Validation of Hazard Control in Building Water Systems

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Abstract

Legionella is a known biological hazard that causes serious disease associated with building water systems. Validation is evidence that hazards have been controlled under operating conditions; it is an essential requirement in the practice of hazard analysis and control. Testing to quantify the hazard in building water systems is the most direct validation evidence. However, results are useful for validation only if precision, accuracy and limit of quantification issues are properly considered. Results from field and laboratory studies of molecular and culture methods are presented, compared and statistically analyzed. Hazard analysis and control recommendations for cooling towers are given.

Executive Summary

Hundreds of millions of dollars have been spent responding to *Legionella* test results. However, misleading interpretations of test results may lead to scientifically indefensible actions that could be counter-productive and may make matters worse in building water systems unless limitations due to low precision, poor accuracy and limit of quantification are properly considered, as follows:

- Only order-of-magnitude numerical values of test results are significant. For example in 121 CFU/ml, the "21" value is not in significant figures. This test result should be simply regarded as 10^2 CFU/ml
- Differences in numerical results less than one order-of-magnitude are not significantly different
- The practical limit of quantification is 10 CFU *Legionella*/ml. Misguided notions about the relevance of far lower theoretical detection limits may lead to potentially dangerous actions
- Numerical test result values that "trigger" recommendations for specific hazard control actions are not supported by the precision and accuracy of the method. They should be not advocated
- Unpredictable changes in shipped or otherwise held water samples cause inaccurate results; this inaccuracy can be eliminated by starting the culture on-site at "time zero", immediately after the sample is removed from the building water system

Facility managers/owners are responsible for hazard analysis and control of cooling towers in their facilities. The hazard analysis and control process requires them to make certain that known hazards, such as *Legionella* bacteria, have been controlled. The burden of testing and decisions about how much testing to do, if any, is on the facility manager/owner. When properly used, *Legionella* test results give reliable and defensible evidence that the hazard has been controlled. Our recommendations for validation and verification of hazard control for cooling towers are:

- All treated cooling towers and any cooling tower that presents risk of exposure to people should be tested once per quarter for *Legionella* and total heterotrophic aerobic bacteria using cultures of the same sample started immediately after the sample is taken from the cooling tower

- Any *Legionella* detection ≥10CFU/ml and/or total heterotrophic aerobic bacteria $\geq 10^5$ in the same sample cultured immediately after removal from the cooling tower system indicates that microbial control is inadequate; the entire treatment program should be immediately reassessed and upgraded
- The cooling tower should be retested after the treatment program has been upgraded; if test results indicate that microbial control is still inadequate, then repeat this process until hazard control has been validated
- Verification that the plan has been implemented including process flow diagrams of the utility water system, cooling tower hazard analysis summaries, treatment monitoring data and validation test results are an absolute requirement; verification materials should be easily retrievable, clear and unambiguous
- Review quarterly with the facility manager/owner all cooling tower validation evidence and verification materials

Introduction

Hundreds of millions of dollars have been spent responding to *Legionella* test results. Thousands of building water systems are regularly tested in the U.S. Tens of millions of building water samples have been analyzed globally since publication of spread plate protocols and an ISO method[1,2].

However, those who respond to these test results are generally unaware that low precision, poor accuracy and mistaken notions about detection limits can result in misleading recommendations. In the worst cases, such misdirected actions can be dangerous by making building water systems more hazardous. The first rule must always be: Do not take actions that make matters worse.

One of the confusing things about *Legionella* test results from building water samples is that, according to much of the data, a vast number of building water systems are dangerously contaminated with the pathogen. Some surveys indicate that 70% of hospital potable water systems and 40% of cooling towers are apparently laden with *Legionella*. Fair questions are: Why then, do so relatively few actual disease cases occur compared to the vast number of apparently contaminated building water systems? And why do disease cases not correlate better with contaminated systems? Part of the answers to these questions may lay in the analytical problems associated with *Legionella* testing of building water systems.

Precision and Accuracy

Precision and accuracy are important because test results are often used to make recommendations related to notions about the risk of disease.

For example, disinfection is recommended in cooling towers in the U.S. when *Legionella* is recovered at or above an arbitrary numerical limit of 100 CFU/ml[3]. Also, 30% percent positive in a set of results from potable water samples is a widely used arbitrary action trigger[4]. These examples illustrate that the most common usage for *Legionella* test results from building water systems is binary: the result is either at or above a specified numerical limit or the result is below it. Thus, precision and accuracy of the numerical result are at issue.

The variation observed in replicate analyses of a sample is referred to as precision. To illustrate this, the targets in Figure 1 show on the left four high-precision shots that severely missed the bulls-eye compared to, on the right, four low-precision shots yielding a nearly dead-center average on the target.

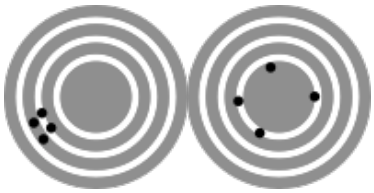

Figure 1. High-precision, low-accuracy results on the left compared to low-precision, high-accuracy results on the right[12].

Precision is the quantitative measure of analytical error. It is usually described as Standard Deviation (Std Dev). Consider two test results, a_1 and a_2. Suppose $a_1 > a_2$. Their mean number μ is the mid-point; the standard deviation σ is the distance from each of the numbers to μ.

For most analytical methods used in water analyses, the Std Dev (σ) is typically about 10-15% of the mean (μ); this ratio is referred to as the Coefficient of Variation (CoV = σ/μ, usually expressed as a percent). For many regulated contaminates in the pharmaceutical, food, and device manufacturing industries, the Standard Operating Procedures (SOPs) for methods will specify that % CoV must be not greater than 20%. Typically, chemists, chemical, mechanical and electrical engineers and certainly most facility managers assume similar precision in *Legionella* test results. This assumption is incorrect and it can be dangerously misleading.

In reality, the Std Dev of *Legionella* test results from building water systems is often more than or equal to the mean. Therefore, the CoV (the analytical error) is very high, about 100%, often far worse. This fact is not widely known because *Legionella* test results are usually reported in such a way as to imply better precision than the methods can actually deliver. Users of test results are thereby led to incorrectly assume high precision results.

An important factor that directly impacts precision is the countable range of colony forming units recovered on spread plates. In order to establish a statistically significant spread plate count, generally 30-300 colony-forming units (CFU) per plate (or in some studies, 25-250 CFU/plate) are require[5,6,7]. Figure 2 shows the dependence of error on the CFU counted per plate; when the number of colonies per plate is small, the error is high. High error means low precision[7].

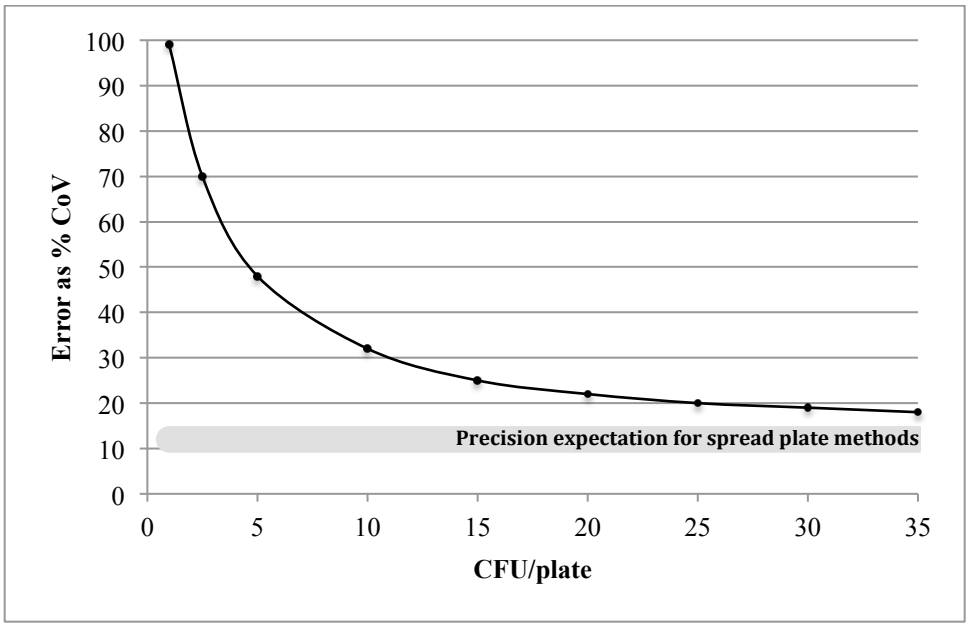

Figure 2. Error (Coefficient of Variation, CoV, which is the ratio of Std Dev to the mean, % σ/μ) in spread plate microbiological methods is highly dependent upon the actual number of colonies recovered per plate[7]. Typically, numerical *Legionella* test results are reported even from plates with a single colony recovered (the ISO protocol does not specify a countable CFU/plate range nor does it require replicates).

This analytical spread plate requirement is generally ignored; it is not even specified, referenced or otherwise mentioned in the ISO Method. Instead, numerical values from plates upon which even single colonies appear are routinely reported out in CFU/ml (or CFU/l). It can be correctly assumed that whenever a numerical value is reported out at near the limit of detection, the analyst has counted just one or a few colonies on the plate. These results are numerically unreliable.

Here's why this is important for *Legionella* test results: Numerical differences that are less than about an order-of-magnitude are not significant and it is potentially dangerous to assume otherwise. The following is a useful example from the "real-world" of water treatment:

> A facility manager was responsible for two systems, A and B. The test result from System A indicated 121 CFU/ml *Legionella*. In response, the facility manager reacted aggressively. Upper management was notified about potential hazardous conditions in System A. An expensive and disruptive "remediation" of the system was undertaken. Meanwhile, the test result obtained from system B was 24 CFU/ml *Legionella*. In response, the facility manager took no action because the result was "well below" the action level trigger.

And yet, there was no scientifically defensible difference between test results from System A and System B. So, based on a misunderstanding about the precision of the method, aggressive action was taken for one system while taking no action for the other system. This situation is potentially dangerous from several perspectives including vulnerability to an accusation of negligence.

Environmental data are extremely variable, much more so than the variation in data from controlled lab studies[6]. Data from environmental tests do not conform to a normal distribution because there are sporadic counts with "zero" CFU predominating. Therefore, the numbers are unreliable. It is only the trend in the data that is important.

The use of test results for action level triggers or for compliance to a regulated specification cannot be justified nor can it be scientifically defended. Quoting from Sutton[7]:

> "Microbiology has a well-deserved reputation for being highly variable. Our lax attention to precision and accuracy in our measurements helps further this perception. We have allowed specifications for environmental monitoring, raw material bioburden, in-process bioburden and finished product bioburden to be imposed by regulation without regard for the ability of the methods to support those specifications."

This statement refers to testing for microbiological hazards in the pharmaceutical, food and medical device industries. We should, in the opinion of the authors, take this lesson to heart so as to avoid making the same mistakes in Standards or Guidelines for the prevention of legionellosis. It does not argue that *Legionella* test results should not be used at all; rather, it argues that validation criteria should be based on realistic and scientifically defensible considerations about the limitations of the methods used.

Limit of Quantification

Just because certain bacteria can be detected in a sample amongst the multitude of other bacteria present does not mean that it is known how many are there or whether they are hazardous. The limit at which the target can be quantitatively discerned is called the limit of quantification (LOQ). The meaning of this parameter is often muddled up with vague or poorly described notions about the theoretical limit of detection (LOD). The following example may help show why these concepts are important.

> Imagine a restaurant with lots of noise from conversations at tables closely situated. If the person next to you speaks quietly, you will probably not hear anything. The volume at which s/he is speaking is below the limit of detection. If s/he speaks a bit louder, you may hear sounds but it is not possible to be certain of what is being said. You would not know how to react and you may not even hear anything that you recognize as meaningful; this describes a voice volume that is above the limit of detection but less than the limit of quantification. If your companion speaks louder, above the background noise, then you can understand what is being said and you can choose to take action or not; this describes a voice volume that is above both the limit of detection and the limit of quantification. These limits are dependent on both the signal intensity (*e.g.,* the voice volume) and the noise (*e.g.,* the surrounding conversations).

Why is this relevant for interpreting *Legionella* test results? The reason is that reacting to test results that are within the "noise" (below the limit of quantification) should be not recommended. Action taken in response to such results may not be defensible. Scientist/experts cannot even say with any certainty what actual risk is indisputably linked to building water systems from which such results have been obtained. In our opinion, it is ill advised to even report numerical values that are within the noise because such results are unreliable.

In studies of standardized samples processed by 20 different US laboratories, reliable numerical test results was at about 10 CFU/ml regardless of how much the sample was concentrated[8]. In the Environmental Laboratory Isolation and Techniques Evaluation (ELITE) program administered by CDC, standardized samples with *Legionella* concentrations less than 10 CFU/ml are regarded as "variable". Either positive or

negative results returned for "variable" samples receive "Pass" scores because not even the reference lab can reliably return numerical results for such samples. This defines the *practical* LOQ; numerical values below the LOQ are within the "noise". Result values below the LOQ are too variable to be numerically reliable.

Theoretical Limit of Detection (LOD)

Users of test results are often unaware of the processing required to concentrate a building water sample in order attain the theoretical LODs required to advocate low detection limits far below the 10 CFU/ml LOQ or, in the UK, for compliance with the Approved Code of Practice, L8.

A large-volume water sample (compared to the volume of water that would be inhaled or aspirated by persons exposed to the building water system) must be pulled (or pushed) through a membrane filter with microscopic pore-size; this is disruptive to the sample. Such samples taken from building water systems are typically not homogenous. They may be laden with massive particulates, suspended pieces of biofilm, large aggregates comprised of thousands of cells, colonized particles and protozoa that may be infected with *Legionella*. It should be here remembered that in order for *Legionella* to cause disease, microscopically very small particles must be inhaled or aspirated deeply into the lungs.

Then, the material on the surface of the filter must be removed, usually by high-speed vortex. The resulting specimen is then suspended into a smaller volume than was filtered in order to achieved the target concentration and then homogenized. This process is further disruptive to the specimen which by this time may have very little or no resemblance to the sample that was removed from the building water system. The re-suspended material is then often further homogenized, and then plated out onto the growth medium.

Low theoretical detection limits do not mean that reliable numbers can be actually obtained. Nor is there necessarily any relevance in the data regarding risk of disease associated with the results. No one can say what risk of disease is predicted by the detection of *Legionella* from such a manipulated produced specimen. Making a puree of all the material in a large volume water sample, especially from cooling towers, and then concentrating it to hunt for *Legionella* in the mix should be not done for hazard control validation testing, in the opinion of the authors. More research would be helpful to describe what actually happens to the microbiological characteristics of building water samples that are processed like this. Much more research would be necessary to determine how analytical results from these specimens relate to the risk of disease from building water systems.

The statement that there is no known "safe concentration" of *Legionella* is true because the infectious dose of *Legionella* is unknown and is variable. However, it is equally true that there is no proof that *Legionella* is hazardous at vanishingly low concentrations in specimens that have been pulled apart by vacuum filtration of massive sample volumes (relatively speaking), vortex-homogenized, re-suspended and then further homogenized before spread-plate analysis.

The practical aspects of this are that 1) numerical differences less than an order-of magnitude are not statistically significant, 2) numerical values below the limit of

quantification are unreliable and should not be regarded as significant and 3) filter concentration, especially of cooling water samples, should not be recommended because the manipulation itself produces specimens that have not been scientifically described and may have very little or no microbiological resemblance to the building water sample from which they were derived.

Accuracy is not the same as precision (refer to Figure 1) but it is of course related to the LOQ. All microbiological methods inherently return highly variable results. Quoting from Sutton[7] in the *Journal of Validation Technology*, 2011:

> "*An unfortunate regulatory trend in recent years is to establish expectations (e.g., specification, limits, levels) for data generated by the plate count method that the accuracy of the method cannot support. This is a real opportunity for modification of current practice to approach the goal of science-based regulations."*

Any discussion of accuracy presumes there is a "true" value and that it is "knowable" within the precision (the analytical error) of the method.

Binary Statistical Description of Accuracy

For *Legionella* tests of building water systems, binary ("positive or negative", "detect or not detect", "greater-than-or-equal-to or less than", "true or false") interpretations relative to a pre-determined detection limit or action trigger level are now widely applied to results [3,4,8,9,10,11].

If there are true-negative results, then logic demands that there must be also the possibility for false-negative results. This is equally so for true- and false-positive results.

A "true-positive" result must be therefore defined as any numerical result greater than or equal to the detection or action limit *at the time the building water system was sampled*. This qualifier is necessary because changes in the sample after it is drawn from the system are uncontrolled.

It follows that a "false-positive" result is any numerical value greater than or equal to the detection or action limit that was not *also* positive when the sample was first drawn from the building water system. It must be noted that false-positive results are further defined as those samples in which *Legionella* was present in the sample below the limit of quantification (LOQ) at t=0 but above the LOQ after some sample holding time; an alternative term for this case is "amplified-positive result".

Accuracy is dependent on the extent of false-positive and false-negative results; it is the balance between specificity and sensitivity. Specificity is the statistical probability that a test result below the limit will be correctly identified as negative; specificity is therefore determined by the extent of false-positive results. The corollary binary statistic is "predictive value". Positive predictive value is the proportion of true-positives to the sum of true-positive and false-positive results; it is the probability that a building water sample with a positive test result really did contain detectable viable *Legionella* when the sample was drawn from the building water system. Negative predictive value and sensitivity are analogously defined; values are high if there are very few false-negative results. For readers interested in more detail about these parameters, see below in **Formulae** for mathematical definitions of the binary statistics.

Holding Time Effects on Accuracy

Holding time effects caused by sample transit and set-up time have been documented to cause inaccuracy in many standardized plate count methods [9,13,14]. Effects of sample storage at 4 °C and 10 °C for 24h were observed to unpredictably alter spread plate results[15]. Even when drinking water samples were held at 5 °C, losses of coliforms as great as 23% after 24h and 33% after 30h were observed; recommendations to analyze drinking water samples on the day of collection were given[13]. Total heterotrophic aerobic bacteria counts of samples from water cooling towers vary remarkably with sample holding time with significant changes occurring even after 1h[16]. Conclusions from published studies of holding time effects are often contradictory[17,18,19].

Sample holding time effects on accuracy are potentially much more severe in *Legionella* tests compared to effects on tests for other waterborne bacteria. This is because of the *Legionella* life cycle in protist hosts and because of complex biofilm effects.

Growth of *Legionella* within infected host cells such as amoeba, ciliated protozoa and many other susceptible protists could result in lytic release of thousands of viable *Legionella* from a single host cell directly into the relatively small volume of a water sample. Thus, an extraordinarily high concentration of *Legionella* could occur in the small-volume sample. This would not resemble the vastly diluted concentration of *Legionella* resulting from lysis of an infected host cell in the building water system itself. The results shown in Figure 3 may have been caused by this effect but it is impossible to prove it as the only explanation for the cases given here.

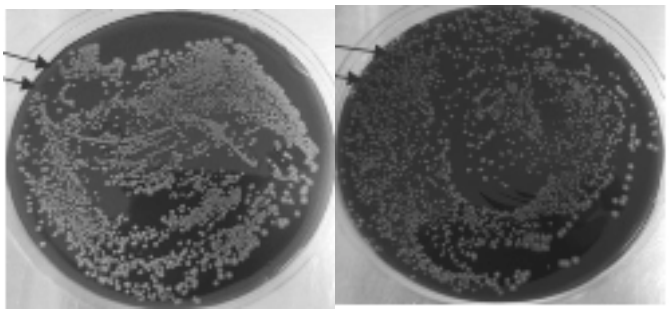

Figure 3. Analyses from two different two cooling tower water systems showing amplified *Legionella* concentrations due to uncontrolled variables that occurred during transit to the laboratory. Samples from both cooling water towers were cultured immediately on-site (data not shown) and then using the same growth media, cultured again from water samples that had been shipped overnight in temperature-insulated boxes. *Legionella* was not detected in either cooling tower sample when the culture medium was inoculated on-site. However, extraordinarily high counts of *Legionella* were observed on plates inoculated from samples shipped overnight (pictured, right and left). *Legionella* counts were similar or greater than the total heterotrophic aerobic bacteria counts (data not shown). Arrows indicate that all the colonies were confirmed *L. pneumophila* far in excess of 10^4 CFU/ml; this is highly unlikely for the cooling towers from which these samples were drawn because the detectable concentrations when the samples were first drawn from the systems was less than 10 CFU *Legionella*/ml.

Occasionally the argument is given that results like these can be interpreted as a harbinger about the *potential* hazards in the building water system from which the sample was drawn. But this would be speculation. Such speculation is to be not recommended for building water system validation testing, in the authors' opinions. The *actual* hazards and

actual risks of disease associated with a *real* building water system should be at issue, not what might be.

Sample holding time effects may also cause the loss of *Legionella* in building water samples and such changes have been documented[9]. Neutralization of biocides and disinfectants with reducing agents such as thiosulfate or thioglycolate may not entirely remedy this problem but inoculation onto growth media immediately after sampling does inactivate them[20]. Inaccurate "false-negative" results due to the loss of viable *Legionella* during sample transit gives misleading information about the extent of hazard control that has been actually achieved in the building water systems.

Extent of Inaccuracies

What is the extent of inaccuracies in *Legionella* test results from real building water systems?

In surveys of building water systems, many unpredictable changes in viable *Legionella* cell counts were observed even after the shortest holding time tested[21]. Forty-two water samples were drawn from building water systems including cooling towers, hot and cold potable water service in hospitals, university facilities, retail stores and office buildings. Samples were inoculated onto standard culture growth media for detection and enumeration of *Legionella*. After a sample holding time, t=t' (h) at ambient room temperature (18-22 °C), samples were inoculated onto growth media of the same type and analyzed again using exactly the same culture method and enumeration protocol. In 22 (52.4%) samples, the changes observed during sample holding time were significant (greater than one order-of-magnitude change). These changes resulted in either a detectable *Legionella* concentration or the loss of a detectable concentration in the water sample after the sample holding time.

It has been noted that these observed changes are the sum of both "analytical error" and "experimental error". The analytical error refers to that which occurs due to the inherent precision limitations in the method and the experimental error is that which occurs due to uncontrolled events during the sample holding time. The experimental error can be reduced or eliminated by culturing the sample immediately after it is removed from the building water system at "t=0".

In another small survey using the ISO 11731 spread plate method, sample holding time was shown to cause significant changes (greater than one order-of-magnitude change at 10CFU/ml LOQ) resulting in detectable *Legionella* concentration or the loss of a detectable concentration in the water sample at t=t'. In this survey, 33% (2 of 6) of the bathroom sinks sampled, 20% (1 of 5) of the cold water sinks, 33% (1 of 3) of the showers and 33% (1 of 3) of the wastewater systems changed significantly during the sample holding time (Table 1).

Table 1. Building water system samples were analyzed following the ISO 11731 spread-plating method at a detection limit of 10 CFU *Legionella*/ml immediately after removal ("t=0") and then after 24h, 48h and 72h (t=t') sample holding times at ambient room temperature.

Building Water System Samples	t=0 Culture Result	t=t' (24h, 48h, 72h) Culture Result
Bathroom Sink #1	Not Detectable	No Change
Bathroom Sink #2	Not Detectable	No Change
Bathroom Sink #3	Not Detectable	No Change
Bathroom Sink #4	Not Detectable	Detectable at t=24h; Not Detectable after t= 48h
Bathroom Sink #5	Not Detectable	Detectable at t=48hr
Bathroom Sink #6	Not Detectable	No Change
Coldwater Sink #1	Not Detectable	No Change
Coldwater Sink #2	Not Detectable	Detectable at t=24h
Coldwater Sink #3	Not Detectable	No Change
Coldwater Sink #4	Not Detectable	No Change
Coldwater Sink #5	Not Detectable	No Change
Shower #1	Not Detectable	No Change
Shower #2	Not Detectable	No Change
Shower #3	Detectable	Not Detectable after t=24h
Wastewater #1	Not Detectable	Detectable at t=48h
Wastewater #2	Not Detectable	No Change
Wastewater #3	Not Detectable	No Change

These surveys, however, are far too small and limited to make any statistical conclusions. It was impractical (far too expensive and time-consuming) to run ISO method spread plates on-site at thousands or even hundreds of building water systems. Therefore, a field culture sampler was developed and used in a survey of over two thousand building water system samples.

Field Culture at "Time Zero"

The field culture device was a dipslide-format sampler and protocol that employs the same growth media and acid-treatment specifications as are given in the ISO spread plate method for *Legionella* detection and enumeration[21]. The dipslide method is a new and promising technique for on-site culturing of *Legionella* bacteria.

Culture media dipslide samplers are used for many other applications and the format is well known. Successful dipslide applications show statistically significant correlations with conventional spread plate methods for accuracy, positive and negative predictive values and with very similar sensitivity and specificity. Successful dipslide culture applications include surgical room diagnostics[22], at-home and in-clinic sampling protocols for urinary tract infections[23,24,25,26], for bronchoalveolar lavage (BAL) specimens to detect pulmonary infections[27], surface hygiene analysis[28], analyses in dentistry[29], hydrocarbon fuel system analysis[30], fecal coliforms analysis for wastewater[31], other water systems in general[32] and of course, in water treatment management for measuring total bacteria counts[16]. These dipslide-format applications are generally more rapid and more accurate (due to elimination of variance during sample transit and holding

time) or otherwise offer valuable benefits compared to conventional spread plates methods.

A concern sometimes expressed about dipslide-format field culture diagnostics is precision: numerical results are limited to order-of-magnitude resolution. This limitation is true and, as discussed previously in this paper, it is equally true for results from spread plate analyses and for precisely the same reasons (see Precision and Accuracy).

Another concern sometimes expressed about dipslide-format field culture diagnostics is in regard to the theoretical LOD; statements like "dipslides are not sensitive enough" are sometimes heard. As discussed previously in this paper, filtering massive volumes of sample and then homogenizing the remains to produce a specimen with substantially altered microbiological characteristics in order to advocate "greater sensitivity" (lower theoretical LOD) which in most cases is below the LOQ anyway, should be not recommended, in the authors' opinions, especially for cooling water samples (see Theoretical Limit of Detection).

Performance of the field dipslide-culture sampling protocol for detection and enumeration of *Legionella* was evaluated using standard working curves of pure cultures and also in the standardized proficiency evaluation program, ELITE, administered by the CDC. Results of this work have been previously published[21]. Table 2 is redrawn and adapted from that work. Correlation of numerical results from the field culture method and the ISO spread plate method were statistically significant ($n=48$, $R^2=0.98$, $P<0.005$). Specificity and sensitivity of the dipslide method based on the numerical results of both methods was 100% and 98%, respectively.

Results in Table 2 are represented by their exponential value (order-of-magnitude) descriptors because, as has been previously described in this paper, precision is not sufficient to claim more significant figures by either method. It would be far better for the users of *Legionella* test results, in the opinions of the authors, if analytical laboratories would refrain from reporting numerical values that are not statistically significant. For example, 121 CFU/ml is not significantly different than 1×10^2 by either method because the "2 and 1 in 21" are not really significant figures. In spite of implying otherwise in the test report, those digits should be ignored and it would be better to not report them at all.

Table 2. Comparison of ISO and FIELD testing methods in the CDC Environmental *Legionella* Isolation Techniques Evaluation (ELITE) program. Correlation between methods was statistically significant correlation (n=48, R^2=0.98, P<0.005) and all samples passed the proficiency certification criteria.

ISO 11731 *Legionella* (CFU/ml)	CDC Reference Lab Result	CDC ELITE Score	FIELD *Legionella* (CFU/ml)
$>10^4$	Positive	Pass	$>10^4$
10^3	Positive	Pass	10^3
10^3	Positive	Pass	10^3
10^2	Positive	Pass	10^2
10^2	Positive	Pass	10^1
10^2	Positive	Pass	10^2
10^2	Positive	Pass	10^1
10^1	Positive	Pass	10^2
10^2	Positive	Pass	10^2
10^1	Positive	Pass	10^2
10^1	Positive	Pass	10^1
10^1	Variable	Pass	<10
10^1	Variable	Pass	<10
10^1	Variable	Pass	<10
10^1	Variable	Pass	<10
<10	Variable	Pass	10^1
<10	Variable	Pass	10^1
<10	Variable	Pass	10^1
<10	Variable	Pass	10
<10	Negative or Variable in 29 samples	Pass	<10

A large survey was undertaken to analyze over two thousand building water system samples. For the entire survey, 9.5% of the samples contained less than detectable concentrations of *Legionella* at t=0 that increased significantly to detectable concentrations by the time samples were received in the laboratory. Nearly 33% of utility water samples returned were in this category. In this data set, 2% of potable water samples analyzed at t=t' were in this category.

There were fewer false-negative results returned in this survey: 1% of samples returned were false-negative results and they were mostly (74%) from potable water samples.

Overall, accuracy of the results for water samples returned to the laboratory was 89.6%. There were many more false-positive results compared to false-negative results (10:1). Therefore, the negative predictive value of the results from the ISO spread plates was very good (99%) but the positive predictive value of results was very poor (36%).

Many of the false-positive ("amplified-positive") results were grossly amplified; that is to say, the difference between the t=0 result and the result after shipment to the laboratory was greater than two orders-of-magnitude. The images given in Figure 2 are examples of cooling water samples in which less than detectable concentrations were observed at t=0 but after 24h, the concentration of *Legionella* had increased four orders-of-magnitude. Table 3 is previously unpublished data showing that of all false-positive samples

observed, 31.3% of them were grossly amplified and this phenomenon was observed more than 2x more often from cooling water samples.

Table 3. Grossly amplified results due to sample holding time effects. There were <10 CFU *Legionella*/ml at t=0 AND $\geq 10^2$ CFU *Legionella*/ml at t=24h in these samples.

Building Water Systems Sampled	**Number of Samples (% of all false-positive results in the survey)**
All building water systems	72 (31.3)
Utility water systems*	65 (34.0)
Potable water systems	7 (14.3)

* mostly cooling tower water samples

Tables 4 shows summary interpretations about culture test results and general conclusions about the occurrence of results in the survey.

Table 4. Interpreting results from *Legionella* culture tests of building water samples.

Culture results from inoculation at t=0	Culture results from inoculation at t=24h	Interpretation Of Results	Occurrence Of Results in the Survey
Negative	Negative	No detectable viable *Legionella* were observed in the sample. The t=t' result is true-negative.	Overall about 84% of sample results were in this category.
Positive	Negative	Viable, detectable *Legionella* were present in the sample when it was first removed from the building water system but were not detectable after shipment to the laboratory. The t=24h culture result is false-negative.	Loss of detectable *Legionella* in shipped potable water samples occurred 3x more often compared to cooling water samples. About 1 of every 10 negative results was positive when the sample was taken from the building water system but negative when spread plate analyzed about 24h later.
Negative	Positive	Less than detectable concentrations of viable *Legionella* were present at t=0 but were amplified to detectable. This result can be called "amplified-positive" or "false-positive"	About 6 of 10 detections in shipped water samples were below detectable levels when cultured immediately after sample removal from the building water system.

Table 5 shows results from both culture and polymerase chain reaction (PCR) analyses to detect the presence of *Legionella* nucleic acid in building water systems. A great deal of useful information can be obtained from samples that have been analyzed by both culture and by PCR. Note that for samples with positive PCR and negative culture results, the indication from the data is highly relevant for systems in which hazard control, such as biocide additions, are being used.

Table 5. Interpreting results from *Legionella* Polymerase Chain Reaction (PCR) and *Legionella* tests of building water samples.

PCR	Time Zero Culture	Interpretation Of Results	Occurrence Of Results in the Survey
Negative	Negative	The most definitive evidence that the sample contains no detectable *Legionella*	Overall, about 9 in 10 negative PCR results were also culture-negative. Therefore, PCR results were a useful negative screen especially for potable water samples (negative predictive value was >90%).
Positive	Negative	The sample contains non-viable "dead" or injured (viable but are not culturable, VBNC *Legionella*) that if not continuously controlled may result in the appearance of viable potentially dangerous *Legionella* in the building water system. Results indicate that hazard control is inactivating a significant quantity of *Legionella*	Overall, about 40% of samples had killed or injured *Legionella* present in the sample that were not viable when the culture was inoculated in the field immediately after removal from the building water system at "time zero". This category of results can be very useful information for water treatment professionals.
Negative	Positive	PCR result not valid. PCR reactions were inhibited. Results not interpretable.	Invalid PCR results occurred in about 1 of 10 potable water samples and in about 3 of 10 cooling water samples. Overall, about 3.6% of PCR results were invalid.

Decisions about hazard control based on realistic and scientifically defensible use of *Legionella* test results are necessary for successful application of hazard analysis and control to prevent building-associated legionellosis.

Hazard Analysis and Control Recommendations for Cooling Towers

Facility managers/owners are responsible for hazard analysis and control of cooling towers in their facilities. The hazard analysis and control process requires them to make certain that known hazards, such as *Legionella* bacteria, have been controlled. The burden of testing and decisions about how much testing to do, if any, is on the facility manager/owner. The authors' recommendations for hazard analysis and control of cooling towers are given as follows.

Hazard Analysis

The cooling water system should be schematically described in a process flow diagram as to its placement and process function within the utility water system of the facility.

The risk of exposure to *Legionella* from the cooling tower should be characterized: the facility manager/owner must decide based on input from technical advisors, suppliers, the literature[33] and in-house professionals whether or not the risk of legionellosis from the cooling tower is significant, Yes or No? (note: since the risk of legionellosis cannot be quantitatively assessed, this is a qualitative characterization). If the risk is judged to be significant, then hazard control measures must be applied and if so, then the cooling tower is to be designated a Critical Control Point (CCP).

Hazard Control

Hazard control measures should include treatments using (for examples) chemical biocides, penetrants, dispersants, deposit control agents, etc., to be determined according to site-specific considerations by water treatment professionals[33] and applied with full knowledge and written approval of the facility manager/owner. The four parameters required for all cooling tower treatments are:

- critical limits (e.g., the concentration ranges),
- method of monitoring the treatment critical limits,
- frequency of monitoring the treatment critical limits and
- the corrective actions* to be taken if any critical limit is violated

*note that "monitoring" and "corrective actions" refer to hazard control treatments, not to results from *Legionella* tests

Validation

Cooling tower systems can be very dynamic. Based on many years of experience with these systems, it is our opinion that all operating cooling towers should be tested for *Legionella* once per quarter by cultured samples inoculated immediately after the sample is removed from the cooling tower ("time zero" culture). Cooling tower samples should not be concentrated by membrane filtration. Total heterotrophic aerobic bacteria (THAB) test results should be obtained from the same cooling tower sample at exactly the same time and from the same sample as the *Legionella* test from inoculations immediately after removal from the cooling tower.

Validation provides defensible evidence (data) that hazard control and good microbiological quality has been achieved. Validation criteria for cooling towers from cultures started immediately after removal from the cooling tower should be, in the authors' opinions, as follows:

- less than detectable *Legionella* at 10 CFU/ml
- less than 10^5 CFU/ml THAB

If these validation criteria are violated, then the hazard control plan should be immediately reassessed and upgraded. After the microbial control program upgrade is implemented, then the cooling tower should be retested. This should be repeated until hazard control has been achieved.

Additional Validation

More evidence can be obtained from testing the same samples with both culture (for viable *Legionella*) and PCR (for total, non-viable+viable *Legionella*). Negative culture results and positive PCR results from the same sample indicate that the hazard control method has resulted in a great deal of productive hazard control (refer to Table 5). This can be helpful to facility managers/owners to prove that microbial control has been achieved. Consider doing this validation test at least once per year.

Verification

Verification is confirmation that the hazard analysis and control plan outlined above has actually been implemented. Water treatment professionals should ensure that adequate records are maintained in easily retrievable formats in full knowledge and regular reviews with the facility manager/owner. Records must include process flow diagrams of the utility water system, hazard analysis summaries, all critical limit monitoring data, corrective action logs and validation evidence.

Formulae

Binary statistical formulae are:

Accuracy = (true-positives+true-negatives)/total samples
Specificity = true-negatives/(true-negatives+false-positives)
Sensitivity = true-positives/(true-positives+false-negatives)
Positive predictive value = true-positives/(true-positives+false-positives)
Negative predictive value = true-negatives/(true-negatives+false-negatives)

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