JVI Accepts, published online ahead of print on 11 June 2008
J. Virol. doi:10.1128/JVI.00857-08
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Novel astroviruses in insectivorous bats

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Key words: astroviruses, bats, zoonosis, coronaviruses, wild-life, evolution.

Running title: Novel astroviruses in insectivorous bats

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Abstract

Bats are increasingly recognized to harbor a wide range of viruses and in most instances these viruses appear to establish long term persistence in these animals. They are the reservoir of a number of human zoonotic diseases including Nipah, Ebola and SARS. We report the identification of novel groups of astroviruses in apparently healthy insectivorous bats found in Hong Kong, in particular bats belonging to the genera *Miniopterus* and *Myotis*. Astroviruses are important causes of diarrhea in many animal species including humans. Many of the bat astroviruses (BatAstV) form distinct phylogenetic clusters in the genus mammastrovirus within the family *Astroviridae*. Virus detection rates of 36%-100% and 50%-70% were found in *Miniopterus magnater* and *Miniopterus pusillus* bats respectively, captured within a single bat-habitat during four consecutive visits spanning one year. There was high genetic diversity of viruses in bats found within this single habitat. Some BatAstV may be phylogenetically related to human astroviruses and further studies in a wider range of bat species in different geographic locations are warranted. These findings are likely to provide new insights into the ecology and evolution of astroviruses and reinforce the role of bats as a reservoir of viruses with potential to pose a zoonotic threat to human health.

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Introduction

The Astroviridae are a family of non-enveloped, positive sense single-stranded RNA viruses of approximately 28-30nm in size with a characteristic star-like surface structure. The genomes of these viruses range in sizes from 6.4 - 7.3 kb and are polyadenlyated at their 3' ends (19). The viral RNA is synthesized by its RNA dependent RNA polymerase (RdRp). The genome consists of 3 open reading frames (ORFs) designated as ORF1a, ORF1b and ORF2 (Figure 1). ORF1a encodes nonstructural polyprotein 1a while the entire ORF1 encodes polyprotein 1ab with a ribosomal frame shift at the ORF1a / 1b junction (9). Efficiency of ORF1b translation is estimated as only 25 - 28% of ORF1a (18). ORF2 encodes the viral structural polyprotein which is required for virion formation. Studies with human astroviruses showed that the structural polyprotein is intracellularly cleaved into functional units including a capsid forming unit and a host binding motif unit (7, 12, 20).

Astroviruses have been identified from a variety of mammals (genus mamastrovirus) and birds (genus avastrovirus) including humans (HAstV), bovine (BAstV), pigs (PAstV), ovine (OAstV), mink (MAstV), dogs, cats, mice, chickens (CAstV) and turkeys (TAstV). In most species, these viruses are associated with gastroenteritis but some avian astroviruses have been associated with both intestinal and extraintestinal manifestations [reviewed in (19)]. Human astroviruses appear to cause milder disease than rotaviruses but are the second or third most common viral agent found in children with diarrhea (5, 8). Astrovirus can also cause significant disease in the elderly (16) and in immunocompromised patients (6).

Most of the surveillance studies of astroviruses focused on humans and domesticated

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72 animals and relatively little is known about the prevalence of astroviruses in wildlife.
73 The role of bats as the reservoirs for zoonotic diseases including rabies, Hendra,
74 Nipah and Ebola has been highlighted in recent years [reviewed in ref. (2)].
75 Insectivorous bats have also been shown to harbor a range of novel coronaviruses
76 including the precursor of SARS coronavirus (15, 25). Some species of bats live in
77 close proximity to human habitation and thus it is important to have a better
78 understanding of the virus ecology found in bats. The range and diversity of
79 coronaviruses found in bats has led to the hypothesis that bat coronaviruses may be
80 the precursors of most other mammalian group 1 and 2 coronaviruses (30). These
81 findings highlight the importance of identifying novel viruses in wildlife in general
82 and bats in particular. We used random primers to detect novel viruses in bat faecal
83 specimens. Here, we report the discovery of novel astroviruses in bats in Hong Kong.
84 The remarkably high prevalence and genetic diversity of astroviruses in various bat
85 species found within a relatively small geographic area highlights the need for study
86 in other species of bats and in other geographic locations.
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Methods

Sample collection

The sampling of bats was carried out in two phases and the sampling methods have been described previously (4, 25). Phase 1 was carried out in 2004 and 2005 and during this phase, swab samples were collected from different species of bats captured in the wild in different habitats in Hong Kong. Phase 2 was carried out in 2005 and 2006 and swab samples were collected from bats of the genus *Miniopterus* captured on four sampling occasions (June, August and December 2005 and March 2006) in a single habitat, an abandoned mine cave in Lin Ma Hang, Hong Kong, near the border with Mainland China (4). In both phases, species of bats captured for sampling were healthy and identified by a bat taxonomist. Rectal and throat swabs, together with fresh fecal samples if available, were collected. Swabs were placed in viral transport medium (Earle's balanced salt solution, 0.2% sodium bicarbonate, 0.5% bovine serum albumin, 200 µg of vancomycin per liter, 18 µg of amikacin per liter, 160 U of nystatin per liter) in screw cap tubes and transported in a cool box to the laboratory for processing. Bats were released at the site after sampling.

Viral nucleic acid extraction and RT-PCR

RNA from 140 µl of sample in transport medium was extracted by QIAamp virus RNA mini kit (QIAGEN) following the protocol provided by the manufacturer. Purified RNA was eluted in 60 µl of Elution buffer provided in the extraction kit. cDNA was generated from RNA using Superscript III reverse transcriptase (Invitrogen) in a 20 µl reaction containing 150 ng of random hexamers or 0.5 µM of a gene specific reverse primer 5'-TTTGGTCCNCCNCTCCAAA-3' targeting the 3' end

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of ORF1b, 10 mM of dithiothreitol, 0.5 mM of deoxynucleoside triphosphate mix, 1x First-Strand buffer (Invitrogen), and 200 U of reverse transcriptase. Reaction mixtures were incubated at 25°C for 5 min, followed by 50°C for 60 min and then the enzyme was inactivated by heating at 70°C for 15 min.

Random hexamer-generated cDNA was screened for the presence of astrovirus using hemi-nested PCR targeting RdRp gene. A 50 µl PCR reaction was set up containing 1 U Accuprime *Taq* DNA polymerase in 1x reaction buffer (Invitrogen), 2 µM each of forward and reverse primers and 2 µl of cDNA or 1 µl of first PCR product as a template. First-round PCR was carried out with a mix of two forward primers 5'-GARTTYGATTGGRCKCGKTAYGA-3' and 5'-GARTTYGATTGGRCKAGGTAYGA-3', and reverse primer 5'-GGYTTKACCCACATNCCRAA-3'. After an initial incubation at 94°C for 1 min, 30 cycles of amplification were carried out consisting of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 68°C for 30 sec. Hemi-nested PCR was carried out with a mix of two forward primers 5'-CGKTAYGATGGKACKATHCC-3' and 5'-AGGTAYGATGGKACKATHCC-3' and the same reverse primers used in the first-round PCR using the same thermocycling conditions as for the first-round PCR except that 40 cycles of amplification were performed. Water controls were included in each run of the RT-PCR assay. PCR products were analyzed by standard agarose gel electrophoresis. The expected product size of the 2nd PCR is 422 base pairs. All positive results were verified by direct DNA sequencing of the PCR amplicons.

Cloning and sequencing of PCR products

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PCR products were purified by QIAquick PCR purification kit (QIAGEN) following the manufacturer's instruction. Long PCR products (product sizes > 1000 bp) were gel purified with QIAquick gel extraction kit (QIAGEN) and then cloned into pCR2.1-TOPO plasmids (Invitrogen) for DNA sequencing. Multiple clones of a PCR product were picked and sequenced by using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Sequencing products were analyzed by PRISM 3700 DNA analyzer (Applied Biosystems).

One astrovirus positive bat specimen, bat astrovirus AFCD337, was chosen for sequencing of the viral genome directly from the original clinical specimen by using cDNA generated by random hexamers and consensus primer RT-PCR. ORF2 region and the 3' end of the virus sample were amplified using 3' Rapid Amplification of cDNA Ends (RACE) System (Invitrogen) following the protocol provided by the manufacturer (primers and PCR conditions available on request). ORF1a (partial) and ORF1b sequences were assembled from multiple overlapping sequences derived from PCR amplicons. Nine additional ORF1a (partial) / ORF1b astrovirus sequences from other samples were obtained using similar method. The deduced sequence of these samples has at least 3-fold sequence coverage.

Phylogenetic analysis

Sequence editing and sequence identities calculation were done using BioEdit version 7.0.4 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Alignments of nucleotide sequences and amino acid sequences were done using Clustal W (29) with default parameters. Phylogenetic trees were constructed using Clustal X V2.0 (14) and Mega 4 (28) by the neighbour joining method with the nucleotide substitution model of

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maximum composite likelihood and default parameters. Bootstrap values of the phylogenetic tree constructed were generated by doing 1000 replicates.

Nucleotide sequence accession numbers.

The sequence of bat astrovirus AFCD337 reported in this paper were deposited in GenBank under accession numbers xxxxx, and RdRp and the partial ORF1b sequences of other strains of bat astroviruses were deposited under accession numbers xxxxx-xxxxx. For the genetic analysis in this paper, other astrovirus genomes were retrieved from GenBank including human astrovirus type 1 strain Oxford (L23513), human astrovirus type 1 strain Dresden (AY720892), human astrovirus type 4 strain Dresden (AY720891), human astrovirus type 4 isolate Goiania/GO/12/95/Brazil (DQ070852), human astrovirus type 4 isolate Guangzhou (DQ344027), human astrovirus type 5 isolate Goiania/GO/12/94/Brazil (DQ028633), human astrovirus type 8 (AF260508), mink astrovirus (NC_004579), ovine astrovirus (NC_002469) and turkey astrovirus type-1 (NC_002470).

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Results

Detection of astroviruses in bats

A total of 262 bats were captured and rectal and throat swabs sampled in the two phases sample collection in Hong Kong in 2004 to 2006. Bats sampled included nine species, *Cynopterus sphinx*, *Hipposideros armiger*, *Miniopterus magnater*, *Miniopterus pusillus*, *Miniopterus schreibersii*, *Myotis chinensis*, *Myotis ricketti*, *Pipistrellus abramus* and *Rhinolophus rouxi*. A hundred and sixteen positives were detected from 250 available rectal samples tested, representing a positive rate of 46% (Table 1). On the other hand, only 19 (8%) positive throat swabs were found in 246 available samples tested. With the exception of two throat swabs collected from *Miniopterus pusillus* and *Rhinolophus rouxi* in 2004, all the throat swabs positive results came from bats with astrovirus detected in rectal samples. All the positive PCR products were sequenced to confirm the identity of the amplicon and since the sequences of the different PCR amplicons were largely non-identical, PCR cross-contamination can be excluded.

Astroviruses were detected in 7 out of the 9 species of bats screened, i.e. *Miniopterus magnater*, *Miniopterus pusillus*, *Miniopterus schreibersii*, *Myotis chinensis*, *Myotis ricketti*, *Pipistrellus abramus* and *Rhinolophus rouxi* (Table 1). The detection rates of astroviruses in these species of bats were remarkably high and ranged from 25% to 100%. However, astrovirus was not detectable in our samples collected from *Cynopterus sphinx* (n=11) and *Hipposideros armiger* (n=10).

These same specimens had previously been tested for bat coronaviruses (3, 4, 25).

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While 6% of bats were co-infected with both a bat astrovirus and a bat coronavirus, such co-infection appears to be randomly distributed and there was no positive or negative statistical association between the presence of these two viruses (Chi square test with Yates correction, p=0.82).

Longitudinal study of astrovirus in bats at a single habitat

The bats listed in Table 1 include 157 *Miniopterus magnater* and *Miniopterus pusillus* bats which were captured at four separate visits over a two year period at one habitat, an abandoned mine cave in Hong Kong. *M. magnater* was found throughout the period while *M. pusillus* was mainly found in two visits carried out in Dec 2005 and Mar 2006 and only 1 found in a visit in Aug 2005. Sixty-two (54%) out of 115 rectal swabs and 2 (2%) out of 116 throat swabs collected from *M. magnater* and 18 (55%) out of 33 rectal swabs and 5 (15%) out of 33 throat swabs collected from *M. pusillus* were positive for astrovirus. The overall positive rate in individual bats (either rectal or throat swab or both positive) for *M. magnater* ranged from 36-100% at each of the four visits which spanned the winter, spring and summer seasons and for *M. pusillus* was 50% and 70% in the two instances when adequate bats were sampled.

Genetic and Phylogenetic analysis of a novel astrovirus from *Miniopterus pusillus*

A bat astrovirus (BatAstV) AFCD337 detected in a rectal specimen from a *Miniopterus pusillus* bat collected in March 2006 was chosen as a representative virus for more extensive genome sequencing. Approximately 74% of the genome of this novel astrovirus was obtained by direct RT-PCR amplification from a rectal swab sample. The partial 5067 nucleotide (excluding the poly-A tail) genome was constructed by aligning sequences from multiple overlapping regions. Amino acid

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sequences deduced from the viral genome include part of the open reading frame 1a (ORF1a) and the complete ORF1b, ORF2 and 3' untranslated region followed by the poly-A tail at the 3' end (Figure 1).

Amino acid sequences encoded by ORFs of the novel bat astrovirus were compared with that of other astrovirus genomes including human astrovirus (HAstV) types 1, 4, 5 and 8 (17, 26) as well as mink astrovirus (MAstV) (22), ovine astrovirus (OAstV) and turkey astrovirus type-1 (TAstV-1) (11) (Table 2). The findings show that the identified bat astrovirus BatAstV AFCD337 is a novel mamastroviruses virus clearly distinct from other known astroviruses. It has <53% and <27% genetic similarity to other known astroviruses in the ORF1b and ORF2 regions, respectively.

The putative ORF1a (partial) and ORF1b of the bat astrovirus sequenced have sizes of 909 nt and 1572 nt respectively. A region at 5' end of ORF1a remains unsequenced so far. ORF1a and ORF1b encodes for non-structural proteins which are essential for virus replication. Characteristic features of BatAstV AFCD337 ORF1a and ORF1b includes the protease motif in ORF1a; an astrovirus "slippery sequence" (AAAAAC) at the junction between ORF1a and ORF1b which is required for inducing a ribosomal shift event; a RdRp motif in ORF1b; and a conserved sequence at the end of ORF1b of astroviruses (11). A conserved stem-loop structure that is predicted at the 3' end of the genomic RNA of human, ovine, porcine and turkey astroviruses type-1 was not found in BatAstV AFCD337 (10).

The putative ORF2 of the virus has a size of 2553 nt, which is the largest astrovirus capsid gene known. The N-terminal half of the ORF2 protein, which was previously

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253 shown to be more conserved among astroviruses and proposed to be the core
254 assembly domain of the viral capsid (12), was also found to be relatively conserved in
255 this bat astrovirus. The amino acid sequence similarities of this N-terminal half of the
256 bat astrovirus capsid protein to HAstV-1 Oxford, OAstV and MAstV are 36.3%,
257 45.0% and 39.5% respectively; compared with <27% similarity for the ORF2 region
258 overall (Table 2). Thus the C-terminal half of this bat astrovirus protein was highly
259 divergent when compared with other astroviruses. This observation supports the
260 speculation that the C-terminal half of the protein is located on the surface of the viral
261 particle and constitutes a region of the capsid that contributes to the species-specific
262 tropism of the virus (12).
263
264 Phylogenetic analysis on astrovirus in bats.
265 Phylogenetic analyses on ORF1a (partial) and ORF1b region and the ORF2 confirms
266 that BatAstV AFCD337 is a novel distinct astrovirus (Figure 2). To better define the
267 genetic diversity within the bat astroviruses by species, time and geographic location,
268 77 PCR RdRp amplicons (422 nucleotides) obtained from the rectal swab samples in
269 the screening PCR assay were selected for genetic sequence analysis. Bat astrovirus
270 gene sequences were aligned with that of other astroviruses including MAstV, OAstV,
271 HAstV-1 Oxford, HAstV-1 Dresden, HAstV-2, HAstV-4 Dresden, HAstV-4 Goiania,
272 HAstV-4 Guangzhou, HAstV-5 Goiania and HAstV-8. An avian astrovirus, Turkey
273 astrovirus was included as an out-group. A phylogenetic tree was constructed from the
274 sequence alignment (Figure 3). The 72 astroviruses detected in bats cluster together to
275 form a novel group of viruses within the cluster of mamastroviruses (Figure 3).
276 Within this group are found two subgroups of viruses, one which include the majority
277 of astroviruses detected from *Miniopterus magnater*, *Miniopterus pusillus* and

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278 *Miniopterus schreibersii* (including BatAstV AFCD337) and another subgroup which
279 includes most of the viruses detected in *Myotis chinensis and Myotis ricketti*. Other
280 than these two major groups of bat astroviruses, a few astroviruses detected in
281 *Miniopterus magnater* and *Miniopterus pusillus* and a virus detected in a *Pipistrellus*
282 *abramus* appear to have an outgroup relationship to the others, albeit with weak levels
283 of statistical confidence in this phylogenetic topology (Figure 3). To further
284 investigate this, a 750 nt region of the RdRp and ORF1b (3'end) was sequenced of
285 these and other representative bat astroviruses. A phylogenetic tree based on the
286 aligned protein encoding sequences was shown (Figure 4). The phylogeny of the
287 major group of viruses related to BatAstV AFCD337 is confirmed. Interestingly
288 however, AFCD11 from *Pipistrellus abramus* and AFCD57 from *Miniopteus*
289 *magnater* appear phylogenetically related to the human astroviruses although with
290 modest bootstrap support. Their close relationship has been also confirmed by
291 phylogenetic analysis using an alternative method, MrBayes (data not shown).
292
293 Occasionally the same virus strain can be found in multiple bats sampled at the same
294 habitat in a single sampling trip (i.e. strains AFCD74 and AFCD79; strains AFCD175
295 and AFCD228). However, most viruses detected even at the same sampling occasion
296 at a single habitat are genetically diverse and no dominant strain could be discerned,
297 even though the detection rates of the virus was remarkably high.

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298 **Discussion**

299 We report the discovery of novel astroviruses in 7 out of 9 species of apparently
300 healthy bats captured in Hong Kong. These astrovirus positive species include
301 *Miniopterus magnater*, *Miniopterus pusillus*, *Miniopterus schreibersii*, *Myotis*
302 *chinensis*, *Myotis ricketti*, *Pipistrellus abramus* and *Rhinolophus rouxi*. Attempts at
303 viral culture were so far unsuccessful (unpublished data). Phylogenetic analysis
304 revealed that 72 out of 77 BatAstV RdRp genes analyzed clustered together to form a
305 novel group of astroviruses. This virus group can be divided into two sub-groups,
306 subgroup A detected from *Miniopterus* species and subgroup B detected from *Myotis*
307 species. In the longitudinal study carried out in the abandoned mine cave habitat, the
308 subgroup A viruses appears to be circulating between *Miniopterus magnater* and
309 *Miniopterus pusillus* bats without any evidence of species restriction. This is in
310 marked contrast to the bat coronaviruses within the same habitat where Bat CoV 1A
311 and 1B appear to have a marked host restriction to *Miniopterus magnater* and
312 *Miniopterus pusillus*, respectively (4). Multiple clones of partial ORF1 sequence
313 (approximately 1000 nt) for 10 representative samples were analyzed and no evidence
314 of multiple infection was found although more systematic studies on this aspect are
315 needed.

316

317 The diversity of astroviruses in bats is remarkable. There is no significant
318 phylogenetic clustering of viruses found within a single sampling occasion. The
319 values of astrovirus RdRp amino acid pair-wise similarity found within a single bat
320 species, i.e. *Miniopterus magnater* captured in a single habitat (mine cave) ranged
321 between 51.1% to 100%, with 97.9% of these values lower than 90%. In contrast, the
322 pair-wise amino acid sequence similarities between the same gene region of human

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astroviruses from geographically diverse regions were estimated to range between 92.6% to 99.1%. It has been previously reported that the amino acid sequence identities of RdRp gene (covering 80% of the RdRp gene regions analysed in this report) between 4 groups of avastrovirus, i.e. turkey astrovirus type 1-like viruses, turkey astroviruses type 2-like viruses, avian nephritis virus-like viruses and chicken-origin astroviruses detected in different regions were also highly diverse ranging from 50.1% and 73.8% (23). The high virus detection rates of BatAstV in our surveillance taken together with the marked genetic diversity of viruses from bats within the same habitat are reminiscent of our observations with bat coronaviruses (4). These findings may suggest that bats are persistently infected with astroviruses although mark-recapture studies are needed to confirm this contention. Other mammalian astrovirus infections tend to be short-lived in immunocompetent humans or other animals. However, type 3 HAstV has been associated with persistent gastroenteritis in immunocompetent children although the same virus serotype was not repeatedly demonstrated over the full period of clinical diarrhea (1).

Five BatAstV sequences from *Miniopterus magnater*, *Miniopterus pusillus* and *Pipistrellus abramus* failed to cluster with the subgroup A and B referred to above and some of these (AFCD11 from *Pipistrellus abramus* and AFCD57 from *Miniopterus magnater*) cluster rather with the human astroviruses although with weak statistical support (Figure 4). Whether these bat viruses are related to the precursor of HAstV is yet to be further investigated. Further sequence data from these strains may help elucidate this phylogenetic association.

Evidence of recombination between astroviruses and also that between coronaviruses

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is well documented (13, 24, 27). It has been reported that a stem-loop motif in 3' untranslated region (UTR) was found conserved in mamastroviruses, TAstV-1, and in avian infectious bronchitis virus which is a group 3 coronavirus (10). However, this stem loop motif was not recognized in the BatAstV AFCD337. This 3' UTR stem-loop motif is also absent in TAstV-2. However a phylogenetic analysis of 3' UTRs did not indicate a close phylogenetic relationship between the two sequences of BatAstV AFCD337 (81 nt) and TAstV-2 (accession no.: NC_005790) (196 nt) which lack the 3'UTR stem-loop structure (data not shown). Recently a report on the identification of a novel coronavirus from liver tissue of a whale with pulmonary disease and terminal acute liver failure showed that the ORF6 of the novel coronavirus possessed significant amino acid similarity to human astrovirus capsid proteins (21). The high rates of infection of bats in the same mine cave habitat with coronaviruses and astroviruses implies frequent co-infection with both viruses. Therefore we searched for sequence of BatAstV AFCD337 similar to bat coronaviruses using BLAST program with algorithms allowing a word-size down to seven bases. However no sequences with significant similarity was detected between bat astroviruses and coronaviruses co-circulating within the same species within the same habitat.

The discovery of novel diverse astroviruses in bats and the genetic analysis of such viruses is likely to provide new insights into the ecology and evolution of astroviruses and reinforces the role of bats as a reservoir of viruses which sometimes pose a zoonotic threat to human health. More extensive surveillance for astroviruses in bats of different species and in different geographic areas is needed to further address these questions.

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Acknowledgements

This project is supported by National Institutes of Health (NIAID contract HHSN266200700005C) and a Research Excellence Award to JSMP from The University of Hong Kong. The study was approved and supported by the Department of Agriculture, Fisheries and Conservation, Hong Kong, Special Administrative Region, People's Republic of China. We thank K. S. Cheung, C. T. Shek and C. S. M. Chan of the Department of Agriculture, Fisheries and Conservation, Hong Kong for facilitating this study.

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Figure Legends

Figure 1. Schematic diagrams of Mink astrovirus genome and BatAstV AFCD337 partial genome. ORFs, protease motif (Pro) and RdRp motif (RdRp) are shown in the diagram. The unsequenced putative 5'end genome region of BatAstV AFCD337 is represented by a dotted line. The 2.5 kb ORF1a (partial), ORF1b region, and ORF2 regions used for phylogenetic analysis in Figure 2 are indicated by arrows.

Figure 2. Phylogenetic analysis of partial ORF1a (~800 nt), ORF1b and ORF2 nucleotide sequences comparing BatAstV AFCD337 with astroviruses of other species. Nine other BatAstV sequences are included in the ORF1a (partial) and ORF1b phylogenetic trees. Alignment was based on the encoded amino acid sequences. Abbreviations used for different astroviruses are bat (BatAstV), mink (MAstV), ovine (OAstV), human (HAstV) and turkey type-1 (TAstV-1).

Figure 3. Phylogenetic tree constructed with RdRp gene sequences (422 nt) amplified by the RT-PCR screening assay. Sequences of 77 bat astroviruses, and other respective sequences of different astroviruses isolated from human (HAstV), mink (MAstV), ovine (OAstV) and turkey type-1 (TAstV-1) were included and aligned based on the nucleotide sequences. For the bat specimens collected during the phase 2 longitudinal study, the sampling dates are indicated in the tree as follows: (1) - June 2005; (2) - August 2005; (3) - December 2005; (4) - March 2006.

Figure 4. Phylogenetic tree constructed with 750 nt sequences of RdRp gene and ORF1b (3' end) of representative astroviruses isolated from bat (BatAstV) human

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503 (HAstV), mink (MAstV), ovine (OAstV) and turkey type-1 (TAstV-1). These
504 sequences were aligned based on the encoded amino acid sequences and reverse
505 translated back to nucleotides for the phylogenetic analysis.

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506 Table 1. Detection of astrovirus in bats by RT-PCR

	Rectal samples			Throat samples			Bats		
	No. tested	No. positive	Positive %	No. tested	No. positive	Positive %	No. tested	No. positive	Positive %
Insectivorous bats									
Hipposideros armiger	10	0	0%	10	0	0%	10	0	0%
Miniopterus magnater	122	67	55%	123	4	3%	132	67	51%
Miniopterus pusillus	73	31	42%	71	6	8%	74	32	43%
Miniopterus schreibersii	3	3	100%	2	1	50%	3	3	100%
Myotis chinensis	9	3	33%	9	1	11%	9	3	33%
Myotis ricketti	12	10	83%	12	5	42%	12	10	83%
Pipistrellus abramus	2	1	50%	1	0	0%	3	1	33%
Rhinolophus rouxi	8	1	13%	8	2	25%	8	2	25%
Fruit bats									
Cynopterus sphinx	11	0	0%	10	0	0%	11	0	0%
Total	250	116	46%	246	19	8%	262	118	45%

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507 Table 2. Amino acid sequence similarities between prototype BatAstV AFCD337, subgroup A (BatAstV AFCD68), subgroup B (BatAstV

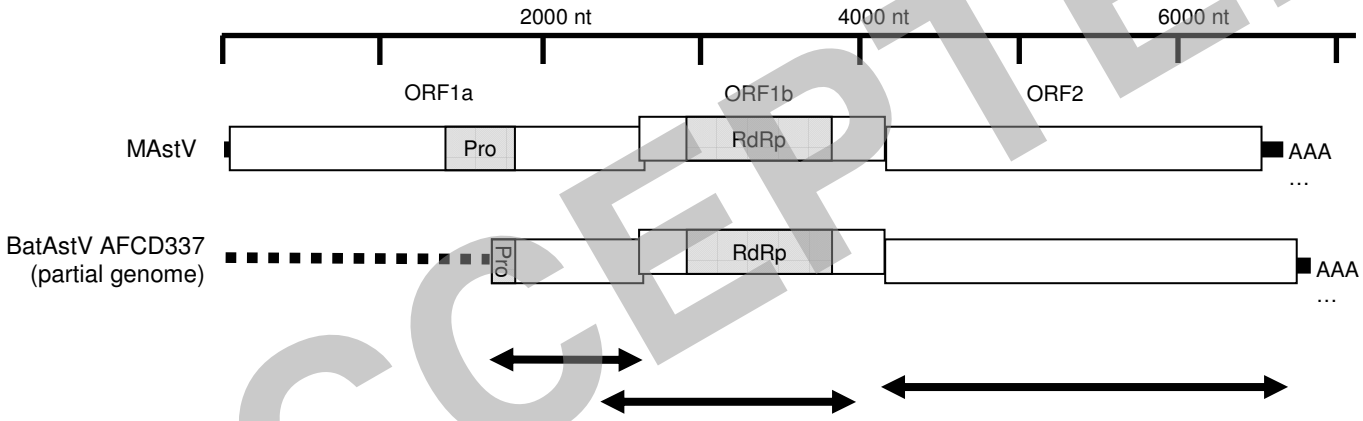
508 WCF140) and other astroviruses.

	BatAstV AFCD337		
Strains	ORF1a (partial)	ORF1b	ORF2
BatAstV AFCD68	79.9%	74.2%	-
BatAstV WCF140	54.0%	68.5%	-
HAstV-1 Dresden	17.2%	48.4%	22.3%
HAstV-1 Oxford	17.9%	49.2%	22.1%
HAstV-4 Guangzhou	17.5%	49.0%	22.3%
HAstV-4 Goiania/95/Brazil	17.9%	49.2%	22.2%
HAstV-4 Dresden	17.9%	48.8%	21.8%
HAstV-5 Goiania/94/Brazil	17.5%	49.2%	20.9%
HAstV-8	17.2%	48.8%	20.5%
MAstV	31.2%	52.7%	24.1%
OAstV	28.9%	52.6%	26.2%
TAstV-1	13.9%	40.2%	14.1%

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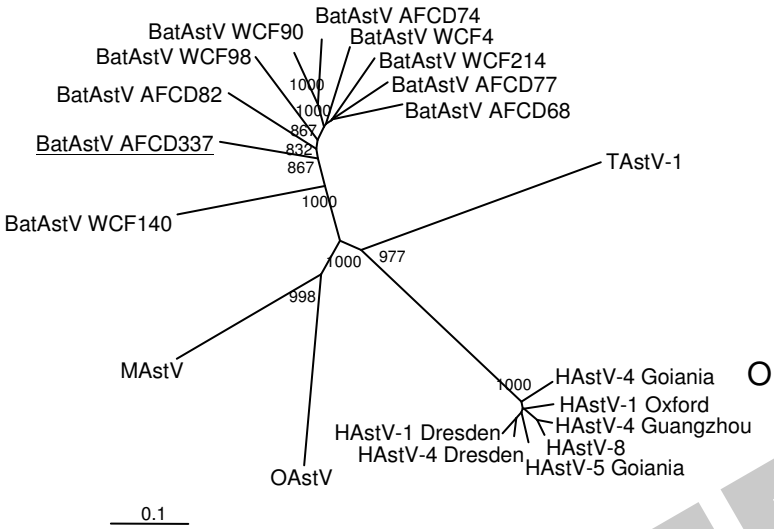
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Figure 1

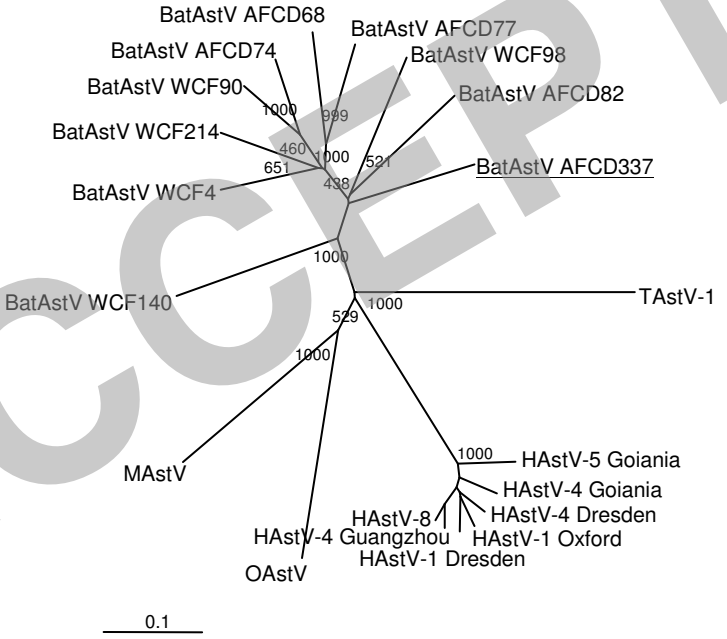


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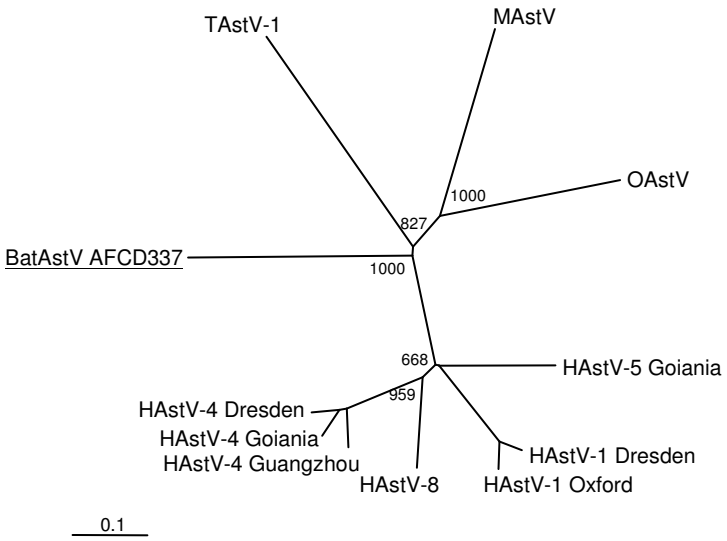
Figure 2



ORF1a (partial)



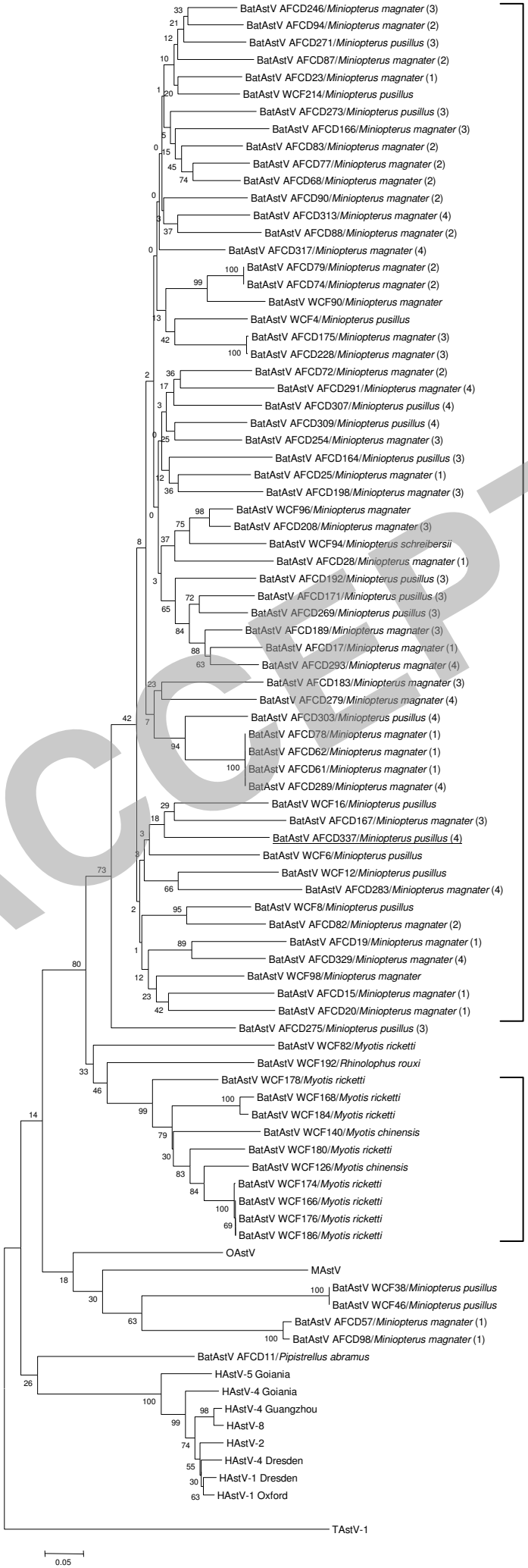
ORF1b (complete)



ORF2 (complete)

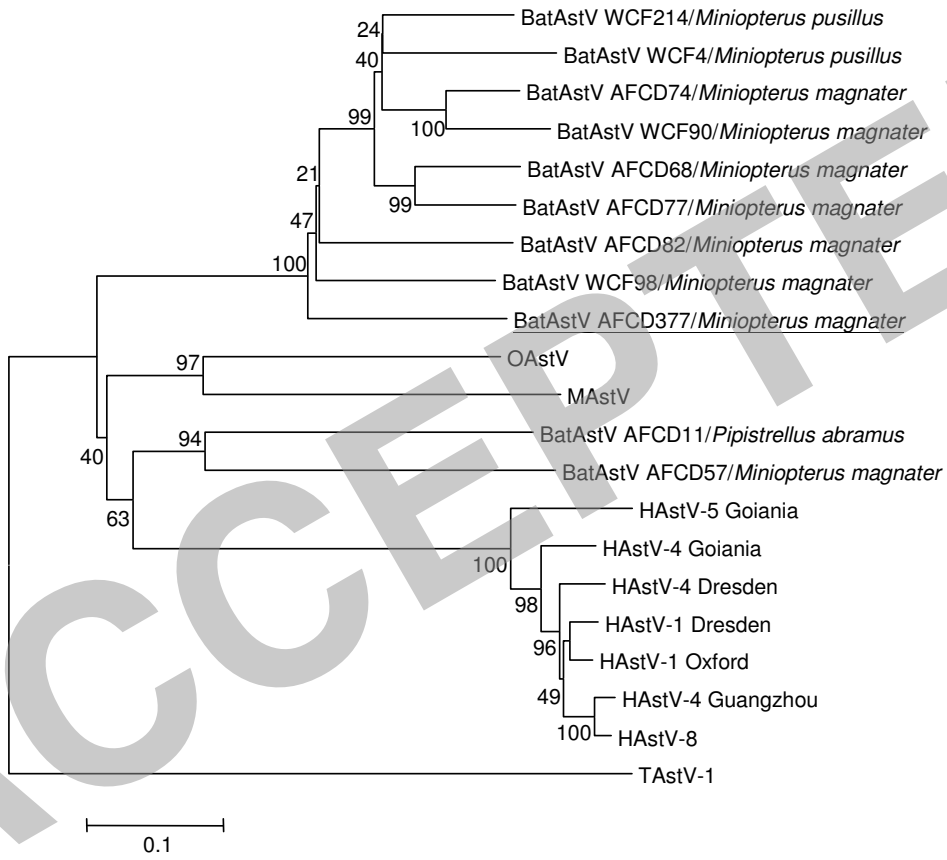
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Figure 3



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Figure 4



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