Published in final edited form as:

*Arch Virol*. 2016 November ; 161(11): 3231–3235. doi:10.1007/s00705-016-3002-9.

# A new densovirus in cerebrospinal fluid of a case of anti-NMDA receptor encephalitis

**Tung Gia Phan**[1,2], **Kevin Messacar**[3], **Samuel R. Dominguez**[3], **Antonio Charlys da Costa**[1,4], **Xutao Deng**[1], and **Eric Delwart**[1,2,*]

[1]Blood Systems Research Institute, San Francisco, CA 94118, USA

[2]Department of Laboratory Medicine, University of California at San Francisco, San Francisco, CA 94118, USA

[3]Children's Hospital Colorado and University of Colorado School of Medicine, Aurora, CO, USA

[4]Institute of Tropical Medicine, School of Medicine, University of São Paulo, São Paulo, Brazil

## Abstract

We characterized the genome of a highly divergent densovirus, tentatively called human CSF-associated densovirus 1 (HuCSFDV1), in the cerebrospinal fluid (CSF) of a human case of encephalitis with anti-N-methyl D-aspartate receptor antibodies. The presence of this viral genome in CSF was independently confirmed. The proposed new species in the *Iteradensovirus* genus of the *Densovirinae* subfamily showed the typical two ORFs encoding nonstructural and structural proteins with low-level identities of 22 and 16% to the closest known densovirus relative. No other eukaryotic viral sequences were detected using deep sequencing. Replication and pathogenicity in humans of a member of a viral subfamily only known to replicate in invertebrates remain to be demonstrated. Alternative explanations for the detection of densovirus DNA in CSF are discussed.

## The study

Densoviruses are small non-enveloped viruses with linear single-stranded DNA genomes ranging from 4 to 6-kb in the subfamily *Densovirinae* within the family *Parvoviridae*. Members of the *Densovirinae* are known to infect invertebrates, and are responsible for several severe diseases in their hosts [2]. The other *Parvoviridae* subfamily is the *Parvovirinae* infecting mammals and birds. These subfamilies differ most prominently in the relative orientation of their two major genes, NS1 and VP. A new densovirus was recently associated with an extensive outbreak of sea-star wasting disease on the west coast of North America [11]. Densoviruses are also frequently detected in mosquitos, showing variable

*Correspondence to: Eric Delwart, Ph.D., Blood Systems Research Institute, San Francisco, CA 94118, USA. delwarte@medicine.ucsf.edu. Tel: 415-923-5762. Fax: 415-567-5899.

Conflict of Interest: All authors declare that there is no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

degrees of pathogenicity [3, 23]. Densovirus sequences are also reported in feces of rodents [19], bats [8, 14], and camels [30]; likely reflecting viruses in their diets.

Viral metagenomics was used to analyze 53 CSF specimens collected from patients with encephalitis without identified infectious etiology at Children's Hospital Colorado (CHCO) in Aurora, CO, USA. This study was approved by the Colorado Multiple Institution Review Board and informed consent was obtained from all subjects. Pools of 3–5 CSF specimens were clarified by 15,000 × g centrifugation for ten minutes, and then filtered through a 0.45-μm filter (Millipore). The filtrates were treated with a mixture of nuclease enzymes to digest unprotected nucleic acids. Viral nucleic acids were extracted using a Maxwell® 16 automated extractor (Promega). cDNA and DNA were then generated by using random RT-PCR followed by the use of the Nextera XT Sample Preparation Kit (Illumina) to construct a DNA library with each pool identifiable using dual barcodes. The library was deep sequenced with the MiSeq Illumina platform using 250 bases paired ends. Viral sequences were identified through translated protein sequence similarity search (BLASTx) to annotated viral proteins available in GenBank's viral database.

Using a BLASTx cutoff of E scores $<10^{-5}$ one pool of five CSF samples containing $3.27\times10^{6}$ sequence reads showed 221 reads related to densovirus (GenBank SRP075630). Total nucleic acids of each individual specimen within that pool were extracted, and the individual specimen containing the densovirus DNA was identified by PCR. Primers Huca-F1 (5′-CTG GAA GCG ACG AAG ATG GAC T-3′) and Huca-R1 (5′-CTT TGT CCA TCT CCT CTG CGG TG-3′) were used for the first round of PCR, and primers Huca-F2 (5′-ACT ATG CTC TCC CTT TCC GAC-3′) and Huca-R2 (5′-CAT ACT TGT CTC CAG GAA TCT TGT C-3′) for the second round of PCR. The PCR conditions were: 95°C for 5 min, 35 cycles 95°C for 30 s, 54°C (for the first round ) or 50°C (for the second round) for 30s and 72°C for 1 min, a final extension at 72°C for 10 min, resulting in an amplicon of 406-bp.

The patient was a 6-year-old girl who presented in February with her second of three episodes of recurrent encephalitis with altered mental status, aphasia, facial droop, and hemiplegia.The patient had not traveled, had no animal exposures, and had no known insect bites. Lumbar puncture was obtained three days after onset and was notable for 52 white blood cells/$mm^3$ (74% neutrophils, 16% lymphocytes, 10% monocytes), 14 red blood cells/$mm^3$, normal glucose and protein. Magnetic resonance imaging demonstrated diffuse bilateral hyperintensity throughout the cerebral sulci on FLAIR sequences and gyral swelling within the right frontal, right occipital, and left insular lobes. Electroencephalogram demonstrated diffuse encephalopathy. Anti-N-methyl D-aspartate (NMDA) receptor antibodies were identified by indirect fluorescent antibody and cell-based assay in CSF [5, 6]. Pelvic ultrasound was negative for ovarian tumors. The patient was treated with high dose intravenous steroids and rituximab with some improvement, but had significant persistent deficits requiring 7 weeks of inpatient rehabilitation. Of note, 20 months later the patient had a third episode of encephalitis with seizures, MRI changes, and CSF pleocytosis, but returned to baseline without treatment in 3 days.

The near complete genome of a new densovirus was determined by *de novo*-assembly of densoviral reads generated from deep sequencing and named human CSF-associated densovirus (HuCSFDV). Putative ORFs were identified using the NCBI ORF finder. Sequences beyond the ORFS were considered 5' and 3' UTRs. Alignments and phylogenetic analyses were based on the translated amino acid sequences. Sequence alignment was performed using CLUSTAL X. Sequence identities were determined using BioEdit. Phylogenetic analyses were performed using NS1 proteins of the most closely related densoviruses [4]. Phylogenetic trees with bootstrap resampling of the alignment data sets were generated using the maximum likelihood method and visualized using the program MEGA version 6 [24]. Bootstrap values for each node are shown if >70%.

The sequence of the HuCSFDV genome generated here (GenBank KX035107) was 4,678 bases in length, including a partial 5′ UTR (317 bases), nonstructural proteins (NS1, NS2 and NS3), structural protein (VP1), and partial 3′ UTR (120 bases). The VP1 of HuCSFDV was 840-aa in length and showed the highest identity of 16% to that of densovirus infecting the cotton bollworm moth (*Helicoverpa armigera*) in the genus *Iteradensovirus* [31]. The VP1 protein contained the phospholipase $A_2$ ($PLA_2$) motif found in other members of the genus *Iteradensovirus*. The putative NS1 (568-aa) resulted from a predicted spliced transcript in which a potential splice donor site CG↓GT and a potential splice acceptor site AG↓G were identified (Figure 1A) using Alternative Splice Site Predictor [28]. The branch site [AATTGAC] was also found in the intron. Upstream (5') from the splice acceptor site AG↓G a region high in pyrimidines [TATCATTTT] was detected. Based on sequence comparison to known iteradensoviruses (Figure S1), two replication initiator motifs [$^{137}$HYHFLHH$^{143}$, and $^{180}$QLFYVHKGA$^{188}$] and three helicase motifs [$^{399}$KENTLQIVSPPSSGKNFFFDPIFLY$^{423}$, $^{449}$ILYWNEPNFE$^{458}$, and $^{489}$TPVIITTN$^{496}$] were identified in the NS1 [12, 26, 27]. A phylogenetic tree based on the NS1 protein sequences of HuCSFDV and the representatives of five genera in the subfamily *Densovirinae* is shown (Figure 1B) [4]. The NS1-based phylogenetic analysis demonstrates that HuCSFDV shared a common root, although in a basal position, with members of the genus *Iteradensovirus*. The NS1 protein showed the highest identity of 22% to that of a densovirus infecting cotton bollworms (*Helicoverpa armigera*) [31] (Figure 1C). Taken together, HuCSFDV (GenBank KX035107) is therefore proposed as prototype of a new species of the genus *Iteradensovirus* pending ICTV review. Members of the genus *Iteradensovirus* are known to infect members of the Lepidoptera genus (butterflies and moths) and related endogenized genome fragments have been detected in the chromosomes of ticks [15].

In order to confirm the presence of HuCSFDV in the CSF sample and exclude the possibility of local DNA contamination, the same PCR protocol was also independently used at CHCO using a CSF aliquot retained on site and confirmed the presence of HuCSFDV DNA in the original CSF sample. The same PCR assay targeting NS1 was then used to determine the detection rate of HuCSFDV in all 53 CSF specimens in Colorado, USA. No other specimens except the one initially detected by deep sequencing were PCR positive yielding a low detection rate of 1.9% (1/53) in this population. To investigate a possibility of HuCSFDV viremia, plasma samples from this patient were also tested by nested PCR for HuCSFDV DNA. The blood specimens in both acute and convalescent phases, which were drawn one

day before and nine months thirteen days after the CSF collection, were PCR negative for HuCSFDV DNA.

In an attempt to identify the presence of other viruses, the HuCSFDV-positive specimen was then individually (i.e. not in a pool) analyzed using the same metagenomics approach (GenBank SRP075630). The HuCSFDV genome was again sequenced and no other eukaryotic viral sequences were detected.

It is possible to speculate about the origin of a densovirus genome in CSF. Does HuCSFDV expand the known cellular host range of densoviruses from invertebrates to include mammals and play a role in this patient's neurological symptoms? The detection of endogenized parvoviruses in numerous vertebrates as well as tunicates (sea squirt) and of endogenized densoviruses in insects as well as crustacean, arachnids, and flatworms indicates that the host range of *Parvovirinae* and *Densovirinae* subfamilies may be greater than previously appreciated [15]. The absence of endogenized densovirus genes in the vertebrate genomes analyzed so far makes it unlikely that densovirus are able to replicate in vertebrates [15].

Could HuCSFDV be a trigger for anti-NMDA receptor antibodies through molecular mimicry similar to what has been theorized for herpes simplex virus [1, 9, 13, 25, 29]. Alignment of the viral proteins with the NMDA receptor protein did not show regions of strong similarity particularly surrounding the N368/G369 region of the GluN1 amino terminal domain of the epitope conferring antigenicity [10]. Could detection of HuCSFDV DNA in CSF reflect its passive transfer from an undetected insect bite [7, 17, 21, 22, 32] or human infection with an invertebrate in which this densovirus is replicating and releasing viral particles into the CSF? Alternatively, sample contamination could also conceivably occur from the skin or an environmental source during CSF sample collection. Another densovirus genome (Acheta domesticus densovirus in the *Ambidensovirus* genus) [16] was recently detected using metagenomics on nearly all tested skin surfaces of a healthy person over a 16 months period [18] The role, if any, and the cellular origin of HuCSFDV in this case of encephalitis therefore remains unknown but further demonstrates the diversity of viral genomes detectable in clinical CSF samples using deep sequencing [20, 33].

## Acknowledgement

We acknowledge NHLBI grant R01 HL105770 to E.L.D, the Blood Systems Research Institute, and NIH/NCATS Colorado CTSA Grant Number UL1 TR001082 for support. Contents are the authors' sole responsibility and do not necessarily represent official NIH views.

Funding: This study was funded by NHLBI grant R01 HL105770 and NIH/NCATS Colorado CTSA Grant Number UL1 TR001082

## References

1. Armangue T, Titulaer MJ, Malaga I, Bataller L, Gabilondo I, Graus F, Dalmau J (2013) Pediatric anti-N-methyl-D-aspartate receptor encephalitis-clinical analysis and novel findings in a series of 20 patients. The Journal of pediatrics 162:850–856 e852 [PubMed: 23164315]
2. Bergoin M, Tijssen P (2000) Molecular biology of Densovirinae. Contributions to microbiology 4:12–32 [PubMed: 10941568]

3. Carlson J, Suchman E, Buchatsky L (2006) Densoviruses for control and genetic manipulation of mosquitoes. Advances in virus research 68:361–392 [PubMed: 16997017]

4. Cotmore SF, Agbandje-McKenna M, Chiorini JA, Mukha DV, Pintel DJ, Qiu J, Soderlund-Venermo M, Tattersall P, Tijssen P, Gatherer D, Davison AJ (2014) The family Parvoviridae. Archives of virology 159:1239–1247 [PubMed: 24212889]

5. Dalmau J, Tuzun E, Wu HY, Masjuan J, Rossi JE, Voloschin A, Baehring JM, Shimazaki H, Koide R, King D, Mason W, Sansing LH, Dichter MA, Rosenfeld MR, Lynch DR (2007) Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma. Annals of neurology 61:25–36 [PubMed: 17262855]

6. Dalmau J, Gleichman AJ, Hughes EG, Rossi JE, Peng X, Lai M, Dessain SK, Rosenfeld MR, Balice-Gordon R, Lynch DR (2008) Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. The Lancet Neurology 7:1091–1098 [PubMed: 18851928]

7. Fediere G, Li Y, Zadori Z, Szelei J, Tijssen P (2002) Genome organization of Casphalia extranea densovirus, a new iteravirus. Virology 292:299–308 [PubMed: 11878932]

8. Ge X, Li Y, Yang X, Zhang H, Zhou P, Zhang Y, Shi Z (2012) Metagenomic analysis of viruses from bat fecal samples reveals many novel viruses in insectivorous bats in China. J Virol 86:4620–4630 [PubMed: 22345464]

9. Gilbert GJ (2014) Herpes simplex virus-1 encephalitis can trigger anti-NMDA receptor encephalitis: case report. Neurology 82:2041

10. Gleichman AJ, Spruce LA, Dalmau J, Seeholzer SH, Lynch DR (2012) Anti-NMDA receptor encephalitis antibody binding is dependent on amino acid identity of a small region within the GluN1 amino terminal domain. The Journal of neuroscience: the official journal of the Society for Neuroscience 32:11082–11094 [PubMed: 22875940]

11. Hewson I, Button JB, Gudenkauf BM, Miner B, Newton AL, Gaydos JK, Wynne J, Groves CL, Hendler G, Murray M, Fradkin S, Breitbart M, Fahsbender E, Lafferty KD, Kilpatrick AM, Miner CM, Raimondi P, Lahner L, Friedman CS, Daniels S, Haulena M, Marliave J, Burge CA, Eisenlord ME, Harvell CD (2014) Densovirus associated with sea-star wasting disease and mass mortality. Proceedings of the National Academy of Sciences of the United States of America 111:17278–17283 [PubMed: 25404293]

12. Ilyina TV, Koonin EV (1992) Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria. Nucleic acids research 20:3279–3285 [PubMed: 1630899]

13. Leypoldt F, Titulaer MJ, Aguilar E, Walther J, Bonstrup M, Havemeister S, Teegen B, Lutgehetmann M, Rosenkranz M, Magnus T, Dalmau J (2013) Herpes simplex virus-1 encephalitis can trigger anti-NMDA receptor encephalitis: case report. Neurology 81:1637–1639 [PubMed: 24089390]

14. Li L, Victoria JG, Wang C, Jones M, Fellers GM, Kunz TH, Delwart E (2010) Bat guano virome: predominance of dietary viruses from insects and plants plus novel mammalian viruses. J Virol 84:6955–6965 [PubMed: 20463061]

15. Liu H, Fu Y, Xie J, Cheng J, Ghabrial SA, Li G, Peng Y, Yi X, Jiang D (2011) Widespread endogenization of densoviruses and parvoviruses in animal and human genomes. Journal of virology 85:9863–9876 [PubMed: 21795360]

16. Liu K, Li Y, Jousset FX, Zadori Z, Szelei J, Yu Q, Pham HT, Lepine F, Bergoin M, Tijssen P (2011) The Acheta domesticus densovirus, isolated from the European house cricket, has evolved an expression strategy unique among parvoviruses. J Virol 85:10069–10078 [PubMed: 21775445]

17. Ng TF, Willner DL, Lim YW, Schmieder R, Chau B, Nilsson C, Anthony S, Ruan Y, Rohwer F, Breitbart M (2011) Broad surveys of DNA viral diversity obtained through viral metagenomics of mosquitoes. PLoS One 6:e20579 [PubMed: 21674005]

18. Oh J, Byrd AL, Park M, Program NCS, Kong HH, Segre JA (2016) Temporal Stability of the Human Skin Microbiome. Cell 165:854–866 [PubMed: 27153496]

19. Phan TG, Kapusinszky B, Wang C, Rose RK, Lipton HL, Delwart EL (2011) The fecal viral flora of wild rodents. PLoS pathogens 7:e1002218 [PubMed: 21909269]

20. Phan TG, Mori D, Deng X, Rajidrajith S, Ranawaka U, Fan Ng TF, Bucardo-Rivera F, Orlandi P, Ahmed K, Delwart E (2015) Small circular single stranded DNA viral genomes in unexplained

cases of human encephalitis, diarrhea, and in untreated sewage. Virology 482:98–104 [PubMed: 25839169]

21. Shi C, Liu Y, Hu X, Xiong J, Zhang B, Yuan Z (2015) A metagenomic survey of viral abundance and diversity in mosquitoes from Hubei province. PloS one 10:e0129845 [PubMed: 26030271]
22. Sivaram A, Barde PV, Kumar SR, Yadav P, Gokhale MD, Basu A, Mourya DT (2009) Isolation and characterization of densonucleosis virus from Aedes aegypti mosquitoes and its distribution in India. Intervirology 52:1–7 [PubMed: 19332994]
23. Suzuki Y, Barik TK, Johnson RM, Rasgon JL (2015) In vitro and in vivo host range of Anopheles gambiae densovirus (AgDNV). Scientific reports 5:12701 [PubMed: 26220140]
24. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular biology and evolution 30:2725–2729 [PubMed: 24132122]
25. Venkatesan A, Benavides DR (2015) Autoimmune encephalitis and its relation to infection. Current neurology and neuroscience reports 15:3 [PubMed: 25637289]
26. Walker JE, Saraste M, Runswick MJ, Gay NJ (1982) Distantly related sequences in the alpha-and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. The EMBO journal 1:945–951 [PubMed: 6329717]
27. Wang J, Zhang J, Jiang H, Liu C, Yi F, Hu Y (2005) Nucleotide sequence and genomic organization of a newly isolated densovirus infecting Dendrolimus punctatus. The Journal of general virology 86:2169–2173 [PubMed: 16033964]
28. Wang M, Marin A (2006) Characterization and prediction of alternative splice sites. Gene 366:219–227 [PubMed: 16226402]
29. Wickstrom R, Fowler A, Cooray G, Karlsson-Parra A, Grillner P (2014) Viral triggering of anti-NMDA receptor encephalitis in a child -an important cause for disease relapse. European journal of paediatric neurology : EJPN : official journal of the European Paediatric Neurology Society 18:543–546 [PubMed: 24742910]
30. Woo PC, Lau SK, Teng JL, Tsang AK, Joseph M, Wong EY, Tang Y, Sivakumar S, Bai R, Wernery R, Wernery U, Yuen KY (2014) Metagenomic analysis of viromes of dromedary camel fecal samples reveals large number and high diversity of circoviruses and picobirnaviruses. Virology 471–473:117–125
31. Xu P, Cheng P, Liu Z, Li Y, Murphy RW, Wu K (2012) Complete genome sequence of a monosense densovirus infecting the cotton bollworm, Helicoverpa armigera. Journal of virology 86:10909 [PubMed: 22966197]
32. Yu Q, Tijssen P (2014) Iteradensovirus from the Monarch Butterfly, Danaus plexippus plexippus. Genome announcements 2
33. Zhou C, Zhang S, Gong Q, Hao A (2015) A novel gemycircularvirus in an unexplained case of child encephalitis. Virology journal 12:197 [PubMed: 26596706]

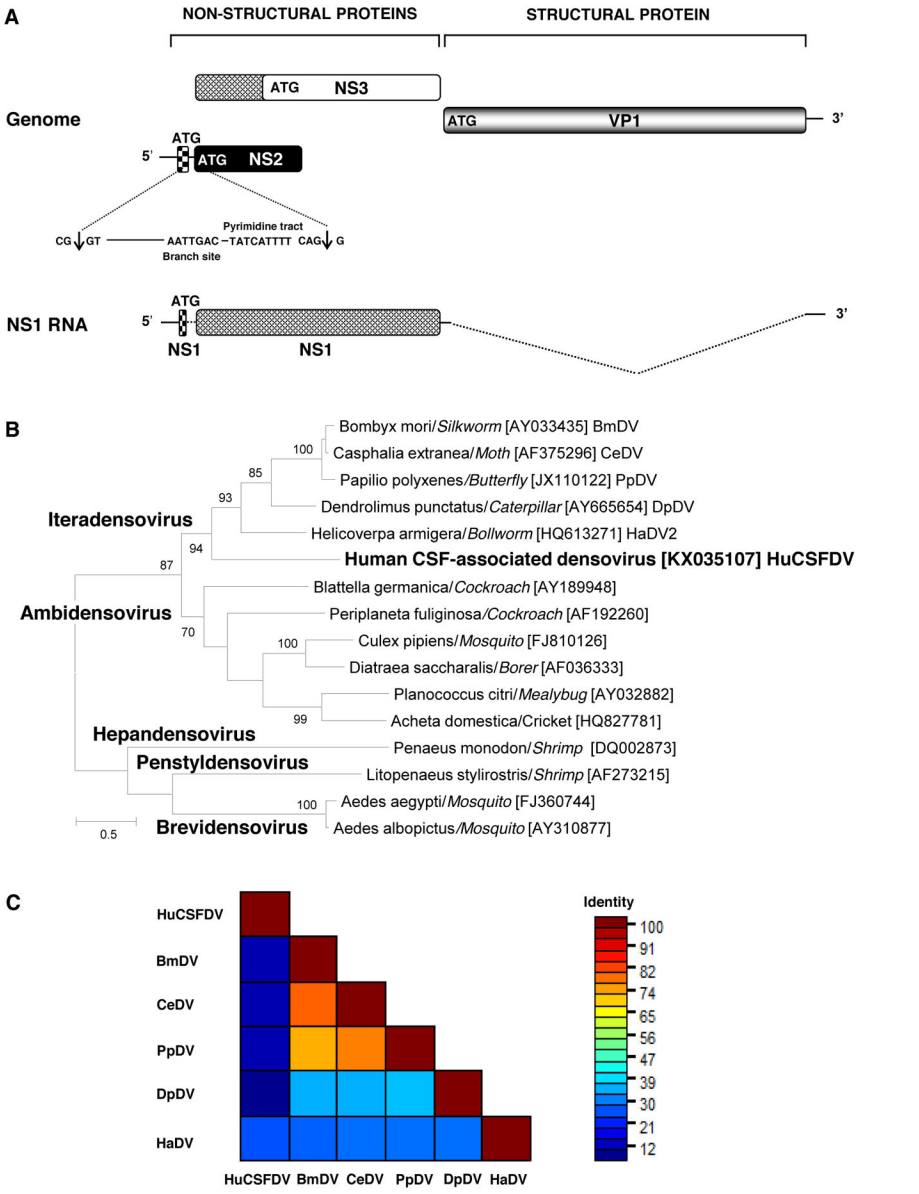


**Figure 1. A new densovirus genome and phylogeny.**

**(A)** Organization of the human CSF-associated densovirus genome. Theoretical splicing for expression of NS1 was shown. **(B)** A phylogenetic tree generated with NS1 of human CSF-associated densovirus and the representatives of five genera in the subfamily *Densovirinae*. The scale indicated amino acid substitutions per position. Bootstrap values (based on 100 replicates) for each node are given if >70. **(C)** Pairwise comparison of NS1 protein of human CSF-associated densovirus and the representatives of five species in the genus *Iteradensovirus*.