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LETTER

Three Novel Avastroviruses Identified in Dead Wild Crows

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Dear Editor,

Astroviruses (AstVs) are non-enveloped, single-stranded RNA viruses with a diameter of approximately 35 nm (Madeley and Cosgrove 1975). The genomes of AstVs range in size from 6.8 to 7.9 kb (De Benedictis *et al.* 2011), consisting of a 5′-untranslated region (UTR), three open reading frames (ORFs), a 3′-UTR, and a poly-adenylated (polyA) tail (Pantin-Jackwood *et al.* 2006). In 1975, AstVs were first discovered in faeces of infants (Madeley and Cosgrove 1975). Until now, a variety of animal species, including cows, sheep, pigs, dogs, deer, rats, bats, chickens, ducks, turkeys, sea lions and seals have been reported to be infected with AstVs (Tzipori *et al.* 1981; Cattoli *et al.* 2007; Chu *et al.* 2008, 2010; Atkins *et al.* 2009; Toffan *et al.* 2009; Rivera *et al.* 2010; Guix *et al.* 2013; Kang *et al.* 2012; Reuter *et al.* 2012a, 2012b; Li *et al.* 2021). Currently, AstVs comprise two genera, *Mamastrovirus* (MAstV) and *Avastrovirus* (AAstV). Herein, we describe three novel AAstVs identified from dead crow tissues. To our knowledge, this is the first report for identification of AstVs in crows.

Over 300 crows were found dead in Henan Province in late December 2017. 35 samples, including the heart (n = 5), liver (n = 5), spleen (n = 5), lung (n = 5), kidney (n = 5), intestine (n = 5), and brain (n = 5) tissues were collected from five dead crows for processing. Total RNA was extracted using the QIAamp RNA Viral Kit following manufacturer's instructions. Samples were tested for West Nile virus, Newcastle disease virus, influenza A virus, Japanese encephalitis virus (Mabsky Biotech Co., Ltd., SKY-8624), and Russian spring–summer encephalitis virus (Mabsky Biotech Co., Ltd., SKY-8630) by RT-PCR or qRT-PCR using specific primers (Supplementary Table S1) or commercial kits (Mabsky Biotech Co., Ltd., SKY-8624 and SKY-8630). However, all samples tested negative for these pathogens. The lungs of the first four crows were mixed and named as sample 1; their brains, kidneys, livers and spleens were mixed and named as sample 2; in which RNAs were extracted from the two samples for next generation sequencing (NGS).

After removing low quality reads, we obtained 133,747,472 and 127,631,716 reads from the two libraries, respectively. The remaining high quality reads were assembled *de novo* using CLC Genomic Workbench

Chunge Zhang and Yongchun Yang have contributed equally to this work.

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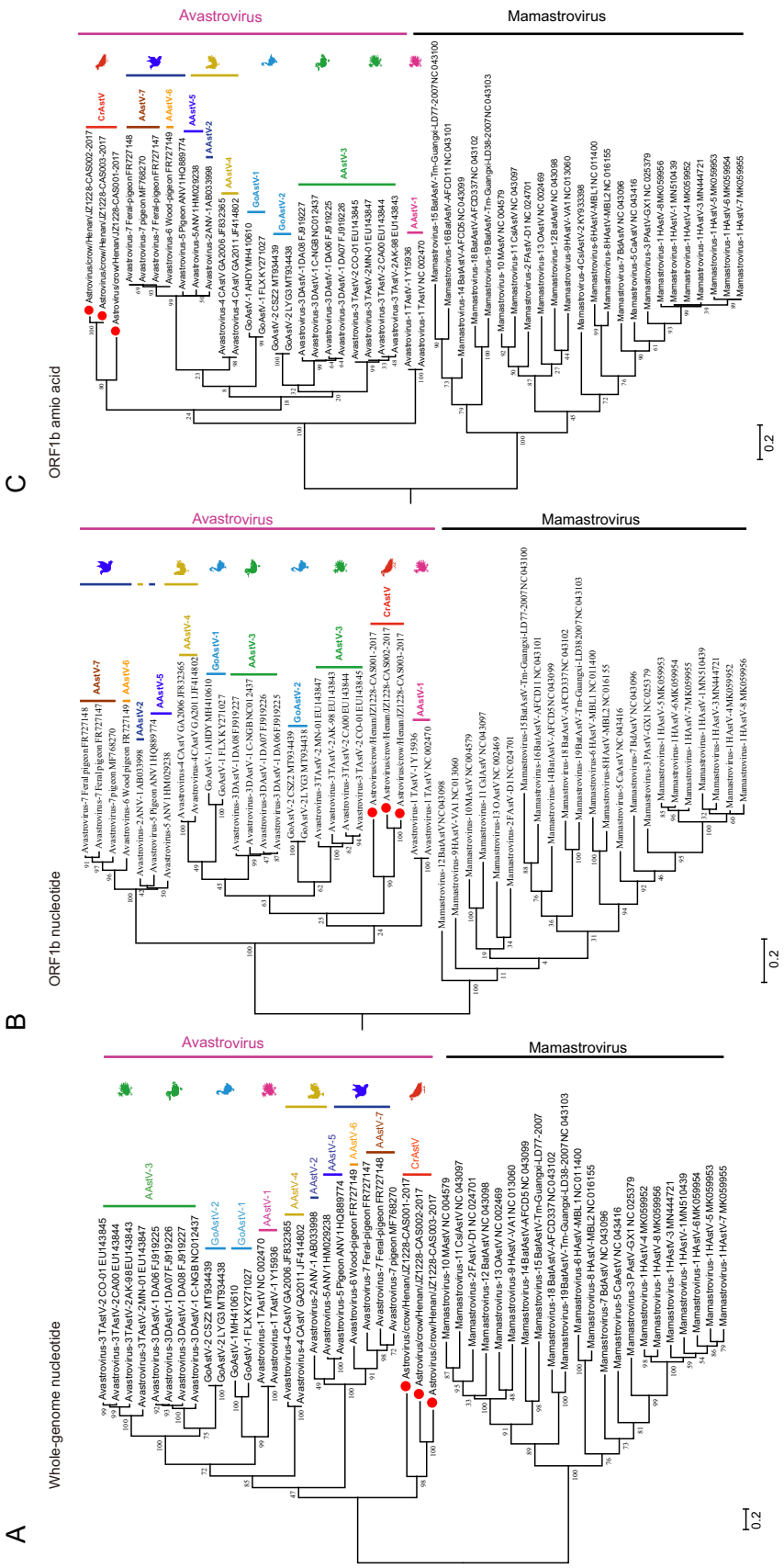

Fig. 1 Phylogenetic analysis of astroviruses. **A** Nucleotide sequence of whole genome. **B** Nucleotide sequence of *ORF1b* gene. **C** Amino acid sequence of *ORF1b* gene. The red points highlighted in the phylogenetic tree represent the viruses identified in the present study. The labels on the right represent different genotypes of *Astrovirus*.

Table 1 Genetic identities among the identified astroviruses and different types of avian astroviruses

Strain	Accession no.	CrAstV-CAS001							CrAstV-CAS002						
		Nucleotide and amino acid identity (%)							Nucleotide and amino acid identity (%)						
		ORF 1a		ORF 1b		ORF 2		Genome	ORF 1a		ORF 1b		ORF 2		Genome
		aa	nt	aa	nt	aa	nt	nt	aa	nt	aa	nt	aa	nt	nt
AAstV-1_TAstV-1	Y15936	26.1	41.5	57.2	56.8	57.1	59.6	52.3	25	41.9	55.8	59.2	52.6	56.3	50.3
AAstV-1_TAstV-1	NC_002470	26.1	41.5	57.2	56.8	57.1	59.6	52.3	25	41.9	55.8	59.2	52.6	56.3	50.3
AAstV-2_ANV-1	AB033998	22.9	40.4	54.6	56.6	54.6	57.7	51.3	23.9	41.1	51.8	55.3	50.9	53.9	49.8
AAstV-3_DAstV-1_C-NGB	NC012437	26.9	42.6	58.6	59.4	51.6	56.6	48.7	26.2	43.2	66.6	60.8	47.6	54.5	48.1
AAstV-3_DAstV-1_DA06	FJ919225	26.6	42.6	58.8	59	51.9	56.7	48.8	26	43.2	66.6	60.6	47.4	54.6	48
AAstV-3_DAstV-1_DA07	FJ919226	26.7	42.7	58.9	58.9	51.9	56.7	48.7	26.1	43.1	66.6	60.6	47.3	54.5	47.9
AAstV-3_DAstV-1_DA08	FJ919227	27.3	42.9	58.8	59	51.8	56.7	49	26.3	43.2	66.6	60.4	47.4	54.6	48
AAstV-3_TAstV-2_AK-98	EU143843	26.5	42.4	58.2	59.3	52.4	56.2	51.4	28.1	43.9	67.1	59.5	46.8	54.4	50.6
AAstV-3_TAstV-2_CA00	EU143844	26.3	43.2	58.9	59	52.5	56.3	51.5	27.5	42.9	67.2	58.4	47.5	54.3	50.5
AAstV-3_TAstV-2_CO-01	EU143845	26.5	43.2	58.4	58.8	52.4	56.2	51.7	27.4	43.1	66.7	58	47.3	54.2	50.4
AAstV-3_TAstV-2_MN-01	EU143847	26.2	42.5	60.5	59.2	52	56.3	51.4	27.6	43.1	64.6	58.3	47.2	54.9	50.5
AAstV-4_CAstV_GA2006	JF832365	26.6	41.6	47.3	57.7	53.1	58.6	50.2	26.4	43.2	44	58	49.4	54.3	48.7
AAstV-4_CAstV_GA2011	JF414802	26.2	41.3	51.3	58.5	50.5	58.1	48.8	26.3	43	48.7	58	48.3	54.9	47.2
AAstV-5_ANV1	HM029238	22.4	39.3	47.5	56.9	54.2	57.8	51.4	23.7	41.5	53	56.6	49.7	53.3	49.6
AAstV-5_Pigeon_ANV1	HQ889774	22	41	47.8	56.1	54	57.9	51.3	23.7	41.3	53.6	57.1	49.6	53.4	49.3
AAstV-6_Wood-pigeon	FR727149	–	–	47.7	56.3	52.1	57	48.2	–	–	54.2	56.6	49.6	53.5	46.5
AAstV-7_Feral-pigeon	FR727147	–	–	45.1	53.4	53	57	47.8	–	–	51.8	56.8	49.7	53.2	46.1
AAstV-7_Feral-pigeon	FR727148	–	–	45.4	55.9	53.7	57.4	48	–	–	52.3	56.7	51.1	54.6	46.2
AAstV-7_pigeon	MF768270	22.6	40.1	50.1	55.9	53.3	57.1	52.1	22.8	40.6	47.7	57.2	50.3	53.4	50.3
GoAstV-1_FLX	KY271027	26.8	42.3	51.6	59.6	54.8	60.6	51.7	26.8	42.3	49.4	59.4	49.7	57.9	50.4
GoAstV-1_AHDY	MH410610	26.6	42.4	51.5	59.1	54.2	60.1	51.8	26.5	42.3	49	59.2	49.7	57.8	50.4
GoAstV-2_CSZ2	MT934439	25.4	41.7	49.9	57	54.5	58	51.8	26.1	41.1	50.1	57.8	49.3	54.9	51
GoAstV-2_LYG3	MT934438	25.4	41.8	49.9	57.2	54.5	58	51.9	26.1	41.3	50.1	57.9	49.3	54.9	51
CrAstV-CAS002	MW385412	46.9	57.3	67.9	68.1	53.6	60.7	65.6	100	100	100	100	100	100	100
CrAstV-CAS003	MW385413	47.4	57.5	68.8	68.7	54.9	59.9	65.6	–	–	–	–	–	–	–
CrAstV-CAS001	MW385414	100	100	100	100	100	100	100	–	–	–	–	–	–	–

Strain	Accession no.	CrAstV-CAS003						
		Nucleotide and amino acid identity (%)						
		ORF 1a		ORF 1b		ORF 2		Genome
		aa	nt	aa	nt	aa	nt	nt
AAstV-1_TAstV-1	Y15936	25.5	42	55.3	57.9	51.9	55	49.5
AAstV-1_TAstV-1	NC_002470	25.5	42	55.3	57.9	51.9	55	49.5
AAstV-2_ANV-1	AB033998	24.5	40.9	51.8	55.2	50.2	52.8	49.9
AAstV-3_DAstV-1_C-NGB	NC012437	26.4	42.8	66.4	60.4	47.5	53.5	47.6
AAstV-3_DAstV-1_DA06	FJ919225	26.1	43.1	66.4	60.8	47.4	53.5	47.9
AAstV-3_DAstV-1_DA07	FJ919226	26.2	43.1	66.4	61.1	47.4	53.4	47.9
AAstV-3_DAstV-1_DA08	FJ919227	26.4	43.3	66.6	61.1	47.5	53.5	48
AAstV-3_TAstV-2_AK-98	EU143843	28.7	44	66.2	60.2	47	53	50.1
AAstV-3_TAstV-2_CA00	EU143844	28.3	42.5	66.4	58.9	47.3	53.2	49.9
AAstV-3_TAstV-2_CO-01	EU143845	28.2	43.2	66	59.1	47.3	52.9	50
AAstV-3_TAstV-2_MN-01	EU143847	28.4	43.1	63.8	59.5	47.4	53.2	49.8
AAstV-4_CAstV_GA2006	JF832365	27	43.3	44.4	59	48.6	54.1	48.8
AAstV-4_CAstV_GA2011	JF414802	27	43.3	48.9	58.5	47	53.5	47.1
AAstV-5_ANV1	HM029238	24.4	40.5	53	56.7	49.5	53.1	49.6
AAstV-5_Pigeon_ANV1	HQ889774	24.2	40.9	53.6	57.8	49.4	53.2	49.6
AAstV-6_Wood-pigeon	FR727149	–	–	54.6	56.5	49.4	53.1	46.2

Table 1 (continued)

Strain	Accession no.	CrAstV-CAS003						
		Nucleotide and amino acid identity (%)						
		ORF 1a		ORF 1b		ORF 2		Genome
		aa	nt	aa	nt	aa	nt	nt
AAstV-7_Feral-pigeon	FR727147	–	–	52	57.2	48.8	52.7	45.8
AAstV-7_Feral-pigeon	FR727148	–	–	52.5	56.6	49.8	53	45.7
AAstV-7_pigeon	MF768270	23.4	40	47.3	57.1	48.8	52.5	50
GoAstV-1_FLX	KY271027	26.8	42.3	49.2	58.5	50.3	55.7	49.5
GoAstV-1_AHDY	MH410610	26.5	42.4	48.9	57.4	50.3	56	49.5
GoAstV-2_CSZ2	MT934439	26.9	42.3	49.6	58.3	49	53	50.4
GoAstV-2_LYG3	MT934438	26.9	42.5	49.6	58.5	49	53	50.5
CrAstV-CAS002	MW385412	96.6	87	97.1	90.4	62.8	66	82.9
CrAstV-CAS003	MW385413	100	100	100	100	100	100	100
CrAstV-CAS001	MW385414	–	–	–	–	–	–	–

aa amino acid sequence, *nt* nucleotide sequence.

(version 10.0). Six contigs were obtained and annotated using multiple BLAST searches. Three genomes were found to be related to AstVs. The NGS results were confirmed by Sanger sequencing with the designed primers (Supplementary Fig. S2 and Table S2). These three genome sequences of AstV from crows have been deposited at the National Microbiology Data Center (accession no. NMDC60014580, NMDC60014581, and NMDC60014582) and GenBank (accession no. MW385412, MW385413, and MW385414).

The near complete genomes of these three AstVs comprised 6152 nt (Astrovirus/crow/Henan/JZ1228CAS001/2017, AstV-CAS001), 6652 nt (Astrovirus/crow/Henan/JZ1228-CAS002/2017, AstV-CAS002), and 6645 nt (Astrovirus/crow/Henan/JZ1228-CAS003/2017, AstV-CAS003), respectively, excluding the polyA tail. These poly-adenylated genomes possessed an astrovirus-like organization, consisting of three sequential open reading frames (ORFs): ORF1a, ORF1b and ORF2. For AstV-CAS001, ORF1a comprised 2625 nt encoding a protein of 874 amino acids (aa) in length. The ORF1b was 1419 nt encoding the RNA dependent RNA polymerase (RdRp) of 472 aa in length. The ORF2 was 2052 nt encoding a capsid protein of 683 aa in length. There was a space of 77 nt between the ORF1a stop codon and the ORF1b start codon, and an overlap of 29 nt between ORF1b and ORF2. The 5′ and 3′ ends comprised UTRs, with 7 nt and 1 nt sequenced, respectively.

Regarding AstV-CAS002, the length of ORF1a was the same as that of AstV-CAS001, while the ORF1b of AstV-CAS002 was 1299 nt, encoding the RdRp of 432 aa. ORF2 was 2370 nt in length, encoding a capsid protein of 789 aa. There was a space of 203 nt between the ORF1a stop codon and the ORF1b start codon, and a space of 3 nt between ORF1b and ORF2. The lengths of 5′ UTR and 3′ UTR were 4 nt and 148 nt, respectively. The genome organization of AstV-CAS003 was highly similar to that of AstV-CAS002, with the only difference in the ORF2, which was 2349 nt in length and encoded a capsid protein of 782 aa. In addition, 1 nt of 5′ UTR and 165 nt of 3′ UTR were obtained for AstV-CAS003. Interestingly, the lengths of the ORF1a, ORF1b or ORF2 were not completely identical among these three strains. However, enough reads were obtained from NGS, and the results have been confirmed by Sanger sequencing. Therefore, the results should be credible, although the full UTRs of these three strains were not obtained in the present study.

Phylogenetic analyses of these three AstVs were performed using the Maximum-Likelihood (ML) method. As shown in the phylogenetic trees, astroviruses were clustered into the *Mamastrovirus* (MAstV) and *Avastrovirus* (AAstV) lineages (Fig. 1A–1C). The AAstVs were further clustered into AAstV-1 (identified in turkey), AAstV-2 (identified in chicken), AAstV-3 (identified in turkey and duck), AAstV-4 (identified in chicken), AAstV-5 (identified in chicken and pigeon), AAstV-6 (identified in pigeon), AAstV-7 (identified in pigeon), GoAstV-1 and GoAstV-2 (identified in goose) sublineages. These three new AstVs grouped within the AAstVs lineage and formed a separate cluster (Fig. 1A) in the tree of whole genomes, which was also found in the phylogenetic trees of the nucleotide and amino acid sequences of ORF1b (Fig. 1B, 1C), ORF1a (Supplementary Fig. S1A–S1B), and ORF2 (Supplementary Fig. S1C–S1D). Therefore, we suggest that these three novel AstVs should be named as crow astrovirus (CrAstV).

These three CrAstVs shared 47.8%–52.3%, 46.1%–51%, and 45.7%–50.5% nucleotide identities to known AAstVs at the scale of whole genome, respectively; and shared 39.3%–44.0%, 53.4%–61.1%, and 52.5%–60.6% nucleotide identities in the *ORF1a*, *ORF1b*, and *ORF2* genes, respectively, and the identities of the corresponding amino acid sequence were 22.0%–28.7% (ORF1a), 44.0%–67.2% (ORF1b), and 46.8%–57.1% (ORF2) (Table 1). In summary, the results from phylogenetic analysis and sequence identities showed that these three novel CrAstVs strains had a distant relationship with known AAstVs, both at the scale of whole genome and single genes.

Specific primers (Supplementary Table S3) were designed to detect the CrAstVs in different tissues of the dead crows. CrAstV-CAS001 was found in the spleen, kidney and intestine from crow 2; CrAstV-CAS002 was found in the spleen and intestine from crow 2; CrAstV-CAS003 was detected in the liver, spleen, lung and intestine from crow 1. Interestingly, both CrAstV-CAS001 and CrAstV-CAS002 were found in the spleen and intestine of the same crow, crow 2 (Supplementary Table S4). The supernatants of different tissues that were RT-PCR positive for the novel CrAstVs were used for virus isolation using specific pathogen free chicken and duck embryos via the chorioallantoic membrane and allantoic cavity inoculation routes. However, no deaths and pathological changes were observed in the embryos after eight passages, and all passages were negative for live CrAstVs, suggesting that these novel astroviruses may have strong host specificity.

In this study, we reported three novel AAstV strains from dead crows, which formed a separate lineage within *Avastrovirus*. However, it is not known whether these novel CrAstVs are the causative agents of death, although they were detected in different tissues. However, our study highlighted the expanded host range of astroviruses and that wild birds (e.g. crows) may play an important host in the maintenance and evolution of astroviruses, which warrant further investigations.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

Ethical statement Samples used in this study were obtained from animal carcasses hence requiring no ethical approval.

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