DNA Connects Biosolids to My Illness

In 2017, I contracted Cryptosporidium (a sewage-indicator parasite) from power-washing outdoor surfaces at my home while preparing to sell it so we could escape living near biosolids. Through DNA analysis, Crypto was positively identified in biosolids that are applied near my home… on our propane tank that I'd power-washed… and in my illness. -Paula B. Yockel

Sampled & analyzed unwashed back side of propane tank after diagnosis.

		Biosolids sample, same source as applied near my home			Power-Washed Surface			Initial Clinical Specimen			Second Specimen, 6 Days After First		
		Biosolid	Biosolid	Biosolid	Propane Tank material	Propane Tank material	Propane Tank material	Stool sample #1	Stool sample #1	Stool sample #1	Stool sample #2	Stool sample #2	Stool sample #2
		A	B	C	A	B	C	A	B	C	A	B	C
Rotavirus		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
***Cryptosporidium* sp.**		Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	(Pos)	(Pos)	ND	ND
Adenovirus		Pos	(Pos)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Norovirus	**G1**	Pos	Pos	(Pos)	ND	ND	ND	ND	ND	ND	ND	ND	ND
	G2	Pos	(Pos)	ND	ND	(Pos)	ND	ND	ND	ND	ND	ND	ND

Note: Pos = DNA or RNA of the organism was definitively detected in the material. (Pos) = the signal was a weak positive that was near the detection limit of the assay and/or below the lowest point on the standard curve. ND = DNA or RNA of the pathogen was not detected by the assay.

Study conducted by highly reputable scientist with expertise in molecular genetics and pathogen detection.
Class B Biosolids sample collected at Oklahoma City, OK, North Canadian WWTP 2016.

Read our Resources for a PubMed Central® paper on Air Transport of Cryptosporidium

Cryptosporidium Study: Biosolids, Contaminated Surface, Paula Yockel Illness

REDACTION: LETTERHEAD OF RESEARCHERS

May 9, 2018

DETECTION of HUMAN PATHOGEN NUCLEIC ACIDS (DNA and RNA) in ENVIRONMENTAL and HUMAN STOOL SAMPLES from OKLAHOMA, USA

Samples

Three samples of sludge (biosolid) material were collected by Accurate Environmental Labs on March 7, 2016 from Veolia North Canadian Wastewater Plant, Oklahoma City, Oklahoma and shipped overnight to the laboratory of [redacted]. Stool samples collected from patient Paula Yockel were shipped overnight from [redacted] Hospital in Oklahoma City, OK and from Paula Yockel to the laboratory of [redacted] On 2/20/2017 Paula Yockel shipped a stool sample on dry ice, which arrived [redacted] on 2/21/2017. This sample had been collected in the office of physician [redacted] M.D., [redacted] Oklahoma City, OK, on 2/20/2017. On 2/21/2017 [redacted] Hospital, [redacted] Oklahoma City, OK sent a stool sample on dry ice that had been collected on 2/14/2017, which arrived [redacted] on 2/22/2017. On March 14, 2017 David and Paula Yockel scraped residue off of a propane tank using a plastic knife and placed it into a clean plastic bag that was zip closed and shipped on dry ice t[redacted] It arrived a[redacted] on March 15, 2017. The tank was located a[redacted] Hefner Rd., Jones, OK. All samples were stored at -80°C until processed. Nucleic acids were extracted in the laboratory o[redacted] and analyzed as described below.

DNA and RNA Extractions

DNA was extracted from the samples using the QIAamp DNA Stool Mini Kit (Qiagen cat. #51504) following the manufacturer's protocol. RNA was extracted from the materials using the MagMAX Viral RNA Isolation kit (ThermoFisher Scientific cat. #AMB18365) following the manufacturer's protocol. DNA was isolated from 180-220 mg of material for each extraction.

- Sludge/Biosolids – a single aliquot of material was taken from each of samples BiosolidA, BiosolidB and BiosolidC for extraction.
- Propane Tank Material – three aliquots of material were taken from the propane tank sample for extraction and were labeled as PtankA, PtankB and PtankC.
- Stool sample #1 – three aliquots of material were taken from stool sample #1, which was the sample shipped from [redacted] Hospital. These aliquots were labeled #1A, #1B and #1C.
- Stool sample #2 – three aliquots of material were taken from stool sample #2, which was the sample collected a[redacted] office and shipped by Paula Yockel. These aliquots were labeled #2A, #2B and #3C.

Adenovirus qPCR

The probe and primers developed by Heim et al. (2003) were used for the adenovirus assay. Ten µl reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA). The final concentration of PCR reagents was as follows: 1X TaqMan® Universal Master Mix (ThermoFisher Scientific cat. #4304437), 0.2 mg/ml bovine serum albumin (BSA), each primer at 0.5 µM, 0.4 µM TaqMan® FAM/TAMRA labeled probe. One µl of sample was added to each reaction. Thermocycling parameters were as follows: 95°C for 20 s, followed by 45 cycles of 95°C for 15 s, 55°C for 10s, 60°C for 1min. Triplicate 10 µl reactions were run for each sample. We used an internal amplification control (IAC) to test for PCR inhibition. Each run included positive controls and "no template controls" (NTC) to ensure that the method was working properly and that there was no contamination. Positive controls were run using 1 µl (0.65×10^6 genome equivalents) of HAdV 41 DNA, strain Tak from ATCC (Manassas, Virginia).

***Cryptosporidium* spp. qPCR**

The probe and primers developed by Hadfield et al. (2011) were used for the *Cryptosproridium* spp. assay. Ten µl reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR system. The final concentration of PCR reagents was as follows: 1X TaqMan® Fast Advanced Master Mix (ThermoFisher Scientific cat. #4444557), 0.4 mg/ml BSA, each primer at 0.9 µM, 0.25 µM TaqMan MGBNFQ labeled probe. One µl of sample was added to each reaction. Thermocycling parameters were as follows: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s, 60°C for 30s. Triplicate 10 µl reactions were run for each sample. Each run included positive controls and "no template controls" (NTC) to ensure that the method was working properly and that there was no contamination. Positive controls were run using 1 µl (> 1 X 10^5 copies/µl) of genomic DNA from *Cryptosporidium parvum* strain ATCC #PRA-67DQ from ATCC (Manassas, Virginia).

Reverse Transcription for the RNA Virus qPCRs

Reverse transcription (RT) was done with 5 µl of RNA from each sample using the Omniscript RT kit (QIAGEN, cat. #205113) in a final volume of 20 µl.

qPCR for Norovirus Detection

RT was done as described above. Assays for noroviruses GI and GII were done separately (Kageyama et al. 2003; Loisy et al. 2005; Svraka et al. 2005; Da Silva et al. 2007). Ten µl reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR system. The final concentration of PCR reagents was as follows: 1X TaqMan® Fast Advanced Master Mix, 0.4 mg/ml BSA, each primer at 1.0 µM, and a 0.25 µM TaqMan FAM-BHQ probe for the G1 reaction, and a 0.25 µM TaqMan FAM-TAMRA probe for the GII reaction. One µl of the RT reaction for each sample was added to for each qPCR reaction. Thermocycling parameters were

as follows: 20 s at 95°C followed by 40 cycles with each cycle consisting in 3 s at 95°C and 30 s at 60°C. We used an internal amplification control (IAC) to test for PCR inhibition (Nordstrom et al. 2007). When PCR inhibition was evident, the samples were diluted 1:5 or 1:10 and reanalyzed. Triplicate 10 µl reactions were run for each sample. Each run included a norovirus positive control that was a GI or GII gBlock® amplicon sequence from Integrated DNA Technologies Inc. (Coralville, Iowa) and "no template controls" (NTC) to ensure that the method was working properly and that there was no contamination.

qPCR for Rotavirus Detection

RT was done as described above. The probe and primers developed by Zeng et al (2008) and as modified by Pang et al. (2011) were used for the rotavirus assay. Ten µl reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR system. The final concentration of PCR reagents was as follows: 1X TaqMan® Fast Advanced Master Mix, 0.4 mg/ml BSA BSA, each primer at 0.9 µM, and 0.25 µM TaqMan MGBNFQ labeled probe. Thermocycling parameters were as follows: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s, 60°C for 30 s. Triplicate 10 µl reactions were run for each sample. Each run included a positive control that was a rotavirus gBlock® amplicon sequence and "no template controls" (NTC) to ensure that the method was working properly and that there was no contamination.

Virus Viability Assays

Integrated cell culture qPCR (ICC-qPCR) assays were done to determine whether viable adenovirus or rotavirus could be found in the sludge (biosolid) sample A based on the results of the analysis that indicated the presence of adenovirus DNA in this sample (see Table). Although rotavirus was not detected in this sample, ICC-qPCR was conducted to determine if viable rotavirus could be detected after four days of incubation.

Preparation of Putative Viral Concentrates from Biosolid Sample A

Thirty grams of sludge (biosolid) sample A was added to 60 ml of 10% beef extract and stirred for 30 minutes, followed by centrifugation at 2500 *X g* for 30 minutes at 4°C. The eluate was collected and the beef extract concentration was diluted to 3% with sterile reagent grade water. The eluate was stirred while the pH was adjusted to 3.5 ± 0.1, causing a flocculate to form. The flocculated suspension was then centrifuged at 2500 *X g* for 15 min at 4°C. The resulting pellet was resuspended with Na_2HPO_4 to a final volume of 1/20 of the volume of the original diluted beef extract suspension. The pH was adjusted to 7.2 ± 0.2. Antibiotic/antimycotic and gentamicin were added to the final concentrate at a concentration of 10^{-1} and 10^{-2} v:v, respectively. The concentrate was incubated for 2 hours at 37°C and stored at -80°C until used to inoculate the cells in ICC-qPCR.

Cell Culture

The ICC-qPCR technique followed a combination of protocols from Choo and Kim (2006) and Gallagher and Margolin (2007). Caco-2 cells were previously tested in the laboratory o[redacted] by a graduate student for their efficiency in adenovirus propagation[redacted] Caco-2 cells were grown to 75-90% confluence in t25 tissue culture flasks using Minimum Essential Medium Eagle (MEM) with 10% fetal bovine serum (FBS). Once the cells reached the appropriate confluence, the medium was removed and the cells were washed with fresh medium. Each sample was cultured in quadruplicate by adding 50 µl of the putative viral concentrate prepared as above flasks containing washed Caco-2 cells. Inoculations for the sample were carried out at full concentration and diluted 1/10. After inoculation, the cells were rocked every 15 minutes for 60 minutes. Following this, 5 ml of MEM was added to each culture flask. Two of the four flask cultures for each sample were frozen (-80°C) immediately (T_0) the remaining two were incubated at 37°C for 4 days (T_4). Following the 4-day incubation period the culture flasks were stored at -80°C until DNA and RNA extraction. Prior to extraction, each culture flask was subjected to three freeze/thaw cycles. Total genomic DNA (for adenovirus) or RNA (for rotavirus) was extracted from 200 µl of the supernatant containing thawed cells and subjected to qPCR analyses as described above. The T_0 and T_4 duplicates for each sample were compared to determine if the number of viral particles increased over the 4-day incubation period. An increase in viral particles would be interpreted as meaning viruses contained in a given sample were viable and therefore remained infective. Negative controls were run for each sample by inoculating control flasks with 50 µl of MEM medium. Positive controls were run for each sample by inoculating control flasks with 50 µl (3.25×10^7 genomic equivalents) of adenovirus (HAdV F41, strain Tak) and 50 µl (5.0×10^6 genomic equivalents) of rotavirus (ATCC #VR-2018) both from ATCC (Manassas, Virginia).

Results

qPCR assays for pathogens

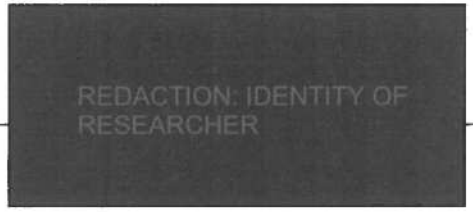
qPCR results for samples are presented on the Table shown on pg. 5.

ICC-qPCR

Both adenovirus and rotavirus replicated over the four days of incubation in the Caco-2 cells as indicated by an approximately 2-fold increase in the amount of viral particle nucleic detected on day 4 in the positive controls. In the cells inoculated with material isolated from the biosolid sample, however, adenovirus DNA and rotavirus RNA was not detected on either day 0 or day 4. This could be because either the virus levels were too low to be detected using this technique or because there was some inhibition by some substance(s) present in the biosolid material that was not removed by the processing before inoculation.

		Biosolid	Biosolid	Biosolid	Propane Tank material	Propane Tank material	Propane Tank material	Stool sample #1	Stool sample #1	Stool sample #1	Stool sample #2	Stool sample #2	Stool sample #2
		A	B	C	A	B	C	A	B	C	A	B	C
Rotavirus		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
***Cryptosporidium* sp.**		Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	(Pos)	(Pos)	ND	ND
Adenovirus		Pos	(Pos)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Norovirus	**G1**	Pos	Pos	(Pos)	ND	ND	ND	ND	ND	ND	ND	ND	ND
	G2	Pos	(Pos)	ND	ND	(Pos)	ND	ND	ND	ND	ND	ND	ND

Note: ND = DNA or RNA of the pathogen was not detected by the assay. (Pos) = the signal was a weak positive that was near the detection limit of the assay and/or below the lowest point on the standard curve. Pos = DNA or RNA of the organism was definitively detected in the material.



May 9, 2018
Date

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