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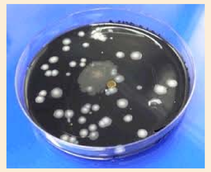
Comparison of the qPCR and the HybriScan *Legionella* assays vs. culture for the identification of *Legionella* sp. in non-potable water samples

Introduction

Legionnaire's disease is a severe form of pneumonia caused by *Legionella* sp., with the majority of cases due to *Legionella pneumophila*. Outbreaks and sporadic cases of legionellosis within communities have been attributed to the inhalation of contaminated, aerosolized water stemming from industrial buildings in close proximity to these communities. In order to evaluate and control the levels of *Legionella* sp. from these industrial buildings, constant monitoring of bacterial levels is recommended. Currently, the gold standard for identification and monitoring of *Legionella* sp. is by culturing samples onto selective and enriched media. However, this conventional method for *Legionella* sp. identification has its drawbacks, including long incubation time (up to 10 days), and its inability to grow viable but non-culturable cells.



NEORSD has two evaporative cooling towers which have a potential risk for employees to become exposed *Legionella* sp. There is a routing monitoring and maintenance schedule in place for these towers. There has been a concern voiced by employees of the length of time it take to obtain results from the culture method. The concern for the health and safety of employees the laboratory began to look at alternative methods that were quicker and could be used to monitor and screen samples along with the confirmation of *Legionella* pneumophila.

The laboratory began researching the methods using Quantitative PCR and other molecular methods like the HybriScan *Legionella* assay. A comparison study using both these methods along side the culture method on cooling water sample collected from the Southerly and Westerly Wastewater Treatment Facilities owned and operated by NEORSD. The samples were collected between January 2016 to April 2016. In addition to these samples comparison studies were also performed using known concentrations of *Legionella* for certified reference standards provided by the CDC

Sample Locations

Samples were collected from two NEORSD facilities, Southerly WWTP (SWWTP) and Westerly WWTP (WWWTP). The SWWTP has an odor control facility serving the solids handling building. The facility uses a fine mist scrubber to capture odors from the building. The water is captured and recirculated through the scrubber. A small portion of water is drained on a regular basis and replenished with city water.

The Westerly cooling tower provides cooling services the maintenance building. Samples were collected in duplicate at three location, feed water, resivior water and drain.

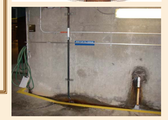
Both facilities have routine a routine maintenance schedule and add bromine tablets every quarter.

Sample Collection

The sample bottles were sterilized and contained sodium thiosulfate to remove and oxidizers.

The samples were checked for residuals at the laboratory prior to analysis. All samples were analyzed within the hold time of 24-48 hours.

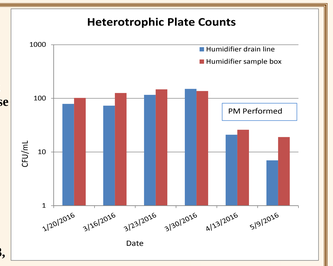
Approximately 500-1000ml of water were filtered through 0.20um polycarbonate filters. The membrane was mixed with 5ml of sterile water in a 50ml conical tube and vortexed for 1 minute. One mL of this sample was treated with 1mL of acid buffer (KCl/HCl solution) for 15—30 minutes. For comparison studies, known *Legionella* samples were mixed in 10.0mL of sterile water, and then serially diluted.

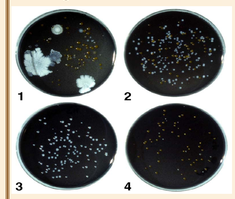


Analytical Methods

HETEROTROPHIC PLATE COUNT

Heterotrophic Plate Count (HPC) estimates the number of viable bacteria as colony forming units per milliliter (CFU/mL) of water using the pour plate or spread plate method with nonselective media. There is no direct correlation between HPC levels and Legionella concentration. However HPC results > 100,000 CFU/mL indicate ineffective microbiological control as sited in Guide to Legionella control in cooling water systems, including cooling towers, August 2003. HPC was used in the project to identify other bacteria that could compete with Legionella and cause a false negative result.





CDC CULTURE METHOD (METHOD #)

The culture method was performed according to the procedures provided by the CDC for ELITE program members. Samples were concentrated and acid treated then 0.1mL of sample was transferred onto BCYE, PCV (BCYE containing polymyxin B, cycloheximide, and vancomycin), and GPCV (PCV containing glycine) agar media and incubated at 35^{O}C. Cultures checked for suspect analyzed at 1, 2, 3, 7 and 10 days post-incubation. Suspect colonies were streaked onto BCYE media without L-cysteine and iron, a required component for Legionella to grow. If the colonies did not grow on this media they are presumptively identified as Legionella

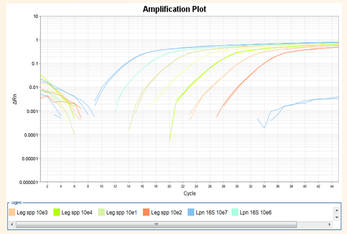
POLYMERASE CHAIN REACTION

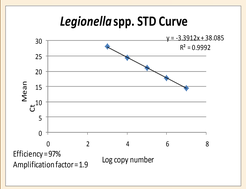
Polymerase Chain Reaction (PCR) is a genetic test which looks for the deoxyribonucleic acid (DNA) that is specific for Legionella. While PCR is not considered the "gold standard" for Legionella analysis, it may be very useful for quickly determining the presence or absence of Legionella in a sample. The samples were analyzed using three separate qPCR assays were utilized an, *Legionella* spp, *Legionella pneumophila* 16S, and serogroup 1.

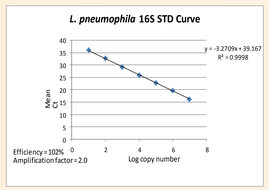
Procedure

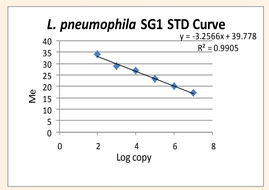
500—1000mL of samples were filtered through a 0.2um filter. The filter was placed into a 2.0mL tube containing glass beads, then mixed with 0.6mL of AE buffer. DNA was extracted using the bead beating method.

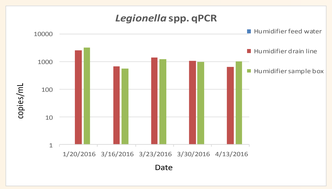
Five ul of sample DNA was added to the PCR reaction containing 1uM of each primer, 0.08uM of probe, and ToughMix master mix in a total volume of 25ul. PCR was performed using an Applied Biosystems StepOne Plus qPCR system using the following parameters: 95^{O}C for 10 minutes, 45 cycles of 10 sec 95^{O}C and 60 sec. at 60^{O}C, then 30 secs. at 40^{O}C. An internal control was included to monitor PCR inhibition.









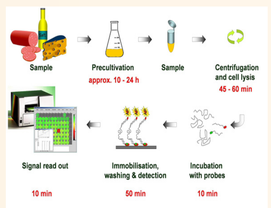


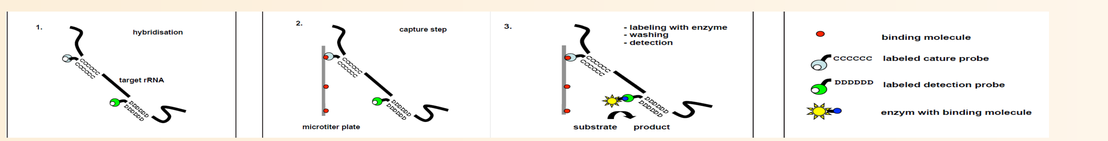
HYBRIDSCAN

The HybriScan kit is a rapid test system for organisms such as Legionella. **Sigma-Aldrich has developed a kit based on the detection of Legionella rRNA. The test is performed on a microtitre plate and takes less than 2.5 hours. Cell count quantification is possible with this kit using photometric methods. Compared to PCR, our system does not count dead cells**

Procedure

4.0mL of the sample filtered through the 0.22um membrane and re-suspended in sterile water was used for the assay. In brief, 4.0mL of sample was transferred into a tube containing glass beads and centrifuged. The pellet was collected and treated with lysis buffer. Extraction for rRNA was completed using a thermoshaker. Detection and capture probes were then added to bind to target rRNA sequences and subsequently to the binding molecules in the 96 well microplate and to the DIG horseradish peroxidase. The bound complex was observed after the addition of the TMB substrate. Results were read out photometrically using the SpectraMax 190 plate reader. Both qualitative and semi-quantitative results were calculated according to manufacturer's directions.





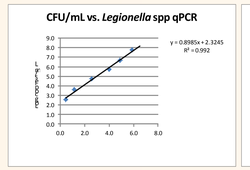
Conclusion

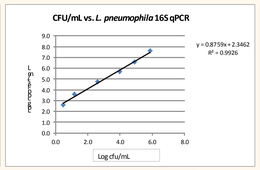
Comparison studies demonstrate good correlation between culture and qPCR, and limited correlation with the HybriScan vs. culture or qPCR. The HybriScan assay, however, detects viable cells only while qPCR can detect DNA from both viable and non-viable cells, which may account for the limited correlation.

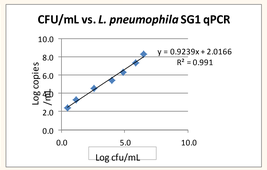
Both qPCR and the HybriScan test were able to detect the presence of *Legionella* spp. and *L. pneumophila* in greater sensitivity than the culture method. Additionally, results were obtained within the same day of testing, significantly decreasing the assay time from 10 days (culture method) to 1-2 days.

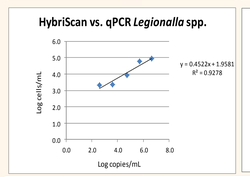
Results demonstrate the ability of the *Legionella* organism to survive over time despite environmental and mechanical changes.

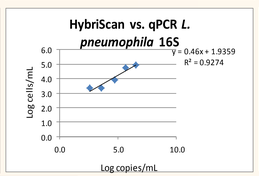
We conclude that qPCR and the HybriScan test are simple and rapid screening/confirmation tools for the assessment of water samples for *Legionella*. Because of their short turn-around-time, the use of one, or both, of these assays along with the culture method can lead to quicker response times for the treatment of facilities that may harbor the organism.

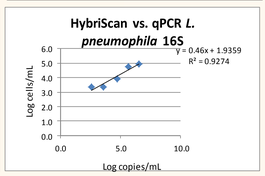






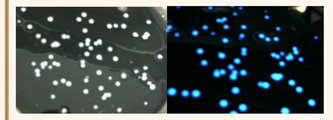


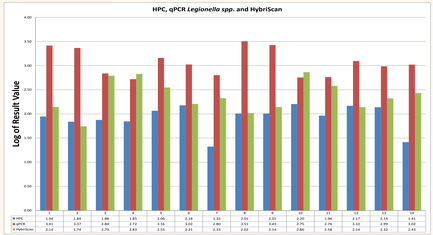


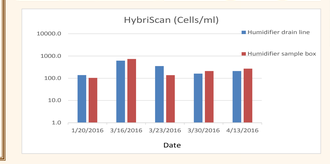


SAMPLE DILUTION	LOG CFU/ML ON PCV	LOG QPCR - LEG SPP	LOG QPCR - LPN 16S	LOG QPCR - LPN SG1	LOG HYBRISCAN - QUANTITATIVE
10^0	6.5	8.7	8.6	8.3	4.9
10-1	5.9	7.7	7.6	7.4	4.9
10-2	4.9	6.6	6.6	6.3	4.9
10-3	4.0	5.7	5.7	5.5	4.8
10-4	2.6	4.7	4.7	4.6	3.9
10-5	1.2	3.6	3.6	3.3	3.4
10-6	0.5	2.6	2.6	2.4	3.3
10-7	0.3	0.0	1.9	0.0	3.4
10-8	0.0	0.0	0.0	0.0	3.3

	Culture	qPCR - *Legionella spp*	qPCR - *L. pneumophila 16S*	qPCR - *L. pneumophila SG1*	HybriScan
Culture	1.00	0.96	0.97	0.97	0.97
HybriScan	0.97	0.90	0.92	0.91	1.00







Unknown Samples Provided by the CDC ELITE Program

SAMPLE ID	Culture Method	QPCR - Legionella spp. (copies/mL)	QPCR - Legionella pneumophila 16S (copies/mL)	QPCR - Legionella pneumophila SG1 (copies/mL)	HybriScan - Qualitative	HybriScan - Quantitative	CDC RESULTS	MALDI-TOF ID
D69-150113106CDC	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	<5000	NEGATIVE	None Identified
D90-150113115CDC	POSITIVE	1.90E+07	NEGATIVE	NEGATIVE	POSITIVE	84903	POSITIVE	*Legionella gormanii*
D93-150113125CDC	POSITIVE	1.60E+10	1.50E+10	NEGATIVE	POSITIVE	144300	POSITIVE	*Legionella pneumophila*
E01-150204122CDC	NEGATIVE	9.70E+07	1.20E+08	7.50E+07	POSITIVE	144300	POSITIVE	No Growth
E06-150204132CDC	POSITIVE	1.90E+07	1.40E+08	1.50E+08	POSITIVE	144300	POSITIVE	*Legionella pneumophila*
E17-150100606CDC	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	<5000	NEGATIVE	None Identified

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