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Proposed Modifications of Environmental Protection Agency Method 1601 for Detection of Coliphages in Drinking Water, with Same-Day Fluorescence-Based Detection and Evaluation by the Performance-Based Measurement System and Alternative Test Protocol Validation Approaches[▽]

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Coliphages are microbial indicators specified in the Ground Water Rule that can be used to monitor for potential fecal contamination of drinking water. The Total Coliform Rule specifies coliform and *Escherichia coli* indicators for municipal water quality testing; thus, coliphage indicator use is less common and advances in detection methodology are less frequent. Coliphages are viral structures and, compared to bacterial indicators, are more resistant to disinfection and diffuse further distances from pollution sources. Therefore, coliphage presence may serve as a better predictor of groundwater quality. This study describes Fast Phage, a 16- to 24-h presence/absence modification of U.S. Environmental Protection Agency (EPA) Method 1601 for detection of coliphages in 100 ml water. The objective of the study is to demonstrate that the somatic and male-specific coliphage modifications provide results equivalent to those of Method 1601. Five laboratories compared the modifications, featuring same-day fluorescence-based prediction, to Method 1601 by using the performance-based measurement system (PBMS) criterion. This requires a minimum 50% positive response in 10 replicates of 100-ml water samples at coliphage contamination levels of 1.3 to 1.5 PFU/100 ml. The laboratories showed that Fast Phage meets PBMS criteria with 83.5 to 92.1% correlation of the same-day rapid fluorescence-based prediction with the next-day result. Somatic coliphage PBMS data are compared to manufacturer development data that followed the EPA alternative test protocol (ATP) validation approach. Statistical analysis of the data sets indicates that PBMS utilizes fewer samples than does the ATP approach but with similar conclusions. Results support testing the coliphage modifications by using an EPA-approved national PBMS approach with collaboratively shared samples.

Ensuring the microbial quality of drinking water, watersheds, processes, and water distribution systems is an important public health control measure designed to prevent the spread of communicable disease (6, 13, 22, 30). Water municipalities that supply drinking water in the United States must comply with the Total Coliform Rule (TCR), which mandates testing for total coliform and *Escherichia coli* contamination to monitor for potential human pathogens (28). Fecal contamination encompasses both bacterial and viral pathogens. The use of the bacterial indicators does not detect or predict viral contaminants; thus, reliance on bacterial indicators alone is inadequate to predict viral contamination (1). Over 50% of waterborne illnesses since 1980 have been caused by viral contamination of source water (23). As a result, coliphages, viruses that infect bacteria of the coliform group, were added as another fecal indicator in the 2006 Ground Water Rule (GWR) to allow direct measurement of a viral surrogate (8).

The GWR is intended for prequalification of groundwater quality in supplies intended for general municipal use. Once qualified, the municipality must comply with the TCR annually, and if ground-sourced waters incur a TCR violation, the municipality must comply with GWR for reinstatement. Regulatory public health authorities are now given a choice of water quality indicators, *E. coli*, coliphages, or enterococci, when deciding which indicator microorganism to use to test GWR compliance. In GWR comments, the National Drinking Water Advisory Council recommended that both bacterial and viral indicators be included in a water safety testing protocol, as each indicator predicts the likelihood of contamination with a specific group of microorganisms and gives a more complete picture of the groundwater quality. In the final promulgated GWR, testing for both bacterial and viral indicators was not considered economically feasible due to the additional expense of coliphage testing methods (26).

Current coliphage detection methods are costly and laborious. They require 2 to 3 days for time-to-result, and there are few laboratories certified to perform coliphage detection (24, 25). There is a need for easier methodologies to attract public health officials to select viral indicator tests to comply with GWR and to complement existing bacterial indicator tests.

Coliphages are classified as somatic or male specific. The

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somatic coliphages are DNA viruses that infect *E. coli* cell walls. Male-specific coliphages are either DNA or RNA viruses that infect through fertility (F) pili of *Enterobacteriaceae* bacteria. The host specified in U.S. Environmental Protection Agency (EPA) Methods 1601 and 1602 for somatic coliphages is nalidixic acid-resistant *E. coli* CN-13. The host bacterium specific for male-specific coliphages in the EPA methods is ampicillin/streptomycin-resistant *E. coli* F_{amp}, and the host specified in European Union (EU) standard methods is *Salmonella* WG 49 (7, 29).

Simple and rapid methods for coliphage detection have been reported with preliminary detection in a single working day (11, 12, 14, 20). Qualitative detection methods, including EPA Method 1601, are multiple-step procedures that involve coliphage replication in exponential-growth-phase cells of the host *E. coli* (enrichment step) followed by a spotting on seeded agar for plaque confirmation. The goals of this study were to simplify, shorten, and reduce the cost of EPA Method 1601; to determine the predictive value of a same-day fluorescence-based assay result in comparison with conventional plaque detection; and to demonstrate that laboratories could meet EPA Method 1601 performance-based measurement system (PBMS) acceptance criteria by using the modified method with their internal water samples and spiking materials.

EPA Method 1601 is a performance-based measurement where acceptance criteria for method performance were developed in collaborative testing and published as part of the method (21). PBMSs were adopted by the EPA to facilitate adoption of method improvements when defined acceptance criteria are met in demonstrated intralaboratory (tier 1) and interlaboratory (tier 2) studies (2, 9). Tier 2 is a national approval of modifications and requires EPA participation and review of a collaborative laboratory protocol before commencement. This work was performed to test if an EPA Method 1601 modification called Fast Phage meets defined PBMS criteria in tier 1 validations. Laboratory participants followed PBMS guideline 14.2.1 in Method 1601 using their own laboratory and groundwater and wastewater sources (4).

Fast Phage incorporates ease-of-use, time, and cost-saving improvements into EPA Method 1601, including convenient, shelf-stable, ready-to-use reagents in a simplified format (18). The same-day coliphage detection method utilizes an enrichment medium containing isopropyl-β-D-1-thiogalactopyranoside (IPTG) to induce transcription of the host *E. coli lac* operon. Coliphage lysis has been shown to be coupled with *lac* operon expression; thus, there are a large amplification and a rapid extracellular beta-galactosidase enzyme release during coliphage-induced lysis of the infected host in comparison to the uninfected exponentially growing host (11). A transfer aliquot of the amplified primary enrichment material into a secondary enrichment/detection medium containing the enzyme substrate 4-methylumbelliferyl-β-D-galactoside (MUG-Gal) will fluoresce under 366-nm UV light when the MUG-Gal is cleaved by the extracellular beta-galactosidase to liberate the fluorescent component MUG and indicate the presence of coliphages. Detectable enzyme is released extracellularly into the medium throughout the prescribed incubation period and during the coliphage-induced lytic cycle. Fluorescence can occur in as little as 45 min to 1 h depending on the growth phase of the bacteria and induction of the lytic properties of the phage (15, 17). The fluorescence step of Fast Phage provides a same-day prediction of the modified Method 1601 plaque assay endpoint measured the next morning.

The EPA oversees and approves microbiological methods for drinking water. The alternative test procedure (ATP) has been used for 20 years to validate new *E. coli* and total coliform detection methods in comparison to reference methods (5, 27). The procedure for qualitative tests involves direct testing of 20 split samples by reference method, at a 50% positive level, compared to testing of the 20 split samples by the proposed method. If the proposed method demonstrates substantial equivalence, the method may be promulgated as a new method. This approach, including promulgation, can take several years, and so a fast-track version that can eliminate the promulgation step when the alternative method has the same endpoint as the reference method was adopted. Currently, there is no ATP-equivalent protocol for coliphage detection methods. As a development exercise, the ATP validation approach is useful to check the robustness of method modifications.

This work was performed to facilitate EPA PBMS tier 2 protocol review and acceptance of modifications to EPA Method 1601. Tier 2 evaluation involves sharing split samples between the participating laboratories, while in this tier 1 evaluation internal laboratory-supplied samples were tested under similar proposed spiking protocols to demonstrate initial laboratory competency.

The somatic coliphage data generated by the two evaluation approaches, ATP and PBMS tier 1 study, are evaluated statistically to determine the significance of any differences observed between Fast Phage modification and EPA Method 1601.

MATERIALS AND METHODS

Presence-absence method for coliphage detection. EPA Method 1601 was performed as described previously (25). The Fast Phage (Charm Sciences, Inc., Lawrence, MA) modifications to EPA Method 1601 are summarized in Table 1. STEP-1 and STEP-2 media are Luria-Bertani (LB Lennox) broth-based media containing an antibiotic to which the host *E. coli* strain is resistant. Included is an additional proprietary formulation to promote faster host growth and coliphage amplification than those with tryptic soy broth (TSB) used in EPA Method 1601. The fluorescent substrate in STEP-2 is MUG-Gal. Host *E. coli* strains are CN-13 (ATCC 700609) and F_{amp} (ATCC 700891) cultured to log phase, freeze-dried, and formed into a shelf-stable ready-to-use reagent tablet containing 10^7 to 10^9 viable CFU/tablet. The modified method spot agar plates use a 1.5% tryptic soy agar (TSA) formulation of Method 1601 containing antibiotic and seeded with host culture grown to log phase conveniently from a reagent tablet for 4 to 5 h in TSB containing antibiotic.

Fast Phage modification. Table 1 contains information on the Fast Phage modification. STEP-1 enrichment medium is packaged in a dissolvable pouch and is added to a 100-ml water sample and rehydrated for 10 min at room temperature. An *E. coli* host tablet is added after medium dissolution. The mixture is warmed in a 38°C ± 1°C water bath for 0.5 h, followed by 4.5 h of air incubation at 39°C ± 1°C. After these 5 h of incubation, a 10-μl aliquot is spotted with a loop on a host *E. coli*-seeded agar plate and incubated overnight for plaque confirmation. The same-day fluorescence-based prediction of the agar spot plate result is performed by transferring an aliquot of the STEP-1 enrichment culture (10 ml for somatic coliphage or 1 ml for F^+ coliphage) into 100 ml of predissolved STEP-2 medium. The STEP-2 medium enrichment is incubated for an additional 3 h (0.5 h in a 38°C ± 1°C water bath followed by 2.5 h of 39°C ± 1°C air incubation for the somatic test or 1 h in a 38°C ± 1°C water bath followed by 2 h at 39°C for the F^+ test). Fluorescence observed under 366-nm UV light is visible within 1 to 3 h. Positive fluorescence in samples is interpreted as coliphage presence, and nonfluorescence at 3 h is interpreted as coliphage absence (<1/100 ml).

PBMS validation approach. Filtered (PALL-Acrodisc 0.45-μm HT Tuffryn low-protein-binding filter) nonchlorinated primary wastewater collected by each

TABLE 1. Summary of Fast Phage modifications compared to Method 1601

Method 1601	Fast Phage modification
Preculture host *E. coli* strain (CN-13 for somatic coliphages and F_{amp} for male-specific coliphages) overnight. Transfer to TSB and bring to optical density at 520 nm of 0.1 to 0.5. Ice	Predispensed dried medium components in dissolvable film. Formulation proprietary for rapid amplification
Prepare 10× TSB, $MgCl_2$, antibiotic solutions in advance and filter autoclave	Predispensed dried medium components in dissolvable film. Formulation proprietary for rapid amplification
Add prepared solutions and *E. coli* to water sample in precise sequence of additions and temperatures	Add STEP-1 medium pouch to water sample in test vessel. Gently swirl to dissolve contents for ~10 min. Add *E. coli* tablet (same host as Method 1601). Swirl to dissolve tablet
Incubate overnight at 36 ± 1°C in air incubator	Incubate for 30 min in water bath at 38 ± 1°C. Incubate for 4 h 30 min in air incubator at 39 ± 1°C
Use precultured exponential-phase *E. coli* to make spot plates. Prepare TSA, autoclave, and add preprepared and sterile-filtered antibiotic solution after tempering	Use *E. coli* tablet as TSB inoculum. Add sterile water to supplied freeze-dried antibiotic (nalidixic acid for somatic coliphages or ampicillin-streptomycin for F^+ coliphages) to make solution. Prepare TSA, autoclave, and add antibiotic solution after tempering
Transfer 10 μl of enrichment culture (24 h) to spot plate. Incubate overnight at 36 ± 1°C in air incubator. Total elapsed time, approx 60 h	Transfer 10 μl of STEP-1 enrichment culture (5 h) to spot plate. Incubate overnight at 39 ± 1°C in air incubator. Total elapsed time, 16 to 24 h
Fast prediction is not part of the method	Transfer STEP-1 enrichment culture (1 ml for F^+ or 10 ml for somatic coliphages) to 100 ml indicator medium STEP-2. Incubate up to 3 h for fluorescence-positive results to predict positive spot result. Total elapsed time, 5.5 to 8 h

testing laboratory was quantified for coliphage content using the double-layer agar (DLA) technique, as described in EPA Method 1601. This is according to the exact protocol that was used to prepare and share samples in the Method 1601 collaborative study (21; Greg Lovelace, personal communication). Laboratory-grade water and groundwater (1.1-liter volumes of each) supplied by each laboratory were artificially contaminated with wastewater-derived coliphages to obtain 1.5 somatic PFU/100 ml or 1.3 male-specific (F^+) PFU/100 ml. Ten replicate 100-ml samples were tested along with a negative control (uncontaminated water source) and a positive control. The positive control was made by adding 1 ml of rehydrated ϕX174 or MS2 (ATCC 13707-B1 or 15597-B1) coliphage supplied with the somatic or F^+ Fast Phage modification. In addition to the STEP-1 plaque determination (modification of EPA Method 1601), the laboratories also performed an overnight plaque determination at the end of the fluorescence-based prediction step and reported it as STEP-2 plaque.

ATP validation approach. The ATP validation approach, studying somatic coliphages only, was completed in the method developer's laboratory with reagent-grade and multiple groundwater sources and with nonchlorinated secondary wastewater effluents collected from geographically different regions of the United States. Secondary wastewater effluent was filtered as previously described and used to contaminate 5-liter volumes of laboratory-grade water or groundwater at 0.5 PFU/100 ml as determined by the coliphage enumeration double agar layer (DAL) method specified in section 11 of EPA Method 1601. For the ATP comparison study, 40 replicate 100-ml samples were split into two groups of 20 each and tested by Method 1601 and by Fast Phage modification with rapid fluorescence-based prediction. Negative- and positive-control assays were performed as in the PBMS approach. Some of the secondary wastewater effluents were disinfected with chlorine prior to testing. A 4-liter test water volume was spiked with a 1-liter wastewater effluent volume to achieve 4- to 5-log levels of total coliforms per 100 ml. The water was mixed with a stir bar and disinfected with 2 ppm total chlorine and 0.5 ppm free chlorine for 20 min, resulting in a 3- to 4-$\log_{10}$ coliform reduction per 100 ml. The chlorine was neutralized with 100 mg sodium thiosulfate/liter. Reduction of coliphage titer was measured at 0.5 to 1 log. The disinfected water was subsequently diluted with test water to achieve the target 0.5-PFU/100-ml level prior to testing. For determination of method specificity, all positive and negative Fast Phage agar spots were excised with a Pasteur pipette and resuspended in 0.5 ml TSB, mixed by vortexing, and respotted as the reference method on EPA Method 1601 confirmation plates. The remainder of the resuspension was filtered to remove host bacteria and spotted as the referee method on EPA Method 1601 agar plates with a layer of host *E. coli* bacteria.

Statistical methods. Modified method fluorescence-based prediction results are compared to next-day STEP-1 or next-day STEP-2 results by using 4-fold tables (3). Agreeing results are scored as true positive and true negative, while positives (fluorescence or plaque) with no plaque in the reference method are scored as false positive, and negatives (no fluorescence or no plaque) with plaque formation in the reference method are scored as false negative. Chi-square and Cohen kappa statistics are determined as a measure of the agreement of the two procedures. Binomial data differences may also be analyzed for significance by using power analysis (16, 19). For any binomial data, equation 1, which is the equation for a power analysis based on binomial data, is solved for the differences that are detectable as related to sample size, percent positivity, and number of samples. This equation can also be solved to determine the number of samples required to detect differences that are observed or anticipated. The differences that are detectable for a particular variability and sample size were calculated as

$$\delta = \sqrt{\left(\frac{[(Z_\alpha + Z_\beta)^2 \times (p_1 q_1 + p_2 q_2)]}{n}\right)} \quad (1)$$

where $\delta = p_1 - p_2$ is the difference between two percentages, $Z_\alpha + Z_\beta$ is the factor determined by the values of α and β, p_1 is percent positive (as a decimal) for site 1 or time 1, q_1 is percent negative (as a decimal) for site 1 or time 1, p_2 is percent positive (as a decimal) for site 2 or time 2, q_2 is percent negative (as a decimal) for site 2 or time 2, and n is number of samples collected at each site and/or time.

RESULTS

Somatic PBMS approach. Results of the somatic method modification according to the EPA Method 1601 PBMS protocol are shown in Table 2. All five testing laboratories reported the appropriate negative- and positive-control data not

TABLE 2. Fast Phage somatic presence-absence testing in 5 laboratories

Lab no.	Spike level (PFU/100 ml)	No. of positive samples for water type and assay:					
		Reagent water			Groundwater		
		Fluorescence	STEP-1	STEP-2	Fluorescence	STEP-1	STEP-2
1	1.5	10	10	10	10	10	10
2	1.5	7	7	7	8	8	9
3-1	1.7	9	10	10	10	9	9
3-2	1.5	10	10	10	10	10	10
4-1	1.7	7	7	9	9	8	9
4-2	1.4	7	7	7	5	6	6
5	1.5	10	7	10	10	6	10

shown. Method modifications successfully met EPA tier 1 performance criteria as defined in section 14 of Method 1601 by detecting more than the minimum of 50% positive samples in reagent and groundwater samples when contaminated at 1.5 PFU/100 ml or less. Testing performed by two laboratories in which the DLA quantification exceeded the specified maximum 1.5-PFU/100-ml level was repeated. The fluorescence-based STEP-2 prediction of coliphage-positive samples was consistent except in lab 5, which had a number of fluorescence-positive results with negative STEP-1 plaque confirmations. However, these same fluorescent samples tested positive by STEP-2 plaque confirmation. These are interpreted as false-positive fluorescence in comparison to STEP-1 plaque and as true-positive fluorescence in comparison to STEP-2 plaque.

Male-specific (F^+) PBMS approach. Table 3 reports results of Fast Phage male-specific coliphage modification tested by five laboratories according to the PBMS protocol. Data reported have proper negative- and positive-control data not shown. Fast Phage modifications successfully met EPA tier 1 performance criteria by detecting at least 50% STEP-1 plaque-positive samples in reagent and ground spiked samples when they were spiked at the specified 1.3-PFU/100-ml level. Data are shown in one instance where lower-than-specified levels yielded more than 50% plaque results with false-negative fluorescence. Lab 2, in one testing instance with a 1.5-PFU/100-ml spike, reached the specified number of positives with groundwater but not with reagent water. A repeat run at a lower spike level of 1.2 PFU/100 ml yielded 50% positive results in reagent water and 90% positive results in groundwater. Both results suggest a possible coliphage clumping effect in the reagent water. Clumping has been observed with other virus dilution steps (10).

TABLE 3. Fast Phage male-specific presence-absence testing in 5 laboratories

Lab no.	Spike level (PFU/100 ml)	No. of positive samples for water type and assay:					
		Reagent water			Groundwater		
		Fluorescence	STEP-1	STEP-2	Fluorescence	STEP-1	STEP-2
1-1	1.1	7	8	8	4	8	8
1-2	1.3	9	9	9	8	8	9
2-1	0.9	2	6	8	1	5	7
2-2	1.4	3	4	6	7	9	10
2-3	1.2	5	5	5	9	9	9
3-1	1.3	10	10	10	10	10	10
4-1	1.1	9	10	10	10	10	10
4-2	1.3	10	10	10	10	10	10
5	1.3	7	5	10	9	6	8

TABLE 4. Somatic coliphage fluorescence-based prediction compared to STEP-1 plaque[a]

Sample category in STEP-1 plaque	No. of samples in STEP-2 fluorescence-based assay		
	Positive	Negative	Total
Positive	113	2	115
Negative	9	16	25
Total	122	18	140

[a] Sensitivity, 98.3%; specificity, 64.0%; overall agreement, 92.1%; Cohen kappa value, 0.699; false-negative error rate, 1.7%; false-positive error rate, 36%; miscalculation rate, 7.9%.

Fluorescence-positive somatic prediction. The fluorescence-based prediction method, STEP-2, provides a same-day result when the sample is set up for testing. There is no equivalent step in EPA Method 1601, but the procedure may have value as an early warning/alarm of water contamination. The results of the Fast Phage fluorescence-based prediction in the PBMS evaluations were correlated with the STEP-1 plaques in 4-fold table comparison. Table 4 presents the somatic coliphage STEP-1 plaque result correlation, and Table 5 presents the male-specific STEP-1 plaque result correlation. The somatic same-day fluorescence-based prediction is 92.1% predictive of STEP-1 plaque formation with a 1.7% false-negative rate and a 36% false-positive rate. The target spike level causes predominantly positive results with a small number of negative samples. The small number of negatives causes the false-positive rate to be overstated, and a better assessment of error is the combined negative and positive misclassification rate of 7.9%. Proof that false positives are overestimated is found with the plaque tests performed from the fluorescing STEP-2 media (STEP-2 plaque). Table 2 shows that 8 of 9 false-positive fluorescence-based results reported in Table 4 were true coliphage positives compared to STEP-2 plaque, indicating that the fluorescence-based false-positive rate may be as low as 1 in 25, or 4%. Positive fluorescence-based correlation with positive plaques from STEP-2 is greater than 98%. The Cohen kappa statistic is 0.699, indicating substantial agreement between the coliphage plaque and fluorescence-based prediction results.

Fluorescence-positive male-specific coliphage prediction. The male-specific fluorescence-based prediction is 83.6% predictive of STEP-1 plaque formation with a 14.7% false-negative rate and a 23.5% false-positive rate (Table 5). Because of the

TABLE 5. Male-specific coliphage fluorescence-based prediction compared to STEP-1 plaque[a]

Sample category in STEP-1 plaque	No. of samples in STEP-2 fluorescence-based assay		
	Positive	Negative	Total
Positive	122	21	143
Negative	8	26	34
Total	130	47	177

[a] Sensitivity, 85.3%; specificity, 76.5%; overall agreement, 83.6%; Cohen kappa value, 0.539; false-negative error rate, 14.7%; false-positive error rate, 23.5%; miscalculation rate, 16.4%.

TABLE 6. ATP approach comparability study: somatic coliphages

Waste location	Chlorine disinfection	Dilution (ml/liters)	No. of samples positive by assay:			Total no. of samples
			EPA 1601	STEP-1	Fluorescence	
M 10-15-08	No	2.25/4.5	15	15	16	20
L 09-09-08	No	4.5/4.5	12	10	11	20
SA 12-18-08	No	0.35/4.5	10	6	7	20
L 06-26-08	Yes	10/4.5	15	13	7	20
O 123008	No	1.9/4.5	10	9	11	20
O 010209	Yes	280/4,220	5	4	3	20
Sum			67	57	55	120
% positive			55.8	47.5	45.8	

small number of negative samples, the false-positive rate is overstated, and a better assessment of the fluorescence-based error is the combined negative and positive misclassification rate of 16.4%. False positives are overstated as evidenced in Table 3, which shows that 7 of the 8 false-positive fluorescence results reported in Table 5 were true coliphage positives compared to STEP-2 plaque. This indicates that the false-positive rate may be as low 1 in 34, or 3%. Positive fluorescence correlation with positive plaques from STEP-2 is greater than 98%. The Cohen kappa statistic of 0.539 indicates moderate agreement between coliphage plaque and fluorescence-based prediction results.

Somatic coliphage ATP approach: comparability. Somatic coliphage comparative data for comparison with Method 1601 according to the ATP validation approach are presented in Table 6. Control data are proper with these test cycles but not shown. These data were generated as manufacturer method validation by using a variety of wastewater spikes and different groundwater sources and include water that went through disinfection. Of the six testing cycles, totaling 120 test results for each method, EPA Method 1601 had 55.8% positive results and the Fast Phage modification STEP-1 plaque had 47.5% positive results. Chi-square analysis of the six water samples using Method 1601 as an expected result and modification as observed results indicates that the two testing populations are equivalent, not significantly different, with a P value of 0.33. A power analysis was applied (see Table 8) to additionally analyze if observed differences were significant. The only sample that is statistically significant is L 06-26-08 and only for the difference between EPA Method 1601 and the fluorescence-based prediction. All other sample differences are not significant. Projecting the number of samples required to achieve significance by the ATP approach indicates that sample testing requirements could become impractically large. The fluorescence-based prediction in the ATP study samples had a 90% correlation with Fast Phage STEP-1 plaque with a 7.9% false-positive rate and a 12.3% false-negative rate. One disinfected water sample had 6 of the 7 observed false-negative fluorescence-based results. The Cohen kappa value of 0.799 indicates substantial agreement between coliphage plaque and fluorescence-based prediction results.

Somatic coliphage ATP approach: specificity. The Fast Phage modification STEP-1 plaque result is the endpoint equivalent to EPA Method 1601. The ATP approach calls for a specificity study where the alternative method endpoint result is tested again with the reference method and again using a third referee method. Table 7 summarizes results where the Fast Phage STEP-1 spot results of the comparability study are cut out, resolubilized, and then respotted onto EPA Method 1601 plates. For a referee method, the resolubilized material was filtered and then spotted. This referee method is consistent with procedures in section 12 of Method 1601 to determine questionable samples containing coliphages (25). The Fast Phage method STEP-1 plaque compared to the reference method had a 90% agreement with a 2.1% false-negative error rate and a 15.7% false-positive error rate with a Cohen kappa statistic of 0.798, indicating substantial agreement between Method 1601 and the modification. The Fast Phage method compared to the referee method had a 92.5% agreement with a 2.0% false-negative error rate and an 11.4% false-positive error rate and a Cohen kappa statistic of 0.849, indicating very close agreement. These results can be compared directly with the reference method agreement with the referee method, which had an overall 90.8% agreement with a 12.0% false-negative error rate, a 7.1% false-positive error rate, and a Cohen kappa statistic of 0.811.

DISCUSSION

The coliphage modifications examined in this study are novel and important method simplifications/conveniences that reduce the preparation, labor cost, and time required to per-

TABLE 7. ATP approach selectivity study: somatic coliphages[a]

Waste location	No. of samples													
	STEP-1 negative	STEP-1 positive	Unfiltered spot repicked to EPA 1601				Filtered spot repicked to EPA 1601				Unfiltered EPA 1601 vs filtered EPA 1601			
			TN	TP	FN	FP	TN	TP	FN	FP	TN	TP	FN	FP
M 0-15-08	5	15	4	10	1	5	4	12	1	3	6	10	3	1
L 09-09-08	10	10	10	8	0	2	10	8	0	2	11	7	1	1
SA12-18-08	14	6	14	3	0	3	14	4	0	2	15	2	2	1
L 06-26-08	7	13	7	13	0	0	7	13	0	0	7	13	0	0
O 123008	11	9	11	8	0	1	11	8	0	1	10	8	0	2
O 010209	16	4	16	4	0	0	16	4	0	0	16	4	0	0
Sum	63	57	62	46	1	11	62	49	1	8	65	44	6	5

[a] Abbreviations: TN, true negative; TP, true positive; FN, false negative; FP, false positive.

TABLE 8. Power analysis: differences between the percent positive (Table 6 data) values observed by the EPA Method 1601, the Fast Phage STEP-1 plaque, and the fluorescence-based prediction[a]

Waste source	1601 vs STEP-1		1601 vs fluorescence		STEP-1 vs fluorescence	
	% difference	No. of samples required to detect observed differences[b]	% difference	No. of samples required to detect observed differences[b]	% difference	No. of samples required to detect observed differences[b]
M 10-15-08	0.0	NA[c]	−5.0	824	−5.0	824
L 09-09-08	10.0	304	5.0	1,019	−5.0	1,043
SA12-18-08	20.0	72	15.0	115	−5.0	931
L 06-26-08	10.0	258	40.0	14	30.0	28
O 123008	5.0	1,234	−5.0	1,043	−10.0	260
O 010209	5.0	862	10.0	199	5.0	725
Summary over all sources	8.3	443	10.0	204	1.7	7,379

[a] The total number of samples was 20.
[b] Number of samples that would be required to detect any differences as significant based on a binomial two-tailed power analysis.
[c] NA, not applicable.

form a coliphage assay. The ability to reliably detect levels of 1.3 to 1.5 PFU/100 ml in less than 8 h or within 1 working day shift provides better public health protection than do assays that require 24 to 48 h. This speed and sensitivity have not yet been achieved with conventional microbiology methods approved for drinking water quality. Other researchers have shown that accelerated amplification with subsequent coliphage detection techniques can meet detection criteria in a single working day (11, 12, 14, 20). The Fast Phage modifications work within the framework of EPA Method 1601 while incorporating the use of beta-galactosidase as an 8-h coliphage prediction method.

Laboratories were able to show that both somatic and male-specific Fast Phage modifications successfully meet Initial Demonstration Capability (IDC) as defined in section 9.3 of EPA Method 1601 by detecting at least 50% STEP-1 plaque-positive samples with waste-spiked reagent water (25). Similarly the laboratories met expanded matrix spike (MS) criteria defined in section 9.8 of EPA Method 1601 by detecting at least 50% positive (somatic) and 40% positive (male-specific) samples with waste-spiked groundwater (25). These are the defining criteria for meeting internal laboratory validation defined in section 14.1.1 as tier 1 validation and as determined by collaborative study of Method 1601 (21). The result of this study is that 5 laboratories demonstrated that the Fast Phage modification meets tier 1 criteria for both somatic and male-specific coliphages.

The next step in approving method modifications for laboratory use under the PBMS protocol is called a tier 2 study. A tier 2 study is a national validation study that would share a single sample with four collaborative laboratories according to criterion objectives similar to those followed in this study. If 3 of 4 laboratories meet the performance criteria, the modified method could be nationally approved and included as acceptable in the prologue of EPA Method 1601. The EPA must first review and accept the testing protocol (2, 9).

The alternative test procedure has been used for microbiological method approvals that can be used in compliance with the TCR. Therefore, there is interest in applying these familiar validation approaches to coliphage methods. There is no EPA-approved ATP protocol for coliphages, and so the experiments and protocols in this study followed designs similar to those of coliform and *E. coli* ATP method validations (5). Since there was no formal EPA approval of the ATP design in this study, and due to the extensive work involved, only somatic coliphages were evaluated in this study by using this ATP comparative approach. In the somatic coliphage comparison study, the modified method detected 57 positive of 120 samples while the reference method detected 67 positive of 120 samples. The fluorescence-based prediction method detected 55 positive of 120 samples. While there appears to be a low recovery bias, statistical analysis of data does not show a significant difference between the modified method results and the reference method results. Furthermore, the number of samples required to determine if the observed differences are significant, as projected by power analysis (Table 8), is impractically large.

The second aspect of an ATP evaluation is the specificity of the alternative method result relative to the reference and referee (gold standard) test. Data comparing somatic Fast Phage plaque recultured onto EPA Method 1601 plates indicate substantial equivalence between the two protocols. There is a low (2%) false-negative error rate compared to either the reference method or the referee method. A false-positive error rate of 15.7% compared to the somatic reference method and a rate of 11.4% compared to the somatic referee method are due to the lack of agreement between the reference and the referee methods, which show a 12.1% false-negative error rate and a 7.1% false-positive error rate between two reference procedures. In this study the modified assay had a population fit, with a kappa value of 0.849, with the reference method that was higher than the reference method fit to the referee method, with a kappa value of 0.811. All these analyses support the idea that the somatic Fast Phage modification is at least equivalent to the reference method with overall 90% or better correlation with the referee and reference methods.

The fluorescence-based positive prediction has value as a positive early warning that could alert users to a positive result in less than 8 h from starting the assay. This early warning has important implications in remediation testing and rapid response to fecal contamination triggered by events such as flooding, distribution main breaks, weather, or other natural disasters. The fluorescence-based indicator is 98% predictive of STEP-2 plaque recovered from fluorescence-based tests. The fluorescence-based prediction has between 83.5 and 92.1% overall agreement with the STEP-1 plaque shown to meet EPA PBMS criteria. ATP

somatic data are also consistent with the somatic PBMS data, showing a 90% correlation with STEP-1 plaque formation. All data indicate a high probability of coliphage presence and plaque confirmation when fluorescence is observed.

Fluorescence-negative results as a prediction of coliphage absence were less predictive than were the positive results, particularly with male-specific coliphages in the PBMS study. Several water samples had test results with a negative fluorescence result but with subsequent plaques observed on the confirmation plates. Laboratory 2 tested a water sample with a low spike of coliphages that produced some false-negative fluorescence in groundwater. Laboratory 1 also observed some false-negative fluorescence with male-specific coliphages in one test of groundwater spiking. These account for the 16.4% misclassification rate of the fluorescence-based prediction observed in male-specific PBMS data. Somatic coliphage ATP data sample L 06-26-08, which was chlorine disinfected after spiking, also had several split samples with no fluorescence but with observed plaques. Based on these multiple false-negative fluorescence-based observations, it is prudent to wait for endpoint plaque confirmation of the predicted fluorescence-negative result. Fluorescence absence may be less predictive of coliphage absence because coliphages under certain growth conditions may be slower to divide, may produce fewer progeny, or may be more difficult to induce into lytic cycles from their lysogenic states.

The results of the somatic coliphage method validation approach comparison indicate that a PBMS approach is most practical. The coliphage modification Fast Phage meets the published acceptance EPA Method 1601 criteria when tested in five different laboratories. Both the tier 1 PBMS data for somatic and male-specific coliphages and the ATP data for somatic coliphages support the idea that the Fast Phage method is a coliphage method equivalent to EPA Method 1601. The 8-h fluorescence-based predictor of the modified method is highly predictive of positive results and can provide users with a very early warning of fecal contamination. These data should be used as justification to help finalize design of EPA validation protocols so that the modified method could be nationally validated to be equivalent to Method 1601 and so that it could be used to comply with the GWR. Selection of coliphage as the indicator of choice to comply with GWR regulations or to complement bacterial indicators is more likely with the convenient and ready-to-use reagents offered by the modified method. Because viruses can survive and persist longer in groundwater than can bacteria, testing for coliphages provides an additional measure of water quality and further protects public health. This modified method will allow utilities to easily use two analytical methods—one viral and one bacterial—in assessing water quality to comply with the Ground Water Rule.

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REFERENCES

1. **Ashbolt, N. J., W. O. K. Grabow, and M. Snozzi.** 2001. Indicators of microbial water quality, p. 289–331. *In* L. Fewtrell and J. Bartram (ed.), Water quality guidelines: guidelines, standards and health. World Health Organization water series. IWA Publishing, London, United Kingdom.
2. **Barbour, M. T., J. Gerritsen, B. D. Snyder, and J. B. Stribling.** 1999. Rapid bioassessment protocols for use in streams and wadeable rivers: periphyton, benthic macroinvertebrates, and fish, 2nd ed. Chapter 4. Performance-based method systems. EPA 841-B-99-002. U.S. Environmental Protection Agency, Washington, DC. http://www.epa.gov/owow/monitoring/rbp/ch04main.html.
3. **Bishop, Y. M., S. E. Fienburg, and P. W. Holland.** 2007. Discrete multivariate analysis—theory and practice. Springer Science and Business Media, New York, NY. [Reprint of 1975 MIT Press edition.]
4. **Clancy, J., E. Conklin, G. Durbin, and R. S. Salter.** 2009. Performance-based method systems (PBMS) validation of a method 1601 same-day detection of coliphage in water, microposter Mon-2. Proc. AWWA Water Quality Technol. Conf., Seattle, WA.
5. **Covert, T. C., E. W. Rice, S. A. Johnson, D. Berman, C. H. Johnson, and P. J. Mason.** 1992. Comparing defined-substrate coliform tests for the detection of *Escherichia coli* in water. J. Am. Water Works Assoc. **84:**89–104.
6. **Dufour, A., M. Snozzi, W. Koster, J. Bartram, E. Ronchi, and L. Fewtrell.** 2003. WSH/DOC assessing microbial safety of drinking water—improving approaches and methods. IWA Publishing, London, United Kingdom. http://www.bvsde.paho.org/bvsacd/WHO3/assess.pdf.
7. **European Committee for Standardization.** 1995. European Standard EN ISO 10705-1. Water quality—detection and enumeration of bacteriophages—part 1: enumeration of F-specific RNA bacteriophages. European Committee for Standardization, Brussels, Belgium.
8. **Federal Register.** 2006. EPA 40 CFR parts 9, 141, and 142: national primary drinking water regulations: ground water rule; final rule. Fed. Regist. **71:** 65574–65659.
9. **Federal Register.** 1997. Performance-based method systems. Fed. Regist. **62:**52098–52100.
10. **Floyd, R., and D. G. Sharp.** 1979. Viral aggregation: buffer effects in the aggregation of poliovirus and reovirus at low and high pH. Appl. Environ. Microbiol. **38:**395–401.
11. **Ijzerman, M., and C. Hagedorn.** 1992. Improved method for coliphage detection based on B-galactosidase induction. J. Virol. Methods **40:**31–36.
12. **Ijzerman, M., J. O. Falkinham III, and C. Hagedorn.** 1993. A liquid, colorimetric presence-absence coliphage detection method. J. Virol. Methods **45:**229–234.
13. **Kroll, D.** 2006. Securing our water supply: protecting a vulnerable resource. Pennwell Corporation, Tulsa, OK.
14. **Love, D. C., and M. Sobsey.** 2007. Simple and rapid F+ coliphage culture, latex agglutination, and typing assay to detect and source track fecal contamination. Appl. Environ. Microbiol. **73:**4110–4118.
15. **Lwoff, A., L. Siminovitch, and N. Kjeldgaard.** 1950. Induction of bacteriophage lysis of and entire population of lysogenic bacteria. C. R. Acad. Sci. **231:**190–191.
16. **McDonald, J. H.** 2009. Handbook of biological statistics, 2nd ed., p. 33–38. Sparky House Publishing, Baltimore, MD.
17. **Oppenheim, A. B., O. Koblier, J. Stavans, D. L. Court, and S. Adhya.** 2005. Switches in bacteriophage lambda development. Annu. Rev. Genet. **39:**409–429.
18. **Salter, R. S., G. W. Durbin, S. Wang, S., R. Abraham, and S. Charm.** 2009. Same day and continuous detection of coliphage in water, abstr. Q-194, p. 483. Abstr. 109th Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, DC.
19. **Sokal, R. R., and F. J. Rohlf.** 1995. Biometry: the principles and practice of statistics in biological research, 3rd ed. W. H. Freeman, New York, NY.
20. **Stanek, J. E.** 1997. Development of a rapid coliphage assay. Ph.D. thesis. Virginia Polytechnic Institute and State University, Blacksburg, VA.
21. **U.S. Environmental Protection Agency.** July 2003. Results of the interlaboratory validation of EPA method 1601 for presence/absence of male specific (F+) and somatic coliphages in water by two step enrichment. Office of Water, U.S. Environmental Protection Agency, Washington, DC.
22. **U.S. Environmental Protection Agency.** 2010. Beaches, beach pollution. U.S. Environmental Protection Agency, Washington, DC. http://www.epa.gov/beaches/learn/pollution.html.
23. **U.S. Environmental Protection Agency.** 2006. Occurrence and monitoring document for the final ground water rule. U.S. Environmental Protection Agency, Washington, DC. http://www.epa.gov/ogwdw/disinfection/gwr/pdfs/support_gwr_occurance-monitoring.pdf.
24. **U.S. Environmental Protection Agency.** April 2001. 821-R-01-029. Method 1602: male-specific (F+) and somatic coliphage in water by single agar layer (SAL) procedure. U.S. Environmental Protection Agency, Washington, DC. http://www.epa.gov/nerlcwww/1602ap01.pdf.
25. **U.S. Environmental Protection Agency.** April 2001. 821-R-01-030. Method 1601: male-specific (F+) and somatic coliphage in water by two-step enrichment procedure. U.S. Environmental Protection Agency, Washington, DC. http://www.epa.gov/nerlcwww/1601ap01.pdf.
26. **U.S. Environmental Protection Agency.** October 2006. Office of Water (4606-M) EPA 815-R-06-014. Economic analysis for the final ground water rule. Office of Water, U.S. Environmental Protection Agency, Washington, DC. http://www.epa.gov/safewater/disinfection/gwr/pdfs/support_gwr_economicanalysis.pdf.

27. **U.S. Environmental Protection Agency.** April 2004. Microbiological alternate test procedure (ATP) protocol for drinking water, ambient water, and wastewater monitoring methods: guidance. EPA no. 821-B-03-004. U.S. Environmental Protection Agency, Washington, DC. http://www.epa.gov/waterscience/methods/atp/EPA821B03004.pdf.
28. **U.S. Government.** 2002. Code of federal regulations. CFR 40 141.4. Total coliform rule. U.S. Government Printing Office, Washington, DC.
29. **Van Regenmortel, M. H. V., C. M. Fauquet, D. H. L. Bishop. E. B. Carstens, M. K. Estes, S. M. Lemon, D. J. McGeoch, J. Maniloff, M. A. Mayo, C. R. Pringle, and R. B. Wickner.** 2000. Virus taxonomy: classification and nomenclature of viruses. Seventh ICTV report. Academic Press, San Diego, CA.
30. **World Health Organization.** 2002. World health report 2002: reducing risks, promoting healthy life. World Health Organization, Geneva, Switzerland. http://www.who.int/whr/2002/chapter1/en/index.html.