Nephros PluraPath™ Detects Low levels of Legionella in Water

Introduction

PluraPath™ is a portable, quantitative polymerase chain reaction (qPCR)-based water-borne pathogen detection system that can provide data onsite in approximately one hour. The PluraPath™ system is designed to provide actionable data to infection control teams on up to 15 different pathogens in one test. In this study, the PluraPath™ system was used to detect different concentrations of *Legionella pneumophila* in water samples. The results were compared to both traditional plate-based culturing method and standardized laboratory-based qPCR test. This study demonstrates that PluraPath™ has comparable sensitivity for the detection of *Legionella pneumophila* in water samples to both plate-based culturing methods and laboratory-based qPCR tests at concentrations as low as 100 CFU/ml and can deliver results in less than an hour.

Methods

Culture: A culture of *Legionella pneumophila* Philadelphia-1 strain was streaked on plates containing ACES-Buffered Charcoal Yeast Extract (ABCYE) Agar and allowed to grow for approximately 72 hours.

Stock solutions: Colonies were removed using a sterile loop and placed in 3 milliliters of sterile, filtered water. 1 ml was used to take an optical density reading on a spectrophotometer set to 600 nanometers (OD600). A sample of the stock solution was used to perform 10-fold dilutions for plate counting to determine the colony forming units (CFU) per milliliter (1). Additional 10-fold dilutions of the stock were made using sterile, filtered water to obtain two additional concentrations of secondary stocks.

Water samples: Three 1-liter water samples were prepared for use with the PluraPath™ system by adding 100 microliters of the stock and two secondary stock dilutions to each of the 1-liter water samples.

PluraPath™ System

Filter concentration: Water samples were concentrated by using a peristaltic pump to pull the water volume through a Nephros Filpath™ Ultrafilter.

Sample lysis: 15 milliliters of a lysis buffer was pushed through the filter using a sterile 20 milliliter Luer-lock syringe, retained for approximately 30 seconds inside the filter, and then the residual lysis buffer was extracted back into the 20-milliliter syringe. The filter concentrate was placed in a sterile 50-milliliter conical centrifuge tube.

qPCR: 25 microliters of the lysed water sample were pipetted into each tube of an 8-tube strip containing dried Roche Custom Biosciences HawkZO5 Fast master mix with enzyme, GenArraytion, Inc., primers and probe for *Legionella pneumophila* (for all serogroups) labeled with FAM, primers and probe for an internal positive control labeled with HEX, and internal positive control target template. The specific target of GenArraytion's assay is proprietary to GenArraytion, Inc., and not identified here. qPCR was performed using a Chai Biosciences Open qPCR™ machine. Two replicate qPCR

reactions were performed on the lysate from the 1-liter water samples for the 10^4 and 10^3 dilutions and four replicate qPCR reactions were performed on the lysate from the 1-liter water sample for the 10^2 dilution.

CFU per milliliter estimation: The CFU per milliliter estimation from Cq and Ct values for each 1-liter water sample and replicates of same was performed by first estimating the starting *Legionella* target DNA copies present in the PCR reaction volume (2), and then performing a series of back-calculations estimating the number of CFU per milliliter of *L. pneumophila* present in each of the three 1-liter samples of water concentrated on the FilPath™ filters.

qPCR using *mip* Gene: a standard curve was generated using genomic DNA from *Legionella pneumophila* Philadelphia-1 quantified using a Nanodrop spectrophotometer and amplification with primers for the macrophage infectivity potentiator (*mip*) gene. Aliquots of the three 1-liter water sample dilutions were also tested using the *mip* gene primers alongside standards.

Results

The OD600 of the stock solution measured .776 and based on this estimated to be approximately 1.09 x 10^9 CFU per milliliter *Legionella* while plate spotting culture yielded an average count of 2.85 x 10^9 CFU per milliliter, ranging from 2.7 x 10^9 to 3.0 x 10^9. The three 1-liter water samples were therefore estimated to contain approximately 2.85 x 10^4, 2.85 x 10^3, and 2.85 x 10^2 CFU per ml, respectively. Spot dilution counts were not performed on each 1-liter water sample dilution as the entire volumes were used to produce lysates for qPCR analyses.

Table 1 below shows the resulting *Legionella* assay Cq values, corresponding internal positive control (IPC) Cq values for each of the eight tests conducted and the associated dilution of the 1-liter water sample tested. The IPC Cq values ranged from 21.64 to 22.76 (mean Cq value 22.24). Sample eight did not produce a Cq value and showed no amplification in channel 1 (FAM), the *Legionella* assay (see Figure 1). Figure 1 below is a screen capture of the Open PCR graphic user interface showing the amplification plots and Cq values. Table 2 below shows the calculated CFU per milliliter estimations from PluraPath™, estimated spot plate counts, and *mip* gene copies per milliliter calculated from results of the qPCR using primers for the *mip* gene. Figure 2 graphically illustrates the average estimated CFU counts for each methodology.

qPCR Tube	Sample Cq	IPC Cq	Dilution
1	26.93	21.64	10^4
2	28.22	21.72	10^4
3	31.33	21.71	10^3
4	32.56	22.48	10^3
5	36.86	22.60	10^2
6	37.34	22.67	10^2
7	36.60	22.76	10^2
8	-	22.41	10^2

Table 1. Sample Cqs and IPC Cqs for three 1-liter water sample dilutions using PluraPath™ system. Two replicate qPCR reactions were performed on the lysate from the 1-liter water samples for the 10^4 and 10^3 dilutions and four replicate qPCR reactions were performed on the lysate from the 1-liter water sample for the 10^2 dilution.

Average Estimated CFU per ml using PluraPath™	**Estimated CFU per ml from Spot Dilutions***	**Average Estimated CFU per ml using *mip* Gene and ABI One Step Plus™**
1.38×10^4	2.85×10^4	1.36×10^4
1.36×10^3	2.85×10^3	2.39×10^3
9.63×10^1	2.85×10^2	2.25×10^2

Table 2. Average estimated CFU per ml of *Legionella* for three 1-liter water sample dilutions using the PluraPath™ system, approximate estimated spot dilutions, and using the *mip* gene on the ABI One Step Plus™.
*Spot dilution counts were made on the stock dilution yielding 2.85×10^9 CFU per milliliter. Spot dilution counts were not performed on each 1-liter water sample dilution as the entire volumes were used to produce lysates for qPCR analyses.

Figure 1. Screen capture of Open PCR™ graphical user interface showing fluorescence curves and Cq values for channel 1 (FAM) and channel 2 (HEX) for *Legionella pneumophila* Philadelphia-1 from 1-liter water sample dilutions. Two replicate qPCR reactions were

performed on the lysate from the 1-liter water samples for the 10^4 and 10^3 dilutions and four replicate qPCR reactions were performed on the lysate from the 1-liter water sample for the 10^2 dilution.

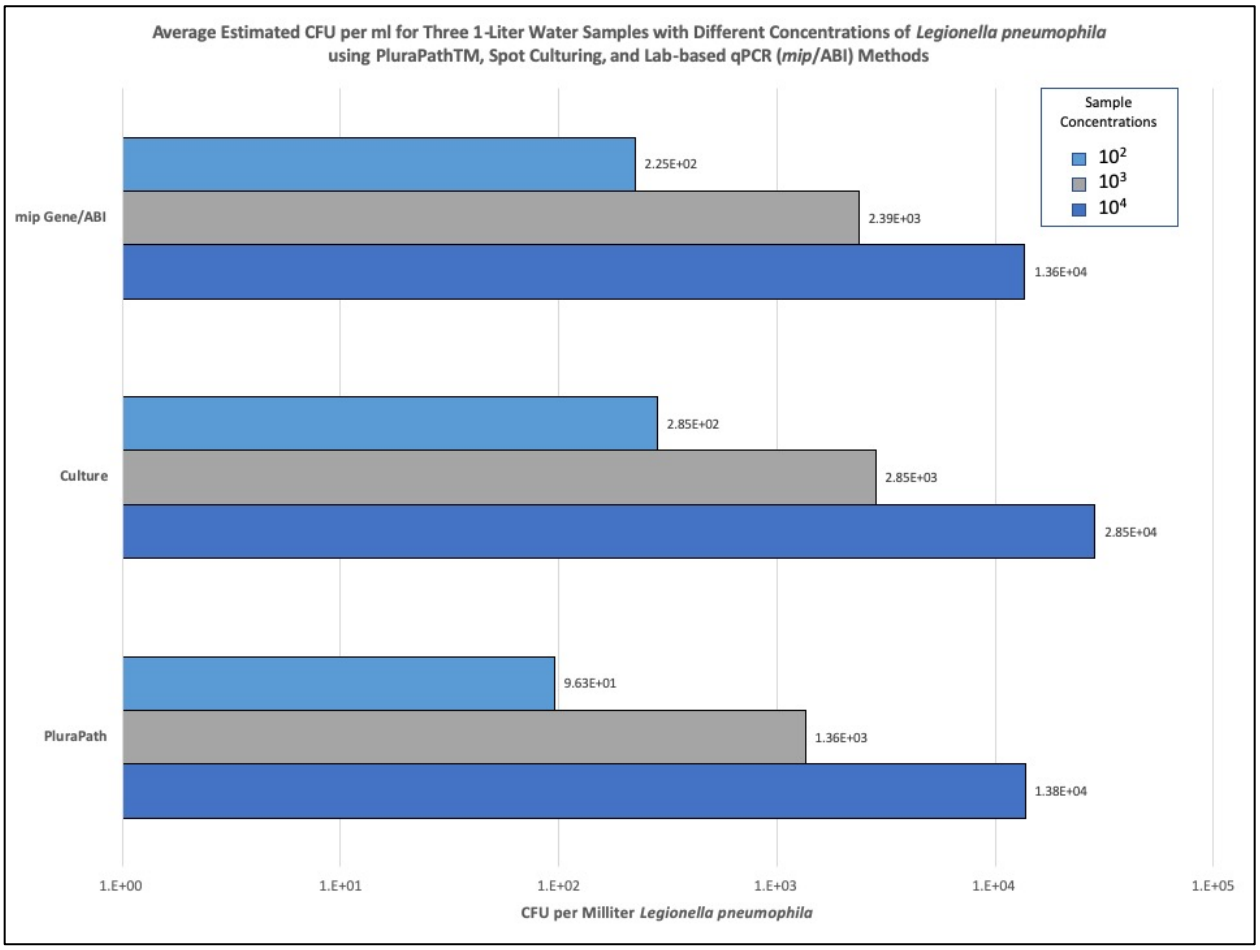

Figure 2. Average estimated CFU per ml of *Legionella* for three 1-liter water sample dilutions using the PluraPath™ system, approximate estimated spot dilutions, and using the *mip* gene on the ABI One Step Plus™.

Discussion

This study compares the results of CFU per milliliter estimates of the waterborne pathogen *Legionella pneumophila* Philadelphia-1 strain using the PluraPath™ system, traditional spot dilution culturing method, and a laboratory-based qPCR method using the *mip* gene and an ABI One Step Plus™ system. The PluraPath™ system incorporates a filter concentration capability

that makes it possible to detect waterborne pathogens at low concentrations with comparable results to culture and qPCR laboratory-based methods. Two clear advantages of the PluraPath™ system are portability, giving the ability to perform testing onsite, without the need to collect and ship samples, and, time saving by performing the entire procedure in about an hour (see Figure 3). Additionally, the PluraPath™ system is complete with no need for additional laboratory support onsite.

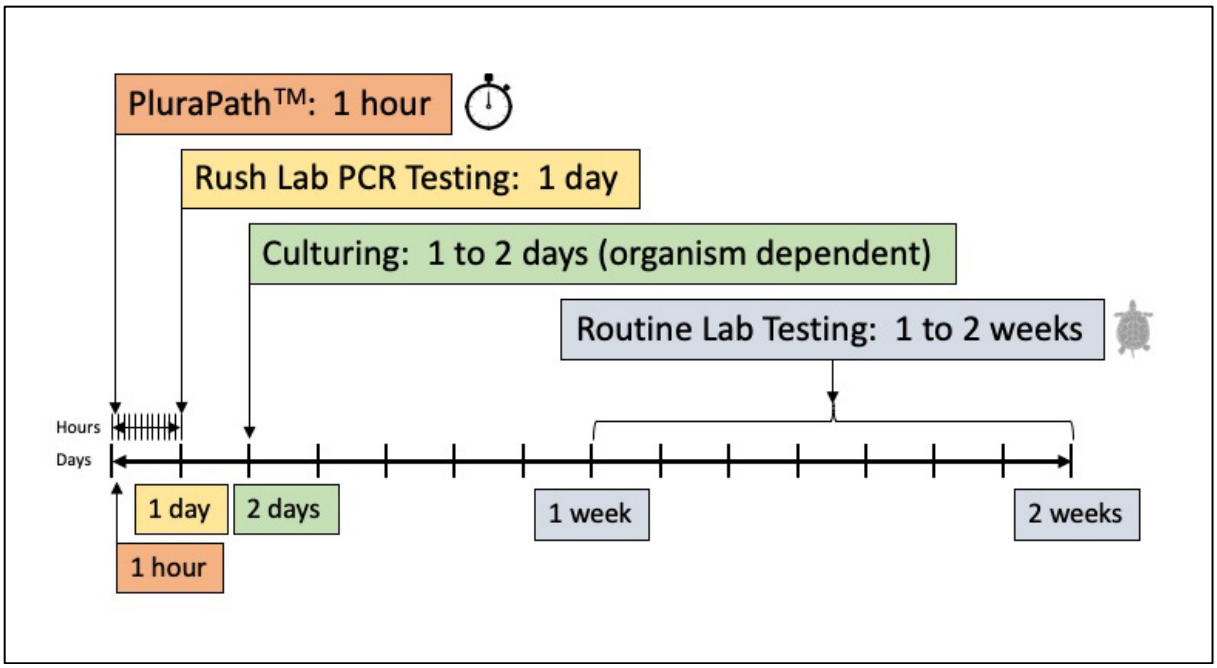

Figure 3. Timeline comparing PluraPath™ to time required for other forms of testing, culture and PCR lab based.

The PluraPath™ system detects the presence and estimated quantities of waterborne pathogens but cannot distinguish between live and dead organisms. qPCR measures DNA (and or RNA) copy numbers – molecular targets – which are not a direct measurement of viable organisms (3). However, monitoring of molecular targets provides precise, quantitative information over several orders of magnitude about organisms viable at some point upstream in the water distribution system, while also capturing viable but nonculturable organisms.

Nephros PluraPath™ testing strips incorporate internal positive controls (IPC) to confirm that the qPCR testing has functioned correctly and specifically provide a quantification of PCR inhibition from substances such as heavy metals in water samples if present (see Table 1, IPC Cq). If PCR inhibitors are present in tested water samples, the IPC Cq value will be elevated over the

expected value or, if severe enough, there may be no IPC Cq value returned from the PCR analysis. In cases of mild PCR inhibition with slight IPC Cq elevation (< 3 Cq), adjustments are made in the calculation of the estimated CFU per milliliter of microbes being tested. In this case the mean IPC Cq was 22.24 +/- 0.32 95% confidence interval (well within the expected range from observed normal testing) with a median value of 22.44. In cases where PCR inhibition is so extreme as to elevate the IPC Cq above 25 or be totally absent, further testing and mitigation must be employed to eliminate heavy metals or other PCR inhibitors before accurate qPCR results can be obtained from the PluraPath™ system.

Conclusions

The PluraPath™ system fits into an overall water safety management program strategy in healthcare facility plumbing in the following ways:

- As a rapid, cost-effective tool to establish the baseline at which microbial organisms are present and at what levels (using PluraPath™ Multi-strip testing for multiple waterborne pathogens) either proactively or in an outbreak response
- As a screening tool to identify where additional, extensive water sampling must be performed using conformational laboratory testing with culturing and molecular techniques
- As a confirmation tool after mitigation and treatment measures have been taken as a routine monitoring tool used monthly or quarterly to ensure microbial presence and level are within compliance limits (using PluraPath™ Mono-strips for single organisms or Custom-strips with only those organisms of concern in specific premises)

This study has examined the results of PluraPath™ system testing compared to spot culture and laboratory-based qPCR methods for estimating CFU per milliliter in 1-liter water samples containing dilutions of *Legionella pneumophila* Philadelphia-1 strain. The results obtained show that PluraPath™ provides roughly equivalent estimations of CFU per milliliter of *L. pneumophila* to lab-based methods with a considerable time savings and the advantage of providing portable testing onsite at the point of need. PluraPath™ system testing provides an integral part of a complete and integrated water safety management plan and program with outbreak response, pathogen presence and quantification baselining, treatment and mitigation assessments, and routine monitoring.

References

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