Comparative assessment of BGM and PLC/PRF/5 cell lines for enteric virus detection in biosolids

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Abstract

The BGM cell line is required for the detection of enteric viruses in biosolids through a total culturable viral assay (TCVA) by the United States Environmental Protection Agency. In the present study, BGM and PLC/PRF/5 cell lines were evaluated for TCVA and for their use in determining the incidence of adenoviruses and enteroviruses in raw sludge and Class B biosolids. Six raw sludge and 17 Class B biosolid samples were collected from 13 wastewater treatment plants from seven U.S. states. Samples were processed via organic flocculation and concentrate volumes equivalent to 4 g total solids were assayed on BGM and PLC/PRF/5 cells. Cell monolayers were observed for cytopathic effect (CPE) after two 14-d passages. Cell lysates were tested for the presence of adenoviruses and enteroviruses by PCR or RT-PCR. The PLC/PRF/5 cells detected more culturable viruses than the BGM cells by CPE (73.9% versus 56.5%, respectively). Fifty-two percent of the samples were positive for CPE using both cell lines. No viruses were detected in either cell line by PCR in flasks in which CPE was not observed. No adenoviruses were detected in 13 CPE-positive samples from BGM lysates. In contrast, of the 17 samples exhibiting CPE on PLC/PRF/5 cells, 14 were positive for adenoviruses (82.4%). In conclusion, PLC/PRF/5 cells were superior for the detection of adenoviruses in both raw sludge and Class B biosolids. Thus, the use of BGM cells alone for TCVA may underestimate the viral concentration in sludge/biosolid samples.

Keywords: biosolids; ICC-PCR; enteric viruses; adenovirus; BGM cell line; PLC cell line

Introduction

In the United States, approximately 5.6 million tons of dry biosolids are generated annually, of which 60% is used for agricultural land application to provide additional nutrients for crops (National Research Council 2002). Biosolids are classified as either Class A or Class B depending upon the concentration of enteric pathogens present (United States Environmental Protection Agency 2003) as measured by mammalian cell culture. Class B biosolids are allowed to contain detectable levels of enteric viruses, but typically have below one most probable number (MPN) per four grams (Pepper et al. 2010). Class A biosolids are required to have less than one plaque forming unit per four grams of total culturable viruses using the Buffalo green monkey (BGM) cell line (United States Environmental Protection Agency 2003).

Class B biosolids are the most commonly generated biosolids in the United States and are produced by mesophilic anaerobic digestion (MAD), a process using bacteria to break down the biodegradable materials in biosolids in the absence of air for at least 15 d at 35°C (Gerba et al. 2002b; Viau and Peccia 2009). Other methods include aerobic digestion (bacterial breakdown of biodegradable materials in the presence of oxygen), lime stabilization (addition of a lime slurry to raise the pH to >12 for 2 h, followed by retention at pH >11.5 for an additional 22 h), and dewatering (concentrates the solids content of sludge to 20-40% via filtration and the use of drying beds) (Gerba and Pepper 2015). Numerous studies have reported the occurrence of enteric viruses in biosolids after the digestion processes (Monpoeho et al. 2004; Bofill-Mas et al. 2006; Guzmán et al. 2007; Viau and Peccia 2009; Pepper et al. 2010; Wong et al. 2010). A variable fraction containing as much as 50% of the enteric virus present in the raw sewage may be associated with the solids (Payment et al. 1986); therefore, the concentration of viruses in the final treated biosolids

can even be higher than in the source wastewater. It is thus important to be able to detect viruses in Class B biosolids to determine the efficacy of the treatment processes.

The genus *Enterovirus* includes a large group of non-enveloped, single-stranded RNA viruses that include poliovirus, human rhinoviruses, enteroviruses, echoviruses, and Coxsackieviruses. Human adenoviruses are non-enveloped, double-stranded DNA viruses with 67 serotypes organized into subgroups A to G (Matsushima et al. 2013); subgroup F is composed of enteric adenoviruses types 40 and 41 that are most associated with diarrhea in children (Strauss and Strauss 2002). Adenoviruses are known to be more resistant to ultraviolet (UV) light and heat than other enteric viruses (Enriquez et al. 1995; Gerba et al. 2002a) and can survive in the environment for prolonged periods of time (Gerba et al. 2002b).

Currently, the BGM kidney cell line is recommended by the United States Environmental Protection Agency for the detection of culturable viruses in biosolids (United States Environmental Protection Agency 2003). However, the A549 (human alveolar basal epithelial cells) and PLC/PRC/5 (primary liver carcinoma) cell lines have been shown to yield greater numbers of infectious enteric viruses (Chapron et al. 2000; Lee and Jeong 2004; Vivier et al. 2004; Sedmak et al. 2005). In addition, unlike with BGM cells, adenoviruses will produce observable cytopathogenic effects (CPE) in these cells (Lee et al. 2004; Rodríguez et al. 2008).

The primary goal of the current study was to compare the BGM and PLC/PRC/5 cell lines for total culturable viruses and for the specific detection of enteric viruses in raw sewage sludge and Class B biosolids. A secondary objective was to assess the occurrence and relative abundance of adenoviruses and enteroviruses in these samples.

Materials and Methods

Sample Processing

Twenty-three samples were collected from 13 different wastewater treatment plants located in seven states: Arizona (three locations), Washington (three locations), Michigan (two locations), Minnesota (two locations), Oregon, Wisconsin, and Wyoming. Six of the samples were raw sewage sludge and 17 were Class B biosolids. The samples were collected during two different periods (Phase I and II). The biosolids were generated using a variety of treatments including mesophilic anaerobic digestion, aerobic digestion, lime stabilization, or drying beds.

The samples were concentrated by organic flocculation using 3% beef extract as described previously (American Society for Testing Materials 1993; Gerba et al. 2011). Sample concentrates were re-suspended in 30 mL of 0.15 M sodium phosphate (final pH adjusted to 7.0 – 7.5 after resuspension). Next, the following solutions were added: 1 mL of an antibiotic mixture (containing 10,000 units/mL of penicillin G, 10,000 µg/mL of streptomycin sulfate, 25 µg/mL of amphotericin B), 2 mL of kanamycin sulfate (5 mg/mL), and 1 mL of gentamicin (5 mg/mL). The concentrates were frozen at -80°C until assayed via cell culture. The efficiency of the organic flocculation averaged 60% using poliovirus type 1 (strain LSc-2ab; obtained from the Department of Virology and Epidemiology, Baylor College of Medicine, Houston, TX).

Cell Culture Infection

The concentrate of each sample was divided into sub-samples (three replicates per volume; typically 2.5 mL, 1.0 mL, and 0.1 mL). The 2.5-mL volumes were used to inoculate 75 cm^2 flasks

containing cell monolayers; the 1-mL and 0.1-mL volumes were used to inoculate 25 cm^2 flasks. Both the PLC/PRF/5 cell line (ATCC #CRL-8024; American Type Culture Collection, Manassas, VA) and the BGM cell line (United States Environmental Protection Agency, Cincinnati, OH) used in this study were between passage 40 and 85. The cells were three to five d old with approximately a 90% confluent monolayer at the time of infection. Inoculated flasks were incubated at 37°C with slow agitation using a platform shaker (Gyratory Shaker-Model G2, New Brunswick Scientific Co. Inc., Edison, NJ) for one h. Following this, the sample liquid was then discarded and Eagles maintenance media (Gibco™ Invitrogen Corporation, Grand Island, NY) containing 2% fetal bovine serum (Hyclone, Logan, UT) was added. The flasks were incubated for 14 d at 37°C, with media changes every four d, and examined each d for viral CPE.

Confirmation of Positive and Negative Cell Culture Flasks

Both positive and negative flasks for viral CPE after 14 d of incubation were frozen at -20°C and thawed twice in order to release any viruses from the cells. These solutions were then filtered through Steriflip® membrane filters with a 0.22 µm diameter pore size (Millipore Corporation, Billerica, MA) to remove cellular debris and from this, 1 mL was inoculated into flasks containing fresh cell monolayers (three to five d old) to confirm the viral CPE result (positive or negative), with incubation for an additional 14 d. The viral MPN/4 g was determined using the EPA Most Probable Number (MPN) Calculator Version 2.0 (United States Environmental Protection Agency 2013). The viruses were harvested from the confirmatory flasks (CPE-positive flasks) or from the second passage flasks (CPE-negative flasks) by three freeze-thaw cycles as before and stored at -20°C until polymerase chain reaction (PCR) or reverse transcription-PCR (RT-PCR) analysis,

respectively, for adenoviruses or enteroviruses.

Detection of Enteroviruses using Semi-Nested RT-PCR

The PCR primers and assay conditions are shown in Table 1. The PCR primers for enteroviruses were obtained from Sobsey et al. (1996). The RT and PCR conditions and the internal nested PCR primer sequence were obtained from Rodríguez et al. (2008). Prior to RT-PCR analysis, the cell lysate was heated to 97°C for five min and chilled on ice. The final PCR products were analyzed by gel electrophoresis using 2% agarose in 0.5X Tris-borate-EDTA buffer containing 5 μg/mL of ethidium bromide. Electrophoretic bands were visualized using an AlphaImager 2000 (Alpha Innotech Corporation, San Leandro, CA). RNA-free water was used as a negative control in all PCR assays.

Detection of Adenoviruses by Nested PCR

The PCR procedure for adenoviruses was obtained from Van Heerden et al. (2003); the primer sequences were obtained from Avellón et al. (2001). Details of the adenovirus PCR assay are shown in Table 1. The PCR mixture consisted of 1X PCR buffer, 2.5 mM $MgCl_2$, a 200 μM concentration of each dNTP, a 1 μM concentration of each primer, 1 U of Amplitaq GOLD polymerase, 10 μL of sample, and water for a total volume of 50 μL. The PCR products were analyzed as described previously. RNA-free water was used as a negative control in all PCR assays.

Statistical Analysis

A Student's t-test was used to compare the MPN/4 g values for the TCVA between the BGM and the PLC/PRF/5 cell lines for the detection of enteric viruses in raw sludge and Class B biosolid samples. Differences between the two cell lines were considered statistically significant if the resulting *P* value was ≤ 0.05.

Results and Discussion

The results of the total culturable virus assay (TCVA) are shown in Tables 2 and 3. A total of 12 of the 23 samples (52.2%) were positive CPE on both cell lines. Thirteen samples out of 23 (56.5%) were positive for culturable viruses on BGM cells, with an average of 39.3 MPN/4 g. Using the PLC/PRF/5 cells, 17 out of 23 (73.9%) samples were positive for culturable viruses, with an average of 76.8 MPN/4 g. CPE was also observed earlier with the PLC/PRF/5 cells than with the BGM cells, averaging three to five vs. eight d, respectively. Even though the difference in the ability to detect culturable viruses between these two cell lines was evident, there was no statistical difference between the total numbers of viruses detected in samples that were positive in both cell lines ($P = 0.145$).

For the raw sewage samples, all six (100%) tested positive for culturable viruses in BGM cells; only five of the six (83.3%) were positive in the PLC/PRF/5 cells. Nevertheless, the PLC/PRF/5 cells proved to be more sensitive for culturable viruses than the BGM cells when testing Class B biosolids (70.6% versus 41.2% positive, respectively). Similar results have been reported for treated sewage effluents for TCVA and subsequent PCR using BGM and PLC/PRF/5

cell lines. Only 8% of effluent samples were positive for CPE in BGM cells, whereas 57% of PLC/PRF/5 cells exhibited CPE (Rodríguez et al. 2008).

As described previously, biosolids were collected during two different periods. During Phase I, all of the samples were assayed using both cell lines; however, the cell lysates were only assayed using PCR for adenoviruses. Based on the results from this first set of samples, during Phase II, RT-PCR was also performed on the cell culture lysates for the detection of enteroviruses. This included both samples positive and negative for CPE on both cell lines. The RNA-free water used as negative controls were verified to be negative for enteroviruses and/or adenoviruses in all PCR assays.

Two of 11 BGM cell lysates (18.2%) were positive for enteroviruses; both of these had previously exhibited viral CPE. No enteroviruses were detected by the RT-PCR / semi-nested PCR in any of the four negative CPE samples tested. No adenoviruses were detected in any of the 23 BGM cell lysates (0%), including 13 samples that were previously positive for CPE. Adenoviruses do not produce plaques and therefore are not detected in plaque assays using BGM cell (Yates 2014). The culturable viruses that produced CPE in BGM cells in the current study were most likely either enteroviruses or reoviruses based on previous studies conducted in our laboratory (data not shown) and by others (Sedmak et al. 2005). For instance, in a study by Sedmak et al. (2005) of wastewater influent and effluent samples collected over a nine-year period using a variety of cell lines (BGM, HEp-2, Caco-2, and RD cells), 90.6% of the samples were positive for reoviruses and 85.0% of the samples were positive for enteroviruses. Most of the reovirus isolates (96.2%) from the samples were found using BGM cells. The majority of adenoviruses in influent samples were detected using HEp-2 cells (87.2%); none of the samples were positive for adenoviruses using BGM cells. Rodríguez et al. (2008) were also unable to detect any adenoviruses

in treated wastewater effluents by PCR of BGM cell lysates, whereas 52% of the samples were positive for adenoviruses by PCR of PLC/PRF/5 cell lysates (Rodríguez et al. 2008).

In the PLC/PRF/5 cell lysates in the current study, one of 11 (9.1%) was found to be positive for enteroviruses. This sample had previously exhibited viral CPE in the PLC/PRF/5 cell monolayers. All 23 PLC/PRF/5 cell lysates were examined for the presence of adenoviruses. Of the 17 samples exhibiting CPE, 14 were positive for adenoviruses by nested PCR (82.4% of the CPE-positive samples; 60.1% of the total number of samples). None of the samples that were negative for viral CPE in PLC/PRF/5 cells monolayers were positive for enteroviruses or adenoviruses using PCR.

Enteric viruses were detected by CPE in six of six (100%) raw sludge samples using BGM cells and in five of six (83.3%) samples using PLC/PRF/5 cells. Nevertheless, none of the six (0%) CPE-positive lysates from BGM cell monolayers were found to be positive for adenoviruses by PCR, whereas four of the five (80.0%) CPE-positive samples from PLC/PRF/5 cell lysates were confirmed as adenoviruses. For Class B biosolids, 41.2% were positive for CPE on BGM cell monolayers and 70.6% were positive on PLC/PRF/5 cell monolayers. Overall, the PLC/PRF/5 cell line was able to detect culturable viruses in more biosolid samples than the BGM cell line (17 samples versus 13 samples, respectively). In addition, the PLC/PRF/5 cell line was superior to BGM cells for the specific detection of adenoviruses. A total of 82.4% of samples with positive CPE in PLC/PRF/5 cell culture were confirmed to be adenoviruses by PCR. In contrast, none of the 13 samples (0%) with positive CPE in BGM cells were found to be adenoviruses using PCR. Grabow et al. (1999) reported similar results in a study comparing the sensitivity of PLC/PRF/5, BGM, PVK and L20B cell lines in which adenoviruses were only detected by PLC/PRF/5 cells in river water. Van Heerden et al. (2003) found adenoviruses in raw and treated wastewater using

PLC/PRF/5 and CaCo-2 cells. Non-CPE producing adenoviruses were also detected by nested PCR in both cell lines. Another study determined that the PLC/PRF/5 cell line was superior to 293 and Chang conjunctival cells for the propagation and enumeration of typical laboratory strains of adenovirus 40 and adenovirus 41 as well as two stool isolates of adenovirus 41. The greater susceptibility of PLC/PRF/5 cells to adenoviruses was reflected by the more rapid appearance of CPE (Grabow et al. 1992). These observations agree with our data in which PLC/PRF/5 also demonstrated more rapid onset of CPE than BGM cells, therefore reducing the time required for virus detection.

To our knowledge, this is the first report of the utilization of PLC/PRF/5 cells for the detection of adenoviruses in raw sludge and Class B biosolids and one of only a few studies on the occurrence of human adenoviruses in biosolids (Lyndholm and Nielsen 1983; Williams and Hurst 1988; Bofill-Mas et al. 2006; Pepper et al. 2010; Rhodes et al. 2015). This study further confirms the survival of infectious adenoviruses after mesophilic and aerobic sludge digestion (Lyndholm and Nielsen 1983). Infectious adenoviruses usually occur in greater numbers than enteroviruses in activated sludge-treated wastewater (Irving and Smith 1981). Our results also found the occurrence of infectious adenoviruses in sewage sludge to be greater than that of enteroviruses, which corresponds to previous studies (Williams and Hurst 1988; Pepper et al. 2010). Studies evaluating the survival of different serotypes of viruses in the environment have shown adenoviruses to be more resistant to temperature and humidity fluctuations than some enteroviruses (Mahl and Sadler 1975; Irving and Smith 1981). Schwarz et al. (2014) also showed that adenoviruses were capable of prolonged survival after land application of biosolids. Since the occurrence of adenoviruses is likely greater than that of enteroviruses in biosolids and considering that adenoviruses do not grow well on BGM cells, the use of BGM cells alone for TCVA most likely underestimates the enteric

viral concentration in sludge/biosolid samples. PLC/PRF/5 cells appear to be superior for the detection of adenoviruses in both raw sludge and Class B biosolids and thus should be considered for use alongside BGM cells for TCVA.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1. PCR primers used to detect enteroviruses and human adenoviruses in raw sludge and Class B biosolids.

Viruses Detected	Primer	Sequence (5' - 3')	Gene Target	Product (primers used)	PCR Assay Conditions	Reference
Enteroviruses	EV-L Forward (external)	CCTCCGGCCCCTGAATG	5' untranslated region	195 bp (EV-L/EV-R)	RT step: 44°C for 60 min, 99°C for 10 min, 50°C for 25 s, 72°C for 45 s. PCR step: 35 cycles of 94°C for 25 s, 55°C for 25 s, 72°C for 45 s, extension of 72°C for 7 min	Sobsey et al. 1996; Rodríguez et al. 2008
	EV-R Reverse (external)	ACCGGATGGCCAATCCAA				
	Ent 33 Reverse (internal)[a]	CCCAAAGTAGTCGGTTCCGC	5' untranslated region	105 bp (EV-L/Ent 33)	Semi-nested PCR step: 94°C for 10 min, 55°C for 25 s, 72°C for 45 s, followed by 35 cycles of 94°C for 25 s, 60°C for 25 s, 72°C for 45 s, extension of 72°C for 7 min.	Rodríguez et al. 2008
Adenoviruses	ADHEX1 F Forward (external)	AACACCTAYGASTACATGAAC	Hexon gene	473 bp (ADHEX1F/ ADHEX1R)	PCR step: 94°C for 10 min, then 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, extension of 72°C for 10 min.	Avellón et al. 2001; Van Heerden et al. 2003
	ADHEX1 R Reverse (external)	ACATCCTTBCKGAAGTTCCA				
	ADHEX2 F Forward (internal)[a]	CCCMTTYAACCACCACCG	Hexon gene	168 bp (ADHEX2F/ ADHEX2R)	Nested PCR step: Same as primary PCR step.	
	ADHEX2 R Reverse (internal)[a]	KATGGGGTARAGCATGTT				

* Includes degenerate primers (B = G+T+C; K = G+T; M = A+C; R = A+G; S = G+C; Y = C+T)
[a] Nested primers

Table 2. Presence and quantity of viruses in different biosolid types as determined by cell culture (BGM or PLC/PRF/5 cells) or ICC-PCR (using primers for enteroviruses or adenoviruses).

Sludge Type	Treatment Method	Virus Detection in BGM cells (MPN/4 g)	Virus Detection in PLC/PRF/5 cells (MPN/4 g)	Virus Detection by BGM ICC-PCR		Virus Detection by PLC/PRF/5 ICC-PCR	
				Enterovirus	Adenovirus	Enterovirus	Adenovirus
Raw	Thickened Waste Activated Sludge	3	370	not tested	-	not tested	+
Raw	Thickened Waste Activated Sludge	44.8	381	not tested	-	not tested	+
Raw	Thickened Waste Activated Sludge	54	31	not tested	-	not tested	+
Raw	Thickened Waste Activated Sludge	64.4	49	not tested	-	not tested	-
Raw	Thickened Waste Activated Sludge	57.4	< 4.8	not tested	-	not tested	-
Raw	Gravity Thickened	5	8.5	+	-	-	+
Class B	Mesophilic, Anaerobic	180.2	180.2	-	-	-	+
Class B	Mesophilic, Anaerobic	24.8	139.3	-	-	-	+
Class B	Mesophilic, Anaerobic	24.4	15.1	+	-	+	+
Class B	Mesophilic, Anaerobic	< 0.9	45	not tested	-	not tested	+
Class B	Mesophilic, Anaerobic	< 1.2	3.3	not tested	-	not tested	+
Class B	Mesophilic, Anaerobic	19.4	4.8	-	-	-	-
Class B	Mesophilic, Anaerobic	9.8	9	-	-	-	-
Class B	Mesophilic, Anaerobic	< 1.4	2.4	-	-	-	+
Class B	Mesophilic, Anaerobic	< 1.4	15.7	not tested	-	not tested	+
Class B	Mesophilic, Anaerobic	< 0.7	5.4	not tested	-	not tested	+
Class B	Mesophilic, Anaerobic	0.9	3.1	not tested	-	not tested	+
Class B	Aerobic Digestion	14.8	34.6	-	-	-	+
Class B	Lime Stabilization	< 0.3	< 0.3	not tested	-	not tested	-
Class B	Lime Stabilization	< 0.3	< 0.3	not tested	-	not tested	-
Class B	Drying Beds	< 0.9	< 0.9	-	-	-	-
Class B	Drying Beds	< 1.6	< 1.3	-	-	-	-
Class B	Drying Beds	< 1.5	< 1.8	-	-	-	-

"<" Sample was below the limit of detection of the assay.

Table 3. Comparative detection of enteric viruses in BGM and PLC/PRF/5 cell lines.

Biosolid Type	Assay with Positive Result	BGM Cells	PLC/PRF/5 Cells	Detection in Both Cell Lines
Raw (n = 6)	Cell Culture CPE	6 of 6 (100%)	5 of 6 (83.3%)	5 of 6 (83.3%)
	PCR for Enteroviruses*	1 of 1 (100%)	0 of 1 (0%)	N/A
	PCR for Adenoviruses*	0 of 6 (0%)	4 of 5 (80.0%)	N/A
Class B (n = 17)	Cell Culture CPE	7 of 17 (41.2%)	12 of 17 (70.6%)	7 of 17 (41.2%)
	PCR for Enteroviruses*	1 of 6 (16.7%)	1 of 7 (14.3%)	N/A
	PCR for Adenoviruses*	0 of 7 (0%)	10 of 12 (83.3%)	N/A
All Samples (n = 23)	Cell Culture CPE	13 of 23 (56.5%)	17 of 23 (73.9%)	12 of 23 (52.2%)
	PCR for Enteroviruses*	2 of 7 (28.6%)	1 of 8 (12.5%)	N/A
	PCR for Adenoviruses*	0 of 13 (0%)	14 of 17 (82.4%)	N/A

* A subset of samples that was negative for cell culture CPE was also found to be negative for both enteroviruses and adenoviruses by PCR (see Table 2) and are not included in this table.

N/A Not applicable