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Accuracy and precision of *Legionella* isolation by US laboratories in the ELITE program pilot study

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ABSTRACT

A pilot study for the Environmental *Legionella* Isolation Techniques Evaluation (ELITE) Program, a proficiency testing scheme for US laboratories that culture *Legionella* from environmental samples, was conducted September 1, 2008 through March 31, 2009. Participants ($n = 20$) processed panels consisting of six sample types: pure and mixed positive, pure and mixed negative, pure and mixed variable. The majority (93%) of all samples ($n = 286$) were correctly characterized, with 88.5% of samples positive for *Legionella* and 100% of negative samples identified correctly. Variable samples were incorrectly identified as negative in 36.9% of reports. For all samples reported positive ($n = 128$), participants underestimated the cfu/ml by a mean of 1.25 logs with standard deviation of 0.78 logs, standard error of 0.07 logs, and a range of 3.57 logs compared to the CDC re-test value. Centering results around the interlaboratory mean yielded a standard deviation of 0.65 logs, standard error of 0.06 logs, and a range of 3.22 logs. Sampling protocol, treatment regimen, culture procedure, and laboratory experience did not significantly affect the accuracy or precision of reported concentrations. Qualitative and quantitative results from the ELITE pilot study were similar to reports from a corresponding proficiency testing scheme available in the European Union, indicating these results are probably valid for most environmental laboratories worldwide. The large enumeration error observed suggests that the need for remediation of a water system should not be determined solely by the concentration of *Legionella* observed in a sample since that value is likely to underestimate the true level of contamination.

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1. Introduction

Legionellaceae are ubiquitous in moist environments and a frequent contaminant of building water systems (Fields et al., 2002). Inhalation of aerosolized water contaminated with legionellae by susceptible individuals results in legionellosis that may present as the acute pneumonia, Legionnaires' disease, or the less severe Pontiac Fever. *Legionella pneumophila* is the most common etiological agent, however all species of legionellae are presumed to be capable of causing disease (Alli et al., 2003; Palusinska-Szysz and Cendrowska-Pinkosz, 2009). Legionellosis cannot be spread person-to-person and is only acquired from environmental sources. The widespread presence of legionellae precludes their removal from the environment so that disease prevention methods instead focus on reducing transmission of bacteria to susceptible hosts (Fields et al., 2002; Sehulster and Chinn, 2003; Freije, 2004; Tablan et al., 2004; Fields and Moore, 2006).

Monitoring levels of legionellae in building water systems by routine environmental sampling has been employed by some as a means of controlling transmission, though the relationship between the presence of legionellae and

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incidence of disease remains unclear (O'Neill and Humphreys, 2005; Den Boer et al., 2007). Environmental sampling for *Legionella* spp. as an approach for primary control can provide useful information to institutions that house persons at high risk for disease, such as chronic care and transplant facilities (Anonymous, 1997; Butler et al., 1997; Yu, 1997; Fiore et al., 1999; Fields and Moore, 2006). However, routine culture in the absence of documented cases of legionellosis is an area of considerable controversy, mostly because there is no generally accepted protocol for the choice of sampling sites, frequency of sampling events, or endpoints for remediation (Den Boer et al., 2007; Stout et al., 2007; Ditommaso et al., 2010). Recommendations that suggest the use of routine sampling for primary control acknowledge that the acceptable limits of contamination, either measured in cfu/ml or as a percentage of positive sites sampled, are based on limited data (Force, 1997; Sehulster and Chinn, 2003; Stout et al., 2007). Another confounding factor that is not frequently mentioned in the discussion on routine sampling is the considerable variability in recovery of legionellae from repeated sampling of sites or even seeded tap water suggesting that interlaboratory enumeration error may be high (Boulanger and Edelstein, 1995; Bentham, 2000; Napoli et al., 2009). The Centers for Disease Control and Prevention (CDC) guidelines for the reduction of legionellosis advise that there is no acceptable level of legionellae contamination and that if *Legionella* spp. are detected a plan to prevent transmission to susceptible individuals should be employed (1997).

Domestic and international organizations have published procedures for the recovery of *Legionella* bacteria from environmental samples but the application of these techniques can still require considerable expertise from the laboratorian (Anonymous, 1998, 2000, 2004, 2005a,b, 2008). Numerous variables contribute to effective recovery including properly taking and transporting samples, the application of sample pre-treatments intended to increase the representation of viable legionellae, and the use of suitable selective media (Reeves et al., 1981; Tesh and Miller, 1981; Shahamat et al., 1991; Lee et al., 1993; Ta et al., 1995; Leoni and Legnani, 2001; Wiedenmann et al., 2001; Bartie et al., 2003; Luck et al., 2004). Characterization of isolates entails the ability to distinguish *Legionella* colony morphology from autochthonous microbiota and serological methods for confirmation of identity, both of which determinations are subjective and sometimes difficult to interpret without considerable prior experience by the laboratorian (Benson and Fields, 1998; Helbig et al., 2007; Wagner et al., 2007). PCR-based methods of isolate identification offer greater sensitivity and specificity but are often cost prohibitive for laboratories with a low volume of tests for legionellae and do not yet provide enough discrimination between strains for epidemiological surveillance (Thurmer et al., 2009; Tronel and Hartemann, 2009).

In the European Union (EU) laboratories that culture environmental samples for *Legionella* spp. are required to participate in a proficiency testing (PT) scheme to ensure baseline quality standards throughout the industry (2005). The EU scheme, in operation since June 2004, requires participants to process PT products as would be performed for potable water and report results to the program administrators for scoring according to ISO standards (1997; 1997). The EU scheme has been successful in assessing the status of the industry and publishes quarterly reports of their trends and performance distributions over time. A new PT scheme, the Environmental *Legionella* Isolation Techniques Evaluation (ELITE) program was created by CDC to capture similar information for US laboratories. A Pilot Program was performed September 1, 2008–March 31, 2009 to determine industry limits of detection and ascertain the accuracy of legionellae enumeration by participating laboratories. The results indicate that US laboratories are generally capable of a qualitative assessment of environmental samples for the presence of legionellae but that quantitation displays significant inter- and intra-laboratory variability.

2. Methods and materials

2.1. Sample creation, panel composition, quality control, and re-tests

Legionellae and heterotrophic bacteria were grown from freezer stocks of less than three passages on BCYE media (BBL agar base plus 10 g/L L-cysteine) for 3–5 days at 35 °C with 2.5% (v/v) CO_2. Samples distributed for PT were removed from plates by suspension in sterile de-ionized water. Bacterial suspensions were diluted in 10% (v/v) AYE broth prior to lyophilization. The formula for full strength AYE broth is as follows: 5 g bovine serum albumin fraction V, 10 g ACES, 10 g yeast extract, 0.4 g L-cysteine, 0.25 g iron pyrophosphate. Bring volume to 1 L with sterile de-ionized water, adjust pH to 6.9 with 1 N potassium hydroxide, and filter to sterilize. The AYE broth helped to stabilize lyophilized cells during storage and formed a consistent pellet regardless of sample composition. Samples were made in batches monthly with 20–25 aliquots per lot. A representative sample from each lot was examined for quality control (QC) one week after lyophilization by plating serial dilutions from the vial reconstituted in 1 ml sterile de-ionized water on BCYE in triplicate at each serial dilution. Plates were incubated 5 days at 35 °C with 2.5% (v/v) CO_2 prior to being read. The total bacterial count and *Legionella*-specific count per plate were determined. At least one representative *Legionella*-like colony per plate was confirmed as a cysteine auxotroph.

PT panels consisted of 6 samples, one of each sample type: pure positive, mixed positive, pure negative, mixed negative, pure variable, and mixed variable (see Table 1). Positive samples were either *Legionella* in pure culture or mixed with a low ratio of heterotrophs. Negative samples contained no viable legionellae, though they might contain heat killed legionellae. Variable samples consisted of either low levels of *Legionella* in pure culture or a mixture of organisms with a high ratio of heterotrophs to legionellae. Laboratories were scored on a pass/fail basis, required to correctly identify both positive and both negative samples for a passing score. Variable samples did not count toward the score but were included in each panel to assess lower limits of detection.

Panels identical to those sent to participants were shipped back to the program administrators for re-tests. Re-test samples were first reconstituted in the vial with 1 ml of sterile,

Table 1 – PT sample characteristics: All PT samples used during the Pilot Program (Panel column: Panels number I–VIII and Accelerated) are listed here with sample composition, QC, and re-test results. Samples were categorized into sample types according to QC results. Re-test treatments specific to each sample are abbreviated: D = direct plating, SD = serial dilutions, A = 15 min acid.

Sample ID no	Status	Panel	Sample composition	QC total cfu/ml ± SD	QC *Legionella* cfu/ml ± SD	Re-test[a] total cfu/ml ± SD	Re-test Legionella cfu/ml	Re-test protocol
A50-08081901	Negative – media	I	Blank media	0	0	0	0	D, BCYE
A49-08071519	Variable – mixed	I	*L. pneumophila* Sg1 + HTs	5 ± 0.3	0.6 ± 0.4	7 ± 0.3	1	D, GPCV
A32-08061701	Variable – low	I	*L. pneumophila* Sg3	Pure	53 ± 13	Pure	52	D, BCYE
A12-08041602	Positive – pure	I	*L. pneumophila* Sg8	Pure	211 ± 14	Pure	139	SD, BCYE
A31-08061718	Negative – mixed	I, A	HTs	1370 ± 374	0	861 ± 5	0	SD, BCYE
A25-08052219	Positive – mixed	I, A	*L. bozemani* + HTs	4400 ± 712	170 ± 11	≥3000	170	SD, PCV
A50-08081905	Negative – media	II	Blank media	0	0	0	0	D, BCYE
A30-08061719	Negative – mixed	II	HTs	703 ± 9	0	≥300	0	SD, BCYE
A49-08071520	Variable – mixed	II	*L. pneumophila* Sg1 + HTs	4 ± 1	0.7 ± 4	4 ± 1	1	D, GPCV
A32-08061707	Positive – mixed	II	*L. pneumophila* Sg3 + HTs	583 ± 26	48 ± 4	≥300	35	SD, A, PCV
A12-08041604	Positive – pure	II	*L. pneumophila* Sg8	Pure	254 ± 17	Pure	344	SD, BCYE
A32-08061702	Variable – pure	II, A	*L. cherrii*	Pure	7 ± 4	Pure	2	D, BCYE
A31-08061704	Negative – pure non-Legionella	III	HTs	3330 ± 471	0	ND	0	SD, BCYE
A21-08052203	Negative – mixed	III	HTs	83,000 ± 3740	0	ND	0	SD, BCYE
A48-08071517	Variable – mixed	III	*L. pneumophila* Sg1 + HTs	7870 ± 309	150 ± 39	ND	13	A, SD, PCV
A65-08091607	Variable – pure	III	*L. pneumophila* Sg1	Pure	593 ± 170	Pure	120	SD, BCYE
A67-08091619	Positive – mixed	III	*L. pneumophila* Sg8 + HTs	4730 ± 624	2000 ± 630	ND	311	A, SD, GPCV
A62-08091601	Positive – pure	III, A	*L. pneumophila*	Pure	7600 ± 1280	Pure	≥3000	SD, BCYE
A20-08052201	Negative – media	IV	Blank media	0	0	0	0	D, BCYE
A28-08052204	Negative – mixed	IV	HTs	15, 200 ± 2380	0	ND	0	SD, BCYE
A6508091615	Variable – pure	IV	*L. pneumophila* Sg8	Pure	61 ± 7	Pure	16	D, BCYE
A48-08071518	Variable – mixed	IV	*L. pneumophila* Sg1 + HTs	883 ± 58	257 ± 38	ND	193	A, SD, BCYE
A6708091617	Positive – mixed	IV	*L. pneumophila* Sg1 + HTs	18, 7000 ± 1250	4000 ± 1410	ND	412	A, SD, PCV
A62-08091609	Positive – pure	IV	*L. pneumophila* Sg8	Pure	3300 ± 804	Pure	≥3000	SD, BCYE
A50-08081904	Negative – media	V	Blank – broth only	0	0	ND	0	D, BCYE
A71-08111906	Negative – mixed	V	HTs	15,600 ± 1130	0	ND	0	SD, BCYE
A38-08061703	Variable – pure	V	*L. rubrilucens*	Pure	10 ± 0.4	Pure	3	D, BCYE
A25-08052218	Positive – mixed	V	*L. dumoffii* ± HTs	39,000 ± 5720	1670 ± 125	ND	1000	A, SD, GPCV
A84-08120201	Positive – pure	V	*L. feelei*	Pure	27,300 ± 5910	Pure	16500	SD, BCYE
A88-08120215	Variable – mixed	V, A	*L. pneumophila* Sg1 + HTs	600 ± 36	33 ± 5	ND	12	A, SD, BCYE
A50-08081908	Negative – media	VI	Blank – broth only	0	0	0	0	D, BCYE
A28-08052207	Negative – mixed	VI	HTs	13,600 ± 8740	0	ND	0	SD, BCYE
A89-08120213	Variable – pure	VI	*L. pneumophila* Sg10	Pure	183 ± 24	Pure	102	SD, BCYE
A14-08041609	Positive – mixed	VI	*L. rubrilucens* + HTs	16,200 ± 9420	1290 ± 173	ND	540	A, SD, PCV
A39-08061716	Variable – mixed	VI	*L. rubrilucens* + HTs	70,700 ± 7040	2400 ± 57	ND	2000	A, SD, BCYE
A86-08120217	Positive – pure	VI	*L. dumoffii*	Pure	74,200 ± 17,000	Pure	34300	SD, BCYE
A31-08061717	Negative – mixed	VII	HTs	7870 ± 141	0	ND	0	SD, BCYE
A89-08120211	Variable – mixed	VII	*L. rubrilucens* + HTs	205 ± 23	35 ± 4	ND	17	SD, BCYE
A62-08091607	Variable – pure	VII	*L. pneumophila* Sg1	Pure	59 ± 17	Pure	60	D, BCYE
A32-08061706	Positive – mixed	VII	*L. pneumophila* Sg3 + HTs	11,700 ± 5350	1480 ± 398	ND	200	A, SD, GPCV

A62-08091603	Positive – pure	VII	*L. pneumophila* Sg1	Pure	3100 ± 804	Pure	1410	SD, BCYE
A90-09010505	Negative – media	VII, A	*L. pneumophila* Sg1 [heat killed]	0	0	0	0	D, BCYE
A90-09010509	Negative – media	VIII	*L. pneumophila* Sg10 [heat killed]	0	0	0	0	D, BCYE
A71-08111907	Negative – mixed	VIII	HTs	7670 ± 858	0	ND	0	SD, BCYE
A89-08120212	Variable – mixed	VIII	*L. rubrilucens* + HTs	1130 ± 17	23 ± 12	ND	5	A, GPCV
A65-08091608	Variable – pure	VIII	*L. pneumophila* Sg1	Pure	≥300	Pure	45	SD, BCYE
A32-08061708	Positive – mixed	VIII	*L. pneumophila* Sg3 + HTs	4430 ± 377	183 ± 37	ND	200	A, SD, BCYE
A62-08091604	Positive – pure	VIII	*L. pneumophila* Sg1	Pure	1330 ± 557	Pure	835	SD, BCYE

a Heterotrophic plate counts were enumerated by re-test only for the first round (Panels I and II). Heterotrophic cfu/ml were not determined (ND) by re-test for panels III through VIII.

de-ionized water then diluted 1:1000 in a bottle of sterile, de-ionized water to yield a 1 L Test Sample. All Test Samples were filtered. Each Test Sample was then processed according to an individual protocol determined by sample composition, which was designed for maximum recovery and enumeration of legionellae (Table 1). Three replicates of each dilution were plated but only the highest count was used as the re-test value for data analysis. All re-test values were within one standard deviation of the mean of the re-test replicate plate counts (data not shown). Test Samples were subjected to combinations of pre-treatment with acid (18 parts 0.2 M KCl to 1 part 0.2 M HCl), serial dilutions prior to plating, and/or plating on selective media PCV (BCYE plus 13.22 mg/L polymixin B, 80 mg/L cyclohexamide, and 5 mg/L vancomycin) or GPCV (PCV plus 2 g/L glycine). QC results, re-test results, and re-test treatment protocols are listed for all samples in Table 1.

2.2. *Pilot participants*

Because current industry capabilities were unknown at the inception of the ELITE Program, a pilot study was conducted September 1, 2008 through March 31, 2009 to generate baseline values. The Pilot Program was limited to ten participating laboratories. A total of 20 laboratories enrolled in the program prior to the August 31, 2008 deadline for inclusion in the Pilot Program. Laboratories that could not be accommodated in the Pilot Program due to space limitations were enrolled as Accelerated Members. Commercial laboratories comprised the majority of enrollees but the Pilot Program was designed to include at least one of each type of laboratory as listed in Table 2. CDC reference laboratory personnel separate from those administering the ELITE Program participated in the pilot. To avoid confusion in the text, CDC reference laboratory Pilot Program participants are designated the "federal" laboratory in the text and figures, while administrators' results are labeled "CDC". For data analysis purposes, State, County, and Hospital laboratories were grouped into the category "Local Public Health."

Participants were asked to process the panels according to their standard in-house protocol and return results as they would be given to clients. Participants were directed to rehydrate the lyophilized pellets in 1 ml sterile water then dilute the suspension 1:1000 in sterile water to yield a test sample of the volume dictated by the participant's in-house protocol. Supplement 1 contains the complete Sample Handling Instructions available to all participants. Pilot Members processed four panels shipped September 3, 2008, November 3,

Table 2 – Pilot and accelerated program demographics: the number and type of each participant in the pilot or accelerated program.

	Pilot	Accelerated	Total
Commercial	6	6	12
Federal	1	0	1
State	1	3	4
County	1	0	1
Hospital	1	1	2
Total	10	10	20

2008, December 29, 2008, and March 2, 2009. Accelerated Members processed a single panel consisting of samples already tested by Pilot Members that was shipped March 20, 2009. All laboratories enrolled during this time were given two months to process a panel and report results.

2.3. Data collection and analysis

Combined results from Pilot and Accelerated Members were captured from the CDC SQL 2005 database June 12, 2009. Reported laboratory concentrations were tabulated and summarized. Differences were computed and tabulated between the reported concentrations and (a) the CDC-validated concentration (re-test values) and (b) the intra-laboratory mean among all laboratories reporting a value. In addition, tabulations of un-quantified identifications were made. Quantitative results were analyzed directly and through log transformations. Reporting laboratories were categorized, respectively, by: type of laboratory (state, local, federal, hospital, commercial); annual volume of *Legionella* tests performed; how long (years) the laboratory has tested for *Legionella*; number of full-time and *Legionella*-dedicated employees; and by protocol-related factors such as incubation time and temperature, water volume, use of CO_2, and standard protocol reference (ASM, ISO, or CDC). State, county, and hospital laboratories were grouped into the category "Local Public Health" for further analyses. For each categorization of laboratories or procedures, Chi-square tests were computed for dichotomous outcomes such as finding *Legionella* or not or being within a specified error, such as one log, of the respective (re-test or interlaboratory mean) reference concentration. In addition, for continuous measures, *t*-tests were performed comparing the errors of mutually-exclusive groups of laboratories, such laboratories which used less than 750 ml of water versus those who used 750 ml or more.

3. Results

3.1. PT sample qualitative identification

Concordance between expected positive or negative results, as determined by QC, and results returned by Pilot or Accelerated Members was high regardless of laboratory type (Table 3). The majority of positive samples (88.5%) were correctly identified. No false positives from a negative sample were reported by any member. In contrast, 36.9% of all variable samples tested ($n = 103$) were incorrectly identified (Table 4). The federal laboratory was most often correct, followed by commercial laboratories, and then local public health laboratories.

Correct identification of positive samples depended on the *Legionella* concentrations in the samples. Samples with less than 10 cfu/ml, as determined by re-test, were identified as negative in 93.1% of reports while samples with 10 cfu/ml or more were reported positive in 85.3% of reports. These results were independent of whether the sample was pure or mixed, positive or variable indicating that 10 cfu/ml is at or near the lower limit of detection. In addition to whether a sample was positive or negative for *Legionella* growth, participating laboratories had the option to report species and serogroup of legionellae recovered. All laboratories that provided optional results correctly identified the species and serogroup of recovered *Legionella*.

Table 3 – Concordance of positive and negative sample reports by laboratory type: displays the agreement between expected results and participants' reported results for all positive or negative samples, both mixed and pure. Variable samples' reported results are not included in this table. Definitions of column headings are: True Negative = expected negative and reported negative, False Negative = expected positive but reported negative, True Positive = expected positive and reported positive.

	True negative	False negative	True positive	Total results (*n*)
Commercial	56 (100%)	3 (6.3%)	45 (93.8%)	104
Federal	8 (100%)	0 (0%)	7 (100%)	15
Local public health	32 (100%)	7 (21.9%)	25 (78.1%)	64
Total results (*n*)	96 (100%)	10 (11.5%)	77 (88.5%)	183

3.2. Accuracy and precision of Legionella quantitation

Pilot and Accelerated Members were encouraged to also provide results for their observed concentration of legionellae in samples ($n = 128$). The expected concentration for each sample was determined by re-test (Table 1). The log error for each report is displayed graphically in Fig. 1. The majority (99.5%) of reported results underestimated the expected concentration by an average of 1.25 logs with a standard deviation of 0.78 logs, standard error of 0.07 logs, and range of 3.57 (−3.30 to 0.27) logs. Centering the results on the inter-laboratory mean forced an overall net difference of zero logs (Fig. 2). The standard deviation was 0.62 logs, standard error was 0.06 logs and the range was 3.22 (−1.89 to 1.33) logs. The standard deviation from the interlaboratory mean of the results from laboratories that entered at least three concentrations for evaluation ($n = 11$) ranged from 0.53 to 0.96 and standard error from 0.14 to 0.58. No sampling protocol, treatment regimen, incubation procedure, or organizational structure type analyzed produced significantly greater accuracy when compared to re-test values or precision when compared to the interlaboratory mean concentration (Table 5).

Table 4 – Accuracy of variable sample reports by laboratory type: Displays the reported results for all variable samples, both mixed and pure. No positive or negative sample results are included in this table.

	Reported positive	Reported negative	Total results (*n*)
Commercial	39 (62.9%)	23 (37.1%)	62
Federal	7 (77.8%)	2 (22.2%)	9
Local public health	19 (59.4%)	13 (40.6%)	32
Total results (*n*)	65 (63.1%)	38 (36.9%)	103

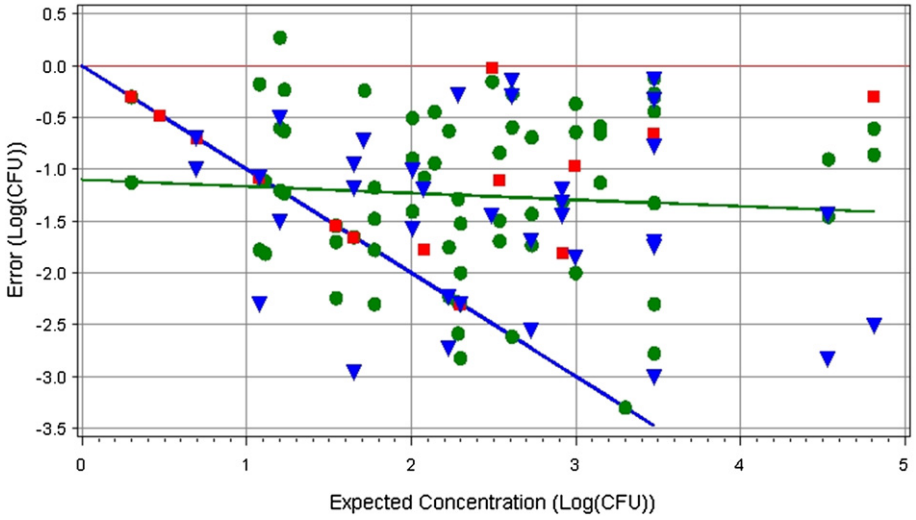

Fig. 1 – Accuracy of quantitative results relative to re-test values for all samples reported positive: Displays the log error of the difference between re-test values and reported concentrations for all samples reported positive. Each marker represents a sample reported positive by a participant ($n = 190$). Markers are coded by laboratory type: commercial (green circle), federal (red square), local public health (blue triangle). Enumeration error [Error(Log(CFU))] was calculated by taking the difference between the log value of the expected concentration in cfu/ml and the log value of the concentration reported by a participant in cfu/ml. Participant responses are plotted as enumeration error (y-axis) vs. expected concentration derived from re-test values (x-axis). Samples reported positive without a concentration value ($n = 62$) are indicated by markers connected with the blue line ($y = -x$) and are not included in calculations measuring accuracy. The green line depicts the mean enumeration error across the range of reported concentrations (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The results from the ELITE Pilot Program indicate that most participating US laboratories are capable of qualitatively identifying *Legionella* spp. from a water sample and that the lower limit of detection is approximately 10 cfu/ml. According to 2009 EU Health Protection Agency external quality assessment for *Legionella* isolation from water samples reports, the accuracy of the European PT participants (Shah et al., 2009a,

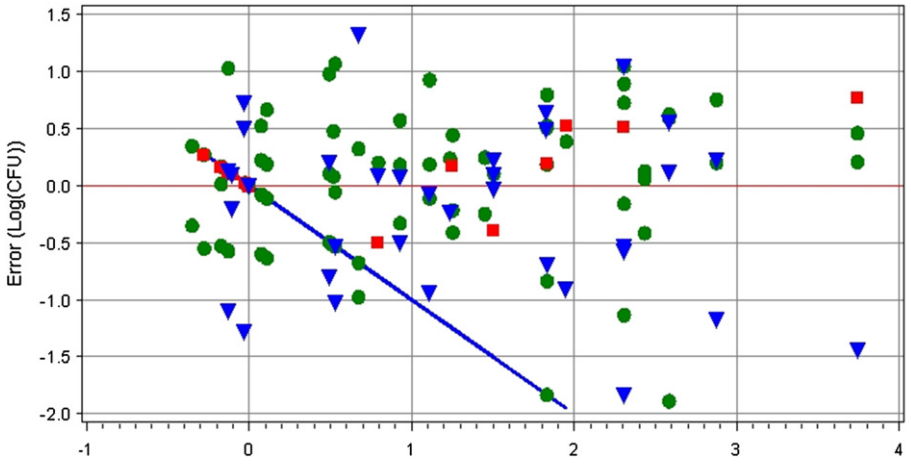

Fig. 2 – Precision of quantitative results relative to interlaboratory means for all samples reported positive: Displays the log error of the difference between interlaboratory mean values and reported concentrations for all samples reported positive. Each marker represents a sample reported positive by a participant ($n = 190$). Markers are color coded by laboratory type: commercial (green circle), federal (red square), local public health (blue triangle). Enumeration error [Error(Log(CFU))] was calculated by taking the difference between the log value of the interlaboratory mean cfu/ml and the log value of the concentration reported by a participant in cfu/ml. Participant responses are plotted as enumeration error (y-axis) vs. expected interlaboratory mean (x-axis). Samples reported positive without a concentration value ($n = 62$) are indicated by markers connected with the blue line (y = -x) and were not included in calculations to determine the interlaboratory mean (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5 – Statistical analysis of accuracy as a function of procedural variables: The enumeration errors associated with treatment, protocol, and experience categories were compared with *t*-tests. The *p*-values are given when the baseline is calculated from either re-test values or the interlaboratory mean.

Measurement	Categories	*p* Value compared to re-test	*p* Value compared to interlaboratory mean
Incubation temperature	35.0 °C or >35.0 °C	0.297	0.311
Incubation with CO_2	<2.5% CO_2 or 2.5–5.0% CO_2 (v/v)	0.537	0.332
Water volume	<750 ml or 750–1000 ml	0.454	0.476
Incubation time	≤7 days or >7 days	0.318	0.562
Sample composition	Pure or mixed sample	0.065	>0.999
Sampling protocol	CDC or other	0.221	0.216
Years of testing	≤10 or >10 years	0.672	0.159
Number of dedicated employees	≤2 or >2 employees	0.993	0.960
Number of samples processed annually	≤200 or >200	0.919	0.565

2009b, 2009c, 2009d) averaged 93% concordance (range 78–98%). US laboratories in this study averaged 86% concordance with positive samples, well within the range of EU performance. On the other hand, with regard to precision, US laboratories demonstrated more extreme variability in enumerating bacteria in the PT samples both within and between participants. US labs underestimated the concentration of *Legionella* by an average of 1.25 (±0.78) logs when compared to re-test values. Further, US labs exhibited 0.62 logs variance around the interlaboratory mean. In contrast, EU laboratory results from 2009 clustered very close to the interlaboratory mean and FEPTU median (a reference standard comparable to re-test values), usually within a 0.5 log range of either score, with a mean standard deviation of 0.44 logs (Shah et al., 2009a, 2009b, 2009c, 2009d). Thus, EU and US laboratories demonstrate similar accuracy in identifying positive samples but US laboratories appear to be less precise than EU laboratories in enumerating viable bacteria.

The apparent greater precision of enumeration by EU laboratories compared to US laboratories is partially due to the bacterial concentrations used in the two sources of PT samples. EU sample concentrations spanned 0.2–110 cfu/ml while US PT samples contained between 1 and 34,300 cfu/ml (as determined by re-test), allowing the possibility for a larger range of enumeration error within US samples. However, a greater contribution to the disparity in variance between EU and US reported results was the different methodologies used to generate reference standards for comparison. US re-test values were calculated from the highest count recovered from a representative sample after treatment with an individualized protocol that took sample concentration and composition into account. Plating serial dilutions from the PT samples in triplicate, a feature of the majority of individualized re-test protocols (Table 1) but no referenced standard protocol (Anonymous, 1998, 2004, 2005a, 2005b, 2008, 2010), resulted in plates that had reduced numbers of obscuring heterotrophs and increased physical distance between colonies, making *Legionella* cfu easier to determine. In contrast, EU FEPT medians were generated from the results of 10 samples treated according to a single standard protocol without regard to sample concentration or composition, making the results inherently less variable. Both reference standards are valuable in assessing participant results but address different laboratory capabilities. Re-tests return the most accurate count of viable bacteria within a sample, independent of protocol bias while the EU FEPT median and ELITE interlaboratory mean values address the precision with which a sample can be enumerated using a (set of) standard protocol(s). Thus, comparison to the re-test value indicates how close a laboratory can come to a 'true' answer while FEPT medians and interlaboratory means illustrate how reproducible the results are between and within a laboratory.

Laboratories that submitted more than three concentration reports were statistically indistinguishable from each other in enumeration error and also demonstrated large intralaboratory variance (0.53–0.96). Two PT samples were quantified with significantly different log error from re-test values compared to the rest. These observations can be explained by sample composition. A62-08091603 (−0.71 vs. −1.27, $p = 0.002$, $n = 5$) was a pure sample with a concentration within the optimum range for allowing distinct colony growth on media from a single 10-fold dilution and so could be measured more precisely than other samples. In contrast, sample A32-08061706 (−2.19 vs. −1.21, $p = 0.006$, $n = 5$) was heavily mixed with heterotrophs that had a spreading colony morphology, obscuring legionellae growth, resulting in a sizeable difference between QC and re-test results (Table 1), and a substantial interlaboratory enumeration error. Enumeration of the other 46 samples was statistically indistinguishable. No sampling protocol, treatment, incubation, or experience level analyzed in this study affected the accuracy or precision of enumeration (Table 5) at the 0.05 significance level. Taken together, these results indicate that intralaboratory and interlaboratory variance in precision and accuracy were similar in degree and magnitude for all pilot study participants.

Two obvious caveats to these results are the small number of participating laboratories and their method of selection. ELITE Program participation is voluntary rather than mandated by legal statutes, as in the EU, and there was little promotion for the creation and implementation of the program. Most Pilot and Accelerated Members discovered the program through scientific meetings and/or word of mouth. Pilot Members were also likely to have a previous relationship with CDC, multiple years experience with *Legionella* isolation,

and/or a large number of well-trained personnel dedicated to *Legionella* testing. Thus, it is possible that Pilot Members do not accurately reflect the capabilities of all US laboratories that culture environmental samples but are a subset of those whose primary focus is *Legionella*.

5. Conclusions

Overall, *Legionella* PT qualitative and quantitative results were similar between US and EU laboratories in 2009 despite differences in sample composition, delivery, and reference standard determination. The observed variability in enumeration by both US and EU laboratories is probably due to the inherent inconsistency in assessing a sample by culture techniques. Given these data no protocol can be recommended to yield more accurate or precise results than any other. Responses from ELITE Program participants will continue to be monitored and analyzed to determine if more data can illuminate practices that contribute to increased accuracy and precision. However, the current findings have several implications for the use of routine sampling as a primary method of legionellosis control. Agreement between EU and US PT schemes suggest these results are applicable worldwide to environmental sampling laboratories. Since a sample qualitatively identified as positive could represent a 3 log cfu/ml range of viable legionellae it would be in the best interests of public health to consider any detectable level a hazard. Therefore, primary legionellosis prevention should consider the risk posed by an individual water system, assessing the likelihood of transmission and population affected, to determine if remediation is required rather than relying on a contamination cutoff level to take action.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.watres.2011.05.030.

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