A Molecular Epidemiological Investigation of Carriage of the Adeno-Associated Virus in Murine Rodents and House Shrews in China

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Keywords

Adeno-associated virus · Rodents · Shrews · Epidemiological investigation · Phylogenetic analysis

Abstract

Objective: To investigate the prevalence of the adeno-associated virus (AAV) in murine rodents and house shrews in 4 provinces of China. **Methods:** A total of 469 murine rodents and 19 house shrews were captured between May 2015 and May 2017. *Cap* gene of AAV sequences was obtained to evaluate the genetic characteristics of rat AAV. **Results:** Rat AAVs were found in 54.7% (267/488) of throat swabs, 14.3% (70/488) of fecal samples, and 18.4% (41/223) of serum samples. Rat AAVs were detected in 3 species of murine rodents including *Rattus norvegicus* (34.8%), *R. tanezumii* (43.0%), and *R. losea* (2.3%), and house shrews (*Suncus murinus*) (26.1%) from the selected sampling sites. Fourteen near-full-length *Cap* gene sequences, ranging in length from 2,156 to 2,169 nt, were isolated from the fecal samples of *R. norvegicus* and *R. tanezumii*. These 14 sequences shared a high identity of 97.4% at the nucleotide level and 99.1% at the amino acid level. Phylogenetic analysis showed that the rat AAV formed a distinct clade, distinguishable from the AAV discovered in humans and in other animals. **Conclusions:** A high preva-

lence of rat AAV that was highly conserved within the *Cap* gene was found in 3 common murine rodents and house shrews in China.

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Introduction

The adeno-associated virus (AAV) is a single-stranded DNA virus, of the genus *Dependovirus* within the family Parvoviridae [1]. The replication of AAV requires a helper coinfection virus such as *Adenovirus*, *Herpesvirus*, or human *Papillomavirus* [2, 3]. When the helper virus is lacking, AAV will integrate into the host chromosome to establish a latent infection. AAV can infect both dividing and nondividing cells with a wide range of tissue tropism. The expression of AAV will last for long periods in host cells with mild immune responses [4–7]. The genome of AAV is approximately 4.7 kb and contains 2 open reading frames, referred to as *Rep* and *Cap*. The *Rep* gene encodes 4 nonstructural replication proteins required for the viral life cycle, and the *Cap* gene encodes 3 structural proteins (VP1, VP2, and VP3) [8]. According to the diversity of *Cap* genes, AAV can be divided into >100 viral variants and 10 serotypes [9]. Although AAV is generally consid-

Table 1. The prevalence of rat adeno-associated virus (rat AAV) in murine rodents and house shrews

Sample type	Species	XM (Oct 2015)	GZ (Feb 2016 to Feb 2017)	MM (Apr 2017)	YY (Mar 2017)	MLP (May 2017)	Total
Throat swab	<i>R. norvegicus</i>	15.0 (3/20)	42.9 (63/147)	61.3 (57/93)	78.7 (59/75)	94.2 (49/52)	59.7 (231/387)
	<i>R. tanezumi</i>	76.9 (10/13)	33.3 (2/6)	–	80.0 (12/15)	–	70.6 (24/34)
	<i>R. losea</i>	4.2 (2/48)	–	–	–	–	4.2 (2/48)
	<i>S. murinus</i>	75.0 (6/8)	36.4 (4/11)	–	–	–	52.6 (10/19)
	Subtotal	23.6 (21/89)	42.1 (69/164)	61.3 (57/93)	78.9 (71/90)	94.2 (49/52)	54.7 (267/488)
Fecal	<i>R. norvegicus</i>	5.0 (1/20)	13.6 (20/147)	14.0 (13/93)	24.0 (18/75)	13.5 (7/52)	15.2 (59/387)
	<i>R. tanezumi</i>	0 (0/13)	16.7 (1/6)	–	60.0 (9/15)	–	29.4 (10/34)
	<i>R. losea</i>	0 (0/48)	–	–	–	–	0 (0/48)
	<i>S. murinus</i>	0 (0/8)	9.1 (1/11)	–	–	–	5.3 (1/19)
	Subtotal	1.1 (1/89)	13.4 (22/164)	14.0 (13/93)	30.0 (27/90)	13.5 (7/52)	14.3 (70/488)
Serum	<i>R. norvegicus</i>	16.7 (2/12)	18.2 (14/77)	17.2 (10/58)	–	54.2 (13/24)	22.8 (39/171)
	<i>R. tanezumi</i>	0 (0/9)	0 (0/2)	–	–	–	0 (0/11)
	<i>R. losea</i>	3.0 (1/33)	–	–	–	–	3.0 (1/33)
	<i>S. murinus</i>	0 (0/2)	16.7 (1/6)	–	–	–	12.5 (1/8)
	Subtotal	5.4 (3/56)	17.6 (15/85)	17.2 (10/58)	–	54.2 (13/24)	18.4 (41/223)

Values are expressed as % (n/N). XM, Xiamen city in Fujian province; GZ, Guangzhou city in Guangdong province; MM, Maoming city in Guangdong province; YY, Yiyang city in Hunan province; MLP, Malipo county in Yunnan province.

ered nonpathogenic in humans, several studies have suggested that AAV infection during pregnancy may be a risk factor for adverse outcomes, and sperm infection with AAV may increase the risk of male infertility [10–12].

In addition to being detected in humans and nonhuman primates, AAVs have been detected in several domestic and wild animals, including pigs, cows, goats, chickens, snakes, bats, and rodents [13–21]. Lochrie et al. [21] used polymerase chain reaction (PCR) and genome-walking strategies to identify AAV sequences from rat and mouse liver samples. They discovered 2 novel AAVs, AAV-ra.1 and AAV-mo.1. Based on phylogenetic analysis of the *Cap* gene, AAV-ra.1 and AAV-mo.1 are most closely related to the AAV-5 group and an AAV strain derived from goats [21].

Rodents comprise the largest population of mammals and are natural reservoirs of a variety of zoonotic pathogens, including viruses, bacteria, and parasites [22]. However, except for the first report of rat AAV by Lochrie et al. [21], to our knowledge, no other related research reports have published. The distribution, prevalence, serotypes, and genetic diversity of AAV in rodents remain unclear. Here, we report the prevalence of AAV in murine rodents and house shrews in 4 provinces of China. Simultaneously, the *Cap* gene was amplified to elucidate the characteristics and genetic diversity of rat AAV.

Materials and Methods

Sample Collection and Nucleic Acid Extraction

A total of 488 animals, including 469 murine rodents and 19 house shrews, were captured using cage traps between May 2015 and May 2017. These animals were captured in or close to human residences in 5 regions of 4 provinces in China (Table 1). The rodents and shrews were anesthetized with diethyl ether, after which samples of cardiac blood were drawn. Serum samples were subsequently obtained using centrifugation. Throat-swab samples were obtained by swabbing the posterior oropharynx, and the swabs were then soaked in PBS. Throat-swab, fecal, and serum samples were immediately frozen at –80 °C for storage, and then thawed at 4 °C prior to processing.

The RNA/DNA of the virus was extracted using a MiniBEST viral RNA/DNA extraction kit (TaKaRa, Japan). A total of 1,199 samples were collected, including 488 throat swabs, 488 fecal samples, and 223 serum samples.

PCR Detection of AAV

A specific nested-PCR was conducted to detect AAV in throat-swab, fecal, and serum samples. Two nested-PCR primer pairs were designed according to the AAV reference sequence (AAV-ra.1, GenBank accession No. KY489985), to amplify 473 bp (Rat-AAV-F1/Rat-AAV-R1) and 373 bp products (Rat-AAV-F2/Rat-AAV-R2) in the first and second rounds of amplification (online suppl. Table S1; see www.karger.com/doi/10.1159/000493799). The DNA samples (2 µL) were added to a total reaction volume of 25 µL containing Premix Taq™, according to the manufacturer's protocol (TaKaRa). Both the first- and second-round amplifications were conducted under the following conditions: 94 °C, 5 min (1 cycle); 94 °C, 30 s; 57 °C, 35 s; 72 °C, 45 s (40 cycles); 72 °C, 10

min (1 cycle); and 4 °C, to infinity. In addition, 4 pairs of primers were designed to amplify the *Cap* gene. Details of these primers are presented in online supplementary Table S1.

Identification of Rodent and Shrew Species

To confirm the taxonomic identities of the murine rodents and shrews, PCR was used for amplification of the mitochondrial cytochrome *b* gene, as previously described [23]. The results were compared to the GenBank database using the Basic Local Alignment Search Tool x (BLASTx) with an *E* value cut-off of $<10^{-10}$.

Phylogenetic and Statistical Analyses

Multiple alignments were performed using the ClustalW multiple-sequence alignment program, which was implemented in MEGA v6.0 software (Oxford Molecular Ltd., UK). Phylogenetic trees were constructed using the neighbor-joining (NJ) and maximum-likelihood (ML) methods based on the results of alignment with MEGA v6.0. The robustness of the tree topology was assessed using 1,000 bootstrap replicates, as was implemented in the program. Lasergene SeqMan software (DNASTAR, Inc., Madison, WI, USA) was used to assemble sequences. After multiple alignments, the percent identity was selected to display the similarity of each sequence pair using Lasergene MegAlign software (DNASTAR, Inc., Madison, WI, USA).

Accession Numbers

The 14 near-full-length *Cap* gene sequences (GZ.512, HD.16, HD.20, HD.43, HD.94, MLP.26, XM.70, YY.12, YY.25, YY.54, YY.78, YY.93, MLP.6, and YY.80) were submitted to the GenBank databases (MH202992–MH203005).

Results

Prevalence of Rat AAV in Rodents and Shrews

According to nested-PCR detection, rat AAV was detected in the throat-swab, fecal, and serum samples, with positive rates of 54.7% (267/488), 14.3% (70/488), and 18.4% (41/223), respectively (Table 1). Among these positive samples, 2 *R. norvegicus* samples were simultaneously AAV-positive in the throat-swab, fecal, and serum samples.

The positive rates were diversified among the 4 species of animals. High positive rates were observed in throat swabs and fecal samples of *R. norvegicus* (59.7 and 15.2%, respectively) and *R. tanezumi* (70.6 and 29.4%, respectively). In addition, rat AAV was detected at relatively high positive rates in the throat swabs (52.6%) and serum samples (12.5%) of house shrews (*Suncus murinus*), but at a rate of only 5.3% in the fecal samples. The positive rates of rat AAV in *R. losea* were, comparatively, the lowest, at 4.2, 0.0, and 3.0% in the throat swabs, fecal samples, and serum samples, respectively. Although variations in prevalence were observed across sampling sites, rat AAV was found in all species of animals under investigation in all 5 regions, which is indicative of its broad distribution (Table 1).

Phylogenetic Analysis

Of the samples that were positive for rat AAV according to nested-PCR, 13 near-full-length *Cap* gene sequences (GZ.512, HD.16, HD.20, HD.43, HD.94, MLP.6, MLP.26, XM.70, YY.12, YY.25, YY.54, YY.78, and YY.93) were isolated from the fecal samples of *R. norvegicus* and 1 (YY.80) was isolated from *R. tanezumi*. The lengths of these 14 *Cap* gene sequences ranged from 2,156 to 2,169 nt. The identity among these sequences was very high, with an overall mean identity of 97.4% (range: 94.9–100%) at the nucleotide level and 99.1% (range: 97.5–100%) at the amino acid level. Furthermore, these 14 sequences had a high similarity to a previously reported rat AAV strain (DQ100363), with an overall identity in the range of 95.3–97.1% at the nucleotide level and 97.4–99.2% at the amino acid level.

A phylogenetic tree was constructed, based on the 14 *Cap* gene sequences obtained and representative *Cap* gene sequences of rat AAV, mouse AAV, AAV1–8, avian AAV, bovine AAV, caprine AAV, porcine AAV, and cynomolgus monkey AAV. The NJ and ML trees had identical tree topology. They showed that the sequences obtained in this study and a previously reported rat AAV strain (DQ100363) fell into a monophyletic lineage (rat AAV) (Fig. 1). Moreover, the rat AAV clade was distinguishable from all the other AAV strains (Fig. 1). The topology of the phylogenetic tree showed that rat AAV is most closely related to mouse AAV, caprine AAV, porcine AAV, and AAV-5.

Two sequences (MLP.6 and GZ.512), which represented the maximum and minimum variation within the 14 AAV sequences, were selected to analyze the genetic diversity of the *Cap* genes. The nucleotide and amino acid pairwise distance comparisons within the AAV are shown in Table 2. High levels of sequence divergence were observed between rat (MLP.6 and GZ.512) and other AAV strains, both at the nucleotide and amino acid levels (Table 2). Even when compared with the most closely related AAV (mouse AAV), the identities of rat AAV were only approximately 64 and 60% at the nucleotide and amino acid level, respectively.

Discussion

To the best of our knowledge, this study is the first epidemiological investigation of AAV in murine rodents and house shrews since that of Lochrie et al. [21] reporting AAV in rats and mice. We detected AAV in 3 species of murine rodents, *R. norvegicus*, *R. tanezumi*, *R. losea*, and

Fig. 1. Phylogenetic tree constructed using the neighbor-joining method based on rat adeno-associated virus *Cap* gene sequences. Rat AAV, rat adeno-associated virus; HD, Huadu district of Guangzhou city in Guangdong province; MLP, Malipo county in Yunnan province; YY, Yiyang city in Hunan province; GZ, Guangzhou city in Guangdong province; XM, Xiamen city in Fujian province.

house shrews. The AAV-positive animals were found in all 4 provinces under investigation (Guangdong, Yunnan, Fujian, and Hunan provinces), with a total positive rate of 54.8% in the throat swabs, 14.7% in the fecal samples, and 18.6% in the serum samples of murine rodents. The AAV-positive rate in the throat swabs, fecal samples, and serum samples of shrews was 52.6, 5.3, and 12.5%, respectively

(Table 1). These results indicate that rat AAV is broadly distributed among murine rodents and shrews in China. This study also suggests that the AAV can effectively infect murine rodents and shrews naturally, thereby further broadening our understanding of the hosts of AAV. Furthermore, in order to clarify the infection status of murine rodents and shrews, an antibody test for rat AAV in serum

Table 2. Pairwise similarity of AAV *Cap* gene sequences at the nucleotide and amino acid levels

Sequence		N																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	MLP.6																		
2	GZ.512		97.6	94.0	64.7	54.7	54.1	60.5	60.9	43.1	48.8	30.5	55.0	55.1	29.8	33.4	35.5	56.5	61.2
3	Rat AAV 1(DQ100363)	A	98.6		93.4	64.2	55.0	54.3	60.6	60.9	43.1	48.6	30.6	55.4	55.2	29.5	33.8	35.6	56.1
4	Mouse AAV 1(DQ100362)		97.8	96.8		65.9	54.6	54.5	60.9	61.7	42.9	48.5	29.9	54.8	55.5	30.1	32.9	35.2	55.7
5	AAV 6(AF028704)		60.5	60.0	60.7		55.4	56.8	58.1	59.2	42.1	50.6	29.5	55.4	56.3	30.6	33.0	35.4	57.2
6	Cynomolgus monkey AAV(AY631966)		53.2	52.6	53.7	58.7		64.0	57.9	57.5	47.9	65.9	30.4	97.1	58.3	28.7	42.9	43.7	58.6
7	Caprine AAV(AY724675)		49.7	49.0	49.7	56.0	68.0		57.8	57.8	47.0	57.4	29.3	64.2	74.6	27.6	41.8	41.7	61.6
8	Porcine AAV(FJ688147)		58.9	58.7	59.2	56.0	58.1	54.7		85.0	45.8	53.0	31.4	58.0	61.2	32.8	37.6	39.0	58.7
9	AAV 2(NC 001401)		59.7	59.6	60.1	56.3	59.6	54.9	89.4		45.4	52.6	29.6	57.4	59.5	32.1	37.5	38.0	57.6
			53.5	53.1	53.8	58.4	83.6	64.0	58.3	59.6		63.0	30.8	47.8	44.2	30.0	38.9	37.7	45.1
10	AAV 3(NC 001729)																		
11	AAV 4(NC 001829)		51.9	51.5	52.1	58.3	86.8	65.2	59.1	60.0	87.5		30.9	65.9	53.9	27.9	39.7	39.3	54.3
12	AAV 1(NC 002077)		49.1	48.4	49.0	54.6	64.9	81.7	53.7	54.2	61.5	63.7		30.4	28.8	43.1	28.1	30.0	28.4
13	Bovine AAV(NC 005889)		53.2	52.6	53.7	58.8	99.2	68.2	58.0	59.4	83.4	86.7	65.0		58.2	28.2	43.3	44.0	58.6
14	AAV 5(NC 006152)		48.8	48.2	48.7	55.7	61.8	79.3	57.0	56.9	60.0	61.1	77.0	61.9		29.3	38.0	37.4	61.1
15	AAV 7(NC 006260)		59.1	59.1	59.7	56.0	58.6	54.7	94.3	87.4	58.9	59.3	54.1	58.4	56.1		28.9	29.5	29.3
16	AAV 8(NC 006261)		53.2	52.7	53.4	58.1	85.6	67.9	58.5	59.4	82.9	84.6	64.6	85.5	61.3	58.7		58.2	37.0
17	Avian AAV(NC 006263)		53.2	52.6	53.2	58.6	84.4	66.6	58.6	59.2	83.2	85.6	64.4	84.2	59.9	58.5	88.3		36.9
18	Bat AAV(NC 014468)		49.7	49.7	49.9	55.5	58.8	56.6	54.9	55.2	59.4	60.1	55.6	58.5	55.8	54.9	60.2	59.1	59.1
			52.5	51.9	52.6	59.3	63.1	58.4	57.4	58.4	62.6	62.1	58.7	63.1	60.1	57.9	64.2	62.2	56.7

Values are percentages. N, pair sequence identity at nucleotide level; A, pair sequence identity at amino acid level; AAV, adeno-associated virus.

needs to be conducted. Considering the lack of a commercialized test kit at present, the establishment of a specific detection system for the rat AAV antibody is essential.

The genome of AAV shows considerable genetic diversity, and there are reportedly >100 viral variants and 10 serotypes based on the *Cap* gene [9]. The novel AAV strains discovered by Lochrie et al. [21] in 2006, referred to as rat AAV (AAV-ra.1) and mouse AAV (AAV-mo.1), show significant differences from those of primates and other nonprimate animal sources of AAV in previous reports. The AAV-ra.1 and AAV-mo.1 capsid amino acid sequences were <60% identical to any other AAV capsid. Moreover, in the 2 very close species, the AAV-mo.1 and AAV-ra.1 capsid amino acid sequences were only 58% identical to each other [21]. We used AAV *Cap* gene sequences from rats, mice, bats, birds, pigs, goats, cows, and primates to perform the phylogenetic tree (Fig. 1). The result showed that viruses isolated from different host species were clustered into distinct clades, indicating that AAV seemingly coevolved with its specific hosts. In the same time, in our study, AAV was detected in 3 species of murine rodents and in shrews with a highly similar *Cap* gene. The overall mean identity of the 14 *Cap* gene sequences that we obtained was 97.4% at the nucleotide level and 99.1% at the amino acid level. These results are inconsistent with previous reports of the considerable genetic diversity of AAV among closely related animals [20, 21]. In our study, murine rodents and shrews were generally captured from the same sites, and there were overlapping niches among dif-

ferent species of animals. Rat AAV can be effectively spread between rodents and shrews, resulting in a high similarity of the virus in different species of animals. Whether AAV coevolved with specific hosts or is transmitted among different hosts needs further investigated.

Owing to its high expression and low pathogenicity, AAV has been widely used as a vector in gene therapy. However, because of its high prevalence in the general population, the existing neutralizing antibodies and phagocytic cells may significantly reduce the transfection efficiency of recombinant (r)AAV vectors in vivo [24]. The utilization of alternative AAV serotypes could facilitate an escape from preexisting neutralizing antibodies, which act against natural infection, or could be applicable prior to treatment with AAV-based vectors. The application of a nonprimate animal-derived novel AAV as a vector is expected to reduce the immune response and increase the transfection efficiency of rAAV vectors in vivo [15]. Bello et al. [15] obtained new AAV capsid sequences from porcine tissues and successfully generated a recombinant vector (AAV2/po1) by transfection. They indicated that the AAV2/po1 vector has a distinct antigenic profile, which is not cross-neutralized by antisera generated against all other commonly used AAVs (serotypes 1–8). For the mouse AAV, Lochrie et al. [21] reported that a recombinant vector, with the mouse AAV capsid and a *lacZ* transgene (rAAV-mo.1 *lacZ*), is able to transduce rodent cell lines in vitro; however, it is not able to transduce 8 human cell lines or primary human fibroblasts in vitro. They

suggested that the AAV-mo.1 capsid could therefore be used as an experimental tool for gene transfer in rodents. Taking the genetic differences between rat AAV and mouse AAV into consideration, whether or not a recombinant vector with the rat AAV capsid is able to transduce human cell lines and avoid cross-neutralization with the serotypes of other AAVs requires further research.

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Statement of Ethics

The study protocol and animal experiments performed in this study conformed to internationally accepted standards and have been approved by the Institutional Animal Care and Use Committee of Southern Medical University.

Disclosure Statement

The authors declare that they have no conflict of interest.

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