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On-site reverse transcription-quantitative polymerase chain reaction detection of rotaviruses concentrated from environmental water samples using methacrylate monolithic supports

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ABSTRACT

Rotaviruses are the leading cause of gastroenteritis in children and they exist widely in water environments. Ingestion of 10–100 viral particles is enough to initiate disease, what calls for extremely sensitive detection methods. In this study we have confirmed the validity of a recently published method for rotavirus concentration and detection based on the combination of methacrylate monoliths and real-time reverse transcription-quantitative PCR (RT-qPCR). The method was used to concentrate rotaviruses from different tap water and environmental water samples collected in Slovenia within years 2007 and 2009. The performance of virus concentration using monolithic supports was improved in comparison to the one of tangential ultrafiltration upon application of both methods on a range of environmental samples. Several samples were successfully concentrated on-site after successful adaptation of the method to field requirements. In such on-site format, the combination of concentration using CIM and detection using RT-qPCR detected as low as 30 rotavirus particles/ml, spiked in an environmental water sample.

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1. Introduction

Rotaviruses are the main cause of acute viral gastroenteritis in children less than 5 years old [1] and are responsible for hundreds of thousands of deaths each year affecting primarily developing countries [1]. Rotaviruses are widely present in aquatic environments such as lakes, rivers and public water supplies, which are contaminated mainly through leaking sewer and septic systems and urban and agricultural runoff [2]. Alternative contamination risks are those derived from natural disasters (earthquakes, floods) or intentioned release (bioterrorism). Rotaviruses, as well as other enteric viruses, are of public health concern due to their low infectious dose [3]. For example, the probability of infection from exposure to one rotavirus particle is 31%, and no more than 1 PFU is required to cause infection in 1% of healthy adults with no antibody to the virus [4], emphasizing the need for sensitive detection methods.

The basic steps of virological analysis of environmental waters are sampling, virus concentration, and detection with cell culture assays, serological methods (ELISA) or molecular methods (PCR) [2]. Glass wool filtration, adsorption–elution using positive or negative filters, and ultrafiltration are among the most commonly used virus concentration methods on environmental waters [2,5]. In most of the cases, due to low viral recoveries and/or high end volumes, an additional concentration step is required, such as ultracentrifugation, PEG precipitation, flocculation or further filtration. A novel method has been recently optimized for the concentration of rotaviruses from water samples [6]. The method relies on the binding of the viruses to Convective Interaction Media® (CIM) methacrylate monolithic chromatographic supports. Once the viruses were bound to a CIM quaternary amine (CIM QA) monolith, close to 100% of the bound particles are recovered upon elution with 1 M NaCl. Binding of viruses and recoveries were not affected by increasing the flow rates up to 100 ml/min, enabling the loading of high sample volumes in short time. Electron micrographs indicated that the recovered viruses consisted of intact particles, what increases the probability of detecting infectious viral particles. CIM QA monolithic columns have also been used for the concentration of other waterborne viruses, such as Calicivirus and Hepatitis A virus [7]. In that work, the virus concentration from spiked bottled water samples using CIM QA monolithic supports was compared with concentration using electropositive filters. Even though the achieved recoveries were not as high as with rotaviruses, they still improved the results obtained by electropositive filters. The use of CIM monoliths in virus applications is rapidly increasing,

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not only for concentration [8], but also for the purification, and in-process control of different viruses [9–11], including phages [12,13]. The improved performance of methacrylate monoliths for virus purification and concentration in contrast to classic bead-based chromatographic supports resides in their structure, which consists of large flow-through channels that enable convective mass transport of molecules, leading to flow-independent dynamic binding capacity and separation [14,15].

Concerning detection, available methods for the detection of viruses concentrated from environmental waters can be divided in cell culture based methods, serological methods and molecular methods. The cell culture is the only method that provides actual information on the infectivity of the detected virus, but its long processing time is a major drawback. Integrated cell culture PCR (ICC-PCR) reduces the time-to-result by coupling cell culture with viral specific PCR detection, but the time needed to obtain the result is still relatively long. Serological methods (ELISA) are faster but they lack the sensitivity of modern molecular methods such as real time quantitative PCR (qPCR) [16]. PCR and specially qPCR are gaining increased popularity, due to their sensitivity, specificity, quantitative potential, genotypization ability and high throughput. In addition, the nucleic acid amplification and visualization technologies are rapidly evolving towards the simplicity and on-site use, in comparison to cell culture or serological methods [17].

In this work, recently published optimized conditions for the binding and elution of rotaviruses to CIM QA monolithic support [6] were chosen for concentrating different environmental water samples collected throughout Slovenia. The methodology was based on the binding of the low amounts of rotaviruses present in a water sample to a CIM QA monolithic support, and posterior elution in a small volume. The concentrated viruses were detected by a rotavirus specific reverse transcription-qPCR (RT-qPCR) [16]. The performance of the CIM based concentration was compared with the tangential-flow ultrafiltration method. Finally, several concentrations were performed on-site after adaptation of the method to field requirements.

2. Material and methods

2.1. Water samples

Tap water was collected directly from the tap in the laboratory and spiked with known concentration of rotaviruses from clarified stool sample. The concentration of rotaviruses in the spike was estimated by counting under electron microscope JEM 1200 EXII (Jeol, Tokyo, Japan) using the latex-negative staining technique [18]. Environmental water from different locations throughout Slovenia with elevated probability of fecal contamination (locations close to wastewater treatment plant and/or close to urban settlements...) were sampled and stored at 4 °C until further analysis. Samples from river Drava at locations designed as Otiški Vrh, Ormož, Tribej, Mariborski otok and Drava, were kindly supplied by Dr Maja Rupnik from the Institute of Public Health in Maribor. In the concentrations performed in field, the water samples were collected directly on-site, and concentrated both directly and after spiking them with known rotavirus concentrations.

2.2. Concentration using CIM QA monolithic supports

Spiked tap water samples and environmental water samples were pumped through 8 ml CIM QA monolithic columns (BIA separations, Slovenia) using either a Milton Roy LMI B71 dosing pump (Milton Roy Europe, Point Saint Pierre, France), a Knauer modular HPLC system (Knauer, Berlin, Germany) or an ÄKTA purifier 100 (GE Healthcare, Uppsala, Sweden). Flow rate was always 80 ml/min or 100 ml/min. The pH of the water sample was fixed by adding the required amount of 10× concentrated mobile phase (50 mM Hepes, pH 7) before loading. In the case of river water samples a 0.8 μm cut-off 142 mm I.D. filter (Sartorius, Goettingen, Germany), in a specially designed stainless steel housing, was placed between the pump and the CIM QA column to prevent the CIM column from clogging. After a washing step, elution of the bound viruses was performed, with 50 mM Hepes, pH 7 containing 1 M NaCl. The elution peak (typically of 10–15 ml volume) was monitored by measuring UV absorption either at 280 nm with the UV detector of the ÄKTA purifier 100, or at 254 nm with a Smartline preparative UV Detector 200 (Knauer, Germany). After each measurement, CIM QA column was sanitized with 1 M NaOH with a contact time of 2 h. In the case of the river water samples (collected during the years 2008 and 2009) used for comparison between CIM QA concentration with the tangential-flow ultrafiltration method (Table 3), the concentrated fraction obtained with CIM QA column was further concentrated by ultracentrifugation at 4 °C, 100,000 × *g* during 1 h, in a Beckman L8-70M ultracentrifuge using a SW65 rotor (Beckman Coulter, CA, USA). For the detection and/or quantification of the viruses a previously published rotavirus specific RT-qPCR assay was used [16]. RNA was isolated from load and elution fractions with TRIzol LS reagent, following manufacturer instructions (Invitrogen, Carlsbad, CA) and applied to the RT-qPCR assay on an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Quantification of relative viral amounts in load and elution fractions for calculation of concentration factors was done as explained in [6]. Some modifications were applied for the environmental samples used in the method comparison (Table 3). In these cases, the RNA was isolated with QIAamp viral RNA mini kit (QIAGEN, Chatsworth, CA, USA) and applied to RT-qPCR in one-step format using Ag-Path one step kit (Applied Biosystems) following manufacturer's instructions. The detection of rotavirus RNA in these samples was performed in the portable Smart Cycler real time PCR thermocycler (Cepheid, Sunnyvale, CA, USA).

2.3. Concentration using tangential ultrafiltration

For the experiment shown in Table 2, 1 l of spiked tap water was concentrated using tangential-flow ultrafiltration with Vivaflow 30,000 Da Molecular Weight Cut-Off (MWCO) Polyethersulfone (PES) ultrafilter (Sartorius, Goettingen, Germany). The estimated recirculation rate was 250 ml/min. A final recovery volume of 50 ml was processed further with the second ultrafiltration step using pressurised Vivacell 30,000 MWCO PES ultrafiltration system (Sartorius, Goettingen, Germany). The filtration process was terminated at the final concentrate volume of 10–15 ml. A 250 μl volume from loads and elution was used for RNA extraction with TRIzol LS reagent, following manufacturer instructions (Invitrogen, Carlsbad, CA). RNA was applied to the RT-qPCR assay, quantified and concentration factors calculated as previously described [6].

For the experiment shown in Table 3, 2 l of river water sample were used for the virus concentration using tangential flow ultrafiltration technique. Water was filtered through 30,000 MWCO PES ultrafilter (Sartorius, Goettingen, Germany) by an estimated recirculation rate of 250 ml/min to the final recovery volume of 20 ml. A 10 ml concentrate was then transferred to two ultracentrifuge tubes (5 ml in each) and further concentrated by ultracentrifugation in a Beckman L8-80M ultracentrifuge (Beckman), for 1 h at 100,000 × *g* and 4 °C. The supernatant was discarded and the pellet was resuspended in 125 μl of sterile PBS (pH 7.4). Concentrated samples were lysed directly in the ultracentrifuge tubes by adding 375 μl of TRIzol LS reagent (Invitrogen, Carlsbad, CA). In this stage the separate concentrates of one sample were merged into one 1.5 ml centrifuge tube and RNA was extracted following the manufacturer instructions (Invitrogen, Carlsbad, CA). Two

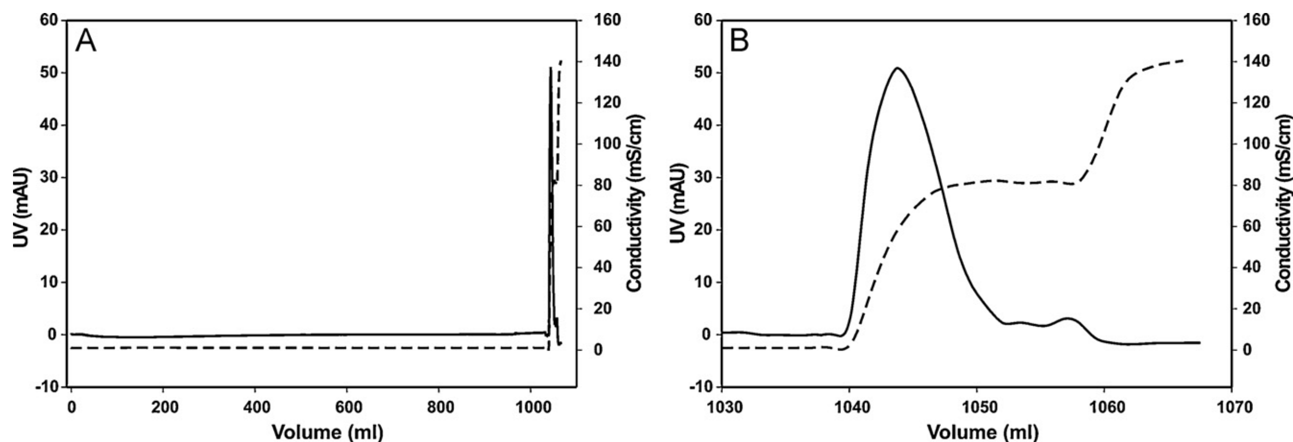

Fig. 1. Chromatogram of a typical concentration on a CIM QA monolithic column. A panel: chromatogram showing the loading of 1 l of tap water on CIM QA 8 ml column and the posterior elution with 1 M NaCl in the mobile phase (50 mM Hepes, pH 7). Solid line corresponds to the UV_{280} signal while the dashed line corresponds to the conductivity measure. B panel: detail of the elution with 1 M NaCl. The obtained peak (10 ml) was applied to rotavirus RT-qPCR detection after RNA extraction. An additional elution with 2 M NaCl elution was performed to elute any molecule remaining in the CIM QA column. The shown run was performed with ÄKTA purifier 100.

microlitres of the extracted RNA were applied to one step RT-qPCR rotavirus specific detection system [16] using Ag-Path one step RT-PCR kit (Applied Biosystems) according to manufacturer instructions. The detection was performed in a StepOne Real-Time PCR System (Applied Biosystems).

2.4. On-site concentration and detection

In the concentration experiments performed in the field, a standard gasoline power generator was used as power supply. CIM QA 8 ml columns were transported to the planned location already equilibrated with the loading buffer (50 mM Hepes, pH 7). Elution buffer and 10× concentrated loading buffer were also previously prepared in the laboratory. The Milton Roy LMI B71 dosing pump (Milton Roy Europe) and the modular Smartline preparative UV Detector 200 (Knauer, Germany) were used for pumping the sample and detecting the elution of the concentrated fraction. Each sample was concentrated both directly and after spiking with rotavirus to a final concentration of 30 particles/ml, in order to assess the success of the concentration procedure. A mini spin microcentrifuge (Eppendorf, Germany) was used in combination with the above mentioned QIAamp kit (QIAGEN) for the viral RNA purification. The RT-qPCR detection was performed on site using the portable Smart Cycler real time PCR thermocycler (Cepheid, Sunnyvale, CA, USA), and the Ag-Path one step kit (Applied Biosystems) following manufacturer's instructions.

3. Results

3.1. Concentration and detection of rotaviruses from tap and environmental waters using CIM QA and RT-qPCR

A variety of tap and environmental water samples collected during year 2007 throughout Slovenia were concentrated in the laboratory, followed by RNA isolation from the concentrated fractions and analysis using two-steps RT-qPCR format (initial RT step to obtain cDNA, followed by the amplification step). The whole procedure lasted approximately 6.5 h for a 4 l water sample: 1 h for virus concentration using CIM QA 8 ml column (at 80 ml/min flow rate), 1.5 h for the RNA extraction (using TRIzol LS reagent), 2.5 h for the reverse transcription, and 1.5 h for the cDNA amplification. A typical chromatogram corresponding to the concentration of a 1 l tap water sample is shown in Fig. 1. Following the elution with the loading buffer containing 1 M NaCl, a10 ml fraction corresponding to the chromatographic peak, was collected in each run. If rotavirus particles were present in the loading sample they eluted in this peak. Such concentration method, in combination with the two-steps RT-qPCR, enabled the detection of rotaviruses in three Slovenian rivers (Krka, Drava, Vogršček) (Table 1). The same samples gave negative result if the concentration step was skipped (Table 1). In the beginning of October 2007 due to heavy rain several suburbs in Ljubljana became flooded. In response to this, a recommendation of boiling the tap water before use was given to the inhabitants of those suburbs, including Črnuče. CIM QA columns were used to concentrate tap water from several houses and water from a stream nearby the same residential area in Črnuče and the adjacent suburb Gameljne (Table 1). The concentration step allowed detecting the presence of rotaviruses in the tap water from the house in Črnuče. Interestingly, another sample from the same tap water was analyzed 2 weeks later, when the boiling of water was not mandatory anymore, giving negative results even after CIM concentration (Table 1).

Table 1
Concentration and detection of rotaviruses from environmental water samples using CIM QA and RT-qPCR.

Sample	Datum	Type (volume)	Load	Concentrate
Krka	October 2007	River (5 l)	–	+[a]
Drava	October 2007	River (5 l)	–	+
Vogršček	October 2007	River (5 l)	–	+
Črnuče	4th October 2007	Tap (3 l)	–	+
Črnuče	20th October 2007	Tap (3 l)	–	–
Gameljne	October 2007	Tap (1 l)	–	–
Gameljne	October 2007	Stream (1 l)	–	–

[a] A result was considered positive when qPCR threshold cycles were lower than 40, and at least two reactions were positive within a given triplicate [16].

3.2. Comparison of CIM QA concentration with tangential ultrafiltration method

In order to further validate the CIM QA potential for concentrating rotavirus, the method was compared to an already established one, tangential-flow ultrafiltration [19–21]. In the first comparison, 1 l tap water samples spiked with decreasing concentration of rotavirus (10^6, 10^3 and 10^1 particles/ml) were concentrated in parallel using both methods. In this experiment, a Knauer modular HPLC system was used to pump the tap water through the CIM QA 8 ml column, ending in an elution volume of ≈10–15 ml. For the ultrafiltration, the 1 l samples were initially concentrated to a 50 ml volume by tangential ultrafiltration on a Vivaflow system

Table 2
Comparison of concentration using CIM QA with tangential ultrafiltration in spiked tap water samples.

	Spike [RoV] (particles/ml)	Load	Concentrate	Concentration factor[b]
CIM QA	9.7×10^6	+[a]	+	80×
	9.7×10^3	+	+	54×
	9.7×10^a	–	+	nm[c]
	Non spiked	–	–	nm
Ultrafiltration	9.7×10^6	+	+	16×
	9.7×10^3	+	+	18×
	9.7×10^a	–	+	nm
	Non spiked	–	–	nm

[a] A result was considered positive when qPCR threshold cycles were lower than 40, and at least two reactions were positive within a given triplicate [16].
[b] Concentration factor was calculated using RT-qPCR for quantifying the virus concentration both in the load and the concentrate as described in [6].
[c] Non measurable.

(Sartorius), followed by and additional ultrafiltration on a VivaCell system (Sartorius) to reach a final 10–15 ml volume. All loads and elutions were applied to quantitative RT-qPCR analysis and concentrations factors were calculated (Table 2). The use of CIM QA columns resulted in concentration factors of 54× to 80×, in good correlation with previously reported values from a similar experiment, 56× to 66× [6]. Such concentration allowed the detection of rotavirus in the sample with the lowest virus concentration (9.7×10^1), which was negative before concentration. Similarly, the combination of ultrafiltration on Vivaflow and Vivacell systems, also allowed the detection of rotavirus in the lowest concentration sample, but in this case, the calculated concentration factors were four times lower than those obtained when using CIM QA column (Table 2). Similar concentration factors, 15× to 17×, were obtained in a repetition of the experiment using the filtration approach (data not shown).

The second comparison was made using river water samples collected throughout Slovenia during years 2008 and 2009. This time, with the goal of improving the ultrafiltration method, the second filtration step using Vivacell system was omitted and the retentate obtained with the tangential-flow ultrafiltration was directly applied to an ultracentrifugation step. For a better comparison, an identical ultracentrifugation step was also applied to the elution fraction from CIM QA column. Each river water sample collected was divided in half and concentrated in parallel at the National Institute of Biology (CIM QA) and at the Institute of Microbiology and Immunology (tangential filtration), both in Ljubljana. In both cases, the presence of rotaviruses was assessed by RT-qPCR, using Ag-Path one-step kit. In a first attempt to search for on-site applicability the samples concentrated with CIM QA were this time analyzed in the portable SmartCycler qPCR system (Cepheid). The results are shown in Table 3. All 12 samples were negative before concentration. Rotaviruses were detected in 10 out of 12 samples after the CIM QA concentration step, while the inclusion of the ultracentrifugation step, enabled a positive result in all of the analyzed samples. In contrast, only 7 out of 12 samples were positive after tangential ultrafiltration and ultracentrifugation. One of the 12 samples showed a qPCR inhibitory effect (Table 3), therefore, it could not be concluded whether this sample was positive or negative. Concerning the results obtained using only the tangential ultrafiltration step, data on just 8 of the 12 samples was available (samples from May 2009 were not analyzed by RT-qPCR due to lack of RNA) being all eight of them negative. These results are quite below the ones obtained using only the CIM QA step, where 6 out of those 8 samples were already positive before ultracentrifugation (Table 3).

3.3. Concentration and detection on-site

Samples were collected at two different locations of the stream Glinščica in Ljubljana (side stream and main stream). The side stream, flows from the Ljubljana ZOO until it joins the main stream. The sample in the main stream was collected in a point before the side stream joins. Three litres of water were concentrated on-site, either directly or after spiking to a final 30 particles/ml rotavirus concentration. Several improvements were introduced progressively within the concentration/detection method since the initial experiments shown in Table 1. Flow rate was increased from 80 ml/min to 100 ml/min. The time needed for RNA isolation was also reduced by using a QIAamp viral RNA isolation kit, instead of TRIzol reagent based method. The two-steps RT-qPCR detection was adapted to the one-step format using the portable qPCR

Table 3
Comparison of the concentration using CIM QA with tangential ultrafiltration in environmental water samples.

Sample	Datum	Type (vol)	CIM QA 8 ml column + ultracentrif.			Ultrafiltration + ultracentrif.		
			Before CIM	After CIM	After Ultrac.	Before VIVA	After VIVA	After Ultrac.
Otiskih Vrh	Dec.08	River (2 l)	–	+[a]	+	–	–	+
Podklanc	Dec.08	River (2 l)	–	+	+	–	–	+
Marib. otok	Dec.08	River (2 l)	–	+	+	–	–	+
Ormoz	Dec.08	River (2 l)	–	–	+	–	–	+
Tribej	Dec.08	River (2 l)	–	–	+	–	–	+
Vipava	May.09	River (2 l)	–	+	+	–	n.a.	+
Lesane	May.09	River (2 l)	–	+	+	–	n.a.	+
Mura	May.09	River (2 l)	–	+	+	–	n.a.	i
Ljubljanica	May.09	River (2 l)	–	+	+	–	n.a.	–
Krka	Sep.09	River (2 l)	–	+	+	–	–	–
Drava	Sep.09	River (2 l)	–	+	+	–	–	–
Vogršček	Sep.09	River (2 l)	–	+	+	–	–	–
			0/12 (0%)	10/12 (83%)	12/12 (100%)	0/12 (0%)	0/8 (0%)[b]	7/12 (58%)

i = inhibition observed in the RT-qPCR, indicated by assessing the amplification of luciferase control RNA spiked in the sample [16].
n.a. = not assayed.
[a] A result was considered positive when qPCR threshold cycles were lower than 40.
[b] Four of the 12 samples were not applied to RT-qPCR analysis before the ultracentrifugation step.

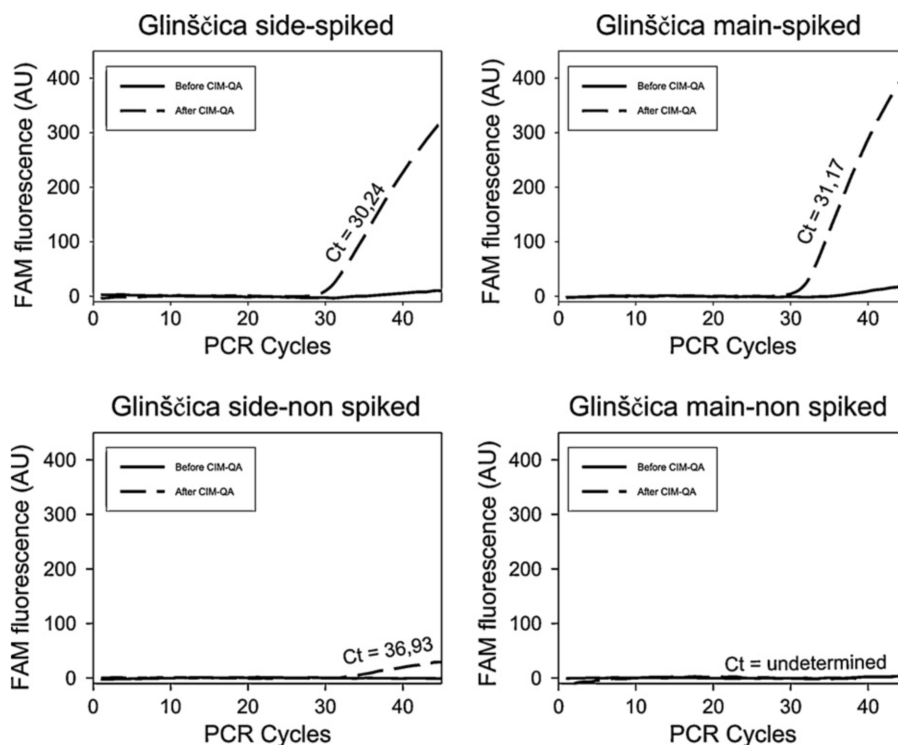

Fig. 2. Real time RT-qPCR fluorescence amplification curves of rotavirus RNA isolated from on-site concentrated environmental water samples. Each panel shows the RNA amplification curves obtained for each sample, before (solid line) and after (dashed line) concentration using CIM QA. The calculated threshold cycles (Ct) for the curves where amplification was observed are indicated beside the corresponding curve. Sampling location is indicated above each panel. The curves were obtained on-site using the portable SmartCycler RT-qPCR thermocycler.

device. In total the time needed from sample to result, was reduced from 6.5 h to 2 h 45 min (45 min for concentration of 3 l water sample using CIM QA, 45 min for RNA isolation, and 1 h 15 min for the RT-qPCR reaction). The RT-qPCR could not detect the presence of rotavirus in any of the samples before concentration. Both spiked samples (side and main streams) showed nice amplification, reflected from an increase of the rotavirus specific qPCR probe fluorescence at PCR cycles close to 30 (Fig. 2). Among non spiked samples only the side stream was rotavirus positive (Ct 36.93) after concentration.

4. Discussion

The objective of this study was to confirm the suitability of CIM chromatographic supports for fast and efficient on-site concentration of rotaviruses present in tap and environmental water samples. In the recent work by Gutiérrez-Aguirre et al. [6], the conditions of binding and elution of rotavirus particles to CIM QA monoliths were defined, and a preliminary concentration experiment was performed using rotavirus-spiked tap and river water samples. In this work, the method has been further validated on a range of environmental samples and compared to an already established concentration method. Tangential-flow ultrafiltration [19–21] is one among the typical methods (binding to positive/negative filters, other filtration techniques...) that are being used in the last decades to concentrate viruses from water samples. Upon comparison of CIM QA concentration, with tangential-flow ultrafiltration, CIM QA performed better both in spiked tap water samples as well as in environmental water samples (Tables 2 and 3). The concentration factors obtained with CIM QA were four times better than those obtained with tangential-flow ultrafiltration using Vivaflow, in combination with Vivacell systems (Table 2). The obtained values (close to 65×) correspond, given a concentration from 1000 ml to 10–15 ml, to a ~100% viral recovery. Such recoveries are in good correlation with the ones reported previously when optimizing the rotavirus binding to CIM QA [6]. The detection of rotaviruses from environmental water samples was also more efficient using CIM QA (10 positive samples out of 12, Table 3), than using tangential filtration even if combined with an additional ultracentrifugation step (7 positives out of 12). In another recent comparison, the performance of CIM QA for the concentration of feline Caliciviruses and Hepatitis A virus from bottled water was proven to be equal or better to electropositive filters [7]. In addition, CIM monoliths have also been successfully applied to the concentration of plant viruses such as tomato mosaic virus [8]. All together these facts confirm CIM monoliths as suitable and promising technology for virus concentration from water samples, what is further supported by the increasing use of CIM monoliths in other virus applications, such as, the purification and in-process control of different viruses and phages [9–13].

In Table 1 it can be seen that the CIM QA concentration step can, in combination with RT-qPCR, detect the presence of rotavirus where RT-qPCR alone was unable. The presence of rotaviruses was detected mainly in river water samples. The RT-qPCR assay used in this work has proven ability for the detection of human, bovine and porcine rotaviruses [16], thus, it was not surprising to observe positive results in the rivers from Tables 1 and 3, especially if we take into account that such rivers are subjected to both urban and agricultural runoff. Among the tap water samples from Table 1, only the Črnuče sample collected during the floods of October 2007 was rotavirus positive after concentration, while the same sample location analyzed 16 days later, as well as Gameljne sample, were negative even after concentration. These facts support the developed CIM based method as a useful tool for the detection of rotavirus and prevention of potential outbreaks.

An added value for a technique dealing with the concentration and detection of viruses in environmental samples is the potential to perform the analysis on-site. This reduces the time from sampling to result and facilitates the adoption of fast measures to prevent a potential risk, while further analysis can be performed later on in the laboratory. In order to adapt the developed method to on-site requirements, special emphasis was given to the reduction in time and to the use of portable devices. For the RT-qPCR analysis, the two-steps RT-qPCR format was adapted to one-step format, and the reaction was performed in aportable qPCR device. This adaptation did not compromise the assay's specificity (data not shown). Such disposition was tested for the RT-qPCR analysis of samples shown in Table 3 with satisfactory results. For CIM QA concentration, a transportable Milton Roy LMI dosing pump for pumping, in combination with a Knauer Smartline modular preparative UV detector for detection were used. Rotavirus detections in water samples collected, concentrated and analyzed on-site were successful and allowed to detect at least as low as 30 particles/ml of rotavirus as deduced from the results with spiked water samples (Fig. 2). The limit of detection may, however, be even lower, because in the non spiked water sample Glinščica side stream, rotaviruses were detected at higher qPCR threshold cycles (Ct) than in the spiked one, indicating a lower initial concentration. The detection of rotaviruses in that side stream, which flows to the main Glinščica stream from the ZOO, allows not only the localization of a most probable animal rotavirus source, but also serves as proof of principle for the on-site rotavirus concentration and detection potential of the CIM QA and RT-qPCR based method. Next steps should go in the direction of designing a user-friendly on-site adapted device for the CIM-based concentration, as well as for the molecular detection. Isothermal amplification procedures, such as loop mediated isothermal amplification (LAMP) [17,22], enable target amplification without the need for expensive thermocylers. Such amplification approach was already developed for animal rotavirus [22]. In addition, LAMP amplification products can be easily detected in a lateral flow device [17], simplifying even more the methodology.

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References

[1] R. Bishop, J. Gastroenterol. Hepatol. 24 (2009) 81.
[2] T.T. Fong, E.K. Lipp, Microbiol. Mol. Biol. Rev. 69 (2005) 357.
[3] C.N. Haas, J.B. Rose, C. Gerba, S. Regli, Risk Anal. 13 (1993) 545.
[4] G.M. Schiff, G.M. Stefanovic, B. Yg, J.K. Pennekamp, in: J.L. Melnick (Ed.), Enteric Viruses in Water, vol. 5, Karger, Basel, 1984, p. 222.
[5] A.P. Wyn-Jones, J. Sellwood, J. Appl. Microbiol. 91 (2001) 945.
[6] I. Gutiérrez-Aguirre, M. Banjac, A. Steyer, M. Poljšak-Prijatelj, M. Peterka, A. Štrancar, M. Ravnikar, J. Chromatogr. A 1216 (2009) 2700.
[7] K. Kovac, I. Gutiérrez-Aguirre, M. Banjac, M. Peterka, M. Poljšak-Prijatelj, M. Ravnikar, J.Z. Mijovski, A.C. Schultz, P. Raspor, J. Virol. Methods 162 (2009) 272.
[8] J. Boben, P. Kramberger, N. Petrovič, K. Cankar, M. Peterka, A. Štrancar, M. Ravnikar, Eur. J. Plant Pathol. 118 (2007) 59.
[9] P. Kramberger, N. Petrovič, A. Štrancar, M. Ravnikar, J. Virol. Methods 120 (2004) 51.
[10] P. Kramberger, M. Peterka, J. Boben, M. Ravnikar, A. Štrancar, J. Chromatogr. A 1144 (2007) 143.
[11] R.J. Whitfield, S.E. Battom, M. Barut, D.E. Gilham, P.D. Ball, J. Chromatogr. A 1216 (2009) 2725.
[12] F. Smrekar, M. Ciringer, M. Peterka, A. Podgornik, A. Štrancar, J. Chromatogr. B 861 (2008) 177.
[13] P. Kramberger, R.C. Honour, R.E. Herman, F. Smrekar, M. Peterka, J. Virol. Methods 166 (2010) 60.
[14] A. Jungbauer, R. Hahn, J. Chromatogr. A 1184 (2008) 62.
[15] A. Podgornik, A. Štrancar, Biotechnol. Ann. Rev. 11 (2005) 281.
[16] I. Gutiérrez-Aguirre, A. Steyer, J. Boben, K. Gruden, M. Poljšak-Prijatelj, M. Ravnikar, J. Clin. Microbiol. 46 (2008) 2547.
[17] H.E. James, K. Ebert, R. McGonigle, S.M. Reid, N. Boonham, J.A. Tomlinson, G.H. Hutchings, M. Denyer, C.A. Oura, J.P. Dukes, D.P. King, J. Virol. Methods 164 (2010) 68.
[18] Y.Z. Zheng, R. Webb, P.F. Greenfield, S. Reid, J. Virol. Methods 62 (1996) 153.
[19] S. Jiang, R. Noble, W. Chu, Appl. Environ. Microbiol. 67 (2001) 179.
[20] J. Olszewski, L. Winona, K.H. Oshima, Can. J. Microbiol. 51 (2005) 295.
[21] S.R. Wickramasinghe, B. Kalbfuss, A. Zimmermann, V. Thom, U. Reichl, Biotechnol. Bioeng. 92 (2005) 199.
[22] M. Nemoto, H. Imagawa, K. Tsujimura, T. Yamanaka, T. Kondo, T. Matsumura, J. Vet. Med. Sci. 72 (2010) 823.