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BRIEF REPORT

Bufavirus in fecal specimens of patients with and without diarrhea in Thailand

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Abstract Bufavirus (BuV) was initially discovered in fecal samples from children with acute diarrhea. In this study, we determined the prevalence, distribution, and genotype(s) of BuV in Thailand. A total of 1,495 diarrheal and 741 non-diarrheal stool specimens were collected and analyzed. A portion of the NS1 gene of BuV was amplified by nested RT-PCR. Phylogenetic analysis was performed to classify the BuV strains found. We detected bufavirus (BuV) in diarrheal (4/1495; 0.27 %) but not in non-diarrheal specimens (0/726). All four strains belonged to BuV genotype 1. BuV could be detected in adults and children, but its role in causing acute diarrhea remains unclear.

Keywords Bufavirus · Diarrhea · NS1 gene · Phylogenetic analysis

The metagenomic approach has been used to identify new viruses from biological samples, including a novel bufavirus (BuV) from fecal samples from children with acute diarrhea in the West African country of Burkina Faso [1]. This virus was subsequently classified as a member of the family *Parvoviridae*, genus *Protoparvovirus* [2]. The BuV genome consists of single-stranded DNA encoding nonstructural protein 1 (NS1) and viral structural proteins 1 and 2 (VP1 and VP2) [1]. Three distinct genotypes (BuV1, BuV2, and BuV3) have been reported [1, 3].

BuV has been identified in several countries, including Tunisia, Burkina Faso [1], Bhutan [3], Finland [4], and the Netherlands [5]. Since many cases of diarrhea present no obvious etiology, we investigated the prevalence of BuV in diarrheal fecal samples compared with non-diarrheal samples obtained from patients with hand, foot and mouth disease (HFMD). The study protocol was approved by the Institutional Review Board of Chulalongkorn University (IRB number 329/57), and the requirement for informed consent was waived because all of the samples were anonymous. We conducted this study to define the prevalence, distribution, and genotype(s) of BuV in Thailand.

Diarrheal specimens (n = 1495) were collected from patients with gastroenteritis from King Chulalongkorn Memorial Hospital (n = 394) and Chumphae Hospital (n = 1101), Khon Kaen province, during January 7, 2009 to April 30, 2014. There were 884 males, 586 females and 25 of unspecified gender (mode of age is 1 year, range 1 day to 97 years) (Table 1). All samples were tested for human rotavirus A (HRoV) at the Center of Excellence in Clinical Virology, Department of Pediatrics, King Chulalongkorn Memorial Hospital in Bangkok [6]. In addition, a total of 741 non-diarrheal stool specimens (193 stools and 548 rectal swabs) were selected from an earlier HFMD study [7] from hospitals in Bangkok (n = 646) and Khon Kaen (n = 95) between February 15, 2010 and July 7, 2014 (Table 1).

Samples were diluted in PBS (1:10) and then centrifuged at 4000*g* at 25 °C for 10 minutes. Total viral nucleic acid was extracted from supernatants using Ribospin vRD II (GeneAll, Seoul, Korea). Nested PCR using primers specific for the NS1 gene of BuV (1) was performed with

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Table 1 Demographic information for individuals whose stool samples were analyzed in this study

	Diarrhea group		Non-diarrhea group (HFMD)	
	Children (1 day-15 yr; mode = 1 yr)	Adults (16 yr-97 yr; mode = 48 yr)	Children (1 mo-15 yr; mode = 1 yr)	Adults (16 yr-39 yr; mode = 16 yr)
Number of samples	1414	81	726 (stool = 183, rectal swabs = 543)	15 (stool = 10, rectal swabs = 5)
Source of sample collection				
Bangkok	313	81	631	15
Khon Kaen	1101	0	95	0
Sex				
Male	848	36	410	9
Female	542	44	316	6
Data missing	24	1	0	0

mo = month; yr = year

PerfectTaq MasterMix (5 PRIME, Darmstadt, Germany). Successful amplification of the human housekeeping gene GADPH served as positive control. Subsequent BuV-positive PCR products (~440 bp) were purified using Expin Gel SV (GeneAll, Seoul, Korea) after 2 % agarose gel electrophoresis. Direct Sanger DNA sequencing in both the forward and the reverse direction with second-round PCR primers was performed by 1st BASE Laboratories (Selangor Darul Ehsan, Malaysia).

Additionally, one-step RT-PCR or semi-nested PCR was used to detect several other viruses associated with acute diarrhea, including HRoV (39.7 %), human norovirus (10.6 %), human sapovirus (3.3 %), human astrovirus (2.6 %), human parechovirus (HPeV) (5.9 %), human Saffold virus (0.2 %), human cosavirus (0.2 %), human bocavirus (3.5 %), and human adenovirus (5.6 %) [8–12]. No test for diarrhea-causing bacteria was done on the samples.

To identify the BuV genotype(s) detected in the samples, multiple sequence alignment was done using ClustalW in BioEdit v7.0.4.1 [13]. The best nucleotide substitution model was evaluated using jModelTest [14]. Genetic distances were determined and phylogenetic trees were constructed by the neighbor-joining method using MEGA v5.0 [15], with Tamura-Nei and G (Gamma) distribution as the substitution model. A bootstrap analysis of 1,000 replicates was done to test the significance of the branching.

Four samples (0.27 %, 4/1495) from the diarrhea cohort tested positive for BuV DNA (Table 2). Despite the relatively large cohort, this prevalence is lower than those found in previous studies, including 1.6 % in Tunisia (n = 63), 4 % in Burkina Faso (n = 98) [1], 1.1 % in Finland (n = 629) [4] and 0.8 % in Bhutan (n = 393) [3]. Although one positive sample (CU-B710) was from a child, three positive samples came from adults. This observation confirmed that BuV infection is not only confined to children, but adults and especially the elderly are also susceptible [4]. However, further investigations are needed because only four samples tested positive in this study. It is not clear if BuV was directly responsible for the observed diarrhea in these patients or it exacerbated other existing underlying conditions, as evidence of bleeding was found in stool samples from two patients (CU-B1493 and CU-B1549) with hemorrhagic gastritis. One patient died (CU-B1549) from congestive heart failure. All BuV-positive samples tested negative for the other enteric viruses, except for CU-B710, which showed co-infection with HPeV genotype 1A (Table 2).

Comparison of the partial NS1 nucleotide sequences showed ~97.6 %-99.5 % identity to published sequences and ~98.7 %-100 % identity at the amino acid level. Although all four BuV strains in this study clustered under genotype 1 (BuV1), the phylogenetic tree showed that the Thai isolates separated into two different clades: those from 2010 (CU-B710 and CU-B1493) and those from 2012 (CU-B1549 and CU1639) (Fig. 1). Due to the limited number of published studies and viral sequences of BuV in the database so far [1, 3, 4], the geographic or chronological specificity of BuV strains deduced from our phylogenetic classification should be regarded as tentative.

In the absence of a cell culture system and animal model studies, the relationship between BuV infection and pathogenicity in human remains unclear. Although we did not include fecal samples from healthy individuals for evaluation, we did screen the available stool samples from patients diagnosed with HFMD, which is caused by a non-diarrheal enterovirus. Since BuV was not detected in any of these samples, it may not be part of the commensal flora of the gut virome. Future studies involving BuV screening

Table 2 Samples obtained from diarrhea patients that tested positive for BuV DNA

Sample	Sex	Age (year)	Source of sample collection	Date of sample collection (dd/mm/yyyy)	Nationality	Underlying disease	Fever	Diarrhea	Stool exam RBC	Stool exam WBC	Other viruses
CU-B710	F	1	KK	10/02/2010	Thai	Acute diarrhea, vomiting	Yes	Yes (acute)	0-1	0-1	Positive for HPeV 1A
CU-B1493	M	48	BKK	05/06/2010	Thai	Hemorrhagic gastritis	No	Yes (acute)	10-20	0-1	All negative
CU-B1549	M	90	BKK	16/02/2012	Thai	Heart failure, renal failure, hemorrhagic gastritis	Yes	Yes (off and on)	5-10	0-1	All negative
CU-B1639	F	29	BKK	26/09/2012	Myanmar	Maculopapular rash, hypoproteinemia, eosinophilia	Yes	Yes (acute)	0-1	3-5	All negative

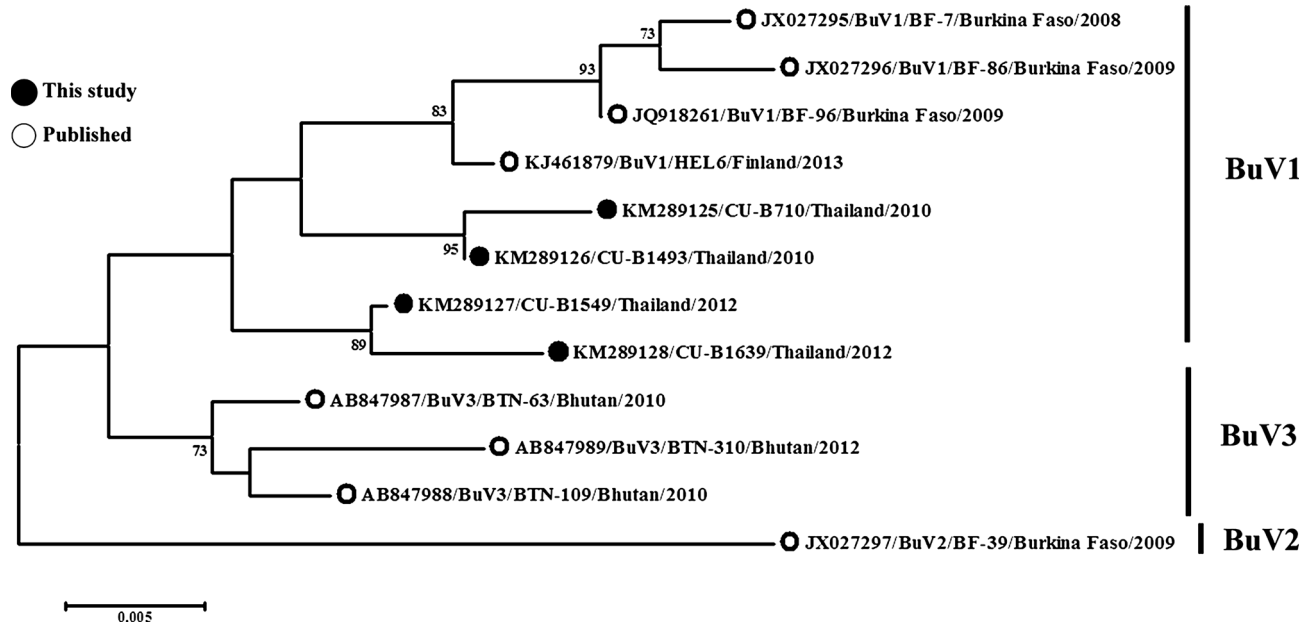

Fig. 1 Phylogenetic analysis of a portion of the NS1 gene of BuV strains isolated from patient stool samples and previous published isolates. The tree was constructed by the neighbor-joining method with Tamura-Nei and Γ distribution as the substitution model. The branching was tested by 1,000 bootstrapping replicates, and only bootstrap values >70 % are shown at the node of each tree branch. The scale bar indicates nucleotide substitutions per site

may require larger cohorts with and without diarrhea. Unequivocal association of BuV infection with acute diarrhea would require further assessment of BuV in healthy individuals, such as a serological survey of BuV in the healthy population.

In conclusion, a survey for ten human viruses associated with diarrhea found BuV genotype 1 among the samples screened from a relatively large Thai cohort. Future studies may provide insight into the extent of BuV genetic diversity in the feces, which will be useful in characterizing the potential pathogenicity of this novel enteric virus.

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Conflict of interest The authors have no conflicts of interest to declare.

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