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Optimizing a Suspension Culture Method with a Decreased Cost to Detect Enteroviruses in Water to Increase Surveillance Access

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Abstract: Enteroviruses are a public health threat due to the high incidence of infections and potential for serious illness or death. Some laboratories in high-income countries detect enteroviruses in water by integrating cell culture and PCR (ICC/PCR). This combined method carries a high financial burden, due in part to specialized cell culture equipment. Therefore, we expanded upon a pilot study to reduce the cost by using common laboratory polypropylene tubes to create a cell culture in suspension. We optimized the protocol by determining minimal incubation periods post-infection as a function of the initial virus concentration. Cells in suspension and traditional monolayers were inoculated with poliovirus and incubated in 8-hour intervals up to 48 hours prior to extraction. Quantitative PCR (qPCR) was used to detect viral nucleic acid targets. Treated and raw water samples were seeded with virus and the suspension ICC/qPCR protocol used to ascertain whether the protocol performed similar to directly seeding cells. No variation in virus detection occurred using the suspension ICC/qPCR or monolayer ICC/qPCR ($p = 0.95$). In surface water samples, viral nucleic acid was successfully detected, with no significant increase after 32 h ($p > 0.05$). Suspension ICC/qPCR is as effective as monolayer ICC/qPCR in detecting enteroviruses in surface waters. Materials used in the suspension ICC/qPCR have a lower monetary cost than traditional cell culture materials without loss of sensitivity. More accessible testing of waters for enterovirus contamination through cost reduction has the potential to reduce human exposure and disease.

Keywords: enteroviruses; detection; waterborne; methods; poliovirus

1. Introduction

Enteroviruses (EVs) are waterborne viruses which can lead to a variety of human diseases [1]. An estimated 30–50 million symptomatic cases occur in the United States every year [2]. Poliovirus, an EV, caused 350,000 cases in over 125 countries by 1988, causing paralysis in 1 out of 200 cases and death in 5% to 10% of those paralyzed [3,4]. The number of poliovirus cases has dropped to 33 cases worldwide in 2018; however, contaminated environmental samples are still found in countries declared polio-free [3–5]. Continued surveillance of water samples is needed to keep countries polio-free leading to global eradication.

No vaccines exist for other enteroviruses, yet these can also result in grave syndromes such as acute flaccid paralysis, encephalitis, meningitis and death [6–8]. This is exemplified by the outbreak of Enterovirus 71 in 2008 where there were 490,000 cases and 126 deaths and acute flaccid paralysis caused by Enterovirus D68 in the USA [9,10]. Due to the potential for severe illness, death, and outbreak potential, The World Health Organization recently declared non-polio enteroviruses

an emerging major public health threat [7,11]. Research and development are the principle tenets by this declaration, which includes understanding the prevalence of these viruses in people and the environment. These activities are crucial because risk of viral disease transmission can be interrupted through active surveillance [12–15].

The icosahedral capsid of enteroviruses enables some tolerance to environmental changes and common water treatments [2,16]. Therefore, using common water treatments may not eliminate all enteroviruses from water sources [16]. Low resource settings are disproportionately affected by infectious diseases and with less infrastructure for environmental pathogen surveillance. It is imperative to provide methods with reduced financial costs to promote surveillance of EVs in water used especially by higher-risk populations [17]. Many of these areas still suffer from poor sanitation and crowded living conditions which promote the spread of waterborne diseases [18,19].

The United States Environmental Protection Agency (EPA) called on water providers to use Method 1615, which uses conventional cell culture and polymerase chain reactions (PCRs) separately, to test for the presence of enteroviruses in water samples [20,21]. Independently, laboratories have developed assays which integrate cell culture and PCR (ICC/PCR) to detect enteroviruses [22,23]. Cell culture propagates infectious virus distinguishing inactivated nucleic acid from replicating viruses, while PCR gives greater sensitivity than culture [15,24–26]. This method remains inaccessible to most low resource areas due to the financial cost and access to specialized materials.

To our knowledge, only one article investigated using cells suspended in media in common laboratory polypropylene tubes compared to traditional monolayer ICC/quantitative PCR (qPCR) to detect EVs in water [27]. This study determined that the suspension method resulted in higher concentrations of virus after 6 days of incubation [27]. The authors noted the suspension culture had increased infectivity, reduced costs of polypropylene tubes versus cell culture flasks, and reduction of cell manipulation, reducing the potential for contamination and labor [27]. However, this study suffered from a small sample size (*n* = 6) and used a long incubation period of 6 days. Therefore, we examined the suspension ICC/qPCR protocol for the detection of EVs to determine the minimal incubation time of buffalo green monkey kidney (BGMK) cells required to detect virus in surface waters varying the starting viral concentration, expecting 6 days to be more than what would be required. In addition, we compared the methods to determine whether either was associated with earlier EV detection, hypothesizing that the suspension ICC/qPCR method would perform better.

2. Materials and Methods

Poliovirus type 1 strain LSc-1 (PV-1) (ATCC, Gaithersburg, MD, USA) was propagated in buffalo green monkey kidney (BGMK) cells (ATCC, Gaithersburg, MD, USA) grown in minimum essential media (MEM) comprised of 43% Leibovitz's L-15 (Sigma-Aldrich, St. Louis, MO, USA), 27% Eagle's minimal essential medium (Sigma-Aldrich, MO, USA), 24% HEPES (Thermo Fisher Scientific, Waltham, MA, USA), 4% sodium bicarbonate (Millipore, Billerica, MA, USA), 2% (*w/v*) L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 1% non-essential amino acids (Thermo Fisher Scientific, Waltham, MA, USA), and 1% kanamycin sulphate (Sigma-Aldrich, St. Louis, MO, USA). The BGMK cells and PV-1 were incubated at 37 °C for 90 min, after which MEM supplemented with 2% Hyclone fetal bovine serum (FBS) (Cytiva, Marlborough, MA, USA) was added prior to returning flasks to the incubator for 24 h. Flasks were observed under an inversion microscope to verify PV-1 infection of the BGMK cells. Stocks were aliquoted in 15% glycerol (Thermo Fisher Scientific, Waltham, MA, USA) and phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −80 °C.

Restored BGMK cells were washed three times with PBS and 1 mL of trypsin-ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific, Waltham, MA, USA) was added to prevent cells from attaching to plasticware used to prepare cell cultures during a 45-minute incubation period on a rocker. Following the incubation period, cells were observed. To prepare monolayer samples, cells were grown to confluency in 25 cm^2 rectangular canted neck cell culture flasks with phenolic-style caps (Corning Incorporated, Corning, NY, USA) with 4mL of MEM supplemented with FBS for a final volume of 5 mL. BGMK cells

in suspension were obtained by placing 1 mL of cells in EDTA into 15 mL conical tubes. MEM with 2% FBS was added to reach a total volume of 5 mL. PV-1 was diluted with PBS to attain desired viral titers of 1, 10, and 100 PFU/mL in a total volume of 100 μL, which was added to flasks and conical tubes containing cells. Beginning immediately after inoculation (T = 0), a minimum of one flask and one tube was frozen and stored. The remaining samples were incubated at 37 °C while continuously rocking. Every 8 h a tube was removed and frozen at −80 °C. This process continued for 48 h. The ICC/qPCR procedure was performed a minimum of 6 times for each time interval in each type of culture (Figure 1).
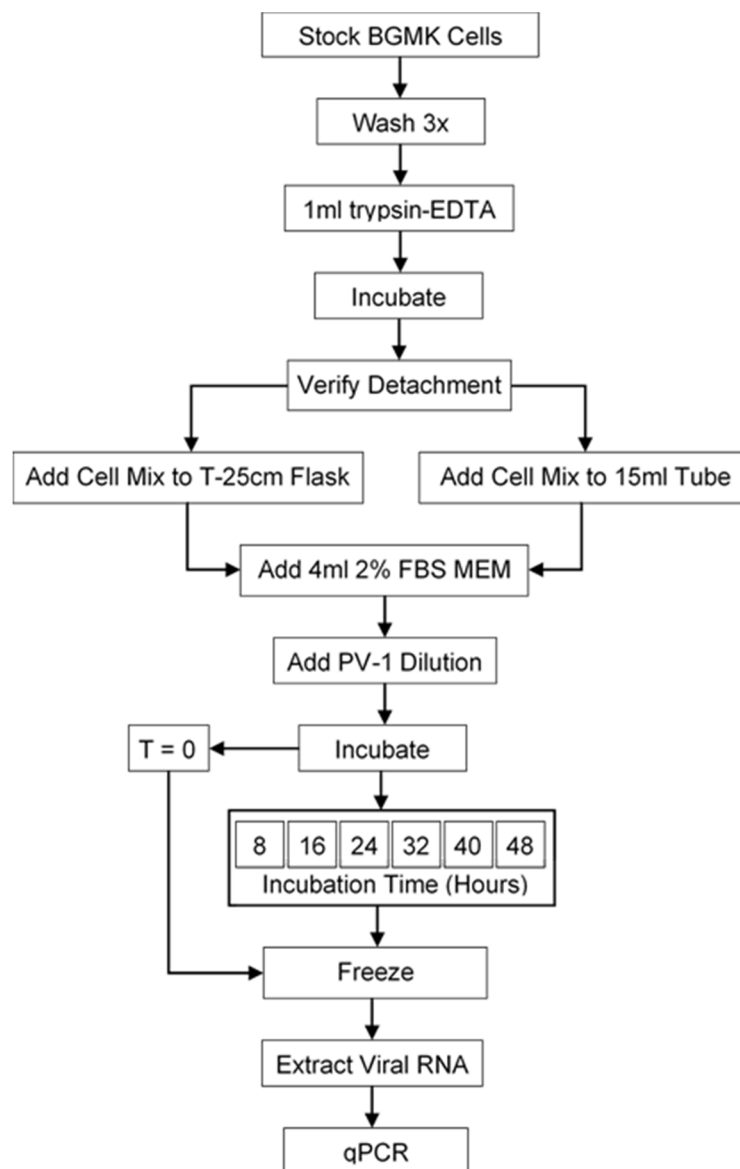

Figure 1. Preparation of monolayer and suspension cultures.

Four water samples (two raw and two treated samples), previously determined to be negative for enteroviruses were seeded with poliovirus. After mixing, 5 mL of each water sample was placed in a 15 mL conical tube (Corning Incorporated, Corning, NY, USA) and inoculated with enumerated PV-1. Seeded samples were serially diluted and added to 5 mL of suspended BGMK cells for a final PV-1/surface water inoculum of 20 PFU/mL. Samples were subjected to the suspension ICC/qPCR method using the method described above, incubating at 37 °C, removing and freezing a tube at −80 °C every 8 h up to 48 h.

Prior to extracting PV-1 RNA from any samples, the cultures were removed from frozen storage and placed in a 37 °C water bath for rapid thawing. Once thawed, QIAamp Blood Mini kits (QIAGEN Germantown, MD, USA) were used to extract the viral RNA. The spin protocol accompanying the kits was followed with minor changes: the amount of ethanol used was increased from 200 to 230 μL, elution buffer was decreased from 200 to 60 μL, and the incubation time was increased from 1 to 5 min [2]. The final elution containing the target nucleic acid sequence was immediately used in qPCR. TaqMan® Fast Virus 1-Step Master Mix (MM), panenterovirus primers, and probes, from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA, USA) as described previously were used for the detection of viral nucleic acid [28]. The probe was labeled with FAM as a reporter dye and TAMRA as a quencher dye. The primers and probes were added to the MM, which was thoroughly mixed and maintained at 4 °C until use. To perform the assay, 15 μL of the prepared MM, primers, and probes were added to separate reaction wells of a 96-well plate. Every well tested received 5 μL of final eluted product from the RNA extraction procedure. The qPCR thermocycling was performed at 48 °C for 45 min, 95 °C for 10 min, 94 °C for 15 s, and 55 °C for 1 min for 40 cycles. Molecular grade water was used as a negative control and considered successful when it did not return a qPCR cycle threshold (Ct) value under 40. The direct addition of 5 μL of 10^5, 10^6, and 10^7 PFU/mL PV-1 was used to determine a standard of detection (Table 1), and 5 μL of 10^7 PFU/mL was included as a positive control to each PCR run. Less than or equal to 10^4 PFU/mL was not detected via qPCR.

Table 1. Mean cycle threshold (Ct) values of poliovirus type 1 strain LSc-1 (PV-1) dilutions used in the standardization of detection values.

PFU/mL	Mean Ct (Standard Deviation)
10^5	34.4 (4.8)
10^6	21.2 (0.4)
10^7	18.1 (0.1)

Ct values 35 or higher were not included in analyses, with the assumption that viral titers less than 10^5 PFU/mL were not able to be detected (Table 1). Analysis of variance (ANOVA) was used to determine the association of Ct values with time of sample incubation, starting viral PFU/mL, or the use of a monolayer T-25 cm^2 cell culture flask versus a polypropylene centrifuge tube. Time of incubation was performed in 8-hour increments up to 48 h. Models were created for seeded surface water samples to determine if the type of water sample was associated with differences in Ct values. These water samples had previously been determined to be negative for enteroviruses. All statistical analyses were considered significant at an alpha of 0.05 with two-tailed 95% confidence intervals (CIs). Data analysis was performed in STATA 14.2/IC, and figures were produced in IBM SPSS Statistics.

3. Results

A total of 120 samples, 62 suspension and 58 monolayer, were processed and analyzed via qPCR. A total of 8 samples had Ct values of 35 or higher and, as described in Appendix A and were treated as not having virus nucleic acid detected to avoid the detection of environmental contamination. Over half of the samples inoculated with a starting concentration of 1 PFU/mL did not return Ct values for analysis and were therefore excluded. Of the remaining samples, 84 samples with Ct values were analyzed, 38 were from monolayer and 46 were from suspension cultures. A total of 36 samples did not have detectable nucleic acid (Table 2), particularly in samples frozen at time 0 h.

Table 2. Number of samples with detectable viral nucleic acid by cell culture method and time.

Time (Hours)	Overall N (%)	Suspension N (%)	Monolayer N (%)
0	4 (28.6%)	2 (25.0%)	2 (33.3%)
8	12 (70.6%)	7 (87.5%)	5 (55.6%)
16	10 (58.8%)	7 (87.5%)	3 (33.3%)
24	14 (73.7%)	8 (80.0%)	6 (66.7%)
32	14 (73.7%)	7 (70.0%)	7 (77.8%)
40	16 (88.9%)	9 (90.0%)	7 (87.5%)
48	14 (87.5%)	6 (75.0%)	8 (100%)

The average Ct value of all samples was 23.3. The mean Ct value was highest in samples with 1 PFU/mL and earlier time points. Nucleic acid from suspension cultures increased between time 0 and 24 h as demonstrated by mean Ct values, whereas values continued to decrease until 40 h, on average, in monolayer samples (Table 3).

Table 3. Mean Ct values of PV-1 nucleic acid extracted from suspension and monolayer cultures.

	Overall ($N = 84$)	Suspension ($N = 46$)	Monolayer ($N = 38$)
Mean Ct (standard deviation)	23.3 (7.4)	23.37 (6.7)	23.3 (8.2)
PFU/mL			
1	28.2 (5.7)	29.9 (3.7)	27.4 (6.4)
10	21.3 (5.6)	22.0 (5.9)	20.4 (6.3)
100	20.3 (5.4)	21.3 (4.5)	18.8 (6.3)
Incubation Time (hours)			
0	27.4 (2.5)	29.4 (1.6)	29.0 (6.4)
8	28.6 (4.5)	29.3 (3.8)	29.5 (7.0)
16	26.8 (4.0)	25.7 (3.8)	29.5 (3.4)
24	21.7 (4.2)	20.9 (3.0)	24.7 (7.4)
32	19.3 (5.4)	21.0 (7.8)	24.1 (10.1)
40	17.7 (3.0)	20.6 (6.5)	16.3 (2.2)
48	18.9 (6.6)	22.8 (9.8)	18.1 (6.4)

Time incubated and starting PV-1 PFU/mL were both significantly associated with mean Ct values ($p < 0.00$) in univariate models. No statistically significant difference was observed in mean Ct values when comparing monolayer or suspension cell cultures ($p = 0.48$). An ANOVA including all three variables of interest maintained the statistical significance of Ct values with time and Ct values with starting viral concentration (both $p < 0.00$); however, the method of cell culture still lacked an association with mean Ct value ($p = 0.18$). We plotted the mean Ct values determined at each 8 h time interval with an initial PV-1 titer of 100 PFU/mL in both culture types (Figure 2).

An ANOVA determined that there was no relationship between Ct values and whether water was raw or treated ($p = 0.15$); however, time incubated was significant ($p < 0.00$). In a model examining the four types of water individually, there was no statistically significant association with Ct values ($p = 0.06$) over time (Figure 3).

We tested whether BGMK cell concentrations increased over time, leading to the decrease in Ct values observed throughout the experiment. Cells were counted every 24 h after following the suspension ICC/qPCR culture (using a starting PV-1 inoculum of 100 PFU/mL) through 144 h. A linear regression was performed to assess the relationship of cell counts and time incubated, which was not significant at any time point. Although not statistically significant, there was an increase in the mean cell/mL in the first 48 h of incubation. A separate experiment was performed to determine the relationship of different starting BGMK cells and Ct values using an inoculum of 100 PFU/mL. Cells in suspension cultures were adjusted to contain 10^5, 10^6, or 10^7 BGMK cells in each sample. Using 10^7 BGMK cells as a reference value, after adjusting for time incubated, starting cell

concentrations of 10^6 BGMK cells were not statistically significant ($p = 0.61$); however, samples with cell concentrations of 10^5 were significantly associated with Ct values compared to 10^7 ($p = 0.01$).
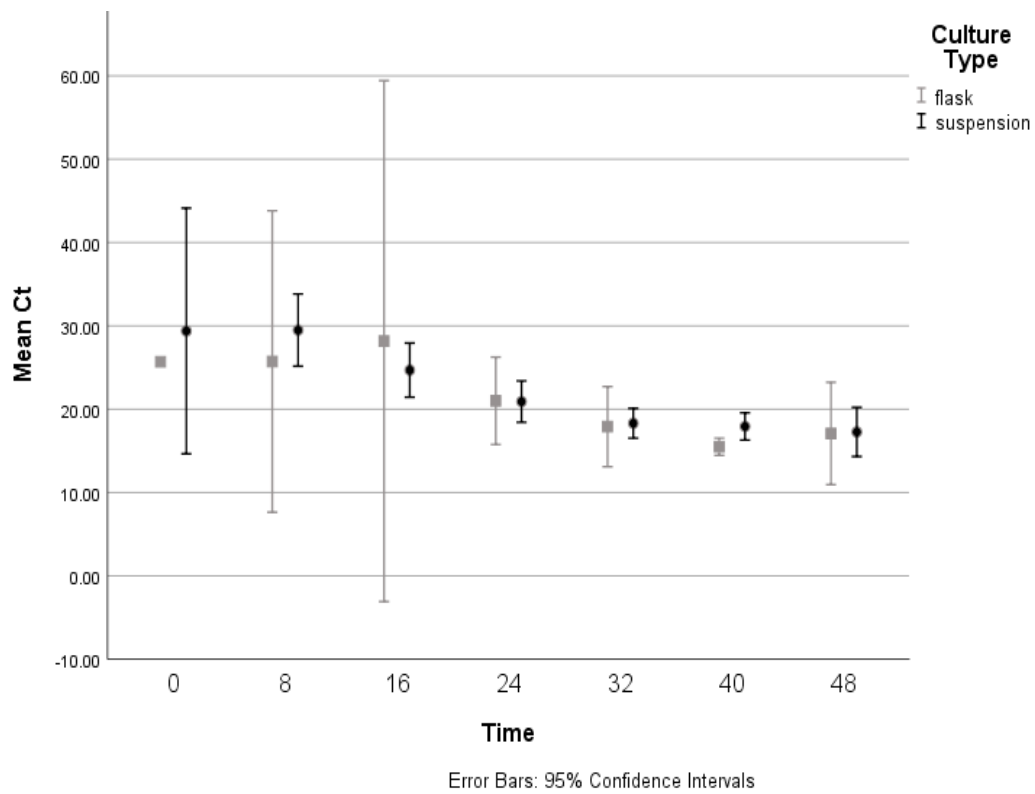

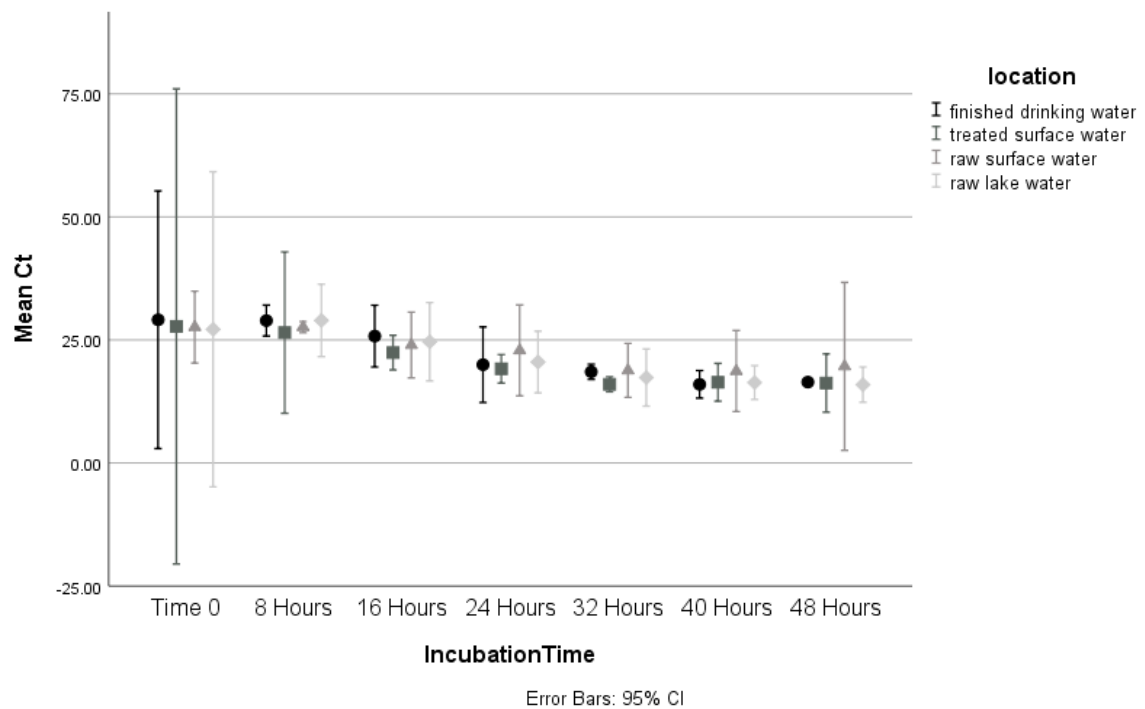**Figure 2.** Mean cycle threshold at 8-h intervals in suspension and monolayer cultures.

Figure 3. Mean cycle threshold in seeded water samples using the suspension integrative cell culture/quantitative polymerase chain reaction method.

4. Discussion

Both the monolayer and suspension cultures showed no statistically significant decrease in Ct values after 24 h of incubation prior to RNA extraction in samples inoculated with 10 or 100 PFU/mL into 5 mL of BGMK cells and media. Starting cell concentrations were approximately 10^6 cells in both monolayer and flask samples and the 24 h incubation period allowed for approximately four to six replication cycles of poliovirus [16,29]. Assuming that the starting concentration doubled during each replication cycle, the ending viral titer would be greater than the number of cells in the sample after four cycles. Since Ct values no longer significantly decreased after 24 h, it is likely that most BGMK cells were infected in both types of cultures and the plateauing of Ct values after 24 h.

In early publications of the ICC/PCR method, it was noted that one of the main benefits is rapid detection, with one publication noting the detection of PV-1 after 24 h versus multiple days when using traditional cell culture [30]. Another study compared the use of ICC/PCR versus traditional cell culture and found that detection was reliable within two days in the ICC/PCR, whereas 14 days were necessary using cell culture alone [28]. We found that PV-1 was consistently detected using the suspension ICC/qPCR protocol within 24 h using low starting concentrations of 10 and 100 PFU/mL.

Along with time post-infection, the optimal starting viral concentration was determined under the prescribed conditions. The suspension ICC/qPCR assay was able to detect less than 100 PFU/mL in 5 mL; although, such low starting titers may require additional time to improve reliability. Increasing the beginning viral concentration would likely result in earlier detection; however, we used small titers of enterovirus to better mimic environmental sources, a strength of our study compared to others [31]. This paper presents evidence that suspension ICC/qPCR culture is able to detect levels of EVs that may be present in environmental water samples.

The T-25 cm^2 monolayer flasks used to obtain the cells for suspensions are estimated to contain about 2.5×10^6 cells; therefore, the lack of statistical difference in Ct values in BGMK cell concentrations of 10^6 compared to 10^7 BGMK cells is likely due to all cells being infected with PV-1. We base this assumption on our standard curve, suggesting approximately 10^6–10^7 PFU/mL before 48 h, halting an increase in viral nucleic acid. Although BGMK concentrations of 10^6 did not result in the lowest Ct values, enterovirus was consistently detected without the use of additional manipulation or larger supplies which would increase the financial burden of the protocol. Therefore, the BGMK concentrations found in T-25 cm^2 vessels of about 2.5×10^6 cells are appropriate for detecting enteroviruses in water samples [27]. Earlier suspension culture results questioned the effect of cell concentration in the suspension ICC/qPCR protocol. It has been theorized that the suspension culture merely allows additional space for cells to replicate, resulting in lowered Ct values. By determining the cell concentrations in both culture methods over time, our results refute this assessment as there was no significant difference in cell counts over time.

This study finds that the suspension ICC/qPCR reliably detects low concentrations of enteroviruses in surface waters within 24 h. This study is the first to determine the minimum time post-infection required to detect enteroviruses at concentrations which may be encountered in surface waters with high consistency. The detection is possible regardless of whether a suspension or monolayer culture is used. Long incubation periods, often one to two weeks, are not necessary to make a final determination of the presence of enterovirus in surface water using the suspension ICC/qPCR method. These extended time periods are a potential source of unnecessary labor, materials, and personnel costs [25,32].

Our investigation differs from earlier work by suggesting that suspension cultures allow for faster detection than monolayer cultures [27]. The pilot study by Balkin and Margolin used 6-day incubation periods, perhaps allowing BGMK cells to be present in significantly greater concentrations in larger vessels, which increased PV-1 replication and reduced Ct values in suspension cultures compared to monolayers. However, in agreement with that study, we demonstrate the utility of suspension cultures, also reducing material costs by using common polypropylene tubes. This study is limited by not performing a formal costing analysis; however, the financial savings are readily apparent. For example, 1000 T-25 flasks from Thermo Fisher Scientific (https://www.thermofisher.com/us/en/home.html),

a major worldwide distributor and partner of laboratory products, currently costs USD 1980 for flasks bearing the Fisher Scientific name. To purchase the same flasks made by Corning, makers of popular cell culture products, the current cost rises to USD 2600 on Thermo Fisher Scientific's website. At the same time, the price for 1000 15 mL conical laboratory tubes is USD 620 for 1000 from the same supplier. This means a saving of USD 1360–USD 1980 per 1000 pieces of plasticware. Scaling up testing to include more samples or locations, especially over time, would make the savings become large.

5. Conclusions

Combining the information collected on time incubated post-infection, initial virus titer, BGMK cell concentration, and the effect on Ct values, it is determined that a minimum of 10 PFU/mL in 5 mL of MEM containing 10^6 BGMK cells incubated for 24 h allowed for the consistent detection of enteroviruses in water. The work presented here demonstrates that a suspension ICC/qPCR protocol is effective at detecting and quantifying enteroviruses in surface water rapidly and with high efficiency while using common polypropylene tubes instead of cell-culture-specific flasks, thereby reducing financial costs and increasing accessibility when compared to current published methods. This study is of particular importance for the surveillance of water quality, and therefore public health, particularly in settings with limited financial resources or access to specialized equipment.

Appendix A

Table A1. Characteristics of samples with Ct values above 35.

Ct Value	Sample Time	Culture Type	Starting PFU/mL
35.5	32 h	Monolayer	1
36.4	Time 0	Monolayer	10
36.9	24 h	Monolayer	1
37.2	Time 40	Suspension	1
39.2	8 h	Monolayer	100
39.4	32 h	Monolayer	1
39.4	48 h	Suspension	10
39.7	32 h	Suspension	1

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