
DETECTION OF SALIVIRUS IN RAW SEWAGE SAMPLES IN RIO DE JANEIRO, BRAZIL

*Marcelle Silva-Sales^{1,2}, Mariana Seglia Caldas¹, Julia Monassa Fioretti¹,
Monica Simões Rocha¹, Tulio Machado Fumian¹ and Marize Pereira
Miagostovich¹*

ABSTRACT

Gastroenteric viruses are important pathogens related to cases of acute gastroenteritis, affecting millions of people worldwide with a major impact on children under five in developing countries. The introduction of metagenomic approach techniques in the 2000s has allowed the description of new viruses, among them Salivirus, which has been associated worldwide with cases of diarrhea. This study aimed to detect salivirus in raw sewage samples from a wastewater treatment plant (WWTP) collected between June 2013 and May 2014 in Rio de Janeiro, Brazil. Fifty-two samples collected weekly were tested by using a real-time quantitative PCR (qPCR). Salivirus genome was detected in 71.1% (37/52) of the samples, with viral concentration ranging from 7.56×10^4 to 7.20×10^6 genomic copies per liter. Higher viral loads were detected in the summer and fall of 2014, although these data were not sufficient to infer seasonality for this virus. The high prevalence of salivirus in sewage samples highlights the importance of viral research in wastewater to generate data on salivirus circulation, increasing understanding regarding its dissemination in the population.

KEY WORDS: Wastewater; viral detection; salivirus; Brazil; raw sewage; qPCR.

INTRODUCTION

Acute gastroenteritis is the cause of millions of infantile deaths each year, and is a public health issue in both high and low-income countries (Tate et al., 2016). Although, the most common viral pathogens associated with gastroenteritis are rotavirus A and norovirus, responsible for most acute gastroenteritis outbreaks as well as sporadic cases worldwide, the number of new viruses associated to gastrointestinal illnesses have been increasing (Li et al., 2009; Santos et al., 2015; Reuter et al., 2017). The introduction of metagenomic approach techniques in the 2000s has allowed the description

1. Laboratory of Comparative and Environmental Virology, Oswaldo Cruz Institute, Fiocruz, Ministry of Health, Brazil.

2. Laboratory of Virology and Cell Culture, Tropical Pathology and Public Health Institute, Federal University of Goiás, Goiânia, Brazil

Corresponding author: Marcelle Silva-Sales. E-mail: marcelle.figueira@ufg.br

Received for publication: 31/3/2020. Reviewed: 6/5/2020. Accepted: 13/5/2020.

of new viruses, among them Salivirus. Since 2009 many studies have reported the prevalence of Salivirus (at that time named Klassevirus) among different age groups, particularly children under the age of five, as well as in polluted environmental samples, including river water, and sewage (Greninger et al., 2009; Holtz et al., 2009; Han et al., 2010; Calgua et al., 2013; Prevost et al., 2015; Santos et al., 2015; Itta et al., 2016; Pei et al., 2016; Badru et al., 2018; Adineh et al., 2019; Mancini et al., 2020).

Salivirus belongs to the *Picornaviridae* family and are non-enveloped, positive-sense RNA viruses with an 8kb genome and icosahedral symmetry encoding only one polyprotein that is subsequently cleaved into structural and non-structural proteins (Greninger et al., 2009; Holtz et al., 2009). Although well characterized, the role of salivirus as a pathogen associated to gastroenteritis still requires elucidation (Bergallo et al., 2018).

This study aimed to detect and quantify salivirus in raw sewage samples over a one-year period, using an environmental approach to demonstrate the circulation of this virus in the metropolitan region of Rio de Janeiro.

MATERIAL AND METHODS

Wastewater samples and virus concentration method

Samples were obtained from the second largest wastewater treatment (WWTP) plant in Latin America which receives 5,000 liters of sewage from 46 districts of Rio de Janeiro, with approximately 1,500,000 inhabitants (24.6% of the population). One raw sewage sample was collected weekly from June 2013 to May 2014 in 500 mL sterile plastic bottles, totaling 52 sewage samples. All samples were spiked with a known concentration of PP7 bacteriophage used as an internal control process. The samples were concentrated using the organic flocculation method (Calgua et al., 2013) and stored at -80°C.

Extraction of the viral genome and cDNA synthesis

Viral genome was extracted from 140 µL of concentrated samples using the QIAmp Viral RNA Mini Kit (QIAGEN®, Valencia, CA, USA) and cDNA synthesis performed with pd(N)₆® random primer (Amersham Biosciences, UK) and SuperScript® III as Reverse Transcriptase (Invitrogen®, Carlsbad, CA, USA), both following the manufacturer's protocols.

Detection and viral quantification

Real-time quantitative PCR (qPCR) using the TaqMan® system was applied for PP7 (Rajal et al., 2007) and salivirus analysis (Haramoto et al., 2013). Salivirus genome concentration was calculated using a standard curve based on 10-fold serial dilutions of synthetic gBlock® Gene Fragments (IDT®) containing salivirus polymerase/capsid junction region, generating a fragment of 202bp. Ten-fold dilutions were used to prepare samples with concentrations ranging from 2×10^0 to 2×10^6 copies per reaction. Samples presenting fluorescence signals that crossed the threshold line in both replicates, with Ct values ≤ 40 and a sigmoidal curve, were considered positive. All qPCR assays included non-template controls, and for all molecular techniques positive and negative (DNase/RNase-free water) controls were used to avoid contamination.

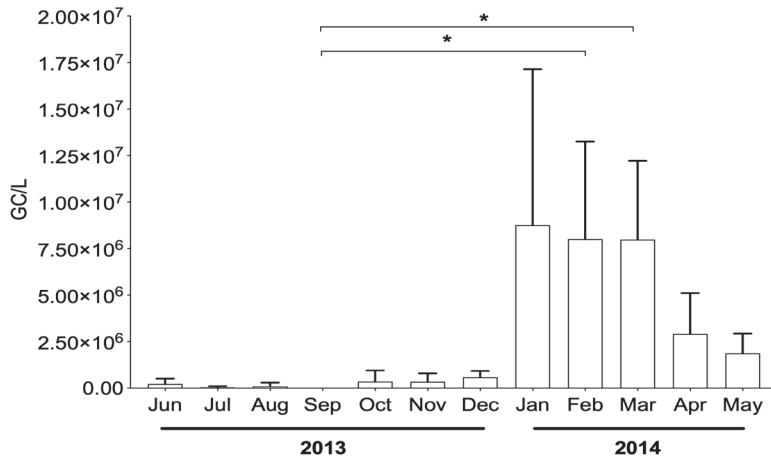
Statistical analysis

Normal distribution and homogeneous variance were tested for all variables. Assuming non-Gaussian distribution data, the following nonparametric tests were used: Kruskal-Wallis and Dunn's post-hoc test. The results were expressed as mean \pm standard deviation (SD). The observed differences were considered significant when $p < 0.05$. Statistical analysis was performed using GraphPad Prism, version 7.0 (La Jolla, CA, USA).

RESULTS AND DISCUSSION

The positivity detected for salivirus was 71.1% (37/52) in the raw sewage samples, with viral load ranging from 7.56×10^4 to 7.20×10^6 genomic copies per liter (GC/L) (Figure). Bacteriophage PP7, used as an internal control for the viral concentration method, was detected in 100% of wastewater samples. Although previous studies conducted in Brazil have shown the circulation of salivirus in environmental samples with detections of 33% (2/6) and 4.6% (2/43) in different areas (Calgua et al., 2013; Fumian et al., 2018), none of them investigated wastewater samples which might explain the higher detection noted in this study. Kitajima et al. (2014) reported 15% positivity for salivirus (7/48) in wastewater samples collected in Arizona, USA and Badru et al. (2018) detected salivirus in 32.6% (31/95) of water samples collected from wastewater, irrigation canals, river, and reservoirs in Thailand from November 2016 to February 2018. Currently, it is not possible to determine if this variability represents true variations in the prevalence of salivirus within those countries or if it is due to the methods used, sampling size, sample volumes, and molecular detection methods.

Figure. Detection of Salivirus in environmental samples from the city of Rio de Janeiro between June 2013 and May 2014.



GC/L: genomic copies/liter (*p < 0.05)

Viral genomic quantification results showed an average value of 7.56×10^4 GC/L during the winter of 2013, with some negative sampling or below detection level during that same year, and 4.05×10^6 to 7.20×10^6 GC/L between the summer and fall of 2014. Similar results were obtained by Kitajima et al. (2014) in USA, with the highest concentration of 2.28×10^5 GC/L in raw sewage samples.

Concerning seasonal distribution, the results revealed high viral concentrations throughout the year, with higher values detected in January (summer) and May (fall) 2014, suggesting no well-established seasonality for this virus in Rio de Janeiro, in accordance with the results obtained in Japan (Haramoto et al., 2013), France (Prevost et al., 2015), Thailand (Badru et al., 2018) and Italy (Mancini et al., 2020).

The high viral positivity rate (71.1%) and average seasonal salivirus concentrations during 2013 (2.12×10^5 /Fall, 7.56×10^4 /Winter, 3.71×10^5 /Spring) and 2014 (7.20×10^6 /Summer, and 4.05×10^6 /Fall) found in wastewater samples showed that this virus circulates in the population and is widespread in the environment in high viral concentrations in the city. To the best of our knowledge, this is the first time that such a high salivirus load was detected in environmental samples. Furthermore, significant differences ($p < 0.05$) were noted in the viral load in samples collected in September 2013 when compared to those obtained in February and March 2014 (Figure 1). Although not significant, it is worth noting the increase in the viral load early in 2014, which could suggest an intensification in the circulation of this virus in Rio de Janeiro during this period due to the large number of tourists for the Soccer World Cup.

Despite high viral positivity in the environment, detection of salivirus in clinical samples is low with an incidence of about 1% in fecal samples of diarrheic children in Rio de Janeiro (Santos et al., 2015). In an investigation conducted by our laboratory using the same qPCR as in this study, it was not possible to detect salivirus in 40 fecal samples from children with gastroenteritis. All samples were collected from children residing in the WWTP area whose samples had previously tested negative for rotavirus A and norovirus (data not shown). The low prevalence of salivirus related to acute gastroenteritis in clinical samples in Rio de Janeiro may result from some aspects such as the lack of a detection protocol for this virus in laboratorial routine, or even due to its association with asymptomatic infections (Aldabbagh et al., 2015; Ayouni et al., 2016).

The main limitations of this study were the small number of samples, along with underreported cases of gastroenteritis associated with salivirus. Yet it is relevant regarding seasonality and the increase in viral load early in 2014.

Finally, the authors understand the need to carry out more clinical and environmental studies to generate data that clarify the impact of salivirus as an environmental contaminant and its association to cases of gastroenteritis.

ACKNOWLEDGMENTS

The authors would like to thank Marcia Araújo Pimenta and Fernando C. Ferreira for their technical support and Helioswilton Sales de Campos for the statistical assistance. The project received the support of the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). This research study is part of the activities of FIOCRUZ as a Collaborating Center for Pan American Health Organization (PAHO) and World Health Organization (WHO) of Public and Environmental Health.

CONFLICT OF INTEREST.

The authors declare that there are no conflict of interest.

REFERENCES

1. Adineh M, Ghaderi M, Mousavi-Nasab SD. Occurrence of Salivirus in Sewage and River Water Samples in Karaj, Iran. *Food Environ Virol* 11: 193-197, 2019.
2. Aldabbagh S, Eckerle I, Müller A, Delwart EL, Eis-Hübinger AM. Salivirus type 1 and type 2 in patients with acute gastroenteritis, Germany. *J Clin Virol* 72: 16-19, 2015.
3. Ayouni S, Estienney M, Hammami S, Neji Guediche M, Pothier P, Aouni M, Belliot G, de Rougemont A. Cosavirus, Salivirus and Bufavirus in Diarrheal Tunisian Infants. *PLoS One* 11: e0162255, 2016.

4. Badru S, Khamrin P, Kumthip K, Yodmeeclin A, Surajinda S, Supadej K, Sirilert S, Malasao R, Okitsu S, Ushijima H, Maneekarn N. Molecular detection and genetic characterization of salivirus in environmental water in Thailand. *Infect Genet Evol* 65: 352-356, 2018.
5. Bergallo M, Daprà V, Rattu M, Bonamin S, Cuccu R, Calvi C, Montanari P, Galliano I. Prevalence and Clinical Profile of Human Salivirus in Children with Acute Gastroenteritis in Northern Italy, 2014-2015. *Intervirolog* 61: 49-52, 2018.
6. Calgua B, Fumian T, Rusiñol M, Rodriguez-Manzano J, Mbayed VA, Bofill-Mas S, Miagostovich M, Girones R. Detection and quantification of classic and emerging viruses by skimmed-milk flocculation and PCR in river water from two geographical areas. *Water Res* 47: 2797-2810, 2013.
7. Fumian TM, Victoria M, Vieira CB, Fioretti JM, Rocha MS, Prado T, Guimarães FR, da Gama NP, de Oliveira JM, Mendes ACO, Gaspar AMC, Santos JDO, Chame M, Leite JPG, Miagostovich MP. Enteric viruses' dissemination in a private reserve of natural heritage. *Let Appl Microbiol* 66: 313-320, 2018.
8. Greninger AL, Runckel C, Chiu CY, Haggerty T, Parsonnet J, Ganem D, DeRisi JL. The complete genome of Klassevirus a novel Picornavirus in pediatric stool. *Virology* 6: 1-9, 2009.
9. Han TH, Kim CH, Chung JY, Park SH, Hwang ES. Klassevirus infection in children, South Korea. *Emerg Infect Dis* 16: 1-3, 2010.
10. Haramoto E, Kitajima M, Otagiri M. Development of a reverse transcription-quantitative PCR assay for detection of Salivirus/Klassevirus. *Appl Environ Microbiol* 79: 3529-3532, 2013.
11. Holtz LR, Finkbeiner SR, Zhao G, Kirkwood CD, Girones R, Pipas JM, Wang D. Klassevirus 1, a previously undescribed member of the family Picornaviridae, is globally widespread. *Virology* 6: 1-7, 2009.
12. Itta KC, Patil T, Kalal S, Ghargi KV, Roy S. Salivirus in children with diarrhoea, western India. *Int J Infect Dis* 52: 14-15, 2016.
13. Kitajima M, Iker BC, Rachmadi AT, Haramoto E, Gerba CP. Quantification and genetic analysis of Salivirus/Klassevirus in wastewater in Arizona, USA. *Food Environ Virol* 6: 213-216, 2014.
14. Li L, Victoria J, Kapoor A, Blinkova O, Wang C, Babrzadeh F, Mason CJ, Pandey P, Triki H, Bahri O, Oderinde BS, Baba MM, Bukbuk DN, Besser JM, Bartkus JM, Delwart EL. A novel Picornavirus associated with gastroenteritis. *J Virol* 83: 12002-12006, 2009.
15. Mancini P, Bonanno Ferraro G, Suffredini E, Veneri C, Iaconelli M, Vicenza T, La Rosa G. Molecular Detection of Human Salivirus in Italy Through Monitoring of Urban Sewages. *Food Environ Virol* 12: 68-74, 2020.
16. Pei N, Zhang J, Ma J, Li L, Li M, Li J, Sun Y, Ji J, Jiang H, Hou J, Xu F, Lu H, Zhang R, Wei X, Xu X, Deng J. First report of human Salivirus/Klassevirus in respiratory specimens of a child with fatal adenovirus infection. *Virus Genes* 52: 620-624, 2016.
17. Prevost B, Lucas FS, Goncalves A, Richard F, Moulin L, Wurtzer S. Large scale survey of enteric viruses in river and waste water underlines the health status of the local population. *Environ Int* 79: 42-50, 2015.
18. Rajal VB, McSwain BS, Thompson DE, Leutenegger CM, Kildare BJ, Wuertz S. Validation of hollow fiber ultrafiltration and real-time PCR using bacteriophage PP7 as surrogate for the quantification of viruses from water samples. *Water Res* 41: 1411-1422, 2007.
19. Reuter G, Pankovics P, Boros A. Saliviruses: the first knowledge about a newly discovered human Picornavirus. *Reviews Medical Virol* 27: 1-10, 2017.
20. Santos N, Mendes GS, Silva RC, Pena GA, Rojas M, Amorim AR, Lima DP. Salivirus and Aichivirus A infections in children with gastroenteritis in Brazil. *Clin Microbiol Infect* 21: 799.e1-799.e3, 2015.
21. Tate JE, Burton AH, Boschi-Pinto C, Parashar UD. Global, regional, and national estimates of rotavirus mortality in children <5 years of age, 2000-2013. *Clinical Infect Dis* 62: S96-S105, 2016.