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Short communication

Characterization of an outbreak of astroviral diarrhea in a group of cheetahs (*Acinonyx jubatus*)

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ABSTRACT

A *Mamastrovirus* was identified in an outbreak of diarrhea in cheetahs (*Acinonyx jubatus*). Five young adult and two adult cheetahs presented with lethargy, anorexia, watery diarrhea and regurgitation over an 11-day period. Fecal samples were submitted for electron microscopy and culture. Electron microscopy results revealed particles morphologically consistent with an astrovirus, and no other viral pathogens or significant bacterial pathogens were identified. The astrovirus was confirmed and sequenced using consensus astroviral PCR, resulting in a 367 base pair partial RNA-dependent-RNA polymerase (RdRp) product and a 628 base pair partial capsid product. Bayesian and maximum likelihood phylogenetic analyses were performed on both the RdRp and the capsid protein segments. All animals were monitored and treated with bismuth subsalicylate tablets (524 mg PO BID for 5 days), and recovered without additional intervention. This is the first report we are aware of documenting an astrovirus outbreak in cheetah.

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1. Introduction

Astroviruses are small round nonenveloped viruses with a positive stranded RNA genome. They were relatively recently discovered, and were first reported in 1975 (Madeley and Cosgrove, 1975). The family *Astroviridae* is divided into two genera, *Avastrovirus*, found in avian hosts, and *Mamastrovirus*, found in mammal hosts (Monroe et al., 2005). Recognized species in the genus *Mamastrovirus* include *Bovine astrovirus*, *Feline astrovirus*, *Human astrovirus*, *Mink astrovirus*, *Ovine astrovirus*, and *Porcine astrovirus* (Monroe et al., 2005). *Human astrovirus* is a significant cause of enteric disease in human children (Dennehy et al., 2001).

In the mammalian order Carnivora, astroviruses associated with enteritis have been characterized from mink (*Mustela lutreola*) (Mittelholzer et al., 2003) and domestic cats (*Felis catus*) (Harbour et al., 1987), and astrovirus-like particles have been seen on electron microscopy in the feces of domestic dogs (*Canis lupus familiaris*) with diarrhea (Williams, 1980). Within the family *Felidae*, only domestic cats have been documented as host species for astroviruses, but infection is cosmopolitan, having been documented in Australia (Marshall et al., 1987), England (Harbour et al., 1987), Germany (Herbst and Krauss, 1988), New Zealand (Rice et al., 1993), and the United States (Hoshino et al., 1981). One study of fecal electron microscopy of cats found that astroviruses were the most common virus particles seen in cats with diarrhea (Marshall et al., 1987). Serum from a human with astrovirus-associated disease was found to react with feline astrovirus, implying similarity in antigenic regions, which are primarily found in portions of the capsid (Marshall et al., 1987; Matsui et al., 1993).

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Distinguishing the small round viruses in feces on electron microscopy can be challenging. Astroviruses, caliciviruses, picornaviruses, and parvoviruses are all morphologically similar and can potentially be confused (Oliver and Phillips, 1988).

While most enteric viruses typically cause inflammation and death of enterocytes, astroviruses usually cause very little inflammation and cell death (Koci et al., 2003). Astrovirus capsid protein interacts with apical enterocyte membranes, increasing permeability and causing a secretory diarrhea while leaving less of a histologic footprint (Moser et al., 2007). Histopathology is therefore a very insensitive test for detection of astroviral diarrhea.

Currently, astrovirus diagnostics are very limited. As stated, there are few histopathologic changes associated with astrovirus infections, and electron microscopy may lack specificity and sensitivity. Despite high prevalence in humans, human astroviruses were not recognized until 1975, and relatively few astroviruses have been identified in other species. Culture of astroviruses is difficult, and the authors are not aware of previously published broad-range PCR protocols for this group of viruses that can cause clinically significant diarrhea. Therefore, improved methods of diagnostic testing for diverse astroviruses were needed. Consensus PCR represents a rapid way to obtain DNA template from clinical samples of novel viruses suitable for sequencing (Wellehan et al., 2004). Astroviruses are not uncommon in humans, the only hosts in which they have been well studied epidemiologically (Dennehy et al., 2001), and it is likely that there are numerous additional astrovirus species associated with disease in other vertebrates. The methods developed in this study will be useful for rapid identification and characterization of Mamastroviruses from diverse hosts.

2. Methods

2.1. Clinical case history

In July 2007, 5 young adult, 1 year old, cheetah and 2 adult, 2 year old, cheetah in a large breeding facility presented with signs of lethargy, partial to complete anorexia, diarrhea and regurgitation over an 11-day period. The index animal was a young adult female housed with 4 other cheetah of similar age in a large outdoor, vegetated enclosure. Her initial presenting signs included mild lethargy and partial anorexia. Two days later she developed watery diarrhea and there was evidence of regurgitation in her enclosure. At this time, fecal samples were submitted for electron microscopy and culture for enteric bacterial pathogens to the state diagnostic laboratory. Culture for enteric pathogens was performed by initially culturing fecal samples on blood agar, MacConkey's and penylethyl alcohol (PEA) media. Subcultures were then placed in tetrathionate enrichment media and incubated for 24 h at 42 °C. Broth cultures were then placed onto brilliant green and chrome agar for salmonella screening. Viral screening via PCR for canine parvovirus, feline panleukopenia (McKnight et al., 2007) and corona virus (Kennedy et al., 2001) was performed by veterinary diagnostic laboratories using standard techni-

ques. Within 6 days of initial onset, she had fully recovered. Over the next 5 days, four additional animals within the same enclosure developed similar clinical signs. Two additional adult animals also developed similar clinical signs within this time frame. Five of the seven affected cats were housed together in a large outdoor enclosure with a natural grass substrate. The last two animals to develop signs were housed together in a separate pen that was not adjacent to the initial site and therefore did not have direct contact with the first group to develop signs. There were no reports from the animal keepers of any gastrointestinal signs amongst themselves or in any of their personal pets during this time. Additionally, no stray animals (domestic felines) had been observed in the area surrounding the cheetah enclosures to provide a route of infection.

2.2. Electron microscopy

Swabs of feces from two affected animals, one from each enclosure, were homogenized in distilled water in a 1.8 ml centrifuge tube. Fecal homogenates were centrifuged at 4000 × g for 10 min. Supernatants were centrifuged at 14,000 × g for 60 min. The resulting pellets were suspended in 25 µl of 2% phosphotungstic acid (PTA) in water, pH 6.8. This suspension was further diluted in 1% PTA to obtain a slightly turbid suspension. This preparation was applied to a carbon coated formvar film on a 400 mesh copper grid and examined in a transmission electron microscope.

2.3. PCR amplification and sequencing

Astrovirus sequences from GenBank (National Center for Biotechnology Information, Bethesda, MD) were aligned. Degenerate primers were designed targeting conserved regions in the RNA-dependent-RNA polymerase (RdRp) and the capsid protein. RNA was extracted from fecal samples of two cheetahs with diarrhea using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). Reverse transcription PCR for the RdRp was performed using the OneStep RT-PCR Kit (Qiagen) according to standard protocol using forward primer Astr4380F (5'-GAYTGRCNCGNTWYGATGGNACIAT-3') and reverse primer Astr4811R (5'-GGYTTNACCCACATNCCAAA-3'). To increase sensitivity, a hemi-nested second round of PCR amplification was run with 2 µl of product from the first reaction was used in a 20 µl with forward primer Astr4380F and reverse primer Astr4722R (5'-ARNCKRTCATCNCATA-3'), and polymerase (Platinum Taq DNA polymerase, Invitrogen, Carlsbad, CA, USA). The mixtures were amplified in a thermal cycler (PCR Sprint, Thermo Hybaid) with an initial denaturation at 94 °C for 5 min, followed by 36 cycles of denaturation at 94 °C for 30 s; annealing at 45 °C for 60 s, DNA extension at 72 °C for 60 s, and a final extension step at 72 °C for 10 min. To obtain further sequence, an alternate second round was run under the same conditions using forward primer Astr4574F (5'-GGNAYCCMTCWGGICA-3') and reverse primer Astr4811R.

For the capsid protein, PCR was performed as for the RdRp, substituting forward primer Astr4811F (5'-TTTGGNATGTGGGTNAARCC-3') and reverse primer Astr5819R (5'-TCATTNGTGTNGTNANCCACCA-3') in the first round.

In the second round, primers Astr5159F (5'-TGGAGG-GGMGGACCAAAG-3') and Astr5819R were used.

The PCR products were resolved in 1% agarose gels. The bands were excised and purified using the QIAquick gel extraction kit (Qiagen). Direct sequencing was performed using the Big-Dye Terminator Kit (PerkinElmer, Branchburg, NJ) and the above second-round primers, and analyzed on ABI 3130 automated DNA sequencers at the University of Florida Interdisciplinary Center for Biotechnology Research Sequencing Facilities. All products were sequenced in both directions. Primer sequences were edited out prior to further analyses.

2.4. Phylogenetic analysis

The sequences were compared to those in GenBank (National Center for Biotechnology Information, Bethesda, MD), EMBL (Cambridge, United Kingdom), and Data Bank of Japan (Mishima, Shizuoka, Japan) databases using TBLASTX (Altschul et al., 1997). Predicted homologous 122–139 amino acid sequences of astroviral RdRp and 186–209 amino acid sequences of astroviral capsid protein were aligned using three methods; ClustalW (Thompson et al., 1994), T-Coffee (Notredame et al., 2000), and MUSCLE (Edgar, 2004).

Bayesian analyses of each alignment were performed using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) with gamma distributed rate variation and a proportion of invariant sites, and mixed amino acid substitution models. The first 10% of 1,000,000 iterations were discarded as a burn in.

Maximum likelihood (ML) analyses of each alignment were performed using PHYLIP (Phylogeny Inference Package, Version 3.66) (Felsenstein, 1989), running each alignment using the program Proml with amino acid substitution models JTT (Jones et al., 1992), PMB (Veerassamy et al., 2003), and PAM (Kosiol and Goldman, 2005) further set with global rearrangements, five replications of random input order, less rough, gamma plus invariant rate distributions, and unrooted. The values for the gamma distribution were taken from the Bayesian analysis. Avian nephritis virus 1 (GenBank accession number AB033998) was designated as the outgroup. The alignment producing the most likely tree was then used to create data subsets for bootstrap analysis to test the strength of the tree topology (200 re-samplings) (Felsenstein, 1985), which was analyzed using the amino acid substitution model producing the most likely tree in that alignment.

3. Results

3.1. Treatment and disease course

All animals were monitored and treated with bismuth subsalicylate tablets (524 mg PO BID for 5 days). All recovered without any additional intervention within 6–11 days of the onset of clinical signs and no additional cases were detected in subsequent months.

Fecal PCR tests for canine parvovirus, corona virus, and feline panleukopenia virus were negative. Fecal cultures for the initial case were positive for *Escherichia*



Fig. 1. Negative stained electron micrograph of cheetah feces showing round virus-like particles measuring 30 nm. Note the presence of star-like patterns typical of astrovirus.

coli and *Plesiomonas shigelloides* but all other fecal cultures from additional animals identified no pathogenic enteric bacteria.

3.2. Electron microscopy

In three random fecal samples of the affected animals with clinical signs, abundant, round virus-like particles measuring 30 nm were observed. Many of the particles exhibited faint star-like patterns typical of astrovirus (Fig. 1).

3.3. PCR amplification and sequencing

PCR amplification of both cheetah astrovirus samples that were tested resulted in a 367 base pair RdRp product and a 628 base pair capsid gene product when primer sequences were edited out. Sequences from both cheetahs were identical. This virus is referred to hereafter as cheetah astrovirus. Sequences were submitted to GenBank under accession numbers EU650331 and EU650332.

3.4. Phylogenetic analysis

TBLASTX results for cheetah astrovirus RdRp showed the highest score with Human astrovirus 5 (GenBank accession number DQ028633). TBLASTX results for cheetah astrovirus capsid protein showed the highest score with Feline astrovirus (GenBank accession number AF056197).

Bayesian phylogenetic analysis showed the greatest harmonic mean of estimated marginal likelihoods using the MUSCLE alignment for both the RdRp and the capsid gene (see Supplementary data). For the RdRp, the Wag model of amino acid substitution was found to be most probable with a posterior probability of 0.962 (Whelan and Goldman, 2001), and a posterior probability of 0.037 for the rtRev model (Dimmic et al., 2002). For the capsid protein, the Wag model was also most probable with a posterior probability of 0.625, and a posterior probability of 0.312 for the rtRev model. Bayesian trees using the MUSCLE alignment for the RdRp and capsid gene are shown (Fig. 2).

ML analysis found the most likely tree from the MUSCLE alignment and the PMB model of amino acid substitution

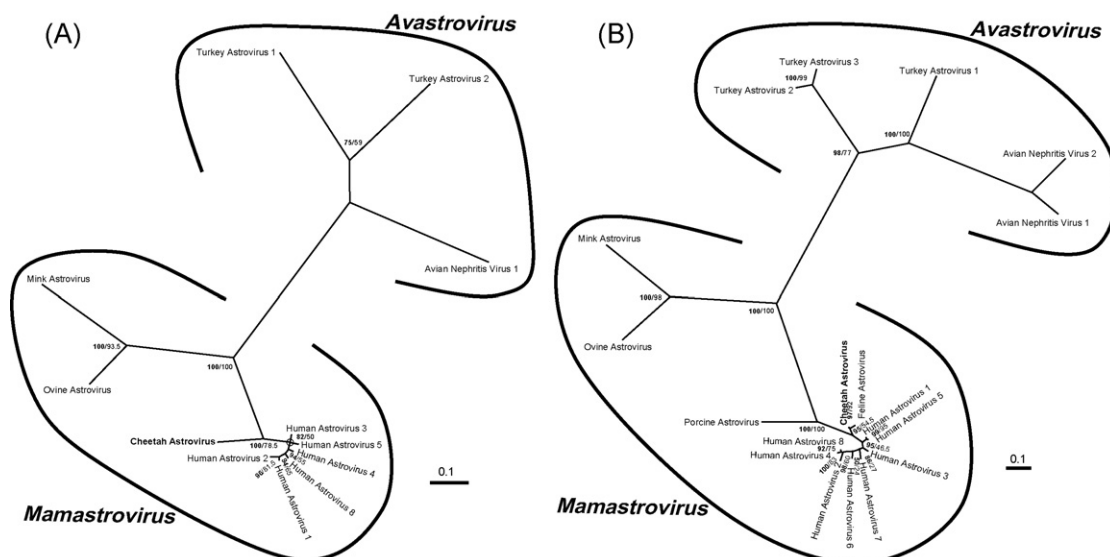


Fig. 2. (A) Bayesian phylogenetic tree of predicted 122–132 amino acid partial astroviral RNA-dependent-RNA polymerase sequences based on MUSCLE alignment. Bayesian posterior probabilities of branchings as percentages are in bold, and ML bootstrap values for branchings based on 200 re-samplings are given to the right. Avian nephritis virus 1 (GenBank accession number NP_620617) was designated as the outgroup. Virus genera are delineated by brackets. Branches with Bayesian posterior probabilities less than 50 are collapsed, and an area of trifurcation is marked by a circle. Cheetah astrovirus is bolded. Sequences retrieved from GenBank include Human astrovirus 1 (GenBank accession number AAW51881), Human astrovirus 2 (AAA65000), Human astrovirus 3 (AAD28539), Human astrovirus 4 (AAY84778), Human astrovirus 5 (AAY46273), Human astrovirus 8 (AAF85963), Ovine astrovirus (NP_059945), Mink astrovirus (AAO32082), Turkey astrovirus 1 (CAB95006), Turkey astrovirus 2 (NP_987087), and Avian Nephritis Virus 1 (NP_620617). (B) Bayesian phylogenetic tree of predicted 186–209 amino acid partial astroviral capsid sequences based on MUSCLE alignment. Bayesian posterior probabilities of branchings as percentages are in bold, and ML bootstrap values for branchings based on 200 re-samplings are given to the right. Avian nephritis virus 1 (GenBank accession number NP_620618) was designated as the outgroup. Virus genera are delineated by brackets. Cheetah astrovirus is bolded. Sequences retrieved from GenBank include Feline astrovirus (GenBank accession number AAC13556), Human astrovirus 1 (BAE97460), Human astrovirus 2 (AAA62427), Human astrovirus 3 (ABG00150), Human astrovirus 4 (BAA93440), Human astrovirus 5 (AAY46274), Human astrovirus 6 (AAK97065), Human astrovirus 7 (AAK31913), Human astrovirus 8 (AAF85964), Porcine astrovirus (CAB95000), Ovine astrovirus (NP_059944), Mink astrovirus (NP_795336), Turkey astrovirus 1 (CAB95007), Turkey astrovirus 2 (NP_987088), Turkey astrovirus 3 (AAV37187), Avian Nephritis Virus 1 (NP_620618), and Avian Nephritis Virus 2 (BAB21617).

for the RdRp, and the MUSCLE alignment and the JTT model of amino acid substitution for the capsid gene. These parameters were used for bootstrap analysis. Bootstrap values from ML analysis are shown on the Bayesian trees.

4. Discussion

This report documents the first identification of astrovirus in cheetahs. Astrovirus species are defined on the basis of host of origin (Monroe et al., 2005), and using these criteria, this would constitute a novel astrovirus species. However, the relationship of the partial capsid sequence to that of *Feline astrovirus* is very close (88.6% nucleotide identity, 97.1% amino acid identity), and this may be the same virus species. Strain diversity in feline astroviruses has not been investigated, and GenBank only contains one partial feline astrovirus capsid precursor sequence. Comparable sequence from the *Feline astrovirus* polymerase, which is expected to be more conserved, is not available. Further sequence from these viruses as well as astroviruses from additional hosts in the family *Felidae* would help resolve this.

When looking at subtyping within astrovirus species, serotypes are defined on the basis of 20-fold or greater two-way cross-neutralization titers (Monroe et al., 2005). However, small RNA viruses have high mutation rates and often behave as quasispecies (Domingo and Gomez, 2007).

Due to this, together with strong positive selective pressure for change in immunologically exposed sites such as capsid protein, serology is fraught with problems, and serological relationships are not as useful as genetic relationships for phylogeny (Iturriza-Gómara et al., 2006). Genotyping may represent a more appropriate method of characterizing subtypes, but criteria have not been established for genotypes within astrovirus species.

If this cheetah astrovirus is determined to be a *Feline astrovirus* strain, it would be the first report we are aware of describing a *Mamastrovirus* crossing host species. *Turkey astrovirus 2*, an *Avastrovirus*, appears to have crossed from turkeys (*Meleagris gallopavo*) to guinea fowl (family Numididae, species not given), since guineafowl sequences nest within *Turkey astrovirus 2* sequences from turkeys (Cattoli et al., 2007). Cheetahs are more closely related to the small cats, including domestic cats, than they are to other big cats (Flynn et al., 2005). A number of other domestic cat viruses have been shown to cause disease in cheetahs. *Feline parvovirus* has been confirmed by sequence identification from two diseased cheetahs in Namibia (Steinel et al., 2000). *Feline coronavirus* has been confirmed by sequence identification from cheetahs (Kennedy et al., 2006). There is serological evidence for *Feline herpesvirus 1* and *Feline calicivirus* in cheetahs (Munson et al., 2004), and *Feline herpesvirus 1* has been confirmed by sequence identification from diseased

cheetahs (Wellehan and Citino, unpublished data). However, sequence analysis has shown that *Feline Immunodeficiency Virus* (FIV) found in cheetahs is distinct from domestic cat FIV (Trojer et al., 2005).

The phylogenetic topology determined in this study is largely in agreement with previous analyses of astrovirus phylogeny (Jonassen et al., 2001; Lukashov and Goudsmit, 2002). The analysis of the capsid region found that cheetah astrovirus clustered relatively more closely with human astroviruses than was found in the analysis of the polymerase. This is surprising, given that rates of change have been found to be higher in the capsid region (van Hemert et al., 2007b). One possible explanation for this would be viral recombination of a capsid from a human-like astrovirus and a polymerase from a more divergent virus.

Viral polymerases have been repeatedly demonstrated to be good choices for long-range phylogeny (Attoui et al., 2002; Gonzalez et al., 2003; Knopf, 1998). Capsids are typically under strong positive selective pressure from the host immune system, and one analysis found that most positively selected sites in astroviruses are present in the capsid (van Hemert et al., 2007b). When looking at evolutionary relationships of more distantly related organisms, the continued accrual of mutations resulting in homoplasy can diminish the ability to correctly resolve phylogeny, making a rapidly mutating gene a poor choice. Astroviral capsid proteins are the most rapidly evolving part of the virus, and analysis of only synonymous nucleotide substitutions from astroviral capsid proteins found that insufficient phylogenetic signal remained for distance based analysis (van Hemert et al., 2007a), and significant compression of tree branches was found using maximum likelihood methods for analysis (van Hemert et al., 2007b), which are generally superior to distance based methods. We therefore chose to analyze predicted amino acid phylogeny in this analysis, which only considers non-synonymous substitutions.

Genes for which there is strong negative selection will have fewer nucleotides with a history of multiple changes, making them a better choice for resolving phylogeny over greater distances. Genes that are critical for replication and are not under heavy immune selection are usually more conserved. Other studies have found that the astroviral polymerase is the most conserved part of the genome (Jonassen et al., 2003), and although the protease was not examined in this study, our data found that the polymerase was more conserved than the capsid. Polymerases are therefore more likely to accurately reflect the deeper history of the virus than capsid proteins. The disadvantage of the astroviral polymerase is the smaller database of sequence for comparison. The availability of more complete data sets for comparison results in greater phylogenetic resolution (Flynn et al., 2005). However, the total number of available astrovirus sequences from any gene is still relatively small, and techniques developed in this study could lead to rapid accumulation of sequence data from the polymerase gene of additional mamastroviruses.

Of the six recognized mamastroviruses, five are from hosts from one mammalian superorder, *Laurasiatheria*. The other speciose placental mammal superorder, *Euarchonto-*

glires, has only one host from which an astrovirus is recognized, humans. Two possible explanations for this include greater distribution of astroviruses in the *Laurasiatheria* and lack of screening of the *Euarchontoglires*. Most agriculturally important or common companion mammals are in the *Laurasiatheria*, with only domestic rabbits falling into these categories from the *Euarchontoglires*. Humans have primarily valued a small number of other *Euarchontoglires*, such as mice, rats, and rhesus macaques, as laboratory animals. Similar reasons may have resulted in only finding avastroviruses in the order *Galliformes*, which are by far the most common agriculturally produced birds. Further screening of diverse species may result in a more complete picture of astrovirus evolution.

It has previously been noted that *Feline astrovirus*, *Mink astrovirus*, *Ovine astrovirus*, and *Porcine astrovirus* sequences are relatively GC rich compared to *Human astrovirus* and the characterized avastroviruses (van Hemert et al., 2007a). This trend was also seen in comparison of the regions of sequence in this study (Table 1). This has previously been interpreted as a drive toward host codon usage. Since birds and humans are more phylogenetically different, it was interpreted as acquisition of GC by non-human mammal astroviruses from AT-rich ancestors (van Hemert et al., 2007a). An alternate hypothesis would be the gain of AT in human and domestic poultry astroviruses from GC rich ancestors. There is evidence that adenoviruses and retroviruses become AT rich after recent host switching events (Poss et al., 2006; Wellehan et al., 2004), and this may also be the case with astroviruses. However, there does not appear to be a difference in %GC of the cheetah astrovirus relative to the reference *Feline astrovirus* sequence. If AT gain is seen after host switching events in astroviruses, three possible explanations for the lack of AT bias in cheetah astrovirus relative to the domestic cat astrovirus include a very recent host switch so that loss of GC has not had time to occur, recombinant acquisition of *Feline astrovirus* capsid by a distinct host-adapted cheetah astrovirus, and sufficient biological similarity between cheetahs and domestic cats such that selective pressures after a host switch are minimal.

In conclusion, we document an outbreak of astrovirus-associated diarrhea in a cheetah collection, and provide initial characterization of the virus. While no obvious source of infection was detected, the fecal–oral route seems to be the most common route of infection in other species. Sources in this outbreak could be dietary, fomite transmission from a source outside of the institution, or an in apparent carrier/shedder in the collection. Screening of the remaining unaffected population was not conducted

Table 1

Percentage GC composition of astroviral partial capsid and partial polymerase nucleotide sequences examined in this study.

| | %GC—capsid | %GC—polymerase |
|---------------------------|------------|----------------|
| Cheetah astrovirus | 52.7% | 46.3% |
| <i>Feline astrovirus</i> | 52.6% | Not available |
| <i>Human astrovirus</i> | 44.6–48.1% | 39.2–42.2% |
| <i>Porcine astrovirus</i> | 49.3% | Not available |
| <i>Mink astrovirus</i> | 52.0% | 47.8% |
| <i>Ovine astrovirus</i> | 54.2% | 44.4% |
| Avastroviruses | 46.3–50.2% | 37.2–43.5% |

during this outbreak but could be done in the future. Based on the molecular results, this represents a novel pathogen for this species, and astrovirus should be considered as a differential diagnosis in cases of diarrhea in cheetahs.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2008.10.035.

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