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RESEARCH ARTICLE

Cosavirus, Salivirus and Bufavirus in Diarrheal Tunisian Infants

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Abstract

Three newly discovered viruses have been recently described in diarrheal patients: Cosavirus (CosV) and Salivirus (SalV), two picornaviruses, and Bufavirus (BuV), a parvovirus. The detection rate and the role of these viruses remain to be established in acute gastroenteritis (AGE) in diarrheal Tunisian infants. From October 2010 through March 2012, stool samples were collected from 203 children <5 years-old suffering from AGE and attending the Children’s Hospital in Monastir, Tunisia. All samples were screened for CosV, SalV and BuV as well as for norovirus (NoV) and group A rotavirus (RVA) by molecular biology. Positive samples for the three screened viruses were also tested for astrovirus, sapovirus, adenovirus, and Aichi virus, then genotyped when technically feasible. During the study period, 11 (5.4%) samples were positive for one of the three investigated viruses: 2 (1.0%) CosV-A10, 7 (3.5%) SalV-A1 and 2 (1.0%) BuV-1, whereas 71 (35.0%) children were infected with NoV and 50 (24.6%) with RVA. No mixed infections involving the three viruses were found, but multiple infections with up to 4 classic enteric viruses were found in all cases. Although these viruses are suspected to be responsible for AGE in children, our data showed that this association was uncertain since all infected children also presented infections with several enteric viruses, suggesting here potential water-borne transmission. Therefore, further studies with large cohorts of healthy and diarrheal children will be needed to evaluate their clinical role in AGE.

Introduction

Diarrhea remains a frequent illness throughout the world and causes the death of almost 6 million children annually, especially in developing countries. Besides well-documented enteric viruses, the list of viral pathogens causing acute gastroenteritis (AGE) is continuously growing with the emergence of new viruses. No less than three new types of virus have been discovered in diarrheal patients these last years: Cosavirus and Salivirus, two new genera in the Picornaviridae family since 2013, and Bufavirus from the Protoparvovirus genus of the Parvoviridae.
Cosavirus (CosV) was first identified in 2008 from children suffering from acute flaccid paralysis but has been later associated with diarrhea. It has been detected in feces from both patients with gastroenteritis and healthy subjects [1]. CosV has a single-stranded RNA genome of around 7.6 Kb organized in a typical picornavirus genome and has a wide genetic diversity: not less than 6 species (noted A to F) have already been described, of which CosV-A includes 24 different genotypes, and CosV-D includes 5 different genotypes [2].

Salivirus (SalV) was first identified in 2009 [3, 4]. SalV has a single-stranded RNA genome of around 7.1 Kb organized in a typical picornavirus genome. Although this virus is related, but distinct, to the Kobuvirus genus, SalV forms a genus that presently includes a single genotype with 2 clusters [5]. Human salivirus (also formerly called klasseviruses) has been associated with diarrhea and detected in feces from both gastroenteritis patients and healthy subjects from all continents, as well as in sewage from Spain and Hong-Kong, suggesting a widespread geographic distribution [4].

Bufavirus (BuV) was first discovered in 2012 in fecal samples from children suffering from diarrhea in Burkina Faso, from which it get its name [6]. Thereafter, BuV was detected in diarrheal stool samples of children from other continents [7–10]. BuV has a single-stranded DNA genome of around 4.9 Kb, which encodes nonstructural protein 1 (NS1) and viral structural protein (VP2). Three genotypes (BuV1, 2 and 3) have been described so far [11], but diversity within the capsid gene suggests the possibility of several other genotypes [6].

Whether these viruses are etiologic agents of human gastroenteritis remains unclear, but knowledge about their distribution and genetic divergence in humans is mounting. In this context, the detection rate and the role of these new viruses in AGE in diarrheal infants remain to be established.

**Methods**

From October 2010 through March 2012, stool samples were collected from 203 children <5 years-old suffering from AGE and attending the Fattouma-Bourguiba Children’s Hospital in Monastir, Tunisia. The children’s median age (MA) was 7.0 mo (ranging 0.5 to 60 mo), and the sex ratio was 1.29. The study and the data collection procedure were approved by the Ethics and Research Committee of the Fattouma-Bourguiba Public Hospital. Informed consents were obtained verbally from the parents of the study participants and consigned in their clinical records in accordance to the Tunisian good clinical practices and hospital clinical investigations guidelines. The samples were anonymized before processing.

For each stool sample, nucleic acids were extracted from 800 μl of 10% fecal suspension in PBS on a Nuclisens® EasyMAG system (bioMérieux, Marcy l’Étoile, France), according to the manufacturer’s instructions. RNA/DNA was eluted in a final volume of 110 μL. CosV and SalV were screened by nested RT-PCR using primer sets targeting the 5’UTR region [1, 12]; and BuV by nested PCR using primer sets targeting the NS1 region [6]. Virus characterization was performed using primer sets targeting various regions: capsid (VP1) and polymerase (3Dpol) for CosV [1, 2]; capsid (VP0), helicase (2Chel) and polymerase (3Dpol) for SalV [4, 13, 14]; and the capsid protein region (VP2) for BuV using the following designed primers: ARUB259: 5’- ATCTCTTTGGTCATTGCGAGAAAAAAAG -3’ and ARUB261: 5’- TTGWTTTTGTGTTTACACCCTGTAGAAAAAAG -3’. Primer details are provided in Table 1. RT-PCRs were performed using the Qiagen OneStep RT-PCR kit (Qiagen GmbH, Hilden, Germany) and PCRs with Novagen KOD Hot Start polymerase kit (EMD Millipore, Darmstadt, Germany) on an Eppendorf MasterCycler PCR machine (Eppendorf AG, Hamburg, Germany), according to the manufacturer’s instructions.

Sequences were obtained with the ABI Big Dye sequencing kit on an ABI 3130XL sequencer (Applied Biosystems, Waltham, USA). Phylogenetic analysis were performed using using
MEGA6 software [15]. After sequence alignment using the MUSCLE programme with a maximum of 64 iterations [16], phylogenetic trees were inferred using the Maximum Likelihood method based on the Tamura-3-parameter model with a discrete gamma distribution, which was the best-fit DNA substitution model for the nucleotide dataset submitted. Bootstrap values were calculated from 1000 replicates. The nucleotide sequences were deposited in the GenBank database under the accession numbers: KU362760 to KU362792.

All samples were tested for norovirus (NoV) and group A rotavirus (RVA) by RT-qPCR. Positive samples for the three screened viruses were also tested for astrovirus (AstV) and sapovirus (SaV) by RT-qPCR, adenovirus (AdV) by qPCR and Aichi virus (AiV) by RT-PCR then genotyped when technically feasible, using the NRC’s PCR screening and typing procedures as reported in S1 Table [17–30]. Secretor status of positive individuals has been determined by genotyping on blood samples by using the methods described in our previous studies [31,32].

### Table 1. Oligonucleotides used for detection and genotyping of cosavirus, salivirus and bufavirus in this study.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Method</th>
<th>Target</th>
<th>Primer</th>
<th>Nucleotide Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cosavirus</strong></td>
<td>RT-PCR (1st round)</td>
<td>5’UTR</td>
<td>DKV-NSU-F1</td>
<td>CGT GCT TTA CAC GGT TTT TGA</td>
<td>Kapoor et al, 2008 [1]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VP1</td>
<td>INO-VP1-F1</td>
<td>GAI CAR GCI ATG ATG GGI AC</td>
<td>Kapusinszky et al, 2012 [2]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3Dpol</td>
<td>DKV-IF1</td>
<td>CTA CCA RAC TTT YCT IAA RGA</td>
<td>Kapoor et al, 2008 [1]</td>
</tr>
<tr>
<td><strong>PCR (2nd round)</strong></td>
<td>5’UTR</td>
<td>DKV-NSU-F2</td>
<td>ACG GTT TTA GAA CCC CAC</td>
<td>Kapoor et al, 2008 [1]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VP1</td>
<td>INO-VP1-F1–2</td>
<td>GCC ATG ATG GGI ACI TWY DCI ATI TGG GA</td>
<td>Kapusinszky et al, 2012 [2]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3Dpol</td>
<td>DKV-IF2</td>
<td>CTA CCA GAC ATT TCT CAA RGA YGA</td>
<td>Kapoor et al, 2008 [1]</td>
<td></td>
</tr>
<tr>
<td><strong>Salivirus</strong></td>
<td>RT-PCR (1st round)</td>
<td>5’UTR</td>
<td>SAL-L1</td>
<td>CCC TGC AAC CAT TAC GCT TA</td>
<td>Shan et al, 2010 [12]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SAL-R1</td>
<td>SAC ACC AAC CTT ACC CCA C</td>
<td>Holtz et al, 2009 [4]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>VP0</td>
<td>LG0119</td>
<td>GCT AAC TAT GCT GCC ACC</td>
<td>Han et al, 2010 [13]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2Chel</td>
<td>KL-2C-F1</td>
<td>CTC GCC GAG GAC ATC AGC GA</td>
<td>Han et al, 2010 [13]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3Dpol</td>
<td>LL-3D-F1</td>
<td>GAA GAT GCC ATT CGT CTC</td>
<td>Li et al, 2009 [14]</td>
</tr>
<tr>
<td><strong>PCR (2nd round)</strong></td>
<td>5’UTR</td>
<td>SAL-L2</td>
<td>ATT GAG TGG TGC ATG TGT TG</td>
<td>Shan et al, 2010 [12]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SAL-R2</td>
<td>ACA AGC CCG AAG ACC ACT AC</td>
<td>Han et al, 2010 [13]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>VP0</td>
<td>KLVPF</td>
<td>GTC ACY CCM AAC ACC TCC ACT GAA G</td>
<td>Han et al, 2010 [13]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2Chel</td>
<td>KL-2C-F2</td>
<td>AAT CTG CTG CCC AGG CCG C</td>
<td>Han et al, 2010 [13]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3Dpol</td>
<td>LL-3D-F2</td>
<td>CTT TCC CAA TCT CCT GGC TAC</td>
<td>Li et al, 2009 [14]</td>
</tr>
<tr>
<td><strong>Bufavirus</strong></td>
<td>RT-PCR (1st round)</td>
<td>NS1</td>
<td>BF.F1</td>
<td>TCA ACA ATC ACT GCA AAT GG</td>
<td>Phan et al, 2012 [6]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS1</td>
<td>BF.R1</td>
<td>AGT TTG CCT GGA TGT TCT TGG A</td>
<td>Phan et al, 2012 [6]</td>
</tr>
<tr>
<td><strong>PCR (2nd round)</strong></td>
<td>NS1</td>
<td>BF.F2</td>
<td>CTA ACA CTG GTC CTT GCT ATG GAC</td>
<td>Phan et al, 2012 [6]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS1</td>
<td>BF.R2</td>
<td>TTT CTT GGT GAT GAT TCT TGT GTC</td>
<td>Phan et al, 2012 [6]</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0162255.t001
Results and Discussion

During the study period, 71 (35.0%) children were infected with norovirus and 50 (24.6%) with rotavirus, of which 11 were mixed infections. In all, 11 (5.4%) samples were positive for one of the three investigated viruses: 2 (1.0%) CosV (MA = 12.0 mo.), 7 (3.5%) SalV (MA = 6.0 mo.) and 2 (1.0%) BuV (MA = 20.8 mo.). Of note, all individuals infected by these new viruses were secretors. No mixed infections involving these viruses were found, but multiple infections with 1 to 4 viruses responsible for GEA in humans were found in all cases (Table 2). These mixtures of several enteric viruses, particularly with non-enteric adenoviruses, suggest here an environmental contamination from soiled waters. While both CosV and BuV infections occurred during winter in 2 different seasons, most SalV infections occurred in the autumn of the second season of the survey (Fig 1). However, the number of tested stools being rather limited, a larger sampling will be required to confirm any seasonality pattern.

Our data showed that the detection rate of CosV in diarrheal children (1%) was lower than observed in China or Brazil, where they were reported in diarrheal children in 2.8% and 3.6% of patients [33, 34], respectively. CosV has been previously reported in up to 33.0% of healthy Tunisian subjects or suffering from non-polio acute flaccid paralysis, most of them being children <6 years old [35]. However, the difference in detection rates is due to different natures of the two cohorts. According to phylogenetic analysis of their VP1 regions, the two CosV strains,

Table 2. Demographic characteristic and detected enteric viruses in patients with cosavirus, salivirus or bufavirus infections.

<table>
<thead>
<tr>
<th>New viruses detected</th>
<th>Genotype</th>
<th>Sample ID</th>
<th>Age, mo / sex</th>
<th>Sample date</th>
<th>Other viruses tested*</th>
<th>GenBank Accession Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CosV</td>
<td>A10</td>
<td>H036</td>
<td>9/M</td>
<td>2011/01/21</td>
<td>GII.g/ GII.1</td>
<td>KU362764⁴, KU362768⁵, KU362784⁶, KX721253⁷</td>
</tr>
<tr>
<td></td>
<td>A10</td>
<td>H226</td>
<td>3/F</td>
<td>2012/03/15</td>
<td>- - - + -</td>
<td>KU362765⁴, KU362785⁵, KX721254⁷</td>
</tr>
<tr>
<td>SalV</td>
<td>A1</td>
<td>H010</td>
<td>27/M</td>
<td>2011/03/09</td>
<td>- G2P [⁴] - - + -</td>
<td>KU362767⁴, KU362773⁵, KU362779⁶, KU362786⁷</td>
</tr>
<tr>
<td></td>
<td>A1</td>
<td>H108</td>
<td>13/M</td>
<td>2011/12/03</td>
<td>- G9P [⁸] + + GII.1</td>
<td>KU362768⁴, KU362774⁵, KU362780⁶, KU362787⁷</td>
</tr>
<tr>
<td></td>
<td>A1</td>
<td>H142</td>
<td>12/M</td>
<td>2011/10/04</td>
<td>GII.P21/ GII.3 G4P [⁸]</td>
<td>KU362769⁴, KU362775⁵, KU362781⁶, KU362788⁷</td>
</tr>
<tr>
<td></td>
<td>A1</td>
<td>H144</td>
<td>2.5/F</td>
<td>2011/09/30</td>
<td>- - + - + -</td>
<td>KU362770⁴, KU362776⁵, KU362782⁶, KU362789⁷</td>
</tr>
<tr>
<td></td>
<td>A1</td>
<td>H159</td>
<td>6/F</td>
<td>2011/10/20</td>
<td>GII.P21/ GII.3 - - -</td>
<td>KU362771⁴, KU362777⁵, KU362783⁶, KU362790⁷</td>
</tr>
<tr>
<td></td>
<td>A1</td>
<td>H169</td>
<td>39/M</td>
<td>2012/02/18</td>
<td>- G3P [⁸] - - -</td>
<td>KU362791⁷</td>
</tr>
<tr>
<td></td>
<td>A1</td>
<td>H214</td>
<td>7/M</td>
<td>2011/11/15</td>
<td>- - HAstV- 5 - - -</td>
<td>KU362772⁴, KU362778⁵, KU362792⁶</td>
</tr>
<tr>
<td>BuV</td>
<td>1</td>
<td>H040</td>
<td>2.5/M</td>
<td>2011/01/30</td>
<td>GII + - - + -</td>
<td>KU362760⁴, KU362762⁴</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>H232</td>
<td>39/M</td>
<td>2012/03/05</td>
<td>- GxP [⁸] - - -</td>
<td>KU362761⁴, KU362763⁴</td>
</tr>
</tbody>
</table>

CosV: cosavirus; SalV: salivirus; BuV: bufavirus; NoV: norovirus; RVA: rotavirus; AstV: astrovirus; AdV: adenovirus; SaV: sapovirus; AiV: Aichi virus

* Genotypes are shown when available; -: negative; +: positive; M: male; F: female

⁴ Polymerase region
⁵ Helicase region
⁶ 5’UTR region
⁷ VP region

doi:10.1371/journal.pone.0162255.t002
H036 and H226, were closely related to the genotype A10. Indeed, they shared 87% and 88% of their nucleotide sequences, respectively, but 98% of their amino acids (aa) sequences with the Nepalese NP8/3 CosV strain (JQ811823) (Fig 2A). Since the 3Dpol sequence of the NP8/3 strain is not known and the length of the sequences is short, their 3Dpol regions were closely related to the Nigerian NG263 CosV strain (JN867756), which belongs to the genotype A20, with 93% and 94% of nucleotide sequence homology, respectively (Fig 2B). Of note, the VP1 sequences of our strains shared merely 56% of their aa sequences with the NG263 strain.

In this study, the detection rate of SalV infections accounted for 3.5% of the Tunisian cases, which is close to the 4.2% observed in Chinese children but much less than the 8.8% of cases in South Korea [12, 13]. Although they are globally widespread, SalV seem to circulate more in Asia that in North Africa. Interestingly, the detection rate of SalV in this study was similar to the detection rate of AiV (3.6%) in Tunisian children [36]. Since Salivirus is a genus phylogenetically close to Kobivirus, both viruses might share some epidemiology characteristics that remain to be defined. Phylogenetic analysis of VP0, 2Chel and 3Dpol regions of 6 of the 7 detected SalV also showed that they belonged to the cluster A1 and were all closely related with various Asian strains, especially from South Korea (Fig 3). Further analysis also showed that the nt and aa sequences of the Tunisian clustering strains (i.e. strains H010, H142, H144, H159 and H214) had 99% of homology with Hungarian strains detected 1 year later in newborns suffering from AGE [5] (data not show).

With a low detection rate (1%), BuV were only found occasionally in Tunisian stools. These findings are similar to those observed in children from Asia, Europe or Africa where detection rates range from 0.5% to 4.0% in patients of all ages [6–10]. Given that this present study was focused only on young children, our results suggest that BuV detection rate is the similar in young children as the rest of the Tunisian population. Complete VP2 sequences of H040 and
Fig 2. Phylogenetic trees of cosaviruses detected in diarrheal Tunisian children. A. VP1 region of cosavirus (904 nt); B. 3Dpol region of cosavirus (400 nt). Phylogenetic trees were inferred using the Maximum Likelihood method based on the Tamura-3-parameter nucleotide substitution model with a discrete gamma distribution. Bootstrap values were calculated from 1000 replicates. Strains of this study are shown in red. Genotypes are shown in bold. doi:10.1371/journal.pone.0162255.g002

Fig 3. Phylogenetic trees of saliviruses detected in diarrheal Tunisian children. A. VP0 region of salivirus (815 nt); B. 2CHel region of salivirus (275 nt); C. 3Dpol region of salivirus (686 nt); Phylogenetic trees were inferred using the Maximum Likelihood method based on the Tamura-3-parameter nucleotide substitution model with a discrete gamma distribution. Bootstrap values were calculated from 1000 replicates. Strains of this study are shown in blue. Genotypes are shown in bold. doi:10.1371/journal.pone.0162255.g003
Cosavirus, Salivirus and Bufavirus in Diarrheal Tunisian Infants

A

B

BuV-2 BF.39(JX027297)-Burkina Faso
BuV-3 BF.189(KM580352)-China
BuV-3 BTN63(AAB847987)-Bhutan
BuV-3 BTN310(AAB847989)-Bhutan
BuV-3 BJ154(KM580348)-China
BuV-1 BU149(KM289127)-Thailand
BuV-1 CUB/1639(KM289128)-Thailand
BuV-1 CUB/1493(KM289126)-Thailand
BuV-1 BF.96(JQ918261)-Burkina Faso
BuV-1 BF.7(JX027295)-Burkina Faso
BuV-3 AHP747(AAB882223)-Turkey
BuV-3 AHP740(AAB982222)-Turkey
H232-(KU362761)-Tunisia
BuV-3 AHP178(AAB982217)-Turkey
BuV-1 BJ133(KM580347)-China
BuV-3 AHP228(AAB982218)-Turkey
BuV-1 HEL6(KJ461879)-Finland

Cutavirus FR/F(KT868815)-France

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H232 BuV strains showed that both strains belonged to genotype 1 and shared 99% of their sequence with published West-African and Finnish strains (Fig 4A). The 2 BuV strains shared 99% of their VP2 aa sequences (7/569 aa substitutions). Although NS1 sequences appeared more closely related to strains from genotype 3 (Fig 4B), aa sequence analysis of BuV polymerase showed small divergence between genotypes. Serological studies will be needed to get a better picture of BuV circulation in Tunisian population.

Conclusion

Although these new viruses are suspected to be responsible for AGE in children, our data showed that this association was uncertain since all infected children also presented infections with several enteric viruses. Nevertheless, these multiple infections exemplified the threat that enteric viruses pose in terms of public health within communities in North Africa and generally in developing countries. Therefore, further studies with large cohorts of healthy and diarrheal children will be needed to evaluate their clinical role in AGE.

Supporting Information

S1 File. GenBank Accession Numbers.
(DOCX)

S1 Table. Oligonucleotides used for detection and genotyping of classic enteric viruses in this study.
(PDF)

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Author Contributions

Conceptualization: SA GB AdR.
Data curation: SA AdR.
Formal analysis: SA ME GB AdR.
Funding acquisition: AdR.
Investigation: SA ME AdR.
Methodology: SA AdR.
Project administration: AdR.
Resources: PP AdR.
Software: GB AdR.
Supervision: SH MNG MA GB AdR.
Validation: SA GB AdR.
Visualization: GB AdR.
Writing – original draft: AdR.
Writing – review & editing: SA ME SH MNG PP MA GB AdR.

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