



Genetic diversity and expanded host range of astroviruses detected in small mammals in Singapore

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ABSTRACT

Astroviruses are a genetically diverse group of viruses that infect a wide range of hosts, including small mammals. Small mammals were trapped at 19 sites across Singapore from November 2011 to May 2014. Pooled oropharyngeal and rectal swabs ($n = 518$) and large intestine tissue ($n = 107$) were screened using a PCR to detect the presence of Astrovirus RNA-dependent RNA polymerase (*RdRp*) gene. Astroviruses were detected in 93 of 625 (14.9%) of samples tested, with eight of 11 species of rats, shrews, and squirrels testing positive. This is the first detection of astroviruses in seven species (*Callosciurus notatus*, *Mus castaneus*, *Rattus tanezumi*, *Rattus tiomanicus*, *Sundamys annandalei*, *Suncus murinus* and *Tupaia glis*). Phylogenetic analysis of 10 *RdRp* gene sequences revealed that astroviruses from Singapore small mammals fall in three distinct clades, one that is specific to the common treeshrew (*Tupaia glis*), and two comprised of multiple species. One of these includes viruses from the cave nectar bat (*Eonycteris spelaea*), two rodent species, and a squirrel, suggesting that virus spillover from bats to small mammals may have occurred. Our results show an increased host range for astroviruses and highlight their potential for intra- and inter-species transmission.

1. Introduction

Astroviruses belong to the *Astroviridae* family, which is genetically diverse and includes two genera, *Avastrovirus* and *Mamastrovirus*, that generally infect avian and mammalian species, respectively [1]. However *Avastrovirus* has been detected in non-human primates [2] and *Mamastrovirus* in birds [3]. Astroviruses are non-enveloped, positive sense, single stranded RNA viruses that often cause co-infections with other enteric viruses such as adenoviruses and noroviruses [4]. The spectrum of disease varies by host and infection can be asymptomatic or can result in gastroenteritis, explosive diarrhea, hepatitis, nephritis and encephalitis [5]. In humans, the main symptom is diarrhea, however, infections in immunocompromised children can be lethal [6] and immunodeficient adults may experience a wide array of symptoms that are more severe and long-lasting [7].

Astroviruses infect a wide variety of species including humans, livestock (sheep, cows, pigs), companion animals (dogs and cats), wild animals (bats, rodents and monkeys), and marine species such as whales,

sea lions and penguins [8]. Astroviruses have been detected in a wide variety of rodent species from Africa, Asia, Europe and North America [9–13], and these animals appear to be an important host that supports high astrovirus diversity. Rodents are important hosts for multiple pathogens [14] due to their high species diversity, broad ecological distribution, rapid reproductive rates and commensal habits [15].

Astroviruses have relatively high mutation rates that are typical of RNA viruses, they frequently undergo recombination between multiple co-circulating strains that generates high genetic diversity and facilitates cross-species transmission [9]. In particular, astroviruses that are associated with human infection have been found in non-human primates, with evidence of recombination between human and non-human primate astroviruses [2]. Non-human primates were also found to harbor *Avastrovirus* [2]. Other examples of cross-species transmission include the detection of human-like astroviruses in brown rats (*Rattus norvegicus*) [10] and the evidence of *Avastrovirus* infection in minks [3]. Taken together, this demonstrates the vagility of astroviruses across host taxa, questioning current taxonomic classification.

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Apart from human astroviruses, in Singapore astroviruses have been described from two bat species (*Rhinolophus lepidus* and *Eonycteris spelaea*) [17] and a feline astrovirus was detected in a water catchment [18]. There are no published reports of astrovirus infections in small mammals from Singapore, and of the 16-recorded resident small mammal species [19], astroviruses have only been previously detected in the brown rat [10]. Here we report the discovery of multiple astroviruses in rats, shrews, and squirrels in Singapore and characterize their ecology and evolution.

2. Materials and methods

2.1. Animal trapping and sample collection

All animal work was conducted with approvals from the National University of Singapore Institutional Animal Care and Use Committee (IACUC B01/12), National Parks Board (Permit no NP/RP12-004) and the Agri- Food and Veterinary Authority of Singapore (Permit no AV16.01.004.0004).

From November 2011 to May 2015, small mammals were trapped in Singapore using baited Tomahawk and Sherman live traps at 19 different sites in four habitat types: forest, parks that are dominated by shrubs, scrubland, and highly urbanised sites where there is a high density of building, houses and roads. Animals were sedated with isoflurane, sexed and identified to species. Morphological measurements (body length, tail length, weight) were recorded and oropharyngeal and rectal swabs were taken and kept at 4 °C in the field before transport to the laboratory. Animals were marked with an ear tag, held until fully conscious and released at the capture location. Tissues were collected from euthanized individuals of common widespread pest species (*Rattus norvegicus*, *Rattus tanezumi* and *Suncus murinus*) and from individuals of non-pest species (*Callosciurus notatus*, *Mus castaneus*, *Rattus exulans*, *Rattus tiomanicus*, *Sundamys annandalei*, *Sundasciurus tenuis* and *Tupaia glis*) that died during handling or donated carcasses. All samples were stored at -80 °C before further processing.

2.2. Nucleic acid extraction, PCR and sequencing

Combined aliquots of oropharyngeal and rectal swabs and large intestine tissue were screened for the presence of astroviruses. Oropharyngeal and rectal swabs were vortexed and pooled, while large intestine tissue was homogenized with silicon carbide beads (BioSpec) using a FastPrep-24 Instrument (MP Biomedicals). Homogenized tissue was centrifuged at 4000 rpm for 1 min and the supernatant was removed for screening. RNA was extracted using a QiaExtractor or a QiaAMP Viral RNA Mini Kit (QIAGEN) following manufacturer's instruction. Complementary DNA was synthesized with random hexamers using Superscript II (Invitrogen). A heminested PCR amplification of the RNA-dependent RNA polymerase gene (*RdRp*) was performed as previously described [10].

PCR products were visualized on a 1.5% agarose gel stained with GelRed Nucleic Acid Gel Stain (Biotium). Amplicons with an estimated size of 422 bp were gel purified with a QIAquick Gel Purification kit (QIAGEN) and cloned using a *pGEM®-T Easy Vector System* (Promega). Plasmids were purified using an E.Z.N.A. Plasmid Mini Kit (Omega Bio-tek) and sequenced in forward and reverse directions by 1st Base DNA Sequencing Services (Axil Scientific, Singapore).

2.3. Phylogenetic analysis

Sequences were manually curated, primers were removed and forward and reverse reads *de novo* assembled to generate consensus sequences. Singapore small mammal astrovirus sequences generated from this study (GenBank accession numbers MT123336 to MT123345) and over 700 global astrovirus RdRp sequences downloaded from NCBI GenBank [16] were aligned using MUSCLE v3.8.31 [20] in Geneious

7.1.7 [21]. Alignments were curated to remove duplicate or redundant sequences, resulting in a final dataset of 459 RdRp sequences. A maximum likelihood phylogeny using the generalized time-reversible nucleotide substitution model with gamma rate heterogeneity and 1000 bootstrap replicates was reconstructed using RAXML v8.0.14 [22]. The phylogenetic tree was visualized and edited in FigTree v1.4.2 [23] and only bootstrap values $\geq 60\%$ are indicated.

3. Results

A total of 625 animals from 11 species were trapped including 136 Asian house rats (*Rattus tanezumi*), 109 Annandale's rats (*Sundamys annandalei*), 101 common tree shrews (*Tupaia glis*), 99 plantain squirrels (*Callosciurus notatus*), 95 Asian house shrew (*Suncus murinus*), 46 Asian house mice (*Mus castaneus*), 19 brown rats (*R. norvegicus*), 15 Malayan field rats (*R. tiomanicus*), three Polynesian rat (*R. exulans*), one brown spiny rat (*Maxomys rajah*) and one slender squirrel (*Sundasciurus tenuis*) (Table 1). Astrovirus was found in 8 of the 11 species tested, with a total of 93 of 625 (14.9%) individuals testing positive. The highest infection rate was observed in *Rattus spp* (10.3 to 26.7%) and tree shrews (23.8%), with the lowest rate in Asian house shrews (7.4%) (Table 1).

A total of 10 astrovirus sequences were generated from six species. The maximum likelihood phylogeny shows that astroviruses circulating in small mammals in Singapore fall into three distinct clades (Fig. 1A). Clade 1 is a monophyletic group of astroviruses from *T. glis* (bootstrap [BS] = 100%) that is most closely related to Mamastroviruses from diverse hosts including bats, sheep, non-human primates and mink (Fig. 1B). Clade 2 consists of astroviruses found in multiple bat pteropodid species from Bangladesh and Laos, along with sequences from three small mammal species (Fig. 1C). Specifically, there is an unsupported monophyletic Clade 2A that consists of sequences from the cave nectar bat (*Eonycteris spelaea*) sampled in Singapore [17] along with astrovirus from *C. notatus*, *M. castaneus* and *R. tanezumi*. The nucleotide sequence identity between the astrovirus RdRp sequences from the three small mammals species and closely related sequences from *Eonycteris spelaea* was 94.4–98.7% (Table 2). In Clade 3 sequences from three rat species (*R. tanezumi*, *S. annandalei* and *R. norvegicus*) form a monophyletic group (BS = 97%) with astroviruses from *R. norvegicus* in Hong Kong (Fig. 1D). This group of *Rattus* astroviruses is then sister group to astroviruses from another rodent (*Niviventer eha*) found in China.

Table 1

Astrovirus prevalence in small mammal species, Singapore.

Host species	Common name	No. Positive/No. Tested (%)		Total (%)
		Oral/Rectal Swabs	Large Intestine	
<i>Callosciurus notatus</i>	Plantain squirrel	16/87 (18.4)	2/12 (16.7)	18/99 (18.2)
<i>Maxomys rajah</i>	Brown spiny rat	0/1 (0)	–	0/1 (0)
<i>Mus castaneus</i>	Asian house mouse	5/31 (16.1)	1/15 (6.7)	6/46 (13)
<i>Sundamys annandalei</i>	Annandale's rat	14/94 (14.9)	1/15 (6.7)	15/109 (13.8)
<i>Rattus exulans</i>	Pacific rat	0/2 (0)	0/1 (0)	0/3 (0)
<i>Rattus norvegicus</i>	Brown rat	2/15 (13.3)	3/4 (75)	5/19 (26.3)
<i>Rattus tanezumi</i>	Asian house rat	12/106 (11.3)	2/30 (6.7)	14/136 (10.3)
<i>Rattus tiomanicus</i>	Malayan field rat	4/12 (33.3)	0/3 (0)	4/15 (26.7)
<i>Suncus murinus</i>	Asian house shrew	7/75 (9.3)	0/20 (0)	7/95 (7.4)
<i>Sundasciurus tenuis</i>	Slender squirrel	–	0/1 (0)	0/1 (0)
<i>Tupaia glis</i>	Common tree shrew	22/95 (23.2)	2/6 (33.3)	24/101 (23.8)
Total		82/518 (15.8)	11/107 (10.3)	93/625 (14.9)

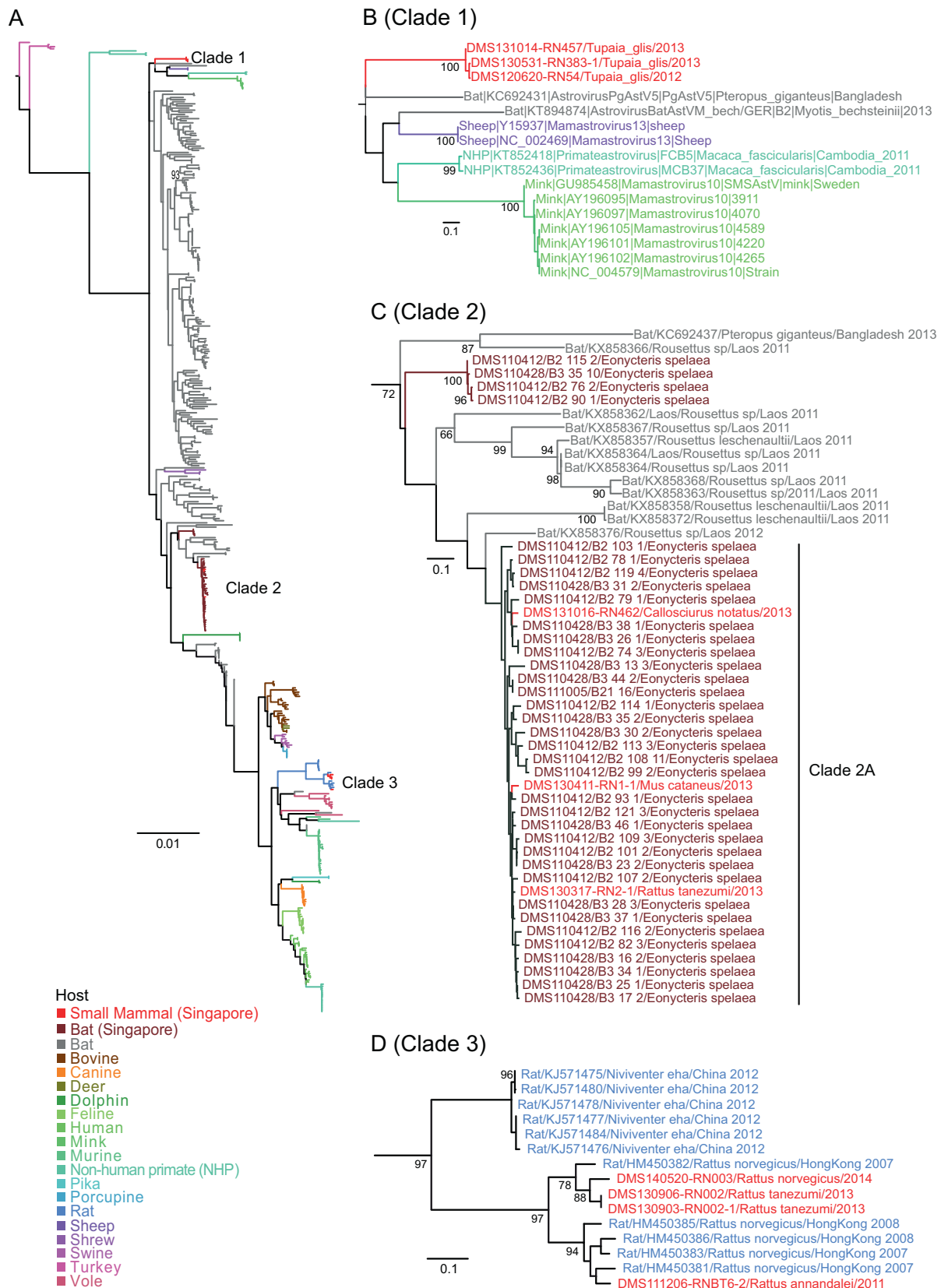


Fig. 1. Maximum likelihood phylogeny tree based on the astrovirus *RdRp* gene (A). Three clades containing the identified astrovirus sequences are shown in panels B, C and D. Branches containing the astrovirus sequences identified from small mammals in this study are shown in red. The other colored branches indicate different host species. The tips are labeled with astrovirus sequence ID, host species and year. The scale bar indicates nucleotide substitutions per site and bootstrap values $\geq 60\%$ are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

Nucleotide similarity (%) between astrovirus RdRp gene sequences from cave nectar bats (*Eonycteris spelaea*) and small mammal species, Singapore.

<i>Eonycteris spelaea</i>		Small mammals		
		DMS131016-RN462 /Callosciurus notatus/ 2013	DMS130411- RN1-1 /Mus castaneus/ 2013	DMS130317-RN2- 1 /Rattus tanezumi/ 2013
DMS110412/ B2_74_3	94.9	–	–	
DMS110412/ B2_79_1	94.4	–	–	
DMS110428/ B3_26_1	95.2	–	–	
DMS110428/ B3_38_1	95.4	–	–	
DMS110412/ B2_93_1	–	95.7	–	
DMS110412/ B2_107_2	–	–	96.8	
DMS110428/ B3_28_3	–	–	98.7	
DMS110428/ B3_37_1	–	–	97.9	

4. Discussion

This study demonstrates the significant genetic diversity of astroviruses present in small mammal species. We report the presence of astroviruses in seven new small mammal species in Singapore and provide evidence of virus spillover from bats into three small mammal species. These astroviruses form both species-specific and multi-host clusters, highlighting the capacity of this virus and its capacity to spillover [8]. Astrovirus sequences from *Tupaia glis* were not closely related to the majority of known Mamastroviruses, grouping with mink, non-human primate and sheep astroviruses. Treeshrews are not true shrews but form an order, Scandentia, that is distantly related to rodents and primates. The phylogenetic relationships of the treeshrew astroviruses may therefore reflect this taxonomic isolation, or may be due to unsampled astrovirus diversity. We also detected astrovirus in three *Rattus* species that form a monophyletic clade with other *Rattus* and *Niviventer* astroviruses from Hong Kong and China, respectively, indicating there may be a genus-specific strain of astroviruses circulating in these closely related genera [13].

There is increasing evidence of cross species transmissions and subsequent adaptation of astrovirus to novel host species [24] that is likely aided because of its high environmental stability [9]. We found astrovirus sequences in three small mammal species (*C. notatus*, *M. castaneus*, and *R. tanezumi*) that are most closely related to astroviruses found in *Eonycteris spelaea*, suggesting that virus spillover from bats to small mammals may have occurred. As the *M. castaneus* and *R. tanezumi* were trapped directly underneath the *Eonycteris* roost, this may represent a novel interspecies transmission of astrovirus. But we can't discount the possibility that we have detected the transient presence of bat astrovirus in our samples after these animals ingested bat excreta while foraging. In contrast, the squirrel was trapped approximately 3 km from the bat roost, however, bats and squirrels both occupy forest canopy that may provide a route for interspecies transmission.

Our study provides additional evidence that host ecology is a major determinant of virus host range and risk of zoonotic spillover [25]. *Mus castaneus*, *R. norvegicus*, *R. tanezumi* and *Suncus murinus*, the Asian house shrew, are peridomestic/synanthropic species often residing alongside humans throughout Southeast Asia [26,27], some of which are known to harbor medically relevant pathogens such as *Bartonella* spp [28] and *Rickettsia* spp [29]. *Rattus tiomanicus* inhabits secondary forest and scrubland [30], while *Sundamys annandalei* is primarily restricted to old secondary forest [31]. Both these rat species host *Rickettsia* spp and are maintenance reservoirs in the transmission cycle of scrub typhus [32]. The

plantain squirrel, *Callosciurus notatus*, resides in forest, scrubland and parkland [30] and is a reservoir of *Escherichia coli* [33]. *Tupaia glis*, the common treeshrew, inhabits forests and scrublands [30] and harbors several pathogens including *Toxoplasma gondii* [34] and *Bartonella* spp [28].

Astroviruses are an excellent candidate virus for studying the intersection of human, animal and environmental health. Astroviruses are highly prevalent with a global distribution and are found in a wide variety of host species [5]. They represent a public health and economic burden to humans and livestock, and there are examples of zoonotic spillover to humans and reverse zoonoses from humans to marine mammals [35,36]. The enzootic spillover from bats to rats described here could result in novel lineages in synanthropic species and increased zoonotic risk from bat-borne astroviruses. This reinforces the need for continued surveillance in sentinel species to proactively detect viruses with the capacity for spillover.

Author contributions

ESN, IHM, and GJDS conceived and designed the study. ESN and SAB conducted field and laboratory work. ESN, IHM, YCFS curated datasets and performed phylogenetic analyses. ESN, IHM, YCFS, and GJDS wrote the paper.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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