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Mark D. Stenglein  
*University of California San Francisco*

Eric Velazques  
*University of California San Francisco*

Cheryl Greenacre  
*University of Tennessee - Knoxville*, cgreenac@utk.edu

Rebecca P. Wilkes  
*University of Tennessee - Knoxville*, beckpen@utk.edu

J. G. Ruby  
*University of California San Francisco*

*See next page for additional authors*

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Authors
Mark D. Stenglein, Eric Velazques, Cheryl Greenacre, Rebecca P. Wilkes, J. G. Ruby, Julia S. Lankton, Donald Ganem, Melissa A. Kennedy, and Joseph L. DeRisi

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Complete genome sequence of an astrovirus identified in a domestic rabbit (Oryctolagus cuniculus) with gastroenteritis

Mark D Stenglein1, Eric Velazquez2, Cheryl Greenacre2, Rebecca P Wilkes3, J Graham Ruby1, Julia S Lankton3, Donald Ganem1,4, Melissa A Kennedy3 and Joseph L DeRisi1,4*

Abstract
A colony of domestic rabbits in Tennessee, USA, experienced a high-mortality (~90%) outbreak of enterocolitis. The clinical characteristics were one to six days of lethargy, bloating, and diarrhea, followed by death. Heavy intestinal coccidial load was a consistent finding as was mucoid enteropathy with cecal impaction. Preliminary analysis by electron microscopy revealed the presence of virus-like particles in the stool of one of the affected rabbits. Analysis using the Virochip, a viral detection microarray, suggested the presence of an astrovirus, and follow-up PCR and sequence determination revealed a previously uncharacterized member of that family. Metagenomic sequencing enabled the recovery of the complete viral genome, which contains the characteristic attributes of astrovirus genomes. Attempts to propagate the virus in tissue culture have yet to succeed. Although astroviruses cause gastroenteric disease in other mammals, the pathogenicity of this virus and the relationship to this outbreak remains to be determined. This study therefore defines a viral species and a potential rabbit pathogen.

Keywords: Rabbit astrovirus, Rabbit virus, Virus discovery, Virochip, Gastroenteritis, Diarrhea virus, Mucoid enteropathy, Enterocolitis

Background
The astroviruses form a family of small, non-enveloped, positive strand RNA viruses that infect a variety of mammalian and avian hosts [1-22]. First identified in human stool samples in 1975, these viruses were named after their star-shaped appearance in some electron micrographs [2,3]. Astroviruses have been shown to replicate in cells of the intestinal tracts of infected organisms, and in some hosts, infection has been demonstrated to cause gastroenteritis [6,13,23-31]. In birds, astroviruses have been linked to intestinal and extra-intestinal pathology [10,32-35].

High throughput, unbiased molecular methods can be used to identify candidate etiologic agents in diseases of unknown origin [36-38]. The Virochip is a DNA microarray that has been used to identify known and to discover novel viruses [39,40]. Next generation sequencing provides a complementary virus discovery method, and is being increasingly used as its cost decreases. The sensitivity of these methods decreases as divergence from known viruses increases and as abundance of the viral nucleic acid in the sample decreases.

In this study, the Virochip microarray was used to screen samples from an outbreak of gastroenteritis in a commercial rabbit colony in Tennessee, USA. A virus was suspected as one possible cause because of virus-like particles in electron microscopic examination of stool from an affected animal, but traditional diagnostic approaches, including virus isolation, failed to identify a candidate viral pathogen. Virochip screening suggested that an astrovirus was present, and subsequently the complete genome was recovered and characterized. Recently, the partial genome sequence of a related virus was recovered from rabbits in Italy [22]. The astroviruses form a diverse family of viruses that infect many animals, and metagenomic investigations such as the
one described here will continue to reveal the full extent of their diversity and host range.

Results
Outbreak and clinical diagnostics
A domestic rabbit colony of about 100 animals of various breeds near Johnson City, TN, USA, experienced an outbreak of enterocolitis. The predominant rabbit breeds were angora, satin and mini rex. No changes in diet had occurred in 1.5 years. The rabbits were housed outdoors, on the ground, in an enclosure measuring 8 x 20 feet with an attached hutch. The owner reported voles in the area. Characteristics of the outbreak included a 1–6 day history of lethargy, bloating and diarrhea, followed by death. Initially, animals under 5 months old were affected (~75 over the next 3 weeks), then the adults were affected (~15 in the first 2 weeks), then the adults were affected (~75 over the next 3 weeks). Consistent clinical signs included lethargy, dehydration, increased intestinal sounds with intestinal gas (bloating) and cecal impaction.

Fort Dodge). Necropsy findings on seven rabbits (2 dead on arrival, 5 euthanized) were all consistent with mucoid diarrhea. Severe intestinal coccidiosis (Eimeria spp.) was identified and the entire colony was placed on sulfamethazine in the water (Sulmet, 12.5%, Fort Dodge). Necropsy findings on seven rabbits (2 dead on arrival, 5 euthanized) were all consistent with mucoid enteropathy and cecal impaction. (Table 1) At necropsy, common gross findings within the intestinal tract included mucoid intestinal contents (5/7), cecal impaction (4/7), serosal hemorrhage (4/7) and gastrointestinal gas distention (4/7). Microscopically, common findings included lymphoplasmacytic enteritis (5/7), necrotizing heterophilic enterotyphlocolitis (2/7), intestinal coccidiosis (5/7), and gastric or cecal candidiasis (2/7). In addition to the intestinal coccidiosis identified in all animals tested, one rabbit had hepatic coccidiosis (Eimeria stiedae), two had Trichuris spp., and 4 of 5 small intestinal cultures grew E. coli that was found not to be an attaching effacing E. coli. 30 nm virus-like particles were observed by electron microscopy in the stool of 1 of 2 animals tested (Figure 1 and Table 1). Follow-up PCR-based testing determined that this virus was not Rabbit Hemorrhagic Disease Virus. Initial attempts to isolate virus from 2 samples in tissue culture were unsuccessful.

Genome recovery and phylogenetic analysis
Because virus-like particles were observed but diagnostic testing failed to identify known viruses, several samples were subjected to Virochip analysis. Total nucleic acid was extracted from the stool of four sick animals and one unaffected control, fluorescently labeled, and hybridized to the Virochip (other case samples lacked sufficient remaining material). To enrich for viral nucleic acid, the stool sample from the EM virus-positive sample was filtered and treated with micrococcal nuclease, to digest non-protected nucleic acid. Virochip analysis of the nuclease-treated sample suggested the presence of astrovirus-related sequence (Table 1 and Figure 2).

To validate this finding, several sets of degenerate primers were designed based on the positive Virochip probe sequences and on conserved regions of astrovirus genomes (Figure 2 and Table 2). PCR using one of these primer pairs produced an amplicon of the expected size, which was cloned and sequenced, confirming that nucleic acid from an apparently divergent astrovirus was present in the sample. Additional degenerate primers and 3’ RACE were used to recover a 5 kbp contiguous sequence. But, repeated attempts using 5’ RACE and other PCR-based strategies failed to recover the full predicted ~7 kb genome.

To recover the remainder of the genome, metagenomic sequencing using the Ion Torrent PGM instrument was performed [41]. This produced 3x10⁶ sequences of median length 125 nucleotides (standard deviation 17.5). The average Sanger quality score of these sequences was 19.5 at base 1, 17.3 at base 100, and

Table 1 Summary of diagnostic findings

<table>
<thead>
<tr>
<th>Animal #</th>
<th>Enteritis</th>
<th>Died / Euthanized</th>
<th>Parasites</th>
<th>Culture</th>
<th>EM VLPs</th>
<th>Astrovirus Virochip</th>
<th>Rabbit astrovirus RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>Died</td>
<td>Intestinal Eimeria spp.</td>
<td>No growth</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>Euthanized</td>
<td>Trichuris spp.</td>
<td>E. coli</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C. perfringens</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>Died</td>
<td>Intestinal Eimeria spp.</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hepatic Eimeria spp.</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>Euthanized</td>
<td>Intestinal Eimeria spp.</td>
<td>E. coli</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>Euthanized</td>
<td>Intestinal Eimeria spp.</td>
<td>E. coli</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>Euthanized</td>
<td>Intestinal Eimeria spp.</td>
<td>E. coli</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>Euthanized</td>
<td>Intestinal Eimeria spp.</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8 (Control)</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

EM VLPs, virus-like particles evident by electron microscopic examination. ND, not determined. E. coli, Escherichia coli. C. perfringens, Clostridium perfringens.
14.4 at base 150, quality values consistent with previously reported metrics for this sequencing platform [42]. Insertions and deletions, which occurred at a frequency of 1.3% (1.3 indels per 100 bases aligned), were more frequent than mismatches, which occurred at a frequency of 0.28%. Retrospective mapping of the sequences revealed that $1.9 \times 10^5$ unique sequences (6% of sequences) derived from the astrovirus, corresponding

**Figure 1** Electron micrograph of virus like particles in the stool of one animal (Table 1). Scale bar indicates 50 nm.

**Figure 2** The rabbit astrovirus genome. (A) A cartoon showing the organization of the viral genome. Scale bar indicates nucleotide position. The location of a putative ribosomal frameshift motif is indicated by an arrowhead. The position of the two overlapping Virochip probes that were originally positive for an astrovirus are indicated. (B) Location of overlapping PCR, 5', and 3' RACE amplicons used to determine and confirm the sequence of the viral genome. (C) A histogram showing the number of Ion Torrent reads ($x1000$ reads) aligning at each nucleotide position in the genome. (D) The average pairwise percent identity between the predicted rabbit astrovirus protein sequences and the sequences of the proteins from the astroviruses shown in Figure 3. The histogram is binned into 20 amino acid windows.
to >1000x average coverage of its genome (Figure 2). The PRICE de novo targeted assembly software (Graham Ruby, http://derisilab.ucsf.edu/software/price/) was used to assemble the entire viral genome, the sequence of which was validated by RT-PCR and Sanger sequencing (Figure 2). 5’ RACE was used to confirm that the Ion Torrent-based assembly extended to the 5’ terminus of the genome (Figure 2). The remainder of the reads in the metagenomic dataset consisted mainly of sequences derived from bacteria (~76% of taxonomically assigned reads), with the Escherichia, Akkermansia, Clostridium, Salmonella, Enterobacter, and Shigella genera being most prevalent. 56 sequences (0.002% of all sequences) aligned best to Eimeria species, consistent with the clinical diagnosis of coccidiosis (Table 1). A small percentage (0.4%) of human sequences were also evident in the data. These were likely laboratory contaminants, as the majority of them aligned to the breast cancer 1 and 2, early onset genes (BRCA1 and BRCA2), and targeted sequencing of these genes was conducted in the laboratory where the sequencing libraries were prepared. Library contamination was also the likely source of 27 reads with nucleotide sequences nearly identical to human immunodeficiency virus-1 (HIV-1) database sequences.

In an attempt to propagate and isolate the virus, stool from the positive sample was filtered and used to inoculate a rabbit kidney cell line (RK) culture and cultures of several other mammalian cell lines previously shown to support the replication of human astroviruses: Vero, HT-29, and Caco-2 [43-45]. These cultures were maintained for 15 days, and culture supernatants were collected on days 1, 2, 4, 6, 10, and 15 post inoculation. Rabbit astrovirus RNA was detected in the day 1 supernatant (in the inoculum) by RT-PCR but not in later time points, suggesting that the virus did not replicate in these cultures.

A phylogenetic analysis of the predicted rabbit astrovirus protein sequences was performed. The rabbit astrovirus

| Table 2 Oligonucleotides used in this study |
|-------------------|-------------------|
| Oligo name   | Sequence (5’ – 3’) |
| MDS-4        | CGCTCTTCGATCTNNNNN |
| MDS-189      | CTGTCGCTGGCTCCCCAGCT |
| MDS-115      | CCATCGGTCAWNTCAACAAC |
| MDS-116      | CTGCTAAHTAYGDDGATGA |
| MDS-117      | GGTITNACCATNCCAAA |
| MDS-118      | GTCCAACCTGADNCCACARAA |
| MDS-119      | ATATAAACATGTCACTTGGCTTC |
| MDS-120      | GACATCTTATATATTTACAACATTTT |
| MDS-156      | GCCCTGTATACAAAAAATCAC |
| MDS-157      | CTCATAGGGCTCTCGTCTG |
| MDS-298      | CGGGCAGAGGCTATTACCC |
| MDS-296      | GGCTAGAGGAATGGGGTCAG |
| MDS-299      | GCTGCGTTCGATAATTTGCTC |
| MDS-300      | GCAACTAACCACGGCAATG |
| MDS-144      | TCTGGACYGARGARGAART |
| MDS-300      | CACTCCATCGAGGTAACCAA |
| MDS-135      | TCCACCGCGGCTCACCCCAA |
| MDS-145      | CCACCCACAATCAGAGGTC |
| MDS-119      | ATATAAACATGTCACTTGGCTTC |
| MDS-125      | CATAACGATGGGAAGGAGGAG |
| MDS-147      | CTTCGAGCCACTCTCTG |
| MDS-137      | TTGTCTCCCTTCCA |
| MDS-138      | TGGTGTTTGT |
| MDS-150      | TCTGAAATGGCAGCTGTTGG |
| MDS-121      | CAATGACGAGGATTGAGGAG |
| MDS-123      | GAGGACTCGAGCTCAACG |

sequences were compared to those of all the astroviruses in GenBank for which a complete genome sequence exists (see Methods). Analysis with the three major viral polyproteins, nsp1A, nsp1B (RdRp), and capsid (see Discussion) produced overall similar tree topologies (Figure 3). The rabbit astrovirus sequences branch basally on the phylogram, with the closest related sequences being those of Astrovirus MLB1, which was isolated from a human [46]. The rabbit virus nsp1A, nsp1B, and capsid proteins share 30%, 59%, and 25% pairwise amino acid identity with the corresponding Astrovirus MLB1 protein sequences. In the phylogenies based on nsp1A and nsp1B, the clade formed by the rabbit virus and Astrovirus MLB1 is supported by 100% and 81% of bootstrap replicates, respectively (Figure 3A and B). In contrast, the rabbit virus capsid sequence does not form a well-supported clade with the astrovirus MLB1 capsid sequences, but instead branches along the lineage leading to the canonical human astroviruses.

Diagnostic primers were designed to specifically detect rabbit astrovirus (MDS-119 and −120; Table 2). RT-PCR using these primers indicated that, of the five stool samples collected (from four sick and one control rabbit), only the EM-positive sick rabbit tested positive for the virus (Table 1).

Discussion
In this study, we describe the identification and genomic characterization of a member of the Astroviridae family of viruses. Traditionally, astroviruses have been named after the host species from which they were identified, so we propose naming this virus species Astrovirus rabbit/TN/2009/USA.

This virus was isolated from the feces of a rabbit suffering from enterocolitis, but it is not clear that the virus caused the disease in this instance. Indeed, samples from three of the other four sick rabbit from the same outbreak tested negative for the virus (Table 1). Nevertheless, astroviruses cause gastroenteritis in other mammals, and it is possible that this virus caused or exacerbated the illness in a subset of the animals [13,24,25,28,29,47]. The coccidian parasites and bacteria evident in the samples may have also contributed to the
enteritis and from this data alone, it is impossible to as-
certain whether this astrovirus is capable of causing dis-
ease. Unfortunately, the sample material was limited, and
tries to propagate the virus in culture were un-
successful, so experiments to directly demonstrate dis-
ease causality were not possible. It may be possible to
generate an infectious clone of the virus, as has been
accomplished for other astrovirus species [48]. Screening
of additional rabbits with diarrhea will provide additional
epidemiological information and another source of virus
for follow-up experiments.

Indeed, as this manuscript was being prepared, the
partial sequence of an astrovirus recovered from rabbits
in Italy was reported [22]. In this report, viral RNA was
detected by RT-PCR in 10 of 23 rabbits (43%) with en-
teritis and in 18% of 139 asymptomatic animals, and the
mean copy number was ~100-fold higher in the feces of
the sick rabbits compared to the apparently healthy ani-
imals. The 3395 nt sequence (accession JN052023) in the
Martella et al. report includes a portion of ORF1B and
ORF2, and is 92% identical at the nucleotide level to the
sequence recovered from the Tennessee rabbit. Thus,
similar astroviruses appear to be a common and geo-
graphically widespread infection of rabbits, and an
increased viral load is associated with enteritis.

Astroviruses were so named because of their star-
shaped appearance in electron micrographs [3]. Although
non-enveloped virus-like particles of a size typical of
astrovirus (~30 nm) were observed in the astrovirus
RNA-positive sample, it is not clear that these particles
belong to the astrovirus. It is important to note that
as few as 10% of particles in a preparation may exhibit
the characteristic star-shaped morphology [3].

The rabbit astrovirus genome contains characteristics
typical of astrovirus genomes, and it is therefore possible
to make predictions about viral gene expression and
function [1]. Like other astroviruses, the rabbit astro-
virus genome is about 7 kb in length and contains
two large open reading frames, named ORF1A, ORF1B,
and ORF2. It is likely that ORF1A can be translated by
itself or in conjunction with ORF1B via a −1 ribosomal
frameshift at a conserved AAAAAAC motif (Figure 2)
[49-51]. ORF1A and ORF1AB encode the viral non
structural polyproteins nsp1A and nsp1ab. ORF2 encodes
the viral structural capsid polyprotein. These polyproteins
are predicted to be proteolytically processed into multiple
domains with discrete functions [52-57].

Astrovirus nsp1A proteins contain protease domains,
but apart from this their function is not well character-
ized. According to NCBI’s Conserved Domain Database
search tool (CDD) the predicted rabbit astrovirus nsp1A
protein contains two conserved domains: a serine prote-
ase motif, and a chromosome segregation protein DNA-
binding domain [58]. This DNA binding-related domain
may bind RNA and be involved in viral replication. As
has been reported for other astroviruses, the nsp1A pro-
tein is predicted to contain several transmembrane and
coiled coil domains [59]. It is likely that the nsp1A con-
tains other functional domains of as yet unknown func-
tion. rabbit astrovirus nsp1A contains a 76 amino acid
insertion between residues 674 and 749 not found in
related protein sequences. These residues contain no ob-
vious identifiable domain.

ORF1B is predicted to encode nsp1B, which results
from proteolytic cleavage of nsp1ab. Nsp1b is the RNA-
dependent RNA polymerase (RdRp), and this is the only
domain identifiable by CDD or BLAST search. The
nsp1B protein is the most conserved of the three poly-
proteins (Figure 2).

ORF2 encodes the viral structural (capsid) precursor
polyprotein. As is the case with other astroviruses, the
structural polyprotein may be translated from a subge-
nomic RNA, although no evidence of such a species is
evident in the deep sequencing coverage levels (Figure 2)
[60,61]. It is possible that the subgenomic RNA is
present at higher levels in infected cells than in extra-
cellular virus particles, which are likely the source of
the filtered material sequenced. The region defined by
the first 70 amino acids of the ORF2-encoded protein is
basic, with a predicted isoelectric point of 13.3. This is
consistent with related astrovirus proteins, in which this
domain has been reported to be involved in nucleic acid
binding [55]. The N-terminal ~450 amino acids of the
capsid protein align by BLAST with other astrovirus
capsid proteins, but no recognizable protein domains
are contained in the C-terminal 400 amino acids, and
the latter half of the protein exhibits a much lower
degree of sequence similarity to other astrovirus pro-
teins sequences (Figure 2). This is in agreement with the
model that the N-terminal half of astrovirus capsid
polyproteins form a conserved core domain and the C-
terminal halves of the proteins are highly variable recep-
tor interacting domains [62]. ORF1A is preceded by a
dis untranslated region, which is short but within the
size range (11–85 nt) previously reported for astro-
viruses [1]. The 87 nt predicted 3’ untranslated region is
followed by a polyA tail.

Conclusions

Here, we identify and fully characterize the genome of
an astrovirus present in the stool of a rabbit suffering
from a fatal intestinal disease. This was enabled by the
combination of two complementary genomics techni-
qules: the Virochip microarray and high throughput se-
quencing on the Ion Torrent PGM platform. Although
the precise contribution of this virus to the observed
pathology remains to be determined, related viruses
cause similar disease in other mammals, and this virus is
For microarray hybridization, a fraction of each library was amplified by PCR as above but with a modified dNTP mixture including 5-(3-aminoallyl)-dUTP (Ambion) in lieu of 75% of the dTTP normally in the mixture. The resulting amino-allyl-containing DNA was purified using a DNA Clean and Concentrator-5 column (DNA-CC-5; Zymo Research). The eluate was heat denatured at 95°C for 2 min, cooled briefly on ice, then fluorescently labeled in reactions containing 100 mM sodium bicarbonate, pH 9, 10% DMSO, and 667 μM Cy3 mono NHS ester (GE Healthcare) for 1 hour at 25 °C. Labeled DNA was purified using the DNA-CC-5 columns and added to hybridization reactions containing 3xSSC, 25 mM HEPES pH 7.4, and 0.25% SDS. Hybridization mixtures were heated at 95°C for 2 minutes then applied to microarrays and hybridized overnight at 65 °C. Following hybridization, arrays were washed twice in 0.57xSSC and 0.028% SDS and twice in 0.057x SSC; then scanned on an Axon GenePix 4000B microarray scanner. Three tools were used to analyze Virochip data: E-predict [32], Z-score analysis [33], and cluster analysis [34].

**PCR/Sanger sequencing**

Oligonucleotide sequences used to amplify the rabbit astrovirus genome are listed in Table 2. PCRs contained 1x reaction buffer, 2 μM primer, 0.25 mM dNTPs, 2 U Taq DNA polymerase, and 2 μl library template. Thermocycling was: 95°C for 2 min, then 30 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 2 min, with a final extension of 72°C for 5 min. Amplicons were purified from agarose gels using the PureLink gel extraction kit (Invitrogen) and cloned into the pCR2.1 TOPO vector (Invitrogen) according to the manufacturer’s protocols. Cloned amplicons were sequenced on an ABI 3700 instrument. For diagnostic testing, primers MDS-119 and −120 (Table 1) were used with reaction and thermocycling as described above.

**5’ and 3’ RACE**

5’ and 3’ RACE were performed essentially as described [64,65], with primers listed in Table 2. RACE amplicons were cloned and sequenced as described above.

**Ion Torrent metagenomic sequencing**

Randomly primed cDNA was prepared in reverse transcription reactions containing 1x reaction buffer, 5 mM dithiothreitol, 1.25 mM dNTPs, 20 pmoles primer MDS-189 (Table 2). PCRs contained 1x reaction buffer, 2 μM primer, 0.25 mM dNTPs, 2 U Taq DNA polymerase, and 2 μl library template. Thermocycling was: 95°C for 2 min; 2 cycles of 95°C for 30 sec, 40°C for 30 sec, and 72°C for 1 min, then 25 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min, with a final extension of 72°C for 5 min.
analysis to eliminate possible PCR duplicates. Sequencing was performed on an Ion Personal Genome Machine on one model 314 chip and one model 316 chip according to the manufacturer’s protocols.

Tissue culture
100 μl of stool positive for rabbit astrovirus RNA by RT-PCR was diluted 1:10 in PBS and filtered through a 0.22 μm filter (Millipore). Filtrate was used to inoculate cultures of RK, Vero, HT-29, or Caco-2 cells, which were grown in DMEM supplemented with 10% fetal bovine serum and 50 units/ml penicillin and 50 μg/ml streptomycin at 37°C and 5% CO2. Cultures were supplemented with 0, 1, 10, or 100 μg/ml trypsin, which has been shown to be necessary for astrovirus infectivity in tissue culture. Culture supernatant was harvested and replaced at the indicated time points and stored at −80°C until processing. RNA was extracted from supernatant and reverse transcribed as described above. PCR using primers MDS-119 and −120 for rabbit astrovirus and MDS-156 and −157 for rabbit rRNA (as a positive RT-PCR control) was used to detect viral RNA, with reaction conditions as described above (See Table 2 and [66]).

Bioinformatics
Astrovirus protein sequences for all astroviruses with complete genome sequences were downloaded from GenBank. The accessions of these sequences are: AB308374, AF260508, AY179509, AY720891, AY720892, DQ028633, DQ070852, DQ344027, EU111742, FJ222451, FJ402983, FJ434664, FJ755402, FJ755403, FJ755404, FJ755405, FJ919225, FJ919226, FJ919227, FJ919228, FJ973620, GQ145660, GQ495608, GQ891990, GU985458, HM237363, HM450380, HQ398856, HQ916313, HQ916314, HQ916316, HQ916317, IF414802, IF755422, I23513, NC_001943, NC_002469, NC_002470, NC_003790, NC_004579, NC_005790, NC_010646, NC_011400, NC_012437, NC_013060, NC_013443, NC_014320, NC_015936, and Y15937. Sequences were trimmed to the point of clear homology. The ORF2 (capsid) alignment is based on the first ~420 amino acids of the conserved core region of the capsid protein. Neighbor joining trees were constructed using PhyML software (version 2.2.12) using 100 bootstrap replicates and default parameters [68]. The sequence of the rabbit astrovirus has been deposited in GenBank, with accession IF7729316.

The BLAST alignment software (version 2.2.25+) was used to taxonomically categorize the sequences in the Ion Torrent dataset [69]. The NCBI non-redundant nucleotide database was searched using the blastn algorithm with an expect value cutoff of 1e-6. For each query producing an alignment, the taxonomic ID of the best alignment was determined and tallied. The Paired-Read Iterative Contig Extension (PRICE, version 0.13) de novo assembler (freely available at: http://derisilab.ucsf.edu/software/price/index.html) was used to assemble the rabbit astrovirus genome from the Ion Torrent dataset.

Several tools were combined to determine coverage, quality, and error metrics. First, CD-HIT was used to collapse identical sequences (cd-hit-est version 4.5.4 run with parameter −c 1), which may be PCR duplicates [70]. Then, the bowtie2 aligner was used to map unique reads to the Sanger-verified viral genome assembly (version 2.0.0 run in −local mode with otherwise default parameters; [71]). The number of gap openings (XO field) and mismatches (XM field) and the total number of aligned bases in the bowtie2 SAM output were tallied to determine the frequency of indels and mismatches. Average per base quality scores were determined directly from the Ion Torrent FASTQ output.

Competing interests
The authors declare no competing interests.

Authors’ contributions
MDS performed library preparation, microarray analyses, genome sequencing, bioinformatic analyses, and drafted the manuscript. EV performed library preparation and microarray analyses. CG extracted nucleic acid and conducted clinical examinations and analyses. RW performed electron microscopy and nucleic acid extraction and clinical diagnostics. JGR performed pathologic examination and analyses. DG, MAK, and JLD oversaw project design and coordination. All authors read and approved the final manuscript.

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Author details
1Departments of Medicine, Biochemistry and Biophysics, and Microbiology, University of California San Francisco, San Francisco, CA, USA. 2Department of Small Animal Clinical Sciences, University of Tennessee College of Veterinary Medicine, Knoxville, TN, USA. 3Department of Biomedical and Diagnostic Sciences, University of Tennessee College of Veterinary Medicine, Knoxville, TN, USA. 4Howard Hughes Medical Institute, Chevy Chase, MD, USA.

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