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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

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Introduction. Two cases of circulating vaccine-derived poliovirus type 1(cVDPV1), from southwestern Ukraine, bordering Romania, were confirmed in 2015 and the environmental enterovirus surveillance was enhanced in our country. The molecular detection of human enteroviruses as a screening test followed by isolation on cell culture lines or sequencing could be proposed as a new diagnosis algorithm.

Material and Methods. The sensitivity of two molecular methods for the detection of enterovirus strains in 10 mL of sewage water (15 samples) was studied with Film Array ME panel BioFire (Biomerieux, France) and Xpert EV assay (Cepheid, USA). These are standardized methods for the detection of microorganisms in the cerebrospinal fluid (CSF).

Results. Of the 15 samples, six enterovirus strains were detected using Film Array ME, four enterovirus strains were detected using Xpert EV assay, while only two nonpolio enterovirus strains were isolated on RD cell line, using the standard WHO algorithm. However, only one of the strains detected by the standard WHO algorithm was detected by one of the molecular methods.

Conclusions. The molecular methods for enterovirus detection are more sensitive than the virus isolation on cell culture lines, but in one case the virus isolated on RD cell line was not detected by the molecular methods. The results could be influenced by the small number of the samples investigated, by the volume and the concentration method used for samples tested, and by the limits of detection (LoD) of the enterovirus species in the samples, depending on the method used.

Key words: poliovirus, sewage water, multiplex PCR, Xpert EV assay, Film Array ME.

INTRODUCTION

Poliovirus (PV), a member of the *Enterovirus* genus, is the etiological agent of poliomyelitis, an acute paralytic disease. Poliomyelitis has been virtually eliminated in most countries by the widespread immunization with the formalin-inactivated vaccine (IPV) and live-attenuated vaccines (OPV). After the decision of the World Health Organization (WHO) to globally eradicate poliomyelitis, there was a reduction in the number of countries where wild poliovirus was still endemic from 125 to three: Afghanistan, Nigeria, and Pakistan [1]. In Romania, poliomyelitis was controlled mostly by using trivalent oral poliovaccine (TOPV) until 2008; vaccination with inactivated polio vaccine (IPV) started in 2009. In 2002, one month after Certification of European region as polio free, a type 1 PV, vaccine derived poliovirus (VDPV) strain, recombinant Sabin1/Sabin2/Sabin1, was isolated from a vaccine associated paralytic poliomyelitis (VAPP) case not vaccinated against poliomyelitis, and from 8 healthy contacts [2]. Evidence of interhuman circulation of Sabin strains was found in 2008 in the same population studied in 2002 [3]. No PV strains were isolated from 2009 to 2017 in Romania. In 2015, 2 cases of circulating vaccine-derived poliovirus type 1(cVDPV1) were confirmed in southwestern Ukraine, bordering Romania, Hungary, Slovakia and Poland [4]. Romania was considered a country at risk because of its lower level of polio vaccine coverage, and the environmental enterovirus surveillance was enhanced. 642 sewage water samples were investigated between 2015-2016 from north and southeast Romania, and 189 nonpolio enteroviruses strains were detected (29.4%).

In the context of the Global Polio Eradication Strategy, the method used in the National Polio Laboratories for the detection of poliovirus and nonpolio enteroviruses in the environment is the virus isolation on cell culture lines. Using this method, the estimated time for isolation is 21 days. In a previous study we compared the standardized WHO method with a molecular rapid one [5]. Both on the large samples (500 mL of sewage water)

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concentrated by the WHO method, and on the small samples (10 mL), the molecular method was more sensitive: in the 74 large samples, the molecular method detected 42 strains of nonpolio enterovirus compared with only 30 by the standardized WHO method, while in the 36 small samples the molecular method detected 10 nonpolio enterovirus strains compared with only 3 detected by the WHO method. No strains were detected by the WHO method in the samples which tested negative by the molecular method. Herein, we compared the detection methods (standardized WHO method with two molecular methods), used after their respective method of sample concentration (large 490 mL sample for the WHO method, and small 10 mL sample for the molecular methods).

MATERIAL AND METHODS

15 sewage water samples were included in our study. Grab sampling was used for collection from different counties of 500 mL environmental samples for virological analysis. Concentrated samples were processed at the Enteric Viral Infections Laboratory, "Cantacuzino" National Institute of Research, Bucharest, Romania.

490 mL of sewage water were concentrated by the WHO method (centrifugation in a refrigerated centrifuge, followed by the two-phase separation method using a mixture of two carbohydrate polymers, dextran and polyethylene glycol (PEG), dissolved in water), and 10 mL of sewage water were concentrated by centrifugation at 1500 g in a refrigerated centrifuge for 10 minutes [6]. 200 µL from the sewage water concentrated using the WHO method were inoculated on each L20B (genetically engineered mouse cell line expressing the human poliovirus receptor PVR), and RD (derived from human rhabdomyosarcoma) cell lines, as recommended by WHO for human enterovirus (HEV) detection. RD cell lines can be infected by most enteroviruses, but L20B cells are sensitive only to poliovirus [7, 8]. The time interval for enterovirus isolation and characterization must be at least 10 days (minimum of 5 days post-inoculation, and minimum of 5 days post-passage, before a reported negative test).

140 µL, respectively 200 µL of concentrated sewage water were tested using Xpert EV assay, and Film Array ME. Xpert EV assay is designed to detect RNA enterovirus genome 5' untranslated region (UTR) between nucleotides 452 and 596, in 2 hours and 30 minutes. The FilmArray Meningitis/ Encephalitis (ME) panel detects 14 pathogens including *Enterovirus*, in one hour.

For the molecular diagnosis, the remained 10 mL of every sewage water sample was concentrated by centrifugation, at 1500 g in a refrigerated centrifuge for 10 minutes. Afterwards, the samples were tested using the Xpert EV assay (Cepheid, USA), and the FilmArray Meningitis/ Encephalitis panel, BioFire (Biomerieux, France), standardized methods only for the detection of the microorganisms in the cerebrospinal fluid (CSF).

RESULTS

In our samples, six enterovirus strains were detected using Film Array ME, and four enterovirus strains were detected using Xpert EV assay (Table 1). Only two nonpolio enterovirus strains were isolated on RD cell line, using the standard WHO method. In one case the virus isolated on RD cell line was not detected by the molecular methods (sample 14, Table 1), and in another case the virus isolated on RD cell line was detected by only one of the two molecular methods (sample 5 detected by Film Array ME).

Table 1

Results of the samples investigation using the cell culture lines, and the molecular detection by Xpert EV assay and Film Array ME

ID/ County	Cell culture lines RD/L20B	Xpert EV assay	Film Array ME
1/SM	Negative RD/ Negative L20B	*EV* not detected	*EV* not detected
2/MM	Negative RD/ Negative L20B	*EV* not detected	*EV* not detected
3/SV	Negative RD/ Negative L20B	*EV* not detected	*EV* not detected
4/B	Negative RD Negative /L20B	*EV* not detected	***EV* detected**
5/CT	**Positive RD/ Negative L20B**	*EV* not detected	***EV* detected**
6/CT	Negative RD/ Negative L20B	*EV* not detected	*EV* not detected
7/TL	Negative RD/ Negative L20B	*EV* not detected	***EV* detected**
8/B	Negative RD/ Negative L20B	*EV* not detected	*EV* not detected
9/BT	Negative RD/ Negative L20B	*EV* not detected	*EV* not detected
10/MM	Negative RD/ Negative L20B	***EV* detected**	***EV* detected**
11/BT	Negative RD/ Negative L20B	***EV* detected**	***EV* detected**
12/ B	Negative RD/ Negative L20B	***EV* detected**	***EV* detected**
13/ BT	Negative RD/ Negative L20B	***EV* detected**	*EV* not detected
14 /CT	**Positive RD/ Negative L20B**	*EV* not detected	*EV* not detected
15 /CT	Negative RD/ Negative L20B	*EV* not detected	*EV* not detected

B – Bucuresti, BT – Botosani, CT – Constanta, MM – Maramures, SM- Satu Mare, SV – Suceava, TL – Tulcea

DISCUSSION

In our previous study, the standardized WHO method was compared with a molecular method on sewage water concentrated by two methods, the WHO method, and an in house method, double centrifugation of 10 mL in a refrigerated centrifuge for 30 minutes after adding 2 mL of chloroform [5]. When compared in samples obtained by similar concentration techniques, the molecular method was more sensitive than the standardized WHO one, and of course much more rapid. In the present study, where the detection method (WHO standardized and molecular) was used on samples concentrated differently (490 mL for the standardized method and 10 mL for the molecular ones), the molecular methods detected more enterovirus strains (6 by Film Array ME and 4 by Xpert EV Assay, *versus* only two by the standardized WHO cellular method). However, the two strains detected by the standardized WHO method were not detected by Xpert EV Assay, and only one was detected by Film Array ME molecular method, therefore here the molecular methods were not more sensitive because they missed those strains. Taking into account the results of our previous study, we could suppose that the decrease in sensitivity of the molecular methods was due to the small samples used (10 mL *versus* 490 mL) and, for an optimal sensitivity, the standardized WHO concentration method of 500 mL samples should be used together with the molecular methods, but this has to be demonstrated in a future study.

The fact that the molecular methods detect many more enterovirus strains shows that probably there is a silent circulation of the enteroviruses in the environment, which cannot be detected by isolation on cell culture lines, and therefore more sensitive methods are necessary. Moreover, in an emergency situation, the use of a small volume of the sewage water for investigation and a simple method for concentration would be essential to give a rapid response, but as this study shows, either the small volume or the in house concentration method have led to a loss of sensitivity, despite the higher sensitivity of the molecular method. In this study, the results could have been influenced by the small number of the samples investigated, by the different volumes and concentration methods used for the samples, and by the limits of detection (LoD) of the enterovirus species in the samples. The molecular methods for enterovirus detection – the Film Array ME and the Xpert EV assay – are more sensitive than the virus isolation on cell culture lines, and could be useds in the screening diagnosis algorithm for poliovirus detection in the sewage water, giving faster results (several hours instead of 21 days).

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Conflict of interest: No conflict of interest declared.

Introducere. *Două cazuri produse de o tulpină a virusului polio tip 1 derivat din vaccin (cVDOV1), din sud-vestul Ucrainei, la granița cu România au fost confirmate în anul 2015, iar supravegherea circulației enterovirusului în mediu a fost sporită în țara noastră. Detecția moleculară a enterovirusurilor umane ca metodă de screening urmată de izolarea pe culturi celulare sau de secvențiere ar putea fi propuse ca nou algoritm de diagnostic.*

Materiale şi metode. *A fost studiată sensibilitatea a două metode moleculare pentru detecția tulpinilor de enterovirus din 10 mL de apă reziduală menajeră (15 probe) şi folosind Film Array ME panel BioFire (Biomerieux, France) şi Xpert EV assay (Cepheid, USA). Aceste metode sunt standardizate pentru detecția microorganismelor din lichidul cefalorahidian.*

Rezultate. *Din 15 probe, au fost detectate 6 tulpini de enterovirus prin metoda Film Array ME, 4 tulpini au fost detectate folosind Xpert EV assay şi numai două tulpini de enterovirus nonpolio au fost izolate folosind linia celulară RD prin algoritmul standard O.M.S. Totuşi numai una din tulpinile detectate folosind algoritmul standard O.M.S a fost detectată prin una din metodele moleculare.*

Concluzii. *Metodele moleculare pentru detecţia enterovirusurilor sunt mult mai sensibile decât izolarea virusului pe linii de culturi celulare, dar într-un caz virsusul izolat folosind tehnica pe linia celulară RD nu a fost detectat prin metode moleculare. Rezultatele ar putea fi influenţate de numărul mic de probe investigate, de volumul redus sau de metoda de concentrare folosită precum şi de limita de detecţie (funcţie de metoda folosită) a tulpinilor de enterovirus din probele analizate.*

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