Design and Validation of a Real-Time PCR Assay to Detect *Legionella pneumophila*

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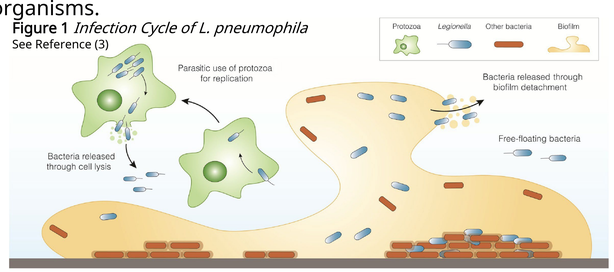
Introduction

Background

- *Legionella pneumophila* is an aerobic, gram-negative, rod-shaped bacterium that lives in warm water systems.
- *L. pneumophila* most commonly causes Legionnaires' disease and Pontiac fever.
- Legionnaires' disease typically consists of symptoms like cough, fever, headaches, muscle aches, and shortness of breath.
- *L. pneumophila* is not spread person to person, but is very prevalent in human-made building water systems due to being a bacterium that occurs naturally in freshwater environments like lakes and streams.
- 97,104 confirmed Legionnaires' disease cases were reported from 2000 to 2021.
- Reported cases of Legionnaires' disease are most prevalent in summer and fall months.
- There is around 5-10% mortality in the general population and up to 25% or more in healthcare-associated outbreaks.
- There is a macrophage infectivity potentiator gene within *L. pneumophila* that encodes for a 24-kDA protein virulence factor which aids in infection of amoebae and macrophages.
- The *mip* gene provides unique DNA sequences that differentiate *L. pneumophila* from other Legionella species as well as other organisms.

Figure 1 *Infection Cycle of L. pneumophila*
See Reference (3)



Aims

- This research aimed to develop a qualitative real-time PCR molecular assay to detect *Legionella pneumophila* that has fast turnaround times and accurate detection.

Methods

- Primers and a probe were designed using the National Center for Biotechnology Information (NCBI) to amplify a section of the *mip* gene in *L. pneumophila*.
- This was multiplexed with *RNase P*, a human internal control gene, to ensure proper amplification.
- Human DNA (Coriell Life Sciences) and *L. pneumophila* DNA (ATCC 33152D-5) were used to ensure that the designed primers and probes amplified the desired targets.
- Samples consisting entirely of Human DNA and *L. pneumophila* DNA were tested, as well as samples with varying quantities of Human DNA and *L. pneumophila* DNA combined.
- Each reaction consisted of multiplexed primers and probes, TaqMan Fast Advanced Master Mix, and nuclease-free water (NFW), totaling 15 µL. The remaining 5 µL was for control or patient DNA, respectively.
- Each run contained a No Template Control, Positive Control, and Negative Control loaded in the following manner:
  - No Template Control: 5 µL NFW
  - Positive Control: 5 µL of 50/50 mix of Human and *L. pneumophila* DNA, equating to 25 ng DNA total
  - Negative Control: 5 µL of Human DNA (25 ng)
- Mock patients were run at 25 ng, which was the ideal loading mass for the assay.

Methods Cont.

- The assay was optimized and validated using the default Fast Real-Time PCR settings from the QuantStudio 5 system, as shown in Figure 2.
- Unblinded and blinded runs were utilized throughout validation, using three different operators to control for individual operator differences.
- Accuracy was determined by comparing the results from the blinded runs and their true results.

Figure 2 *Real-Time PCR Settings from QuantStudio 5*

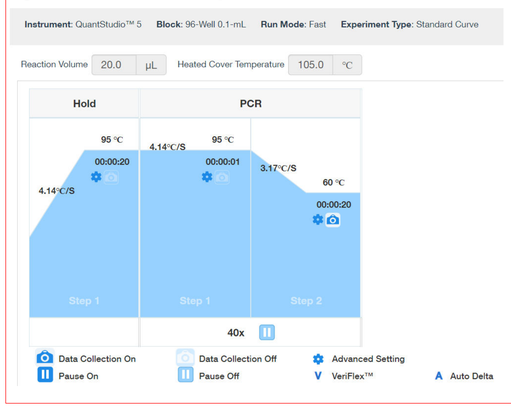


Table 1 *L. pneumophila Primers and Probe Sequences*

L. pneumophila Primers and Probe	
Forward Primer	5' GTTATCCTGGATGGACAGAAG 3'
Reverse Primer	5' TTATGTTCCCTCAGGTCTTGC 3'
Probe	5' ATGCCAGCTGGATCAACTTGGGAA 3'

Table 2 *RNase P Primers and Probe Sequences*

RNase P Primers and Probe	
Forward Primer	5' GCGTAGCCATCCTGGAAGTT 3'
Reverse Primer	5' TGGCAAAATCAGAGCCAGGT 3'
Probe	5' CAAGCCTGTACACACTAGCATTGTC 3'

Results

Table 3 *Assay Performance Criteria*

Criteria	Performance
Accuracy	99.35%
Intra-Run Precision	1.06 %CV
Inter-Run Precision	1.06 %CV
Specificity	100%
Sensitivity	2.5 x 10^{-6} ng

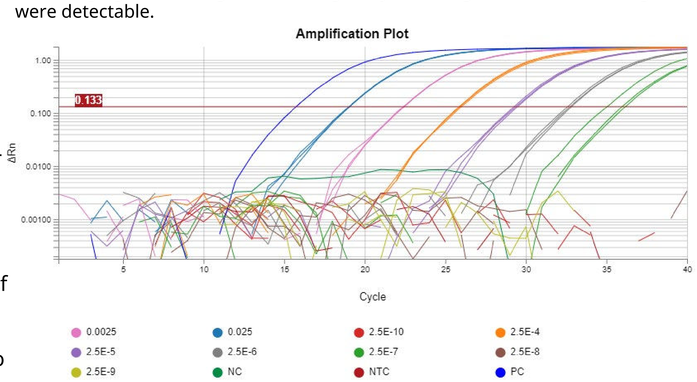
Accuracy, Precision, and Specificity

The concordance was 99.35% in both positive and negative samples (n=459). Inter-run precision and Intra-run precision for *L. pneumophila* (n=126) was determined to be 1.06% over 7 validation runs. *L. pneumophila* did not experience cross-reactivity with other relevant respiratory pathogens. The pathogens tested were *Acinetobacter baumannii, Pseudomonas aeruginosa, Aspergillus fumigatus*, and *Shigella flexneri.* Pathogens were selected based on symptom similarity. There was no interference with the expected *L. pneumophila* amplification in the presence of other pathogens. Therefore, specificity was determined to be 100%.

Limit of Detection

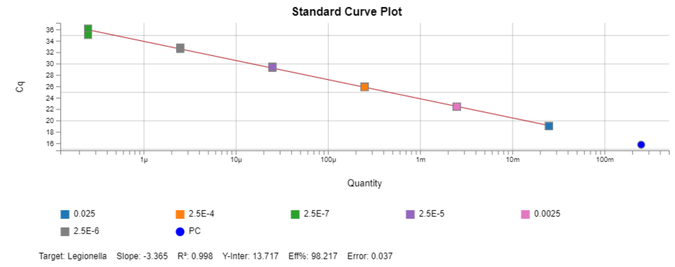
- Standard curves of 1:10 dilutions were analyzed and established the point at which our assay detected the dilution ≥95%
- The number of samples run for the generation of a standard curve was 126 over 7 runs
- At a mass of 2.5 x 10^{-6} ng, 23 out of 24 samples were detected, for a detection rate of 95.83%
- Because of this, the sensitivity of our assay was determined to be at 2.5 x 10^{-6} ng

Figure 3 *Amplification Plot of Legionella pneumophila Standards.* 1:10 dilutions with DNA ranging from 2.5 ng to 2.5E-10 ng. Only masses greater than 2.5E-7 ng were detectable.



Results Cont.

Figure 4 *Standard Curve Plot of Legionella pneumophila.* The following figure was generated during a 1:10 standard dilution, with a DNA mass range of 2.5 ng to 2.5E-10 ng. Only masses greater than 2.5E-7 ng amplified.



- Blinded, unblinded, and standard curve runs with known and unknown samples to the technologist were run to ensure accurate detection. The blinded samples consisted of samples that had 1ng, 2.5ng, and 4ng of *L. pneumophila* DNA. In total, 459 samples were tested during validation.

Table 4 *Expected Results vs Actual Results for All Validation Data* The following table was generated from all the results collected during the validation of the assay, and how the results were classified for data analysis.

	Positive (Expected)	Negative (Expected)
Positive (Actual)	269	3
Negative (Actual)	0	187

Results Summary / Discussion

Our data showcases an analytical specificity of 100%, an analytical sensitivity of 2.5 x10^{-6} ng , precision at 1.06 %CV for Inter- and Intra-run, and accuracy at 99.35%, which shows that this assay has great sensitivity and specificity for detecting *Legionella pneumophila*. In our accuracy study, the 3 false positives that were detected were below our LOD of 2.5 x10^{-6} ng of *L. pneumophila*. Values that are below our LOD would not be resulted as positive. Any results below our LOD would not be accurately detected 99.35% of the time and would need to be classified as negative for this specific assay. This research demonstrates that this qualitative Real-Time PCR assay can serve as a useful method in the detection of *Legionella pneumophila*.

Conclusion/ Future Studies

- Our assay shows promising analytic sensitivity and specificity for the detection of *Legionella pneumophila*.
- If we had more time, our next step would be to test reagent stability and potential PCR inhibitors such as dirt.
- Testing different extraction methods on different sample types could also benefit for clinical applications.
- Ideal sample types would include Sputum, Bronchoalveolar lavage fluid (BAL), and Tracheal aspirate.
- To prepare this assay for a clinical application, real patient samples need to be ran to prove the test can accurately identify, diagnose, or predict a *L. pneumophila* infection
- With more clinical testing and validation, this assay could be utilized in diagnosis and personalized patient treatments.

References

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2. Wilson, D. A., Yen-Lieberman, B., Reischl, U., Gordon, S. M., Procop, G. W. (2003). Detection of *Legionella pneumophila* by real-time PCR for the *mip* gene. *J. Clin Microbial, 41*, https://journals.asm.org/doi/full/10.1128/jcm.41.7.3327-3330.2003
3. Gonçalves, G. I., Simões, C. L., & Simões, M. (2021). Legionella pneumophila. *Trends in Microbiology, 29*(9), 860-861.