Detection of a Novel Astrovirus in Brain Tissue of Mink Suffering from Shaking Mink Syndrome by Use of Viral Metagenomics

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Received 24 May 2010/Returned for modification 23 June 2010/Accepted 24 September 2010

In 2000, farmed mink kits in Denmark were affected by a neurological disorder. The characteristic clinical signs included shaking, staggering gait, and ataxia. The disease, given the name shaking mink syndrome, was reproduced by the inoculation of brain homogenate from affected mink kits into healthy ones. However, the etiology remained unknown despite intensive efforts. In this study, random amplification and large-scale sequencing were used, and an astrovirus was detected in the brain tissue of three experimentally infected mink kits. This virus also was found in the brain of three mink kits naturally displaying the disease but not in the six healthy animals investigated. The complete coding region of the detected astrovirus was sequenced and compared to those of both a mink astrovirus associated with preweaning diarrhea and to a recently discovered human astrovirus associated with a case of encephalitis in a boy with x-linked agammaglobulinemia. The identities were 80.4 and 52.3%, respectively, showing that the virus described in this study was more similar to the preweaning diarrhea mink astrovirus. For the nonstructural coding regions the sequence identity was around 90% compared to that of the astrovirus, which is associated with preweaning diarrhea in mink. The region coding for the structural protein was more diverse, showing only 67% sequence identity. This finding is of interest not only because the detected virus may be the etiological agent of the shaking mink syndrome but also because this is one of the first descriptions of an astrovirus found in the central nervous system of animals.

The discovery of novel viruses frequently is hindered by the limitations of the conventional techniques for virus detection. Some viruses are difficult to isolate in cell cultures, and the small amount of viral nucleic acid compared to that of cellular nucleic acid is also a problem. Furthermore, sequence information often is lacking or is insufficient. Accordingly, many viruses remain undetected both by conventional methods like virus isolation and by molecular diagnostic techniques such as PCR. To overcome these problems, viral metagenomic approaches have been developed. These methods have been shown to be able to detect both DNA and RNA viruses in different types of material obtained from a vast number of different animals as well as from humans. Many of these approaches are based on the random amplification and sequencing of nucleic acids (2) or on different panviral microarray chips (18, 21). New sequencing technologies, such as 454, Solid, and Solexa, have facilitated the possibility of discovering unknown and emerging novel viruses (14). These technologies circumvent the need for the in vitro replication of the virus and primer design. They are, however, dependent on the availability and use of databases encompassing large amounts of nucleic acid sequences, including those similar to the genomes of the novel agents present in the clinical specimens.

In 2000 a neurological disease, termed shaking mink syndrome (SMS), was observed in farmed minks in Denmark. In 2001 the disease also appeared in Sweden and Finland. The main clinical symptoms were neurological signs, such as shaking, staggering gait, and ataxia. At postmortem examination no gross lesions were observed. Lesions common to nonsuppurative encephalomyelitis were observed by histopathology. The experimental inoculation of healthy mink kits with brain homogenates from minks suffering from SMS led to the development of the same symptoms, indicating that this is a transmissible infectious disease having the central nervous system (CNS) as its main target. Attempts to find the causative agent through virus isolation and bacterial cultures were performed, and in total 17 different agents (viruses and bacteria) known to cause different neurological signs were investigated, with negative results (9).

In this study, random amplification and large-scale sequencing were used to study the presence of possible viral agents in the brain tissue of three experimentally intracerebrally inoculated mink kits. By using this technology, the presence of an astrovirus (SMS-AstV) was discovered in the brain tissue of the minks. Similarly, the PCR analysis of the brain tissue of three mink kits naturally displaying SMS demonstrated the presence of astrovirus in these as well. To elucidate this finding, the detected astrovirus was genetically characterized and compared to other known astroviruses. Astroviruses are small, nonenveloped, single-stranded RNA viruses that can infect humans as well as a number of animals, such as mink, chicken, and calves. In children, astrovirus is known to be a major cause of diarrhea, and in animals astrovirus also is associated with enteric disorders (17). Through a case-control study, mink astroviruses have been shown to be a significant risk factor for preweaning diarrhea (5), and an astrovirus detected in a mink with this disorder has been molecularly characterized (15, 16).

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Published ahead of print on 6 October 2010.
Metagenomic approaches have been used in humans to detect novel divergent astrovirus in patients with diarrhea (6–8), and recently an astrovirus (HAstV-PS) was detected in the brain of an immunosuppressed boy with encephalitis (19).

**MATERIALS AND METHODS**

**Samples.** Brain tissue samples from three experimentally infected mink kits were used for random amplification and large-scale sequencing. To verify the results from the 454 sequencing, brain tissue from three mink kits with the natural occurrence of SMS was also investigated, as well as brain tissue obtained from six healthy mink kits.

**Sample preparation.** The brain tissue was mechanically homogenized in 3 ml of 1× DNase buffer (Roche, Mannheim, Germany) before being centrifuged for 10 min at 4,000 × g. The supernatant was collected and filtered through a 0.45 μm syringe filter (Millipore, Bedford, MA) and treated with RNase and DNase for 2 h at 37°C. After the treatment, half of the sample was used for RNA extraction and the other half for DNA extraction.

**Nucleic acid extraction.** The DNA was extracted using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the blood and body fluid spin protocol and eluted in 50 μl elution buffer (EB). The RNA was extracted using a combination of TRIsol (Invitrogen, Carlsbad, CA) and an RNaseasy mini kit (Qiagen, Hilden, Germany) and eluted in 50 μl EB.

**Tag labeling of cDNA and DNA.** Double-stranded cDNA synthesis, which involves labeling the cDNA at both ends with a special tag sequence, was performed using the primer FR-2nRV (GCC GGA GCT CAG ATA TCG AAT GGA CCG TGG TGA C) according to the manufacturer’s instructions. After the inactivation of superscript at 70°C for 10 and 2 min on ice, the second-strand synthesis was performed with the addition of Klenow fragment (3′ to 5′) reaction using the same primer and temperature exo-) reaction using the same primer and temperature.

**Random amplification.** PCR amplification was performed on both samples using the primer FR-2nRV (GCC GGA GCT CAG ATA TCG AAT GGA CCG TGG TGA C) according to the following procedure: 1× PCR buffer, 2.5 mM MgCl2, 2.5 mM deoxyribonucleoside triphosphates (dNTP), 0.4 mM F primer, 0.4 mM R primer, and 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The amplification was initiated with a 10-min heating step at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 58°C, and 90 s at 72°C. The reaction was run for 1 h at 37°C before a 10-min termination step at 75°C. The DNA was labeled during a Klenow fragment (3′ to 5′) reaction using the same primer and temperature as those for the cDNA reaction. After the labeling reaction, both samples were treated in an identical way.

**Large-scale sequencing.** The sample was sequenced on a shared plate using GS-FLX 454 technology (Roche, Mannheim, Germany) at the Royal Institute of Technology (KTH) in Stockholm, Sweden, according to their standard operation protocol. Briefly, the PCR product was sonicated before adaptors were ligated, and the emulsion PCR was carried out. The emulsion products then were sequenced through pyrosequencing on a picotiter plate.

**Bioinformatic analysis of the data.** The data were analyzed (through quality check and the removal of very short sequences) and then assembled using Lasergene 7.2 (DNastar, Madison, WI). Blastn and Blastx searches were done using PLAN (10) and CLC bio (http://www.clcbio.com/index.php) to identify the contig identity.

**Astrovirus detection.** Based on the sequences obtained from the 454 run, primers were designed to fill the gaps between the contigs. To get the sequence of the 5′ end of the genome, a primer was designed based on the sequence of a mink astrovirus (AY179509) (MiAstV) associated with preweaning diarrhea. The PCR assays were performed, with its respective primer pair (Table 1), using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) under the same conditions as those described for random amplification. The 3′ end of the genome was amplified with a FirstChoice RLM-RACE kit (Invitrogen, Maryland) according to the 3′ rapid amplification of cDNA ends (RACE) protocol provided by the manufacturer. The PCR products were either sequenced directly or cloned and then sequenced. The sequencing was done by Sanger sequencing at Macrogen (Korea).

**Sequence analysis.** The sequences were edited and assembled with Lasergene 7.2. Protein and nucleotide comparisons were done using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A neighbor-joining tree with a bootstrap of 1,000 was constructed with MEGA 4 (20).

**Accession number.** The sequence of astrovirus described in this study has been deposited in GenBank under accession number GU985458.

**RESULTS**

**454 Sequencing data.** The random PCR resulted in a smear of products that ranged from 200 bp to more than 20 kb in size. After the 454 sequencing, a total of around 49,000 sequences with an average length of 212 bases was obtained. After assembly, 1,403 contigs consisting of 38,027 sequences (matched) were created, and 10,756 sequences remained unique (singletons) (Table 2). Four singletons and one contig, consisting of four sequences, were of viral origin. All of these sequences showed similarity to mink astrovirus. The obtained sequences were dispersed over the mink astrovirus genome (Fig. 1).

**Astrovirus detection in SMS brain tissue.** To confirm the astrovirus finding, a PCR assay was set up and run on cDNA obtained from the experimentally infected minks. PCR also was run on cDNA collected from minks with shaking mink syndrome and from healthy minks. All of the healthy minks were negative for astrovirus, while all of the animals suffering

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**TABLE 1. Primers used for the detection of astrovirus and full-length sequencing**

| Primer name | Primer sequence (5′→3′) |
|-------------|-------------------------|
| F12         | TGT GTG TGG CCG TTA TG |
| F364        | ACA AGC GCA CAG CAC TCG |
| R497        | CAA GGC CTC ATA GTG ATG TC |
| F1662       | GTT GGG ATT TCT GAA GGT GT |
| R1890       | CAA GCT CAG CCA CCT TAG C |
| F2335       | CTG ATG TCA CCA AGA CGC T |
| R2354       | GCA GGG TCT TGG TGA CAT C |
| R3373       | AAG GGC TTC AAT CCT ACG CT |
| F3689       | AGC ATT GAA GAG CTA GGG GA |
| R5903       | TAG GTC TGG TCC AAC TGT GA |
| R4501       | CAG AAA GGT GGC AAT CTG CA |
| F4869       | GTC TTA CCG AGG CCA TGG A |
| R4900       | CGG TGA TCT TTT CAT CGT TGA |
| R6443       | AAT CAC GGA GAG CCT CAT AC |

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**TABLE 2. Results of 454 sequencing and assembly of sequences**

| Parameter | No. of sequences | Avg length (bp) | Total no. of bases |
|-----------|------------------|-----------------|--------------------|
| Reads     | 48,786           | 212             | 10,363,149         |
| Matched   | 38,027           | 216             | 8,200,060          |
| Singletons| 10,756           | 201             | 2,163,089          |
| Contigs   | 1,403            | 272             | 382,321            |

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**FIG. 1. Genetic organization of astrovirus and the relative position of the different astrovirus sequences obtained by large-scale sequencing.**
from SMS, as well as the experimentally infected minks, were positive.

**Full-length sequencing and genetic features.** The complete coding region was determined by PCR and by sequencing. The sequence has been deposited in GenBank (GU985458). The sequence was 6,614 nucleotides (nt) in length and displayed a poly(A) tail at the 3' end. The primer designed to the 5' end was positioned 12 nucleotides into the sequence of MiAstV, and therefore around 12 nucleotides are missing at the untranslated region (UTR) of the 5' end. Showorf (EMBOSS) displayed three open reading frames (ORFs), corresponding to ORF1a (2,631 nt), ORF1b (1,557 nt), and ORF2 (2,328 nt). The coding region was flanked with a short UTR on both the 5' and 3' end. The complete coding region was compared to that of the newly described human astrovirus (HAstV-PS; GenBank number GQ89199) connected with a case of encephalitis (19), and the identity was 53%. SMS-AstV showed a higher similarity (80.4%) to MiAstV in analyses of the complete sequence. The comparison of ORF1a and ORF1b from SMS-AstV to those from MiAstV showed that they display 88 to 98% sequence identity on nucleotide and protein levels. ORF2 of SMS-AstV was more diverse, with around 60% identity compared to MiAstV (Table 3). The 5' region of ORF2 was more conserved than the 3' region, as shown in Fig. 2. A heptameric AAAAAC region followed by a secondary stem-loop region was found in ORF1a; this region is thought to be involved in the frameshift between ORF1a and ORF1b. Another conserved region detected was the catalytic triad in ORF1a, which is important for the ability of the serine protease to cleave its substrates.

**Phylogenetic studies.** The constructed neighbor-joining tree showed that SMS-AstV grouped together with MiAstV when using the complete genome sequence (Fig. 3a). The comparison between different mink isolates in Sweden and Denmark was done using a partial region of ORF1b, and in this tree SMS-AstV grouped in a clade different from that of different mink astroviruses associated with preweaning diarrhea (Fig. 3b).

**DISCUSSION**

By utilizing random amplification and large-scale sequencing, we have detected an astrovirus in mink kits experimentally infected with brain homogenate originating from animals suffering from SMS. We confirmed the presence of this virus in minks with a natural occurrence of SMS. However, we did not detect the virus in brain tissue from six healthy mink kits, indicating an etiological connection between the development of SMS and the detected astrovirus.

The genome of SMS-AstV was genetically characterized. Comparative studies of SMS-AstV, MiAstV, and HAstV-PS showed that SMS-AstV is more genetically related to MiAstV than to HAstV-PS. The studies revealed that the ORF1a and ORF1b regions, coding for the nonstructural proteins, are similar between SMS-AstV and MiAstV. On nucleotide and protein levels, the sequence identity covering these regions was 88 to 98%.

Because of the high similarity in ORF1a and ORF1b, it might have been possible to detect the astrovirus in the minks suffering from SMS using a PCR based on the genome sequence of MiAstV. In the study of Gavier-Widén et al., a number of infectious agents were targeted when first trying to find a possible agent causing SMS, such as sarcocystis, canine distemper virus, and parvovirus (9). However, the astrovirus detection assay was not included. The reason for this may be that the focus in 2004 was on known agents associated with encephalitis, and mink astrovirus had at that point never been described in relation to neurological disease, nor had it been shown to be present in the CNS. Recently, an astrovirus was linked to a case of encephalitis seen in a boy with agammaglobulinemia using an approach similar to the one described here (19). The astrovirus in that study is genetically different from the one described in this study, but it shows, together with this work, that astrovirus might have a role in diseases of the CNS. The strength with viral metagenomics, i.e., that these new methods do not target any specific virus but have a general capacity to detect a wide range of infectious agents, including unknown emerging novel viruses, are shown by these examples. Furthermore, metagenomic approaches can be used to understand diseases with a more-complex etiological background, where several agents might be acting together as has been shown in a previously published study (3). After the detection and characterization of detected viruses it is important to make further investigations to determine a possible clear correlation between the presence of the detected agent(s) and the etiology of the disease.

By studying the genome of SMS-AstV, it was found that ORF2, coding for the capsid protein, differed more from

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**TABLE 3. Comparison between SMS-AstV and MiAstV genome**

| Region       | Identity | Nucleotide | Protein |
|--------------|----------|------------|---------|
| Complete     | 0.804    | ND*        |         |
| ORF1a        | 0.853    | 0.926      |         |
| ORF1b        | 0.937    | 0.976      |         |
| ORF2         | 0.668    | 0.594      |         |

* ND, not determined.
MiAstV than did the nonstructural region. The sequence identity was 67 and 59% on nucleotide and protein levels, respectively. It has previously been shown, as well as in this study, that the N terminal of the capsid protein and the end of the C-terminal half is more conserved than the C-terminal half (12). Whether the differences in ORF2 alter the cell tropism relative to the mink preweaning diarrhea associated astrovirus is not known.

Overall, the sequence had certain genetic features that have been shown to be present in many astroviruses, such as a conserved heptamer region followed by a stem-loop region, that have been shown to be involved in the frameshift between ORF1a and ORF1b (11, 13). Other features of the sequence were a transmembrane region, a nuclear localization signal, and the catalytic triad in ORF1a. In studies of astroviruses from different species, SMS-AstV grouped closest with MiAstV and ovine mink astrovirus.

The fact that the clinical symptoms and the histopathological lesions were reproduced through the experimental inoculation of healthy animals with the brain homogenate of kits with a natural disease indicates that SMS is an infectious, transmissible disease (9). SMS-AstV was detected in both the three naturally infected SMS mink kits studied and the three experimentally infected ones, but not in the brain of the six clinically healthy animals. This finding indicates a correlation between the development of the neurological disease and the presence of the newly detected astrovirus. The detection of an astrovirus in the brain of a boy with encephalitis (19) further strengthens the possibility that astrovirus is connected not only to enteric disease but also has a possible role in neurological disorders as shown in this paper. The fact that we did not detect any other viruses in the diseased mink kits through large-scale sequencing further supports the involvement of SMS-AstV in SMS. Considering that these are the first preliminary observations, it is clear that further studies are needed to study in detail the role of astroviruses as a possible agent connected to the etiology of SMS.

In conclusion, we have detected, through viral metagenomics, an astrovirus in the central nervous system of mink kits suffering from SMS. This is one of the first descriptions of an
astrovirus detected in the CNS and associated with neurological disease. Further studies of the possible association between the presence of astroviruses in CNS and the development of neurological disorders are, however, needed.

ACKNOWLEDGMENTS

This work was supported by SIDA/Sarec (SWE-2005-469) and by the Award of Excellence provided to Sandor Belak by the Swedish University of Agricultural Sciences, Uppsala, Sweden.

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