Short Communication

Detection of novel astroviruses in urban brown rats and previously known astroviruses in humans

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Several novel astroviruses have been recently discovered in humans and in other animals. Here, we report results from our surveillance of astroviruses in human and rodent faecal samples in Hong Kong. Classical human astroviruses (n=9) and a human MLB1 astrovirus were detected in human faecal samples (n=622). Novel astroviruses were detected from 1.6 % of the faecal samples of urban brown rat (*Rattus norvegicus*) (n=441), indicating the prevalence of astrovirus infection in rats might be much lower than that recently observed in bats. These rat astroviruses were phylogenetically related to recently discovered human astroviruses MLB1 and MLB2, suggesting that the MLB viruses and these novel rat astroviruses may share a common ancestor.

The family *Astroviridae* comprises non-enveloped, positive-sense, single-stranded RNA viruses. The members of the family are classified into mamastroviruses and avastroviruses and are known to infect mammalian and avian species, respectively (Mendez & Arias, 2007). In human, the classical human astroviruses are genetically closely related and can be classified into eight serotypes (HAstV1–8). In addition, several genetically distinct human astroviruses (e.g. MLB1) have been recently identified in stool samples from patients suffering from gastroenteritis (Finkbeiner *et al.*, 2008a, 2009a, c; Kapoor *et al.*, 2009). Although astrovirus is one of the major causative agents for gastroenteritis, there is relatively little information on the ecology and evolution of these viruses. The recent discoveries of genetically diverse astroviruses in bats (Chu *et al.*, 2008b; Zhu *et al.*, 2009) and other animals (Atkins *et al.*, 2009; Rivera *et al.*, 2010) highlight the genetic diversity of astroviruses in nature and suggest that there might be many more novel astroviruses circulating in peridomestic and wild animals.

Bats represent the second largest group of mammals (comprises 20 % of all mammals). The remarkably high detection rate of astroviruses in bats prompted us to perform a similar surveillance study on rodents, which is the largest group of mammals accounting for 40 % of known mammalian species. As we were primarily interested in viruses that might be at the animal–human interface, we specifically targeted rodent species that have a long history of living in close proximity to human populations (Sullivan, 2003). Here, we report the discovery of rat astroviruses (RAstVs) from urban rats. In addition, we also report our recent surveillance of astroviruses in humans.

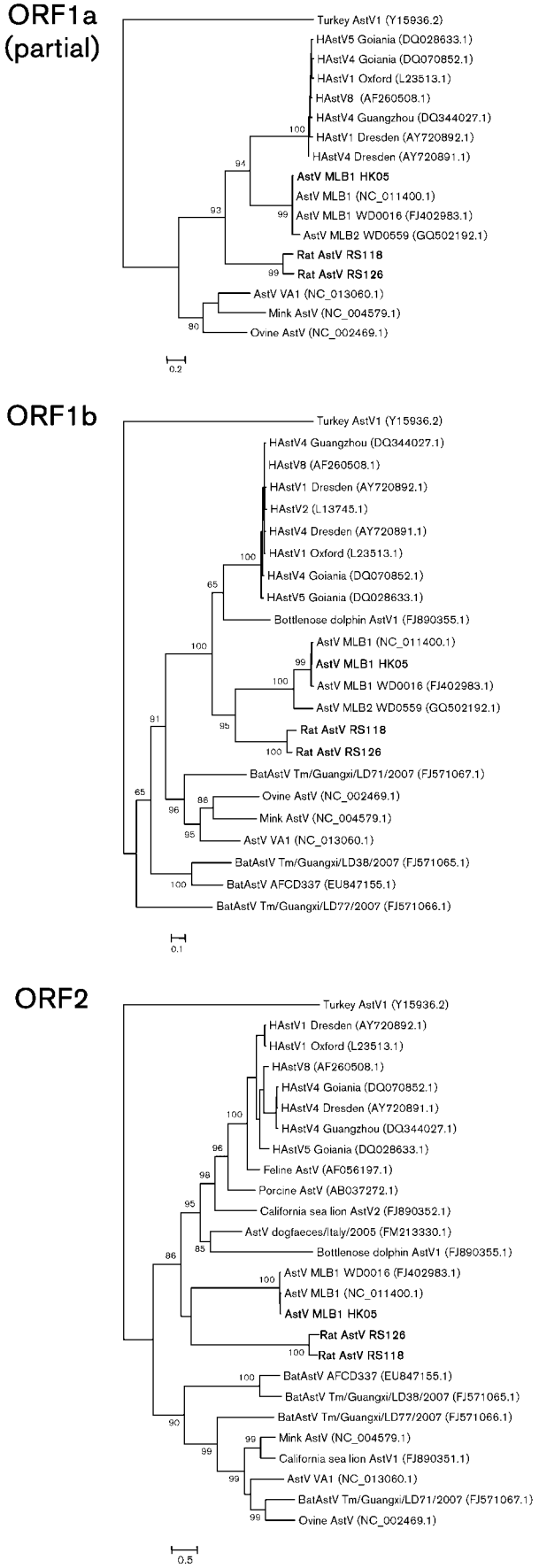
A total of 622 human stool samples collected from patients with diarrhoea admitted to the Queen Mary Hospital in Hong Kong during the years 2004 and 2005, and a total of 441 rat faecal samples collected from *Rattus norvegicus* (brown rat, n=371) and *Rattus rattus* (black rat, n=70) captured from urban areas in Hong Kong during the years 2007 and 2008 were resuspended in 1 ml of viral transport medium and tested as described before (Chu *et al.*, 2008b). RNA was extracted using QIAamp virus RNA mini kit (Qiagen) following the protocol recommended by the manufacturer. Hemi-nested RT-PCR assay with degenerated primers designed to detect the RNA-dependent RNA polymerase (RdRp) gene of all known human and other mammalian astroviruses (Chu *et al.*, 2008b) were used for the screening of astroviruses in these samples. All PCR amplicons obtained were subsequently analysed by sequencing.

Ten (1.6 %) of 622 samples from patients with diarrhoea were positive for astroviruses. Direct sequencing of the RT-PCR products revealed that nine (1.4 %) of these were classical human astroviruses and one (0.2 %) was MLB1 astrovirus. Though sequence identity suggests that the primers employed in our PCR screening test should detect other recently discovered human astroviruses MLB2, HAstV-VA1, VA2, VA3 (Finkbeiner *et al.*, 2009a, c) and HMO astroviruses (Kapoor *et al.*, 2009), none of these

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are HM450380–HM450386.

A supplementary table is available with the online version of this paper.

(a)


ORF1a
(partial)
Turkey AstV1 (Y15936.2)
HAstV5 Goiania (DQ028633.1)
HAstV4 Goiania (DQ070852.1)
HAstV1 Oxford (L23513.1)
HAstV8 (AF260508.1)
HAstV4 Guangzhou (DQ344027.1)
HAstV1 Dresden (AY720892.1)
HAstV4 Dresden (AY720891.1)
AstV MLB1 HK05
AstV MLB1 (NC_011400.1)
AstV MLB1 WD0016 (FJ402983.1)
AstV MLB2 WD0559 (GQ502192.1)
Rat AstV RS118
Rat AstV RS126
AstV VA1 (NC_013060.1)
Mink AstV (NC_004579.1)
Ovine AstV (NC_002469.1)
100
94
93
99
99
90
0.2
ORF1b
Turkey AstV1 (Y15936.2)
HAstV4 Guangzhou (DQ344027.1)
HAstV8 (AF260508.1)
HAstV1 Dresden (AY720892.1)
HAstV2 (L13745.1)
HAstV4 Dresden (AY720891.1)
HAstV1 Oxford (L23513.1)
HAstV4 Goiania (DQ070852.1)
HAstV5 Goiania (DQ028633.1)
Bottlenose dolphin AstV1 (FJ890355.1)
AstV MLB1 (NC_011400.1)
AstV MLB1 HK05
AstV MLB1 WD0016 (FJ402983.1)
AstV MLB2 WD0559 (GQ502192.1)
Rat AstV RS118
Rat AstV RS126
BatAstV Tm/Guangxi/LD71/2007 (FJ571067.1)
Ovine AstV (NC_002469.1)
Mink AstV (NC_004579.1)
AstV VA1 (NC_013060.1)
BatAstV Tm/Guangxi/LD38/2007 (FJ571065.1)
BatAstV AFCD337 (EU847155.1)
BatAstV Tm/Guangxi/LD77/2007 (FJ571066.1)
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ORF2
Turkey AstV1 (Y15936.2)
HAstV1 Dresden (AY720892.1)
HAstV1 Oxford (L23513.1)
HAstV8 (AF260508.1)
HAstV4 Goiania (DQ070852.1)
HAstV4 Dresden (AY720891.1)
HAstV4 Guangzhou (DQ344027.1)
HAstV5 Goiania (DQ028633.1)
Feline AstV (AF056197.1)
Porcine AstV (AB037272.1)
California sea lion AstV2 (FJ890352.1)
AstV dogfaeces/Italy/2005 (FM213330.1)
Bottlenose dolphin AstV1 (FJ890355.1)
AstV MLB1 WD0016 (FJ402983.1)
AstV MLB1 (NC_011400.1)
AstV MLB1 HK05
Rat AstV RS126
Rat AstV RS118
BatAstV AFCD337 (EU847155.1)
BatAstV Tm/Guangxi/LD38/2007 (FJ571065.1)
BatAstV Tm/Guangxi/LD77/2007 (FJ571066.1)
Mink AstV (NC_004579.1)
California sea lion AstV1 (FJ890351.1)
AstV VA1 (NC_013060.1)
BatAstV Tm/Guangxi/LD71/2007 (FJ571067.1)
Ovine AstV (NC_002469.1)
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viruses was detected from our human samples. To determine the sequence variation between MLB1 found in Hong Kong and the viruses previously detected from other continents, the complete genome sequence of MLB1 virus identified in this study (MLB1 HK05) was deduced. Primers used for determining the MLB1 HK05 genome were designed based on other MLB1 virus genomes previously reported in GenBank (available upon request). The MLB1 HK05 genome was found to be similar to other reported MLB1 genomes detected in Australia (Finkbeiner *et al.*, 2008b) and USA (Finkbeiner *et al.*, 2009b) (Fig. 1a), with overall nucleotide sequence identities of 95.2 and 91.8 %, respectively. The complete genome sequence of MLB1 HK05 can be accessed through GenBank (accession no. HM450380).

The screening by the RT-PCR assay of astrovirus in rats revealed that six (1.6 %) of 371 faecal samples from *R. norvegicus* and none of the samples from *R. rattus* were positive. The positive specimens were collected in 2007 (n=4) or 2008 (n=2) and were collected from different geographical locations in Hong Kong.

Phylogenetic analysis on the partial RdRp sequences of these rat astroviruses revealed a group of previously unknown astroviruses. The rat astrovirus sequences deduced in this study were found to have a remarkable

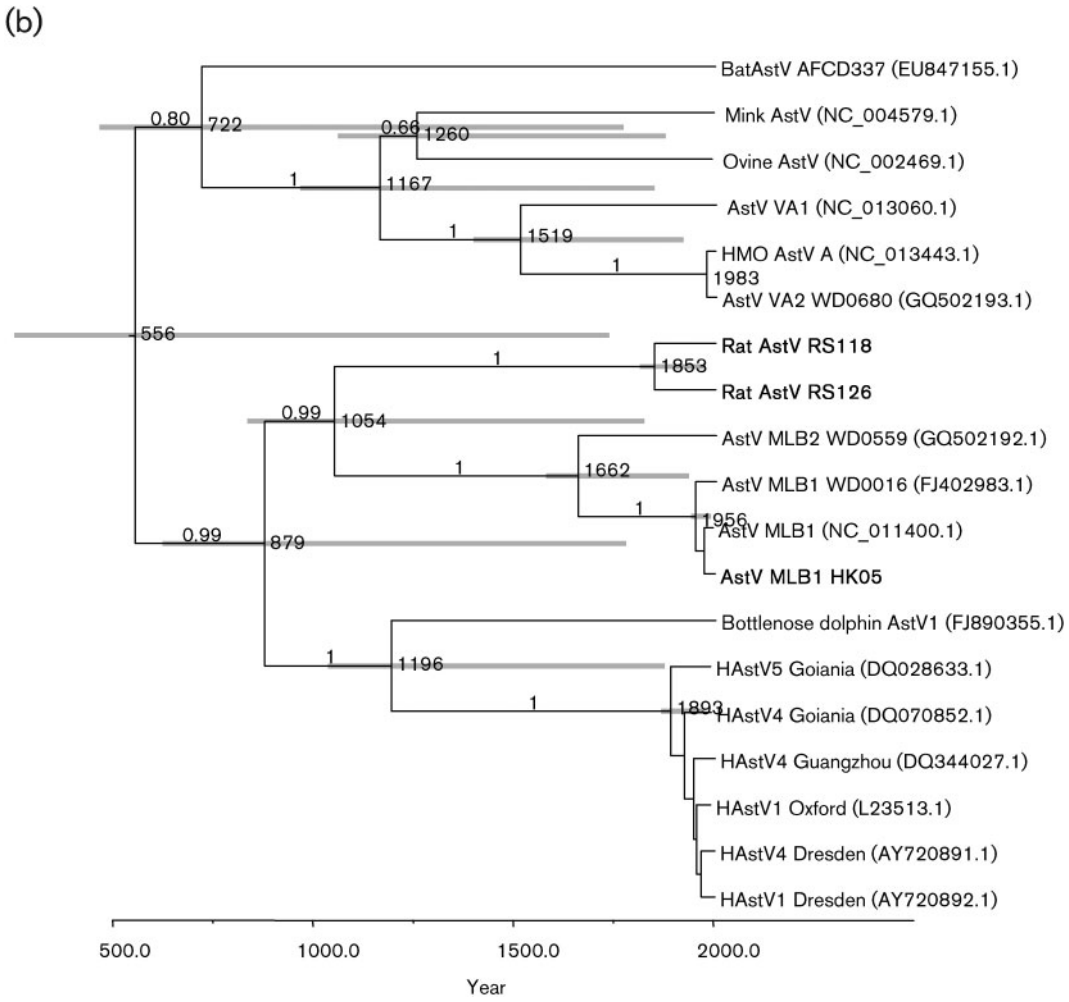

Fig. 1. (a) Phylogenetic trees for ORF1a (partial), ORF1b and ORF2 regions of representative astroviruses. Representative rat astroviruses (RS118 and RS126) and astrovirus MLB1 HK05 are in bold. Alignments of amino acid sequences were done by using CLUSTAL W with default parameters and edited manually for accuracy. Phylogenetic trees were constructed with maximum-likelihood method by PHYML. The reliability of nodes in the trees was estimated by the approximate likelihood ratio test method. Bars show the estimated genetic distance of these viruses. (b) Phylogenetic trees for ORF1b nucleotide sequences with dating estimated by BEAST. Mean date (in years) and the estimated posterior probabilities of nodes are shown. Ninety-five per cent highest posterior density regions of node dating are indicated by node bars. GenBank accession numbers of the reference sequences are indicated.

degree of sequence variation (82–93 % for the RdRP gene) and they formed two genetically closely related, but distinct, clusters in a phylogenetic analysis (represented by RS118 and RS126 in Fig. 1a). Samples RS118 and RS126 were selected for further sequence analyses. Primers used in determining the sequences of these viruses are summarized in Supplementary Table S1 (available in JGV Online). All nucleotide sequences of the RAstVs generated in this study can be accessed through GenBank (accession nos HM450381–HM450386). Attempts in culturing these viruses in BHK, CaCo-2, Vero and HEK 293 cells with or without trypsin (1–10 μg ml^{-1}) and attempts in amplifying the 5′ end of these RAstVs (predicted length: ~1500 nt) by using consensus primers deduced from other AstVs sequences were unsuccessful.

Excluding the poly(A) tail, the partial genome sequences of RS118 (4918 nt) and RS126 (4853 nt) were constructed by aligning multiple overlapping PCR fragments. Each of these assembled sequences represented the 3′ two-third of the corresponding viral genome, which includes partial ORF1a, complete ORF1b, complete ORF2 and 3′ UTR regions. Serine protease gene and RdRp gene can be identified in ORF1a and ORF1b, respectively, from both RAstV sequences. A heptameric slippery sequence (AAAAAAC) can be identified at the putative ORF1a/1b junction of each of these RAstVs. Similar to the MLB1 virus (Finkbeiner *et al.*, 2008b), a stem–loop II-like motif (s2m), which can be commonly found in the 3′ UTR of many astroviruses, could not be found in the RAstVs.

Pair-wise amino acid identities of different ORFs between astroviruses indicated that these rat viruses are genetically distinct from other previously known astroviruses (Table 1). Between the two representative RAstVs, sequence identities of ORF1a (partial), ORF1b and ORF2 regions were 79.4, 91.6 and 70.9 %, respectively (Table 1). These representative rat viruses are phylogenetically distinct from each other and have a sister-clade relationship, which is supported by high posterior probability values in all ORFs (Fig. 1a). Sequence analysis of these and other astroviruses by using Recombination Detection Program 2 (Martin *et al.*, 2005) and Simplot (Lole *et al.*, 1999) did not reveal strong evidence of recombination events in the rat viral genomes. Overall, our results suggested that these are two groups of astroviruses circulating in *R. norvegicus* in Hong Kong.

Interestingly, these RAstVs are shown to be phylogenetically closely related to the human MLB1 and MLB2 astroviruses (hereinafter MLB viruses) with strong statistical support (Fig. 1a, ORF1b and ORF2). It should be noted that this sister-clade relationship does not have a strong statistical support in the ORF1a sequence analyses

Table 1. Protein sequence identities between representative astroviruses

Only the 3′ end of ORF1a region (~350 aa near 3′ end) was used in the analysis. ND, Not done; ID, identical.

ORF	RS118	RS126	HastV1 Oxford	AstV MLB1	AstV MLB2	Ovine AstV	Mink AstV
ORF1a (partial)							
Rat AstV RS118	ID						
Rat AstV RS126	0.794	ID					
HastV1 Oxford	0.348	0.344	ID				
AstV MLB1	0.384	0.399	0.444	ID			
Ovine AstV	0.304	0.299	0.281	0.316	ND	ID	
Mink AstV	0.34	0.301	0.277	0.308	ND	0.443	ID
Turkey AstV1	0.165	0.156	0.151	0.152	ND	0.177	0.178
ORF1b							
Rat AstV RS126	ID						
Rat AstV RS118	0.916	ID					
HastV1 Oxford	0.562	0.549	ID				
AstV MLB1	0.555	0.549	0.534	ID			
AstV MLB2	0.551	0.551	0.531	0.785	ID		
Ovine AstV	0.463	0.465	0.491	0.463	0.449	ID	
Mink AstV	0.485	0.482	0.492	0.45	0.456	0.633	ID
Turkey AstV1	0.343	0.338	0.355	0.37	0.381	0.365	0.366
ORF2							
Rat AstV RS118	ID						
Rat AstV RS126	0.709	ID					
HastV1 Oxford	0.214	0.219	ID				
AstV MLB1	0.196	0.203	0.242	ID			
Ovine AstV	0.174	0.183	0.211	0.202	ND	ID	
Mink AstV	0.2	0.189	0.209	0.194	ND	0.421	ID
Turkey AstV1	0.136	0.128	0.146	0.16	ND	0.178	0.16

(Fig. 1a). However, the lack of resolving power in the ORF1a analysis is very probably due to the lack of other viral sequences available for analysis.

We further selected ORF1b gene, which is the most conserved gene in astrovirus genomes, for a dating analysis using BEAST (Drummond & Rambaut, 2007). Complete ORF1b nucleotide sequences of selected mamastroviruses were aligned at the amino acid level. Dating analysis was done using the uncorrelated relaxed clock model, with rates at each branch being allowed to vary independently from a lognormal distribution. Markov chain Monte Carlo sample chains were run for 60 000 000 generations with sampling every 3000 generations under GTR nucleotide substitution model and allowing gamma-rate heterogeneity for all datasets. As indicated in Fig. 1(b), RAstVs and MLB viruses formed a unique clade which is supported by a posterior probability of 0.992. This strongly indicated that RAstVs and MLB viruses might share a common ancestor virus. The mean date of the most recent common ancestor (MRCA) virus was estimated to be AD 1054 (95 % highest posterior densities: AD 837–1827). In addition, the aligned ORF1b sequences were subjected to a d_N/d_S analysis by GA-Branch method incorporated in Datamonkey (http://www.datamonkey.org/) (Pond & Frost, 2005). This analysis failed to find an accelerated positive selection for the RAstVs or for the MLB viruses (not shown), suggesting that these RAstVs and MLB viruses have been circulating in and adapted to rats and humans, respectively, over a long period of time.

Phylogenetic analyses on ORF1b strongly indicated that the newly found rat astroviruses are genetically related to human astrovirus MLB viruses. *R. norvegicus* and *R. rattus* are two of the most commonly found rodent species in human settlements and are long known as major reservoirs for some zoonotic diseases (Morse, 1995). Our results suggest that the human MLB viruses and the rat astroviruses shared a common ancestor. However, with the limited sequencing data available at present, questions concerning the evolutionary history of these viruses cannot be answered in greater detail. Further surveillance in rodent species might help to improve the preciseness of the MRCA dating and better explain the origins of the rat astroviruses and MLB viruses.

We previously reported the detection of genetically highly diverse astroviruses and coronaviruses in bats (Chu *et al.*, 2008a, b; Poon *et al.*, 2005; Zhu *et al.*, 2009). The prevalence of these novel RNA viruses in bat faecal samples was found be extremely high (10–50 %) (Chu *et al.*, 2008a, b; Poon *et al.*, 2005; Zhu *et al.*, 2009). By contrast, less than 2 % of the studied rodent faecal samples were found to be positive for astrovirus. We also screened about 60 % of these rodent samples (n=403) for coronavirus, but none of them was positive in the assay (data not shown). These findings suggest that bats are unusual in their propensity to harbour astroviruses and coronaviruses and may have a more prominent role in shaping the evolution of these groups of viruses. However, as rats are more likely to have close and repeated contact to human populations, further systematic surveillance of novel viruses in rodent populations in different geographical locations should be encouraged.

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