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Isolation and molecular characterization of a second serotype of the encephalomyocarditis virus

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ABSTRACT

Encephalomyocarditis virus (EMCV) and *Theilovirus* are the two species of the *Cardiovirus* genus. Whereas theiloviruses comprise several sero-/genotypes, all known EMCV isolates are serologically very similar and are thought to belong to one serotype, named EMCV-1. Here, a novel EMCV type is described. Strain RD 1338 (D28/05) was isolated from a wood mouse (*Apodemus sylvaticus*) in Germany and can be distinguished from EMCV-1 by serological and molecular means. Failure of EMCV-1 specific hyperimmune sera to neutralize RD 1338 suggests a distinct serotype. The viral genome was de novo sequenced using next-generation Illumina/Solexa technologies. Considerable differences of the BC-loop/loop I/loop II sequences of VP1, the VP2 puff and the VP3 knob provide a structural basis for deviant serological properties. Sequence alignments reveal amino acid identities of 75 percent for the P1 region and 84 percent for the P2 and P3 regions when comparing RD 1338 to EMCV-1 strains and some 60 percent and less than 50 percent amino acid identities, respectively, for comparisons with theilovirus strains. Phylogenetic analyses of the P1, 2C and 3CD gene regions support the establishment of an EMCV-2 serotype. In contrast to the theilovirus sero-/genotypes that show a narrow host range, EMCV-1 infects a wide variety of hosts. The host range of EMCV-2 remains to be determined.

1. Introduction

Cardioviruses of the *Picornaviridae* family are non-enveloped icosahedral RNA viruses. The RNA genome has positive strand polarity and a length of roughly 7700–8100 nucleotides; the 5′-end is covalently linked to a small virus-encoded peptide (3B, VPg), the 3′-end is polyadenylated (Knowles et al., 2012). A single open reading frame encodes a polyprotein that is co- and posttranslationally processed into 12 viral proteins. Most cardiovirus proteins have homology to other picornaviral proteins. Among them are four capsid proteins (1A–1D) and several nonstructural proteins (2B and 2C, 3B–3D). Unique cardioviral proteins are the leader (L) protein, the 2A protein and the 3A protein. The L protein was recently described to inhibit the assembly of stress granules upon cardiovirus infection and to trigger nucleoporin hyperphosphorylation in order to inhibit nucleocytoplasmic transport (Borghese and Michiels, 2011; Bardina et al., 2009; Porter et al., 2010). The 2A protein has at least two functions in cardiovirus replication. Firstly, it mediates the processing of the polyprotein precursor by a translation elongation arrest and re-initiation at the next in-frame codon leading to an apparent "cleavage" at the 2A C-terminus (Donnelly et al., 2001); this function is achieved by some twenty amino acid residues at the C-terminus of the protein. Secondly, 2A inhibits translation initiation in cardiovirus-infected cells by binding to

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40S ribosomal subunits (Groppo and Palmenberg, 2007). The function of 3A is largely obscure. A recent study suggests co-localization of 3A with cellular Atg8/LC3, a key protein of autophagy (Zhang et al., 2011). Most nonstructural proteins and their precursors are multifunctional and disrupt the cellular transcription, translation, DNA synthesis and nucleocytoplasmic trafficking. Upon infection, the virus suppresses several cellular pathways and usurps the functions of cellular proteins and supramolecular structures in order to generate an environment that is favorable for genome replication and progeny virus production (Belov and Ehrenfeld, 2007; Cameron et al., 2010).

Picornavirus taxonomy is still in flux as numerous candidates for novel picornaviruses have been described in recent years (Knowles et al., 2012). Previous investigations led to the establishment of two *Cardiovirus* species. First, *Encephalomyocarditis virus* (EMCV) comprising the Columbia SK virus (Jungeblut et al., 1942), the MM virus (Jungeblut and Dalldorf, 1943), the virus of encephalomyocarditis (Helwig and Schmidt, 1945), the Mengo encephalomyelitis virus (Dick, 1948) and mouse Elberfeld (ME) virus isolated by Gönnert in 1949 (Franklin et al., 1959). These strains were derived from various hosts including mice, hamster, chimpanzee and rhesus monkey and are serologically identical (Dick, 1949; Warren et al., 1949). Therefore, it is assumed that they belong to a single serotype. Second, the species *Theilovirus* includes Theiler's murine encephalomyelitis virus (Theiler, 1934), the theravirus of rats (Ohsawa et al., 2003; Drake et al., 2008), the Vilyuisk human encephalomyelitis virus (Sarmanova and Chumachenko, 1960) and several human cardioviruses known as Saffold viruses (Jones et al., 2007; Abed and Boivin, 2008). In the present study, isolation, de novo genome analysis using Illumina/Solexa next-generation sequencing (NGS) and molecular and serologic characterization of a second EMCV serotype is described.

2. Material and methods

2.1. Viruses and cells used in the analysis

Virus strain RD 1338 (D28/05) was isolated from a captive wood mouse (*Apodemus sylvaticus*) and propagated in mouse neuroblastoma NA 42/13 cells. EMCV-1 strains Greece 432, Bulgaria 47/98 and Cuba 1990 served as positive controls in neutralization assays. Strain EMCV-1 LC 75 Cuba was used to generate hyperimmune sera in a guinea pig and a rabbit.

Mouse neuroblastoma cells NA 42/13 (cat. no. 229) were propagated in Eagle Minimum Earle medium supplemented with Earle's salts, 10 percent fetal bovine serum, 100,000 IU/l penicillin, and 100 mg/l streptomycin.

2.2. Neutralization assay

For neutralization assays, hyperimmune sera specific to EMCV-1 strain LC 75 Cuba were generated in a guinea pig and a rabbit. Virus neutralization assays were performed as follows: virus was adjusted to 100–1000 TCID50/100 μl and neutralized with antisera of a known type specificity diluted in twofold steps. The maximum serum dilution which neutralized the virus completely was determined. For controls, immune sera were replaced by normal rabbit sera. After 2–3 days, cells were surveyed for CPE and infectivity titers were calculated.

2.3. RNA isolation

Virus RNA was purified from virus-infected NA42/13 cells. Briefly, Petri dishes with infected cells showing cytopathic effect were freeze-thawed three times to release virus. The medium was transferred to 15 ml tubes and were centrifuged at 4000 × *g* for 20 min. The medium was decanted and virus was precipitated from the supernatant by ultracentrifgation at 100,000 × *g* for 3 h at 4 °C. Viral RNA was extracted from the pellets using the RNeasy® Mini Kit (QIAGEN) according to the manufacturer's instructions and stored at −80 °C.

2.4. Sample preparation and sequencing with Illumina GAIIx

Sample preparation was done with around 500 ng of purified RNA using Illumina's RNA sample preparation kit (RS-100-0801) and Multiplexing Sample Preparation Oligo Kit (PE-400-1001). All steps were done as described in the manufacturer's description except poly-A purification. The isolated RNA was used for library preparation without prior poly-A purification. Sequencing was performed on a GAIIx (Illumina) to create reads with lengths of 76 nt. For assembly, two strategies were followed. First, the reads were mapped to the NCBI nucleotide collection database (nr/nt) to identify picornavirus similar reads. These were used for a de novo assembly using ABySS (Birol et al., 2009) with a k-mer length of 50. Second, the reads were mapped to a set of 12 known EMCV sequences with a low stringency to identify EMCV similar reads. These reads were used in a de novo assembly approach using CLC Genomics Workbench (CLC bio, Cambridge, MA).

2.5. Fragment amplification and cycle sequencing

Reverse transcription was performed with 5 μg viral RNA, 40 U RevertAid Premium reverse transcriptase (Thermo Fisher Scientific/Fermentas) and 20 pmol T-RACE primer (5′-CCGATCGCTCGAGAATAGCCCTTTTTTTTTTTTTTTTTTT-3′), in a final reaction volume of 20 μl. Two microliters of cDNA were subjected to PCR amplification using specific oligonucleotide primer pairs (Table 1). The PCR cycling conditions were as follows: 1 cycle of 94 °C for 5 min; 40 cycles of 94 °C for 30 s, 55 °C for 50 s, 72 °C for 1:30 min, and a final cycle of 72 °C for 7 min, followed by holding at 4 °C. Amplification products were analyzed by gel electrophoresis and gel-extracted employing the QIAquick Gel Extraction kit (Qiagen). Purified amplification products were sequenced with a set of primers (Table 1) by cycle sequencing using the CEQ DTCS Quick Start kit (Beckman Coulter) and analyzed on a CEQ8000 sequencer (Beckman Coulter).