

Adsorption-elution techniques and molecular detection of enteric viruses from water

Abstract

It is generally accepted by municipalities and public health authorities that monitoring microbial water quality is performed by enumerating faecal coliforms. These bacterial indicators of faecal contamination are used because of the assumption that they have the same behaviour in the water as enteric pathogens, which is not true with regards to viruses. Therefore, there is need for a supplementary viral indicator or to directly detect viruses from water. Detection of viruses in water is complicated because of occurrence of viruses in low numbers. There is need for concentration of the viruses from large volumes water to aliquots that can be used in PCR or cell culture. We present the adsorption-elution techniques for the recovery and concentration of enteric viruses from water and subsequent detection by quantitative real time RT-PCR/PCR.

Keywords: Enteric viruses, RT-PCR, Recovery, PCR, Waterborne pathogens

Volume 3 Issue 6 - 2016

Vurayai Ruhanya,¹ Landry Kabego,² Josiah Otwoma Gichana³

¹Department of Medical Microbiology, University of Zimbabwe, Zimbabwe

²Universite Catholique de Bukavu, Democratic Republic of Congo

³Department of Oral and Maxillofacial Surgery, Oral Pathology and Medicine, Faculty of Dental Sciences, University of Nairobi, Kenya

Correspondence: Vurayai Ruhanya, Department of Medical Microbiology, College of Health Sciences, University of Zimbabwe, Box A178, Avondale Harare, Zimbabwe, Tel +2634 791 631 Email vruhanya@gmail.com

Received: September 05, 2016 | **Published:** September 19, 2016

Introduction

Rationale for virological analysis of water

Water quality monitoring and public health assurance is performed routinely by enumerating faecal indicator bacteria in drinking water. Faecal coliforms are used as indicators because of the assumption that the fate and transport of faecal coliforms reflect that of the waterborne pathogens.¹ However, accumulating evidence on waterborne disease outbreaks shows that absence of faecal coliform indicators does not entirely eliminate possibilities of the presence of other pathogens with a potential to cause disease outbreaks.² For example some enteric viruses are resistant to many disinfectants and remain viable after disinfection.³ Studies have also demonstrated that there is no relationship between current bacterial indicator detection and the presence of viruses in treated water.⁴ There is therefore need to have multiple or supplementary indicators which include enteric viruses and if possible to directly monitor the levels of viral pathogens in surface waters, irrigation water, sewage effluent as well as treated drinking water for public health safety and quality assessment.⁵ However due to the low concentration of viruses in water matrices and presence of inhibitors, efficient concentration methods from large quantities are essential.^{5,6}

Virological analysis of water plays an important role in research on the incidence and behaviour of viruses in the water environment and evaluation of the efficiency of water treatment and disinfection processes.⁷ Routine monitoring of virological quality of water is also done to test compliance with public health guidelines and specifications.³ In addition the information is necessary to study the public health impact of waterborne viral infections and for the identification of strains isolated from water as a tool to study epidemiology of viral infections.⁸ Detection and quantification of viral pathogen levels in water is rarely performed because of the technical challenges involved in viral assays.⁹ One of the major difficulties encountered in the detection of viruses in water sources is

the occurrence of low virus particle numbers in water.^{10,11} This makes analysis of viruses in water samples a complex and more expensive process than detecting bacteria.

Recovery of viruses from water

The analysis of water for enteric viruses is a two stage process: the first step is to apply efficient viral recovery and concentration procedures from large volumes (10-1000 L) of water.^{12,13} thereafter, a range of isolation and/or detection methods may be applied.^{6,8,14,15} Important criteria to be considered when concentrating viruses from water are:

1. The technique should be easy to complete in a short time; ii) have high virus recovery rate;
2. Should concentrate large range of viruses;
3. Be less costly;
4. Be capable of processing large volumes of water; and
5. Be repeatable within a laboratory.

For this reason, different methods have been used for the recovery of viruses from water in different studies. Each method has its own variations, advantages and disadvantages.¹⁴ The cost of virus recovery technologies is a very important issue, particularly in resource poor settings like those found in developing or underdeveloped regions, low-income countries. The recovered and concentrated viruses are then detected using different methods like conventional cell culture techniques, conventional qualitative molecular-based assays such as nucleic acid hybridisation and polymerase chain reaction (PCR), and qualitative or quantitative real-time (*rt*) RT-PCR/PCR. However, the problem with the molecular detection of viruses in water is that these techniques only indicate the presence of viral genomes and do not necessarily correlate with the presence of intact/infective virions.

In order to monitor the virological quality of water an efficient combination of techniques has to be assessed for optimal recovery and

detection of low virus titres in water.^{10,14} A number of concentration methods have been used in viral recovery studies with recovery efficiencies as high as 100%.¹⁶ Based on the different properties of the viruses, four main principal approaches are used for viral recovery and concentration. These include ultrafiltration, ultracentrifugation, and two-phase separation with polymers or flocculation, and adsorption-elution using filters, membranes, glass wool or glass powder.^{14,17,18}

Adsorption-Elution techniques

The adsorption-elution method, based on the ionic properties of the viruses is widely applied in the primary concentration of enteric viruses from water. Due to the unique surface properties of viruses, they readily adsorb to a number of substances including starch, minerals, fabrics alumina gel and various resins.¹ The adsorption-elution theory stems from the work of Wallis and his colleagues in 1967 who reported the first application of virus adsorbing filters when they described a method for concentrating viruses from sewage.^{1,14} The principle is that virus containing sample is brought in contact with solid matrix to which virus will adsorb under specific pH and ionic strength. When the virus is adsorbed, the residual water is discarded. Thereafter the virus is released from the matrix by elution into a smaller volume, usually one tenth of the original volume. The choice of the adsorbing matrix, eluting fluid and processing conditions are influenced by the nature of the sample. Adsorption and elution using charged membranes or filters is applied worldwide for the concentration of different enteric viruses and bacteriophages.^{18,19}

Membranes and filters

The virus is bound to the filters by electrostatic forces and not by size exclusion. The virus containing sample is passed under positive pressure or vacuum through a cellulose nitrate membrane. Since the viruses and filter materials are both negatively charged at neutral pH, the water samples have to be preconditioned by adjusting the pH to pH 3.5 with the addition of aluminium or magnesium ions to allow electrostatic binding.¹⁴ Viral recoveries using negatively-charged membranes range from 60% to 70% with river water.¹⁴ They are less expensive and can be obtained in a sterile cartridges in a disposable form. The membranes however are prone to clogging and cannot be used for turbid waters.²⁰

Electropositive membranes and cartridges are commonly used for the recovery of enteric viruses from water and other materials without prior conditioning of the sample because most enteric viruses are negatively charged at ambient pH.^{21,22} The other advantage of the electropositive membranes is the ability to recover rotaviruses (RVs) and coliphages which are sensitive to the low pH needed for electronegative membranes.²³ Viral recoveries from the electropositive filters are comparable to the electronegative filters.²⁴ but they have low recovery rates for viruses from marine waters and they clog easily.²⁵

Glass wool

Glass wool is an economic alternative to the microporous filters and charged membranes. The technique was pioneered in France by Vilaginés and colleagues, 1993 who applied it to the concentration of a range of viruses from drinking, surface and waste waters.¹⁴ Oiled sodocalcic glass wool, packed in holders at a density of 0.5g/cm³, is washed with 1 M hydrochloric acid (HCl), sterile distilled water, 1 M sodium hydroxide (NaOH) in sequence and finally with sterile distilled water again to bring the glass wool to neutral pH before the sample is passed through the filter.^{15,17} Elution is done using a solution of buffered beef extract or skimmed milk, both at high pH (pH 9.5) so that the negatively charged viruses adsorbed on the matrix

would be displaced by the buffer. The only pre-treatment necessary is dechlorination for the recovery of viruses from treated drinking water.¹⁹ Glass wool has been used to recover viruses from wastewater, treated and untreated drinking water,^{4,17,26} groundwater,^{1,4,27} and river water.²⁹ Compared to charged membranes glass wool is cheaper with a good efficiency of recovery (EOR) for a wide range of enteric viruses: namely 29% for norovirus from drinking water,¹⁹ 56% for norovirus in treated waste water, 3.4% for norovirus from acidified fresh water. The data available show that the highest recoveries using glass wool were obtained using seeded enteroviruses (EVs) in drinking water, with 102.5% for poliovirus (PV), 75% for Coxsackie B4.¹⁷ An Efficiency of Recovery (EOR) of 87% was reported for the recovery of simian RV SA11 from estuarine waters and while the EOR for human RV (WA) from fresh water was 77%.³⁰ These studies however, used different quantities of glass wool, different volumes of water matrices, different volumes of elution buffer, as well as different virus quantification methods.¹⁹ There is therefore a need for more standardised data on the EOR of enteric viruses from both surface and drinking waters for quantitative virus monitoring, risk and exposure analyses.

The glass wool recovery of viruses from large volumes of water is a primary process and the volume is too large for direct detection and also contains some inhibitors which compromise viral assays such as cell culture-based plaque assays and molecular-based assays. Therefore there is need for secondary concentration which reduces the volume of the eluate as well as getting rid of the possible inhibitors which may interfere with the detection methods. Many methods for reconcentration/secondary concentration of viruses from various eluates are available. Most are based on acid precipitation,⁶ and organic flocculation.¹⁴ One disadvantage for these two methods is operating pH range for the procedures (pH 3.0-4.5). The low pH compromises the viability of viruses like RVs.⁶ Polyethylene glycol 600 (PEG600) precipitation was found to be an effective secondary concentration method for detection of human enteric viruses.^{6,30} The advantage of PEG is that it is simple and inexpensive, non-destructive to viruses,⁶ and has been applied to concentrate many different enteric viruses, namely Pv, Rv, hepatitis A (HAV) and norovirus (Nov) from water.³¹ One of the best advantages of PEG is the ability to obtain precipitation at neutral pH and high ionic concentration.¹⁴ Part or all of the secondary concentrate may be used to detect the virus.

Detection of viruses in water

There are several approaches to detection of viruses from water samples.^{6,8,14} Isolation in cell culture and enumeration by plaque assay is the gold standard for infectious virus quantification.⁶ Tissue culture infectious dose (TCID₅₀) or most probable number (MPN) units may also be used to quantify the viruses.¹⁴ Viruses that do not produce cytopathic effects (CPE) can be detected by immunofluorescence staining. However viral culture is costly, labour intensive and time consuming, taking several days or weeks making it not very suitable for routine monitoring of viruses in water.

Viruses are usually present in low numbers in water.¹⁰ therefore direct detection by Electron microscope (EM), Immuno-Electron Microscopy (IEM) or Enzyme-linked Immunoassays (EIA) is limited by low sensitivity. Their detection limit is too high (105 particles or more per mL) to be used for detection of viruses from water.⁶

Molecular methods have permitted faster detection and have become very useful for viruses that cannot be propagated in cell culture.³² Molecular methods have the highest degree of sensitivity and retain a good specificity and are mostly applied in food and environmental samples.⁶ The increased sensitivity of molecular-

based assays enables the detection of low numbers of enteric virus pathogens in the water. The PCR assay is now widely used to monitor the presence of virus contamination in water samples.^{31,33} Quantitative real time polymerase chain reaction *rt* PCR which is faster, more reliable and more efficient than conventional PCR is used more often for monitoring viruses in environmental samples.^{8,33,34}

First introduced in 1992 by Higuchi and co-workers, real time reverse transcriptase (*rt* RT-PCR/PCR) allows precise quantification of nucleic acids in a complex mixture even if the starting amount of nucleic acid is in very low concentrations.³⁶ The *rt* PCR measures the amount of amplicon produced at each cycle of amplification using fluorescence based technology.^{34,37} The amplicon is measured in 'real time' or as it is being produced by labelling and detecting the accumulating product with a fluorescently tagged substrate during amplification procedure.³⁸ In *rt* RT-PCR/PCR the amplicon is measured early in the reaction during the exponential phase of PCR when amplification is proceeding most efficiently.³⁸ Therefore *rt* PCR has increased speed due to reduced cycle number, is more sensitive due to higher sensitivity of fluorescent dyes used for the detection of the amplicon and more specific by use of DNA/RNA strand specific probes like Taqman or molecular beacons.^{33,35,36} Real time quantitative (*rt* qRT-PCR/PCR) provides numerical data on the presence of enteric viral genomes which is important in estimating the public health risks of low levels of enteric viruses in environmental samples.¹¹

Real time RT-PCR/PCR is a multi-stage protocol that requires high quality nucleic acid extraction/purification of RNA/DNA, optimal conversion of RNA to cDNA and accurate detection of PCR products. One of the most important obstacles to successful PCR-based analysis of environmental samples is co-purification of the nucleic acid with inhibitory compounds like polysaccharides, humic acids, fulvic and tannic acid present in environmental samples.^{33,39} The presences of inhibitors in purified nucleic acids inhibit RT and or polymerase reactions.³² It is therefore important that nucleic acid extraction systems applied must get rid of all possible inhibitors. A variety of 'home-brew' and kit-based extraction systems are available which rely on ethanol to precipitate nucleic acids.^{23,40} Some of the more modern extraction methods are based on the Boom principle of binding nucleic acids to silica beads for additional purification.⁴¹ and removal of inhibitors.⁴² However there is no nucleic acid extraction method that removes all the inhibitors in the environmental samples.

As a result the *rt* PCR/RT-PCR analyses for environmental samples which are easily affected by inhibitors have to find ways to mitigate the effect of inhibitors on the assays.⁸ Several methods for the removal or relief of *rt* RT-PCR/PCR inhibition of virus detection in water sample concentrates have been evaluated.^{33,39,43} The primary way is to improve sample concentration and nucleic acid extraction and purification techniques to avoid co-concentrating inhibitors and ablate extracted inhibitors.³⁹ Gel filtration resins like Sephadex or Sepharose are generally effective in removing inhibitors but there is loss of nucleic acids in the process.^{33,39} To avoid loss of nucleic acids that can arise from chromatographic techniques and binding resins, bovine serum albumin (BSA) has been added to PCR mixtures to reduce inhibition by binding to inhibitors.^{8,11,39} Methods including the use of internal or external controls are also available for identifying and monitoring inhibition.^{18,19,36,39} Another approach to identify inhibition is to analyse shifts in the amplification efficiency of *rt* qRT-PCR/PCR reactions.³³ The presence of inhibitors is shown by a shift or increase in the quantification cycle (C_q) which is the cycle at which target nucleic acid has been amplified enough to reach a defined threshold.³⁹ In some situations it may be adequate to identify sample containing inhibitors from their flat curves or from the complete absence of

amplification products.⁴⁴ Inhibition makes interpretation of public health risk of enteric virus pathogen difficult, because inhibition can result in underestimating exposure and consequently health risk.⁵

Since enteric viruses are present in low quantities in water samples, effective quality assurances and quality control are required to avoid false positives and false negatives.⁸ To generate useful quality, reliable and comparable data with *rt* RT-PCR/PCR it is important to monitor the efficiency and accuracy of the critical steps in the recovery and detection assays which are acceptable to regulatory authorities.⁸ In order to monitor the critical steps in the recovery and molecular detection of enteric viruses in water and other environmental samples, process controls were introduced.⁴⁵⁻⁴⁷ The process control should have similar features to the target virus, it must not be associated or naturally present in water or food samples and must not be infectious to human beings and must be added to the sample prior to processing.⁶ The process control is therefore co-recovered, concentrated, co-extracted with the target enteric virus and then detected in the same nucleic acid extract.^{6,45} Feline calicivirus and mengovirus have been proposed as process controls for recovery, concentration and detection of enteric viruses from food and environmental samples.^{47,48}

To ensure accuracy of data generated by *rt* qRT-PCR/PCR, quality control has to be done on all critical reagents including optimisation. Primers are critical reagents in PCR analyses, hence appropriate concentrations need to be ensured to enhance detection of PCR products.³⁶ Too high concentrations lead to non-specific products such as primer-dimers. Tests are usually done with varying amounts of each primer where optimal concentrations which may not be the same for both primers are established. The primer concentrations with the lowest cycle threshold (CT)/crossing point (CP) values should be selected since lower CT values correspond to efficient production of the amplicon.^{36,44} To verify the specificity of the primers in the optimisation process, PCR products are run on the gel where one band will be visible when primers are specific. Using real time (*rt*) thermocyclers, amplification specificity is verified by melting point analysis. This is done using the melting curve analysis feature, where target amplicons are distinguished from PCR artefacts like primer dimers and misprimed products. Melting point analysis is also done to optimise primer annealing temperature if it becomes necessary.

When *rt* RT-PCR tests are run, quality control steps are done to exclude false positives and false negatives.⁸ Negative and positive controls are included in each run. Depending on the analyses, at least one negative control must be included. A negative control where a run includes the master mix without template confirms that PCR reagents are not contaminated when the result is negative.⁴⁴ If the no template control becomes positive, it means that one or more reagents are contaminated with template or previously amplified product.³⁶ No reverse transcriptase control helps the detection of contaminating DNA in the RNA. Positive control makes sure that all reagents are working properly. The positive control could be quantified DNA or RNA containing the target sequence.

Quality and reliability of *rt* qRT-PCR/PCR for enteric viruses from environmental samples depends on the correct setting of thresholds for detection. When setting thresholds, the level of fluorescence signal that is sufficiently above background/ noise to be considered reliable is determined.³⁶ The cycle at which the threshold is met or exceeded is called the CT value and is used to make comparison between samples.^{36,44,49,50} The point at which the product's detected fluorescence crosses the threshold is called CT or CP value. It is important that thresholds are set to allow detection of amplicons

when amplification is in the exponential phase.^{2,36,44,49} If the thresholds are too low, it results in pre-mature 'detection' of the amplicon and too high thresholds results in detection of the product above the exponential phase leading to inaccurate quantification of the virus genome copies.³⁶

There are two methods of *rt* qRT-PCR/PCR, namely absolute quantification and relative quantification. Absolute quantification results in a measure of the amount of target sequence in the sample expressed as genome copies.^{6,44} Relative quantification provides a relative value expressed a ratio of initial target sequence between control and experimental treatments.^{2,44,49} Absolute quantification requires generating a standard calibration curve using a standard that has been carefully quantified as to the copy number.^{2,40} The approach is more accurate but more labour intensive. Accurately quantified standard is serially diluted in increments of 3-10 fold and in a range sufficient to cover the quantities of viruses expected in experimental samples. The standard curve generated from average CT values of each dilution plotted against the absolute amount of the standard is used for comparison with experimental CT values.⁴⁴ This produces an estimate of the amount of target present in the experimental sample.² Quality of standard curves should be monitored for accurate quantification. Wide dynamic range, limit of detection which determines sensitivity as well as the efficiency.^{2,49}

Currently most water quality standards including WHO guidelines do not specify the level of viruses considered acceptable for drinking water, irrigation water, recreational or treated waste water. As result the burden of infectious diseases emanating from water polluted by enteric viruses, particularly in resource limited settings like developing countries have not been documented, reported or properly investigated.^{51,52}

Conclusion

The main challenge in the surveillance and monitoring of enteric viruses in water is the recovery/concentration of low titre viruses from large volumes to small volumes which can be used for either cell culture or molecular detection. Available methods are either too costly for routine water quality monitoring or limited to small volumes. The sodocalcic glass wool adsorption-elution technique is a cost effective and easy-to-use method to recover and concentrate viruses from water but more data is required on its reliability and efficiency. However, the problem with the molecular detection of viruses in water is that they only indicate the presence of viral genomes and do not necessarily correlate with the presence of intact/infective virions.

Acknowledgments

None.

Conflicts of interest

None.

References

- Powell KL, Barret MH, Pedley S et al. Enteric virus detection in groundwater using glass wool trap. Final report. West Midlands: Robens Centre for Public and Environmental Health, University of Surrey, Guildford, United Kingdom. 2000
- Forlenza M, Kaiser T, Savelkoul HFJ et al. The use of real time quantitative PCR for the analysis of cytokine mRNA levels. *Methods Mol Biol.* 2012;820:7–23.
- World Health Organisation Guidelines for drinking water quality. (3rd edn), Recommendations. World Health Organisation, Geneva, Switzerland. 2004
- Ehlers MM, Grabow WO, Pavlov DN Detection of enteroviruses in untreated and treated drinking water supplies in South Africa. *Water Res.* 2005;39(11):2253–2258.
- Gibson KE, Schwab JK, Spencer SK et al. Measuring and mitigating inhibition during quantitative real-time PCR analysis of viral nucleic acids from large volume environmental samples. *Water Res.* 2012;46(13):4281–4291.
- Mattison K, Bidawid S Analytical methods for food and environmental viruses. *Food and Environmental Virology.* 2009;1:107–122.
- Dechesne M, Soyeux E Assessment of source water pathogen contamination. *J Water Health.* 2007;5(Suppl 1):39–50.
- Bosch A, Sanchez G, Abbaszadegan M et al. Analytical methods for virus detection in water and food. *Food Analytical Methods.* 2011;4(1):4–12.
- Bosch A, Guix S, Sano D et al. New tools for the study and direct surveillance of viral pathogens in water. *Curr Opin Biotechnol.* 2008;19(3):295–301.
- Soule H, Genoulaz O, Gratacap-Cavallier B et al. Ultrafiltration and reverse transcription polymerase chain reaction: an efficient process for poliovirus, rotavirus and hepatitis A virus detection water. *Water Research.* 2000;34(3):1063–1067.
- Rutjes SA, van den Berg HH, Lodder WJ et al. Real time detection of norovirus in surface water by use of a broadly reactive nucleic acid sequence-based amplification assay. *Appl Environ Microbiol.* 2006;72(8):5349–5358.
- Köster W, Egli T, Ashbolt N et al. Analytical methods for microbiological water quality testing. OECD/WHO guidance document. In: Ronchi & Bartram (Eds.). Assessing Microbial Safety of Drinking Water: Improving Approaches and Methods. *OECD/WHO, Paris, France, pp.* 2003;237–292.
- Gerba CP Virus occurrence and survival in the environmental waters. *Perspectives in Medical Virology.* 2007;17:91–108.
- Wyn-Jones AP The detection of waterborne viruses. In: Bosch A (Edr.), Human Viruses in Water. *Amsterdam: Elsevier B.V., USA, pp.* 2007;177–203.
- Wyn-Jones AP, Carducci A, Cook N et al. Surveillance of adenoviruses and noroviruses in European recreational waters. *Water Research.* 2011;45(3):1025–1038.
- Hill VR, Kahler AM, Jothikumar N et al. Multistate evaluation of an ultrafiltration-based procedure for simultaneous recovery of microbes in 100-litre tap water samples. *Appl Environ Microbiol.* 2007;73(13):4218–4225.
- Vilaginés Ph, Sarrette B, Husson G et al. Glasswool for virus concentration from water at ambient pH levels. *Water Science and Technology.* 1993;27(3–4):299–306.
- Albinana-Gimenez N, Miagostovich MP, Calgua B et al. Analysis of adenoviruses and polyomaviruses quantitative PCR as indicators of water quality in source and drinking water treatment plants. *Water Research.* 2009;43(7):2011–2019.
- Lambertini E, Spencer SK, Bertz PD et al. Concentration of enteroviruses, adenoviruses, and noroviruses from drinking water by use of glass wool filters. *Applied and Environmental Microbiology.* 2008;74(10):2990–2996.
- Gerba CP Recovering viruses from sewage, effluents and water. In: Berg (Edr.), Methods for Recovering Viruses from the Environment. *Boca Raton, Florida: CRC Press, Inc., USA, pp.* 1987;1–23.
- Haramoto E, Katayama H, Ohgaki S Detection of noroviruses in tap water in Japan by means of a new method for concentrating enteric viruses in large volumes of freshwater. *Appl Environ Microbiol.* 2004;70(4):2154–2160.

22. Gassilloud B, Duval M, Schwartzbrod L et al. Recovery of feline calicivirus infectious particles and genome from water: Comparison of two concentration techniques. *Water Science and Technology*. 2003;47(3):97–101.
23. Kim D, Kim SR, Kwon KS, Lee JW, Kwon OH, et al. Detection of hepatitis A virus from oyster by nested PCR using efficient extraction and concentration method. *J Microbiol*. 2008;46(4):436–440.
24. Sobsey MD, Jones BL Concentration of poliovirus from tap water using positively charged microporous filters. *Appl Environ Microbiol*. 1979;37(3):588–595.
25. Lukasiak J, Scott TM, Andryshak D, Farrah SR. Influence of salts on virus adsorption to microporous filters. *Applied and Environmental Microbiology*. 1979;66(7):2914–2920.
26. Vivier JC, Ehlers MM, Grabow WO Detection of enteroviruses in treated drinking water. *Water Res*. 2004;38(11):2699–2705.
27. van Zyl WB, Williams PJ, Grabow WO et al. Application of molecular methods for the detection of group A rotaviruses in raw and treated water. *Water Sci Technol*. 2004;50(1):223–228.
28. van Zyl WB, Page NA, Grabow WOK et al. Molecular epidemiology of group A rotaviruses in water sources and selected raw vegetables in South Africa. *Appl Environ Microbiol*. 2006;72(7):4554–4560.
29. Taylor MB, Cox N, Vrey MA et al. The occurrence of hepatitis A and astroviruses in selected river and dam waters in South Africa. *Water Res*. 2001;35:2653–2660.
30. Lewis GD, Metcalf TG Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A and human rotaviruses, from oyster water and sediment samples. *Applied and Environmental Microbiology*. 1998;54(8):1983–1988.
31. Huang PW, Laborde D, Land VR et al. Concentration and detection of caliciviruses in water samples by reverse transcription–PCR. *Applied and Environmental Microbiology*. 2000;66(10):4383–4388.
32. Dubrou S, Kopecka H, Lopez–Pila JM et al. Detection of hepatitis A virus and other enteroviruses in waste water and surface water samples by gene probe assay. *Water Science and Technology*. 1991;24(2):267–272.
33. Rodriguez–Lazaro D, Cook N, Ruggeri FM et al. Virus hazards from food, water and other contaminated environments. *FEMS Letters*. 2012;36(4):786–814.
34. Beuret C Simultaneous detection of enteric viruses by multiplex real–time RT–PCR. *J Virol Methods*. 2004;115(1):1–8.
35. Gregory JB, Litaker RW, Noble RT Rapid one step reverse transcriptase PCR assay with competitive internal positive control for detection of enteroviruses in environmental samples. *Applied and Environmental Microbiology*. 2006;72(6):3960–3967.
36. Fraga D, Meulia T, Fenster S Real–time PCR: Overview and principles. *Current Protocols Essential Laboratory Techniques*. 2008;10:1–33.
37. Guo F, Cheng A, Wang M et al. Development of TaqMan MGB fluorescent real–time PCR assay for the detection of anatis herpesvirus 1. *Virol J* 2009;6:71.
38. Kricka LJ Stains, labels and detection strategies for nucleic acid assays. *Ann Clin Biochem*. 2002;39(Pt 2):114–129.
39. Gibson KE, Borchardt MA Basic quality assurance/quality control information required for reporting real time quantitative PCR data. *Journal of American Water Works Association*. 2013;105(1):45–51.
40. Ruhanya V, Diez–Valcarce M, D’Agostino M et al. Monitoring of Extraction Efficiency by a Sample Process Control Virus Added Immediately Upon Sample Receipt. *Food Environ Virol*. 2015;7(4):413–416.
41. Boom R, Sol CJ, Salimans MM et al. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 1990;28(3):495–503.
42. Dubois E, Hennechart C, Merle G et al. Detection and quantitation by real–time RT–PCR of hepatitis A viruses from inoculated tap waters, salad vegetables and soft fruits: Characterisation of the method performances. *Int J Food Microbiol*. 2007;117(2):141–149.
43. Opel KA, Chung D, McCord BR A study of PCR inhibition using real time PCR. *Journal of Forensic Science*. 2010;55(1):24–33.
44. Saunders NA Quantitative real–time PCR. *Current PCR*. 2008
45. da Silva K, Le Saux JC, Parnaudeau S et al. Evaluation of norovirus removal during wastewater treatment, using real time reverse transcription PCR: Different behaviours of genotypes I and II. *Applied and Environmental Microbiology*. 2008;73(24):7891–7897.
46. Comelli HL, Rimstad E, Larsen S et al. Detection of norovirus genotype 1.3b and II.4 in bio accumulated blue mussels using different virus recovery methods. *International Journal of Food Microbiology*. 2008;127:53–59.
47. Pintó RM, Costafreda I, Bosch A Risk assessment in shellfish–borne outbreaks of Hepatitis A. *Appl Environ Microbiol*. 2009;75(23):7350–7355.
48. Costafreda MI, Bosch A, Pintó RM Development , evaluation and standardization of a real–time Taqman reverse transcription–PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. *Appl Environ Microbiol*. 2006;72(6):3846–3855.
49. Bernard PS, Wittwer CT Real time PCR technology for cancer diagnostics. *Clinical Chemistry*. 2002;48(8):1178–1185.
50. Watzinger F, Suda M, Preuner s et al. Real time quantitative PCR assays for detection and monitoring of human pathogenic viruses in immunosuppressed paediatric patients. *J Clin Microbiol*. 2004;42(11):5189–5198.
51. Okoh AI, Sibanda T, Gusha SS Inadequately treated wastewater as a source of human enteric viruses in the environment. *International Journal of Environmental Research and Public Health*. 2010;7(6):2620–2637.
52. Martinez–Martinez M, Diez–Valcarce M, Hernandez M et al. Design and application of nucleic acid standards for quantitative detection of enteric viruses by real time PCR. *Food Environ Virol*. 2011;3(2):92–98.