EPA Public Access

Author manuscript

Ground Water. Author manuscript; available in PMC 2018 November 01.

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Published in final edited form as:

Ground Water. 2017 November ; 55(6): 778–783. doi:10.1111/gwat.12531.

Applying Quantitative Molecular Tools for Virus Transport Studies: Opportunities and Challenges

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Abstract

Bacteriophages have been used in soil column studies for the last several decades as surrogates to study the fate and transport behavior of enteric viruses in groundwater. However, recent studies have shown that the transport behavior of bacteriophages and enteric viruses in porous media can be very different. The next generation of virus transport science must therefore provide more data on mobility of enteric viruses and the relationship between transport behaviors of enteric viruses and bacteriophages. To achieve this new paradigm, labor intensity devoted to enteric virus quantification method must be reduced. Recent studies applied quantitative polymerase chain reaction (qPCR) to column filtration experiments to study the transport behavior of human adenovirus (HAdV) in porous media under a variety of conditions. A similar approach can be used to study the transport of other enteric viruses such as norovirus. Analyzing the column samples with both qPCR and culture assays and applying multiplex qPCR to study cotransport behavior of more than one virus will provide information to under-explored areas in virus transport science. Both nucleic acid extraction kits and one-step lysis protocols have been used in these column studies to extract viral nucleic acid for qPCR quantification. The pros and cons of both methods are compared herein and solutions for overcoming problems are suggested. As better understanding of the transport behavior of enteric viruses is clearly needed, we strongly advocate for application of rapid molecular tools in future studies as well as optimization of protocols to overcome their current limitations.

Introduction

Enteric viruses such as adenovirus, enterovirus, and norovirus have been found in various water environments and land-applied biosolids (Katayama et al. 2002, 2008; Fong et al. 2007; Xagorarakis et al. 2007; Wong et al. 2010). Exposure to them causes a variety of diseases in humans and as a consequence, they have been included on the U.S. Environmental Protection Agency Drinking Water Contamination Candidate List. Since they are much more mobile than bacteria in porous media due to their small size (usually below

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100 nm) (Maier et al. 2009), infiltration of biosolid-associated enteric viruses into groundwater is thus a major concern. To protect the water environment, tremendous effort has been invested over the last several decades to gain a solid understanding of the fate and transport behavior of viruses in the subsurface (Schijven and Hassanizadeh 2000; Jin and Flury 2002; Zhuang and Jin 2003; Attinti et al. 2010; Horswell et al. 2010; Syngouna and Chrysikopoulos 2013; Kokkinos et al. 2015).

The transport of viruses in subsurface environments depend on the processes of advection, dispersion, diffusion, size exclusion, retention, and release from the soil-water and air-water interfaces (Bradford et al. 2014). Mathematical models have been developed to understand and predict the complex virus transport process in subsurface. Soil columns have been used in numerous studies to generate breakthrough curve (BTC) data for grasping the effect of these processes on virus transport and developing models that predict virus mobility in the subsurface (Schijven and Hassanizadeh 2000; Jin and Flury 2002; Attinti et al. 2010; Bradford et al. 2014). A reliable BTC requires frequent measurements of effluent samples and replication of column experiments. Because it is much more time- and resource-consuming to perform culture analysis for enteric viruses than bacteriophages assays, most virus transport studies have only focused on bacteriophages, under the assumption that phages are accurate surrogates for enteric viruses (Pang et al. 2014; Wong et al. 2014; Stevenson et al. 2015).

Advanced molecular techniques such as polymerase chain reaction (PCR) are much less time-consuming than culture methods and numerous monitoring studies have already applied molecular tools to detect enteric viruses in different water systems (Wong et al. 2012a). Indeed, the recent increase in literature reporting the occurrence of enteric viruses is mostly attributed to the availability of PCR viral assays. It is time for virus transport studies to incorporate advanced molecular methods, particularly quantitative PCR (qPCR), to monitor the concentration of viral contaminants of high public health concern in complex environmental matrices. In this issue paper, the main goal therefore is to discuss the needs for a substantial increase in transport data of enteric viruses, and discuss the opportunities and challenges of applying qPCR to column transport experiments.

Needs for Understanding the Transport Behavior of Enteric Viruses

Three recent studies by independent research groups (Shi et al. 2012; Pang et al. 2014; Wong et al. 2014), applied qPCR to column filtration experiments and they all found mobility of human adenovirus (HAdV, the most prevalent enteric virus in water environments) (Wong et al. 2012a) to be much lower than MS2 (the most frequently used bacteriophage in virus transport studies) (Jin and Flury 2002; Wong et al. 2014) in sand media. Another study also found mass recovery of HAdV from glass beads column effluents lower than coliphage reported in previous studies (Kokkinos et al. 2015). One study, however, reported higher retention of MS2 than HAdV in fine granular limestone aquifer material; and suggested that PRD1 was a better transport indicator than MS2 for HAdV in this type of media (Stevenson et al. 2015). Besides HAdV, rotavirus was also found to be much less mobile than MS2 (Pang et al. 2014). This information is extremely valuable because it indicates that assuming MS2 is an accurate transport surrogate for all enteric

viruses in soil systems can lead to significant error. Moreover, risk associated with HAdV and rotavirus can be under- or over-estimated (depending on the media types) if MS2 transport data are used to determine HAdV levels in groundwater systems.

Besides HAdV and rotavirus, no studies have applied qPCR to report BTC data of other enteric viruses. Human norovirus (HNoV) is recognized as the leading cause of acute gastroenteritis for all age groups (Hall et al. 2013), having caused numerous waterborne and foodborne outbreaks (Vega et al. 2014). There is thus a strong need to study mobility and attachment behavior of norovirus in the subsurface environment. qPCR will be the best tool for quantifying HNoV in column studies because there is no practical culture system for HNoV (Koo et al. 2012). Since animal noroviruses such as feline and murine norovirus can be cultured to a high titer, they can be used as a surrogate for HNoV in column study and their qPCR primers have already been developed (Baert et al. 2008; Di Pasquale et al. 2010) and applied in other studies to investigate the persistence of norovirus in water and under thermal treatment (Seitz et al. 2011; Zonta et al. 2015).

Because numerous areas in environmental virology have benefited from application of qPCR, including recent HAdV transport studies, new and exciting information will be added to virus transport science if more studies apply molecular techniques to investigate enteric virus motilities in column systems and compare transport behavior of bacteriophages and enteric viruses, side-by-side. This will be critical to identify bacteriophages that can be accurate surrogates for the presence, fate, and transport of enteric viruses in surface and ground waters. Until then, it is only a guessing game – with relative approximations of mobility at best – which can lead to significant under- and/or over-estimation of human exposure to pathogenic viruses.

Analyzing Column Samples Using Both qPCR and Culture Assays

Even though culture assays are tedious, future column studies should consider analyzing the results using both qPCR and culture methods since attachment to soil and soil organic matter can cause virus inactivation (Schijven and Hassanizadeh 2000; Silva et al. 2015). Culture method is only able to detect viable viruses but qPCR is able to detect naked nucleic acid (NA), capsid-damaged viruses, viable but non-culturable viruses, and culturable viruses. Therefore, if filtration processes can cause viruses to become inactivated or damaged, the difference between virus count by qPCR and culture assay can be attributed to inactivation. Both qPCR and culture methods have been used to analyze samples from HAdV sorption isotherm experiments (Silva et al. 2015). In this study, the authors reported that although organic matter did not lower gene copy numbers in the liquid phase, it reduced the infectivity of HAdV, indicating that organic matter did not enhance attachment of HAdV, but inactivated them instead. This information is extremely useful since a comprehensive fate and transport model includes both attachment/detachment and inactivation factors (Bradford et al. 2014). Because culturing enteric viruses is labor intensive, comparison of bacteriophage BTCs obtained from qPCR and culture assays can offer a fast and cost effective approach, especially because until now, no phage column study has reported BTC results using both culture and qPCR techniques. Having phage BTC results obtained by both

techniques will improve our understanding on the factors causing the removal of virus during groundwater filtration.

Application of Multiplex qPCR for Cotransport Study

Previous transport studies have investigated virus transport influenced by environmental factors such as ionic strength, ion composition, pH, organic matter, and soil types which are very relevant since they strongly influence virus deposition to soil particles (Jin and Flury 2002; Zhuang and Jin 2003; Yuan et al. 2008; Wong et al. 2012b, 2014). However, many different types of viruses including bacteriophage and human, plant, and animal pathogenic viruses are present in the natural water and wastewater environments (Rosario et al. 2009) and their coexistence can lead to competition for the binding sites in porous media, consequently influencing transport of individual viruses. Virus transport behavior determined by previous column filtration studies have mostly focused on a single virus; however, a variety of studies have shown that transport of virus or bacteria can be influenced by the copresence of clay colloids (Vasiliadou and Chrysikopoulos 2011; Syngouna and Chrysikopoulos 2013). This finding implies that copresence of biocolloids, such as other viruses, can also influence individual virus transport behavior. Therefore, there is a need to investigate the effect of copresence of multiple microbes on the transport behavior of individual viruses. We recommend that future studies focusing on virus transport consider including copresence of bacteria and viruses as well as high and low binding viruses. Multiplex qPCR detects and quantifies multiple organisms in one qPCR run, using multiple primers and probes specific to the targeted microbes. It has been applied in previous studies to detect and quantify multiple enteric viruses in the water environment (Wolf et al. 2010; Wong and Xagorarakis 2010). Because analytical measurement of multiple microbes is required in cotransport studies, multiplex qPCR will definitely be more efficient and cost effective than culture or single qPCR assays. The outcome of these studies will enhance our understanding of the interactions between mixed microbial/viral communities and virus transport in groundwater.

Challenges and Solutions Associated with Applying qPCR for Virus Soil Column Studies

While we strongly support this new paradigm of virus transport, there are still technical challenges associated with applying qPCR to study virus filtration in soil column systems. In our opinion, the major challenge applying qPCR to virus column studies is preparing high-quality NA templates.

Typical NA extraction kits such as mini column filtration can prepare high-quality NA by lysing almost any virus capsid and removing PCR inhibitors such as soil humic acid (Wilson 1997). Because multiple steps (e.g., lysing, cleaning, filtration, and elution) are required with kits, the primary deterrent to this technique is its inconsistent recovery rate due to accumulation of error from each step (Table 1). Inconsistent NA recovery reduces the accuracy of calculating the virus retained in soil columns and smoothness of the BTC, which are key results in determining virus attachment/detachment behavior and model development. It can also be very time-consuming and costly to extract NA using kits, especially with the large number of effluent samples usually generated from column experiments. However, when dealing with very inhibited samples, extraction kits are the

most effective method for removing inhibitors. To overcome inconsistent recovery rates, replicate or triplicate extractions are highly recommended. Replicate samples are especially important for the influent sample since the mass recovery for the column experiment is determined by measuring the area under the plot of C_e/C_0 vs. pore volume (or BTC) (Pang et al. 2014; Wong et al. 2014), where C_0 is the influent concentration and C_e is the concentrations of effluent samples collected at different pore volumes. Extra attention should therefore be given to the accuracy of C_0 since it involves calculating all values in the breakthrough plot. To lessen the labor effort, high throughput 96-well extraction kits (e.g., PureLink® Pro 96 Viral RNA/DNA Purification Kit, ThermoFisher Scientific, Waltham, Massachusetts) can be considered for extracting virus NAs from large numbers of effluent samples; and a multichannel pipette is also highly recommended to take full advantage of the high-throughput feature of 96-well extraction kits.

An alternative way to release NA from virus capsids for direct qPCR analysis without using extraction kits is offered by following the QuickExtract DNA (Epicentre, Madison, Wisconsin) protocol. This quick protocol in combination with 95 °C heating resulted in effective adenovirus and MS2 capsid lysis, respectively, producing high-quality NAs suitable for immediate qPCR analysis (Wong et al. 2014). We believe the one-step lysis method provides more consistent NA extraction recovery than the kit-based method since only a lysing step is involved (Table 1). This method has only been tested with HAdV and MS2 (Wong et al. 2012b, 2014), therefore, future studies should focus on testing and developing protocols that can effectively lyse other enteric viruses for direct qPCR analysis. Because clean-up is not involved, the PCR inhibition caused by environmental inhibitors such as humic acid is a major hurdle to overcome when using this method. One way to overcome PCR inhibition drawbacks is through dilution with molecular grade water (Pang et al. 2014; Wong et al. 2014). The recently developed qPCR platform, digital PCR (dPCR), can also be considered because it is less susceptible to inhibition (Hindson et al. 2013; Ra ki et al. 2014; Pavši et al. 2016). Moreover, no calibration curve is needed for quantification by dPCR since it is based on a Bayesian Probabilistic framework (Hindson et al. 2013). This can be extremely useful in analyzing effluent samples with multiple aqueous chemistry matrices since our previous study showed water chemistry (e.g., ionic composition) can slightly shift qPCR threshold values (Wong et al. 2012b, 2014), meaning that separate standard curves are needed to quantify the virus in each water matrix.

Overall, we recommend using one-step lysis protocol only when inhibition from environmental contaminants is not too strong to interfere with the qPCR quantification. Since application of molecular techniques to column studies is still in the early stages, it is worthwhile for future studies to compare the BTCs and mass recovery from extraction kits and one-step lysis protocol, and determine the effectiveness of each under different conditions.

In addition to issues associated with NA extraction, PCR is able to detect the capsid-free NA (cfNA) so it is important to run the column experiment using a stock composed of mostly intact virus since transport of viruses and their NAs can be very different. This is especially important when studying DNA viruses since capsid-free RNA usually degrades very quickly in the environment. We recommend using well-preserved or freshly grown stocks to conduct

experiments so numbers of cfNA will be minimal. Another way to reduce cfNA in the stock is to add DNase or RNase, but they must be removed prior to column experiment and qPCR analysis (Kokkinos et al. 2015). The difference between the qPCR threshold values of virus stock before and after lysing can determine intactness of viruses. The threshold value before lysing should be higher than afterward and the bigger the difference, the higher the portion of intact virus in the stock. Also, DNA-labeled protein-coated silica nanoparticles were recently used to mimic actual virus transport through a column system; no capsid lysis was needed because DNA is covalently attached to the silica nanoparticles (Pang et al. 2014). Enteric viruses may not be allowed for field scale column studies such as lysimeters due to biosafety concerns. Protein-coated silica nanoparticles can therefore serve as a surrogate in field studies since they are not considered pathogenic to human health (Pang et al. 2014). Since only one study has applied this technology in virus transport, more studies are needed to explore and validate this engineered nanomaterial's ability to mimic virus transport.

Conclusion

Based on the extensive development of molecular detection technology in the last couple decades, virus transport science should be able to provide more detailed data on mobility of enteric viruses in subsurface porous media. Considering how studies on waterborne pathogen detection have increased since development of PCR-related technology (Girones et al. 2010; Aw and Rose 2012), studies on mobility of enteric viruses in porous media are surprisingly scarce. We believe there is a great opportunity for scientists and engineers to contribute valuable, exciting breakthrough results in this under-explored area and to make a significant contribution preventing groundwater pollution by enteric viruses.

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Table 1.

Comparison Between Using Extraction Kits and One-Step Lysis Protocol to Prepare Virus Nucleic Acid from Soil Column Samples for qPCR Analysis

	Advantages	Disadvantages/Problems	Recommendations to Lessen the Problems
Extraction kit	Eliminates most inhibition	Inconsistent recovery can affect accuracy of mass recovery and smoothness of breakthrough curve	Replicate extractions, particularly on influent samples
	Extracts NA from most viruses	Long labor time and high cost	96-wells extraction plate
One-step lysis	Minimal concern over inconsistency in extraction recovery rates between samples	Cannot handle highly inhibited samples	Dilution, digital PCR, switch to extraction kits
		Conditions vary for different enteric viruses	Specific optimization for each virus, switch to extraction kits
		Different standard curves are needed for samples having different aqueous chemistry matrices	Dilution, digital PCR, switch to extraction kits