Short Communication

Intervirology

Intervirology 2018;61:49–52
DOI: 10.1159/000490568

Received: March 12, 2018
Accepted: June 3, 2018
Published online: July 18, 2018

Prevalence and Clinical Profile of Human *Salivirus* in Children with Acute Gastroenteritis in Northern Italy, 2014–2015

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Keywords
Salivirus · Real-time polymerase chain reaction · Gastroenteritis · RNA extraction · Coinfection

Abstract
Objective: Human *Salivirus* (SalV) has been associated with gastroenteritis on all continents. ***Methods:*** This paper presents the real-time RT-PCR assay for the detection of SalV in clinical fecal samples collected from 192 hospitalized children with acute gastroenteritis in Piedmont, Italy. ***Results:*** The most commonly detected virus was *Norovirus* genogroup II (GII) (33.8%), followed by *Rotavirus* (21.3%), *Sapovirus* (10.9%), *Parechovirus* (8%), *Norovirus* GI (6.7%), and *Adenovirus* (1%). PCR detected SalV in 1 (0.5%) subject. ***Conclusions:*** Our data show that the detection rate of SalV in diarrheal children (0.5%) is lower than that observed in other countries, where it is reported in diarrheal children in 8.6–1.2% of patients.

Introduction

Diarrhea remains a frequent illness throughout the world and causes the death of almost 6 million children annually, especially in developing countries. Diarrheal disease can be caused by a wide spectrum of parasitic, bacterial, and viral pathogens. Acute gastroenteritis (AGE) is mostly caused by viruses, among which *Rotavirus* (RV), *Calicivirus* (*Norovirus* [NoV] and *Sapovirus* [SaV]), *Adenovirus* (AdV), and *Astrovirus* figure prominently. Besides the well-documented enteric viruses, the list of viral pathogens causing AGE is continues to grow due to the emergence of new viruses.

In the past decade, there has been a great shift from conventional methods to identify viral pathogens to the utilization of polymerase chain reaction (PCR) assays, which have proven their advantages over previously used techniques such as rapid antigen detection and viral culture [1]. The major advantages of molecular diagnostic methods are the lower detection limits and therefore higher analytical sensitivity for common viruses such as RV and AdV, and their ability to detect uncultivable viruses such as NoV, SaV, and *Astrovirus* [2–5] or the more

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difficult cultivable viruses such as human *Parechovirus* (HPeV) [6]. *Salivirus* (SalV) was first identified in 2009 [7, 8]. SalV has a single-stranded RNA genome of around 7,633 nt organized in a typical *Picornavirus* genome. Although this virus is related, but distinct, to the genus *Kobuviru*s, SalV forms a genus that presently includes a single genotype with 2 clusters [9]. SalV (previously called *Klassevirus*) is associated with diarrhea and has been detected in feces from both gastroenteritis patients and healthy subjects on all continents, as well as in sewage from Spain and Hong Kong, suggesting a widespread geographic distribution [8].

This paper presents the real-time RT-PCR assay for the detection of SalV in clinical fecal samples collected from hospitalized children with AGE in Piedmont, Northern Italy, from December 2014 to November 2015.

Material and Methods

A total of 192 fecal specimens, previously screened for RV, AdV (by means of immunochromatographic antigenic testing; DIAQUICK Dialab, Eastleigh, UK), NoV, HPeV, and SaV (by real-time PCR as previously described by Bergallo et al. [10, 11]), were tested for SalV. The samples were collected from children under the age of 5 years with AGE in the Regina Margherita Children's Hospital, Turin, Italy, from December 2014 to November 2015. The study was approved by our institutional ethics committee on 24 November 2014 (protocol No. 116918). Informed consent was obtained from all individual participants included in the study.

The study aim was to develop a real-time RT-PCR assay for the highly sensitive and specific detection of SalV/*Klassevirus*. The 5′-UTR was chosen as a target to design a probe that reacted specifically with this virus (it has been widely used for the primer/probe design for *Picornavirus* [12]). The 5′-UTR nucleotide sequences of 13 SalV isolates were obtained from the GenBank database (accession Nos. GU245894, GQ184145, GQ179640, JN379039, and GU376738–GU376746). A forward and reverse primer were previously described [10], and a TaqMan TAMRA probe (SALIP 5′-FAM-TGCGGGAGTGCTCTTCCCCAACTC-TAMRA-3′) was designed from sequences that are highly conserved only in SalV isolates. We used the Primer Express software v3.0 (Applied Biosystems, Foster City, CA, USA). A nucleotide BLAST search of each primer and probe yielded no significant homology to non-SalV sequences. These results confirm the specificity of the designed primers and probe for SalV detection. GAPDH was used as an internal control. The primers for GAPDH have been published [13].

Total RNA (50 μg) was extracted from the fecal specimens and reverse-transcribed as previously reported [12]. The optimization of the PCR reaction was performed by determining the optimal amount of primers and probe, as well as the cycling condition. The optimized SalV PCR reaction contained 5 μL cDNA, 900 nM of each primer, 200 nM of the SalV FAM probe, and 1× PCR Master Mix (Promega, Madison, WI, USA), in a total 20-μL reaction. The PCR amplification was carried out for 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C, and 1 min at 60 °C on the 7500 ABI real-time instrument (Applied Biosystems).

SalV and GAPDH PCR amplification was performed in simplex reactions to evaluate the potential interference between the PCR targets. A negative SalV result was considered valid only when the internal control GAPDH probe showed amplification. Information was extracted from the medical record database on the following: age, gender, RV, and AdV. HPeV, SaV, and NoV were detected as previously reported [12–14].

The analytical specificity of the RT-PCR assay was determined by testing *Coxsackievirus* types B1 (ATCC VR-28), B2 (ATCC VR-29), and B3 (ATCC VR-30); *Enterovirus* types 68 (ATCC VR-561), 69 (ATCC VR-785), 70 (ATCC VR-836), and 71 (ATCC VR-1432); human respiratory syncytial virus (RSV-A [ATCC VR-26]); human parainfluenza viruses (PIV 1 [ATCC VR-94], PIV 2 [ATCC VR-92], and PIV 3 [ATCC VR-93]); influenza viruses (*Influenza virus A* H1N1 [ATCC VR-95], H3N2 [ATCC VR-547], and *Influenza virus B* [ATCC VR-101]), human AdV 3 (ATCC VR-3), 5 (ATCC VR-5), and 7 (ATCC VR-7); *Herpes simplex virus 1* (ATCC VR-260); human *Cytomegalovirus* (ATCC VR-538); Epstein-Barr virus (ATCC VR-602); human *Coronavirus* types 229E (ATCC VR-740) and OC43 (ATCC VR-1558); *Legionella pneumophila* (ATCC 33152); *Bacillus cereus* (ATCC 14579); *Enterococcus faecalis* (ATCC 29212); *Escherichia coli* (ATCC 25922); *Haemophilus influenzae* (ATCC 49766); *Staphylococcus aureus* (ATCC 25923); and *Lactobacillus plantarum* (ATCC 8014).

Results

A total of 192 fecal specimens (from 112 males and 80 females) previously screened for RV, AdV, NoV, HPeV, and SaV were collected from infants and children with AGE at the Regina Margherita Children's Hospital, Turin, Italy, from December 2014 to November 2015, and tested for SalV. The mean age of the tested cases was 21.6 months, with the majority of the children (86%) being 1–36 months of age; 104/192 (54.2%) samples were positive for at least 1 virus. The most commonly detected virus was NoV genogroup II (GII) (33.8%), followed by RV (21.3%), SaV (7.8%), NoV GI (6.7%), HPeV (5.7%), and AdV (1%). The monthly distribution of the viral pathogens is described in Figure 1, and suggests that gastrointestinal virus detection peaked during the winter. However, with the number of tested stools being rather limited, a larger sampling is required to confirm any seasonality pattern. Multiple infections with 2–3 viruses responsible for the gastroenteritis in human stools were found in 34/192 (17.8%) samples (27 with a double infection and 7 with a triple infection) (Table 1). Real-time PCR detected SalV in 1 subject (0.5%). The virus was detected in March in a child of Bangladeshi origin, in coinfection with RV and without fever; the sample tested resulted positive for SalV, but we are unable to distinguish whether it was type A or type B.

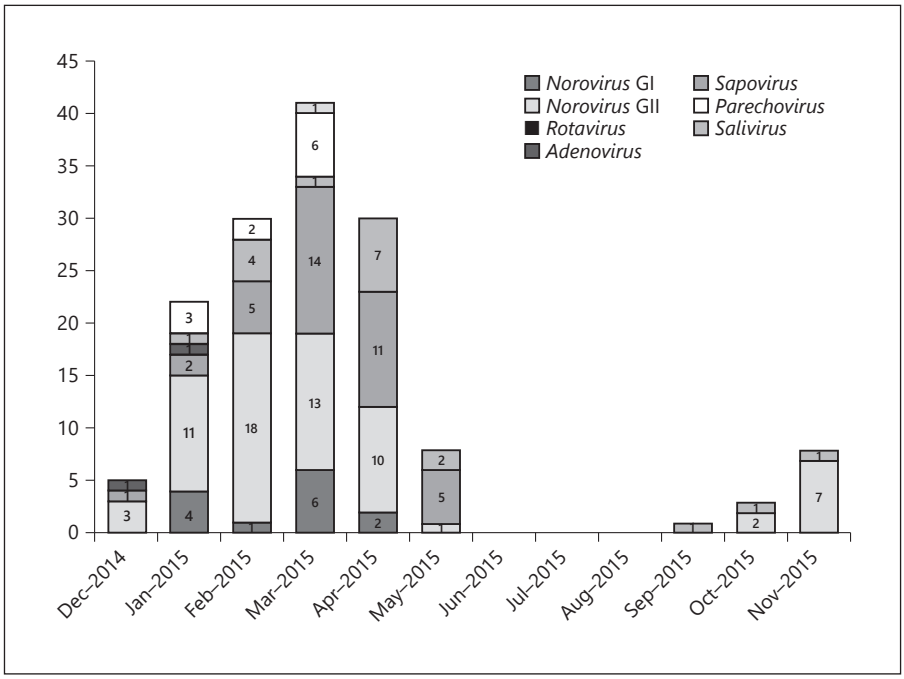

Fig. 1. The monthly distribution of major viral pathogens in pediatric patients with gastroenteritis.

Table 1. Enteric virus coinfection data from 192 patients

Viral pathogen	Patients with infected stools, *n*	Patients with coinfection, *n* (%)
Norovirus GI	13	7 (53.8)
Norovirus GII	65	30 (46.1)
Rotavirus	41	19 (46.3)
Adenovirus	2	1 (50)
Parechovirus	11	10 (90.9)
Sapovirus	15	10 (66.6)

Discussion

Currently, RT-PCR is widely used and considered to be a convenient, useful, and powerful method for molecular diagnosis, by detecting pathogens in clinical specimens. With the development of real-time PCR assays, many of the limitations related to performing PCR amplification in a clinical laboratory have been overcome. In this study, stool specimens (n = 192) obtained from Italian children <5 years of age who presented with acute diarrhea were retrospectively analyzed for SalV using real-time RT-PCR. The stool specimens were collected from 2014 to 2015 in the city of Turin, Italy.

SalV was first described in 2009 in the USA [7] and has been reported worldwide [8, 15–18]. Our results confirmed that enteric viruses play an important role in pediatric diarrhea, and that the most common viral agent associated with gastroenteritis is NoV, followed by RV, SaV, HPeV, and AdV. The prevalence rates of RV, SaV, HPeV and AdV were similar to those reported previously [12, 13, 19], but the detection rate (40.5%) of NoV was higher with the use of RT-PCR [20] or real-time PCR [5, 21]. Our study suggests there is a high prevalence of *Calicivirus* in this area of Italy. Moreover, RV detection peaked during the spring months, in conflict with the results of previous studies [22].

Since its identification, SalV has been detected frequently in stool samples of children with gastroenteritis worldwide, with the highest prevalence rates in Nepal (8.6%), Shanghai/China (4.2%), Korea (4%), Germany (3.8%), Tunisia (3.1%), Brazil (1.7%), and western India (1.2%) [23–27]. Our data show that the detection rate of SalV in diarrheal children (0.5%) was lower than that observed in other countries, where they have been reported in 8.6–1.2% of diarrheal children [17, 23–27]. The only positive sample was from a child of Bangladeshi origin, confirming that SalV is rare in European countries, and that, although widespread, this virus seems to circulate more in Asia and North Africa.

Although this new virus is suspected to be responsible for AGE in children, our data show that this association is not certain, since all of the infected children pre-

sented with infection by several enteric viruses. Therefore, further studies with large cohorts of healthy and diarrheal children will be needed to evaluate the clinical role of viruses in AGE. Interestingly, in a recent case-control study in China on 461 paired stool samples from children with diarrhea and healthy controls, a lack of association between SalV and gastroenteritis was also found [22]. A limitation of our study is the small number of stool samples from AGE cases and the lack of samples from healthy controls. Given the low prevalence of SaV in our population, the SalV result will require confirmation using a much larger sample size from healthy individuals.

Statement of Ethics

All procedures performed in studies involving human participants were in accordance with the ethical standards of University of Turin research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Disclosure Statement

The authors declare that they have no conflict of interest.

Funding Source

This study was funded by University of Turin (BERM_RIC_LOC_15_06).

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