HUMANITARIAN INNOVATION FUND
Final Report

Organisation Name	University of Barcelona

Project Title	Development of an affordable point-of-use test for detection of enteric viruses and viral fecal indicators in water
Problem Addressed / Thematic Focus	Viral contaminants in water, such as HEV, may be present even in very low concentrations or absence of bacteria. If the analysis of virus in water is made easier, water sources and treatments may be better characterized reducing the transmission of viral infections in areas where water-borne viral outbreaks are identified.
Location	Barcelona
Start Date	01/01/2015
Duration	18 months+ 6 months extension
Total Funding Requested	£146.911

Partner(s)	£17.400
Total Funding	£164.311

Innovation Stage	**Development** of an equipment for the analysis of virus in water
Type of Innovation	Product innovation
Project Impact Summary	The developed portable low-cost, easy to use, equipment for the analysis of viruses in water, provides the organizations involved in water sanitation with an affordable system for detecting the presence of viral pathogens and viral contamination in water. The equipment, tools and protocols developed will facilitate the improvement of water safety management practices contributing to the reduction of the incidence of viral

	diseases in humanitarian crisis contexts.

Reporting Period	01/01/2015-31/12/2016
Total Spent	£140.722

PROJECT ACTIVITIES AND OUTPUTS

What have been the key achievements of the project?

1. A low-cost equipment and a protocol for the analysis of viruses in water, has been adapted to be portable and applied to the point of use.

2. The first step of this protocol is a concentration method developed for viruses useful for the concentration of viruses, bacteria and protozoa and that may be applied to the study of pathogens in all types of water.

3. A magnetic nucleic acids extraction protocol and PCR assays were adapted to be used without power supply or large laboratory equipment.

4. Single-tube nested-PCR assays for the detection of human adenoviruses using a visual colorimetric assay (gold nanoparticle-conjugated probes) was developed and is useful for the detection of viruses in sewage, although it is not enough robust for the detection of the low concentrations of viruses expected in source and drinking water.

5. A cleansing step with a detergent containing a diversity of surfactants, and oxidative products showed to remove viruses from some materials tested. These results suggest the interest of using this type of detergents for improving hygiene and sanitation practices in the context of viral outbreaks in humanitarian crisis.

6. The developed method can be performed at the point-of-use, for non-experimented users, by following the provided tools and instructions.

What were the major activities and outputs of the project (this may include a description of the activities conducted and how they related to the work plan)?

Development of the innovation:

According to the work plan, the activities are distributed in 4 workpackages.

Workpackage 1. Development of an innovation which integrates a concentration, NA extraction and detection methods for evaluation of the presence of Human adenoviruses (HAdV) and Hepatitis E Virus in-situ

1.1. Concentration protocol

As described in the proposal, efficient and low-cost protocols for the concentration of viruses have been developed in the laboratory and validated in international studies. Those protocols have been adapted to be used at the point-of-use.

1.1.1. Protocols tested

Different low-cost methods, described in the literature, based on membrane filtration or flocculation have been assayed.

- An electronegative membrane method was tested for the concentration of viruses from 10 litre water samples (Haramoto et al., 2014). Viruses were spiked in water samples with $MgCl_2$ as caution solution and adsorbed on an electronegative membrane, a mixed cellulose ester membrane with a pore size of 0,45µm (Millipore). Viral nucleic acids were then extracted directly from membrane and analysed by qPCR. Viral recoveries using $MgCl_2$ and spiking 4 x 10-liter ground water samples with HAdV and the process control (bacteriophage MS2) were low and showed a low level of repeatibility. Only two out of four samples presented measurable viral recoveries, with a mean value of the recovery in the positive samples of 9%.

- A low-cost organic flocculation, that uses a skimmed milk to adsorb the viruses and concentrate them in a small volume sediment (Calgua et al., 2013)., was analysed and the possibility of reducing time and avoiding a final centrifugation step were analysed. Briefly, one hundred millilitres of flocculated skimmed milk solution (1% (w/v)) is added to previously acidified (pH 3.5) 10-liter water samples. Samples are stirred for 8 h at room temperature, and the flocks are allowed to settle by gravity for another 8 h. The supernatant is removed and the sediment collected and transferred to a 500 mL centrifuge container and centrifuged at 8000 ×g for 30 min at 4 °C. The obtained pellet is suspended in 5 mL and frozen -80°C until the extraction of nucleic acids and the analysis of virus. This method has been recently characterized as very useful for viruses and also for bacteria and protozoa in the paper: Characterization of the efficiency and uncertainty of skimmed milk flocculation for the simultaneous concentration and

quantification of water-borne viruses, bacteria and protozoa (Gonzales-Gustavson, et al. 2017).

Based on this skimmed milk flocculation method routinely used in our laboratory, several steps were evaluated for adaptation to the use in the field, e.g. substitution of hydrochloric acid that is difficult to transport by citric acid to be used for the acidification of water samples. Two main modifications were studied among other minor technical details:

i) Substitution of the sedimentation step required for the flocks to be in a pellet (takes about 8 hours) by a quickest filtration protocol. Four different membranes were tested: nitrocellulose filters of 0.45 and 2 um, polycarbonate filters of 5 um and nylon 10 µm filter. This filtration would substitute the sedimentation and the centrifugation step concentrating all flocks with the viruses in the filter. The 10 µm filter showed best performance, and was considered promising, however after more data was accumulated and after the field studies, the conclusion was that the size of the flocks could be variable and mean recovery values were low and even more important showed low repeatability. The results of 21 different experiments, showed levels for MS2 phage, Rotavirus and Human adenovirus of 1.68% (SD 0,8), 1.42% (SD 1,7) and 5.14% (SD 3,7) respectively.

ii) Modification of the published Skimmed Milk Flocculation protocol, collecting the flocculated sediment with the viruses and extracting directly from this concentrate the viral nucleic acids. This modification enabled higher recoveries of viruses from water similar to what has been previously published, a range between 13 to 37% without using a centrifugation step.

1.1.2. Avoiding cross-contamination between samplings when re-using some materials

In the initial prototypes that included a filtration step in order to facilitate the possibility of reusing the equipment and to avoid cross contamination between samples, various tests were performed to find the most feasible and efficient cleaning method. The studied treatments included: thermal treatments, hot water (95°C), and chemical treatments (0,5L- 1L of Rely+On™ Virkont® (Antec Inernational, Ref, 60007), soap powder (0,4 w/v), chlorine solution (200 ppm). The tests performed with Ariel™, a detergent containing a mixture of chemical compounds as surfactants, hydrogen peroxide, or percarbonate, which were described as efficient for virus disinfection by Heinzel, et al. (2010), showed a complete cleansing of the system in spiking experiments. The washing powder Ariel™ at 4w/v with water was the most effective, cheap and with less risk for the handler.

1.1.3. Stability of viruses in the sample concentrates at room temperature before its nucleic acid extraction and detection.

The flocks obtained after filtration have to be preserved for the final detection of the viral particles. Thus, the stability of the concentrated viruses at room

temperature for further analysis was studied by adding viral preservatives or nucleic acid preservatives after the viral lysis. The three viral preservatives tested were: sodium benzoate 2% with calcium propionate 0,2% (Sigma Aldrich) and Sodium Azida 0,1% (Sigma Aldrich). The results showed significant decreases for both DNA and RNA viruses (Figure 1).

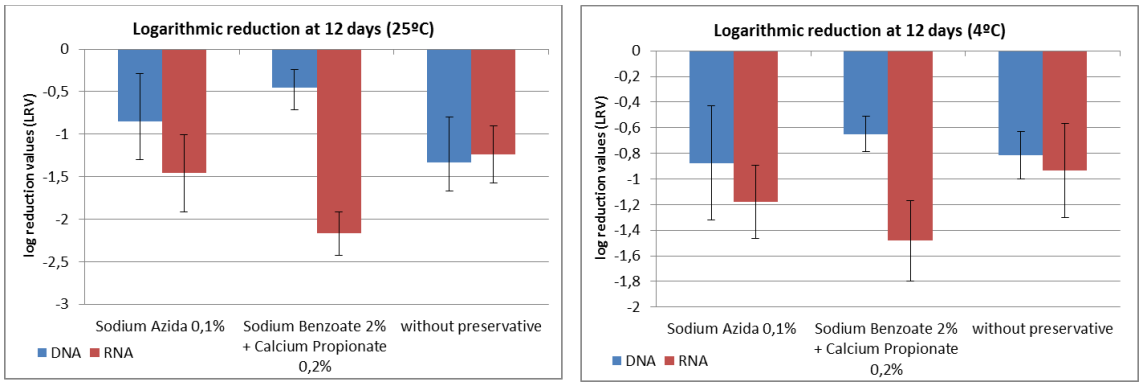


Figure 1: Viral concentrations expressed as logarithmic reduction values after 12 days at 25°C (left) and 4°C (right) with three different preservatives: sodium azida, sodium benzoate with calcium propionate and without preservative

The addition of a nucleic acid preservative, DNA/RNA Shield™ (Zymo Research), maintained the viral titres after 9 days of storage at 25°C. No significant reduction of viral genomic copies was detected at different times and temperatures, for the high efficiency proved, this preservative is included in the final protocol for keeping the concentrated viruses stable at room temperature before their quantification by q(RT)PCR.

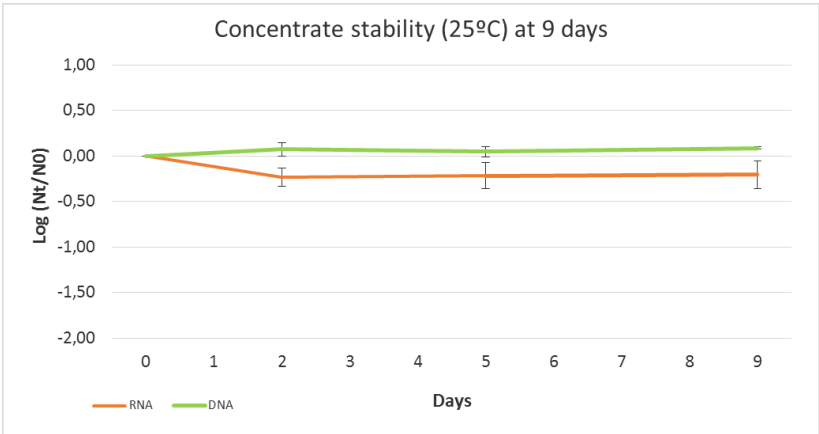


Figure 2: Viral concentrations expressed as logarithmic reduction values after 2, 5 and 9 days at 25°C with viral preservative DNA/RNA Shield™ (Zymo Research)

References:

Calgua, Byron; Fumian, Tulio; Rusiñol, Marta; Rodriguez-Manzano, Jesús; Mbayed, Viviana; Bofill-Mas, Sílvia; Miagostovich, Marize; Girones, Rosina. Detection and quantification of classic and emerging viruses by skimmed-milk flocculation and PCR in river water from two geographical areas. Water research 47 (8), 2797-2810

Gonzales-Gustavson, Eloy ; Cárdenas-Youngs, Yexenia ; Calvo, Miquel ; Da Silva, Marcelle Figueira Marques ; Hundesa, Ayalkibet ; Amorós, Inmaculada ; Moreno, Yolanda ; Moreno-Mesonero, Laura ; Rosell, Rosa ; Ganges, Llilianne ; Araujo, Rosa ; Girones, Rosina. Characterization of the efficiency and uncertainty of skimmed milk flocculation for the simultaneous concentration and quantification of water-borne viruses, bacteria and protozoa. Journal of Microbiological Methods, 2017, Vol.134, pp.46-53

Haramoto, Eiji ; Katayama, Hiroyuki ; Asami, Mari ; Akiba, Michihiro. Development of a novel method for simultaneous concentration of viruses and protozoa from a single water sample. Journal of Virological Methods, 2012, Vol.182(1-2), pp.62-69

Michael Heinzel, Andrea Kyas, Mirko Weide, Roland Breves, Dirk P. Bockmühl. Evaluation of the virucidal performance of domestic laundry procedures. International Journal of Hygiene and Environmental Health 213 (2010) 334–337

1.2. Extraction of nucleic acids

Once the viruses are concentrated from the water samples, nucleic acid extraction is required before the final detection of the viruses. A magnetic-based method was adapted to be performed at room temperature and without any centrifuge. The nucleic acid extraction procedure was tested using silica magnetic particles in order to avoid the need of a centrifuge and of keeping the reagents at low temperatures. Different experiments were carried out to optimize and to determine the sensitivity of this new method compared to the column centrifugation-based extraction method commonly used. The variables tested in these assays included: the RNA carrier molecule, the washing procedures and the incubation times during the adsorption and elution steps.

1.2.1. RNA carrier

Commercial nucleic acid extraction kits include a molecule called carrier to increase recovery and thus yield of DNA and RNA extraction. After the carrier addition, the sample must be kept in cold (4ºC) and thus difficults its application in the field. To evaluate the need of use of an RNA carrier, four water concentrates were extracted by adding different amounts (0, 20 and 68 nanograms) of a poly-A RNA, and subsequently analysed by Real-Time PCR for HAdV and MS2.
The results showed a difference between 0.02 and 0.65 log for a same sample when extracted adding different amounts of carrier, as shown in the Table 1.

A

	Carrier Amount		
HAdV	0 ng	20 ng	68 ng
Sample 1	4,99E+08	1,41E+08	5,12E+08
Sample 2	4,39E+08	9,79E+08	4,96E+08
Sample 3	3,15E+08	2,99E+08	7,28E+08
Sample 4	2,88E+08	4,50E+08	4,84E+08

B

	Carrier Amount		
MS2	0 ng	20 ng	68 ng
Sample 1	1,05E+09	3,65E+08	1,63E+09
Sample 2	1,21E+09	7,95E+08	1,35E+09
Sample 3	9,95E+08	1,40E+09	6,76E+08
Sample 4	9,56E+08	7,01E+08	6,06E+08

Table 1. Quantification values for HAdV (A) and MS2 (B), expressed in genome copies per litre, for the extractions carried out without adding and adding 20 or 68 nanograms or RNA carrier

Although the competition between the carrier RNA and the nucleic acids for the unspecific binding sites suggest a positive correlation between the carrier amount and the nucleic acid recovery, the results obtained don't show a clear tendency into this direction. However, they concur with previously published results (Shaw *et al*., 2009) that indicate that even in the absence of carrier the extraction efficiency reach the theoretical efficiency of 100% when the amount nucleic acid is below 5 ng. Extraction is then perfectly feasible in the absence of carrier.

1.2.2. Washing steps

Washings are the extraction steps that require more manipulation. Keeping the magnetic particles on the tip during the washings makes the manipulation easier, but releasing them into the solution during the washing time could maximize the surface of magnetic particles in contact with the washing solution, which helps to remove cellular and protein debris thus optimizing the washing.

Twelve river water were analysed as representative samples of those that may be tested in the field, the samples were spiked with HAdV and MS2, viruses were concentrated and the nucleic acids were extracted following different washing procedures to determine which provided a higher recovery while being easy to carry out.

i) Procedure A: Releasing the magnetic particles into the solution for 30 seconds.
ii) Procedure B: Keeping the magnetic particles on the tip for 1 minute while leaving the tip still into the washing solution.
iii) Procedure C: Keeping the magnetic particles on the tip for 30 seconds while the tip is shaked in the washing solution.

Eight samples were processed to compare procedures A and B while 4 samples were processed to compare procedures C and D.

A

	Procedure A	Procedure B
Sample 1	2,77E+03	-
Sample 2	3,48E+03	-
Sample 3	2,61E+03	-
Sample 4	2,61E+03	-
Sample 5	5,33E+04	-
Sample 6	4,07E+04	-
Sample 7	2,36E+04	-
Sample 8	5,49E+03	-

B

	Procedure A	Procedure C
Sample 9	2,08E+05	-
Sample 10	3,87E+05	5,74E+01
Sample 11	5,38E+05	-
Sample 12	7,86E+04	9,83E+03

Table 2. Values of quantification for MS2 expressed as genome copies per litre for the extractions carried out through different washing procedures

As shown in the Table 2, the comparison between procedures A and B showed that the procedure A was better, allowing higher recoveries. The same outcome was obtained when comparing procedures A and C. The procedure A was thereby shown to be the better for this extraction method.

1.2.3. Incubation times

The incubation required during the adsorption and elution steps can be performed in the filed either by using an orbital powered by the sunlight (procedure A) or by manual inversion of the tubes (procedure B). These two procedures were evaluated, showing similar results, with a maximum difference in the recovery of 0.52 logs, as shown in Table 3.

	Procedure A	Procedure B
Sample 1	-	-
Sample 2	9,21E+04	2,94E+04
Sample 3	1,03E+05	3,40E+04
Sample 4	1,64E+05	6,81E+04

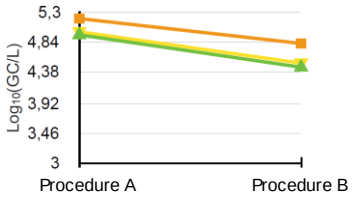


Table 3. Values of quantification for MS2 expressed as genome copies per liter for the extractions carried out through different incubation procedures. ( ) Sample 1, ( ) Sample 2, ( ) Sample 3, ( ) Sample 4

This outcome could be related to the fact that the heating of the elution solution during some nucleic extraction methods carried out in laboratories often improves the efficiency of the extraction, as holding and inverting the tubes with the hands leads the solution to get warmer. Given these results the chosen procedure was the use of a solar-powered orbital, since its use is more standardisable than the manual inversion, which is depending on each person.

1.2.4. Description and validation of the magnetic nucleic acid extraction protocol

The presented results have allowed the development of a nucleic acid magnetic extraction protocol, which has been validated regarding the standard method used in the laboratory (Figure 3 and 4).

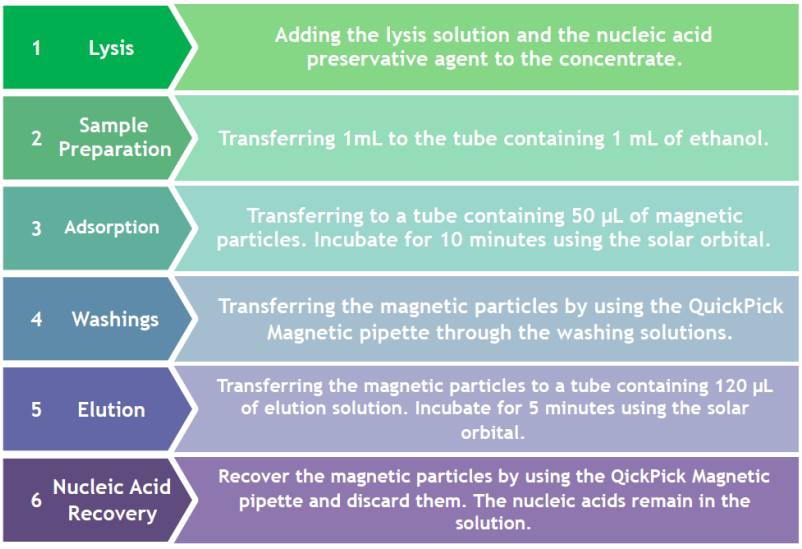


Figure 3. Schematic representation of the steps for the developed nucleic acid extraction method

The extraction starts from the tubes containing the concentrate. Using a disposable Pasteur pipette 1 mL of the concentrate is added to a 5 mL tube containing 1 mL of ethanol, which, as a caotropic agent, increases the binding of the nucleic acids to the silica. The volume of ethanol plus the concentrate is homogenized by inversion for 10 seconds and then transferred to another 5 mL tube containing 50 µL of magnetic particles (QuickPick™XL gDNAMagnetic Particles, BioNobile). The solution is then incubated for 10 minutes at room temperature to homogenize it by using a solar-powered orbital. After the incubation the magnetic particles are recovered by using a pipette that has a magnet at the end of the plunger and uses disposable plastic tips (QuickPick Magnetic Tool and QuickPick Tips, BioNobile). When pressing the plunger the magnetic particles in the solution are attracted to the tip. The magnetic pipette is then moved away from the tube to the tube containing the first washing solution. The plunger is then retracted so the magnetic particles are released into the tube containing 500 µL of the first washing solution (Washing Buffer BAW, BioTools). This solution is composed of caotropic agents, which allow removing the contaminants the solution may have. The magnetic particles are kept for 30 seconds in the solution shaking it with the pipette. The same procedure is followed for the second and third washing solutions, containing 600 and 200 µL of the second and third washing solutions (Washing Buffer BAV3, BioTools). After the third wash the magnetic particles are transferred to a tube containing 120 µL of a low-ionic power elution solution (BRE Elution Solution, BioTools). The tube is incubated at room temperature for 5 minutes, continuously mixing the solution by using a solar-powered orbital, so that the nucleic acids are released from the magnetic particles. The magnetic particles are then recovered using the magnetic pipette and then discarded, while the nucleic acids remain in the solution ready to proceed with the detection step.

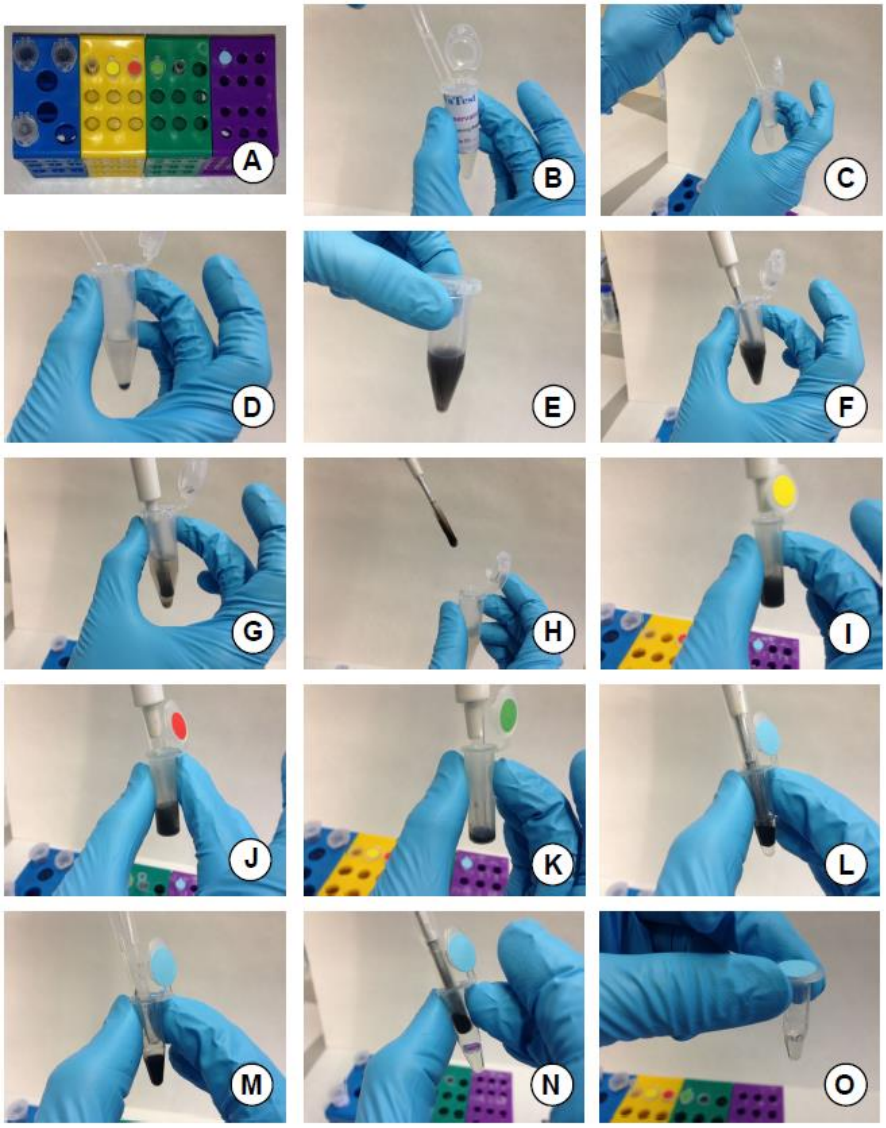


Figure 4. Nucleic acid magnetic extraction method implemented for the VirWaTest project: (A) Tubes containing the samples, the ethanol, the magnetic particles, the washing solutions and the elution solution (B, C) Adding the concentrate to the tube containing the ethanol (D) Adding the concentrate + ethanol to the tube containing the magnetic particles (E) Concentrate + Ethanol + Magnetic Particles (F, G, H) Magnetic particles gather around the tip when the plunger is pressed (I) Transferring the magnetic particles into the first washing solution (J) Transferring the magnetic particles to the second washing solution (K) Transferring the magnetic particles to the third washing solution (L) The magnetic particles are transferred to the tub containing the elution buffer (M) The plunger is retracted, releasing the magnetic particles into the solution (N, O) The magnetic particles are removed from the solution by using the magnetic pipette while the nucleic acids remain in the solution

For this validation, a total of 33 river and well water samples were extracted using both methods. Reverse Transcription Quantitative Real-Time PCR were performed for HAdV and MS2 respectively. The results obtained from these validation assays show that the amount of nucleic acids recovered after the extraction, expressed as genome copies per 10 litters, was higher for the magnetic extraction method over the standard extraction method for 23 out of the 33 samples, when analysed for the detection of HAdV. When analysed for the detection of MS2, again 23 out of 33 samples showed higher recoveries for the magnetic extraction method, although not all these 23 samples matched the 23 ones from the detection of HAdV. The high SD observed in Table 4 is related to the low level of repeatibility of the first concentration prototype used in the study.

	Column-Based Extraction	Magnetic Extraction
Median	2,53E+03	2,43E+03
Mean	1,74E+04	3,12E+04
SD	5,66E+05	2,23E+06

	Column-Based Extraction	Magnetic Extraction
Median	4,74E+04	5,13E+04
Mean	4,24E+04	5,97E+04
SD	4,49E+04	1,63E+05

Table 4. Quantification values, expressed in genome copies per 10 liters, for HAdV (left) and MS2 (right) when performing either the standard method used in the laboratory (column-based method) or the magnetic extraction method developed for the VirWaTest project

Overall, the recoveries obtained with the magnetic extraction method are comparable to those obtained with the column-based method, making it a suitable method to be used when performing the extraction in the field in place of the column-base method, which needs a centrifuge.

References:

Shaw KJ, Thain L, Docker PT, Dyer CE, Greenman J, Greenway GM, Haswell SJ. The use of carrier RNA to enhance DNA extraction from microfluidic-based silica monoliths. Anal Chim Acta. 2009 652(1-2):231-3.

1.3. Detection and quantification of viruses

In the experiments related to the detection method, isothermal amplification, PCR and nested-PCR and q(RT)PCR assays were tested.

1.3.1. Isothermal amplification

Experiments for the development and application of isothermal amplification techniques, specifically Loop-mediated isothermal amplification (LAMP) for the detection of Human adenovirus (HAdV) and Hepatitis E virus (HEV) have been performed considering previously published studies (Ziros et al. 2015; Chen et al. 2014). Alignment of 68 types of human adenovirus and design of a diversity of primers, as well as the application of previously described protocols to the

detection of diverse HEV strains, showed finally that LAMP was useful for specific strains but it was not feasible to use a LAMP test for the detection of all human adenoviruses or all human HEV strains.

1.3.2. PCR assays with reagents stable at room temperature

A PCR protocol was developed using HAdV, a human pathogen also used as viral indicator of human fecal contamination in water. Mixtures of dried reagents were prepared containing Taq DNA polymerase, $MgCl_2$, dNTPs, KCl and Tris-HCl.

1.3.2.1 Stability of primers

The stability of the primers in a dried state was evaluated to determine their structure and activity stability over time. PCR tubes, containing with dried reagents at their bottom and serial viral genome copies dilutions, were tested at times 0, 3 and 4 weeks after preparation. The results showed that drying and rehydrating process doesn't affect negatively the primer integrities. The reagents can be stored dried for at least 4 weeks (Figure 5).

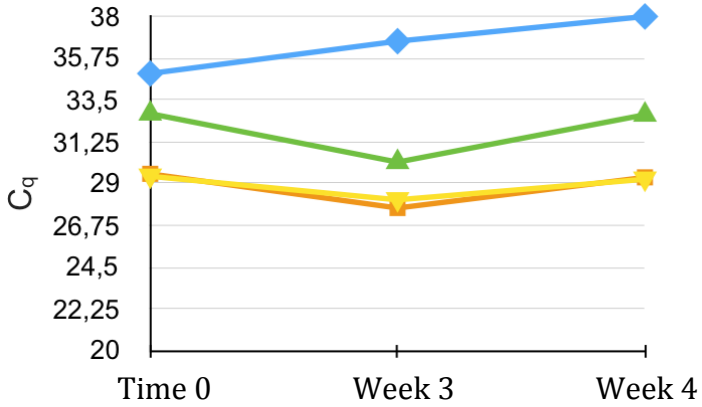


Figure 5. Cq values for the different HAdV standard dilutions using dried primers and probe. (◆) 1E+01 Standard, (▲) 1E+02 Standard, (▼) 1E+03 Standard 3, (■) 1E+03

1.3.2.2 Single-tube nested PCR and colorimetric test

In order to increase sensibility when analyzing low concentration of viruses in water samples, a "two round" PCR or nested PCR assay (nPCR) in a single-tube was also considered (da Silva et al., 2013). Performing the nPCR assay in a single-tube minimizes the possibility of a cross-contamination event and simplifies manipulation. This assay was coupled to a colorimetric test in order to read the results.

The single-tube nPCR assay was achieved by drying separately the internal and the external primers, at the bottom and at the lid of the tube respectively. After the first amplification step the tubes were inverted by hand thus resuspending

the external primers into the solution and then the second amplification step started.

The colorimetric assay was based on the use of a gold nanoparticle-conjugated probe that binds specifically to a region of the amplicon of the HAdV nPCR assay. While the probes tend to aggregate in high ionic strength conditions (achieved through the addition of $MgCl_2$) leading the solution to acquire a light-purple color, the binding of the probes to their complementary regions within the amplicons stabilizes them thus preventing the aggregation and leading the solution to acquire a dark-pink color. Different nPCR product volumes and gold-conjugated probes concentrations were tested (Figure 6).

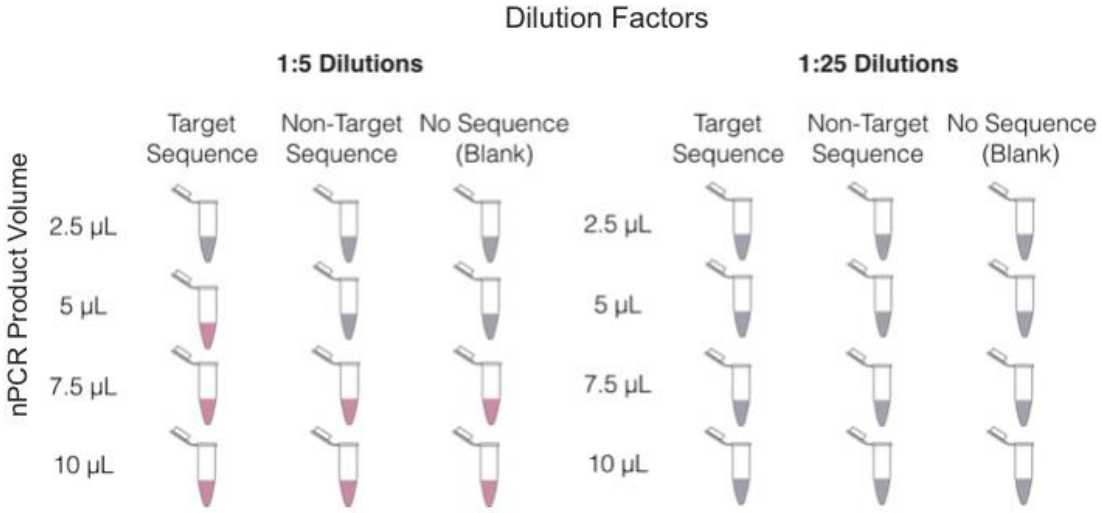
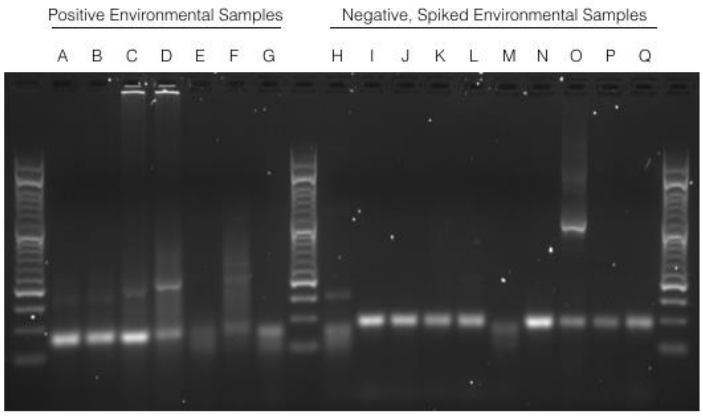


Figure 6. Colour of the solution after adding the probe and the $MgCl_2$ for the different volumes and dilution factors tested. (■) Positive, (■) Negative

The colorimetric solutions were additionally measured by spectrophotometry to set the minimum absorbance ratio between the aggregated (max. Abs at 585 nm) and the not aggregated (max. Abs at 525 nm) species. A Abs_{525}/Abs_{585} ratio over 1 indicates that the predominant species is the non-aggregated, while a ratio below 1 indicated that the predominant species is the aggregated. After running the nPCR all the products were separated by agarose gel electrophoresis so the results could be compared with those from the colorimetric assay (Figure 7). After comparing the results with those from the electrophoresis the volume and dilution factor that proved to be optimal were 5 µL and 1:5.



Sample	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
Gel Electrophoresis	+	+	+	+	-	-	-	-	+	+	+	+	-	+	+	+	+
Colorimetric	-	-	+	+	-	-	-	-	+	+	+	+	-	-	+	-	+

Figure 7. Results from electrophoresis and detection though a labeled probe for positive environmental samples and negative environmental samples spiked with HAdV standard

Colour-observation, spectrophotometry and gel-electrophoresis results showed 100% coincidence when comparing results in sewage samples (data not shown), and 76% of coincidence when analysing low contaminated samples (e.g. ground water or river water) (Figure 7). Considering that low concentrations of viruses are commonly detected in water samples, the colorimetric test described, although interesting and useful for sewage samples, was not incorporated to the final protocol for the analysis of viruses in water.

1.3.2.3 Quantitative (RT) PCR and low-cost qPCR equipment

The final detection and quantification of viruses, human adenoviruses and hepatitis E virus, is achieved using the tubes with dried primers and probes developed as described in the previous section and a quantitative PCR reaction using commercial kits with reagents that will be stable at room temperature. A search of different companies, products and availabilities has been done. Initial selected kits were not manufactured after several months and new kits were tested in the laboratory that may be used at room temperature if the production continue. Facilities as a small refrigerator would facilitate the choice of commercial kits for the q(RT)PCR.

The reagents needed to do Quantitative PCR assays are often supplied as a solution containing all the components mentioned above needed for the amplification. These pre-mixed reagents are easy to use but require to be stored at -20ºC. This requirement makes them unsuitable for using them in the field. These reagents thereby have been replaced by lyophilised formats. The lyophilisates can be transferred to tubes containing the dried primers and probe, so there is only the need to resuspend both with nuclease-free water and then add the sample volume, thus making this process easier.

Regarding the amplification of DNA the TaqMan® Fast Environmental Master Mix Beads (ThermoFisher Scientific) were chosen since they are optimized for environmental samples, which contain high amounts of PCR inhibitors. The results of the assays carried out comparing them with the reagents used at the laboratory showed that they showed equivalent sensitivity, both when amplifying synthetic DNA and real samples. These lyophilized beads have been discontinued a short time ago by the manufacturer, and other alternatives have been evaluated. The qPCR ProbesMaster lyophilisate from Jena Bioscience has been evaluated with HAdV standard and with environmental samples obtaining variable results, since most of the available lyophilisates in the market are not optimized for environmental samples. The illustra PuReTaq Ready-To-Go PCR beads from General Electric, that contain Bovine Serum Albumin which contributes to decrease the effects of some inhibitors, have also been tested, resulting in similar values, all within the same logarithm, both for wastewater samples and environmental samples when comparing the standard method with the General Electric beads both adding and without adding the ROX dye to normalize the fluorescence been this a useful alternative for performing qPCR at the point of use.
Regarding the amplification of RNA, there are some kits available in the market, but many of them are either not optimised for Quantitative PCR or the lyophilised is not distributed in PCR tubes. Therefore we tested the RTqPCR Oasig™ lyophilised 2X qPCR Mastermix (Primerdesign). This kit is based on a mastermix lyophilisate that includes all the reagents needed for the amplification and that is stable for 18 months at room temperature. After resuspending the lyophilisate, it must be kept in the freezer in order to preserve its activity. This kit was combined with HEV-specific primers and probe dried at the bottom of the PCR tube as done in the qPCR for HAdV. This kit was then compared with the reagents used at the laboratory (RNA UltraSense™ One-Step Quantitative RT-PCR System, Applied Biosystems), which doesn't include the different reagents in a same mastermix. The amount of genome copies per liter detected by using the UltraSense kit was between 3E+05 and 1E+06 for the 6 samples tested, while these values for the Oasig kit were between 1E+07 and 5E+07, therefore seeming to be more sensible than the UltraSense kit. The RNA to cDNA EcoDry Premix (Takara Bio) was also tested, combined with the DNA the TaqMan® Fast Environmental Master Mix Beads (ThermoFisher Scientific), allowing to detect from 1E+06 to 7E+06 genome copies per liter for this same 6 samples. These values, however, were only obtained for the 1:10 diluted samples, while the direct ones were negative, suggesting a inhibition effect, making the Oasig kit more convenient and the selected kit to be used for the point of use assays.

The small company collaborating in this project IUL S.A. is interested in developing ready-to-use tubes stable at room temperature for the q(RT)PCR for the quantification of both viral pathogens, HAdV used also as indicator of human contamination and the HEV, and if their work is successful will also be applicable to other viral pathogens.

The developed protocol allows the concentration of the viruses from water and preparing the viral nucleic acids to be:

i. Analysed using a stable commercial kit and a qPCR with a battery at the point of use. The qPCR mini-8 qPCR with battery from Coyote Bioscience Company Co., Ltd has an affordable cost and is available to be used with this innovation.
ii. Send the concentrated or the extracted nucleic acid with the selected preservative at room temperature to a laboratory having the PCR equipment, many laboratories all over the world may be able to detect or quantify the viruses using a PCR or a q(RT)PCR equipment also used in clinical hospitals.

The final objective of this WP has been achieved and Standard Operational Protocols for the innovation have been prepared and converted to easy-following brochures.

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Workpackage 2. Evaluation of the performance of the developed/adapted methods

Experimental results related with the performance of the concentration and the detection efficiency of the methods developed have been described in the previous sections. The equipment and protocols to be used in field conditions have been prepared and an initial prototype has been distributed for validation in two different countries.

The method for virus concentration in water does not require sophisticated laboratories or specialized staff, the equipment has been adapted to the field requirements and will be delivered in one transportable package with detailed protocols and all reagents and material needed. The protocol finally defined at the end of the project, with a simple organic flocculation of the 10L water sample using skimmed milk, collection of the sediment produced that is where all viruses will be, the concentrated of the viruses (and/or other pathogens) and this sediment is used for the direct extraction of the nucleic acids and detection of viruses.

This protocol shows a very high simplicity and good efficiency and repeatability levels and has a limit of sensitivity of the assay of 415 viral genomic copies/L; this limit of detection may be reduced by testing larger volumes of the concentrates.

Alternatively viral concentrates or extracted viral nucleic acids may be analysed with new commercially available instruments recently developed, presenting however all of them higher costs that the nucleic acid extraction protocol developed in this study and the test in the qPCR equipment.

Moreover, the method has been demonstrated in studies developed in parallel in our laboratory during this project, useful for the concentration as expected of other viral pathogens as rotavirus, but also bacteria and protozoa (Gonzales-Gustavson et al., 2017). The efficiency of the method has been validated in environmental samples also as a method of identification of the source of the fecal contamination by microbial source tracking (MST) detecting viral indicators of humans and animal contamination in water (Rusiñol et al. 2014).

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Workpackage 3. Construction of a prototype of the innovation

A protocol has been developed and a prototype for the detection of viruses in field in humanitarian settings has been created. The VirWaTest kit consists in portable and affordable equipment for the detection of 3 samples simultaneously by organic flocculation. The equipment is divided in 3 parts, each part containing:

1. Concentration protocol:

- Materials (figure 8): 10L containers (x7), a holder (x3), peristaltic pump (x1), magnetic stirrers (x3), outlet tube (x1), 2 batteries and 1 car adaptor, glass magnets (x12), laboratory tweezers (x1), 100ml container (x2), 500ml container (x1), calibrated stainless steel spoons (x1) and marker (x1).

Preliminary prototypes included a step of filtration of the flocculated water samples and consequently a filter holder (x1), fixing key 105mm (x1), and a filter holder support; last refined prototypes do not include this filtration process.

Except the containers, tweezers, calibrated stainless steel spoons and the marker, all the materials have been designed and produced by our collaborator in the project GenIUL.

Figure 8. VirWaTest material in the initial prototype (last version of the prototype has been simplified without the filtration step)

- Consumables & Reagents: disposable Pasteur pipettes (x10), pH indicator strips pH 2,8-4,4, collection tubes (x9), preservative tubes (x9), sachets of pre-flocculated skimmed milk (PSM) (x3), Sodium hydroxide (NaOH 4%) (x2), gloves of different sizes (x6), artificial seasalts (170g), citric acid 1-hydrate (130g). Preliminary prototypes included also : Nylon net 10µm membrane Millipore ref, NY100900 (x10), soap sachet (6), neutralizing soap sachet (x6), all these material is not needed in the last version of the prototype.

2. Extraction:

- Materials: Rota-Tube Rack (x1), Magnetic pipette (x1), Solar-revolving platform
- Consumables & Reagents: Tips, ethanol tubes (x9), magnetic particles (x9 reactions), washing tubes (x9 reactions), elution tubes (x9 reactions)

3. Detection:

First Prototypes sent did not include the qPCR reagents. Final detection has been performed in laboratories after shipment from the three validation sites. Instructions, tools and reagents are also available for the complete extraction of nucleic acids in the field.

A VirWaTest user manual has been written in an easy-to-read way, in English, in French and in Spanish. A tutorial video on the initial prototype and methods, available in English and French, has been created and uploaded in the VirWaTest webpage (www.virwatest.org) for further information and consultation.

Workpackage 4. Evaluation of the innovation performance in the laboratory at under point-of-use conditions by expertise and non-expertise users

4.1. Validation partners

1. Central African Republic, by OXFAM , non-experts in the method: Oxfam Wash personnel was trained for the use of the VirWaTest protoype and a suitcase containing the concentration prototype was sent to Bangui, African Central Republic (RCA) to perform validation tests, one of them in September of 2016, and the other in March of 2017. The responsible was Jean-Baptiste Richard (Wash advisor for West Africa, OXFAM).

2. Equator, by an expert user Laura Guerrero, trained at the laboratory of the University of Barcelona: Another suitcase containing all the material necessary for performing the entire process from sample collection to viral detection were sent to Dr. Guerrero from La Universidad de las Américas (UDLA) in Quito, in February of 2017.

Figure 9. Suitcases sent to RCA and Ecuador

4.2. Field validation program

The VirWaTest method is designed for his application in humanitarian settings. For this reason, the field validations have been performed in two different scenarios: Bangui (Central African Republic, RCA) and Pedernales (Ecuador).
A designed program for the validation users was established:

Day 0: Training on the use of the VirWaTest kit (showing kit, reading protocol and watching video) and planning the sample program

Day 1: Groundwater (sampling and flocculation)

- Morning: Choose one source of groundwater used as a drinking water (before treatment). Take 3 samples of 10 Litres each to concentrate in parallel.
- Afternoon: Condition samples (pH, conductivity) and start flocculation

Day 2: Groundwater (filtration)

- Morning: Filters 3 samples (max. 20 hours of flocculation) and collect concentrates.

Day 3: Surface water (sampling and flocculation)

- Morning: Choose one source of surface water with a potential use of drinking water, cooking or irrigation proposes (<30 NTU). Take 3 samples of 10 Litres to concentrate in parallel.
- Afternoon: Condition samples (pH, conductivity) and start flocculation

Day 4: Surface water (filtration)

- Morning: Filter 3 samples (max. 15 hours of flocculation) and collect concentrates.
- Afternoon: Fill the questionnaire on usage of VirWaTest kit and discuss its potentialities and weaknesses.
- Only for the validation in Ecuador: all concentrates obtained were stored in a cool dry place (<25ºC) before shipping. Samples arrived in Barcelona for their final detection in the Vircont laboratory (University of Barcelona)

Day 5: Extraction (only for the validation in Ecuador)

- Morning: Training on the use of the VirWaTest Extraction kit (showing kit and reading protocol).
- Afternoon: Perform the extraction of the 6 concentrates plus an Extraction Negative Control (ENC, 1 ml of distilled water).
- Fill the questionnaire on usage of VirWaTest Extraction kit and discuss its potentialities and weaknesses.

Day 6: Detection (only for the validation in Ecuador)

- Morning: Training on the use of the VirWaTest Detection kit (showing kit and reading protocol).
- Afternoon: Perform the detection of the 7 eluates plus a Negative Control of PCR (NCPCR, follow protocol).
- Fill the questionnaire on usage of VirWaTest Extraction kit and discuss its potentialities and weaknesses.
- The remaining volume of the concentrates after the extraction and the extractions obtained in Quito was sent to Barcelona laboratory for their final detection.

Figure 9: Pictures from the validation in Pedernales, Ecuador

4.3 Validation results

All questionnaires on usage from the first users of VirWaTest Kit have been useful to detect weakness of the different parts of the method, including:

- understanding of the written protocols and the video tutorial.
- feasibility of application of the method,
- difficulties that different users have had,
- robustness of the materials
- preservation of the reagents.

This information has been useful to improve the equipment and protocol, considering the complexity of the entire process. Table 5 summarises the results obtained in the validations. Human adenoviruses have been detected in 3 water samples in Pedernales, Equator and in one water sample in Bangui, RCA, all indicating the presence of human viruses in the water samples analysed.

		internal control	Viral indicator of human pollution	
date	location	MS2 recovery (%)	HAdV (CG/L)	
September 2016	Bangui, RCA	1,48%	n.d.	(0/6)
February, 2017	Pedernales, Equator	7,22%	3,82E+01	(3/5)
March, 2017	Bangui, RCA	n.d.	3,64E+02	(1/5)

Limit of Detection (LOD):

HAdV	*4,16E+01*	*GC/L*
MS2	*4,63E+02*	*GC/L*

Table 5: Viral results from three validation programs

What adjustments and adaptations were made through the course of the project? Why were these needed and how were these made?

Considering all information collected from the characterization and the validation sections, the final protocol and the final equipment has been simplified in the last phase of the project removing the filtration step that introduced high variability in the recoveries. The final SOP shows higher and more consistent recoveries.

We have faced problems with some of the tested temperature stable PCR reagents that have been removed from production. The availability of room temperature PCR reagents may be a limitation that may be solved with a small refrigerator or sending the extracted nucleic acid with the preservative to laboratories in the proximity for the PCR test.

Please explain any budget various greater than 15% of the original budget headlines.

The availability of funds has been very irregular and the expenses needed to be adapted to the available funds.

INNOVATION OUTCOMES

What were the outcomes of the project (positive or negative) and how did these follow from activities and outputs described above?

The outcomes are very positive since as expected a low-cost equipment and a protocol for the analysis of viruses in water, has been adapted to be portable and applied to the point of use.

Major outcomes are:

The first step of this protocol is a concentration method developed for viruses useful for the concentration of viruses, bacteria and protozoa and that may be applied to the study of pathogens in all types of water.

A magnetic nucleic acids extraction protocol and PCR assays were adapted to be used without power supply or large laboratory equipment.

The developed method can be performed at the point-of-use, for non-experimented users, by following the provided tools and instructions.

Has the project demonstrated the success of the innovation?

Yes, although some methodological innovations were evaluated and were found not to be applicable to the detection of viruses in water matrices with the low concentrations of viruses expected in many water samples. The final equipment and protocols were selected for been low-cost, applicable to many viral pathogens and with good level of efficiency, repeatability and applicability.

If yes, what evidence is there for the performance of the innovation?

If no, what are the key lessons about the innovation or area of practice?

The equipment showed to be useful in the laboratory experiments with good recoveries and in the field validation assays to concentrate viruses from water samples, in addition we have simplified and improved the final protocol to be able to supply a practical low-cost equipment with good levels of repeatability. We have studied also the protocols for the concentration of viruses used in this equipment in depth and has been very well characterized during the period of the study in terms of recoveries, repeatability and uncertainty.

The method has been used in-the-field by members of IO and of the University of Quito showing that it could be implemented in the filed by non-experimented users by following the provided instructions.

Do the outcomes support the initial rationale for the innovation?

Yes.

How has your understanding of the innovation changed through the project period?

Initially we considered to include in the innovation some specific techniques for the detection and quantification of the target viruses (HAdV and HEV). However the approaches analysed were not practical to be used for detecting diverse strains of a viral group or for analysing samples with low numbers of viruses, for this reason we decided to work for facilitating the use of q(RT)PCR techniques

that have proved the highest sensitivity. Also we evaluated the repeatability of the protocols to include in the final VirWaTest equipment, SOPs that will produce a good level of recovery and low uncertainty as characterized in our laboratory for the method used (recoveries between 32 and 86%, SD 17,4).

Did the innovation lead to any unexpected outcomes or results? How were these identified and managed?

No unexpected outcomes were identified

What are the key lessons learnt relating to the innovation (this should relate to the innovation itself, rather than project implementation

Some protocols depend on the project and on our research and we may control them, but some others depend on commercial available products and are more difficult to control. Our criteria were to define efficient protocols and equipment low cost and easy to use for non-experienced personal and applicable to a diversity of water matrices with more or less turbidity, and those points limited the application of some very new technologies (still expensive) for the rapid detection/quantification of viruses. We know that the innovation produced will be useful also in the future since it may allow the preparation of water samples for the concentration of viruses and other pathogens and the final step of the quantification could be in the future combined with the use of new technical developments for the quantification of viral nucleic acids if the costs became more affordable.

METHODOLOGY

Was the methodology successful in producing credible evidence on the performance of the innovation?

Yes, the validation exercises in the point-of-use as well as the tests done in the laboratory were useful to produce data on the performance of the innovation. In the laboratory more than 40 environmental samples, have been tested.

What adjustments were made to the methodology during the course of the project? Why were these needed and how were they made?

The methodology has been based in experiments in the laboratory and in the field validation with collaborators and end-users. The methodology is considered correct and has not been changed during the project.

PARTNERSHIPS AND COLLABORATION

Describe the partnership arrangements and how these may have changed during the course of the project.

We sign an agreement with GenIUL that is the company who manufactured the filter holder, the pumps, and the collection bottle supports.

Our collaborators in the validation phase were the wash team of Oxfam Intermon in RCA and their coordinator in Barcelona, Mariona Miret.

Laura Guerrero, a previous member of our team, now working in Equador in UDLA was very interested in participating in the validation part of the project as end-user.

DISSEMINATION

Indicate the steps taken to disseminate the outcomes of the project.

What dissemination activities have or will be conducted (whether or not included in the budget)?

Information on the advance of the innovation has been regularly updated in the project blog.

VirWaTest will be presented at the UNC Water Microbiology Conference 2017, International Symposium on Health-Related Water Microbiology: May 15-19 where the presentation has been accepted

We have the objective of preparing a tutorial video with last modifications that will include the simplified concentration protocol with last modifications and the viral nucleic acid extraction and quantification.

We plan to invite several ONGs to test the prototypes to have feedback on their use and applicability.

The website, which is now restricted to permitted users, will be opened to everybody: www.virwatest.org

The web contains description of the project, instructions of use, video tutorials and FAQs, that will be reviewed in the next weeks to be updated.

What publications have resulted from the project, or are forthcoming (i.e. research and policy reports, journal articles, case studies, evaluations etc.)?

We are at this moment writing a scientific paper describing the method.

Has the project received any third party coverage during the project (from news media, third party blogs, researchers or academics etc.)?

Bosch and Gimpera, a University of Barcelona Foundation, showed their interest in cover and disseminate information on the project but we agree in doing it once the project was finished.

TRANSFERABILITY

Please indicate if there is any potential to replicate the project and how.

The project is not expected to replicate.

What are the plans for scale-up beyond the pilot?

The preparation of more prototypes will depend on the demand after the dissemination of the innovation.

The UB is interested in continue improving the innovation produced in the future to facilitate the study of virus and bacteria simultaneously in water or for the application of new technologies, as NGS techniques and metagenomics, or new technological developments and devices. The small company collaborator is interested in continue working for the development of products to facilitate the use of this innovation, as could be the production of ready-to-use q(RT)PCR tubes stable at room temperature and with all regents included.

Are any other organisations planning to use or adapt the innovation?

We will contact and offer the innovation to different organizations that have shown interest MSF, Oxfam and other smaller organizations in Latin America.

What steps have been taken to ensure the transfer of the innovation and the learning from the project?

The steps for transferability are related to the dissemination plans that we have and also the support that we provide since many years ago to the interested NGOs involved in Wash when requesting our advice and/or contribution in the evaluation of the dissemination of faecal contamination of water and the identification of possible water sources of viral infections in a context of humanitarian crisis and viral outbreaks. As we described before in the section on dissemination, we will present the innovation in a conference and disseminate the information as a scientific paper and contacting NGOs.