Copyright © 2019 University of Bucharest
Printed in Romania. All rights reserved
ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(5): 820-825
doi: 10.25083/rbl/24.5/820.825

Received for publication, November, 2, 2018
Accepted, March, 2, 2019

Original paper

Monitoring Enterovirus and Norovirus circulation in sewage water using isolation on cell culture lines and GeneXpert system

ANDA BAICUS[1,2]

[1]Carol Davila University of Medicine and Pharmacy, Bucharest, Romania
[2]Cantacuzino Medico Military National Institute of Research and Development, Bucharest, Romania

Abstract

The co-circulation of the enterovirus and norovirus species was evaluated by culture and molecular methods, in the framework of the risk assessment for importation of vaccine-derived poliovirus type 1(VDPV1), from Ukraine in Romania. 56 sewage water samples collected from the north and southeast Romania, between April and August 2017 were investigated. Xpert EV assay and Xpert Norovirus assay, standardized methods for the detection of human enterovirus (HEV) in the cerebrospinal fluid respectively for the detection of norovirus genogroups in stool samples, were used for the molecular detections of these viruses in the concentrated sewage water. Additionally, the cell culture lines were used for human enterovirus isolation. 18 human enteroviruses (32,14%) were detected by using the Xpert EV assay and 17 (30,35%) nonpolio enteroviruses (NPEV) were isolated on cell culture lines. Norovirus genogroup G II was detected in 20 samples (35,7%), followed by norovirus genogroups GI and GII, in 8 samples (14,28%). Taking into account our results, a new diagnostic algorithm could be evaluated in an emergency situation, the sewage water concentration by using the WHO method, and screening by molecular detection.

Keywords Enterovirus, norovirus, PCR, cell culture lines.

To cite this article: BAICUS A. Monitoring Enterovirus and Norovirus circulation in sewage water using isolation on cell culture lines and GeneXpert system. *Rom Biotechnol Lett*. 2019; 24(5): 820-825. DOI: 10.25083/rbl/24.5/820.825

*Corresponding author: ANDA BAICUS Cantacuzino Medico Military National Institute of Research and Development, Bucharest, Romania
E-mail: abaicus@cantacuzino.ro

Introduction

The environmental habitats, such as rivers and streams, are ideal vectors for selected genetic modified viruses and the bacteria with antibiotic resistance, creating new scenarios for the potential outbreaks (MARINESCU & al [1]). In our study was evaluated the co-circulation of enteroviruses and noroviruses in the sewage water in the framework of the circulation of the vaccine-derived poliovirus type 1(cVDPV1) strain in Ukraine in 2015, at the border with Romania (MORALES & al [2]). Human enteroviruses and noroviruses infect the human intestinal system, spread mainly by the fecal-oral route and the mean duration of viral shedding is four, six weeks after viral ingestion. These viruses can remain viable in contaminated soil and water for weeks or months (COHEN [3]) and sewage contamination of water supplies, can result in outbreaks (BERGIER & al [4]). The accidental release of live poliovirus, a human enterovirus by a vaccine producer may have consequences for the worldwide polio eradication (DUIZER & al [5]). The environmental surveillance for poliovirus circulation takes place when there is the risk of reintroduction of this virus in polio "free" country. No poliovirus strains were isolated from 2009 to 2019 in Romania (BAICUS & al [6]).

Human enteroviruses (HEVs) are single-stranded RNA viruses, within a non enveloped icosahedral symmetric protein capsid, in the genus Enterovirus, from the Picornaviridae family. Poliovirus, a member of the Enterovirus genus is the etiological agent of poliomyelitis, an acute paralytic disease. Most of the enteroviral infections are asymptomatic or are associated with milder respiratory illness or a rashes disease. Some strains (HEV 68, 71, Coxsackieviruses A9, Echovirus 9) were isolated from patients with viral bronchopneumonia (CHANG & al [7], OBERSTE & al [8], JACQUES & al [9]) and other strains are responsible for severe illness, meningitis (Echovirus 30) myocarditis (Coxsackieviruses B3, B5).

Norovirus is single-stranded RNA, non-enveloped virus in the genus Norovirus, from the Caliciviridae family, which cause acute gastroenteritis in humans (ROBILOTTI & al [10]). Genus is subdivided into at least seven genogroups based upon sequence homology. Genogroups GI, GII, and GIV include human pathogens with multiple genotypes within each genogroup (VINJE [11]). The most common human norovirus involved in infection is GII, followed by GI and GIV. GII.4 viruses have been associated with epidemic infection (LOPMAN & al [12], CDC [13]). From 2005 to 2006, two GII.4 variants were responsible for outbreaks in Australia and New Zealand, in 2012, GII.4 Sydney strain becomes the predominant strain in the United States (CDC [14]) and in 2014 a GII.17 variant emerged in Japan and has spread worldwide (MATSUSHIMA & al [15], de GRAAF & al [16], LEE & al [17]). In 2015, GII.P17-GII.17 strain was detected in Arad, a county in the western part of Romania, during an outbreak of acute gastroenteritis (DINU & al 18]). Enteroviruses resist to ether and alcohol but inactivated at temperatures above 50°C, and Noroviruses resist to chlorine, alcohol, and heating to 60°C (ATMAR & al [19], KESWICK & al [20], CHAN & al [21]).

The objectives of this study were to assess the occurrence of the enterovirus and norovirus strains in the sewage water samples, collected from the north and southeast Romania, between April and August 2017 and to evaluate the efficacy use of the molecular methods in the environment surveillance system.

Material and Methods

Grab sampling was used for monthly collection of 1000 ml sewage water for virological analysis from different counties/towns, Botosani (BT)/ (Stefanesti, Darabani, Botosani), Bucuresti (B), Constanta (CT) (North, South collection points), Maramures (MM)/ (Sighetu Marmatiei, Viseu, Borsa), Satu Mare (SM)/ (Tarna Mare, Tasnad, Agris, Turt), Suceava (SV)/ (Balca, Radauti, Siret, Veresti), Tulcea (TL)/ (Babadag) Tab. 1. Several (56) sewage water samples were concentrated and virological investigated at the Enteric Viral Infections Laboratory, Cantacuzino Medico Military National Institute of Research and Development, Bucharest, Romania. From each sample, 490 ml of sewage water were concentrated by the two-phase separation method [Polyethylene Glycol (PEG)-dextran] and decontaminated by chloroform extraction, as recommended by the WHO guidelines for environmental surveillance (WHO [22]). After treatment, 6 ml of concentrated samples were obtained, corresponding to an approximately 80 fold volume reduction. 10 ml of sewage water were concentrated by centrifugation at 1500 g in a refrigerated centrifuge for 10 minutes, and 8 ml of concentrated samples were obtained, corresponding to an approximately 1,25 fold volume reduction.

For human enterovirus detection, they have used two methods, first, the virus isolation on cell culture lines, RD and L20B, the WHO method (WHO [23], WHO [24]) and the second, the molecular detection by using the Xpert EV Assay and the GeneXpert System. This system integrates sample purification, nucleic acid amplification, and detection of the target sequence in samples using real-time PCR and RT-PCR assays. The Xpert EV assay is designed to detect RNA human enterovirus genome 5' untranslated region (UTR) between nucleotides 452 and 596, in the cerebrospinal fluid, in 2 hours and 30 minutes.

The Xpert Norovirus assay which detects norovirus genogroup I and genogroup II RNA, in stool specimens in less as one hour, it was used for the norovirus detection in the sewage water.

Enterovirus Detection

0,8 ml from 6 ml obtained after concentration of 490 ml sewage water using the WHO method, were inoculated on two cell monolayers RD tubes (0,2 ml/tube) (derived from human rhabdomyosarcoma) (selective for all enterovirus strains) and two cell monolayers L20B (murine cell line L, genetically modified, transfected with receptor for human poliovirus, selective for poliovirus) (0,2 ml/tube). The time interval for enterovirus isolation and characterization on cell culture lines must be at least 10 days (minimum of 5 days post-inoculation, and a minimum of 5 days post-passage, before a reported negative test).

0,14 ml from 8 ml obtained after concentration of 10 ml sewage water by centrifugation, at 1500 g in a refrigerated centrifuge for 10 minutes, were tested using the Cepheid GeneXpert System and Xpert EV assay in according with the manufacturer protocol for enterovirus detection.

Norovirus Detection

0,2 ml from the sewage water concentrated using the WHO method were add in the Sample Reagent bottle from the for Xpert Norovirus assay kit and it was tested in according with the manufacturer protocol for norovirus detection.

Results and Discussions

18 HEV (32,14%) were detected by using the Xpert EV assay, and 17 (30,35%) nonpolio enteroviruses (NPEV) were detected by isolation on cell culture lines. The enteroviruses were detected in samples collected from different sites: Siret (SV), Constanta North, Babadag (TL), Darabani (BT), Viseu (MM), Bucuresti, Sighetu Marmatiei (MM), Agris (SM), Tab. 1.

Concerning the norovirus detection, the genogroup G II was mostly detected in 20 samples (35,7%), the genogroup G I was detected in 3 samples (5,35%) and both norovirus genogroups GI and GII were detected in 8 sewage water samples (14,28%). The circulation of the norovirus genogroup G II was detected in Babadag (TL), Darabani (BT), Siret, Veresti, Balca (SV) Viseu (MM), Agris (SM), Bucuresti, Constanta North, Constanta South, Sighetu Marmatiei (MM), the genogroup G I was detected in Constanta South, and both norovirus genogroups GI and GII were detected in a sample collected from Babadag (TL), Darabani (BT), Viseu (MM), Tarna Mare (SM), Sighetu Marmatiei (MM), Tasnad (SM). Constanta North. The co-circulation of enteroviruses and noroviruses was observed in May in Suceava and Constanta, in June in Tulcea, Botosani, Maramures, and Bucuresti, in July in Maramures and Satu Mare, and in August in Maramures.

The intense co-circulation of enteroviruses and noroviruses was observed in June and in July in the investigated areas. In this study could observe that from 17 nonpolio enteroviruses identified on cell culture lines, only 11 HEV (64,70%) were detected by molecular method, and 6 strains (35,29%) were not detected, because of the small volume of the sample concentrated (10 ml). From 18 HEV identified by molecular method, 7 enteroviruses were not isolated on cell culture lines. Taking into account the number of nonpolio enteroviruses isolated on cell culture lines and those identified by molecular method, a total of 24 enteroviruses were recorded (42,85%). When compare with our anterior study, from 15 sewage samples investigated, 2 nonpolio enteroviruses were identified on cell culture lines and 4 HEV (26,66%) were detected by Xpert EV assay, but not the same with the isolated strains, a total of 6 enteroviruses were recorded (40%) (BAICUS [25]). The comparable percents of the enterovirus detection were recorded in both studies, 42,85% versus 40%. The molecular method used for the norovirus detection was very sensitive, 31 (55,35%) from 56 samples were positive. The norovirus genogroup G II was mostly detected in samples (35,7%).

Table 1. Results of the samples investigations

County / Town	Sampling date* 2017 day.month	Xpert Norovirus assay	Xpert Enterovirus assay	Isolation on cell culture lines RD/L20B**
TL/Babadag	19.04	GI-negative; GII-negative	Negative	Negative
	10.05	GI-negative; GII-positive	Negative	Negative
	13.06	GI-negative; GII-positive	Negative	NPEV
	12.07	GI-positive; GII-positive	Negative	Negative
BT/Stefanesti	26.04	GI-negative; GII-negative	Negative	Negative
	19.06	GI-negative; GII-negative	Negative	Negative
	18.07	GI-negative; GII-negative	Negative	Negative
BT/Darabani	26.04-	GI-negative; GII-positive	EV positive	Negative
	22.05	GI-positive; GII-positive	Negative	Negative
	19.06	GI-negative; GII-positive	EV positive	NPEV
	18.07	GI-positive; GII-positive	EV positive	Negative
BT/Botosani	26.04	GI-negative; GII-negative	negative	Negative
MM/Sighetu Marmatiei	2.05	GI-negative; GII-negative	EV positive	Negative
	6.06	GI-negative; GII-negative	EV positive	NPEV
	4.07	GI-negative; GII-positive	EV positive	Negative
	1.08	GI-positive; GII-positive	EV positive	NPEV
MM/ Viseu	2.05	GI-negative; GII-negative	negative	Negative
	6.06	GI-negative; GII-positive	negative	NPEV
	4.07	GI-positive; GII-positive	EV positive	Negative
	1.08	GI-negative; GII-positive	EV positive	NPEV
MM/Borsa	6.06-	GI-negative; GII-negative	negative	Negative
	4.07	GI-negative; GII-negative	negative	Negative
	1.08	GI-negative; GII-negative	negative	Negative
SV/Balca	8.06	GI-negative; GII-positive	negative	Negative
SV Radauti	12.07	GI-negative; GII-negative	negative	Negative
SV /Siret	10.05	GI-negative; GII-positive	negative	NPEV
SV /Veresti	10.05	GI-negative; GII-positive	negative	Negative
	8.06	GI-negative; GII-negative	negative	Negative
	12.07	GI-negative; GII-positive	negative	Negative
Bucuresti	10.04	GI-negative; GII-positive	negative	Negative
	23.05	GI-negative; GII-positive	negative	Negative
	22.06	GI-negative; GII-positive	EV positive	NPEV
	5.07	GI-negative; GII-positive	EV positive	Negative
	3.08	GI-negative; GII-positive	EV positive	NPEV
SM / Tarna Mare	11.07	GI-negative; GII-negative	negative	Negative
	18.07	GI-negative; GII-negative	negative	Negative
	26.07	GI-negative; GII-negative	negative	Negative
	1.08	GI-negative; GII-negative	negative	Negative
SM/Tasnad	4.07	GI-positive; GII-positive	negative	NPEV
SM /Agris	18.07	GI-positive; GII-negative	EV positive	NPEV
	31.07	GI-positive; GII-negative	EV positive	Negative
	7.08	GI-positive; GII-positive	negative	Negative
	11.07	GI-negative; GII-negative	negative	Negative
	18.07	GI-negative; GII-positive	EV positive	NPEV
	1.08	GI-negative; GII-positive	EV positive	NPEV
	8.08	GI-negative; GII-negative	negative	Negative
SM /Turt	11.07	GI-negative; GII-negative	negative	Negative
	18.07	GI-negative; GII-negative	negative	Negative
	26.07	GI-negative; GII-negative	negative	Negative
	1.08	GI-negative; GII-negative	negative	Negative
CT/ N	18.05	GI-negative; GII-positive	negative	NPEV
	14.06	GI-negative; GII-negative	negative	NPEV
	31.07	GI-positive; GII-positive	EV positive	NPEV
CT/S	18.05	GI-positive; GII-negative	negative	negative
	14.06	GI-negative; GII-positive	negative	negative
	31.07	GI-negative; GII-positive	EV positive	NPEV

*Samples were transported at 4°C from the collection site to the laboratory; **The isolate are recorded as nonpolio enterovirus (NPEV) if the samples were positive on RD and negative on L20B cell culture lines, and the samples were recorded as negative if there were negative on RD and L20B cell culture lines .

Conclusion

The molecular detection of enterovirus and norovirus strains in the sewage water help us in the evaluation of the viruses circulation. This study demonstrates that we can use in an emergency situation these standardized molecular methods for the detection of human enterovirus (HEV) and norovirus genogroups in the sewage water. Taking into account our results, a new diagnostic algorithm could be evaluated in an emergency, the sewage water concentration by using the WHO method, and screening by molecular detection.

Conflict of Interest

Nono to declare

Acknowledgements

We thank our colleagues from the National Institute of Public Health Bucharest and from the Public Health Authorities: Botosani, Constanța, Maramures, Satu Mare, Suceava, Tulcea, Romania; Chear Solomon T, Beleca F, Ibrahim S, Pop R, Leahu D, Zorescu C, Secarea A, for the epidemiological support in the environmental enterovirus surveillance in Romania.

Funding

The molecular investigations were funded by the National Authority of Scientific Research and Innovation, Romania, Core project PN 16 39 01 03, "Risk assessment for poliovirus importation by rapid detection of the enterovirus circulation in the sewage water and in healthy children."

References

1. F. MARINESCU, L. MARUTESCU, I. SAVIN, V. LAZAR. Antibiotic resistance markers among Gram-negative isolates from wastewater and receiving rivers in South Romania. *Rom Biotechnol Lett*, 20 (1) (2015).
2. M. MORALES, C.D. NNADI, R.H TANGERMANN, S.G. WASSILAK. Notes from the Field: Circulating Vaccine-Derived Poliovirus Outbreaks – Five Countries, 2014-2015. *Morb Mortal Wkly Rep,* 65: 128-29 (2016).
3. J.I. COHEN, *Enteroviruses* and *Reoviruses*. in Harrison's, Principle of Internal Medicine 17 th ed., eds., A.S. FAUCI, E. BRAUNWALD, D.L. KASPER, D.L. HAUSER, D.L. LONGO, J.L. JAMESON, J. LOSCALZO, McGrawHill Medical, 2006, pp. 1208-1213.
4. E.M. BERGIER, M.S. OBERSTE, M. L. LANDRY, T. BRENNAN, D.MLYNARSKI, P.A. MSHAR, K. FRENETTE, T. RABATSKY-HER, K. PURVIANCE, A. NEPAUL, W.A. NIX, M.A. PALLANSCH, D. FERGUSON, M.L. CARTTER, J.L. HADLER. An outbreak of concurrent *echovirus 30* and *coxsackievirus A1* infections associated with sea swimming among a group of travelers to Mexico. *Clin Infect Dis*, 47(5): 616-23 (2008).
5 E. DUIZER, S. RUTJES, A.M. HUSMAN de RODA, J. SCHIJVEN. Risk assessment, risk management and risk-based monitoring following a reported accidental release of *poliovirus* in Belgium, September to November 2014. *Euro Surveill,* 21(11): pii=30169 (2016).
6. A. BAICUS, A. PERSU, S. DINU, M.L. JOFFRET, F. DELPEYROUX, G. OPRISAN. The frequency and biodiversity of poliovirus and non-polio enterovirus strains isolated from healthy children living in a limited area in Romania. *Arch Virol*. 156(4):701-706 (2011).
7. L.Y. CHANG, T.Y. LIN, K.H. HSU, Y.C. HUANG, K.L. LIN, C. HSUEH. S.R. SHIN, H.C. NING, M.S. HWANG, H.S. WANG, C.Y. LEE. Clinical features and risk factors of pulmonary oedema after e*nterovirus-71*-related hand, foot, and mouth disease. Lancet, 354:1862-66 (1999).
8. M.S. OBERSTE, K. MAHER, D. SCHNURR, M.R. FLEMISTER, J.C. LOVCHIK, H. PETERS, W. SESSIONS, C. KIRK, N. CHATTERJEE, S. FULLER, J.M. HANAUER, M.A. PALLANSCH. Enterovirus 68 is associated with respiratory illness and shares biological features with both the *enteroviruses* and the *rhinoviruses*. *J Gen Virol,* 85:2577-84 (2004).
9. J. ACQUES, H. MORET, D. MINETTE, N. LEVEQUE, N. JOVENIN, G. DESLEE, F. LEBARGY, J MOTTE, L. ANDREOLETTI. Epidemiological, molecular, and clinical features of enterovirus respiratory infections in french children between 1999 and 2005. *J Clin Microb*, 46: 206-13 (2008).
10. E. ROBILOTTI, S. DERESINSKI, B.A. PINSKY. *Norovirus*. Clin Microbiol Rev, 28:(1) 134-64 (2015).

11. J. VINJÉ. Advances in laboratory methods for detection and typing of *norovirus*. *J Clin Microbiol,* 53:373-81(2015).
12. LOPMAN B, VENNEMA H, KOHLI E, POTHIER P, SANCHEZ A, NEGREDO A, et al Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. Lancet. 2004; 363 (9410): 682-8.
13. CENTERS FOR DISEASE CONTROL AND PREVENTION. Norovirus activity – United States, 2006-2007. *Morb Mortal Wkly Rep.* 56 (33): 842-6 (2007).
14. CENTERS FOR DISEASE CONTROL AND PREVENTION. Emergence of new *norovirus* strain GII.4 Sydney –United States, 2012. *Morb Mortal Wkly Rep*. 62(3): 55(2012).
15. Y. MATSUSHIMA, M. ISHIKAWA, T. SHIMIZU, A. KOMANE, S. KASUO, M. SHINOHARA, K. NAGASAWA, H. KIMURA, A. RYO, N. OKABE, K. HAGA, Y.H. DOAN, K. KATAYAMA, H. SHIMIZU. Genetic analyses of GII.17 *norovirus* strains in diarrheal disease outbreaks from December 2014 to March 2015 in Japan reveal a novel polymerase sequence and amino acid substitutions in the capsid region. Euro Surveill; 20 (26) (2015).
16. M. DE GRAAF, J.VAN BEEK, H. VENNEMA, A.T. PODKOLZIN, J. HEWITT, F. BUCARDO, K. TEMPLETON, J. MANS, J. NORDGREN, G. REUTER, M.LYNCH, L.D. RASMUSSEN, N. IRITANI, M.C. CHAN, V. MARTELLA, K. AMBERT-BALAY, J. VINJÉ, P.A. WHITE, M.P. KOOPMANS. Emergence of a novel GII.17 *norovirus* – End of the GII.4 era? *Euro Surveill*, 20 (26)(2015)
17. C.C. LEE, Y. FENG, S.Y.CHEN, C.N. TSAI, M.W. LAI, C.H. CHIU. Emerging *norovirus* GII.17 in Taiwan. *Clin Infect Dis,* 61:1762-64 (2015).
18. S. DINU , M. NAGY, D.G. NEGRU, E.D. POPOVICI, L. ZOTA, G. OPRIȘAN. Molecular identification of emergent GII.P17-GII.17 *norovirus* genotype, Romania. *Euro Surveill,* 2015; 21(7) (2015).
19. R.L. ATMAR, A.R. OPEKUN, M.A. GILGER, M.K. ESTES, S.E. CRAWFORD, F.H. NEILL, S. RAMANI, H. HILL, J. FERREIRA, D.Y. GRAHAM. Determination of the 50% human infectious dose for Norwalk virus. *J Infect Dis*, 209(7): 1016-22 (2014).
20. B.H. KESWICK, T.K. SATTERWHITE, P.C. JOHNSON, H.L. DUPONT, S.L. SECOR, J.A. BITSURA, G.W. GARY, J.C. HOFF. Inactivation of Norwalk virus in drinking water by chlorine. *Appl Environ Microbiol,* 50:261-64 (1985).
21. M.C. CHAN, J.J. SUNG, R.K. LAM, P.K. CHAN, N.L. LEE, R.W. LAI, W.K. LEUNG. Fecal viral load and norovirus-associated gastroenteritis. *Emerg Infect Dis,* 12:1278 (2006).
22. WHO. Guidelines for environmental surveillance of poliovirus circulation. WHO/V&B/03.03. WHO, 2003. Geneva, Switzerland.
23. WHO. Polio Laboratory Manual. WHO/IVB/04.10. Geneva, Switzerland: World Health Organization. 2004. Retrieved from http://polioeradication.org/wp-content/uploads//Polio Lab_Manual04.pdf.
24. WHO. Supplement to the WHO Polio Laboratory Manual. 2014. Retrieved from http://polio eradication. org/wp-content/uplods/ NewAlgorithm For Poliovirus Isolation Supplement1.pdf.
25. A. BAICUS. The integration of the molecular methods in the diagnosis algorithm for the poliovirus detection in the sewage water: comparing concentration and detection methods. A Pilot Study. *Rom J Intern Med*, Dec 1; 55(4): 245-248 (2017).