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U.S. Department of the Interior
U.S. Geological Survey

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Conversion Factors and Abbreviations

Inch/Pound to SI

Multiply	By	To obtain
	Length	
meter (m)	3.281	foot (ft)
millimeter (mm)	0.03937008	inch (in)
micrometer (µm)	0.00003937	foot (ft)
	Volume	
liter (L)	33.8140227	ounce, fluid (fl. oz)
milliliter (mL)	0.03381	ounce, fluid (fl. oz)
microliter (µL)	0.00003381	ounce, fluid (fl. oz)
	Mass	
gram (g)	0.03527	ounce, avoirdupois (oz)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:

°F=(1.8×°C)+32

Abbreviations

ANOVA	analysis of variance
ARPD	absolute relative percent difference
BSA	Bovine serum albumen
Ct	cycle threshold
CTAB	hexadecyltrimethylammonium bromide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
MST	microbial source tracking
NTC	sno-template controls
qPCR	quantitative polymerase chain reaction
USGS	U.S. Geological Survey
WVDA	West Virginia Department of Agriculture

Interlaboratory Comparison of Results for Three Microbial Source Tracking Quantitative Polymerase Chain Reaction (qPCR) Assays from Fecal-Source and Environmental Samples

By Erin A. Stelzer[1], Kriston M. Strickler[2], and William B. Schill[3]

Abstract

During summer and early fall 2010, 15 river samples and 6 fecal-source samples were collected in West Virginia. These samples were analyzed by three laboratories for three microbial source tracking (MST) markers: AllBac, a general fecal indicator; BacHum, a human-associated fecal indicator; and BoBac, a ruminant-associated fecal indicator. MST markers were analyzed by means of the quantitative polymerase chain reaction (qPCR) method. The aim was to assess interlaboratory precision when the three laboratories used the same MST marker and shared deoxyribonucleic acid (DNA) extracts of the samples, but different equipment, reagents, and analyst experience levels. The term assay refers to both the markers and the procedure differences listed above. Interlaboratory precision was best for all three MST assays when using the geometric mean absolute relative percent difference (ARPD) and Friedman's statistical test as a measure of interlaboratory precision. Adjustment factors (one for each MST assay) were calculated using results from fecal-source samples analyzed by all three laboratories and applied retrospectively to sample concentrations to account for differences in qPCR results among labs using different standards and procedures. Following the application of adjustment factors to qPCR results, ARPDs were lower; however, statistically significant differences between labs were still observed for the BacHum and BoBac assays. This was a small study and two of the MST assays had 52 percent of samples with concentrations at or below the limit of accurate quantification; hence, more testing could be done to determine if the adjustment factors would work better if the majority of sample concentrations were above the quantification limit.

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Introduction

Microbial source tracking (MST) tools are currently (2012) being used as a means to differentiate fecal contamination sources (U.S. Environmental Protection Agency, 2005). MST tools are divided according to two basic approaches: library-dependent methods, which rely upon large collections of known-source fecal-indicator bacteria; and library-independent methods, which utilize host-associated genetic markers. The library-dependent methods are applicable to an unlimited range of hosts, but are prone to false-positive results (Griffith and others, 2003; Stoeckel and others, 2004). Library-independent methods are limited in the range of hosts, but are less prone to false-positive results than library-dependent methods (Griffith and others, 2003).

A commonly used group of library-independent MST markers relies on the detection of 16S rRNA-based genetic markers from the fecal anaerobes of the order *Bacteroidales*. These obligate anaerobes make up about one-third of the human fecal flora, far outnumbering coliforms (Holdeman and others, 1976; Salyers, 1984). *Bacteroidales'* host-associated markers have been used in several studies to detect human and ruminant fecal contamination (Boehm and others, 2003; Bower and others, 2005; Shanks and others, 2006; Seurinck and others, 2005; Kildare and others, 2007).

An emerging library-independent MST method is quantitative polymerase chain reaction (qPCR). This technique cannot only be used to detect, but also to estimate the amount of MST marker present in samples. Many systems capable of running qPCR are now commercially available, and there is concern about assuming comparability of results generated by different laboratories using protocols without common equipment, reagents, and standards.

The U.S. Geological Survey (USGS), in cooperation with West Virginia Department of Agriculture (WVDA), assessed interlaboratory precision in MST assay concentrations when the same MST markers were applied to shared samples; however, there were differences in the equipment, reagents,

and standards between the analytical laboratories. Due to the lack of standardized protocols and quality control, results from different laboratories may vary and at this time there is little guidance on how to determine the true MST marker concentration by qPCR. Twenty-one samples were analyzed by three laboratories using three MST assays. All analyses by the different laboratories were performed on deoxyribonucleic acid (DNA) extracts from the same set of samples to eliminate variability associated with samples, sample handling, and DNA extraction. Each laboratory/analyst had a different level of qPCR experience: one was a novice, another had approximately 5 years of experience, and another had more than 10 years of experience. Understanding the interlaboratory precision when using different equipment, reagents, and experience levels would help MST researchers to compare data from multiple laboratories within one study or even compare similar MST data among multiple studies, which can be rather complicated.

Methods of Study

Analytical Laboratories

Three laboratories participated in this study: a West Virginia state laboratory and two Federal laboratories. A single analyst at each of the laboratories handled all of their laboratories' analytical work for this study.

Sample Collection

During September 2010, personnel from WVDA and the USGS in Charleston, West Virginia, collected seven river samples and six fecal-source samples in south-eastern West Virginia. All river samples were collected approximately 0.3 m below the water surface into sterile bottles using a grab-sampling technique (Myers and others, 2007). Fecal-source samples from chickens, cows, influent, and two effluent samples from a wastewater-treatment plant and a discharge pipe into a river were collected using sterile bottles or bags. Fecal-source samples were taken from farms or wastewater-treatment plants in the area, and chicken and cow feces were composited by species. Influent was collected by grab-sampling from the settling pond, and effluent was collected by grab-sampling from the wastewater-treatment plant.

The USGS in Leetown, West Virginia, provided eight additional river samples that were collected as part of another study during summer 2010. These samples were from sites in the Shenandoah Valley and were selected to be representative of surface-water samples that were thought to contain fecal contaminants from cattle, chicken, and human sources. These samples were collected in sterile bottles (as previously mentioned) and kept frozen until they were filtered and extracted.

Filtration and DNA Extraction

River and fecal-source samples collected in September 2010 were filtered and extracted by WVDA within 48 hours of collection. The chicken and cow source samples were made into slurries by adding 1 g of feces to 99 mL of sterile-phosphate buffer. All samples were concentrated by use of 47-mm diameter, 0.4-µm pore size polycarbonate filters (Whatman, Florham Park, New Jersey). After filtration, each filter was aseptically folded and placed into a 2.0-mL screw-cap tube containing 0.3 g of acid-washed glass beads (Sigma, St. Louis, Missouri). Samples were extracted using a DNA-EZ extraction kit (GeneRite, North Brunswick, N.J.) according to manufacturer's instructions. The final extract volume for each sample was 200 µL, which was split into three tubes. One tube was kept by the laboratory that filtered and extracted and the other two tubes were shipped on dry ice to the other laboratories. All DNA extracts were stored at −20 or −70°C until qPCR analysis.

Samples of river water (1 L) provided by USGS in Leetown, were filtered and extracted using a hexadecyltrimethylammonium bromide (CTAB) method (Karl and Bailiff, 1989; Ishii and others, 1998). Crude DNA was further purified using a Clean and Concentrator™ – 5 Kit (Zymo Research, Irvine, California). Purified DNA was eluted in 500 µL of IDTE, pH 8.0 (Integrated DNA Technologies, Inc., Coralville, Iowa) and stored frozen at −70°C until distribution to the other laboratories.

Interlaboratory Differences

The aim of this study was to do an interlaboratory comparison of results from MST assays. Sample processing up to qPCR was constant since samples were filtered and extracted by one laboratory and then distributed to the other laboratories. All laboratories had different analysts and used different qPCR systems and reagents. One laboratory used different qPCR reaction volumes and different DNA standard material; however, with accurate quantification these differences should not affect precision. Table 1 contains the differences that could influence the interlaboratory precision in this study.

qPCR Analyses

In all three laboratories, DNA extracts were analyzed for the same three MST markers using primer and probe sets as well as run conditions from previously published protocols: in each sample the AllBac marker (Layton and others, 2006) was used to estimate total *Bacteroidales*, the BacHum marker (Kildare and others, 2007) was used to measure human-associated *Bacteroidales*, and the BoBac marker (Layton and others, 2006) was used to measure ruminant-associated *Bacteroidales*. The term MST assay, when used in this report, refers to both

the standardized marker listed above and the differences in protocol from each laboratory (table 1). In laboratory 1, all qPCR analyses were done using 5 µL of DNA extract and 20 µL of master mix containing Quantitect Probe PCR kit (Qiagen, Valencia, Calif.) and Bovine serum albumen (BSA) (Invitrogen, Carlsbad, Calif.) in Smartcycler or Smartcycler II (Cepheid, Sunnyvale, Calif.) thermocyclers. In laboratory 2, all qPCR analyses were done using 5 µL of DNA extract and 20 µL of master mix containing 2x Universal TaqMan Master Mix (Applied Biosystems, Foster City, Calif.) and BSA (Invitrogen, Carlsbad, Calif.) in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, Calif.) thermocycler. In laboratory 3, all qPCR analyses were done using 2 µL of DNA extract and 10 µL of master mix containing QuantiFast Multiplex PCR + R kit (Qiagen, Valencia, Calif.) and BSA (Sigma, St. Louis, Mo.) in a Corbett Rotor-Gene 3000 (Qiagen, Valencia, Calif.) thermocycler. See table 1 for all the differences that could influence the interlaboratory precision in this study.

After completion of the qPCR analyses, some qPCR systems offer the ability for the researcher to manually designate a cycle threshold (Ct) value to determine at what point in the exponential phase the samples will be measured and the start of the baseline (the initial cycles of PCR during which there is little change in fluorescence signal) (Kephart and Bushon, 2009). All three laboratories used an automatic baseline determined by the individual system and, if allowed, manually set the threshold at a value they determined to be the best fit in the exponential phase, which then was used for all qPCR runs of the same assay in that laboratory. Sample concentrations were calculated as assay copy number per 100 mL using the standard curves from each laboratory:

$$Conc = (\text{sample } Ct - y\text{-intercept})/\text{slope } x \text{ (extraction elution volume/volume added to qPCR reaction) } x \text{ (100 mL/volume filtered).} \quad (1)$$

Standard Curves and DNA Standards

A standard curve is generated by plotting cycle threshold values against known quantities of DNA from serially-diluted standards. Results from this set of standard reactions then are used to determine a best-fit regression line. DNA concentrations of environmental and fecal-source samples are calculated using this standard curve regression line (Applied Biosystems, 2010).

Laboratories 1 and 2 used plasmid DNA standards created at laboratory 2 to develop their standard curves. A plasmid is a DNA molecule contained in some type of bacteria that is separate from, and can replicate independently of, chromosomal DNA. Plasmids promote gene transfer, and therefore, easily incorporate and retain non-native genes. A positive control for each MST assay was amplified by PCR and cloned into a PCR-TOPO plasmid (Invitrogen, Carlsbad, Calif.). The plasmid was transformed into *Escherichia coli (E. coli)* TOP10 cells, and recombinant bacteria were selected on

Table 1. Equipment and analytical differences among laboratories.

[qPCR, quantitative polymerase chain reaction; DNA, deoxyribonucleic acid]

Category	Differences by laboratory		
	Laboratory 1	Laboratory 2	Laboratory 3
Thermocycler	Cephied Smartcycler and Smartcycler II	Applied Biosystems 7500 Real-Time PCR System	Corbett Rotor-Gene 3000
qPCR reagents	Quantitect Probe PCR kit	2x Universal TaqMan Master Mix	QuantiFast Multiplex PCR + R kit
qPCR reaction volumes	5 microliters	5 microliters	2 microliters
DNA standards	Plasmids created by laboratory 2	Plasmids created by laboratory 2	Commercially synthesized standards
Analyst experience levels	Novice	5 years	10+ years

ampicillin-containing LB agar. Real-time TaqMan PCR was used to screen colonies for presence of the target insert, and isolated colonies were selected for overnight liquid-culture propagation. Plasmids were extracted from the transformed *E. coli* using UltraClean 6 Minute Mini Plasmid Prep kit (MoBio, Carlsbad, Calif.), and inserts were sequenced to confirm identity. Plasmids were quantified using the PicoGreen™ assay (Molecular Probes, Inc., Eugene, Oregon) and were serially diluted (10-fold dilutions) to generate standard curves.

Laboratory 3 purchased synthesized standards for each MST assay from Integrated DNA Technologies (Coralville, Ia.). Synthesized standards are commercially produced by artificially adhering a prescribed sequence of bases together to produce the target gene sequence. After quantification by laboratory 3, these synthesized standards were serially diluted 100-fold to generate standard curves. A single standard curve was analyzed with each batch of test samples in all three laboratories.

Quality Assurance/Quality Control

Quality-control samples for qPCR included no-template controls (NTCs) and qPCR positive controls. The NTCs were processed once per qPCR batch by using sterile molecular-grade water in place of DNA template. A standard curve was added to every qPCR batch as a qPCR positive control.

Matrix inhibition of the environmental and fecal-source samples was measured by use of the SPUD assay by laboratory 1 before laboratories 2 and 3 analyzed the samples (Nolan and others, 2006). Ct values for all samples were within two cycles of the expected value for the positive control, and thus, were not considered to be matrix inhibited.

Data Analysis

Precision refers to the ability of a measurement to be consistently reproduced. If a sample of water is analyzed two times, measurements that are close together in magnitude are said to have "good precision" (Kinzelman and others, 2011). The measurement of precision used in this report is the absolute relative percent difference (ARPD), determined as follows:

$$ARPD = ABS(X1-X2)/Xavg \times 100 \quad (2)$$

where

X1 is concentration observed with the first replicate,

X2 is concentration observed with the second replicate, and

X_{avg} is average concentration = *((X1 + X2) / 2)*.

When interpreting these results, the lower the relative percent difference, the greater the precision. The ARPD values were calculated using copy number per 100 mL. Geometric means of ARPDs for a given assay were calculated to facilitate a qualitative comparison across laboratories.

Three different statistical tests were performed in order to have a more quantitative comparison of laboratories. Friedman's test (Friedman, 1940) was used to test the null hypothesis that the median concentrations from the laboratories for a given assay were equal, versus the alternative hypothesis that at least one of the medians is different. Concentrations from a given assay were ranked within blocks corresponding to the 21 unique samples, and each laboratory was considered a separate treatment within the block. An analysis of variance (ANOVA) test was run on the ranked laboratory data along with posthoc Tukey's Studentized Range multiple comparison tests (when ANOVA indicated significant differences) to facilitate pair wise comparisons of median concentrations among laboratories. All tests of significance were based on an alpha level of 0.05.

It is common practice for researchers to use standardized controls that are analyzed by all laboratories participating in an interlaboratory comparison of methods (Vermeulen and others, 2009). Although not ideal, this practice may enable adjustment factors to be calculated and retrospectively applied to a laboratory's results if they significantly differ from the results of another laboratory. In this study, the fecal-source samples were used as standardized controls. Ratio adjustment factors were computed for laboratory 3 for each MST assay. The adjustment factors (*F*) were computed (equation 3) and multiplied by the original results from laboratory 3 to account for its differences in method and controls.

$$F = \frac{\sum_{n}^{i=1} \frac{X1_i}{X3_i} + \sum_{m}^{j=1} \frac{X2_j}{X3_j}}{m+n} \quad (3)$$

where

$X1_i$ is concentration for fecal source *i* by laboratory 1,

$X2_i$ is concentration for fecal source *i* by laboratory 2,

$X3_i$ is concentration for fecal source *i* by laboratory 3,

and *n* and *m* are the numbers of fecal-source samples analyzed by laboratories 1 and 2, respectively.

Interlaboratory Comparison of Standard Curves

Standard-curve performance characteristics data from all three laboratories are provided in table 2. Guidelines for interpreting standard-curve data are available in the Applied Biosystems StepOne and StepOnePlus Real-Time PCR Systems Reagent Guide (Applied Biosystems, 2010). The amplification efficiency of the qPCR should be 90–110 percent; an efficiency of 100 percent means an exact doubling of the target DNA sequence at each cycle. This corresponds to a slope of about −3.1 to −3.6. In this study, the range of amplification efficiencies for all laboratories and all MST assays was 89 to 116 percent. Slopes ranged from −2.992 to −3.630. The dynamic range describes the lowest and highest standards analyzed by each laboratory in copies per qPCR reaction. Laboratories 1 and 2 have similar dynamic ranges since they shared the same lot of plasmid standards. The y-intercept is the Ct value where the standard curve crosses the y-axis at 1 copy of the target sequence DNA. The y-intercepts in this study ranged from 34.58 to 44.74. The correlation coefficient (R^2) is used to assess the fit of the standard curve to the plotted data points. The closer the R^2 value is to 1, the better the fit. In this study, the R^2 values ranged from 0.976 to 1.000.

Interlaboratory Comparison of Results from Microbial Source Tracking Assays

Replicates of the 21 DNA extracts for the same three MST qPCR targets were analyzed by three laboratories. DNA concentrations for each sample, by laboratory and assay, are provided in table 3. One or more of the differences in protocol from the three laboratories likely caused laboratory 3 to report much higher sample concentrations than did laboratories 1 and 2. In order to bring sample concentrations for laboratory 3 more in line with sample concentrations of laboratories 1 and 2, adjustment factors were calculated and applied as described in the *Data Analysis* section of this report. The mean adjustment factors for AllBac, BacHum and BoBac were determined to be 0.42, 0.38, and 0.54, respectively. The adjustment factors and original and adjusted sample concentrations for laboratory 3 are provided in table 3.

Table 2. Performance characteristics of quantitative polymerase chain reaction (qPCR) standard curves for each laboratory and microbial source tracking (MST) assay.

[R^2, correlation coefficient; AllBac, general fecal indicator; BacHum, human-associated fecal indicator; BoBac, ruminant-associated fecal indicator]

Assay	Laboratory	Slope	y-intercept	R^2	Dynamic range (copies/qPCR reaction)	Amplification efficiency (in percent)
AllBac	1	-3.630	42.85	0.991	46.6 - 4.66E+07	89
AllBac	2	-3.584	38.44	0.999	46.6 - 4.66E+07	90
AllBac	3	-2.992	34.58	0.996	120 - 1.20E+10	116
BacHum	1	-3.477	39.93	0.999	45.1 - 4.51E+07	94
BacHum	2	-3.527	38.72	0.999	22.3 - 2.23E+06	92
BacHum	3	-3.230	43.59	0.999	120 - 1.20E+10	104
BoBac	1	-3.336	44.74	0.976	46.3 - 4.63E+07	99
BoBac	2	-3.540	42.59	1.000	36.2 - 3.62E+06	92
BoBac	3	-3.315	38.01	0.999	120 - 1.20E+10	100

Table 3. Sample concentrations for all three microbial-source tracking (MST) assays from each laboratory.

[mL, milliliter; AllBac, general fecal indicator; BacHum, human-associated fecal indicator; BoBac, ruminant-associated fecal indicator; <, less than; --, sample not analyzed; bold values indicate concentration is at or below the limit for acurate quantification]

Sample name	Volume filtered (mL)	Extraction elution volume (mL)	AllBac copies/ 100 mL				
			Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 3 adjusted	Computed adjustment factors
River 1	450	0.2	**44**	**45**	120	**50**	
River 2	500	0.2	**42**	**40**	99	**41**	
River 3	100	0.2	180	190	480	200	
River 4	100	0.2	200	210	480	200	
River 5	100	0.2	190	180	470	196	
River 6	100	0.2	200	190	390	162	
River 7	100	0.2	190	190	410	171	
River 8	1,000	0.5	48	49	120	**50**	
River 9	1,000	0.5	54	54	130	54	
River 10	1,000	0.5	**33**	**32**	**70**	**29**	
River 11	1,000	0.5	57	56	140	58	
River 12	1,000	0.5	55	53	130	54	
River 13	1,000	0.5	**44**	**43**	**98**	**41**	
River 14	1,000	0.5	53	51	120	50	
River 15	1,000	0.5	60	59	140	58	
Source 1	150	0.2	127	120	320	133	0.39
Source 2	10	0.2	3,000	3,000	7,200	2,996	0.42
Source 3	50	0.2	620	620	1,500	624	0.41
Source 4	20	0.2	1,300	1,200	2,900	1,207	0.43
Source 5	100	0.2	170	170	450	187	0.38
Source 6	100	0.2	170	170	360	150	0.47

Table 3. Sample concentrations for all three microbial-source tracking (MST) assays from each laboratory.—Continued

[mL, milliliter; AllBac, general fecal indicator; BacHum, human-associated fecal indicator; BoBac, ruminant-associated fecal indicator; <, less than; --, sample not analyzed; bold values indicate concentration is at or below the limit for acurate quantification]

BacHum copies/100 mL					BoBac copies/100 mL				
Laboratory 1	**Laboratory 2**	**Laboratory 3**	**Laboratory 3 adjusted**	**Computed adjustment factors**	**Laboratory 1**	**Laboratory 2**	**Laboratory 3**	**Laboratory 3 adjusted**	**Computed adjustment factors**
9	**10**	**25**	**10**		**17**	**21**	**43**	**23**	
22	24	**52**	**20**		**20**	**22**	**36**	**19**	
34	**14**	**110**	**42**		120	140	270	146	
79	68	150	58		110	110	220	119	
78	**14**	**110**	**42**		93	120	230	124	
100	120	250	96		74	87	**110**	**59**	
110	120	240	92		67	93	140	76	
10	**9**	**15**	**6**		**27**	**33**	**57**	**31**	
36	39	**88**	**34**		**35**	38	**69**	**37**	
10	**4**	**18**	**7**		**16**	**26**	**43**	**23**	
25	28	**59**	**23**		**45**	50	**110**	**59**	
<1	**12**	**13**	**5**		**44**	47	**100**	**54**	
12	**17**	**41**	**16**		**31**	**36**	**68**	**37**	
10	**6**	**16**	**6**		**43**	46	**96**	**52**	
24	27	**61**	**23**		51	56	120	65	
44	35	**74**	**29**	0.53	**39**	45	**78**	**42**	0.54
8	140	480	185	0.15	2,700	--	6,400	3,454	0.42
490	530	1,200	462	0.43	480	490	930	502	0.52
1,000	1,000	2,400	925	0.42	810	780	1,100	594	0.72
33	39	**110**	**42**	0.33	57	39	**64**	**35**	0.75
100	100	220	85	0.45	70	74	320	173	0.23

Prior to adjustments, the geometric means of ARPDs for all three laboratories, by MST assay, were 23.1, 48.9, and 35.2 for AllBac, BacHum, and BoBac, respectively (table 4). Friedman's test indicated that at least one of the unadjusted laboratory concentrations was statistically different from the others for all three assays (table 5). The geometric means of ARPDs were substantially lower at 2.9, 19.4, and 11.2 for AllBac, BacHum, and BoBac, respectively (table 4), after adjustment of results for laboratory 3. Friedman's test on the adjusted concentrations did not indicate statistically significant differences among laboratories for the AllBac assay results; however, statistically significant differences still were indicated for both the BacHum and BoBac assays (table 5).

The differences in protocol used in laboratory 3 may have resulted in low precision of sample concentrations compared to laboratories 1 and 2. Adjustment factors applied to sample concentrations for laboratory 3 helped to heighten the precision across all three MST assays; however, BacHum and BoBac still showed statistically significant differences among the three laboratories. One possible reason why BacHum and BoBac still showed statistically significant differences

Table 4. Geometric means of absolute relative precent differences (ARPDs) for all samples and laboratories for each microbial source tracking (MST) assay before and after applying adjustment factors to sample concentrations for laboratory 3.

[n, number of values included in the ARPD calculation; AllBac, general fecal indicator; BacHum, human-associated fecal indicator; BoBac, ruminant-associated fecal indicator]

Assay	n	Geometric mean ARPD	
		Unadjusted	Adjusted
AllBac	21	23.1	2.9
BacHum	21	48.9	19.4
BoBac	20	35.2	11.2

Table 5. Friedman's and Tukey's studentized range multiple comparison statistical results for each microbial source tracking (MST) assay before and after applying adjustment factors to sample concentrations for laboratory 3.

[AllBac, general fecal indicator; <, less than; ND, not done; BacHum, human-associated fecal indicator; BoBac, ruminant-associated fecal indicator; a p value ≤ 0.05 is considered statistically significantly different; Tukey studentized range grouping are presented as letters, and laboratories with at least one letter in common do not difffer significantly]

Assay	Laboratory	Friedman test p value	Tukey studentized range grouping
Unadjusted			
AllBac	1		
	2	<0.0001	
	3		
BacHum	1		
	2	<0.0001	ND
	3		
BoBac	1		
	2	<0.0001	
	3		
Adjusted			
AllBac	1		
	2	0.3041	ND
	3		
BacHum	1		A B
	2	0.0309	A
	3		B
BoBac	1		A
	2	0.0012	A B
	3		B

could be that 52 percent of the concentrations for these two assays were at or below the limit of accurate quantification and very near or at the detection limit of the assays, making them slightly more uncertain than concentrations for AllBac, which were almost all above the limit of quantification and well above the detection limit. All concentrations at or below the limit of accurate quantification are indicated in bold type in table 3.

In the interlaboratory comparison, laboratories 1 and 2 had the lowest geometric means of ARPDs for all three MST assays when using the unadjusted concentrations (table 6). After adjustments, the geometric means of ARPDs were lowest for laboratories 1 and 2 for AllBac (1.9), for laboratories 1 and 3 for BacHum (19.0), and for laboratories 2 and 3 for BoBac (8.0) (table 6). These results could be an artifact of how the adjustment factors were computed; therefore, more statistical tests were run. Tukey's Studentized Range test was run as a posthoc test if the ANOVA test indicated a significant difference. Tukey's Studentized Range test ranked laboratories against each other in terms of statistical similarities based on their sample concentrations. These tests were run only on adjusted concentrations from laboratory 3 to further explore the nature of the differences indicated in Friedman's tests using the adjusted concentrations for laboratory 3. Differences among laboratory results for AllBac were not statistically significant. Results for BacHum indicated that laboratories 2 and 3 had statistically significant differences, whereas results for BoBac indicated that laboratories 1 and 3 had statistically significant differences (table 5).

In both cases of statistically significant differences after adjustment (BoBac and BacHum assays), laboratory 3 was one of the laboratories singled out. This may be because many of the sample concentrations for these two assays were below the lowest point on the standard curve, and therefore, not as accurately calculated.

Summary and Conclusion

Microbial source tracking (MST) tools are commonly being used to help differentiate sources of fecal contamination in environmental waters. Currently (2012), there is a wide array of commercially available quantitative polymerase chain reaction (qPCR) systems and reagents, which has led to a concern about comparability of data generated by different laboratories using these different equipment and reagents. The U.S. Geological Survey, in cooperation with the West Virginia Department of Agriculture, assessed interlaboratory variability in MST assay concentrations when using the same MST markers and shared samples. Three laboratories shared deoxyribonucleic acid (DNA) extracts from 15 river and 6 fecal-source samples and ran each sample with three MST assays to determine if qPCR concentrations are interchangeable among laboratories. Each laboratory used different qPCR systems and reagents and one laboratory used different standards and qPCR reaction volumes. Results indicated that geometric mean ARPDs showing qPCR precision for laboratory 3 were all at or above 58.3, whereas the geometric mean ARPDs for just laboratories 1 and 2 were all at or below 19.2. Although not ideal, through the use of control samples that were analyzed by all participating laboratories, adjustment factors were applied to sample results for laboratory 3, which brought the geometric mean ARPDs down to at or below 20.1. Technically, the use of different standards (if accurately quantified) and different qPCR reaction volumes should not have caused the large precision issue that was discovered in this study. As previously stated in this report, the use of the adjustment factors was not ideal, but needed in order to compare data from all the laboratories for this report. Therefore, additional study is needed to test interlaboratory variability with only one variable changing at a time and with all samples above the assay limit of quantification.

Table 6. Geometric means of absolute relative percent differences (ARPDs) for all samples for individual laboratories for each microbial source tracking (MST) assay before and after applying adjustment factors to sample concentrations for laboratory 3.

[n, number of values included in the ARPD calculation; AllBac, general fecal indicator; BacHum, human-associated fecal indicator; BoBac, ruminant-associated fecal indicator]

Assay	Laboratories 1 and 2		Laboratories 1 and 3		Laboratories 2 and 3	
	n	Geometric mean ARPD	n	Geometric mean ARPD	n	Geometric mean ARPD
			Unadjusted			
AllBac	21	1.9	21	80.3	21	81.0
BacHum	21	19.2	21	78.5	21	77.7
BoBac	20	11.5	21	65.8	20	58.3
			Adjusted			
AllBac	21	1.9	21	4.7	21	2.4
BacHum	21	19.2	21	19.0	21	20.1
BoBac	20	11.5	21	15.5	20	8.0

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