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Dead-end hollow-fiber ultrafiltration for concentration and enumeration of *Escherichia coli* and broad-host-range plasmid DNA from wastewater

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Abstract

Broad-host-range plasmids can facilitate dissemination of antibiotic resistance determinants among diverse bacterial populations. We evaluated hollow-fiber ultrafiltration for increases in detection efficiency of broad-host-range plasmids and *Escherichia coli* DNA in wastewater. Ultrafiltration followed by PCR showed limited increases in DNA detection and quantification in effluent compared with membrane filtration alone.

Keywords

ultrafiltration; qPCR; *Escherichia coli*; plasmid; wastewater

Dissemination of antibiotic resistance determinants among a wide range of bacterial species has led to increased resistance in human and animal pathogens, providing steadily increasing threats to public health and effective treatment of infections worldwide (Alanis, 2005; Cohen, 1992; Levy and Marshall, 2004). Mobile genetic elements (MGE^a) such as broad-host-range (BHR) plasmids are known to facilitate the movement of antibiotic and metal resistance genes throughout bacterial populations, but the presence and persistence of BHR plasmids and other MGE in dynamic, surface water environments such as wastewater treatment plants (WWTPs) are not well characterized (Aminov and Mackie, 2007; Dröge et al., 2000).

Because low numbers of BHR plasmids can potentially replicate, spread, and persist in bacterial communities under widely varying conditions, a need for monitoring the release of plasmids from WWTPs is clear. Broad-host-range plasmids can exist in low concentrations in the final effluents of WWTPs, making them difficult to detect in 100-mL or even 1-L samples. Methods that focus on the concentration of diffuse microorganisms will help alleviate detection difficulties to address topics like plasmid presence and persistence.

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^a**Abbreviations:** WWTP, wastewater treatment plant; BHR, broad-host-range; MGE, mobile genetic elements; MWCO, molecular weight cutoffs; TMP, transmembrane pressure; qPCR, real-time quantitative polymerase chain reaction

Hollow-fiber ultrafiltration using hemodialysis filters adapted from the medical industry offers a relatively non-labor-intensive approach for sample concentration. While available in a variety of specifications, polysulfone dialyzers with molecular weight cutoffs (MWCO) from 20 to 70 kDa have shown to be effective in the concentration of indicator bacteria, protozoa, and viruses from drinking water, surface water, and recreational beaches (Holowecky et al., 2009; Hernandez-Morga et al., 2009; Hill et al., 2007). The feasibility of ultrafiltration in detection of genetic elements in treated wastewater effluents has yet to be investigated in detail.

Our objective was to determine the impact on detection frequency and quantification of a single-pass, dead-end, hollow-fiber ultrafiltration method in the concentration of indicator bacteria harboring BHR plasmids in municipal WWTP effluents. Quantification of *E. coli* and IncP BHR plasmid and detection frequency for three other BHR plasmid gene fragments (Inc A/C, IncN, and IncW) were field tested in two WWTP effluents.

A dead-end, single-pass ultrafiltration/concentration method was used in this study (Figure 1) (after Hill et al., 2007; Leskinen and Lim, 2008). A new Asahi REXEED-25S hollow fiber dialyzer with a MWCO of 20 kDa, fiber inner diameter of 185 μm and 2.5 m^2 total filter surface area was used for each water sample (Asahi Kasei Kuraray Medical Co., Ltd., Tokyo, Japan). For an elution buffer, Antifoam Y-30 Emulsion (Sigma-Aldrich Corporation, St. Louis, MO, USA) was added to a final concentration of 0.001% to an autoclaved solution containing 0.5% Tween 80 (OmniPur, EMD Chemicals, Merck KGaA, Darmstadt, Germany) and 0.01% NaPP (Hill et al., 2005). The peristaltic pump was modulated to keep system pressure at about 33 kPa (0.5X maximum manufacturer recommended transmembrane pressure (TMP)) during concentration and raised to 66 kPa (TMP) during elution steps. A 500-mL back flush of the system was collected immediately after concentration for membrane filtration and further processing.

Final treated effluent was collected in sterile polycarbonate 1-L vessels (VWR International, Radnor, PA, USA) or sterile 20-L polypropylene carboys (Nalgene – Thermo Fisher Scientific, Rochester, NY, USA) from Fayetteville (n = 3) and Springdale (n = 6), AR, municipal WWTPs between October 2010 and March 2011. Ten-liter samples were subjected to ultrafiltration followed by serial filtration through 5.0 and 0.22- μm pore-size membranes, while 1-L samples were membrane filtered only. Ultrafilter back flushes were membrane filtered in two 250-mL portions and recombined after DNA extraction. Serial membrane filtration, total DNA extraction and ethanol precipitation were performed as previously described (Akiyama et al., 2010).

The real-time quantitative polymerase chain reaction (qPCR) method to quantify a 187-bp region of the *E. coli*-specific uidA gene (encodes β -glucuronidase enzyme) was adapted for use (Heijnen and Medema, 2006). The 241-bp amplicon specific to the trfA gene of IncP plasmids was optimized for qPCR detection using the primers developed by Götz et al. (1996) and used in Dröge et al. (2000) and Akiyama et al. (2010). Assays had lower detection limits of 64 and 23 gene copies for uidA and trfA, respectively. Real-time qPCR runs were performed on an Applied Biosystems StepOnePlus real-time PCR system (Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA). Each 20- μL reaction contained SYBR® GreenER master mix, 200 nmol each of primer, BSA (final concentration 0.04%), ROX (Invitrogen, Life Technologies Corp., Carlsbad, CA, USA) passive reference dye (final concentration 1000 nM), and 1 μL of DNA extracts or dilutions. Amplification run temperature profiles, melt curve analyses, and quality control factors were performed as recommended by the manufacturer. Statistical comparisons of appropriate log-transformed values were made using One-way Analysis in JMP 9.0 software (SAS Institute Inc., Cary, NC, USA) and Tukey's HSD ($\alpha = 0.05$).

DNA increased significantly by about 5-fold (1.6×10^4 and 1.1×10^4 ng total DNA recovered from Springdale and Fayetteville effluents, respectively) when increasing sample size from 1-L to 10-L (Table 1). Abundance of IncP trfA replicons was higher (about 10 – 100X) than uidA replicons, which is not unsurprising given that IncP plasmids are distributed among multiple bacterial populations. Host ranges of individual plasmids may vary widely (Sota et al., 2010). Stability and segregation of IncP BHR plasmids also vary widely in different hosts (De Gelder et al., 2007). Additionally, Akiyama et al. (2010) observed limited association between BHR plasmids and antibiotic-resistant *E. coli* isolates in WWTP effluent, with only 5% of isolates testing positive for IncP plasmids. The lack of a consistent relationship between IncP BHR plasmids and *E. coli* (prominent fecal indicator bacteria) in WWTP effluent further justifies independent investigations of the prevalence and persistence of BHR plasmids that may be released into receiving streams.

The only significant increase in either qPCR amplicon was an approximate 4-fold increase of 1.1×10^5 uidA copies at Springdale WWTP. An approximate 4-fold increase of 1.1×10^5 uidA copies was also observed at Fayetteville, but was not statistically significant. Three-fold (8.5×10^6 copies, Springdale) and 2-fold (1.2×10^6 copies, Fayetteville) increases in trfA amplicons in 10-L compared to 1-L samples were not significant ($\alpha = 0.05$). We expected 10-L ultrafilter concentrates to display 10-fold increases in all parameters tested when compared with 1-L samples of the same effluent. Surprisingly, the maximum increase in any parameter was only 5-fold (total DNA, both WWTPs), and as low as 2-fold in some samples (trfA, Fayetteville). It is difficult to compare these proportions with the work of others as we are not currently aware of any studies that compare ultrafiltration of large effluent water volumes directly with smaller sample volumes for quantification of uidA or trfA amplicons.

While enumeration of the gene fragments did not increase in proportion to changes in sample volume (10 L vs. 1 L), precision of detection (data not shown) was also not improved for *E. coli* chromosomal DNA or BHR plasmid DNA as evidenced by the few statistically significant differences detected in quantification despite two- to fourfold increases in numbers of copies. Ultrafiltration methods that effectively allow for larger water volumes to be sampled may be subject to, or amplify, the same biases and problems noted in smaller samples such as nucleic acid extraction inhibition (Rajal et al., 2007) or possibly assay inhibition.

To allow qualitative comparison of amplification of BHR plasmid amplicons, standard PCR was used to assess the presence or absence of gene fragments corresponding to the IncA/C, IncN, and IncW groups. Total DNA (10 - 50 ng) from each extraction was amplified by PCR and visualized and compared to bands of reference plasmid sequences on agarose gels as described previously (Akiyama et al., 2010). Utilizing ultrafiltration did increase incidence of detection for IncN plasmid amplicons at Fayetteville WWTP and IncW plasmid amplicons at Springdale WWTP, but caution must be used when interpreting these results (Table 2). If a target is consistently present in 1-L samples (e.g. IncP and IncA/C plasmid amplicons (data not shown) and IncN at Springdale), there is no utility in increasing sample size. Additionally, while IncW plasmid amplicons were detected more often in 10-L samples at Springdale WWTP, particularly in the planktonic fraction (0.22- μ m pore-size), results from Fayetteville WWTP indicate that this trend may be site-specific. Moura et al. (2010) observed variable hybridization of IncW, IncN, and IncP probes when examining wastewater from two distinct sources. Specific characteristics of a sample site may impact the utility of using ultrafiltration to concentrate and PCR to detect specific gene fragments.

This study is the first to evaluate the use of ultrafiltration as a method to concentrate and detect BHR plasmids in the bacterial community of treated wastewater effluent. Dead-end

ultrafiltration with membrane filtration to increase water sample volume (10-L) showed limited increases in quantification of uidA and frequency of detection of some BHR plasmid DNA in WWTP effluent when compared with membrane filtration of 1-L water.

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Figure 1. Ultrafiltration apparatus showing configurations for concentration (top, flow A to B) and elution (bottom, flow B to A).

Table 1

Wastewater treatment plant (WWTP) total DNA, uidA (*Escherichia coli*), and trfA (IncP broad-host-range plasmid) amplicon recovery.

WWTP	Sample size ^a (L)	Amount of genetic element recovered ^{b,c}		
		Total DNA (ng)	uidA (# of copies)	trfA (# of copies)
Springdale	1	3.9×10^3 ^a	3.1×10^4 ^a	5.2×10^6 ^a
	10	1.9×10^4 ^b	1.4×10^5 ^b	1.4×10^7 ^a
Fayetteville	1	3.0×10^3 ^a	3.7×10^4 ^a	8.0×10^5 ^a
	10	1.4×10^4 ^b	1.5×10^5 ^a	2.0×10^6 ^a

^aSpringdale 1-L, $n = 6$; Springdale 10-L, $n = 5$; Fayetteville 1-L and 10-L, $n = 3$.

^bValues represent combined amounts collected on 5.0- and 0.22- μ m pore-size membranes.

^cGeometric means from the same WWTP within a column not followed by the same letter are significantly different using Tukey-Kramer HSD ($\alpha = 0.05$).

Table 2

Amplification percentage of BHR plasmid amplicons in wastewater treatment plant (WWTP) effluent.

WWTP	Sample size (L)	Filter size ^a (μm)	Occurrence of DNA band with expected size ^b (%)	
			IncN	IncW
Springdale	1	5.0	100	33
		0.22	100	50
	10	5.0	100	67
		0.22	100	83
Fayetteville	1	5.0	67	33
		0.22	67	33
	10	5.0	100	0
		0.22	100	66

^a $n = 6$ for each Springdale sample-filter combination, $n = 3$ for each Fayetteville sample-filter combination.

^b Amplification percentage of each amplicon calculated by numbers of positive reactions divided by number of samples (n) multiplied by 100.