Q-330

Simultaneous Quantification of Coxsackieviruses and Echoviruses in Disinfection Studies Using an Integrated Cell Culture-qPCR Assay

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Abstract

The Contaminant Candidate List (CCL) includes microbes warranting priority research due to limited data availability. One reason for this limitation is that standard *in-vitro* cell culture methods, which are used for the detection and quantification of viruses (including two CCL enteroviruses: coxsackieviruses and echoviruses), are expensive and time consuming. We have developed an alternative strategy for the simultaneous quantification of enteroviruses in disinfection studies using integrated cell culture–quantitative RTPCR (ICC-qPCR). Traditional cell culture assays require separate experiments for each enterovirus since they infect the same cells. ICC-qPCR enables simultaneous quantification of different enteroviruses, thereby yielding a substantial cost and time savings. The applicability of ICC-qPCR for the simultaneous quantification of coxsackievirus B6, echovirus 10, and poliovirus type 1 was demonstrated in this study. To determine whether 24-well trays can accommodate high virus levels using ICC-qPCR, BGM cells were inoculated with a series of 10-fold dilutions (10^1 to 10^6 $TCID_{50}$/ml of each virus) of mixed enteroviruses and were incubated for 0, 4, 6, 8, and 24 hours post inoculation (h.p.i.). At 24 h.p.i., there was sufficient virus replication to differentiate between initial virus concentrations as low as 10^1 $TCID_{50}$/ml for echovirus and poliovirus and 10^2 $TCID_{50}$/ml for coxsackievirus. An approximate 10-fold increase was observed for dilutions from 10^1 through 10^4 $TCID_{50}$/ml. As a result of well capacity limitations, the ICC-QPCR quantities for the 10^5 and 10^6 $TCID_{50}$/ml samples leveled off in comparison to those with lower initial concentrations. To avoid this effect, samples that are expected to have high viral concentrations (low inactivation in disinfection samples) should be diluted when applied to cells. Most ICC-qPCR results were greater than the inactivated virus (false positive) control. QA/QC controls demonstrated that the Taqman probes prevented cross-reactivity by amplifying only the target virus. The results indicate that ICC-qPCR can be used to simultaneously quantify enteroviruses in disinfection studies.

Background

***Contaminant Candidate List (CCL)*:**

- List of unregulated contaminants warranting priority research regarding their occurrence, health effects, and treatment/disinfection such that informed decisions regarding their future regulatory status can be made.
- The CCL is revised every five years. The second CCL, published in 2005, identified 9 microbial contaminants, including two enteroviruses: coxsackieviruses and echoviruses.

***Enteroviruses*:**

- The enterovirus genus consists of more than 70 serotypes of small, non-enveloped, positive strand RNA viruses, including coxsackieviruses, echoviruses, polioviruses, and numerically-identified enteroviruses.

Coxsackievirus

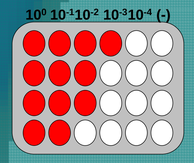
Echovirus

Poliovirus

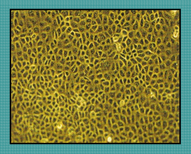
- Human enteroviruses are the second most common cause of viral infections and are annually responsible for 30 – 50 million infections in the United States.
- Enterovirus infections are often manifested subclinically, but may also be associated with a wide range of clinical outcomes, including the common cold, hand-foot-and-mouth disease, conjunctivitis, meningitis, and poliomyelitis.

Standard Assay for Enteroviruses : In-Vitro Cell Culture:

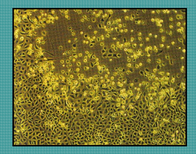
- Infectivity assay which depends on visual differentiation between infected host cells demonstrating cytopathic (CPE) effects and healthy, uninfected cells.
- One sample may require 24 wells.
- Cells are observed for up to 14 days and statistical methods are used to estimate the viral concentration of the original sample based on the number of positive and negative wells.
- Method is time- and cost-intensive, and somewhat subjective.



24-Well Tray Per Sample



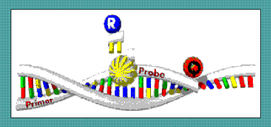
Non-Infected BGM Cells



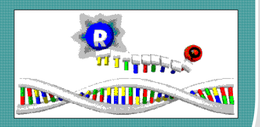
Infected BGM Cells Showing CPE

Quantitative Real-Time qPCR using Taqman Probes:

Primer and Probe Annealing



Primer Extension



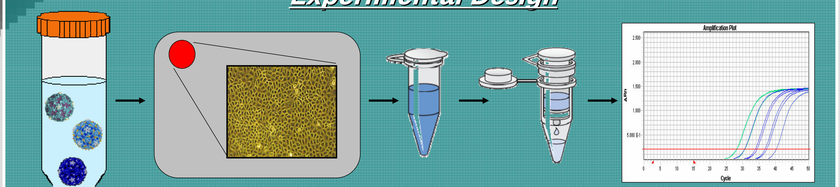
Probe Cleavage and Fluorescence

- Reverse transcription at 48ºC for 30 min; RT inactivation and initial denaturation at 95ºC for 10 min; 40 cycles of denaturation at 95ºC for 15 sec, annealing and extension at 60ºC for 30 sec.

Materials and Methods

- A mixed enterovirus sample was prepared using equal volumes of 10^6 $TCID_{50}$/ml coxsackievirus B6, echovirus 10, and poliovirus type 1. Dilutions ranging from 10^6 through 10^1 $TCID_{50}$/ml were prepared.
- The dilutions were applied to BGM cells in 24-well trays and allowed to incubate for 1 hr. The supernatant was then removed using three washes with TRIS buffer. An overlay of 1 ml 2% FBS MEM was applied.
- The trays were incubated at 37ºC for different time points: 0, 4, 6, 8, and 24 hours post inoculation. Following incubation, the wells (including supernatant, cells, trypsin, and wash buffer) were harvested.
- The cell harvests were subjected to one freeze/thaw cycle (-80ºC/20ºC) to facilitate the release of viruses from the cells.
- A QIAamp Viral RNA Mini Kit was used to purify the samples and extract the viral RNA.
- Real-time quantitative RTPCR was used to quantify the number of each of the viruses using specifically-designed probes.

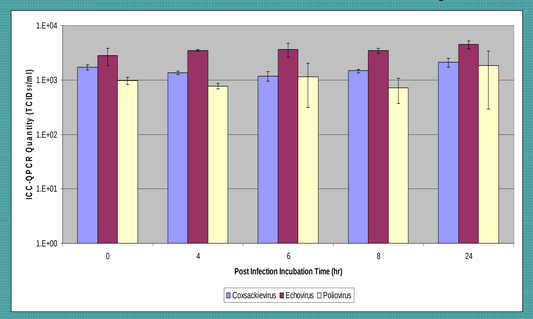
Experimental Design



Mixed Virus Sample — *In-vitro* Cell Culture — Well Harvest — Purification — Real-time Quantitative PCR (qPCR)

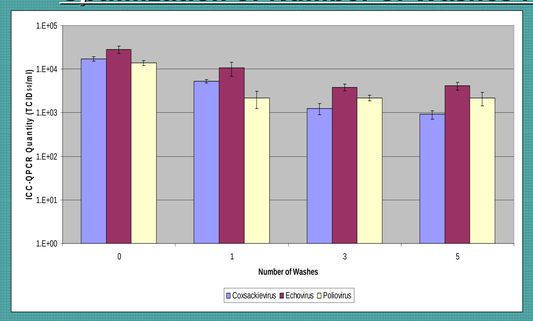
Results

Non-Infectious Virus ICC-qPCR Experiment



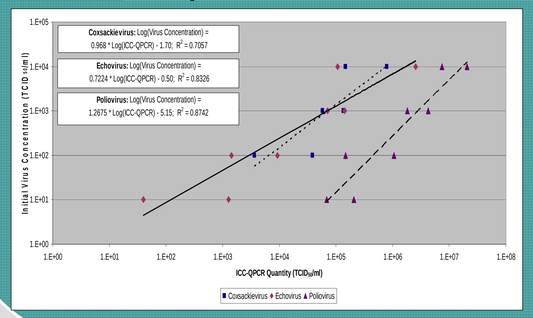
No significant replication was observed for an inactivated enterovirus sample. The results illustrated the need to remove non-infectious viruses in order to prevent false positives. This was accomplished by washing the wells after the 1 hr virus incubation period.

Optimization of Number of Washes for ICC-qPCR



An inactivated enterovirus sample was washed 0, 1, 3, or 5 times using Tris buffer to facilitate removal of non-infectious viruses. The results indicated that 3 washes removed a significant number of viruses.

ICC-qPCR Standard Curves for 24 h.p.i.

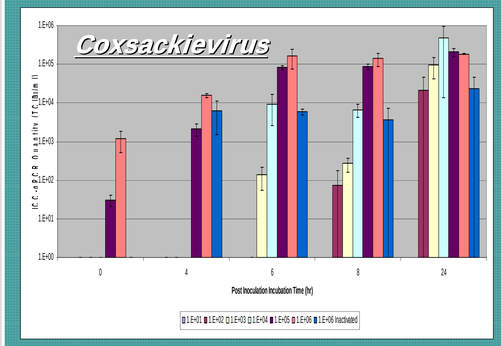


A standard curve is used to quantify the initial virus concentration for unknown samples (such as those from disinfection studies) using the ICC-qPCR quantities.

Results (Cont.)

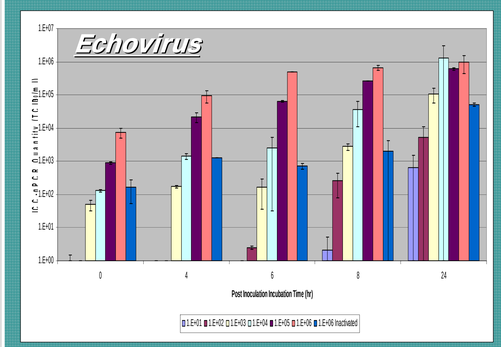
Time Optimization experiments were performed to determine the optimal incubation time for the mixed enteroviruses. Serial dilutions (10^6 through 10^1 $TCID_{50}$/ml) were applied to cells and harvested after different incubation times (0, 4, 6, 8, and 24 hours post inoculation).

Coxsackievirus



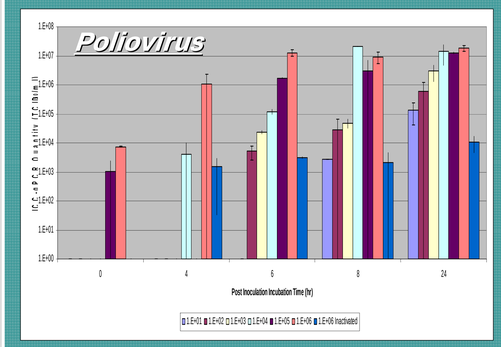
- Some enteroviruses may infect cells very rapidly, such that not all viruses are washed out, as shown by the 0 h.p.i. samples.
- At 0 and 4 h.p.i., only the highest spiking concentrations were detected for coxsackievirus and poliovirus.
- At 6 and 8 h.p.i., less concentrated virus samples were detected. For echovirus, there was approximately 1 log difference between each dilution from 10^3 - 10^6 $TCID_{50}$/ml. This trend was not consistent for coxsackievirus and poliovirus.

Echovirus



- At 24 h.p.i., there was sufficient replication to differentiate between initial concentrations as low as 10^1 $TCID_{50}$/ml for echovirus and poliovirus and 10^2 $TCID_{50}$/ml for coxsackievirus.
- At 24 h.p.i., the ICC-qPCR quantities leveled off as an effect of well capacity, but about 1 log difference is seen for each dilution from 10^1 - 10^4 $TCID_{50}$/ml.

Poliovirus



- At 24 h.p.i., most ICC-qPCR quantities were greater than the inactivation control. This control represents the false positive detection limit and is specific to each experiment.
- 24 h.p.i. is optimal for the detection of enteroviruses at concentrations from 10^1 to 10^4 $TCID_{50}$/ml. For samples in which a high concentration of viruses is expected, dilutions are recommended in order to avoid well capacity limitations.

Summary

- The integrated cell culture-quantitative real time RTPCR (ICC-qPCR) assay was successfully applied for the simultaneous quantification of the CCL viruses coxsackievirus and echovirus as well as poliovirus.
- The assay represents a significant time savings compared to conventional *in-vitro* cell culture techniques (24 hrs of incubation vs. 2 weeks). It also introduces less subjectivity into the quantification of infectious viruses vs. conventional cell culture.
- Well washing experiments demonstrated that 3 post-infection washes removes a significant number of non-infectious virus particles.
- Incubation time optimization experiments indicated that 24 h.p.i. was optimal for the simultaneous quantification of coxsackievirus, echovirus, and poliovirus.

For additional information regarding the use of this ICC-qPCR assay in disinfection studies, please visit Poster Q-440: *Photocatalytic Inactivation of Viruses Using Low-Pressure Ultraviolet Light in a Titanium Dioxide Suspension*

Acknowledgements

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