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1 **Novel astroviruses in insectivorous bats**

2

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18

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25

26 **Abstract**

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

44

45

46

47 **Introduction**

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

60  
61 Astroviruses have been identified from a variety of mammals (genus mamastrovirus)  
62 and birds (genus avastrovirus) including humans (HAstV), bovine (BAstV), pigs  
63 (PAstV), ovine (OAstV), mink (MAstV), dogs, cats, mice, chickens (CAstV) and  
64 turkeys (TAstV). In most species, these viruses are associated with gastroenteritis but  
65 some avian astroviruses have been associated with both intestinal and extraintestinal  
66 manifestations [reviewed in (19)]. Human astroviruses appear to cause milder disease  
67 than rotaviruses but are the second or third most common viral agent found in  
68 children with diarrhea (5, 8). Astrovirus can also cause significant disease in the  
69 elderly (16) and in immunocompromised patients (6).

70

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

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.

87

88 **Methods**

89

90 Sample collection

91 The sampling of bats was carried out in two phases and the sampling methods have  
92 been described previously (4, 25). Phase 1 was carried out in 2004 and 2005 and  
93 during this phase, swab samples were collected from different species of bats captured  
94 in the wild in different habitats in Hong Kong. Phase 2 was carried out in 2005 and  
95 2006 and swab samples were collected from bats of the genus *Miniopterus* captured  
96 on four sampling occasions (June, August and December 2005 and March 2006) in a  
97 single habitat, an abandoned mine cave in Lin Ma Hang, Hong Kong, near the border  
98 with Mainland China (4). In both phases, species of bats captured for sampling were  
99 healthy and identified by a bat taxonomist. Rectal and throat swabs, together with  
100 fresh fecal samples if available, were collected. Swabs were placed in viral transport  
101 medium (Earle's balanced salt solution, 0.2% sodium bicarbonate, 0.5% bovine serum  
102 albumin, 200 µg of vancomycin per liter, 18 µg of amikacin per liter, 160 U of  
103 nystatin per liter) in screw cap tubes and transported in a cool box to the laboratory  
104 for processing. Bats were released at the site after sampling.

105

106 Viral nucleic acid extraction and RT-PCR

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

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

117

118 Random hexamer-generated cDNA was screened for the presence of astrovirus using  
119 hemi-nested PCR targeting RdRp gene. A 50 µl PCR reaction was set up containing 1  
120 U Accuprime *Taq* DNA polymerase in 1x reaction buffer (Invitrogen), 2 µM each of  
121 forward and reverse primers and 2 µl of cDNA or 1 µl of first PCR product as a  
122 template. First-round PCR was carried out with a mix of two forward primers  
123 5'-GARTTYGATTGGRCKCGKTAYGA-3' and  
124 5'-GARTTYGATTGGRCKAGGTAYGA-3', and reverse primer

125 5'-GGYTTKACCCACATNCCRAA-3'. After an initial incubation at 94°C for 1 min,  
126 30 cycles of amplification were carried out consisting of denaturation at 94°C for 30  
127 sec, annealing at 50°C for 30 sec and extension at 68°C for 30 sec. Hemi-nested PCR  
128 was carried out with a mix of two forward primers

129 5'-CGKTAYGATGGKACKATHCC-3' and 5'-AGGTAYGATGGKACKATHCC-3'

130 and the same reverse primers used in the first-round PCR using the same

131 thermocycling conditions as for the first-round PCR except that 40 cycles of

132 amplification were performed. Water controls were included in each run of the

133 RT-PCR assay. PCR products were analyzed by standard agarose gel electrophoresis.

134 The expected product size of the 2nd PCR is 422 base pairs. All positive results were

135 verified by direct DNA sequencing of the PCR amplicons.

136

137 Cloning and sequencing of PCR products

138 PCR products were purified by QIAquick PCR purification kit (QIAGEN) following  
139 the manufacturer's instruction. Long PCR products (product sizes > 1000 bp) were  
140 gel purified with QIAquick gel extraction kit (QIAGEN) and then cloned into  
141 pCR2.1-TOPO plasmids (Invitrogen) for DNA sequencing. Multiple clones of a PCR  
142 product were picked and sequenced by using BigDye Terminator v3.1 Cycle  
143 Sequencing kit (Applied Biosystems). Sequencing products were analyzed by PRISM  
144 3700 DNA analyzer (Applied Biosystems).

145  
146 One astrovirus positive bat specimen, bat astrovirus AFCD337, was chosen for  
147 sequencing of the viral genome directly from the original clinical specimen by using  
148 cDNA generated by random hexamers and consensus primer RT-PCR. ORF2 region  
149 and the 3' end of the virus sample were amplified using 3' Rapid Amplification of  
150 cDNA Ends (RACE) System (Invitrogen) following the protocol provided by the  
151 manufacturer (primers and PCR conditions available on request). ORF1a (partial) and  
152 ORF1b sequences were assembled from multiple overlapping sequences derived from  
153 PCR amplicons. Nine additional ORF1a (partial) / ORF1b astrovirus sequences from  
154 other samples were obtained using similar method. The deduced sequence of these  
155 samples has at least 3-fold sequence coverage.

#### 157 Phylogenetic analysis

158 Sequence editing and sequence identities calculation were done using BioEdit version  
159 7.0.4 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Alignments of nucleotide  
160 sequences and amino acid sequences were done using Clustal W (29) with default  
161 parameters. Phylogenetic trees were constructed using Clustal X V2.0 (14) and Mega  
162 4 (28) by the neighbour joining method with the nucleotide substitution model of

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

165

166 Nucleotide sequence accession numbers.

167 The sequence of bat astrovirus AFCD337 reported in this paper were deposited in  
168 GenBank under accession numbers xxxxx, and RdRp and the partial ORF1b  
169 sequences of other strains of bat astroviruses were deposited under accession numbers  
170 xxxxx-xxxxx. For the genetic analysis in this paper, other astrovirus genomes were  
171 retrieved from GenBank including human astrovirus type 1 strain Oxford (L23513),  
172 human astrovirus type 1 strain Dresden (AY720892), human astrovirus type 4 strain  
173 Dresden (AY720891), human astrovirus type 4 isolate Goiania/GO/12/95/Brazil  
174 (DQ070852), human astrovirus type 4 isolate Guangzhou (DQ344027), human  
175 astrovirus type 5 isolate Goiania/GO/12/94/Brazil (DQ028633), human astrovirus  
176 type 8 (AF260508), mink astrovirus (NC\_004579), ovine astrovirus (NC\_002469)  
177 and turkey astrovirus type-1 (NC\_002470).

178 **Results**

179

180 Detection of astroviruses in bats

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

194

195 Astroviruses were detected in 7 out of the 9 species of bats screened, i.e. *Miniopterus*  
196 *magnater*, *Miniopterus pusillus*, *Miniopterus schreibersii*, *Myotis chinensis*, *Myotis*  
197 *ricketti*, *Pipistrellus abramus* and *Rhinolophus rouxi* (Table 1). The detection rates of  
198 astroviruses in these species of bats were remarkably high and ranged from 25% to  
199 100%. However, astrovirus was not detectable in our samples collected from  
200 *Cynopterus sphinx* (n=11) and *Hipposideros armiger* (n=10).

201

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

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

207

#### 208 Longitudinal study of astrovirus in bats at a single habitat

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

220

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

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

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

231

232 Amino acid sequences encoded by ORFs of the novel bat astrovirus were compared  
233 with that of other astrovirus genomes including human astrovirus (HAstV) types 1, 4,  
234 5 and 8 (17, 26) as well as mink astrovirus (MAstV) (22), ovine astrovirus (OAstV)  
235 and turkey astrovirus type-1 (TAstV-1) (11) (Table 2). The findings show that the  
236 identified bat astrovirus BatAstV AFCD337 is a novel mamastroviruses virus clearly  
237 distinct from other known astroviruses. It has <53% and <27% genetic similarity to  
238 other known astroviruses in the ORF1b and ORF2 regions, respectively.

239

240 The putative ORF1a (partial) and ORF1b of the bat astrovirus sequenced have sizes of  
241 909 nt and 1572 nt respectively. A region at 5' end of ORF1a remains unsequenced so  
242 far. ORF1a and ORF1b encodes for non-structural proteins which are essential for  
243 virus replication. Characteristic features of BatAstV AFCD337 ORF1a and ORF1b  
244 includes the protease motif in ORF1a; an astrovirus "slippery sequence" (AAAAAC)  
245 at the junction between ORF1a and ORF1b which is required for inducing a  
246 ribosomal shift event; a RdRp motif in ORF1b; and a conserved sequence at the end  
247 of ORF1b of astroviruses (11). A conserved stem-loop structure that is predicted at the  
248 3' end of the genomic RNA of human, ovine, porcine and turkey astroviruses type-1  
249 was not found in BatAstV AFCD337 (10).

250

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

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).

#### 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

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 *Miniopterus*  
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.

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

323 astroviruses from geographically diverse regions were estimated to range between  
324 92.6% to 99.1%. It has been previously reported that the amino acid sequence  
325 identities of RdRp gene (covering 80% of the RdRp gene regions analysed in this  
326 report) between 4 groups of avastrovirus, i.e. turkey astrovirus type 1-like viruses,  
327 turkey astroviruses type 2-like viruses, avian nephritis virus-like viruses and  
328 chicken-origin astroviruses detected in different regions were also highly diverse  
329 ranging from 50.1% and 73.8% (23). The high virus detection rates of BatAstV in our  
330 surveillance taken together with the marked genetic diversity of viruses from bats  
331 within the same habitat are reminiscent of our observations with bat coronaviruses (4).  
332 These findings may suggest that bats are persistently infected with astroviruses  
333 although mark-recapture studies are needed to confirm this contention. Other  
334 mammalian astrovirus infections tend to be short-lived in immunocompetent humans  
335 or other animals. However, type 3 HAsV has been associated with persistent  
336 gastroenteritis in immunocompetent children although the same virus serotype was  
337 not repeatedly demonstrated over the full period of clinical diarrhea (1).  
338  
339 Five BatAstV sequences from *Miniopterus magnater*, *Miniopterus pusillus* and  
340 *Pipistrellus abramus* failed to cluster with the subgroup A and B referred to above and  
341 some of these (AFCD11 from *Pipistrellus abramus* and AFCD57 from *Miniopterus*  
342 *magnater*) cluster rather with the human astroviruses although with weak statistical  
343 support (Figure 4). Whether these bat viruses are related to the precursor of HAsV is  
344 yet to be further investigated. Further sequence data from these strains may help  
345 elucidate this phylogenetic association.  
346  
347 Evidence of recombination between astroviruses and also that between coronaviruses

348 is well documented (13, 24, 27). It has been reported that a stem-loop motif in 3'  
349 untranslated region (UTR) was found conserved in mamastroviruses, TAstV-1, and in  
350 avian infectious bronchitis virus which is a group 3 coronavirus (10). However, this  
351 stem loop motif was not recognized in the BatAstV AFCD337. This 3' UTR  
352 stem-loop motif is also absent in TAstV-2. However a phylogenetic analysis of 3'  
353 UTRs did not indicate a close phylogenetic relationship between the two sequences of  
354 BatAstV AFCD337 (81 nt) and TAstV-2 (accession no.: NC\_005790) (196 nt) which  
355 lack the 3'UTR stem-loop structure (data not shown). Recently a report on the  
356 identification of a novel coronavirus from liver tissue of a whale with pulmonary  
357 disease and terminal acute liver failure showed that the ORF6 of the novel  
358 coronavirus possessed significant amino acid similarity to human astrovirus capsid  
359 proteins (21). The high rates of infection of bats in the same mine cave habitat with  
360 coronaviruses and astroviruses implies frequent co-infection with both viruses.  
361 Therefore we searched for sequence of BatAstV AFCD337 similar to bat  
362 coronaviruses using BLAST program with algorithms allowing a word-size down to  
363 seven bases. However no sequences with significant similarity was detected between  
364 bat astroviruses and coronaviruses co-circulating within the same species within the  
365 same habitat.

366

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

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380

ACCEPTED

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478 **Figure Legends**

479

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

485

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

492

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

500

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

503 (HAsV), mink (MAstV), ovine (OAsV) and turkey type-1 (TAsV-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 %
<u>Insectivorous bats</u>									
<i>Hipposideros armiger</i>	10	0	0%	10	0	0%	10	0	0%
<i>Miniopterus magnater</i>	122	67	55%	123	4	3%	132	67	51%
<i>Miniopterus pusillus</i>	73	31	42%	71	6	8%	74	32	43%
<i>Miniopterus schreibersii</i>	3	3	100%	2	1	50%	3	3	100%
<i>Myotis chinensis</i>	9	3	33%	9	1	11%	9	3	33%
<i>Myotis ricketti</i>	12	10	83%	12	5	42%	12	10	83%
<i>Pipistrellus abramus</i>	2	1	50%	1	0	0%	3	1	33%
<i>Rhinolophus rouxi</i>	8	1	13%	8	2	25%	8	2	25%
<u>Fruit bats</u>									
<i>Cynopterus sphinx</i>	11	0	0%	10	0	0%	11	0	0%
<u>Total</u>	250	116	46%	246	19	8%	262	118	45%

507 Table 2. Amino acid sequence similarities between prototype BatAstV AFCD337, subgroup A (BatAstV AFCD68), subgroup B (BatAstV  
508 WCF140) and other astroviruses.

Strains	BatAstV AFCD337		
	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%

509

Figure 1

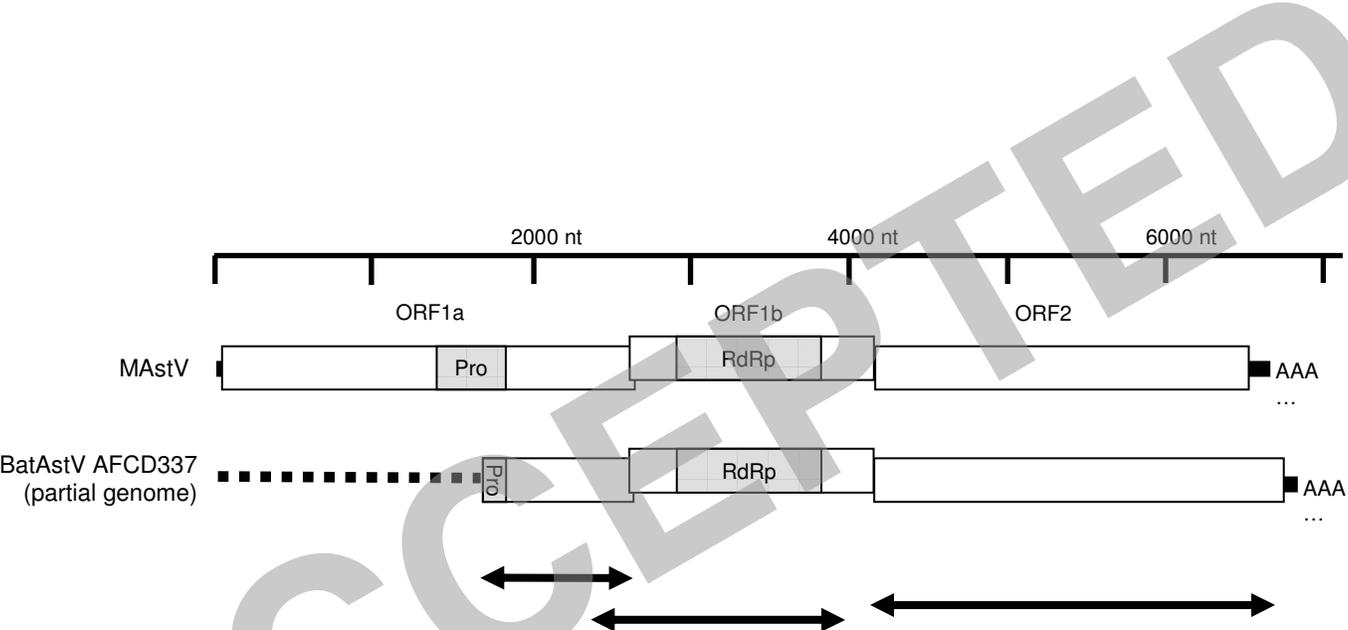


Figure 2

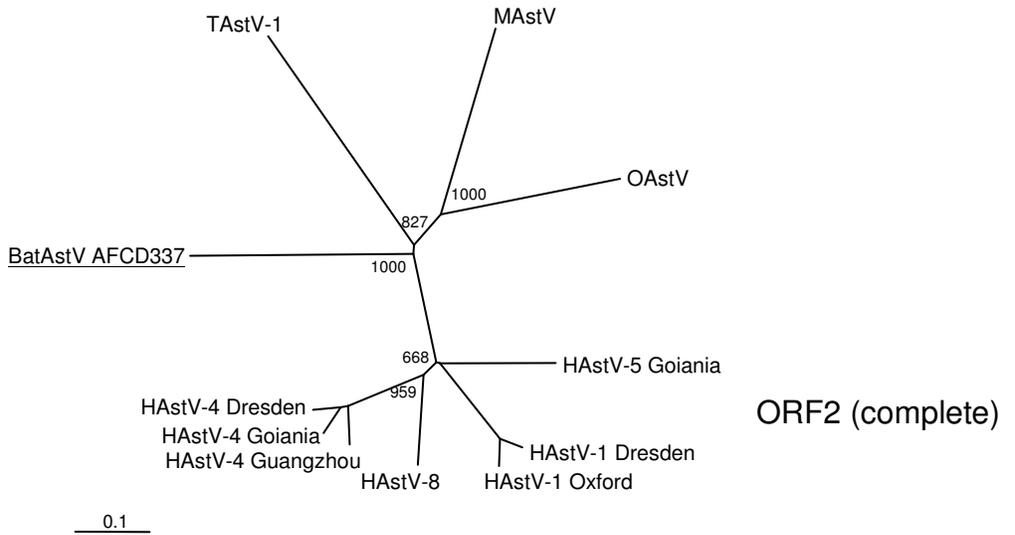
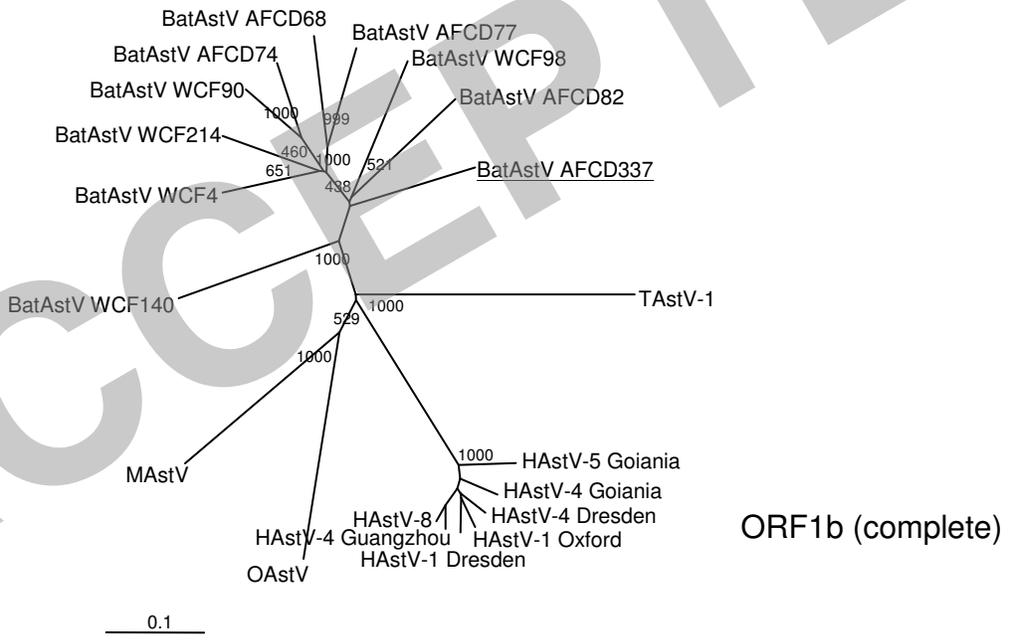
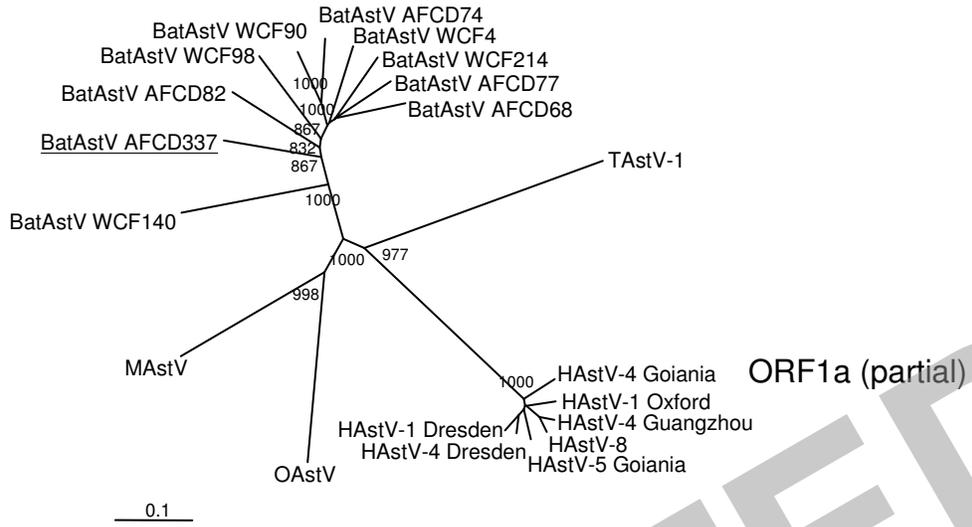
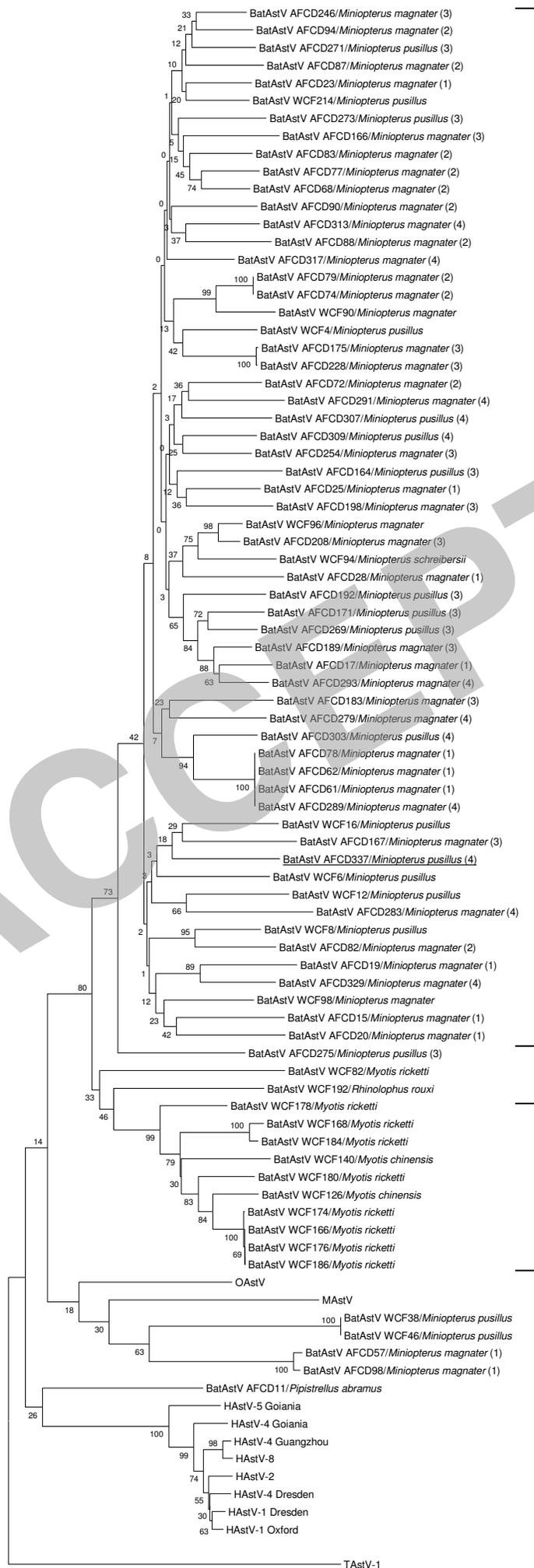


Figure 3



Subgroup A

Subgroup B

0.05

Figure 4

