INTRODUCTION

Organic lettuce (Lactuca sativa) has been acknowledged as an important transmission source of enteric viruses, since it is traditionally eaten raw or receives minimal processing (Gerba & Choi, 2006). Due to their resistance to adverse conditions, the low infectious dose and the large number of infectious particles, these viruses have been described as important environmental contaminants (De Giglio et al., 2017; Grassi et al., 2010). Among those viruses are the human enteroviruses (EVs), which are associated with asymptomatic infections or mild diseases, such as the common cold or minor undifferentiated febrile illnesses. Yet under certain conditions, EVs also cause serious human diseases such as poliomyelitis, meningitis, encephalitis, myocarditis, and hand, foot, and mouth disease (European Centre for Disease Prevention and Control (ECDC), 2016; Faustini et al., 2006; Khetsuriani, Lamonte-Fowikes, Oberst, & Pallansch, 2006; Starlin et al., 2001; Zhu et al., 2007).

Originally EV were classified into four groups: poliovirus (PV), coxsackie A virus (CA), coxsackie B virus (CB), and human orphan cytopathic enteric virus (echovirus), but it was noted that there were significant overlaps in the biological properties of those viruses in different groups. Currently, the Enterovirus genus, classified within the Picornaviridae family, consists of 13 species including Enterovirus A-J and Rhinovirus A-C, in which the species of Enterovirus A, B, C, D and Rhinovirus A, B, C, infect humans (http://www.picornaviridae.com/enterovirus/enterovirus.htm; International Committee on Taxonomy of Viruses (ICTV), 2017).

EV was associated with the first transmission of food-borne viruses reported in 1914, when polio outbreaks were associated with milk consumption (Jubb, 1915). More recently, there are no reports
of food-borne outbreaks associated with EV (Todd and Greig 2015). However, studies have detected EV in several matrices including sewage, irrigation water, and shellfish, some of them from environmental surveillance studies of polioviruses and nonpolio enterovirus (Cheong et al., 2009; Connell et al. 2012; De Oliveira Pereira et al., 2016; Espinosa, Arias, Sánchez-Colón, & Mazari- Hiriart, 2009; Ndiaye, Diop, & Diop, 2014). Elsewhere, some studies have shown that EV can be transferred onto the surface of vegetables through spray irrigation water resulting in viral contamination of the vegetables (Allende & Monaghan, 2015; Cheong et al., 2009; Pachepsky, Shelton, McLain, Patel, & Mandrell, 2011; Uyttendaele et al., 2015).

This study aims to investigate the environmental dissemination of human Enterovirus species in the production chain of organic lettuce in a small farm located in the region of Brejal, municipality of Petropolis, in the state of Rio de Janeiro, Brazil.

# MATERIALS AND METHODS

## Study site, sampling collection, and viral concentration methods

Water and lettuce samples were obtained at different lettuce production stages between August 2010 and August 2011 in an organic production site located in Brejal region, municipality of Petropolis, in the mountains of Rio de Janeiro. Ninety-six samples including seedling irrigation water (W1), catchment spring water (W2), dam water (W3), lettuce irrigation water (W4), lettuce wash water (W5), and three lettuce samples as seedlings (L1), lettuces grown (L2), and washed lettuce (L3), previously concentrated and investigated for rotavirus, norovirus, and human adenovirus (Werneck et al., 2017) were processed for EV investigation.

Water samples (2 L) were processed for viral concentration using an adsorption-elution method with a negatively charged membrane as described previously by Katayama, Shimasaki, and Ohgaki (2002). For lettuce samples, 25 grams (g) was concentrated using the same method adapted for small volumes (approximately 120 ml) using a 0.45 μm negative charge membrane with a Stericup® filter (250 ml) (Nihon, Millipore, USA) and ultrafiltration was performed with Centriprep Concentrator® 50 Nihon, Millipore to give a final volume of 2 ml (Fumian, Leite, Marin, & Miagostovich, 2009; Katayama et al., 2002).

RNA extraction and One-step reverse transcriptase quantitative polymerase chain reaction (RT-qPCR).

Nucleic acids were extracted using QIAamp Viral RNA Mini Kits (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer’s instructions.

RT-qPCR for EV was developed and standardized by the National Enterovirus Reference Laboratory. With an aim to improve the detection and quantification of EV by qPCR, primers and probes corresponding to the 5′NC region of human EV were designed and evaluated for species A to D detection (Da Silva, E.E. - Unpublished data). For a qPCR standard curve the RNA from poliovirus Sabin 3 (NIBSC code: 01/532) obtained from human RD cell cultures was extracted and diluted from $10^{-1}$ to $10^{-9}$. To determine the limit of detection standard RNA was serially diluted in a pool of negative water concentrates. The concentrates were extracted as described above and tested in duplicate.

RT-qPCR was performed using AgPath-ID™ One-step RT-PCR Kit (Applied Biosystems, California, USA). Nine microliter of RNA extracted was added to the reaction mix according to manufacturer’s instructions. Assays were placed into an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) on the following conditions: incubation at 50°C for 2 min to activate UNG, initial denaturation at 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 56–60°C for 1 min. Amplifications were analyzed in duplicated, and positive and negative controls were included in each run. Virus concentration results are present as gc L$^{-1}$ and gc 25 g$^{-1}$ for water and lettuce samples, respectively.

## Virus isolation

According World Health Organization (2004), all positive samples were inoculated into three different cell lines sensitive to the isolation of the genus Enterovirus: human RD, MRC-5, and HEp 2c. Cells were supplied by the Center for Disease Control and Prevention (CDC; Atlanta, USA) to the Fiocruz/RJ Enterovirus Laboratory. Briefly, 200 μl of concentrate was inoculated in duplicate into 12 ml cell culture tubes (Nalgene™ Thermo Fischer Scientific, USA) containing 1.0 x 10^5 cells/ml. Culture cells were kept at 36°C with daily observation using an invert microscope for the appearance of cytopathic effect. Three serial passages were conducted with a 7-day interval. Control cultures cells were used to report toxicity, degeneration, or contamination. After 21 days, the qPCR was performed with paired samples (initial concentrates and inoculated cultures). The same procedure was accomplished for the three cell types used.

# RESULTS AND DISCUSSION

This study investigated the presence of human Enterovirus species using one-step reverse transcriptase quantitative TaqMan® PCR (RT-qPCR) from water and lettuce samples obtained from a chain of organic lettuce. The limit of detection in 100 μl of water of RT-qPCR assays that were used in this study was found to be 168 gc for EV following the FSA 2006 guidelines (Armbruster & Pry, 2008; FSA 2006). The RT-qPCR assay detected EV in 12.5% (12/96) of total samples, 13% (8/60) from water, and 11% (4/36) from lettuce samples. EV load ranged from 3.37 x 10^3 to 4.72 x 10^6 genomic copies per liter (gc L$^{-1}$) and from 2.14 x 10^4 to 5.56 x 10^4 genome copies per 25 grams (gc 25 g$^{-1}$) for water and lettuce samples, respectively (Table 1). Previously, a study carried out with these same samples revealed high contamination with the main gastroenteric viruses (i.e., rotavirus, norovirus and, adenovirus) with detection of at least one of them in all the samples studied and with the rotavirus concentration reaching 10^4 gc 25 g$^{-1}$ in samples of lettuce ready for consumption (Werneck et al., 2017).
results shows that the EV were obtained in a higher concentration in the seedling’s irrigation waters (10^6 gc L⁻¹). However, in the lettuce samples, mean values of EV were detected in concentrations similar to RVA data, and higher than HAdV (10^2 gc L⁻¹), which was the best indicator since they were present in at least one sample from each point of the production chain. The detection of viral concentration ranging from 10^2 to 10^4 gc 25 g⁻¹ in adult lettuce suggests that sometimes the level of viral contamination increased at the end of production. However, it is not possible to state whether there is a growing process of contamination for all viruses, mainly due to the heterogeneous distribution of them commonly observed in environmental samples. It was also observed that lettuces washed at the last stage of production do not represent a benefit for virus removal, since the waters used are also contaminated. Enteric viruses including EV have been used as indicators of sanitation and hygiene practices at food production sites on dairy and swine farms (Fongaro et al., 2014; Kokkinos et al., 2012; Lachapelle, Letellier, Fravalo, Brassard, & L’Homme, 2017; Maunula et al., 2013; Staggemeier et al., 2015; Yavarmanesh, Alum, & Abbaszadegan, 2015).

In order to demonstrate EV infectivity, all 12 EV-positive samples by RT-qPCR were processed to attempt virus isolation following the World Health Organisation (WHO) protocol described previously (World Health Organization, 2004). No EV characteristic cytopathic effects were observed after three consecutive passages of 7 days each in cell cultures. After this period, RT-qPCR was carried out for all different culture cell lines in paired samples (initial concentrates and cultures) with no reduction in threshold cycle (Ct) values, revealing no virus replication. Human rhabdomyosarcoma (RD), human diploid cells derived from lung (MRC-5) and human epithelial carcinoma cells (HEp 2c) are susceptible to EV infection and were used to improve successful isolation as recommended by WHO. This protocol is routinely performed by EV laboratories that usually inoculate specimens into a minimum of three cell lines (World Health Organization, 2004, World Health Organization (WHO), 2015). Although it has not been possible to demonstrate the infectivity of the EVs detected, the presence of the viral genome is sufficient to demonstrate the potential risk of infection from the consumption of those raw products. It was also not possible to demonstrate HAdV infectivity from those same samples, corroborating the idea previously mentioned that a long interval for performing assays may have influenced these results (Werneck et al., 2017).

In conclusion, our results point out the need of considering and monitoring enteric viruses, as environmental contaminants, mainly in food producing areas that do not meet the requirements of adequate sanitation. Worldwide, food security has been a growing concern for authorities that are stepping up efforts in an attempt to minimize harm to health (World Health Organization, 2002; Centers for Disease Control and Prevention (CDC), 2016). In this context, viruses emerge as a challenge since their contamination in the environment is difficult to eliminate, reinforcing the importance of basic sanitation in food security (Cook, Knight, & Richards, 2016).

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CONFLICT OF INTEREST
No conflict of interest declared.

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REFERENCES

Allende, A., & Monaghan, J. (2015). Irrigation water quality for leafy crops: A perspective of risks and potential solutions. *International Journal of Environmental Research and Public Health, 12*(7), 7457–7477. https://doi.org/10.3390/ijerph120707457

Armbruster, D. A., & Pry, T. (2008). Limit of blank, limit of detection and limit of quantitation. *The Clinical Biochemist Reviews, 29*(Suppl 1), S49.

Centers for Disease Control and Prevention (CDC) (2016). Surveillance for foodborne disease outbreaks, United States, 2014. *Annual report*. Atlanta, Georgia: US Department of Health and Human Services, CDC.

Cheong, S., Lee, C., Song, S. W., Choi, W. C., Lee, C. H., & Kim, S. J. (2009). Enteric viruses in raw vegetables and groundwater used for irrigation in South Korea. *Applied and Environment Microbiology, 75*(24), 7745–7751. https://doi.org/10.1128/AEM.01629-09

Cook, N., Knight, A., & Richards, G. P. (2016). Persistence and elimination of human norovirus in food and on food contact surfaces: A critical review. *Journal of Food Protection, 79*(7), 1273–1294. https://doi.org/10.4315/0362-028X.JFP-15-570

Connell, C., Tong, H. I., Wang, Z., Allmann, E., & Lu, Y. (2012). New approaches for enhanced detection of enteroviruses from Hawaiian environmental waters. *PloS one, 7*(5), e32442.

De Giglio, O., Caggiano, G., Bagordo, F., Barbuti, G., Brigida, S., Lugoli, F., ... Montagna, M. T. (2017). Enteric viruses and fecal bacteria indicators to assess groundwater quality and suitability for irrigation. *International Journal of Environmental Research and Public Health, 14*(6), 558. https://doi.org/10.3390/ijerph14060558

De Oliveira Pereira, J. S., da Silva, L. R., de Meireles Nunes, A., de Souza Oliveira, S., da Costa, E. V., & da Silva, E. A. (2016). Environmental surveillance of polioviruses in Rio de Janeiro, Brazil, in support to the activities of global polio eradication initiative. *Food and Environmental Virology, 8*(1), 27–33. https://doi.org/10.1007/s12560-015-0221-5

Espinosa, A. C., Arias, C. F., Sánchez-Colón, S., & Mazari-Hiriart, M. (2009). Comparative study of enteric viruses, coliphages and indicator bacteria for evaluating water quality in a tropical high-altitude system. *Environmental Health, 8*(1), 49. https://doi.org/10.1186/1476-069X-8-49

European Centre for Disease Prevention and Control (ECDC) (2016). Rapid Risk Assessment - Enterovirus detections associated with severe neurological symptoms in children and adults in European countries. Stockholm: ECDC.

Faustini, A., Fano, V., Muscillo, M., Zaniratti, S., La Rosa, G., Tribuzi, L., & Perucci, C. A. (2006). An outbreak of aseptic meningitis due to echovirus 30 associated with attending school and swimming in pools. *International Journal of Infectious Diseases, 10*(4), 291–297. https://doi.org/10.1016/j.ijid.2005.06.008

Fongaro, G., Viancelli, A., Magri, M. E., Elmahdy, E. M., Biesus, L. L., Kich, J. D., ... Barardi, C. R. (2014). Utility of specific biomarkers to assess safety of swine manure for biofertilizing purposes. *Science of the Total Environment, 479*, 277–283. https://doi.org/10.1016/j.scitotenv.2014.02.004

Food Standards Agency. (2006). Review of the analytical terminology for codex use in the procedural manual. *FOOD STAND AGENCY Inf Bull METHODS Anal Sampli Foodst, 68*, 1–15.

Fumian, T. M., Leite, J. P. G., Marin, V. A., & Miagostovich, M. P. (2009). A rapid procedure for detecting noroviruses from cheese and fresh lettuce. *Journal of Virological Methods, 155*(1), 39–43. https://doi.org/10.1016/j.jviromet.2008.09.026

Gerba, C. P., & Choi, C. Y. (2006). Role of irrigation water in crop contamination by viruses. In Sagar M. Goyal (Ed.), *Viruses in foods* (pp. 257–263). New York, NY: Springer. https://doi.org/10.1007/0-387-29251-9

Grassi, T., Bagordo, F., Idolo, A., Lugoli, F., Gabutti, G., & De Donno, A. (2010). Rotavirus detection in environmental water samples by tangential flow ultrafiltration and RT-nested PCR. *Environmental Monitoring and Assessment, 164*(1), 199–205. https://doi.org/10.1007/s10661-009-0885-x

International Committee on Taxonomy of Viruses (ICTV) (2017). Virus taxonomy. *Picornaviridae. Genus Enterovirus.* [online] Available at: https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/picornaviridae/w/picornaviridae/681/gener-enterovirus [Accessed 25 Sept 2017].

Jubb, G. (1915). A third outbreak of epidemic poliomyelitis at West Kirby. *The Lancet, 185*(4767), 67. https://doi.org/10.1016/S0140-6736(01)6366-1

Katayama, H., Shimasaki, A., & Ohgaki, S. (2002). Development of a virus concentration method and its application to detection of enterovirus and Norwalk virus from coastal seawater. *Applied and Environment Microbiology, 68*(3), 1033–1039. https://doi.org/10.1128/AEM.68.3.1033-1039.2002

Khetsuriani, N., Lamonte-Fowlkes, A., Oberst, S., & Pallansch, M. A.; Centers for Disease Control and Prevention (2006). Enterovirus surveillance—United States, 1970–2005. *MMWR Surveillance Summary, 55*, 1–20.

Kokkinos, P., Kozyr, I., Lazic, S., Bouwknecht, M., Rutjes, S., Willems, K., ... Vantarakis, A. (2012). Harmonised investigation of the occurrence of human enteric viruses in the leafy green vegetable supply chain in three European countries. *Food and Environmental Virology, 4*(4), 179–191. https://doi.org/10.1007/s12560-012-9087-8

Lachapelle, V., Letellier, A., Fraval, P., Brassard, J., & L’Homme, Y. (2017). Dynamics of virus distribution in a defined swine production network using enteric viruses as molecular markers. *Applied and Environment Microbiology, 83*(4), e03187-16.

Maunula, L., Kaupke, A., Vasikova, P., Söderberg, K., Kozyr, I., Lazic, S., ... Cook, N. (2013). Tracing enteric viruses in the European berry fruit supply chain. *International Journal of Food Microbiology, 167*(2), 177–185. https://doi.org/10.1016/j.ijfoodmicro.2013.09.003

Ndíaye, A. K., Diop, P. A. M., & Diop, O. M. (2014). Environmental surveillance of poliovirus and non-polio enterovirus in urban sewage in Dakar, Senegal (2007–2013). *The Pan African Medical Journal, 19*, 243.

Pacheypsh, Y., Shelton, D. R., McLean, J. E., Patel, J., & Mandrell, R. E. (2011). Irrigation waters as a source of pathogenic microorganisms in produce: A review. *Advances in Agronomy, 113*(7), 73–138.

Staggemeier, R., Bortoluzzi, M., da Silva Heck, T. M., da Luz, R. B., Fabres, R. B., Soliman, M. C., ... de Matos Almeida, S. E. (2015). Animal and human enteric viruses in water and sediment samples from dairy farms. *Agricultural Water Management, 152*, 135–141. https://doi.org/10.1016/j.agwat.2015.01.010

Starlin, R., Reed, N., Leeman, B., Black, J., Trulock, E., & Mundy, L. M. (2001). Acute flaccid paralysis syndrome associated with echovirus 19, managed with pleconaril and intravenous immunoglobulin. *Clinical Infectious Diseases, 33*(5), 730–732. https://doi.org/10.1086/322624

Todd, E. C., & Greig, J. D. (2015). Viruses of foodborne origin: a review. *Virus Adaptation and Treatment, 7*, 25–45.

Uyttendaele, M., Jaykus, L. A., Amoah, P., Chiodini, A., Cunliffe, D., Jaccsens, L., ... Medema, G. (2015). Microbial hazards in irrigation water: Standards, norms, and testing to manage use of water in fresh produce primary production. *Comprehensive Reviews*
in Food Science and Food Safety, 14(4), 336–356. https://doi.org/10.1111/1541-4337.12133
Werneck, L., Vieira, C. B., Fumian, T. M., Caetano, T. B., Emilio dos Santos, J., Ferreira, F. C., ... Miagostovich, M. P. (2017). Dissemination of gastroenteric viruses in the production of lettuce in developing countries: A public health concern. FEMS Microbiology Letters, 364(9), 1–7.
World Health Organization. (2002). The world health report 2002: Reducing risks, promoting healthy life. Geneva, Switzerland: World Health Organization.
World Health Organization. (2004). Polio laboratory manual, 4th ed.. Geneva: WHO.
World Health Organization (WHO). (2015). Enterovirus surveillance guidelines. Guidelines for enterovirus surveillance in support of the Polio Eradication Initiative. Geneva: WHO.
Yavarmanesh, M., Alum, A., & Abbaszadegan, M. (2015). Occurrence of Noroviruses and Their Correlation with Microbial Indicators in Raw Milk. Food and Environmental Virology, 7(3), 232–238. https://doi.org/10.1007/s12560-015-9185-5
Zhu, Z., Xu, W., Xu, A., Wang, H. Y., Zhang, Y., Song, L. Z., ... Ji, F. E. N. G. (2007). Molecular epidemiological analysis of echovirus 19 isolated from an outbreak associated with hand, foot, and mouth disease (HFMD) in Shandong Province of China. Biomedical and Environmental Sciences, 20(4), 321–328.

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