X 1.8 (Thompson et al., 1997). Calculations of nucleotide and amino acid sequence identities were performed using GeneDoc and DNASTAR (Lasergene). When the complete coding sequence of CppDNV-JZ-16 was compared with those of brevidensoviruses, we found that nucleotide sequence identity ranged between 82 and 93 % (Table 1). The highest identity was found between CppDNV and AaeDNV, with 93 and 90 % at the nucleotide and amino acid levels, respectively. In ORF1 the identity between CppDNV and other brevidensoviruses was 81–95 % at nucleotide and 77–95 % at the amino acid level. CppDNV ORF2 showed the highest identity with those of AaeDNV and AalDNV-2 (95 and 94 %), respectively. The difference in ORF3 between CppDNV and other brevidensoviruses was the highest, with only 79–89 % identity at nucleotide and 76–87 % at amino acid level. The highest identity was also found between CppDNV and AaeDNV (89 % nucleotide identity) in ORF3. The identity between CppDNV and HeDNV, AalDNV-1 and AalDNV-3 was lower (81.6–81.7 % in ORFs 1, 2 and 3). The identity between HeDNV and AalDNV-3 was the highest (98.0 %) as previously described by Roekring et al. (2002).

Moreover, we constructed neighbour-joining phylogenetic trees based on NS1, NS2 and VP genes using MEGA version 3.1 (Kumar et al., 2004). The robustness of phylogenetic constructions was evaluated by bootstrapping using 500 replications. From the results of the phylogenetic trees, we found that CppDNV-JZ-16 strictly clustered within the group constituted by other members of the genus Brevidensovirus, confirming the status of CppDNV as a member of this genus (Fig. 1a, b and c). All three trees gave identical clustering of CppDNV-JZ-16. The virus was closely related to AaeDNV and bootstrap values of more than 80 % were found within the group of brevidensoviruses. Values higher than 95 % confirmed the robustness of branching of CppDNV-JZ-16 and AaeDNV. Within the genus Brevidensovirus, two distinct clusters could be identified. One contains CppDNV, AaeDNV and AalDNV-2, while the other contains AalDNV-1, AalDNV-3 and HeDNV.

Table 1. Percentage sequence identity among brevidensovirus sequences

The complete coding region sequences from GenBank (accession nos are shown within parentheses) for CppDNV (EF579756), AalDNV-2 (AY095351), AalDNV-1 (X74945), AaeDNV (M37899), AalDNV-3 (AY310877) and HeDNV (AY605055) were aligned by CLUSTAL_X and analysed for percentage sequence identity by the MEGALIGN program within the DNASTAR software.

<table>
<thead>
<tr>
<th>CppDNV</th>
<th>AaeDNV</th>
<th>AalDNV-2</th>
<th>HeDNV</th>
<th>AalDNV-1</th>
<th>AalDNV-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CppDNV</td>
<td>93.0</td>
<td>90.0</td>
<td>81.7</td>
<td>81.6</td>
<td>81.6</td>
</tr>
<tr>
<td>AaeDNV</td>
<td>93.0</td>
<td>90.4</td>
<td>81.9</td>
<td>81.6</td>
<td>81.7</td>
</tr>
<tr>
<td>AalDNV-2</td>
<td>90.4</td>
<td>82.4</td>
<td>82.4</td>
<td>82.4</td>
<td>82.0</td>
</tr>
<tr>
<td>HeDNV</td>
<td>90.4</td>
<td>82.4</td>
<td>96.3</td>
<td>96.3</td>
<td>98.0</td>
</tr>
<tr>
<td>AalDNV-1</td>
<td>90.4</td>
<td>82.4</td>
<td>96.3</td>
<td>96.3</td>
<td>98.0</td>
</tr>
<tr>
<td>AalDNV-3</td>
<td>90.4</td>
<td>82.4</td>
<td>96.3</td>
<td>96.3</td>
<td>98.0</td>
</tr>
</tbody>
</table>
In the Eighth report of the International Committee on Taxonomy Viruses, the genus *Brevidensovirus* is reported to contain two species that are designated *AaeDNV* (type species) and *AalDNV* (Fauquet *et al.*, 2005). From our analyses, it is possible to distinguish these two species. We found *CppDNV* clustered within the species represented by *AaeDNV* with high identities between the members (*AaeDNV, AalDNV-2, CppDNV: 90–93 %*). Within the other species represented by *AalDNV*, high identities were also depicted (*AalDNV-1, AalDNV-3 and HeDNV: 95–98 %*). In the past years, three distinct densovirus isolates were obtained from the *C6/36* cell line and are now all identified in databases as *AalDNV-1, AalDNV-2* and *AalDNV-3*. However, it is noteworthy that these three *AalDNVs* belong to two distinct species. The nucleotide identities between members of the two species were 81.6–82.4 %.

*CppDNV* was first obtained from mosquito homogenates collected initially from the Liaoning province of China in the year 2000. Interestingly, wild caught *Culex* and *Anopheles* mosquitoes from other provinces in China in 2005, were also found to contain variants of *CppDNV*. These various isolates were found to show a high degree of conservation (98 and 100 %). Special care was taken to make isolations from each pool on separate days and in separate safety cabinets to avoid contamination and virus carryover. The isolation of *CppDNV* from various locations in the Chinese territory showed that it has a widespread distribution in nature.

The *brevidensoviruses* are widespread in wild mosquitoes (Becnel, 2006; Jousset *et al.*, 2000; O’Neill *et al.*, 1995). These viruses could dramatically interfere with arborvirus isolation from wild mosquitoes. Burivong *et al.* (2004) reported that *AalDNV-1* interfered with dengue virus serotype 2 (DEN-2) replication in *C6/36* cells. The CPE in cell cultures was much less severe, virion production was retarded, and no obvious CPE or cell mortality were observed. The authors concluded that sensitivity of DNV contaminated cell line to DEN-2 infection was depressed. Another example was found in a separate study, where DEN-2 titre was found to be 100-fold less in *AalDNV-2*-infected mosquitoes than in non-infected ones (Wei *et al.*, 2006).

In conclusion, molecular and phylogenetic data showed that *CppDNV* is widespread in China and can infect a wide variety of mosquitoes. The analysis of the phylogenetic relationships and the genome organization of *CppDNV* clearly showed that this virus is a novel member of the species *AaeDNV* alongside the previously identified *AalDNV-2*.

**Acknowledgements**

We thank Dr Wu-yang Zhu and Dr Huan-yu Wang in our department for helpful comments on the manuscript. This work...
References


was supported by grants (to G.-d. L.) from the Ministry of Science and Technology of China (no. 2003BA712A08-01).