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Development of an integrated cell culture—Real-time RT-PCR assay for detection of reovirus in biosolids

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Abstract

The current method for viral detection in biosolids is a plaque assay, as specified by the EPA in the 40 CFR Part 503 rule. Development of an integrated cell culture-polymerase chain reaction (ICC-PCR) assay has allowed detection of viruses that are under-detected and undetected by the plaque assay. This study examined the efficiency of the ICC-PCR method to detect mammalian orthoreovirus, a virus typically under-detected in biosolids. Biosolid samples seeded with mammalian orthoreovirus type 1 (Lang) detected to 3×10^5 plaque forming units (pfu) with a plaque assay, 10^2 pfu equivalents with real-time RT-PCR and no incubation, and 10^8 pfu equivalents with real-time RT-PCR after 7 days incubation. More infectious virus was detected using ICC-real-time RT-PCR than a plaque assay. Twenty-four environmental samples from three locations around the United States did not plaque with the EPA method; however the ICC-PCR detected infectious reovirus in 13 of the samples. Raw biosolids samples accounted for 12 of the positive samples, and 1 positive was from an aerobically digested sample.

Keywords: ICC-PCR; Real-time PCR; Reovirus; Biosolids

1. Introduction

1.1. Reovirus

Mammalian orthoreovirus is a member of the family Reoviridae and the genus *Orthoreovirus*. The virus has two round icosahedral capsids, and in the intestinal lumen of the mammals, the outer capsid is proteolytically uncoated and made infectious (Golden et al., 2002). The double protein capsids make mammalian orthoreovirus resistant to disinfection. The virus is hardy, remaining infectious in water for long periods of time (Matsuura et al., 1988). There are three serotypes that infect humans which are identical to the serotypes that infect other mammals. Although the pathogenic effects of reovirus are unknown, mammalian orthoreovirus has been isolated from patients with respiratory infections, gastroenteritis, or rashes (Ward and Ashley, 1978). The majority of adults have serum antibodies to all three types of this virus. The virus is shed by infected individuals in feces for several weeks (Fenner and White, 1986). Reovirus is frequently detected in environmental water and is usually the most abundant virus detected (AWWA, 1999).

1.2. Biosolids

Sludge is the solid waste byproduct of the municipal sewage treatment process. One popular method of sludge disposal is land application. The EPA 503 regulations (EPA, 1992) were established in 1993 to define how sewage sludge, known as biosolids, must be processed in order to be land applied. Biosolids must be treated according to approved processes or tested for viable helminth ova and enteric viruses. The enteric virus testing is performed by a plaque assay.

1.3. Detection methods

Cell culture is considered by some experts to be the best way to isolate and determine infectious virus from an environmental sample (Fong and Lipp, 2005). Plaques assays utilizing cell culture are typically used with wastewater and biosolid samples. However, many viruses do not produce plaque and some will only plaque after several passages on a cell line. In addition, a

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cell line with a mixed population of viruses may be infected by only the fastest growing virus (Spinner and DiGiovanni, 2001). Another drawback is the inability to conclusively identify the plaque as originating from viral lysis by visual inspection. Plaque assays have also been shown to be dependant on the amount of cells seeded in the cultureware, the amount of viral inoculum added, and the incubation time (Payment and Trudel, 1985).

PCR detection can be sensitive and specific. The efficiency of viral amplification from environmental samples by PCR is influenced by the ability to recover the virus from the environmental matrix and the purity of the recovered nucleic acid (Metcalf et al., 1995). Traditionally, PCR gives a positive or negative result, however real-time PCR can quantify the amount of virus in the sample. Other advantages include a smaller time frame to obtain results because an agarose gel is not necessary and a closed system which is less likely to be contaminated (Fong and Lipp, 2005). Like traditional PCR, real-time PCR does not indicate infectivity.

Integrated cell culture-PCR (ICC-PCR) overcomes the individual disadvantages of cell culture and PCR. The use of cell culture helps to dilute out any PCR inhibitors that would otherwise have to be removed using techniques, such as columns, which while removing PCR inhibitors, also simultaneously reduce the concentration of virus. In addition to diluting out PCR inhibitors, cell culture provides an in-vitro amplification system which increases the numbers of viruses and enhances the sensitivity of the assay while providing a means of differentiating between infectious and non-infectious virus (Reynolds, 2004). Additionally, ICC-PCR permits evaluation of a much larger percentage of the original sample as compared to traditional PCR. Several studies have considered the sensitivity, efficiency, and ease of ICC-PCR and found it be better than either traditional PCR or cell culture methods alone (Blackmer et al., 2000; Chapron et al., 2000; Greening et al., 2002; Jiang et al., 2004; Ko et al., 2003; Lee and Jeong, 2004; Lee et al., 2005; Reynolds et al., 1997, 1996, 2001).

2. Methods and materials

2.1. Propagation of reovirus type 1 (Lang)

Reovirus type 1 (Lang) was chosen for the seeded experiments. The virus was obtained from the American Type Culture Collection (ATTC), Manassas, VA (catalog no. VR-230). BGMK cells (ATTC, Rockville, MD) were grown in 175 cm^2 closed cell culture flasks to confluency using MEM supplemented with 10% fetal bovine serum. Prior to infection, the cells were washed and inoculated with reovirus stock at an MOI of 2 pfu/cell. Flasks were incubated at 37 °C for 90 min with periodic rocking. Post absorption, MEM supplemented with 2% fetal bovine serum was added and the flasks were returned to the incubator. Flasks were checked daily and when approximately 75% of the cell monolayer was exhibiting cytopathic effects (CPE) and the cell layer was sloughing off, the flasks were placed in the freezer until the media was frozen and then removed from the freezer and placed at room temperature to thaw. The process of freeze-thawing was repeated three times to liberate the virus. Cellular debris were removed by centrifugation two times at 1000 × *g* (2100 rpm) with a Beckman JA14 rotor for 15 min and the supernatant containing the virus was aliquoted and stored at −80 °C until use.

LLC-MK2 cells were also used to propagate mammalian orthoreovirus type 1 (Lang). The virus was adapted to this cell line with five passages. Similarly to the initial propagation, the cells were grown in 175 cm^2 closed cell culture flasks to confluency using MEM supplemented with 10% fetal bovine serum. After inoculation at an MOI of 4, the propagation proceeded in the exact same manner except on the first four passages instead of storing the supernatant it was used to infect more flasks. On the final passage, the supernatant containing the virus were aliquoted and stored at −80 °C until use.

2.2. Sludge collection

For seeded experiments, 5 l of biosolids were collected from the end of the secondary treatment train at a Concord, MA wastewater treatment plant. This plant serves about 5000 people and treats 1.2 million gallons per day at capacity during the summer months. The treatment process consists of a single-stage trickling filter with intermittent sand beds for winter season polishing. The sludge collected was 3.4% solids. The biosolids were stored at 4 °C until use.

Raw and treated sludge was collected from three different sites: Texas, Pennsylvania, and New Hampshire. The Texas plant uses anaerobic digestion for treatment, the Pennsylvania plant uses liming, and the New Hampshire plant uses composting. One liter of sludge was collected and sent to the laboratory in New Hampshire via overnight mail with the exception of the New Hampshire samples which were collected by the research team and driven to the laboratory. All samples were stored at 4 °C until use.

2.3. Elution of viruses from sludge samples

Viruses were recovered from the sludge samples as designated by the EPA part 503 rules (EPA, 1992). The procedure for recovery of viruses from wastewater solids was an adsorption process reliant upon adsorption of viruses from the liquid phase to the sludge solids, which are concentrated by centrifugation and subsequently eluted. The supernatant was discarded and viruses were desorbed from the solids by physiochemical means and further concentrated by organic flocculation. Decontamination was accomplished by incubation with antibiotics after the viruses were eluted. The concentrated eluent was frozen at −80 °C until evaluation. The losses due by to procedure have been outlined in Katz (2005) and are not significant for reovirus.

2.4. Sample preparations and spiking

Every sludge sample was thawed at 37 °C and vortexed. For each experiment 5 ml of sample was removed from the larger sample and 0.1 μl chloroform per 1 ml sample was added. The sample was then spun at 10,000 × *g* in a micro-centrifuge

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Table 1
Primers used to detect the three types of mammalian reovirus

Serotype	Forward primer	Reverse primer	Position of amplified region
1 (Lang)	gaggagggacacgcgtagtg	ccagatccagaacgaatctcatc	1114–1176
2 (Jones)	cggctacggtgtcaggatct	cgcgcgacgctattttg	1766–1824
3 (Dearing)	ctaccgctgtaccatcgttaagct	tggtacccctccgggatt	112–170

Nucleotides are listed in 5′-3′ direction.

for 5 min. After centrifugation the sample was split into three aliquots. These aliquots were then individually diluted three times in PBS to become a dilution series.

From the 5 l collected in Concord, MA for the seeded experiments, three different aliquots were removed. Spiking of the seeded samples occurred before the first step of the elution procedure. 0.1 ml of reovirus at 10^{-6} was added to the 200 ml in the blender to bring the final concentration of the virus to approximately 10^{-5}. Each of these aliquots were seeded and eluted separately.

2.5. Reovirus plaque assay

Buffalo Green Monkey Kidney (BGMK) cells were grown to 95–100% confluency in six well plates. The concentrations from the dilution series done under sample preparations were tested. Each concentration was enumerated in triplicate and then averaged to determine the pfu/ml. 0.1 ml of the sample being tested was used as an inoculum and added to each well, and incubated for 90 min with periodic rocking for viral adsorption. After adsorption, 4 ml of agar overlay containing 2% bacto-agar and 2× MEM was added to each well. To enhance plaque formation, 100 μl of 1 mg/μl trypsin was added to each well. The agar overlay was then permitted to harden and plates were incubated at 37 °C for 7 days. After 7 days, 1 ml formalin was added to each well, and the well plates were placed in the incubator for 24 h. After 24 h, the agar overlay was removed with warm water and gentle tapping, and 1 ml crystal violet was added to visualize the plaque forming units in the cell layer (Brabants, 2003).

2.6. Integrated cell culture

Each of the samples were done in duplicate; one which was incubated on the cells for 0 days ($T = 0$) and one which was incubated for 7 days ($T = 7$). LLC-MK2 cells were grown to 75–90% confluency in six well plates using MEM with 10% FBS. The media was then removed, the cells were washed, and 100 μl of the specified dilution was added. The cells were rocked every 15 min for 90 min. After rocking, 4 ml MEM was added. For the $T = 0$ plates, the media was immediately removed and 1 ml trypsin added. The plates were incubated for 20 min at 37 °C to loosen the cells and then placed in the freezer. The $T = 7$ plates were placed in the incubator and after 24 h, 100 μl of 1 mg/ml trypsin was added to each well and the plates were returned to the incubator for an additional 6 days. On the 7th day, the media was removed, trypsin was added, the plates were incubated for 20 min and then placed in the freezer. After thawing, RNA extraction was performed on the LLC-MK2 cells in both the $T = 0$ and 7 plates to retrieve any replicated virus.

2.7 RNA extraction procedure

Qiagen QIAamp viral RNA mini kit was chosen for RNA extraction from the samples (Qiagen, 1999). The spin-column procedure was performed with the maximum amount of recommended sample, 140 μl. The virus elution was tested with real-time PCR immediately following extraction and then stored at −80 °C for further use.

2.8. Real-time PCR primer design

The primers and probe sets were designed using Applied Biosystems Proprietary software, PrimerExpress. They were targeted to sections of the genome which diverged in the three types of reovirus. All three sets of primers (Table 1) and probes (Table 2) were based on outer capsid protein (mu-1) in the m2 segment of the genome. NCBI blast was used to determine that the primers detected only the intended target organism.

2.9. Quantitative real-time reverse transcription polymerase

Primers were received from Applied Biosystems dry and desalted at 80,000 pmol and diluted to 50 μm/l with molecular grade water. The probe was received from Applied Biosystems at 6000 pmol in 60 μl buffer and was diluted to 10 μM/l with molecular grade water. Five μl of the extracted DNA was combined with 25 μl of master mix. The master mix contained 1× of TaqMan® One-Step RT-PCR Master Mix supplied by Applied Biosystems (part number: 4309169) containing AmpliTaq Gold® DNA polymerase, Passive Reference I, and optimized buffer components. The master mix also contained 1× of MultiScribe™ Reverse Transcriptase and RNase Inhibitor from Applied Biosystems, 900 nM of the forward primer, 900 nM of the reverse primer, 250 nM of the probe, and sterile molecular grade water. The sample was placed in the ABI Prism 7700 real-time PCR thermocycler. The run consisted of 48 °C for 45 min,

Table 2
Probes used to detect the three types of mammalian reovirus

Serotype	Probe	Position
1 (Lang)	cttggatcagattgctc (tagged with FAM)	1137–1153
2 (Jones)	taatccgaaaggtattttgt (tagged with VIC)	1787–1806
3 (Dearing)	atcacctggaatgct (tagged with FAM)	137–151

They coordinate with the primers listed in Table 1. Nucleotides are listed in 5′-3′ direction.

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95 °C for 10 min, and 50 cycles of 94 °C for 20 s, 55 °C for 1 min, and 72 °C for 30 s. The fluorescence was detected at the end of each cycle to determine the cycle threshold value (CT) which is the cycle number at which the fluorescence generated within the reaction crossed the threshold. The CT values for the $T=7$ samples were compared to the corresponding CT values for the $T=0$ samples. For the seeded samples, each sample was combined with the reovirus type 1 primers. For the environmental samples, each sample was combined in separate tubes with each of the three primers.

2.10. Correlation

To determine the relationship of plaque forming units to cycle threshold values without cell culture, reovirus type 1 was diluted three times. Aliquots from each concentration were analyzed directly by a plaque assay and by real-time PCR. This entire analysis was repeated four times and plotted to determine the numerical relationship between the variables. To compare the same amount in the plaque assay and the quantitative PCR, the plaque forming units in the 5 μl of sample, which was the amount tested on the PCR, were determined from the plaque forming units per ml found in the plaque assay. This calculation assumed 100% recovery for the extraction method.

2.11. Quality control

The laboratory maintained strict quality assurance methods. The ABI Prism 7700 real-time PCR thermocycler was calibrated monthly using a calibration plate provided by Applied Biosystems. In addition, weekly the background fluorescence was examined and instrument wells emitting light were cleaned with alcohol. In the plaque assay, each run had three wells that served as the negative control and three wells for the positive control. Additionally, each time a sample was diluted into a dilution series; the PBS was tested on a plaque assay to confirm that it did not produce any plaques. For the cell culture portion of the ICC-PCR, a positive and negative well were done each time the assay was run. The positive control was reovirus type 1 and the negative control was a well with no virus at all. For the RNA extraction, the positive and negative controls from the cell culture portion were included. An additional negative control was added, which is referred to as the spin control. This was a tube that had all the buffers added and was placed in the centrifuge each time a spin was required in the protocol. These three controls were included as part of the real-time PCR assay. An additional negative control was added that was just the master mix, and an additional positive control was added that was the master mix with non-manipulated reovirus. These five controls were included with every PCR assay.

3. Results

3.1. Seeded experiment

In the seeded experiments, plaque forming units, the CT value at $T=0$, and the CT value at $T=7$ were compared. The most dilute sample in which reovirus was found in each assay was 10^{-2} for $T=0$, 10^{-8} for $T=7$, and 10^{-5} for the plaque assay (Fig. 1). The comparison between the $T=0$ days and the $T=7$ days shows that more dilute portions of the sample contained virus after incubation in cell culture (Fig. 2). The $T=7$ days compared to the plaque forming units shows that the ICC-PCR method detected the most dilute amount of virus (Fig. 3).

3.2. Environmental samples

When environmental samples were tested, 54% of the samples were positive by ICC-PCR and none were positive by the plaque assay method (Table 3). Each location had positive samples but of the treatments only aerobic digestion had a positive sample (Table 4). The positive samples summarized by treat-

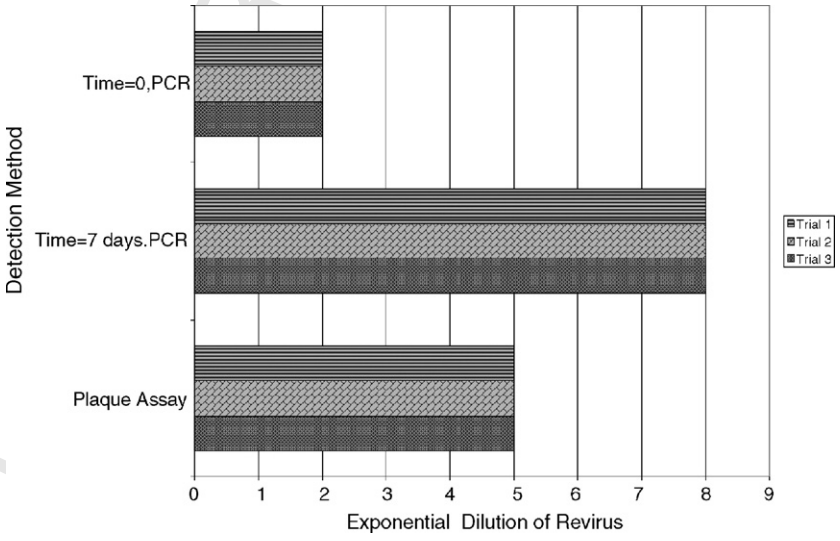

Fig. 1. The sensitivity of the ICC-PCR vs. the plaque assay. In the seeded experiments, the more dilute sample in which reovirus was detected was 10^{-2} for the $T=0$ ICC-PCR, 10^{-8} for the $T=7$ ICC-PCR and 10^{-5} for the plaque assay. This shows the sensitivity of the ICC-PCR vs. the plaque assay.

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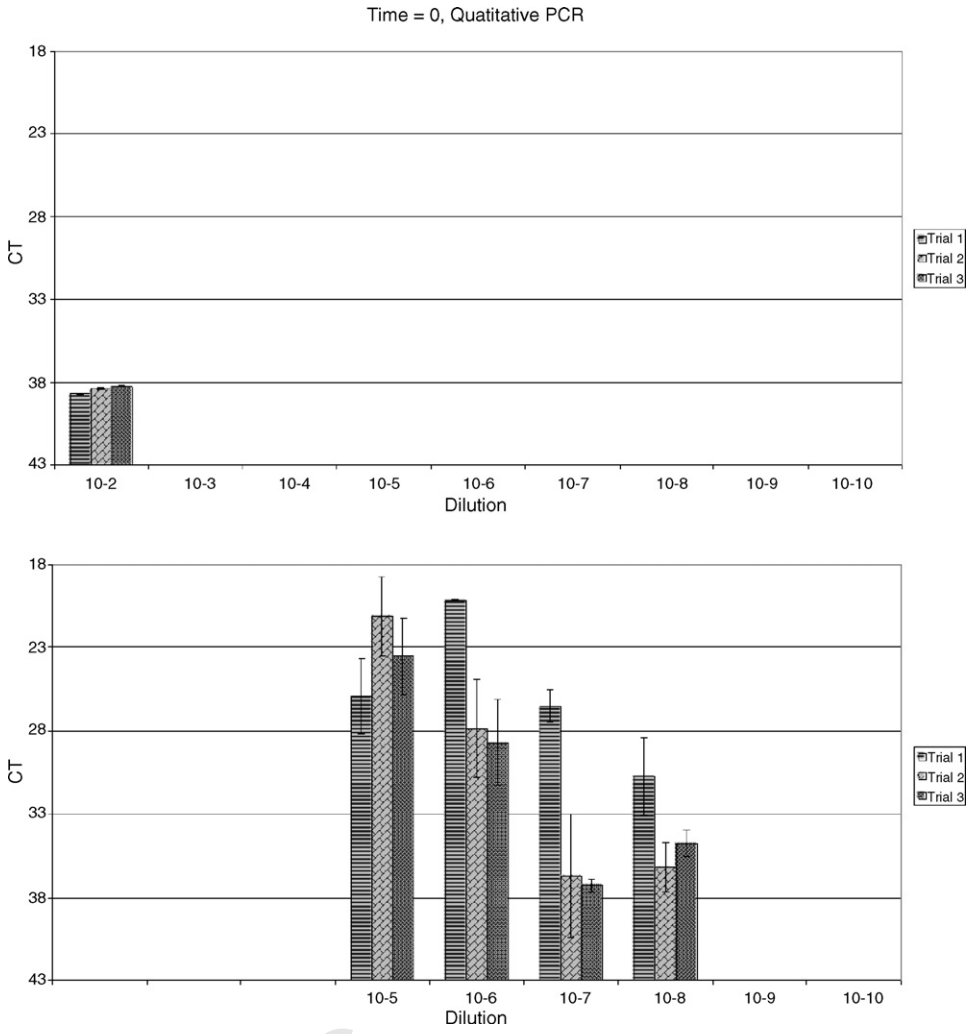

Fig. 2. ICC-PCR detected infectious virus. The $T=7$ ICC-PCR detected reovirus in each of the dilution 10^{-3} to 10^{-8} where reovirus was not detected in the $T=0$ ICC-PCR. This indicates that each of these 10-fold dilutions contained infectious virus.

ment show that 80% of the untreated samples were positive and 11% of the treated samples were positive (Table 5). The types of reovirus varied among the different locations, reovirus type 3 was detected at the Texas location and reovirus type 1 detected at the New Hampshire and Pennsylvania locations (Table 6). The PFU from the plaque assay was plotted as a scatter plot along with the CT values from the real-time PCR to show the rela-

Table 3
Out of the 24 environmental biosolids samples that were tested for reovirus, 13 tested positive by ICC-PCR, and none tested positive by the plaque assay technique

States tested	Number of samples tested	Positive for infectious reovirus by	
		ICC-PCR	Plaque assay
NH	9	5	0
TX	9	6	0
PA	6	2	0
Total	24	13	0
Percent positive (%)		54	0

Table 4
The environmental samples are divided into the treatment types which shows how many untreated are positive and how many treated are positive broken out by location

Location	Treatment	Undiluted (positive/total)	10^{-1} (positive/total)
Texas	Untreated	3/3	3/3
	Limed	0/3	
New Hampshire	Untreated	3/3	2/3
	Composted	0/3	
Pennsylvania	Untreated	1/3	
	Digested	1/3	

Table 5
When the samples are grouped by the treatment type (disregarding the locations), 80% of the untreated samples are positive and 11% of the untreated samples are positive

Treatment	Positive by ICC-PCR/ total samples	Percent positive by ICC-PCR (%)
Untreated	12/15	80
Treated	1/9	11

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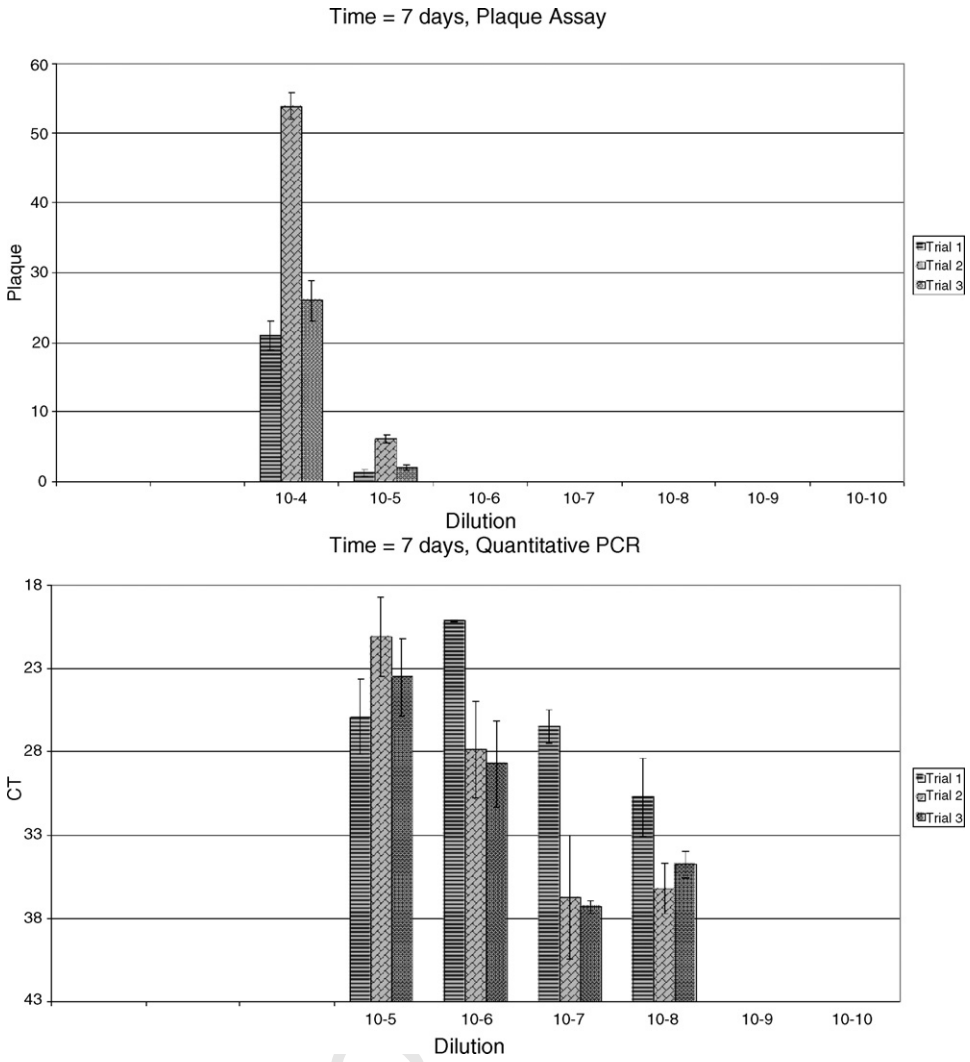

Fig. 3. ICC-PCR detected the most viruses. The top graph shows the plaque assay detected reovirus to a dilution of 10^{-5} and the bottom graph shows the ICC-PCR detected the virus to 10^{-8}.

Table 6
The type of reovirus detected based on sample location

State	Reovirus type(s) found
Texas	Type 3 (Dearing)
New Hampshire	Type 1 (Lang)
Pennsylvania	Type 1 (Lang)

tionship between the two variables. The environmental samples can then be plotted on the line to estimate the final concentration of virus (Fig. 4). The $T=0$ samples are not listed for the environmental samples because they were all negative.

4. Discussion

It is clear that integrated cell culture with real-time RT-PCR detects more mammalian orthoreovirus than the plaque assay method. To determine infectivity using ICC-PCR, the CT value for a $T=7$ sample was compared to the corresponding CT value for the $T=0$ sample. If the CT value for $T=7$ was lower, it was concluded that the lower CT value was from an increased concentration of target viral nucleic acid, indicating viral replication and infectious virus. Evaluation of the results shows that incubation on cells yielded infectious virus and upon comparison to the plaque assay, demonstrates the limitations of using the plaque assay as the method of choice when evaluating biosolids for infectious virus.

All of the seeded dilutions from 10^{-3} to 10^{-8} contained no detectable virus at $T=0$ in the ICC-PCR and all contained detectable virus at $T=7$, which means that each of the dilutions contained at least one virus particle that was able to replicate itself. While it is possible that there was only one infectious virus particle in each of the sample tubes, it is unlikely given that each sample tube was a 10-fold dilution of the previous. Of the dilutions that contained virus at $T=7$, dilutions 10^{-6}, 10^{-7}, and 10^{-8} did not form plaques in the plaque assay. The samples were taken from the exact same tube to minimize dilution inaccuracies. However, there is a larger sample amount tested in a plaque assay then in a PCR tube, and therefore this comparison is somewhat biased to the plaque assay and underestimates the sensitivity of the ICC-PCR. If the three logs detected at $T=0$ are subtracted from the eight logs detected at

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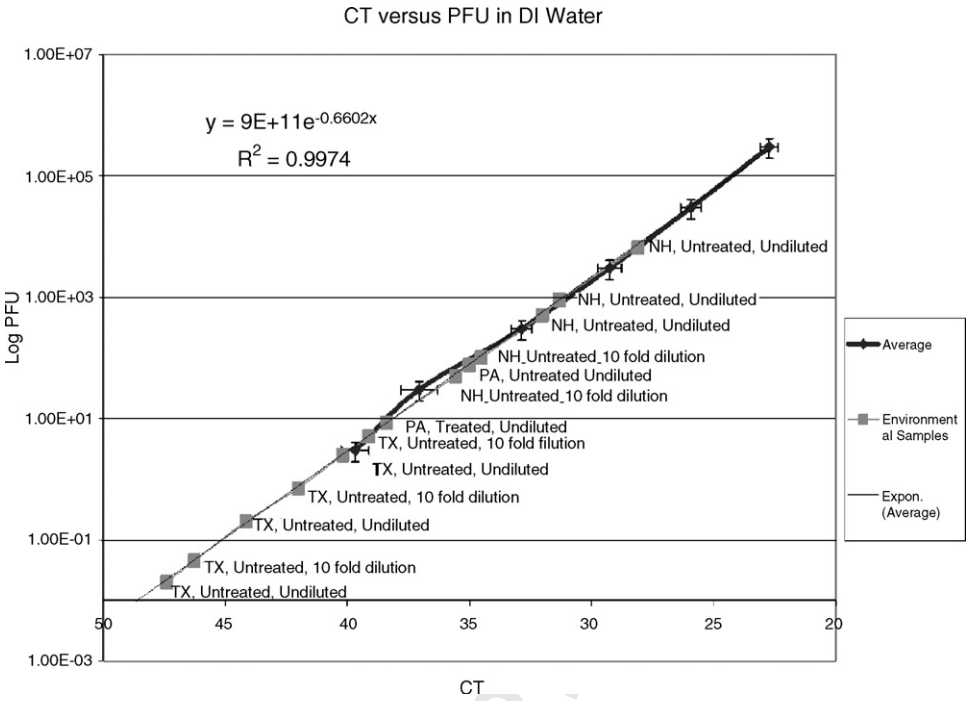

Fig. 4. Relationship between PFU and CT. The scatter plot of the average PFU vs. the CT demonstrates a relationship. The environmental samples are placed on the line created by this relationship and their final concentrations can be estimated.

$T=7$, this is an increase of six logs. This data could be interpreted as only a one log difference between the ICC-PCR and the plaque assay. However, because each tube was a 10-fold dilution and contained decreasingly smaller amounts with more than one infectious virus present in each dilution, it is better to compare the $T=0$, $T=7$, and plaque assay within each dilution. Even if only one log more was detected, the seeded samples demonstrated that the ICC-PCR was a more sensitive method.

One of the problems with plaque assays is cellular damage due to toxicity. This is a problem with the plaque assay because it does not directly detect any part of the virus, but rather points to viral presence by cell lysis. This makes interpretation of the plaque assay more questionable than the ICC-PCR method as described in the study. The ICC-PCR directly detects the viral nucleic acid and for the final analysis the sample can be cleaned to a greater degree because removing the viral capsid proteins does not affect PCR. If there were some toxic effects of the sample, they would have occurred in the cell culture portion of the experiment, and the amount of cells available for infection overcomes this issue.

Similar to the seeded samples, the ICC-PCR method was more sensitive than the plaque assay with the environmental samples. The samples did not plaque, but 54% tested positive for reovirus. For locations where the first three samples of a treatment type yielded a positive result when undiluted samples were used, the sample was diluted 10-fold. A large portion of the samples were positive at the more dilute concentrations indicating the potential for a very small amount of sample to multiply to detectable levels of virus. Based on a positive result, it is not possible to know the exact amount of virus in the biosolids and the small sample size should not be misleading as it relates to risk when consideration is given to the total amount of material applied to a field.

The three sets of primers and probes allowed for differentiation between the types of reovirus detected. In addition to regional variation, the differential detection of the virus types may also be a reflection of different original starting concentrations or differential growth rates among the types after 7 days of incubation.

The correlation between the CT from the real-time PCR and the PFU from the plaque assay can be seen on the scatter plot. The line was extended and the environmental samples were plotted on the line to estimate the amount of virus detected in the ICC-PCR. This is the amount of virus present in the sample after 7 days incubating in cell culture. There should exist a relationship between the amount of virus in the sample before incubation and the amount of virus in the sample after incubation, but that relationship was not determined in this study and needs further exploration. In this study, samples were evaluated on a semi-quantitative basis using a series of dilutions. Ultimately it may be possible to correlate the amount of virus at some incubation time to an original starting concentration to make ICC-PCR truly quantitative.

Very few studies have used ICC-PCR to detect wastewater viruses. Only one other study known to the authors has used a quantitative ICC-PCR method. Astrovirus was quantified using a dilution technique and detected by PCR (Grimm et al., 2004). This study did not compare the results to a cell culture method.

Previously published work clearly demonstrates that ICC-PCR is more effective than both the plaque assay method and the total culturable virus assay-most probable number assay (TCVA-MPN) for the detection of virus.

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Several other studies have been done in a water matrix as compared to a sludge or bisosolids matrix. In one such study, poliovirus and hepatitis A virus was detected by ICC-PCR quicker than cell culture alone (Reynolds et al., 1997). Another study found ICC-PCR to be more rapid and more sensitive for virus detection than looking for cellular cytopathic effects (CPE) (Reynolds et al., 1996). In another study, ICC-PCR detected enterovirus and adenovirus in 13 samples while the TCVA-MPN assay did not detect any virus (Lee et al., 2005). When water samples from across the United States were tested for enteroviruses, adenoviruses and astroviruses comparing the TCVA-MPN method to ICC-PCR, ICC-PCR detected viruses in 48% more samples than TCVA-MPN (Chapron et al., 2000).

Previous work using seeded samples has also been done. In water seeded with adenovirus, ICC-PCR occurred more rapidly than virus detection by the TCVA-MPN assay (Ko et al., 2003). In a study where samples seeded with poliovirus were UV disinfected and then tested for virus using ICC-PCR and TCVA-MPN, ICC-PCR detected poliovirus at the later time points, where TCVA-MPN did not (Blackmer et al., 2000). In another seeded study, researchers compared two different ICC-PCR methods of virus detection to a $TCID_{50}$ assay for the detection of hepatitis A virus. They demonstrated that hepatitis A virus was still infectious by ICC-PCR but not by the more traditional $TCID_{50}$ method 60 h after treatment (Jiang et al., 2004).

In samples of sewage, marine water, and surface water Hepatitis A virus and enteroviruses were detected by ICC-PCR more rapidly than TCVA-MPN (Reynolds et al., 2001). In detecting enteroviruses and adenoviruses in sewage, sludge, river water, and shellfish in New Zealand, ICC-PCR detected more adenoviruses than the plaque assay, although more enteroviruses were detected by the plaque assay than ICC-PCR. However, only the media was removed for testing and therefore the cell layer was not tested, and this may have affected the ICC-PCR results as viruses are intracellular (Greening et al., 2002).

Reovirus has been studied extensively because of its presumed prevalence in water and biosolids (AWWA, 1999). Prior to this study, detection by plaque assay has underestimated the amount of reovirus present. This study has shown that the ICC-real-time RT-PCR method can be used to determine reovirus presence and concentration. With this new method for detection and quantification of infectious reovirus, further work needs to be done to estimate the risk of land applied biosolids.

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