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Read the Expanded Summary

Modified USEPA method 1601 to indicate viral contamination of groundwater

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The 2006 Ground Water Rule allows three indicators for detection of fecal contamination: *Escherichia coli*, enterococci, and coliphages. Although each indicator has its benefits and limitations, testing for all three ensures the safest potable water supply. Somatic and male-specific coliphages are viral indicators. Current coliphage detection methods approved by the US Environmental Protection Agency (USEPA) require more preparation and expense compared with rapid bacterial indicator tests. This study describes a tier 2 validation of commercially prepared reagents used in a modified USEPA method 1601 format for the qualitative detection of coliphages. Four water laboratories participated in a USEPA alternative test procedure format using method 1601 performance-based measurement system guidelines to test four geographically diverse groundwaters spiked with wastewater. Results indicated that the modifications to method 1601 have value in predicting the presence of coliphages in 8 h with subsequent overnight plaque confirmation; the total assay time of 16 to 24 h was equivalent to the 48 to 72 h of USEPA method 1601.

KEYWORDS: *coliphage, Ground Water Rule, microbiological alternative test protocol, performance-based, viral indicator*

Ensuring the sanitary 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 (Kroll, 2006; Dufour et al, 2003; WHO, 2002). Fecal contamination of source water presents both bacterial and viral pathogenic threats to public health (Ashbolt et al, 2001). The Total Coliform Rule prescribes the assessment of potable drinking water for the presence of bacterial indicator organisms, total coliform, and *Escherichia coli* based on municipality populations (USEPA, 2002). When coliform indicators are detected in groundwater sources or when groundwater is initially tested for potability, the Ground Water Rule (GWR) provides the analyst a selection of three fecal indicators—*E. coli*, enterococci, and coliphages—for use in testing (USEPA, 2006b). *E. coli* and enterococci are bacterial fecal indicators, whereas coliphages (viruses of coliform bacteria) are viral indicators of fecal contamination.

BACKGROUND

GWR testing. Groundwater contamination is caused by a variety of factors and stress (e.g., population encroachment, proximity to farms, well depth, recharged water sources, sediment types, porosity, rock fissures) that influence the composition of bacterial and viral microbiota. The GWR microbial indicator choices recognize that waterborne public health threats and documented waterborne disease are both bacterial and viral in etiology. In GWR comments, the National Drinking Water Advisory Council recommended that both bacterial and viral indicators be included in a water safety testing protocol because each can predict the likelihood of contamination with a specific group of microorganisms and provide a more complete picture of the groundwater quality (USEPA, 2006b). In the final GWR, however, testing for both bacterial and viral indicators was not considered economically feasible because of the additional expense of labor and time to perform coliphage testing methods (USEPA, 2006a). US Environmental Protection Agency (USEPA) method 1602 describes a 24-h single agar layer coliphage plaque quantification using a 100-mL water sample and 10 petri plates per sample using precultured *E. coli* in a logarithmic growth phase. USEPA method 1601 describes a two-step coliphage presence–absence determination using 24-h sample pre-enrichment of 100-mL or 1-L samples followed by 24-h spot plate confirmation (USEPA, 2001a, 2001b). Both methods involve detailed hands-on procedures that require skilled microbiologists and preparation of host bacterial cultures and media in advance.

Study design and objective. The current study was conducted as a national tier 2 validation of a commercially available qualitative coliphage method[1] based on modifications to USEPA method 1601 using ready-to-use reagents in an accelerated 16- to 24-h time-to-result format (Clancy et al, 2009). The modified method (referred to here as FPMM) had previously shown initial demonstration of capability (IDC) in a five-laboratory tier 1 study (Salter et al, 2010), accurately predicted coliphage-positive sample results by fluorescence in less than 8 h, and confirmed coliphage qualitative presence–absence results by conventional plaque analysis 16–24 h after initiating the assay.

USEPA method 1601 is a performance-based measurement system (PBMS) in which acceptance criteria for method performance are developed in collaborative testing and published as part of the method (USEPA, 2003). USEPA developed PBMS in order to facilitate adoption of method improvements when defined acceptance criteria are met in demonstrated intralaboratory tier 1 and interlaboratory tier 2 studies (Barbour et al,

1999; USEPA, 1997). Tier 2 is a national approval of modifications and requires USEPA participation and review of a collaborative laboratory protocol before commencement. The current investigation was performed as a USEPA-acceptable tier 2 study based on previous IDC tier 1 work.

In addition to requiring that the study design meet the PBMS criteria described in section 14 of method 1601, USEPA requested that the study include direct method comparison of the FPMM and reference method using split samples with followup precision tests of the modified method according to the microbiological alternative test procedure (ATP) protocol for drinking water (USEPA, 2010). Consequently, the resulting study design is a hybrid protocol of PBMS and ATP criteria. This collaborative comparability study design used four independent, qualified water laboratories and four geographically diverse groundwater sources spiked with wastewater-associated coliphages to PBMS targets levels. On receipt of shared and geographically diverse wastewaters, the laboratories spiked their groundwater to specified coliphage levels and tested 10 replicates per diluted spiked bulk sample with both the modified and the reference methods. The precision study involved the reculture of the modified method spot agar results (both filtered and unfiltered) on a method 1601 spot agar plate to determine true and false results of the FPMM.

Coliphage indicator test results in combination with bacterial indicator test results have demonstrated their value in detecting water supply vulnerabilities in groundwater (Verstraeten et al, 2005; Karim et al, 2004). Somatic coliphage detection also correlates with intrusion of fecal indicators into chloramine-disinfected distribution systems (LeChevallier et al, 2006). Monitoring a suite of indicator organisms is more predictive of the presence of certain pathogens in reclaimed water, suggesting that testing for both viral and bacterial indicators would be more effective at protecting public health (Harwood et al, 2005). The significance of the current work is that if the method modifications were shown to be equivalent to method 1601, a fast, simple-to-use, shelf-ready, and less labor-intensive method would be added to the toolbox of available USEPA-accepted coliphage methods. The FPMM could be useful to assess the public health of ground source water supplies including water production processes, intrusion into distribution systems, and recharged waters.

MATERIALS AND METHODS

Presence–absence method for coliphage. In the study, USEPA method 1601 was performed according to the mandated procedure (USEPA, 2001b). The modifications to method 1601 (i.e., FPMM) were performed as described elsewhere (Salter et al, 2010). Participating laboratories from geographically diverse areas—Arizona, New Jersey, Vermont, and Wisconsin—performed and qualified performance of both the modified and reference methods using four local sourced groundwaters and local wastewaters per the quality control (QC) and IDC requirements in section 9 of USEPA method 1601 (USEPA, 2001b).

Groundwaters and wastewaters. Charm Sciences of Lawrence, Mass., the manufacturer of the modified method, was the coordinating laboratory to obtain, filter, and split wastewater samples collected from Massachusetts (two sites), Arizona, and Wisconsin and to coordinate the simultaneous analyses test schedule. Primary unchlorinated wastewater samples were collected and transported on gel ice by the coordinating laboratory or by participant laboratories in Arizona and Wisconsin and shipped in a cooler with gel ice overnight to the coordinating laboratory. Samples were subsequently filtered through a 0.45-µm filter with a low protein-binding 25-mm membrane,[2] enumerated by double agar layer (DAL) technique, split, and shipped in a cooler with gel ice overnight to all participant laboratories. On receipt of the shared sample filtrate, the laboratories performed DAL assay and used their coliphage titer determination to spike a 2.2-L volume of local groundwater as specified in expanded matrix spiking target levels described in section 9.8 of method 1601 (USEPA, 2001b). The laboratories also performed DAL assay on the day of spiking to calculate the actual number of coliphage spiked into the groundwater sample. At the end of the week of testing, a retention sample of the initial wastewater sample filtrate was DAL-tested by the coordinating laboratory to show a 96-h, 4°C storage result.

Method comparison. The laboratories analyzed replicates (n = 10 each) by FPMM and USEPA method 1601 after spiking groundwater with wastewater filtrate. Somatic coliphage assays were performed June–July 2011, and male-specific assays were performed August–October 2011. Each laboratory obtained qualified local groundwaters and performed ongoing demonstration of capability (ODC) and negative control–testing to meet method 1601 QC requirements.

Laboratory procedures. Detailed protocols and recording sheets were supplied to laboratories following review and approval by USEPA. In addition, laboratories received weekly log sheets to document equipment calibrations, temperatures, and procedural times; log sheets were returned to the coordinating laboratory for review and collating.

Precision. FPMM precision was determined by excising the circular spot area (with or without a lytic zone) with the wide bore end of a Pasteur pipette and transferring it into 0.5 mL of tryptic soy broth (TSB). The suspension was respotted to a USEPA method 1601 agar plate seeded with respective host *E. coli* as the comparative reference. It was then filtered through a 0.45-µm filter with a low protein-binding 25-mm membrane[2] and spotted to a USEPA method 1601 spot agar plate as the reference method specified in section 12 of method 1601 (USEPA, 2001b).

Statistical computations. Calculations for method comparability and precision were as described in the ATP protocol (USEPA, 2010). In the comparative studies, the Fisher exact test was performed to determine if the differences were statistically significant. In addition, an overall Cochran-Mantel-Haenszel (CMH) chi-square analysis was used to evaluate statistical significance of the population of differences as a single degree of freedom. The statistical review of precision data used two-by-two contingency table comparability analyses for false-negatives and false-positives and overall agreement. Analyses were facilitated by web-based inter-rater and statistical computation spreadsheets (McDonald, 2009; Mackinnon, 2000).

RESULTS AND STATISTICAL ANALYSIS

DAL determinations. The results of the DAL determinations of the shared wastewater samples are shown in Table 1. Throughout the comparison study, the Wisconsin and the Vermont laboratories obtained lower plaque-forming-units-per-millilitre counts than did the New Jersey and Arizona laboratories and the coordinating laboratory. DAL results also indicated that the male-specific coliphage was less stable over the week of testing than was the somatic coliphage and that the male-specific coliphage titer trended lower during overnight shipping on gel ice. The end-of-week male-specific DAL titer determinations completed by the coordinating laboratory were almost 50% degraded after 96-h storage postfiltering, whereas the somatic DAL titers were more stable and showed only 15–30% degradation. The wastewater samples therefore met the USEPA method 1601 specifications for raw sewage filtrate because the filtrate titers were determined within 24 h of collection, did not degrade by more than 50%, and were retitered and used within 72 h of receipt by the laboratories.

The laboratories obtained their own groundwater source and spiked it with the filtered sewage at the prescribed plaque-forming unit spike levels (1.5 pfu/100 mL for somatic coliphages and 1.3 pfu/100 mL for male-specific coliphages) as specified by method 1601 for PBMS validation. They used their day-before-assay DAL results (Table 1) in the spiking calculations. The DAL determination was based on duplicate plates of 4-log serial dilutions of the wastewater. The laboratories spiked the 2.2 L of groundwater based on the calculated day-before-assay coliphage concentration, then mixed and aliquoted 100-mL test samples alternatively between the FPMM and USEPA method 1601 and completed each sample as per method protocol. The laboratories also repeated the DAL assay of the wastewater sample that same day; the reported spiked coliphage plaque-forming units per 100 mL based on this day-of-assay DAL result is shown in Table 1. The global day-to-day DAL repeatability within labs was 15% of the average, whereas the reproducibility of DAL results of the same samples shared among the labs was 40% of the average.

Coliphage method comparisons. *Somatic coliphage.* Results of the somatic coliphage method comparison are shown in Table 2. The range of somatic coliphage spikes (0.8–1.4 plaque-forming units per 100 mL) met the target spiking level of < 1.5 pfu/100 mL specified by method 1601 (USEPA, 2001b). The FPMM, complete through plaque confirmation, identified 77% (92 of 120 samples) as positive, compared with 85% (102 of 120 samples) identified as positive by method 1601. The 8-h fluorescence prediction, a component of the FPMM, identified 73% (87 of 120 samples) as positive. At least five positives in 10 replicates at the targeted plaque-forming units per 100 mL were detected in all somatic cycles, which met the PBMS acceptance criteria described in method 1601, section 14.2, for national approval of method modifications (USEPA, 2001b). The comparative method 1601 performance likewise met the extended matrix spike QC criteria as described in section 9 of the method. In all cases, internal positive and negative controls and concurrent method 1601 ODC in all the laboratories met quality specifications for reporting results. In any one somatic cycle, the maximum difference observed in the number of plaque-positive samples by USEPA method 1601 and the 16- to 24-h modified method was 30%.

Male-specific coliphage. Table 3 shows results of the male-specific coliphage method comparison. The range of male-specific coliphage spikes was 0.9 to 1.5 pfu/100 mL, targeting the specified USEPA method 1601 spiking level of < 1.3 plaque-forming units per 100 mL and simulating the range of studied sample spikes from the USEPA method 1601 collaborative study (USEPA, 2003). The FPMM, complete through plaque confirmation, identified 89% (107 of 120 samples) as positive, compared with 96% (115 of 120 samples) identified as positive by method 1601. The 8-h fluorescence prediction identified 88% (105 of 120 samples) as positive. At least five positives in 10 replicates at the targeted pfu/100 mL were detected in all male-specific cycles, which met the PBMS acceptance criteria described in USEPA method 1601, section 14.2, for national approval of method modifications. The comparative method 1601 performance likewise met the extended matrix spike QC criteria as described in section 9 of the method. In all cases, internal positive and negative controls and concurrent method 1601 ODC in all the laboratories met quality specifications for reporting results. In any one male-specific cycle, the maximum difference observed in the number of plaque positives by method 1601 and the 24-h FPMM was 40%. Each comparability table included the actual spike level, the plaque-forming units per 100 mL as calculated from the amount of filtered sewage added, and the day-of-assay DAL result (Table 1) performed on the day of spiking.

Fisher exact test. Because of the number of sample replicates (n = 10), Fisher exact tests were performed on all comparative method somatic and male-specific test cycles reported in Tables 2 and 3 to determine if there were significant differences between the FPMM and the reference method. Fisher exact tests did not indicate a significant difference between any one comparative set of data. When the data sets were considered as whole data sets with a single degree of freedom, CMH chi-square analysis delivered probabilities of p = 0.13 (somatic) and p = 0.06 (male-specific) that the 12 data sets were homologous. These probabilities do not indicate significant differences between the FPMM and the reference method ($p < 0.05$). Although not significant, the p = 0.13 and p = 0.06 may indicate a lower trend in positive determinations. When probabilities were recalculated excluding the single most different study cycle from the chi-square determinations, the resulting probabilities—p = 0.29 (somatic) and p = 0.38 (male-specific)—indicated that one study cycle's strong influence on the overall p determination and further supported the conclusion that the methods were not significantly different. Excluding one of 10 method comparison cycles in determining a probability of method difference is consistent with acceptable ATP comparability requirements that at least 80% of comparison cycles be equivalent.

Precision study results. All FPMM spot plate circular spot areas (with or without a lytic zone) were transferred to TSB broth and respotted to a new method 1601 agar spot plate to calculate the precision of the FPMM in terms of agreement with method 1601. This experiment determined the associated false-negative and false-positive results of the modified method. Results were scored provided the negative- and positive-control samples of the transferred plaques tested appropriately. Appropriate QC results

TABLE 1 DAL coliphage enumeration to demonstrate compliance with spiking specifications of method 1601

Cycle ID	Wastewater Source	Coliphage Type	Laboratory and Groundwater Source	Collection DAL *pfu/mL*	Receipt DAL *pfu/mL*	Day-Before-Assay DAL *pfu/mL*	Day-of-Assay DAL *pfu/mL*	End-of-Week Assay DAL *pfu/mL*
S1	Lawrence, Mass.	Somatic	Mass.	1,382	NP	1,354	1,171	973
S1-1			Vt.	NA	NA	1,636	1,727	NA
S1-2			N.J.	NA	NA	1,216	1,164	NA
S1-3			Ariz.	NA	NA	1,009	820	NA
S1-4			Wis.	NA	NA	901	856	NA
S2	Tucson, Ariz.	Somatic	Mass.	NA	7,273	6,455	6,636	5,182
S2-1			Vt.	NA	NA	11,727	8,454	NA
S2-2			N.J.	NA	NA	6,487	6,667	NA
S2-3			Ariz.	5,909	NA	9,000	8,545	NA
S2-4			Wis.	NA	NA	3,909	4,090	NA
S3	Madison, Wis.	Somatic	Mass.	NA	4,090	3,400	3,636	3,636
S3-1			Vt.	NA	NA	4,636	4,181	NA
S3-2			N.J.	NA	NA	4,505	4,054	NA
S3-3			Ariz.	NA	NA	4,636	4,727	NA
S3-4			Wis.	2,909	NA	2,727	2,454	NA
F+1	Lowell, Mass.	Male-specific	Mass.	2,685	NP	2,720	2,387	1,018
F+1-1			Vt.	NA	NA	946	1,180	NA
F+1-2			N.J.	NA	NA	1,892	2,306	NA
F+1-3			Ariz.	NA	NA	2,189	2,351	NA
F+1-4			Wis.	NA	NA	738	604	NA
F+2	Madison, Wis.	Male-specific	Mass.	NA	4,009	3,649	3,387	1,645
F+2-1			Vt.	NA	NA	865	847	NA
F+2-2			N.J.	NA	NA	3,919	3,108	NA
F+2-3			Ariz.	NA	NA	5,649	5,855	NA
F+2-4			Wis.	2,820	NA	1,468	1,658	NA
F+3	Tucson, Ariz.	Male-specific	Mass.	NA	279	251	243	120
F+3-1			Vt.	NA	NA	140	38	NA
F+3-2			N.J.	NA	NA	204	223	NA
F+3-3			Ariz.	205	NA	142	119	NA
F+3-4			Wis.	NA	NA	101	79	NA

DAL—double agar layer, ID—identification, NA—measurement not applicable to the noncollecting laboratories, NP—not performed (i.e., the collecting laboratory was also the coordinating laboratory and therefore no shipment of sample took place)

DAL calculations are a single numeric calculation based on duplicate plates of 4-log serial dilutions of a single test sample. Collection of DAL was performed by the laboratory collecting the sample and on the day of collecting the sample. Receipt DAL was performed by the coordinating laboratory on receipt of sample to verify < 50% loss of plaque-forming units per millilitre over shipment. Day-before-assay DAL was performed on the split wastewater samples on the day of receipt of sample, and when compared with collecting laboratory collection DAL, demonstrated < 50% loss of plaque-forming units per millilitre. Day-of-assay DAL was performed on the split wastewater sample on the day of spiking into groundwater and took place within 72 h of receipt of the sample. End-of-week assay DAL was performed on the wastewater sample by the coordinating laboratory at the end of study week.

occurred in all test cycles except for a single somatic coliphage cycle in which the negative controls tested positive; those results were recorded as a laboratory accident. Tables 4 and 5 show precision results for somatic coliphage and male-specific coliphage, respectively. In these analyses (and according to USEPA agreed-on convention), the respotted sample without filtration was considered the compared method, and the sample with filtration was considered the reference method. In the two-by-two contingency table scoring, a true-positive produced a lytic zone in both the compared and reference methods, a true-negative produced an intact lawn of bacteria in both the compared and reference methods, a false-negative did not produce a lytic zone in the FPMM but produced a lytic zone on the USEPA method, and a false-positive produced a lytic zone in the FPMM but not on the USEPA method.

Somatic coliphage. Somatic coliphage precision experiment analysis (Table 4) showed a 94% observed agreement with the compared method and a 93% observed agreement with the reference method. False-negative rates for both the compared and reference methods were 4.6%, whereas false-positive rates were 13.6% for the compared method and 17.4% for the reference method. These false rates can be compared with those of the unfiltered USEPA method versus the filtered USEPA method, which had false-negatives of 2.3% and false-positives of 4.6%.

TABLE 2 Somatic coliphage comparability study (n = 10 replicates per cycle)

Cycle ID	Spiked *pfu/100 mL*	48-h Method 1601 Positive—*n*	16- to 24-h FPMM Positive—*n*	8-h Fluorescent Positive—*n*
S1-1	1.4	9	7	7
S1-2	1.4	9	10	10
S1-3	0.8	6	7	5
S1-4	1.1	9	6	6
S2-1	1.3	10	8	8
S2-2	1.2	10	8	8
S2-3	0.8	6	5	4
S2-4	1.2	8	8	8
S3-1	1.1	10	9	9
S3-2	1.1	8	10	8
S3-3	1.1	8	7	7
S3-4	1.3	9	7	7
Total		102	92	87
Percent positive (n = 120)		85%	77%	73%

DAL—double agar layer, ID—identification, FPMM—modified method evaluated in the study

Spiked level was made by each laboratory by spiking shared wastewater (Table 1) into local groundwater. The plaque-forming unit per 100 mL was calculated based on volume of wastewater added to 2.2 L and using each laboratory day-of-assay DAL plaque-forming units per millilitre of wastewater to calculate plaques added to groundwater.

TABLE 3 Male-specific coliphage (F+) comparability study (n = 10 replicates per cycle)

Cycle ID	Spiked *pfu/100 mL*	48-h Method 1601 Positive—*n*	16- to 24-h FPMM Positive—*n*	8-h Fluorescent Positive—*n*
F+1-1	1.0	10	10	9
F+1-2	1.5	10	10	10
F+1-3	1.2	10	8	7
F+1-4	1.3	10	9	9
F+2-1	1.2	10	10	8
F+2-2	1.0	10	10	10
F+2-3	1.0	10	10	10
F+2-4	1.3	9	9	9
F+3-1	0.9	10	9	9
F+3-2	1.5	10	10	10
F+3-3	0.3	10	6	8
F+3-4	1.2	6	6	6
Total		115	107	105
Percent positive (n = 120)		96%	89%	88%

DAL—double agar layer, ID—identification, FPMM—modified method evaluated in the study

Spiked level was made by each laboratory by spiking shared wastewater (Table 1) into local groundwater. The plaque-forming unit per 100 mL was calculated based on volume of wastewater added to 2.2 L and using each laboratory day-of-assay DAL plaque-forming units per millilitre of wastewater to calculate plaques added to groundwater.

The somatic coliphage false-positive rates in the FPMM analysis were consistent with earlier published data but might be overstated because of the small number of true-negative samples in the study (Salter et al, 2010). For example, if the negative control and matrix negative control data were included in the analysis, the false-positive rate of the FPMM to method 1601 decreased from 17.4 to 8.9%, which more closely approximated the compared performance of the unfiltered and filtered USEPA methods. The smaller number of negative samples was a consequence of the 1.5-pfu/100 mL somatic coliphage spike level specified for the PBMS comparison. The limited number of false results in the tables did not allow Yates or Pearson chi-squared analysis to determine if the observed differences were significant, but a ϕ = 0.78 indicated the modified method precision was not significantly different from the reference method (ϕ = 0.92). The odds ratio of the modified method was about half that of the comparative method, indicating about twice the probability of a false result; again, however, this analysis was influenced by the smaller number of negative samples and was not considered significantly different from the reference method by Breslow–Day analysis. The false-negative rate of < 5% and the overall 93–94% agreement of the FPMM with method 1601 were measures of equivalence between the methods.

Male-specific coliphage. Male-specific coliphage precision analysis (Table 5) showed a 99% observed agreement with the compared method and a 98% observed agreement with the reference method. False-negative rates of the modified method were 0.9% in the compared method analysis and 2.7% in the reference method analysis. False-positive rates were 0% for both the compared and reference method analyses. These false rates can be compared with those of the unfiltered versus filtered two-by-two contingency table analysis, which had false-negatives of 1.8% and false-positives of 0%. The false-positives in the current analyses could not be accurately determined because of the small number of negative samples in the study data. The smaller number of negative samples was caused by the spike level of 1.3 pfu/100 mL specified in USEPA method 1601 for PBMS comparison. The false-negative rate of < 3% and the overall 98–99% agreement of the FPMM with method 1601 were measures of equivalence between the methods.

Fluorescence results. Table 6 shows the predictability of the same-day fluorescence warnings for both coliphage types. Fluorescence detected 73% of the samples positive for somatic coliphage, whereas the FPMM plaque process found 77% of the samples positive. Fluorescence detected 86% of the samples positive for male-specific coliphage, whereas the FPMM plaque process found 89% of the samples positive. In the table scoring, a true-positive fluorescence produced a positive plaque on the spot plate, a true-negative fluorescence produced a negative plaque on the spot plate, a false-negative did not fluoresce but produced a positive plaque on the spot plate, and a false-positive fluoresced but produced a negative plaque on the spot plate.

Somatic coliphage. Comparative table analysis showed an observed somatic agreement of the fluorescent result and the FPMM of 93%, with a false-negative rate of 7.5% and a false-positive rate of 7.4%. The false-negative rate of the fluorescence

TABLE 4 Precision study showing number of FPMM somatic coliphage-positive and -negative plaque results picked into TSB and recultured to USEPA method 1601 unfiltered (compared method) and filtered (reference method)

Cycle ID	FPMM Negative *n*	FPMM Positive *n*	Unfiltered Spot Re-picked to Method 1601 (Compared Method)—*n*				Filtered Spot Re-picked to Method 1601 (Reference Method)—*n*				Unfiltered Method 1601 Compared With Filtered Method 1601—*n*			
			TN	TP	FN	FP	TN	TP	FN	FP	TN	TP	FN	FP
S1-1	3	7	3	7	0	0	3	7	0	0	3	7	0	0
S1-2	0	10	0	10	0	0	0	10	0	0	0	10	0	0
S1-3	3	7	2	7	1	0	3	6	0	1	2	6	0	2
S1-4	4	6	4	6	0	0	3	6	1	0	3	6	1	0
S2-1	2	8	2	6	0	2	2	6	0	2	4	6	0	0
S2-2	2	8	0	8	2	0	0	8	2	0	0	10	0	0
S2-3	5	5	LA	LA	LA	LA	LA	LA	LA	LA	LA	LA	LA	LA
S2-4	2	8	2	8	0	0	2	8	0	0	2	8	0	0
S3-1	1	9	1	9	0	0	1	9	0	0	1	9	0	0
S3-2	0	10	0	10	0	0	0	10	0	0	0	10	0	0
S3-3	3	7	3	6	0	1	3	6	0	1	4	6	0	0
S3-4	3	7	2	7	1	0	2	7	1	0	2	8	0	0
Total	28	92	19	84	4	3	19	83	4	4	21	86	1	2
Sensitivity			95%				95%				97%			
Specificity			86%				83%				95%			
Efficiency*			94%				93%				97%			
PVP			97%				95%				99%			
PVN			83%				83%				91%			
FP rate			13.6%				17.4%				4.6%			
FN rate			4.6%				4.6%				2.3%			
Cohen's kappa (κ)			0.8045†				0.7801‡				0.9162†			
Observed agreement			0.94				0.93				0.97			
Positive agreement			0.96				0.95				0.98			
Negative agreement			0.84				0.83				0.93			

ID—identification, FN—false-negative (i.e., negative results recultured as positive), FP—false-positive (i.e., positive results recultured as negative), FPMM—modified method evaluated in this study, LA—laboratory accident (i.e., QC samples disqualified the selectivity portion of this cycle), *n*—number, PVN—predictive value of negative test, PVP—predictive value of positive test, QC—quality control, TN—true-negative (i.e., negative results recultured as negative), TP—true-positive (i.e., positive results recultured as positive), TSB—tryptic soy broth, USEPA—US Environmental Protection Agency

*Represents the correct classification rate
†Indicates almost perfect statistical agreement
‡Indicates substantial statistical agreement

Inter-rater calculations of sensitivity, specificity, overall agreement, and Cohen's kappa are based on summed TN, TP, FP, and FN in two-by-two contingency table comparability analysis.

prediction was higher than that of the FPMM complete with plaque confirmation relative to method 1601. This indicated an elevated risk of relying solely on a fluorescent negative result without the plate confirmation. The positive fluorescent results were highly correlative to a positive plate confirmation, with a predictive value of a positive result of 98%.

Male-specific coliphage. The male-specific fluorescent prediction showed 95% overall agreement compared with the FPMM plaque result, with a false-negative rate of 1.9% and a false-positive rate of 27%. The high calculated false-positive fluorescence level could have been influenced by the small number of negative results in this data set. However, the false-positive rate was higher than the false-positive rate of the completed FPMM with plaque confirmation relative to the USEPA methods and as such indicated the importance of performing plate confirmation. The male-specific fluorescent positive results were highly correlative to a positive plate confirmation, with a predictive value of a positive result of 96%.

DISCUSSION

In the comparative experiments, at least five positives of 10 replicates at the targeted plaque-forming units per 100 mL were detected in all somatic and male-specific test cycles. Therefore, the FPMM met the PBMS acceptance criteria described in method 1601, section 14.2, for national approval of method modifications (USEPA, 2001b). The comparative USEPA method met the extended matrix spike QC criteria specified in section 9 of method 1601 and met all QC and ODC criteria for accepting data. The

TABLE 5 Precision study showing number of FPMM male-specific (F+) coliphage-positive and -negative plaque results picked into TSB and recultured to USEPA method 1601 unfiltered (compared method) and filtered (reference method)

Cycle ID	FPMM Negative *n*	FPMM Positive *n*	Unfiltered Spot Re-picked to Method 1601 (Compared Method)—*n*				Filtered Spot Re-picked to Method 1601 (Reference Method)—*n*				Unfiltered Method 1601 Compared With Filtered Method 1601—*n*			
			TN	TP	FN	FP	TN	TP	FN	FP	FN	TP	FN	FP
F+1-1	0	10	0	10	0	0	0	10	0	0	0	10	0	0
F+1-2	0	10	0	10	0	0	0	10	0	0	0	10	0	0
F+1-3	2	8	1	8	1	0	0	8	2	0	0	9	1	0
F+1-4	1	9	1	9	0	0	1	9	0	0	1	9	0	0
F+2-1	0	10	0	10	0	0	0	10	0	0	0	10	0	0
F+2-2	0	10	0	10	0	0	0	10	0	0	0	10	0	0
F+2-3	0	10	0	10	0	0	0	10	0	0	0	10	0	0
F+2-4	1	9	1	9	0	0	1	9	0	0	1	9	0	0
F+3-1	1	9	1	9	0	0	1	9	0	0	1	9	0	0
F+3-2	0	10	0	10	0	0	0	10	0	0	0	10	0	0
F+3-3	4	6	4	6	0	0	3	6	1	0	3	6	1	0
F+3-4	4	6	4	6	0	0	4	6	0	0	4	6	0	0
Total	13	107	12	107	1	0	10	107	3	0	10	108	2	0
Sensitivity			99%				97%				98%			
Specificity			100%				100%				100%			
Efficiency*			99%				98%				98%			
PVP			100%				100%				100%			
PVN			92%				77%				83%			
FP rate			0%				0%				0%			
FN rate			0.9%				2.7%				1.8%			
Cohen's kappa (κ)			0.9554†				0.8560†				0.9000†			
Observed agreement			0.99				0.98				0.98			
Positive agreement			0.995				0.986				0.991			
Negative agreement			0.96				0.87				0.91			

ID—identification, FN—false-negative (i.e., negative results recultured as positive), FP—false-positive (i.e., positive results recultured as negative), FPMM—modified method evaluated in this study, LA—laboratory accident (i.e., QC samples disqualified the selectivity portion of this cycle), *n*—number, PVN—predictive value of negative test, PVP—predictive value of positive test, QC—quality control, TN—true-negative (i.e., negative results recultured as negative), TP—true-positive (i.e., positive results recultured as positive), TSB—tryptic soy broth, USEPA—US Environmental Protection Agency

*Represents the correct classification rate
†Indicates almost perfect statistical agreement

Inter-rater calculations of sensitivity, specificity, overall agreement, and Cohen's kappa are based on summed TN, TP, FP, and FN in two-by-two contingency table comparability analysis.

PBMS criteria are based on the target spike level and the lower limit of qualitative positive results required to demonstrate that variability in modified results does not exceed the original collaborative study results of method 1601 (USEPA, 2003). In that study the average positive rate ranged from 60 to 90% with average spike levels of 1.0–1.4 pfu/100 mL, which were consistent with the spike levels and positive rates obtained by the FPMM and method 1601 in the current study.

Performing the actual reference method to generate comparative data is not part of PBMS criteria as described in USEPA method 1601 but was part of ATP protocol required by the agency as part of the current study design. The ATP protocol considers the two comparative methods as unrelated. The current study design is a hybrid of two study designs—PBMS and ATP—and therefore 120 comparative somatic and male-specific method analyses using split samples were performed. The modified methods (FPMM somatic and male-specific) did not show statistically significant differences from the reference method 1601, using any of the study design criteria, PBMS, or ATP comparability and precision. When ATP design comparability criteria were used, none of the modified method results for the 24 cycles differed significantly from the method 1601 results using Fisher's exact tests. Taken as a single degree of freedom with CMH chi-square analysis, the differences between comparison cycles of the two methods were not significant, although the modified methods exhibited a trend to yield fewer positives. This lower positive rate may be related to the shorter enrichment time used in the FPMM. However, given that the shorter modified incubation did not yield significantly more false-negative results, it does not appear to be detrimental to coliphage detection.

TABLE 6 Fluorescence results for somatic and male-specific (F+) FPMM at 8 h compared with FPMM plaque (FPMM negative and positive) results after pick and overnight confirmation of plaque

Somatic Cycle ID	FPMM Negative *n*	FPMM Positive *n*	Fluorescence Compared With Somatic FPMM—*n*				Male-specific Cycle ID	FPMM Negative *n*	FPMM Positive *n*	Fluorescence Compared With Male-specific FPMM—*n*			
			TN	TP	FN	FP				TN	TP	FN	FP
S1-1	3	7	3	7	0	0	F+1-1	0	10	0	9	1	0
S1-2	0	10	0	10	0	0	F+1-2	0	10	0	10	0	0
S1-3	3	7	3	5	2	0	F+1-3	2	8	2	7	1	0
S1-4	4	6	4	6	0	0	F+1-4	1	9	1	9	0	0
S2-1	2	8	2	8	0	0	F+2-1	0	10	0	8	2	0
S2-2	2	8	2	8	0	0	F+2-2	0	10	0	10	0	0
S2-3	5	5	4	5	1	0	F+2-3	0	10	0	10	0	0
S2-4	2	8	2	8	0	0	F+2-4	1	9	1	9	0	0
S3-1	1	9	1	9	0	0	F+3-1	1	9	1	9	0	0
S3-2	0	10	0	8	2	0	F+3-2	0	10	0	10	0	0
S3-3	3	7	2	6	1	1	F+3-3	4	6	2	6	0	2
S3-4	3	7	2	6	1	1	F+3-4	4	6	4	6	0	0
Total	28	92	25	86	7	2		13	107	11	103	4	2
Sensitivity			92%				Sensitivity			98%			
Specificity			93%				Specificity			73%			
Efficiency*			93%				Efficiency*			95%			
PVP			98%				PVP			96%			
PVN			78%				PVN			85%			
FP rate			7.4%				FP rate			26.7%			
FN rate			7.5%				FN rate			1.9%			
Cohen's kappa (κ)			0.7982†				Cohen's kappa (κ)			0.7576†			
Observed agreement			0.93				Observed agreement			0.95			

ID—identification, FN—false-negative (i.e., negative results recultured as positive), FP—false-positive (i.e., positive results recultured as negative), FPMM—modified method evaluated in this study, n—number, PVN—predictive value of negative test, PVP—predictive value of positive test, TN—true-negative (i.e., negative results recultured as negative), TP—true-positive (i.e., positive results recultured as positive)

*Represents the correct classification rate
†Indicates almost perfect statistical agreement

Inter-rater calculations of sensitivity, specificity, overall agreement, and Cohen's kappa are based on summed TN, TP, FP, and FN in two-by-two contingency table comparability analysis..

According to the study plan, the precision portion of the ATP design was to determine if there were statistical disadvantages associated with the alternative method when there was an observed difference in the comparative study. Although statistical differences were not observed in either comparability study, the lower *p* value might be considered a trend. Therefore, the precision analysis could provide useful information regarding that trend. The inter-rater statistical precision analyses of Tables 4 and 5 indicated fewer than 5% false-negatives by both the somatic and male-specific studies and overall 93–99% agreement with the compared and reference USEPA methods and FPMM. These results were consistent with those of the published tier 1 study (Salter et al, 2010).

CONCLUSION

The FPMM's shorter incubation time has the effect of cutting by half the time required for the method 1601 coliphage assay. The modified method is a qualitative result for a 100-mL sample. The time of assay and sample volume make the modification similar in time and volume to the quantitative method 1602 without the need to preculture host bacteria or use a large number of petri plates. Concentration of larger sample volumes > 100 mL may be needed to reach detection of a low-level viral contamination. The fluorescence prediction aspect with better than 90% correlation to end result has the added benefit of providing a rapid, same-day response.

The FPMM simplifies USEPA method 1601 with pre-prepared reagents and shortens the time to result to less than 24 h, resulting in savings in time and preparation that make coliphage testing less labor-intensive and easier to perform. If nationally approved, the modification offers the water industry additional low-cost options for GWR compliance.

By providing rapid, same-day response, the FPMM aids in early identification of contaminated samples needing plate confirmation and could facilitate early action and water remediation in water emergencies such as floods and distribution system breaks. This type of sensitive rapid fecal monitor and early warning has

not been technically achieved with other conventional microbiological assays for fecal indicators in drinking water.

From a public health standpoint, the viral coliphage indicator is complementary to bacterial indicators in determining fecal contamination of water. In cases such as disinfectant-treated waters or recharged water supplies, coliphage indicators may offer advantages such as greater chemical resistance and further source diffusion distances for viral pathogen threats. With the availability of easier and lower-cost methods for performing coliphage testing, the water industry is more likely to perform a variety of fecal indicator tests when determining the health and safety of groundwater in compliance with the GWR.

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PEER REVIEW

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FOOTNOTES

[1]Fast Phage™, Charm Sciences, Lawrence, Mass.
[2]Pall-Acrodisc® 0.45-µm filter with HT Tuffryn® low protein-binding 25-mm membrane, Pall, Port Washington, N.Y.

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