Rapid communication

Detection and genetic diversity of water buffalo astrovirus in Guangxi province of China reveals neurotropic, genetic recombinant and possible interspecies transmission

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Summary

Astroviruses (AstVs) are major causative agents of gastroenteritis in children and had been detected worldwide. Recently, the novel neurotropic AstV associated with encephalitis and meningitis has been found in different species including human, bovine and ovine. However, little is known about the prevalence of neurotropic AstVs in water buffalo of China. In this study, we examined fecal samples from water buffalo in the Guangxi province of China and found different lineages of Water Buffalo Astrovirus (BufAstV) infections. In addition, we confirmed that the BufAstV infection of the brain tissues of a dead calf by immunohistochemistry technology in this study. Based on the 3'RACE and next-generation sequencing technologies, 2 full-length genomes (BufAstV-NNA-14 and BufAstV-NNA-12) and 2 ORF2 genes (BufAstV-NND-s2 and BufAstV-NNA-17) of AstVs from this source were sequenced. Phylogenetic analysis of the ORF2 indicated 3 major lineages of BufAstVs including a novel neurotropic BufAstV, a BufAstV which is related to Bovine Astrovirus (BoAstV) and a classical BufAstV. Moreover, the occurrence of genomic recombination between BufAstV and BoAstV strains have been identified. This is the first report to found a BufAstV infected in brain of water buffalo in China and details of the epidemiology, genetic diversity and possible interspecies transmission of BoAstV and BufAstV in

water buffalo from the Guangxi province of China are described.

Keywords: astrovirus, water buffalo, bovine, neurotropic, genetic diversity, Guangxi province

Introduction

Astroviruses (AstVs) are non-enveloped, single-stranded positive-sense RNA viruses (Alfred et al., 2015) which are 6.4kb-7.7kb in length and usually contains three consecutive open reading frames (ORFs): ORF1a, ORF1b and ORF2 (Bosch, Pintó, & Guix, 2014). Both ORF1a and ORF1b encode non-structural protein and ORF2 is expressed from the subgenomic RNA and encodes a capsid structural protein (Bosch et al., 2014). AstVs have a broad range of hosts and are classified with different genotypes according the similarity of nucleotides and amino acids within the ORF2 that encode for the capsid protein (De Benedictis, Schultz-Cherry, Burnham, & Cattoli, 2011). AstVs were generally considered to be the major causative agents for diarrhea in children and other immunodeficient hosts (Cydney, Virginia, Valerie, Victoria, & Stacey, 2017). However, the novel VA/HMO cluster found in AstVs could cause extra-gastrointestinal diseases such as hepatitis, nephritis, meningitis, encephalitis and other neurological symptoms in humans as well as in several animal hosts (Celeste & Dhanasekaran, 2017).

BoAstV was first discovered in calves with diarrhea in 1978 (Woode & BRIDGER, 1978). However, the pathogenicity of BoAstV is not clear. Recently, as with human AstVs, several novel nerve-related tropism bovine AstV strains such as BoAstV NeuroS1 (KF233994.1), BoAstV CH13 (NC 024498.1), BoAstV kagoshima SR28 (LC341267) and BoAstV BH89/14 (LN879482.1) have been identified from the United States, Switzerland, Japan and Germany. These are able to infect the central nervous system (CNS) and cause meningitis and encephalitis which subsequently lead to serious neurological signs (Yoshimasa et al., 2018). Phylogenetic analysis of the major neurotropic AstV strains found them to be clustered into the same clade, namely the VA/HMO clade, which indicates that these strains have the same origin (Yoshimasa et al., 2018). However, the interspecies transmission and recombination cases of AstVs are noteworthy. Because the species barrier of AstVs is not strong (Celeste &

Dhanasekaran, 2017), the frequency of genetic recombination of the ORF2 between different species is the reasons to cause interspecies transmission of these viruses, particularly between similar genetic hosts (e.g., ovine and bovine, wild boar and swine and primate and human).

The Guangxi province has one of the largest capacity to breed water buffaloes in China. The prevalence and genetic diversity of BufAstV in China are still poorly documented. In particular, very little is known regarding the prevalence of different neurotropic strains of AstVs in China. Therefore, in this study, 297 water buffalo fecal samples from 15 different scale farms in five regions of Guangxi province were examined for AstVs. Here we described the discovery of some new neurotropic AstVs from water buffalo feces and brain, and the evidence of genetic recombination of these viruses.

Materials and Methods

1. Samples collection

297 feces and 40 serum samples were collected from water buffaloes reared in 15 different farms in Nanning, Guigang, Beihai, Hengxian and Linshan regions of the Guangxi province in 2019 (Table 1). Samples were collected in autoclaved centrifuge tubes and diluted as 10% suspension in phosphate-buffered saline (PBS) (pH 7.2) and centrifuged for 10 min at 12,000 rpm at 4 °C. The supernatants from fecal samples were used to extract viral nucleic acids and were stored at -80℃.

In September 2019, in a buffalo farms of Nanning, A 5-month-old calf buffalo died with severe neurological symptoms including convulsion, opisthotonos and trembling and also accompanied by severe malnutrition. Veterinarians performed an autopsy on the calf and took part of the brain tissue for further researches. The brain tissue was divided three parts, soaked in PBS, 10% neutral formalin and 4% DEPC-containing paraformaldehyde and used for RT-PCR, HE-staining and immunohistochemistry, respectively.

2. RNA extraction and RT-PCR

RNA was extracted from rectal swab supernatants using the RNAiso Plus kit (Takara Bio, Inc., Dalian, China) by following the manufacturer’s instructions. The

first-strand cDNA was synthesized by the PrimeScript II 1st strand cDNA synthesis kit (Takara Bio, Inc., Dalian, China). The partial RNA-dependent RNA polymerase (RdRp) gene specific for AstVs was amplified by nested PCR (Chu, Poon, Guan, & Peiris, 2008) (STable 1) and sequenced as described previously (Alfred et al., 2015).

3. 3'-RACE and the next-generation sequencing

The ORF2 genome of AstVs was amplified by 3'RACE PCR kit (Takara, Bio, Inc., Dalian, China) by following the manufacturer's instructions. Specific primers were designed according to the RdRp regions and are listed in STable 1. Next-generation sequencing was performed in order to obtain the full-length gene of AstVs. A cDNA library was constructed for each sample using TruSeq™ DNA Sample Prep Kit (Illumina, San Diego, CA, USA). Bridge PCR was performed by using the TruSeq PE Cluster Kit (Illumina, San Diego, CA, USA). Sequencing was carried out on an Illumina TruSeq instrument using TruSeq SBS Kit v3 (Illumina, San Diego, CA, USA).

4. Phylogenetic and genome analysis

All the obtained sequences in this study were aligned against other AstVs reference sequences in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and uploaded to the GenBank and aligned with published reference sequences of AstVs by the ClustalW (1.6) method in MEGA 7.0 software. The same software was used to reconstruct phylogenetic trees from evolutionary distances using the neighbor-joining (NJ) method with p-distances for nucleotide sequences with 1000 replicates for bootstrap test which evaluated their clustering stability. The accession numbers of the nucleotide sequences obtained in this study and reference sequences are shown in STable2.

5. Recombinant analysis

The full-length of ORF2 sequences of AstVs were screened for recombinant signals by using the RDP4 recombination program v.4.39 with RDP, GENOCONV, Bootscan, Maxchi, Chimaera and Siscan recombinant algorithm methods. At least three methods with p values of less than 0.05 were considered potential recombinant events and needed to be further subjected to similarity plots and bootscan analysis with Kimura (2-parameter) methods and the NJ model with 1000 bootstrap replicates by Simplot v3.5.1, respectively. In order to analyze the potential recombinant sequences at both ends of

the breakpoints, the phylogenetic trees from the breakpoints between different portions of recombinant regions were reconstructed by the NJ method.

6. Preparation of the polyclonal antibody

According to the ORF2 sequences of BufAstV-NNA-14 strain, a pair of primers (Stable 1) were designed to construct the prokaryotic expression plasmids. The partial ORF2 gene was amplified and subcloned to pET32a vector (Novagen, Germany) by using the ClonExpress II One Step Cloning Kit (Vazyme Biotech, China). Expression of the recombinant protein by *E.coil* BL21 (DE3) was conducted. The purification of the recombinant protein used His- Tagged Protein Purification Kit (CWBio, Beijing, China) by following the manufacturer's instructions and the purified protein was confirmed by western-blot with an anti-His monoclonal antibody (proteintech, Wuhan, China). Before immunization, 5mL of serum was collected from New Zealand white rabbit as negative control. 500ug of the purified protein was mixed with Freund's adjuvant (Sigma-Aldrich, CA, USA) at a ratio of 1/1 (v/v) and injected in the rabbits. The other 3 injections were performed in the same way at intervals of 2 week. The antiserum that was named BufAstV-NNA-14 capsid polyclonal antibody was collected and isolated at 7 days after the last injection.

7. Histopathology and Immunohistochemistry

After 48h post fixation in neutral buffered formalin, various sections of the brains were trimmed, processed and embedded in paraffin. 4μm sections were cut and fixed on microscope slides, stained with hematoxylin and eosin (H&E) then analyzed for histopathological changes.

After paraformaldehyde fixation, the brain tissues were rinsed for 12h in embedding boxes. The relevant steps of dehydration, transparency, waxing, embedding and cutting are as described above. Antigen retrieval was accomplished with EDTA Antigen Retrieval solution (Boster Biological tech, Beijing, China) in boiling water for 20 min and then was cooled to room temperature. The slides were soaked in 3% hydrogen peroxide (Solarbio, Beijing, China) for 10min followed by three changes of distilled water. After blocking with 5% BSA buffer (Solarbio, Beijing, China) for 30min at 37℃, rabbit polyclonal antibody to capsid protein of BufAstV-NNA-14 strain was used for

the detection of BufAstV antigen. The antibody was diluted 1:200 in PBST (phosphate-buffered saline (PBS) containing 0.1% Tween 20) and transferred to 37 ˚C incubator for 1h. Goat anti-rabbit IgG conjugated HRP (CWBio, Beijing, China) was diluted 1:200 in PBST and added and the slides transferred to a 37 ˚C incubator for 1h DAB (3,3′N-diaminobenzidine tetrahydrochloride) substrate chromogen (CWBio, Beijing, China) was applied to the slides for 5 min followed by a distilled water rinse for 15 min. The slides were then counterstained with hematoxylin, mounting medium added and cover-slipped

Results and discussion

In this study, all the fecal samples were obtained from water buffaloes which had no significant clinical symptoms. The AstVs positive rate of the farm was 40% (6/15), the positive rate of feces was 11% (33/297) and the AstVs-positive rate of serum was 0. The positive rate of calves (less than 150 days old) was higher than the positive rate of adult water buffaloes. All the relevant information related to the samples are shown in Table 1.

Two full-length genomes of AstVs (BufAstV-NNA-12, GenBank accession: MT499771) and (BufAstV-NNA-14 GenBank accession: MT499772) were obtained by next-generation sequencing in this study. The length of BufAstV-NNA-12 and BufAstV-NNA-14 were 6230 nt and 6406 nt, respectively, and contains 3 open reading frames (ORF1ab and ORF2), 2 untranslated regions (5' UTR and 3'UTR) and a poly A tail. The similarity of ORF1ab between BufAstV-NNA-12 and BufAstV-NNA-14 was 52.9%. Except for BufAstV-NNA-12 and BufAstV-NNA-14, two full-length sequences of ORF2 named BufAstV-NND-s2 (GenBank accession: MT521688) and BufAstV-NNA-17 (GenBank accession: MT521687) was found. The nucleotide and amino acid identities between the four ORF2s in this study were 41.1%-57.5% and 20.7%-47%, respectively. The highest nucleotide and amino acid identities between BufAstV-NND-s2 and BufAstV-NNA-12 was only 57.4% and 47%, respectively, and the BufAstV-NNA-14 had a much lower identity with the other three ORF2 sequences.

Based on the NJ phylogenetic tree of ORF2, BufAstV-NNA-14 which is similar to the ovine AstV (GenBank: NC002469), BoAstV/JPN/KagoshimaSR28-462

(GenBank: LC341267) and bovine AstV CH13 (GenBank: NC_024498) which cause meningitis and encephalitis, all belong to the Mamastrovirus 13 clade, also named the VA/HMO clade, and these contain the major neurotropic AstVs strains (Figure 1A). In addition, the major neurotropic AstV strains found in different hosts are closely related to the human AstV VA strains, suggesting the possibility of interspecies transmission of neurotropic AstVs in the VA/HMO clade (Reuter, Pankovics, & Boros, 2018). Interestingly, BufAstV-NND-s2 clustered into the bovine AstV clade and is closely related to the classical bovine AstVs like BoAstV B76/HK (GenBank: HQ916317) and BoAstV GX/G1 (GenBank: KJ476833). The identity with the BoAstV B76/HK strain was much higher than the identity with the other BufAstV strains. The p-distance of ORF2 between BufAstV-NND-s2 and BoAstV B76-2/HK was only 0.181, suggesting the former could be classified as a BoAstV despite the fact that it was isolated from water buffalo feces. In addition, the BufAstV-NNA-17 and BufAstV-NNA-12 isolated strains were clustered with other water buffalo AstVs such as MAstV/Buf/ITA/2013/750 (GenBank: KT963070) and MAstV/Buf/ITA/2013/619 (GenBank: KT963069).

In order to confirm whether the BufAstV-NNA-14 strain could invade the nervous system, A 5-month-old calf buffalo that died with convulsion, opisthotonos and severe malnutrition for diagnostic necropsy. Viral and bacterial pathogens including rabies virus, Japanese encephalitis and other herpesviruses have not been detected. The histopathology revealed neuronal and ganglionic degeneration and necrosis with astrogliosis especially in the gray matter of the brain, cerebral capillary hyperemia and lymphocytic hyperplasia, the nerve fibers around the cerebral capillary had vacuolated (Figure 2). On basis of the ORF2 sequences information of BufAstV-NNA-14, a special polyclonal antibody has been prepared to determine the presence of viral antigen in tissues. The strong antigen labeling was appeared in the degeneration neurons and microglia cells that correlated with the lesion area in the brain (Figure 2). These results are similar to the published for neurotropic astrovirus infections in cows and sheep (Boujon et al., 2017; Schlottau et al., 2016).

NJ phylogenetic analysis is based on studying the nucleotide sequences from the

3’-terminal conserved regions of the partial ORF1b gene segments. These are amplified from detecting primers which is consistent with the results of the complete ORF2 genome. There were three different genetic lineages of water buffalo AstVs circulating in Guangxi province (Figure 1B). BufAstV lineage 1 is related to the classical BufAstVs strains, BufAstV lineage 2 is closely related to the classical bovine AstV strains and BufAstV lineage 3 has been classified as the VA/HMO cluster, which includes the major neurotropic AstVs seen in different species. The identity of isolated strains in BufAstV lineage 2 has a closer phylogenetic distance with bovine AstVs than the other BufAstV strains, suggesting that these strains might be classified into bovine AstVs. These results indicate the possibility of interspecies transmission of BoAstV in water buffalo, suggesting that both BoAstV and BufAstV might be susceptible to water buffalo. Because of the close relationship between water buffaloes and cattle, it may be appropriate to consider the presently identified BoAstV and BufAstV as the different genotypes of the same species of AstVs despite their different hosts. This is similar to the situation with feline and cheetah AstVs (Lawler et al., 2018).

The full-length of ORF2 sequences of AstVs included in this study and the reference sequences were screened for recombinant signals by using RDP4 and Simplot software. Strong recombination signals between partial BoAstV sequences were found in RDP4 which were further investigated using other recombination analysis tools, and these were re-confirmed with Simplot. Based on the Simplot and Bootscan analysis, we found that the sequences of bovine AstV B76-2-HK had a recombination with bovine Astorivirus GX1 and BufAstV-NND-s2 as well as with BAstV GX1 has and bovine AstV GX-J27 in their original ORF2 sequences (Figure 3). Based on the breakpoint positions, 1225 and 2175, the ORF2 was divided into two portions, 1-1224 and 1225-2175, which were consistent with the divisions of the conserved and hypervariable regions of AstVs ORF2 (Arias & Rebecca, 2017). These regions translated the capsid protein which subsequently influenced the properties related to viral virulence, tropism and epitope content of the resultant virus (Arias & Rebecca, 2017). Recombination in these regions may generate diversity of capsid proteins and enable the AstVs to escape host immunity and expand tropism or host range. Moreover, two phylogenetic trees

were constructed for separating different recombinant regions of AstVs ORF2 by the breakpoints. The results confirmed that different recombinant regions had inconsistent topologies, respectively (Figure 3).

In summary, this study shows the water buffalo herds from different regions were infected with three lineages of BufAstV. This is the first report of the identification of a novel BufAstV infected brain of water buffaloes in China. Two full-length genomes of AstVs and two extra complete ORF2 of BufAstV were sequenced. Furthermore, frequent recombinations between BoAstV and BufAstV-NND-s2 were identified. This study described the details of the epidemiology, genetic diversity and possible interspecies transmission of BoAstV and BufAstV in water buffalo in China.

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Ethics statement and conflict interests

This study was approved by the Animal Care & Welfare Committee of Guangxi University and the approval was recorded and supervised (GXU2018-044). The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends

Figure 1: The neighbor-joining phylogenetic trees of full-length ORF2 (Figure 1A) and partial ORF1b gene (Figure 1B) of AstVs with p-distances for nucleotide sequences with 1000 replicates for the bootstrap test. The black dots indicated the sequences from this study.

Figure 2: Histopathological lesions and immunohistochemistry (IHC) of BufAstV-NNA-14 protein from the brains of a 5-month-old calf buffalo with neurologic symptoms. Brains sections showed glial cell aggregation (*), neuronal degeneration (**), cerebral capillary hyperemia, lymphocytic hyperplasia and cavitation of perivascular nerve fibers (***) in HE-stained in Figure 2A (10×) and Figure 2B (40×). The brains sections had incubated with BufAstV-NNA-14 capsid polyclonal antibody (1:200) by IHC at magnification of 40× (Figure 2C). The strong antigen labeling was shown around the damaged neuron cells (*) and the area where microglia aggregates (**) in Figure 2C. The antibody control group (Figure 2D) used the negative antibodies for IHC in these brain sections under the same conditions.

Figure 3: The Bootscan recombination analysis based on the ORF2 gene of bovine Astrovirus B76-2-HK (Figure 3A) and bovine Astrovirus GX1 (Figure 3B) using the two-parameter (Kimura) distance model and the neighbor-joining model with 1000 bootstrap replicates. The neighbor-joining phylogenetic trees of 1-1224nt (Figure 3C) and 1225-2175nt (Figure 3D) of ORF2. In the clade of classical intestinal tropism AstVs, different topologies of phylogenetic trees are shown between portions 1-1224 and 1225-2175. The constructed method is described above and the black dots indicated the sequences obtained in this study.