FULL PAPER *Virology*

Molecular epidemiology and phylogenetic analysis of diverse bovine astroviruses associated with diarrhea in cattle and water buffalo calves in China

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ABSTRACT. Astroviruses are the principal causative agents of gastroenteritis in humans and have been associated with diarrhea in other mammals as well as birds. However, astroviral infection of animals had been poorly studied. In the present study, 211 rectal swabs collected from cattle and water buffalo calves with mild to severe diarrhea were tested for bovine astrovirus (BAstV) by RT-PCR. Results: 92/211 (43.6%) samples were positive for BAstV, at a rate of 46.10% (71/154) in cattle and 36.84% (21/57) in water buffalo. Phylogenetic analysis based on the partial and full-length of 25 ORF2 amino acid sequences obtained in this study classified the Guangxi BAstVs isolates into five subgroups under the genus of *Mamastrovirus*, genotype *MAstV33*, which suggested that the water buffalo was a new host of this genogroup that previously included only cattle and roe deer. Despite the origin of the host, the Guangxi BAstV isolates were closely related to the BAstV Hong Kong isolates (B18/HK and B76-2/HK), but highly divergent from the BAstV NeuroS1 isolate previously associated with neurologic disease in cattle in the U.S.A. Nucleotide sequence-based characterization of the ORF1b/ORF2 junction and corresponding overlapping regions showed distinctive properties, which may be common to BAstVs. Our results suggested that cattle and water buffalo are prone to infection of closely related astroviruses, which probably evolved from the same ancestor. The current study described astroviruses in water buffalo for the first time and is thus far among the largest epidemiological investigations of BAstV infection in cattle conducted in China.
KEY WORDS: bovine astrovirus, cattle, China, phylogenetic analysis, water buffalo

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Astroviruses are small and non-enveloped with a single-stranded, positive-sense RNA genome of 6.4 to 7.3 kb in length consisting of three open reading frames (ORFs): ORF1a, which encodes the non-structural polyprotein 1a; ORF1b, which encodes the polyprotein 1ab, including the RNA-dependent RNA polymerase (RdRp) that is expressed by a ribosomal frameshift at the ORF1a/1b junction [1, 15]; and ORF2, which encodes a viral capsid structural polyprotein. The current International Committee on Taxonomy of Viruses (ICTV) system divides the *Astroviridae* family into two genera, *Mamastrovirus* and *Avastrovirus*, which infect mammals and birds, respectively [5, 21]. Astrovirus was first identified in 1975 as the causative agent of infantile diarrhea in a maternity ward in England [1]. In 2011, astrovirus infection has expanded to 22 animal species or families, including various terrestrial domestic animals, wild animals and aquatic animals, (turkey, chicken, pig, cattle, dogs, cats, deer, ducks, mink, rabbit, sea lion, bats, etc.) [3, 5, 18, 21, 24, 28]. Bovine astrovirus (BAstV) was first described in England in 1978 from acute enteritis of calves [24] and initially considered to be avirulent, as experimental infection of two gnotobiotic calves with astrovirus-like virus did not cause diarrhea [24]. However, in 1984, two bovine astrovirus isolates from the U.S.A., US1 and US2, which were found to be antigenically related to an isolate in England (U.K.), were shown to cause infection and cytopathology of M cells of the dome epithelium covering the Payer's patches of the calf ileum [25]. In addition, astroviruses in co-infection with other enteric pathogens, such as bovine rotavirus (BRV) and bovine torovirus (BToV; Breda virus), were shown to increase the severity of astrovirus infection [26]. In 1985, a serologically based investigation classified bovine astroviruses into two serotypes: BoAstV-1 and BoAstV-2. Meanwhile, a study on the earliest three isolates of bovine astroviruses (UK, US1 and US2) implied that these isolates were different serotypes, suggesting that multiple serotypes of bovine astroviruses may exist in nature [25]. Astroviruses, as well as noroviruses, hepatitis A and E, and rotaviruses, are commonly reported as major causes of foodborne illnesses [2]. Astroviruses are commonly detected alongside other enteric viruses, especially noroviruses in alimentary specimens. A previous study suggested that BAstV may be excreted by up to 60–100% of calves on farms [4], but a later study showed that only 5 (2.4%) of 209 rectal swabs collected from asymptomatic adult cattle tested positive for BAstV [21]. Unlike other enteric viruses, bovine astrovirus is characterized by asymptomatic viral shedding in stool

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samples without heavy diarrheal disease [4, 24].

Even though bovine astrovirus was one of the earliest mammalian astroviruses to be discovered and studied in detail, no further research has been performed on this important enteric pathogen over the past three decades, and its genomic sequence remained uncharacterized until its rediscovery and genomic characterization in 2011 in Hong Kong [21]. The lack of large-scale epidemiological surveillance of bovine astrovirus has continued the divergence of opinions on the morbidity of bovine astrovirus in cattle, especially considering that there is no report of astrovirus infection of water buffalo in the current literature. Furthermore, little molecular information about bovine astrovirus is available, and only limited information is available regarding animal astroviruses in general [9]. Therefore, the diversity, epidemiology and molecular characterization of animal astroviruses remain unclear due to the lack of studies, likely because of the difficulty to grow these viruses in cells and tissue cultures.

Here, we report our results of a relatively extensive epidemiological survey to elucidate the diversity of bovine astroviruses circulating among cattle and water buffalo in Guangxi province, China, by characterization of subgenomic sequences of the ORF1b/ORF2 overlap region and the 3′-end of ORF2.

MATERIALS AND METHODS

Samples: A total of 211 rectal swabs were collected from calves, aged 0–6 months, with mild to severe diarrhea from five industrial farms (3 dairy and 2 water buffalo) in Guangxi province, China. All samples were collected in sterile tubes containing 10 m*l* of 1/100 antibiotic 10 mM phosphate-buffered saline and transported on ice to our laboratory within 24 hr. Samples were homogenized by vortexing for 5 min at room temperature and centrifuged at 3,000 × g for 15 min at 4°C, and then, the supernatant was aliquoted and stored at −80°C until assayed.

Nucleic acid extraction and cDNA bank synthesis: RNA was extracted from 300 μl of the supernatant obtained from each rectal swab using the RNAiso PLUS kit (Takara Bio, Inc., Dalian, China) following the manufacturer's instructions. Extracted RNA was eluted in 35 μl of RNase-free H_2O (Takara Bio, Inc.) and used for first strand cDNA bank synthesis with the PrimeScript II 1st strand cDNA Synthesis kit (Takara Bio, Inc.) in accordance with the manufacturer's instructions.

Detection of bovine astrovirus and genome cloning and sequencing: Bovine astrovirus was detected by reverse transcriptase polymerase chain reaction (RT-PCR) analysis using the degenerate primer pair (forward) DPF 5′-GAYTG-GACBCGHTWTGATGG-3′ and (reverse) DPR 5′-KYT-TRACCCACATNCCAA-3′ to target a 418-bp fragment of the RdRp region common to astroviruses, as described previously [21] with some modifications. Some of the RT-PCR-positive samples were sequenced and primarily analyzed by comparison of nucleotide sequences using the online Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the detection of astroviruses. Subsequently, we amplified the 3′-end of ORF2 and the ORF1b/ORF2 regions, which have been confirmed by subgenomic RNA analysis for characterization of astroviruses. Since the direct amplification with gene specific primers (GSPs) failed, the Rapid Amplification of cDNA Ends (RACE) method was implemented to amplify the 3′-end of the ORF1b/ORF2 region. The 3′-end RACE amplification was performed as described previously [16] with the following modifications to the primer pair: QT 5′-CCAGTGAGC AGAGTGACGAGGACTCGAGCTCAAGCT $(T)_{16}$-3′ and QO 5′-CCAGTGAGCAGAGTGACG-3′. The RACE products were reused as templates in a nested PCR performed to amplify desired sequences using GSPs designed in this study based on the B76-2/HK sequence available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) under the accession number HQ916317.1.The GSPs were designed using OLIGO 7 software (Molecular Biology Insights, Inc., Colorado Springs, CO, U.S.A.). The nucleotide sequences of the primer pairs used to amplify the partial 3′-end of ORF2 were as follows: (forward, B1205F and B1350F) 5′-CAG-GTCACCCCAGGCAACAC-3′ and 5′-ATCATACAGGC-GGGCACGAGT-3′, respectively, and (reverse, B1205R) 5′-CCCTTCACCTATGCTAATCAAATC-3′ (expected products length, 1,200 and 1,426 bp, respectively). The two primer pairs used to amplify the ORF1b/ORF2 region were as follows: (1) (forward, B-GSP2-2F) 5′-AACAGC ACAGGGAGAGGTATAAGCA-3′ and (reverse, B5130R) 5′-TGGCACGTGTTTCATAGGTCT-3′ (expected product length, 1,850 bp); and (2) (forward, B1350F) 5′-ATCATA CAGGCGGGCACGAGT-3′ and (reverse, B1205R) 5′-CCCTTCACCTATGCTAATCAAATC-3′ (expected product length, 1,426 bp). For PCR, the final reaction volume of 25 μl consisted of 2.5 μl of 10x L.A *Taq* Polymerase buffer, 4.0 μl of dNTP mixture (2.5 mM each), 0.25 μl of LA *Taq* Polymerase (Takara Bio, Inc.), 0.5 μl forward (DPF) and reverse (DPF) primers each, 2.5 μl of cDNA template, and 14.75 μl of ddH2O. The PCR cycling conditions consisted of 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min in an automated thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). 3′-RACE PCR and amplification of the ORF1b/ORF2 region were performed under the same conditions as for the PCR analysis described above with cycling set at 58°C as the annealing temperature for 3′RACE and 56°C as the annealing temperature for amplification of the ORF1b/ORF2 region and an elongation step at 72°C for 1 min for each 1 kb.

The PCR products of expected sizes were purified and then cloned into pMD18-T plasmid vectors (Takara Bio, Inc.), which were then used to transform competent *Escherichia coli* Top 10 cells. The recombinant plasmids were submitted in duplicate for DNA sequencing to Invitrogen Biotech (Beijing, China). Editing and assembly of nucleotide sequences were performed using the SeqMan program included in the Lasergene 7 Genomics Suite (DNAstar, Inc., Madison, WI, U.S.A.).

Assessment of the co-infection status of BAstV with other bovine gastrointestinal viruses: The status of a combination

Table 1. Sample collection and RT-PCR detection of BAstV

Origin	Date of sampling	Age/ Months	Type of samples	Clinical status	Total samples tested	RT-PCR positive samples	Positive rate%
A	June, 2013	≤6	Rectal swab	Samples were collected from calves suffering from mild to severe diarrhea	19	8	42.10
B	July, 2013	≤6	Rectal swab		46	26	56.52
	August, 2013	≤6	Rectal swab		36	17	47.22
C	September, 2013	≤6	Rectal swab		31	13	41.93
	October, 2013	≤6	Rectal swab		22	7	31.81
D*	July, 2013	≤6	Rectal swab		32	13	40.62
E*	December, 2013	≤6	Rectal swab		25	8	32.00
Total					211	92	43.60

*Water buffalo Farms, For privacy request, Farms concerned by the study were annotated ABCDE.

Table 2. Primer sequences used for detection of other bovine gastrointestinal viruses

Pathogen	Primer ID	Oligonucleotide sequence	PCR product length (bp)	Annealing temperature (°C)	Ref.
BVDV	BVD-1	5′-GGTAGCAACAGTGGTGAGTTC-3′	471	56	[27]
	BVD-2	5′-CTCTGCTATTACCCGACCTGC-3′			
BCoV	BCoV-F	5′-GCAATCCAGTAGTAGAGCGT-3′	730	58	[11]
	BCoV-R	5′-CTTAGTGGCATCCTTGCCAA-3′			
BEV	BEV-F	5′-GGGGAGTAGTCCGACTCCGC-3′	286	57	[8]
	BEV-R	5′-CAGAGCTACCACTGGGGTTGTGG-3′			
	NBEV-F	5′-ACGGAGTAGATGGTATTCCC-3′	222		
	NBEV-R	5′-CGAGCCCCATCTTCCAGAG-3′			
BRV	RTVP7-F	5′-GATCCGAATGGTTGTGTAATCCAAT-3′	304	55	[20]
	RTVP7-R	5′-AATTCGCTACGTTTTCTCTTGG-3′			
BToV	BToV-F	5′-GTGTTAAGTTTGTGCAAAAATG-3′	741	55	[7]
	BToV-R	5′-TGCATGAACTCTATATGGTGT-3′			

of astrovirus with other gastrointestinal viruses was assessed by RT-PCR.

Phylogenetic and genome analysis: Phylogenetic trees were constructed based on the nucleotide sequences of both partial and full-length ORF2 sequences using the Bootstrap Test Phylogeny algorithm with the neighbor-joining method included in the MEGA 4.1 software package (http://www.megasoftware.net/). Sequences were compared with those of other mammalian astroviruses. Pair distances of nucleotide sequences between bovine and water buffalo astroviruses, as well as the ORF1b/ORF2 junction, were analyzed using DNAstar software (Lasergene 7; MegAlign Program with the ClustalW sequence alignment algorithm).

RESULTS

Screening and detection of BAstV in cattle and water buffalo: Of the 211 rectal swabs tested, 92 (43.6%) were positive for bovine astrovirus (cattle: 46.10%, 71/154; water buffalo: 36.84%, 21/57) (Table 1). These results indicate that astroviruses are widely distributed among cattle and water buffalo populations in Guangxi province, China.

The status of co-infection of BAstV with other bovine gastrointestinal viruses: To assess the status of co-infection with BAstV and other bovine gastrointestinal viruses, 32 samples randomly selected from our study stock were subjected to RT-PCR analysis. Six viruses: bovine astrovirus (BAstV), bovine enterovirus (BEV), bovine viral diarrhea virus (BVDV), bovine coronavirus (BCoV), bovine rotavirus (BRV) and bovine torovirus (BToV) were analyzed in accordance with the results of previous studies [7, 8, 11, 20, 27]. The primer sequences used for detection of gastrointestinal viruses other than astrovirus are shown in Table 2. As shown in Table 3, our results clearly demonstrated BAstV co-infections with one or more gastrointestinal viruses in 87.5% (28/32) of cases. Interestingly, 4 (12.5%) of 32 samples were only positive for BAstV and negative for the all other gastrointestinal viruses tested in this study. Thus, the co-infection status was as follows: BEV, 53.1% (17/32); BAstV, 46.9% (15/32); BVDV, 18.7% (6/32); BCoV, 15.6% (5/32); BRV, 6.25% (2/32); and BToV, 0% (0/32).

Gene cloning and sequencing: We attempted to amplify the genomes of the 92 BAstV-positive samples. However, sequencing proved difficult due to the limited volume of the clinical samples and to the fact that virus cultivation was unsuccessful. We only successfully amplified and cloned 18 partial nucleotide sequences of approximately 1,200–1,426 bp in length in most 3′-ends of ORF2 (of the18 sequences, 12 were derived from cattle and six from water buffalo). Besides, seven fragments of approximately 3,000 bp in length (five derived from cattle and two from water buffalo) partially covering ORF1b and the full length of ORF2 were successfully amplified. In all, 25 sequences were obtained and submitted to the NCBI GenBank under the accession

Table 3. The status of co-infection of BAstV with other bovine gastrointestinal viruses

Sample ID	BAstV	BCoV	BEV	BVDV	BRV	BToV
J21	—	—	+	—	—	—
J22	+	—	—	+	—	—
J23	+	—	—	—	—	—
J24	—	—	—	—	—	—
J25	+	+	+	—	—	—
J26	+	—	+	—	+	—
J27	+	+	+	+	—	—
J30	—	+	—	—	—	—
BF1312	+	—	+	+	—	—
BF4502	+	—	+	—	—	—
BF4503	+	—	—	—	—	—
BF6835	—	—	+	+	—	—
BF6834	—	—	—	—	—	—
BF4506	—	—	+	—	—	—
BF1306	+	—	—	—	—	—
BF4508	+	—	—	—	—	—
G3	—	—	+	—	+	—
G5	—	—	—	—	—	—
G6	+	—	+	—	—	—
G8	—	—	—	—	—	—
G11	+	+	+	—	—	—
G13	+	+	—	—	—	—
G15	—	—	—	—	—	—
G17	—	—	+	+	—	—
G18	—	—	—	—	—	—
SB10	+	—	+	—	—	—
SB12	—	—	+	+	—	—
SB14	—	—	+	—	—	—
SB20	—	—	—	—	—	—
SB21	—	—	—	—	—	—
SB28	—	—	+	—	—	—
SB29	+	—	+	—	—	—
Total samples	32	32	32	32	32	32
Positive samples	15	5	17	6	2	0
Rate%	**46.90%**	**15.60%**	**53.10%**	**18.70%**	**6.25%**	**0%**

+ Cases of self detection of BAstV.

numbers KJ476832–KJ476856.

Phylogenetic and genomic analysis: To identify strains of astroviruses circulating among cattle and water buffalo populations in Guangxi province, phylogenetic tree analysis was performed based on both partial and full-length ORF2 sequences (1). Phylogenetic analysis of partial ORF2 sequences showed that three isolates (BAstGX S7-7, BAstGX S12-7 and BAstGX S17-7) from Dairy Farm A clustered together with one isolate (BAstGX J25) from Dairy Farm B forming a subgroup, which had a high similarity with BAstV-B76-2/HK and BAstV-B18/ HK isolated from cattle in Hong Kong. Five of six isolates from water buffalo (BufAstGX M52, BufAstGX M53, BufAstGX M54, BufAstGX M541 and BufAstGX M552) clustered together appeared to form a new independent subgroup. However, one water buffalo isolate (BufAstGX 141) formed a new subgroup and appeared to be very closely related to the CcAstV-1/DNK/2010 isolate from a roe deer in Europe [17], suggesting that the Guangxi isolates from water buffalo could be classified into two subgroups. Seven of eight isolates (BAstGX J7, BAstGX J8, BAstGX J22, BAstGX J23, BAstGX J27, BAstGX J251 and BAstGX 252) from Dairy Farm B formed a new subgroup, while one isolate (BAstGX G1) from Dairy Farm C formed a new subgroup on its own from other cattle isolates. Regardless of origin, the isolates from cattle were divided into three subgroups, while those from water buffalo were divided into two subgroups. However, five subgroups of the Guangxi cattle and water buffalo astrovirus isolates belonged to one genogroup previously composed of bovine and roe deer astroviruses (BAstv-B76-2/HK, BAstV-B18/HK, CcAstV-1/DNK/2010 and CcAstV-2/DNK/2010) in the genus *Mamastrovirus*, genotype *MAstV33* (Fig. 1a). To confirm this classification, we next constructed another phylogenetic tree based on the seven predicted full-length ORF2 sequences deduced from amino acid sequences (five from cattle and two from water buffalo) obtained in this

study together with other full-length ORF2 sequences of other mammalian astroviruses retrieved from the GenBank database. The phylogenetic relationships within the complete ORF2 sequences of different mammalian astroviruses were addressed in reference to the taxonomic proposals for astrovirus classification criteria as described by the *Astroviridae* Study Group in 2010, and each genotype was represented at least by one prototype strain. The obtained results confirmed the classification under the genus *Mamastrovirus*, genotype *MAstV33*, which, thus far, includes only cattle and roe deer astroviruses (Fig. 1b). Both the partial and full length ORF2 sequences of water buffalo isolates constituted new subgroups in the clade of the genotype *MAstV33*. The above results classified water buffalo as new host species of the genus *Mamastrovirus*, genotype *MAstV33* (Fig. 1a and 1b).

Analysis of the ORF1b/ORF2 junction identified a highly conserved 17-nt sequence motif (5′-cgctccttgcctaaaat- 3′) upstream of ORF2 within the ORF1b/ORF2 region (Fig. 2a). The ORF1b/ORF2 overlapping region contained 56 nt that overlapped between the ORF2 start codon and the ORF1b stop codon, with the exception of BAstV NeuroS1 (a divergent bovine astrovirus). The most 3′-end of ORF2 of the bovine-related astroviruses was highly conserved by 42 nt (Fig. 2b), while this region has been reported as conserved by only 19 nt in human astroviruses [12].

DISCUSSION

Here, we detected a high prevalence of bovine astrovirus by RT-PCR from rectal swabs collected from young calves of cattle and water buffalo with diarrhea. However, more studies are needed to determine whether the persistent diarrhea observed in these calves was mainly associated with the high prevalence of astroviruses, as previous studies reported that bovine astroviruses were not directly associated with severe diarrhea in calves under natural conditions [4, 24]. However, other reports [14, 26] indicated that bovine astrovirus may evolve to severe diarrhea in co-infections with other gastrointestinal viruses, as in the case of BAstV co-infection with BRV or BToV (Breda virus) [26]. Nonetheless, the epidemiology of bovine astrovirus remains unclear, especially considering the limited number of studies of cattle and water buffalo. While excretion of BAstV may occur in up to 60–100% of calves on farms [4], only 5 (2.4%) of 209 rectal swabs collected from asymptomatic adult cattle were positive for BAstV [21]. In the present study, sampling at different time points demonstrated a high prevalence of up to 56.52% among calves with diarrhea. Moreover, in our complementary study, astrovirus was detected alongside other gastrointestinal viruses, including BEV, BCoV, BRV and BVDV, in 87.5% of cases. Surprisingly, 12.5% of these cases were positive for bovine astrovirus, but yet negative for other tested gastrointestinal viruses. In contrast, titers of BRV and BToV, which have been previously reported as principal gastrointestinal co-infecting viruses with bovine astrovirus, were minimal or undetectable in this study. From these results, it is clear that astrovirus may be directly associated with diarrhea or possibly linked to other factors, such as poor hygienic conditions or associated with other non-viral pathogens, such as bacteria or parasites, which are known causative agents of diarrhea in calves. Similar results were reported in Korea, where co-infection of bovine astrovirus with gastrointestinal viruses, other than BRV and BToV, was associated with clinical symptoms of diarrhea in 20- and 14-day-old calves [14].

Our phylogenetic analysis showed that all isolates in this study were closely related to BAstV-B76-2/HK, BAstV-B18/HK, CcAstV-1/DNK/2010 and CcAstV-2/DNK/2010, but highly divergent from a BAstV NeuroS1 isolate previously associated with neurologic disease in cattle in the U.S.A. [10]. Nonetheless, our results support that proposal [21] that BAstV and CcAstV may be different strains of the same virus, and water buffalo may be a new host of the BAstV variant of this virus. Moreover, most sequences derived from one farm belonged to one subgroup, although sequence analysis clearly demonstrated origins from different strains. In addition, no significant genetic differences were observed between the bovine and water buffalo astrovirus strains investigated in this study (Table 4). These results further support previous evidence that one farm could serve as a reservoir of different bovine astrovirus strains, suggesting possible outbreaks of novel astroviruses due to mutation or recombination events. Notably, considering the history of astroviruses, recombination events have been described in cattle, swine, humans and poultry [19, 21, 22], and co-infection of two different astroviruses in one head of cattle has been previously reported as well [21]. Consequently, it is important to screen for and control astrovirus infection in order to prevent eventual outbreaks of highly pathogenic astroviruses resulting in mutation or recombination events on farms. Furthermore, the astrovirus isolates from cattle and water buffalo isolated in this study displayed relatively close relationships, indicating that these animals were infected with diverse astroviruses, which probably evolved from the same viral ancestor. Consequently, given these close relationships, evidence exists of possible cross-infection between the two hosts; therefore, control measures against bovine astrovirus should also be taken into consideration when screening of viruses in water buffalo populations.

Appreciation of the genetic diversity and evolution of astroviruses among wild and domestic animal populations is important to fully understand this challenging gastrointestinal virus [21]. However, the organization of the BAstV genome has not yet been fully described. In this study, a sequence motif upstream of ORF2 was predicted to be the signal of the putative promoter for subgenomic RNA (sgRNA) synthesis in bovine astrovirus as well as that for the ORF1b/ORF2 overlap sequence and the most 3′-end of ORF2. It has been suggested that in astroviruses [13, 23], a conserved sequence acts as a putative promoter for sgRNA synthesis upstream of ORF2. In human astroviruses, the ORF1b/ORF2 overlap is 8 nt in length, while in duck astroviruses, the ORF1b/ORF2 junctions are not overlapped [6, 13, 23]. In contrast, the overlap in the ORF1b/ ORF2 junction of bovine astroviruses may be longer (56 nt) than that of the other known astroviruses. Moreover, compared

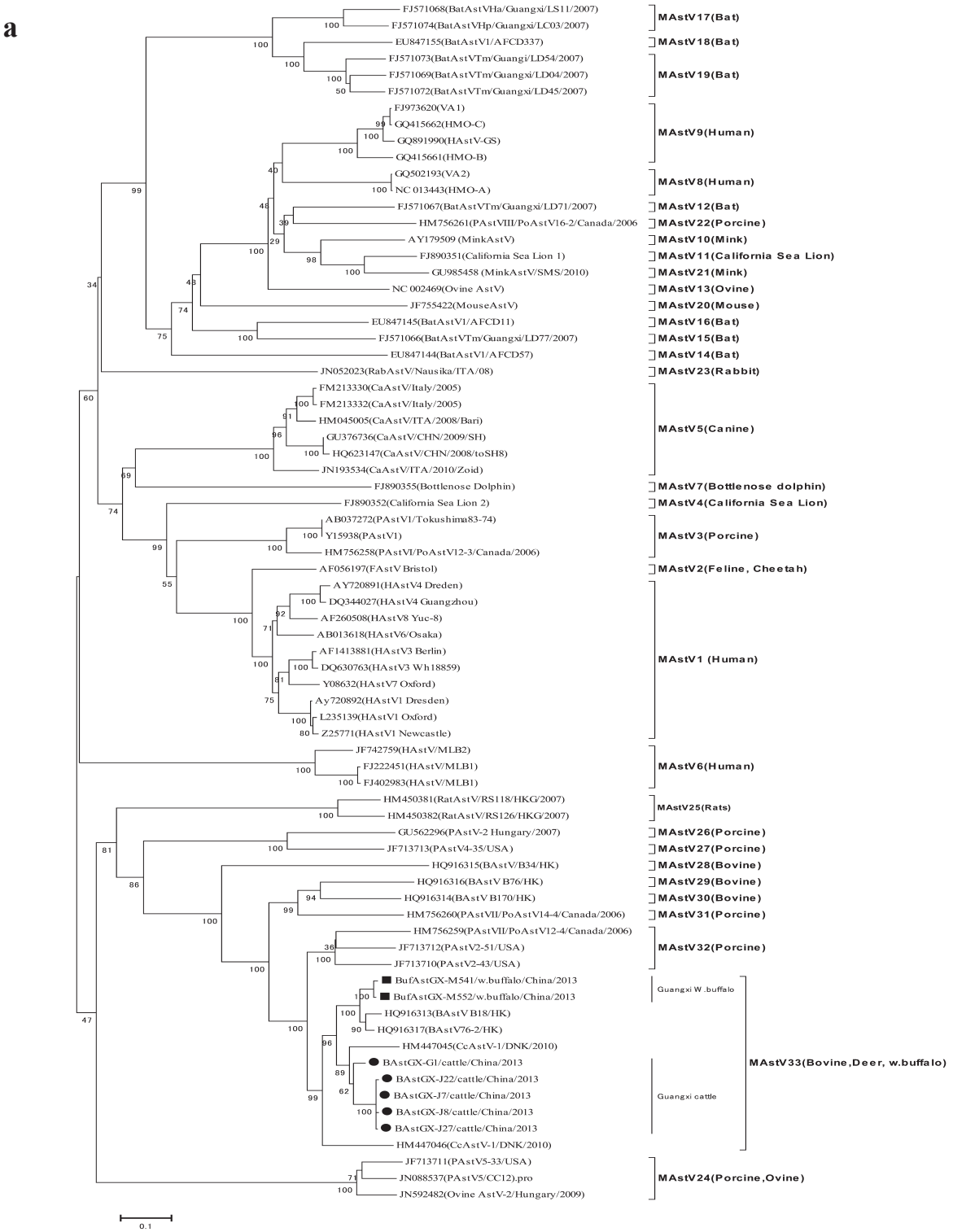
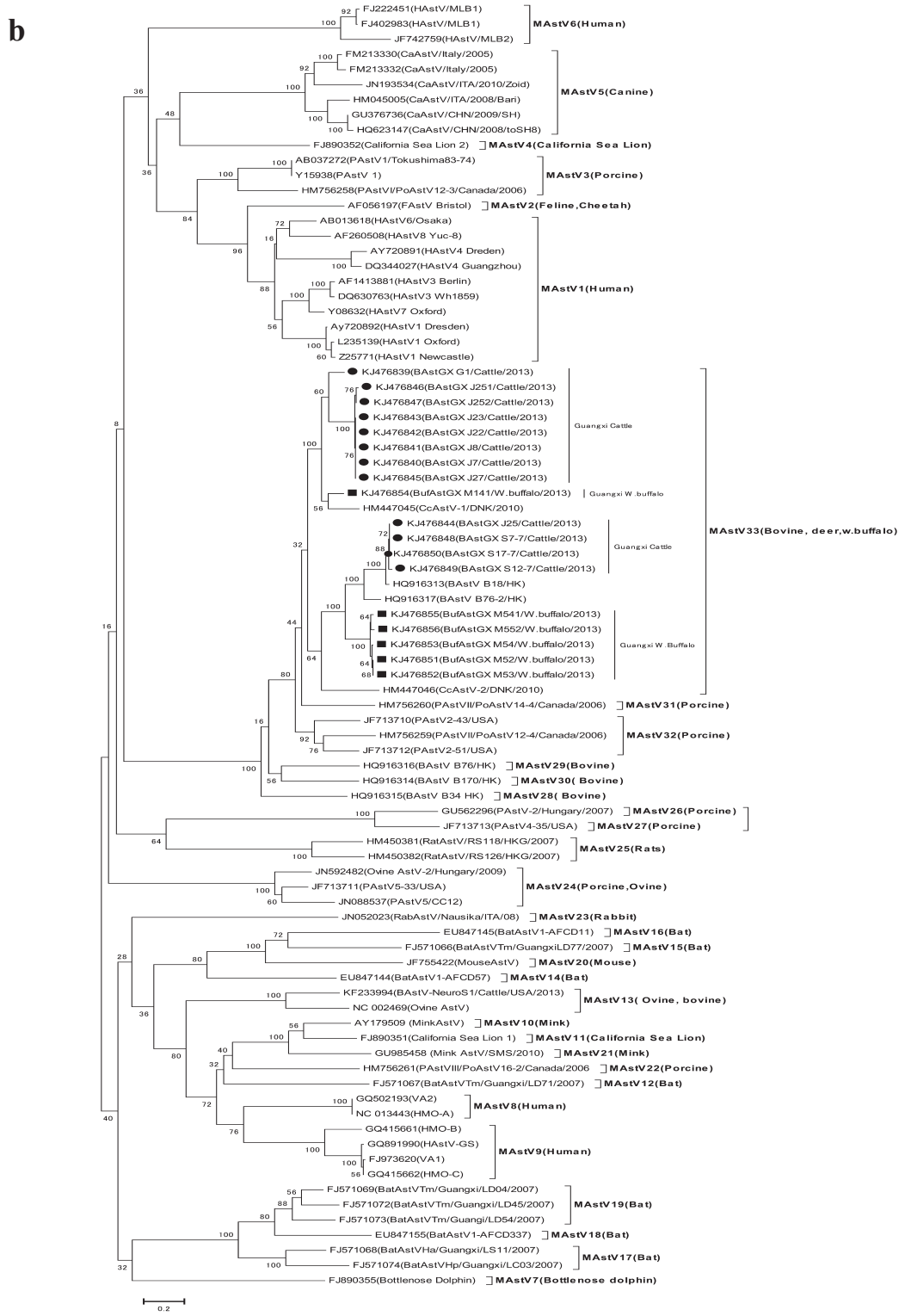


Fig. 1. Phylogenetic relationships between the Guangxi bovine astroviruses and other mammalian astroviruses retrieved from the NCBI database. Phylogenetic trees were constructed based on both predicted partial ORF2 (Fig.1a) and full-length ORF2 (Fig.1b) amino acid sequences of the Guangxi bovine astrovirus and documented full-length ORF2 sequences from other mammal astroviruses. The phylogenetic trees were generated using the Bootstrap Test Phylogeny algorithm with the neighbor-joining method included in the MEGA 4.1 software package. Each genotype of the genus *Mamastrovirus* was represented at least by one or more prototype strains, and genotypes are highlighted. Classification was based on the Taxonomic Proposals submitted to the ICTV by the Astroviridae Study Group in 2010. The Guangxi isolates derived from cattle are marked by (●), and isolates derived from water buffalo are marked by (■).



with a previous report [12], the 3′-end of ORF2 of bovine astroviruses was highly conserved, although our results suggested further studies and analysis of more sequences from several novel bovine astroviruses to fully characterize ORF2 of bovine astrovirus.

In summary, this study is of interest at both the epidemiological and genetic levels. These results will certainly contribute to the understanding of the evolution and pathol-

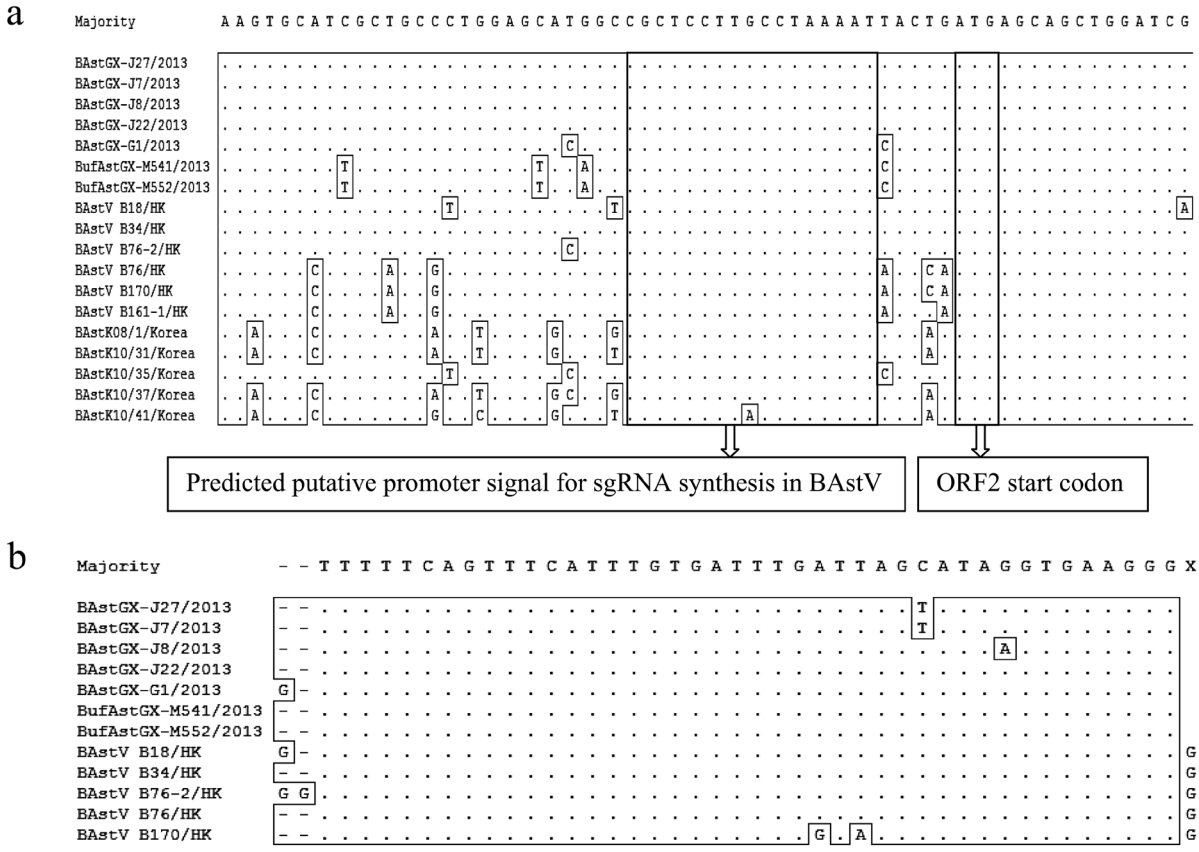


Fig. 2. a: Alignment of the Guangxi cattle and water buffalo astrovirus isolates and NCBI sequences comprising the ORF1b/ORF2 junction identified a highly conserved sequence motif upstream of the ORF2 start codon. Given its site and conservation among strains, this sequence was predicted to be related with the putative promoter for sgRNA synthesis in bovine astroviruses (DNAstar, MegAlign Program: ClustalW Method). b: Alignment of the Guangxi cattle and water buffalo astrovirus isolates and bovine astroviruses full-length ORF2 sequences retrieved from the NCBI database with a 42-nt, highly conserved sequence in the most 3′-end of ORF2 (DNAstar, MegAlign Program: ClustalW Method).

ogy of bovine astrovirus in cattle and water buffalo, as not only did we provide useful reference material for further studies, but also isolated bovine astrovirus in water buffalo for the first time. The present study is thus far among the largest epidemiological investigations of bovine astrovirus conducted at the farm level in the dairy industry in China.

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Table 4. Pair distances of nucleotide sequences between bovine and water buffalo astrovirus strains (MegAlign, ClustalW)

1	BAstGX J25	***	100	99.4	99.3	72.9	73	72.9	72.2	72.3	58.5	59	59	60	59.7	59.7	59.6	59.8	62.9	BAstGX J25
	BAstGX S7-7		***	99.4	99.3	72.9	73	72.9	72.2	72.3	58.5	59	59	60	59.7	59.7	59.6	59.8	62.9	BAstGX S7-7
	BAstGX S17-7			***	99.4	73.1	73.2	73.1	72.4	72.6	58.8	58.9	58.9	59.9	59.6	59.6	59.5	59.7	62.8	BAstGX S17-7
	BAstGX S12-7				***	73	73.1	73	72.3	72.5	58.9	59.1	59.1	59.9	59.8	59.8	59.7	59.9	63.2	BAstGX S12-7
2	BufAstGX M52*					***	99.6	99.5	98.9	99.1	57.5	57.9	57.8	58.4	58.6	58.4	58.3	58.5	59.6	BufAstGX M52*
	BufAstGX M53*						***	99.3	98.6	98.8	57.8	57.5	57.4	58	58.2	58	57.9	58.1	59.5	BufAstGX M53*
	BufAstGX M54*							***	98.6	98.8	57.6	58	57.9	58.5	58.7	58.5	58.4	58.6	59.7	BufAstGX M54*
	BufAstGX M541*								***	99.3	56.8	62.3	62.2	57.7	57.9	57.7	57.6	57.8	58.8	BufAstGX M541*
	BufAstGX M552*									***	57.1	62.3	62.2	57.8	58	57.8	57.7	57.9	58.9	BufAstGX M552*
3	BufAstGX M141*										***	68.8	68.7	69.4	69.4	69.6	69.4	69.6	74	BufAstGX M141*
4	BAstGX J251											***	99.4	98.9	99	99.2	98.9	99.1	74.8	BAstGX J251
	BAstGX J252												***	98.7	98.8	98.9	98.7	98.9	74.9	BAstGX J252
	BAstGX J23													***	99.6	99.7	99.5	99.6	75.4	BAstGX J23
	BAstGX J22														***	99.8	99.6	99.7	75.4	BAstGX J22
	BAstGX J8															***	99.7	99.9	75.6	BAstGX J8
	BAstGX J7																***	99.6	75.3	BAstGX J7
	BAstGX J27																	***	75.6	BAstGX J27
5	BAstGX G1																		***	BAstGX G1

*water buffalo.

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