ABSTRACT

TIMOTHY J. MUKODA. A Feasibility Study for the Detection of Airborne *Legionella pneumophila* Using the Polymerase Chain Reaction. (Under The Direction of Dr. LORI A. TODD)

Current methods to sample and analyze bioaerosols require that organism viability and culturability be maintained. This can lead to an underestimation of airborne bacterial concentrations when the sampling method has inherent qualities which damage or kill fragile microbes. The polymerase chain reaction (PCR) and associated analytical techniques require only intact cellular DNA to identify organisms contained in a sample; thus eliminating the requirement to maintain viability and culturability. This investigation has shown it is feasible to capture airborne *Legionella pneumophila* using either an AGI-30 impinger or a polycarbonate membrane filter, and identify and quantify the organism using PCR coupled with reverse dot-blot hybridization analysis and/or gel electrophoresis. In addition, the comparison of plate counts with acridine orange direct counts (AODCs), underscored the fact that traditional methods of analysis underestimate airborne bacterial concentration.

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Introduction

The term "Sick Building Syndrome" (SBS) has been used to describe a phenomenon related to indoor air quality that is characterized by symptoms including runny nose, eye and sinus irritation, sore throat, headache, fatigue, and dizziness. Complaints related to SBS usually originate in buildings that were designed to be energy-efficient and have climate control systems and permanently sealed windows. Many different indoor air pollutants have been implicated as causes of SBS including volatile organic compounds, combustion products, and bioaerosols. (13) In many instances, it has been difficult to link specific pollutants to the complaints; this is in part due to the fact that concentrations are usually orders of magnitude below documented adverse health effects and occupational exposure limits. For a given SBS episode, identification of a specific cause is complicated by the presence of many pollutants at very low concentrations in air.

In particular, specific bioaerosols can be difficult to identify and link to symptoms. Bioaerosols are known to elicit a variety of acute and chronic adverse health effects at low concentrations, they are always present in ambient air, and there are no widely accepted methods for detecting and quantifying low concentrations that may be contributing to SBS complaints. Bioaerosols, defined as vegetative microbial cells with reproductive units and metabolites that can be dispersed in air, include bacteria, viruses, fungi, algae and protozoa. (13) Bioaerosols can act as sensitizing agents eliciting an allergic response,

or as infectious agents causing disease in the affected individual. (13) Indoors, there may be an even greater risk of infection as a result of reduced dilution by air or inadequate ventilation, which increases the concentration of bioaerosols. (23) Factors such as crowding and degree of activity in an indoor environment may also contribute to levels of airborne bacteria , and any subsequent infectious risk. (26) An excess of airborne microbes, including bacteria, can have either little to no adverse health effect or a marked increase in adverse health effect. In the case of an innocuous bacterium, such as a member of the genus *Micrococcus*, increased airborne concentrations would not cause building occupants to suffer adverse health effects. However, when a bacterium is an agent of infectious disease, an airborne presence can have a markedly different effect. This can occur when bacteria normally found in air are present in excess, or when infectious bacteria, not normally present, are found in air. This would be the case for *Legionella pneumophila (L. pneumophila)*.

Airborne bacteria usually exist in droplets of respirable size as droplet nuclei. (39) It is extremely rare that they exist naturally as an aerosol of single cells; it is more likely they exist as aggregates of individual cells. (32) In many cases aerosols containing bacteria originate from liquid splashes or sprays, agitation of dusts, or coughs and sneezes. (27) Individual biological particles usually range in size from 1.0 to 50 microns. (21) Therefore, in most cases a droplet must have an aerodynamic diameter of at least 1.0 micron to contain an individual bacterium. (23)

Microorganisms, including many bacteria, thrive in any environ

mental reservoir that contains adequate moisture. (13) Therefore, any appliance or mechanical system with a water reservoir or drip pan is a potential source for microbial growth. (13) Devices such as humidifiers or vaporizers are especially problematic since they actively spray water droplets into the air, some of which may contain microorganisms. Any disruption of such a microbe-rich environment can cause the organisms to become airborne. In most cases, they will only survive a short time in the airborne state; however, many are opportunistic and, in some instances, parasitic, requiring a host organism for growth and reproduction. (13) Therefore, those microorganisms which are inhaled by a living host and are of respirable size stand some chance of survival. An inhaled dose of an aerosol, which is a non-infectious type or quantity, can be neutralized by the host organism's intrinsic defense mechanisms. However, if the dose exceeds an infectious level, and the defense mechanisms are overwhelmed, disease may result. It has proven difficult to determine precise levels at which disease results from exposure to an airborne agent. This underscores the need to develop techniques to sample and detect bioaerosols at the lowest possible detection limits.

The ubiquitous nature of bioaerosol, and the fact that there are no official exposure limits such as Threshold Limit Values (TLVs), (15,31) has caused health care professionals and industrial hygienists to be concerned with the presence of bioaerosols in concentrations greater than background. Unfortunately, an outbreak of infectious disease usually provides the first hint that there is an excess of

microbes in air. In addition, traditional bioaerosol sampling and analyses methods are not always sensitive enough to detect and quantify the low-levels of microorganisms in air that may be contributing to SBS related complaints.

At present, there are no direct-reading instruments to detect or measure airborne microorganisms, and few standardized integrated sampling methods. (15) Current sampling methods involve collecting organisms in a liquid medium or onto solid or semi-solid media (2) and culturing the cells to achieve observable numbers. If collected cells are not culturable, there can be significant underestimation of airborne microbial concentrations, or false negative results. The reliance on viable, culturable cells, dictates that collection and plating techniques result in minimal cell damage. (15) Obtaining accurate counts of organisms which can be cultured by plating may be complicated by a) specialized growth requirements of an organism which are absent in the culture media, b) metabolic byproducts formed by one organism which serve as growth inhibitors to another organism being cultured on the same plate, or c) contact suppression by adjacent growth points in neighboring colonies preventing further colony development. (31) An underlying assumption of quantifying viable, culturable organisms, is that non-culturable, viable or non-viable organisms do not cause disease; for many organisms this assumption is false. Viability, not culturability, is the measure of infectious potential for a bacterium such as *L. pneumophila*.

This paper reports on research to investigate the potential of a

new bioaerosol analysis method that may provide the ability to accurately quantify microorganisms at much lower levels of detection than is possible by current techniques. This technique, which is a major departure from current methods, uses deoxyribonucleic acid (DNA) sequences amplified by the polymerase chain reaction (PCR), and subsequent nucleic acid hybridization with DNA probes, to identify and quantify target microorganisms. PCR used with probe hybridization could provide an analytical option for use with current bioaerosol sampling methods. In theory, under optimum conditions of sampling and analysis, intact DNA from only a single organism could provide a template for DNA amplification. PCR has been used extensively as a research tool for the identification of microorganisms from environmental water samples. (5,6,7,8,9,10,11,40) This is the first report investigating the feasibility of adapting PCR to microbial cells captured from air.

For this study, *L. pneumophila* was selected as the target organism, and three different airborne concentrations of *L. pneumophila* were generated using a Collison 3-jet nebulizer in an airtight chamber. Chamber air samples were collected at each concentration using AGI-30 impingers and membrane filters. These methods were selected in the hope they would not interfere with subsequent PCR amplification. Samples were analyzed by PCR, and amplification was determined to be successful using gel electrophoresis and reverse dot-blot hybridization. The samples were also analyzed for culturable colony forming units (CFU) by the plate count method, and for total microbial counts using the acridine orange direct count (AODC) method. Results obtained by these analytical techniques were then compared.

Background

The Polymerase Chain Reaction

The Polymerase Chain Reaction, developed in 1987, is a procedure used to rapidly amplify specific DNA sequences. PCR mimics the natural DNA replication process to produce accurate copies of DNA sequences from single-stranded template DNA. (33) Assuming 100% efficiency, a succession of repetitive PCR cycles generates an exponential increase of a particular DNA sequence, with twenty cycles potentially yielding an approximate million-fold amplification of a given sequence. (4)

To use PCR, sequence information must be identified for a specific target DNA segment, see Figure 1. (3) For amplification to be effec-

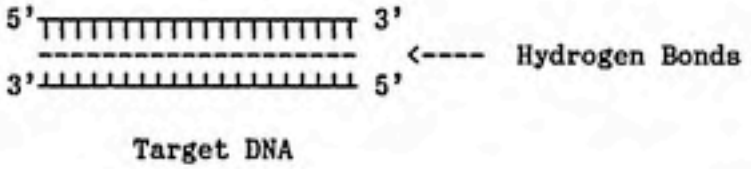


Figure 1. Segment of Target DNA Prior to PCR Cycle 1

tive, this segment must be unique to the organism of interest. It can be either a segment within an intact piece of DNA, or a DNA fragment generated by cleavage of an organism's genomic DNA using a restriction endonuclease. (4) Generally, the most effective PCR occurs when the

sequence of interest is between 100 to 500 base pairs (bp) in length. (29,37)

Once an appropriate DNA sequence has been identified, primers must be generated that are specific to the sequence of interest. Primers are oligonucleotides or short, single-stranded chains, made up of some combination of nucleotides (adenine, thymine, cytosine or guanine). They attach to a single-stranded DNA segment and provide a free 3' end to initiate the addition of nucleotides. The sequence of a particular primer is determined by the sequence of the DNA template at the boundaries of the region to be amplified. (33) The primers attach to complementary sites on the DNA template immediately flanking the sequence on which amplification is desired.

PCR consists of three steps, denaturation, annealing and extension, that are collectively known as a cycle. A typical PCR mix contains: a) target DNA, the template identified for amplification; b) extension primers which attach to sites flanking the target DNA sequence; c) DNA polymerase, an enzyme which performs the copying process; d) magnesium chloride which enhances the reaction; and e) an overlay of mineral oil which prevents evaporation of the reaction mix. (19) PCR is performed in an automated thermal cycler because rapid temperature changes are necessary at each step in a PCR cycle.

Denaturation, the first cycle step (Figure 2) involves high temperature incubation of double-stranded sample DNA, typically at 95° C. (33) The high temperature causes the hydrogen bonds between the individual DNA strands to break, creating separate, single strands of DNA, known as template.

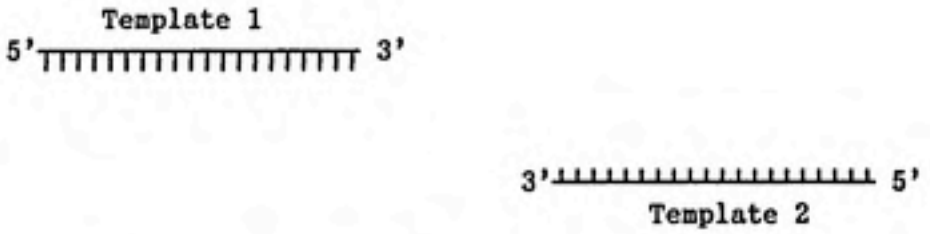


Figure 2. Hydrogen Bonds Broken in Target DNA During Denaturation in Cycle 1

Denaturation is followed by a lowering of the reaction temperature to facilitate annealing. During this step, the oligonucleotide primers anneal, or attach, to the separated DNA strands. Primers attach to opposite DNA strands, such that the 3', or "growing ends", are facing each other, see Figure 3. (8)

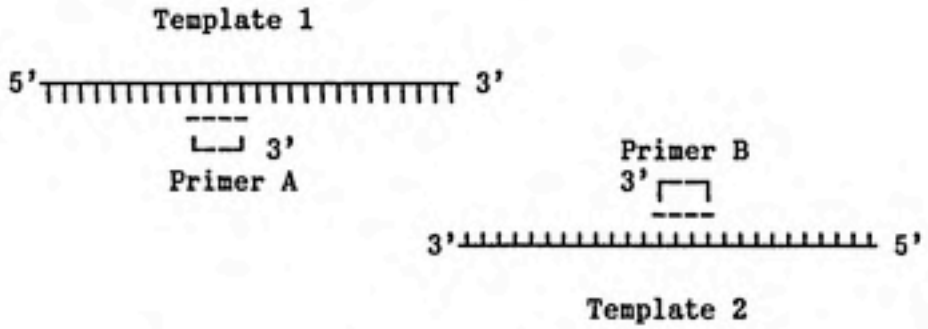


Figure 3. Primers Annealing to Denatured Target DNA in Cycle 1

PCR product is generated during extension, the final step of a PCR cycle. DNA synthesis proceeds from the free 3' end of each primer to the opposite end of the DNA strand, see Figure 4. (8) Extension is

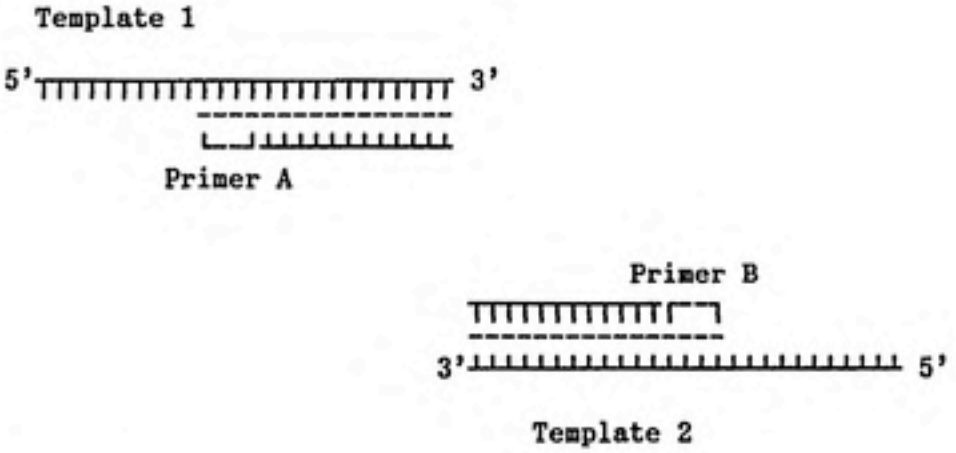


Figure 4. Extension of Target DNA and Formation of Long Product in Cycle 1

catalyzed by *Taq* DNA polymerase, an enzyme which initiates the copying process and sequentially adds individual nucleotides at the free 3' end of the primer/template complex. In Cycle 1, only long product is generated. This is double-stranded DNA which is made up of one complete and one shortened DNA template.

Denaturation of the double-stranded DNA formed in Cycle 1 creates two shortened single-stranded templates (Templates 3 and 4) having primer at one end, as well as complete single-strands of DNA (Templates 1 and 2; Figure 5).

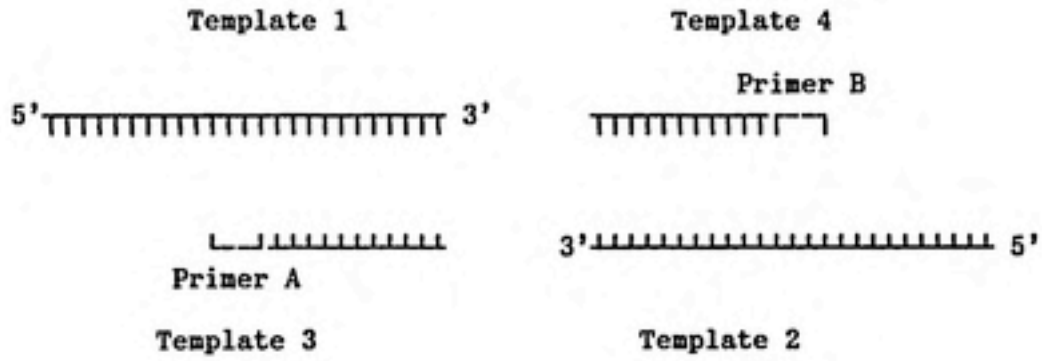


Figure 5. Denaturation of Long Product in Cycle 2

When annealing occurs in Cycle 2, primers bind to both the intact single strands of DNA and the abbreviated template strands, see Figure 6.

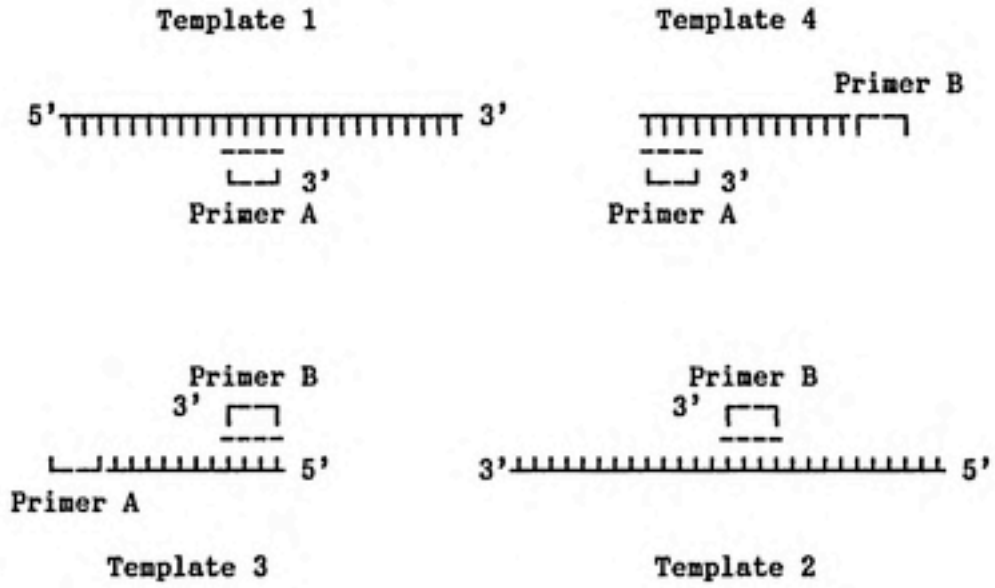


Figure 6. Annealing of Primers to Denatured PCR Product in Cycle 2

During extension in Cycle 2, long product is again formed, but in addition, short product is generated. In forming short product, DNA synthesis is initiated at the free 3' sites and terminates at the primer sites, see Figure 7. Short product is comprised of the lengths of the 2 primers plus the distance of the target DNA between the them. (20) This length is known as the target sequence. (14) Throughout the amplification process the quantity of original template remains constant because long product increases arithmetically, as opposed to exponentially. (33) In theory, following denaturation and reannealing in successive PCR cycles, all denatured strands of short product are available to act as substrates for further DNA synthesis. (41) Ideally, the amount of short product will double after every cycle leading to an exponential accumulation, so short product will be overwhelmingly abundant in comparison to long product. The PCR product can be analyzed using gel electrophoresis and/or nucleic acid probe hybridization to determine if the DNA target sequence was present in the sample and successfully amplified.

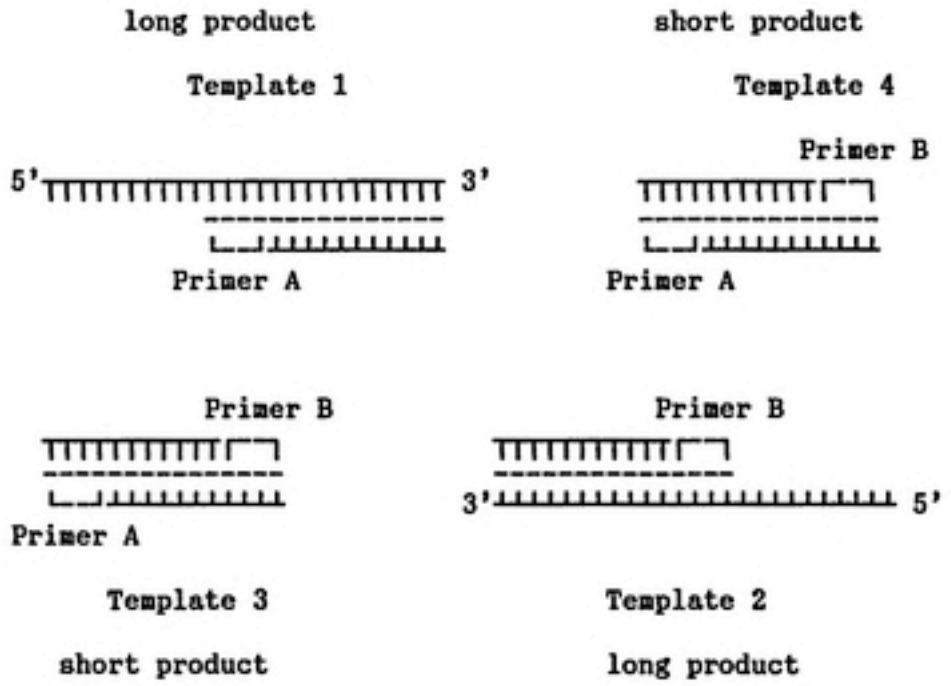


Figure 7. Extension and Formation of Long and Short PCR Product in Cycle 2

Materials and Methods

Target Organism

L. pneumophila was selected for this study because it is both an important agent of human disease and inhalation of contaminated aerosols is the suspected route of infectivity for this organism. (25) *Legionella* species are ubiquitous in all aqueous environments, including potable water supplies, and have been positively identified as the causative agent of Legionellosis, a respiratory disease afflicting humans. Several species of *Legionella* have been identified as causing respiratory disease, but approximately 85% of all documented cases are attributed to *L. pneumophila.* (30) The assumption that inhalation of aerosols generated from contaminated water is the route of transmission is based upon particle size analysis performed on aerosols containing *Legionella*, which have clearly shown the bacteria are contained in droplets of respirable size (16,24), and epidemiological studies that have associated presence of contaminated aerosols with outbreaks of disease. (17) The EnviroAmp^{tm} *Legionella* Sample Preparation, PCR Amplification and PCR Detection Kits were developed to detect *Legionella* directly from water, the environmental source of contamination. *L. pneumophila* was a natural choice for this investigation because of its airborne route of transmission and the existence of a kit to detect its presence by PCR.

Bacterial Growth and Count Determination

A freeze dried culture of *L. pneumophila*, subsp. *pneumophila* was obtained from American Type Culture Collection. A yeast extract broth (YEB) medium was used to rehydrate and grow the bacteria, as described in Appendix I. Approximately forty hours were allowed for exponential cell growth, after which time serial dilutions of stock culture were used to determine the number of colony forming units (CFU) and total organisms per milliliter of broth by plating (Appendix II) and AODC analysis (Appendix III) (28), respectively. Each serial dilution was plated on charcoal yeast extract (CYE) agar. 5.0ul aliquots at each dilution were plated and the cultures were incubated at 35° C and 2.5% CO_2. (38) Growth was allowed to proceed for 1 to 3 days, after which time CFU counts were made and CFU per ml quantities were estimated using equation 1:

Equation 1.

$$\frac{(CFU)}{(5.0ul)} \times \frac{(1 \times 10^3\ ul)}{ml} \times \frac{1}{\text{dilution factor}} = \frac{CFU}{ml}$$

CFU and total organism per ml bacterial stock count results are located in Appendix IV.

Chamber Design and Aerosolization

Aerosolization experiments were performed in a plexiglass glovebox with an interior volume of approximately 253 liters, see Figure 8. Air entering and exiting the glovebox passed through HEPA filters with a rated efficiency of 99.99% capture for particles $\geq$ 0.30 um. The exhaust

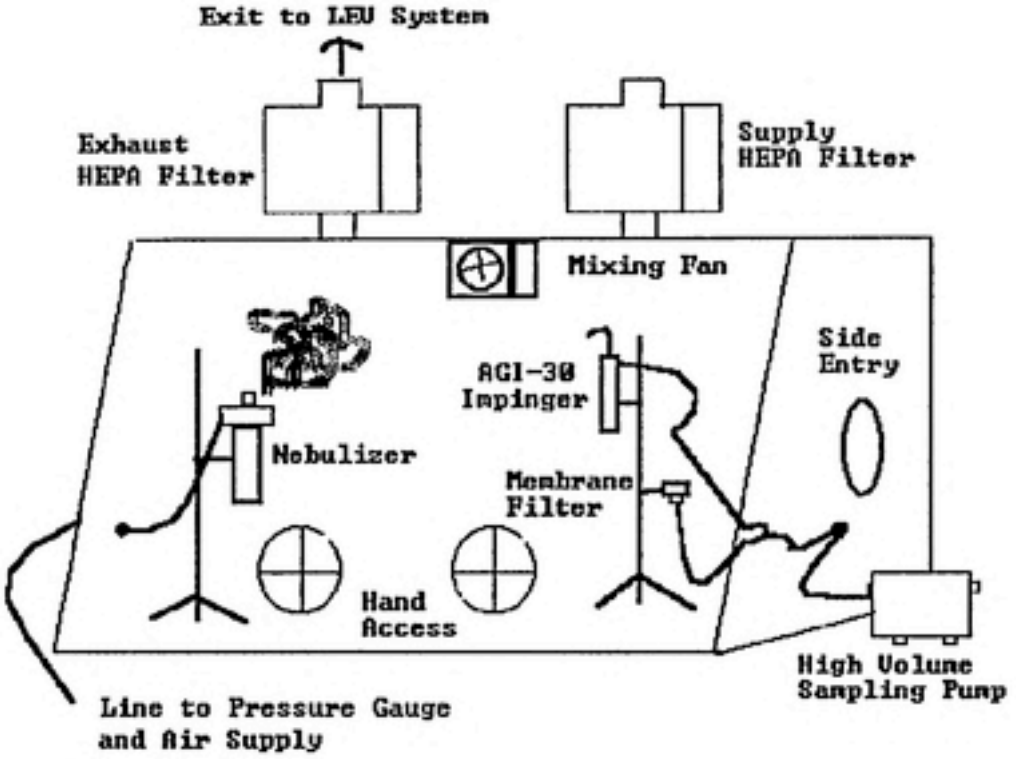


Figure 8. Experimental Set-up

HEPA filter was connected to an existing local exhaust ventilation (LEV) system operated at a flow rate of approximately 100 cubic feet per minute. Prior to each sampling period the glovebox was purged for 10 minutes (approximately 10 air changes) using the LEV system to provide a clean sampling environment. To provide adequate mixing of the air a small fan was mounted to the top of the glovebox. Two side portals with removable covers provided access to the interior of the glovebox. Aerosols containing *L. pneumophila* were generated using a Collison 3-jet nebulizer. Air was supplied to the nebulizer at 20.0 psig; at this pressure the nebulizer is reported by the manufacturer to generate droplets with a mass median diameter of 2.0 um and liquid at a rate of 9.0 ml/hour. (12) Dilutions for aerosolization were prepared by mixing varying amounts of stock culture with distilled water; three trials were conducted, each at a different dilution. The nebulizer was run for 15 minutes prior to each sampling period. Table 1 lists bacterial concentration per ml in the nebulizer solution for trials 1, 2, and 3, as estimated by plate count and AODC.

Trial	Bacterial Stock Volume (ml)	Diluent Volume (ml)	Bacterial Concentration by Plate Count (CFU per ml)	Bacterial Concentration by AODC (organisms per ml)
1	10 ml	10 ml	3.38 E +08	2.90 E +09
2	5 ml	15 ml	1.88 E +08	1.45 E +09
3	2.5 ml	17.5 ml	9.69 E +07	6.75 E +08

Table 1. Estimates of Bacterial Concentration in the Nebulizer Solution for Plate Count and AODC

Sampling Methods and Protocol

Chamber air samples of *L. pneumophila* were captured into AGI-30 impingers and onto Costar^{tm} polycarbonate, 47mm diameter, 0.45um pore size membrane filters. AGI-30 impingers were used since they serve as reference samplers in the development and testing of new bioaerosol samplers. (2) Each impinger contained 50 ml of distilled water as the capture medium. Polycarbonate filters were selected because their smooth filter surface should maximize release of the bacteria into solution following collection. The filters were held in place during sampling by a 47mm diameter, open-faced filter holder.

Air was drawn into the impinger or across the filter face at a target flow rate of 12 lpm using a high-volume air sampling pump. The pump was calibrated using a Gilabrator^{tm}; calibration data are listed in Appendix V. Calibration was performed prior to and following each sampling period. Each sampler was run for 22 minutes, with duplicate samples collected for both the impinger and membrane filter at the three different aerosol concentrations. Following sampling, exposed filters were placed into individual, sterile polypropylene bottles containing 50 ml of distilled water. The bottles were capped and shaken vigorously to release any bacteria trapped on the filter. Sampling parameters are listed in Appendix VI. A detailed listing of equipment used in this investigation is listed in Appendix VII.

Detection of Legionella pneumophila by PCR

PCR sample preparation, amplification, and detection were accomplished using the three part Perkin-Elmer Cetus EnviroAmp^{tm} *Legionella*

PCR Kit. The specificity of the kit is based upon DNA sequences unique to the organism *L. pneumophila*. The genus *Legionella* is identified by amplifying a DNA sequence in the 5S ribosomal RNA (rRNA) gene. (35) Identification of the species *pneumophila* is dependent on the presence of a macrophage infectivity potentiator (*mip*) gene specific for *L. pneumophila*. (35) Other species of *Legionella* contain *mip*-like genes, but the one used in the kit is unique to *L. pneumophila*. (35) The specific sequence of the 5S rRNA and *mip* gene, and their respective primers and, are listed in Appendices VIII and IX.

Sample Preparation for Bacterial Count and PCR Analysis

Sample analysis was performed in a Class II biological safety hood. A 9 ml aliquot was removed from each membrane filter and impinger sample for later analysis by AODC. Serial dilutions of the membrane filter and impinger solutions were plated on BCYE agar. This was done to determine the culturable concentrations in each sample. Each plate was incubated at 35° C and 2.5% CO_2 for 1 to 3 days. The serial dilutions used were as follows:

a. 1×10^{0} (undiluted sample)
b. 1×10^{-1} (0.10 ml of sample, 1.0 ml of distilled water)
c. 1×10^{-2} (0.10 ml of dilution a, 1.0 ml distilled water)

Plate count and AODC data are listed in Appendix X. PCR amplification of prepared samples was performed using the protocols outlined in Appendices XI, XII, XIII, and XIV.

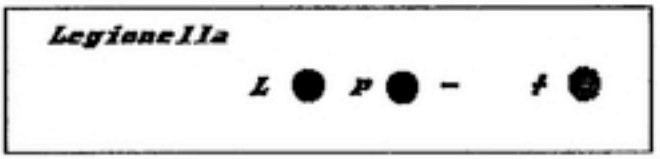
Analysis by Reverse Dot-blot Hybridization

Reverse dot-blot hybridization analysis was used to detect and

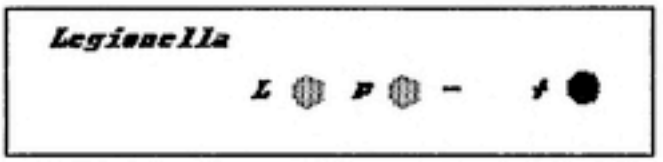
quantify PCR products using DNA probes immobilized on a nylon strip. (35) DNA probes are single-stranded, synthetic oligonucleotides which are complementary to a portion of the DNA sequence contained within the PCR product. Double-stranded PCR product was denatured and hybridized to a specified probe. Sequences for the 5S rRNA and *mip* probes are located in Appendix XV. The primers used in the EnviroAmp^{tm} *Legionella* PCR Kit were chemically marked with Biotin^{tm} to allow for detection of the target sequences. (35) The biotinylated PCR products were incubated with streptavidin-horseradish peroxidase conjugate, washed, and a substrate for the horseradish-peroxidase was added. (35) A series of blue dots appearing on the nylon membrane indicated quantity and presence/absence of the target organism, see Figure 9. Blue dots appearing next to the "*L*" or "*p*" symbols indicated detection of genus *Legionella* and species *pneumophila*, respectively. The color intensity of the dots quantified organisms detected per ml of sample fluid at the following concentrations: a) > 1000 per ml, b) approximately 1000 per ml, and c) 100 < x < 1000 per ml. A blue dot appearing next to the "positive" symbol indicated that PCR was performed properly, based on an internal positive control contained in the reaction mix. A blue dot appearing next to the "negative" symbol indicated PCR had not been performed properly, or inhibitors to PCR were present in the reaction mix. Reverse dot-blot and color development analysis were performed using the protocols outlined in Appendices XVI, XVII, and XVIII.

Analysis by Gel Electrophoresis

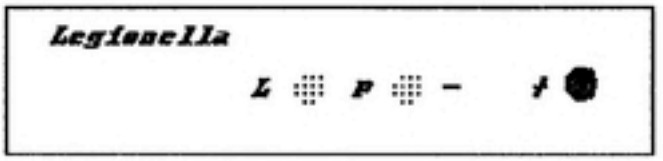
Gel electrophoresis was used to verify that PCR product was



L. Pneumophila detected at > 1000 organisms per ml



L pneumophila detected at approximately 1000 organisms per ml



L. pneumophila detected at > 100 but < 1000 organisms per ml

Figure 9. Interpretation of Reverse Dot-blot Hybridization Detection Strips

indeed present, and that the DNA fragments were the correct size. To use this technique, PCR product mixed with loading buffer was pipetted into individual wells in an agarose gel, and a standard molecular weight marker was inoculated into a separate well. The gel was immersed in a buffer solution and electric current was passed through it. DNA, being negatively charged, moved through the gel toward the positive electrode. The distance a fragment traveled was determined by its length, with smaller fragments moving farther than long ones. After a predetermined time, the current was interrupted and the gel was stained with a solution containing ethidium bromide. Following repeated washings with distilled water the DNA fragments in the gel remained stained. DNA fragments in the gel fluoresced when viewed under ultraviolet light, and fragments of the desired length were identified by comparing them with the lane containing standard DNA marker. The size of the PCR products for the 5S rRNA and *mip* gene are 108 and 168 base pairs, respectively. (35) Gel electrophoresis was performed using the protocols outlined in Appendices XIX, XX, and XXI.

Results

Detection and Quantification using PCR

L. pneumophila was successfully detected and quantified in chamber air samples using AGI-30 impinger and membrane filters. As shown in Table 2, PCR product was positively identified for eleven out of twelve samples by both reverse dot-blot hybridization and gel electrophoresis, for all three trials.

Trial	Sampling Method	Gel Electrophoresis Result (+/−)	Hybridization Analysis Result (+/−)
1	Impinger	+	+
1	Filter	+	+
1	Impinger	+	+
1	Filter	+	+
2	Impinger	+	+
2	Filter	+	+
2	Impinger	+	+
2	Filter	−	−
3	Impinger	+	+
3	Filter	+	+
3	Impinger	+	+
3	Filter	+	+

Table 2. Results of Gel Electrophoresis and Reverse Dot-blot Hybridization Analysis

Reverse dot-blot hybridization analysis showed specific detection of organisms in the genus *Legionella* and species *pneumophila* for all positive samples. Bands corresponding to the 108 and 168 base pair DNA fragments were visible by gel electrophoresis analysis for all positive samples. There was no consistent relationship observed between the intensity of the band present in the gel and the estimated recovery concentration. The single negative result was expected; during the DNA extraction protocol the filter being used to concentrate the sample became unseated in its holder, resulting in loss of most of the cells.

Detection and Quantification by Plate Count and AODC

L. pneumophila was detected and quantified by both plate count and AODC analysis methods using impingers and membrane filters. Figure 10 is a plot of estimates for the mean bacterial CFU count per ml of sample fluid versus trial number for the impinger and filter results. As the concentration in the chamber decreased from trial 1 to 3, the mean count decreased.

Figure 11 is a plot of the mean total count estimated by AODC versus trial number for impinger and membrane filter results. In contrast to the CFU count results, there was no consistent relationship between AODC results and chamber concentration; in fact, trial 2 resulted in the greatest mean recovery by AODC, not trial 1, in which the highest chamber concentrations were generated.

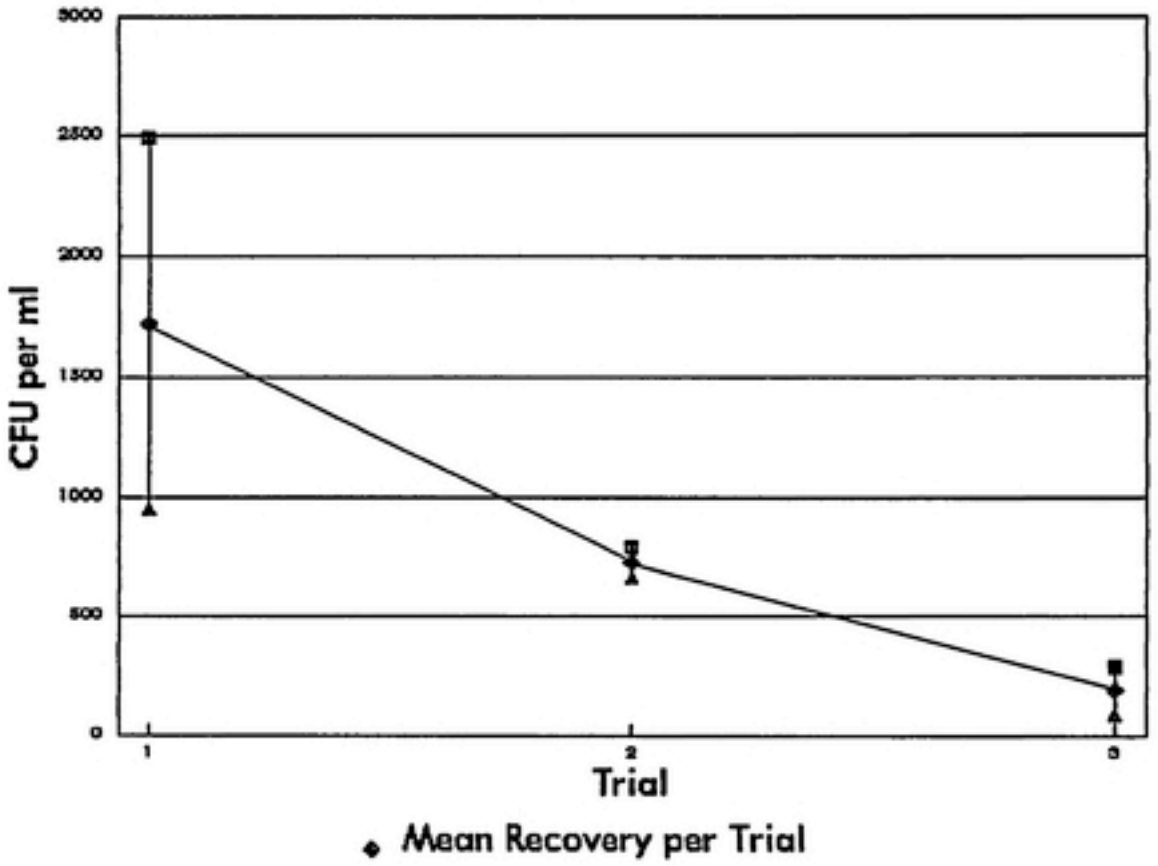


Figure 10. Mean Bacterial Concentrations per ml Estimated by Plate Count

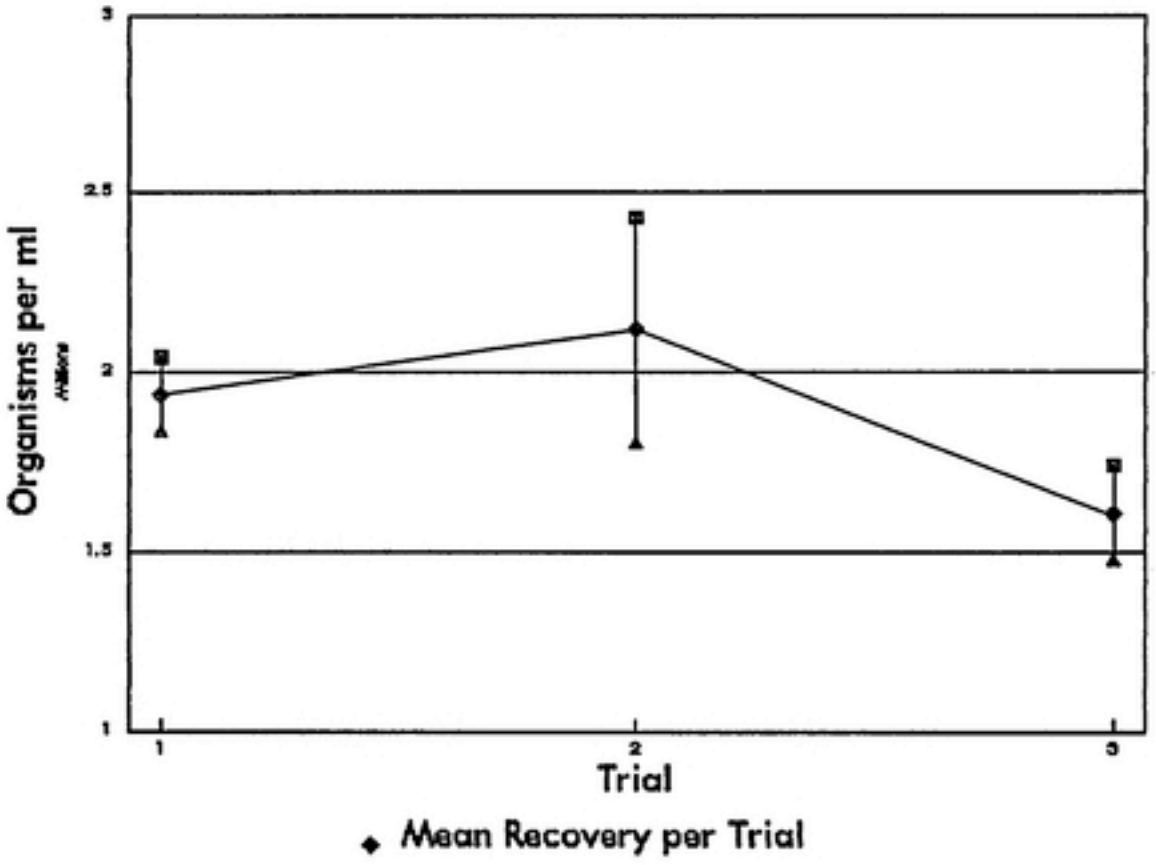


Figure 11. Mean Bacterial Concentrations per ml Estimated by AODC

As shown in Figures 10 and 11, counts recorded by the plating and AODC analysis methods, respectively, were orders of magnitude different from one another. A ratio of the AODC to plate count results was over a thousand and, as the bacterial concentration in the chamber decreased, the magnitude of the discrepancy between the methods increased, see Figure 12.

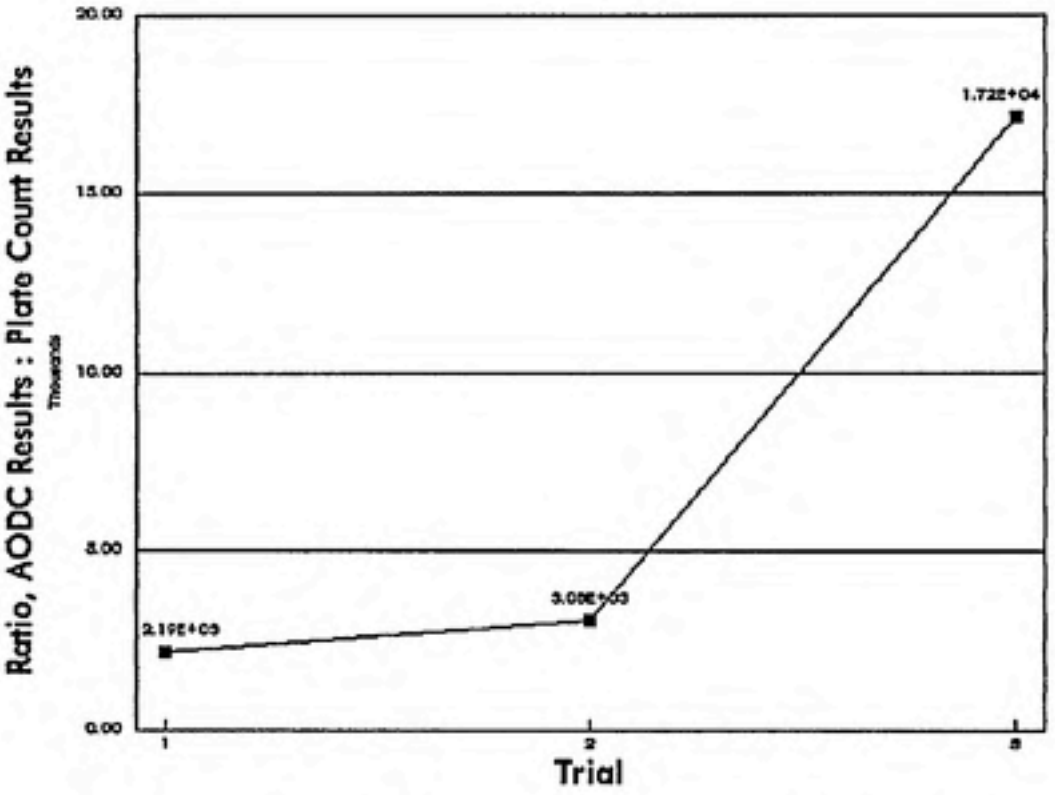


Figure 12. Ratios of AODC to Plate Count Results, Mean Bacterial Recovery per Trial

Overall, this ratio obtained after sampling was not similar to the ratio of the AODC to plate count results obtained from the aerosolized stock solution.

Figure 13, a plot of the ratio of AODC to plate count results for each trial, shows only about a two-fold difference between these counting methods; for the stock solution, as the concentration in the stock decreased, there was no difference in the ratios for the different solutions. One reason for this difference is that some fraction of organisms contained in the stock will always be non-culturable. Differences in the ratios of plate count to AODC results before and after aerosolization may indicate losses of viability and culturability occurred as a result of aerosolization and/or collection.

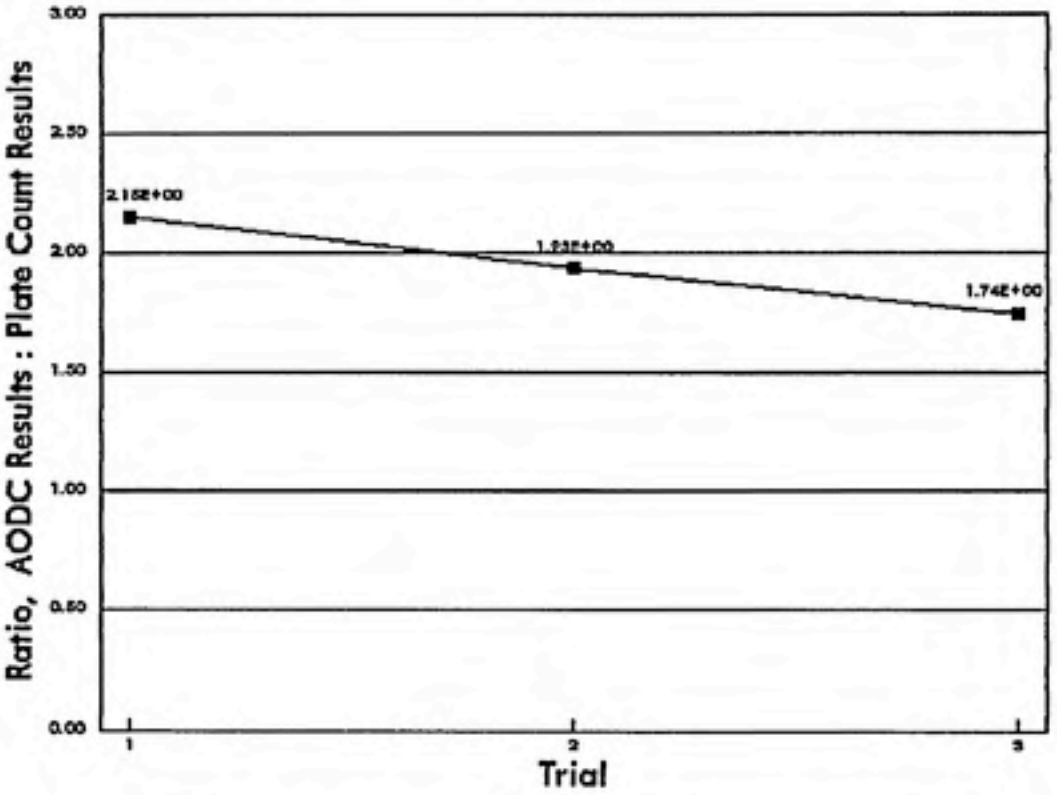


Figure 13. Ratios of AODC to Plate Count Results Prior to Aerosolization

Comparison of Bacterial Count and Reverse Dot-Blot Results

A comparison of results obtained by plate count, AODC, and reverse dot-blot hybridization analysis is shown in Table 3.

Trial	Sampling Method	Bacterial Concentration by Plate Count (CFU per ml)	Bacterial Concentration by AODC (organisms per ml)	Concentration Estimate Based on Hybridization Results (organisms per ml)
1	Impinger	1970	2.12 E +06	> 1000
1	Filter	3800	1.94 E +06	> 1000
1	Impinger	433	2.02 E +06	> 1000
1	Filter	667	1.66 E +06	> 1000
2	Impinger	800	1.19 E +06	> 1000
2	Filter	533	2.51 E +06	> 1000
2	Impinger	800	2.32 E +06	> 1000
2	Filter	767	2.47 E +06	None Detected
3	Impinger	133	1.62 E +06	> 1000
3	Filter	567	1.23 E +06	> 1000
3	Impinger	66	1.73 E +06	> 1000
3	Filter	68	1.84 E +06	> 1000

Table 3. Comparison of Plate Count, AODC, and Reverse Dot-blot Hybridization Estimates of Bacterial Concentration per ml

The hybridization and AODC results both estimated counts to be greater than 1000 organisms per ml of sample fluid. In contrast, only 2 of 12 samples analyzed by plating resulted in greater than 1000 CFU per ml of sample fluid. For the negative PCR sample in trial 2, positive results were obtained using the counting methods. This was expected because the sample was split before PCR analysis was performed.

Discussion

Detection of airborne *Legionella pneumophila* was accomplished using a combination of traditional bioaerosol sampling devices, PCR for amplification of extracted cellular DNA, and gel electrophoresis and reverse dot-blot hybridization as analytical techniques. Stresses placed on the organisms during aerosolization and collection did not prevent DNA amplification by PCR or subsequent detection of PCR product at the airborne concentrations tested.

The primary concern in selecting a sampling methodology for use in conjunction with PCR was capture of the target organism while preserving the integrity of the cellular structure and DNA. Organism viability and culturability were secondary considerations because an assumption was made that any organisms captured could have potential viability, and thus could be considered an infectious risk. This investigation showed that impingement and membrane filtration are capable of recovering *L. pneumophila* with its cellular DNA intact and in adequate quantities for amplification by PCR.

The main advantage offered by PCR is extreme specificity, which gives it potential as a powerful analytical tool for use in air sampling. (4) This specificity allowed precise positive identification of *L. pneumophila* in this study. Even though a single organism was examined in this investigation, PCR has no requirement to rigorously purify samples of non-targeted DNA before subjecting them to analysis. As long

as one copy of the target sequence is unbroken in the region of interest, and the sample does not contain inhibitors to PCR, amplification should occur. (14) Other benefits of using PCR include its ability to amplify target DNA at picogram quantities or less (33) and the commercial availability of automated equipment which can readily synthesize oligonucleotide primers. (34) A final strength of PCR is that it can be fully automated, and 30 cycles of PCR can usually be completed in just a few hours, making it possible to perform sample collection, preparation, and analysis in a single day. This provides an advantage over analysis by plating, which usually take 24 hours or more for adequate incubation of samples.

The extreme sensitivity of PCR proves to be a drawback; contamination of samples with minute quantities of DNA can lead to false positive results. (36) Therefore, to prevent carry-over of PCR product into sample preparation areas, laboratory areas used for PCR reaction preparation must be maintained separately from areas used for PCR product analysis.

The use of PCR is constrained by the requirement for pre-existing knowledge of DNA sequence information, such as the *mip* gene sequence. (8) Only organisms which have been characterized for wholly unique DNA sequences can be used for the design of primers. In addition, primers must be homologous to all known examples of a particular DNA sequence. (14) If only a single example of a particular organism has been sequenced, a high probability exists for false negative results. The choice of a target sequence for amplification by PCR requires extensive background research to ensure uniqueness. If similar sequences occur in

the nucleic acid of unrelated organisms, and both the target and unrelated organism exist in a given sample, analysis by PCR may give false positive results. (14)

An important issue in the use of PCR which has not been resolved is precise quantification of the amount of target sequence in the initial sample based on the amount of PCR product. (14) It is often desirable, especially in environmental sampling, to have knowledge of the concentration of organisms present in a given sample, as well as a test of presence/absence. To date, their are no methods which can precisely relate the amount of PCR product generated to the number of organisms contained in the original sample.

Bacterial counts obtained by AODC were consistently greater than those obtained by plate counts. However, the AODC method is a non-specific technique. Even in an investigation such as this, where a predetermined concentration of a known bacterial species was introduced into a controlled, HEPA filtered environment, an assumption was made that all cells identified by AODC were, in fact, the target organism. Ambient air can contain numerous different species, ranging in concentration from thousands to millions of viable microorganisms per cubic meter (15). The utility of AODC is not in its ability to differentiate organisms or determine viability, but to provide accurate information on total counts. Some other type of assay, such as culture on a selective media, must be performed if positive organism identification is desired, giving the AODC method limited utility in a field investigation. Results from this study indicated that the AODC method was not sensitive

to the changes in chamber bacterial concentration introduced in the three trials. This may have been related to the high bacterial concentrations generated for this study.

Comparison of plate count and AODC results indicated that plating may underestimate the actual number of bacteria contained in a given sample. In addition, as airborne concentrations of bacteria decreased, plate count results became increasingly less accurate. It is widely accepted that, due to a number of factors, culture plate assays often underestimate actual levels of microorganisms collected during air sampling. (31) In this investigation some factors which may have led to plate count underestimation were: a) limited viability of an organism in an airborne environment (31), b) desiccation of, or damage to an organism during collection (31), c) choice of nebulizer diluent and/or impingement collection medium, d) choice of growth medium used to plate sample solutions. *L. pneumophila* is found naturally in water, not air. It is possible that some culturable counts were lost during aerosolization as the bacteria were rapidly transferred from a natural liquid environment to the air. Both collection methods have inherent qualities which make loss of culturability likely. Rapid deceleration of the organisms in the liquid collection medium may have damaged some organisms and contributed to lower recovery by plating. Bacteria collected on membrane filters are susceptible to desiccation from the large volume of air passing across the filter face. Distilled water was not an ideal choice as a diluent or as the impinger collection medium. Bacterial survival and recovery may have been enhanced had a buffered solution been substituted. Finally, the use of a selective medium

(buffered charcoal yeast extract) for plating may have decreased count results by inhibiting repair of minimally damaged cells. A less selective plating medium, such as trypticase soy agar, may have promoted better growth and recovery results. All of the plate counts results observed in this investigation were within documented CFU detection limits for both impinger and membrane filter sampling methodologies. (1)

The count underestimation by plating is significant because some fraction of the bacteria in the aerosol can be viable, yet non-culturable. This is of clinical significance for a bacteria such as *Legionella pneumophila* where viability, not culturability, is the measure of infectious potential. Underestimation by plate count can have serious consequences when false negative results are reported, because the volume and mass of material in an infectious dose of a pathogenic organism can be very small. (15) It may be prudent to reconsider some of the current methods employed for analysis and enumeration of collected airborne microorganisms based on the potential for unacceptable count underestimation.

This investigation was limited to detection of high airborne bacterial concentrations. While the question of detection feasibility has been addressed, the issue of a quantifiable limit of detection for airborne microorganisms using PCR still remains. Follow-up studies should include work to determine the lowest levels at which airborne microorganisms can be detected using PCR in conjunction with various sampling methodologies. A lower detection limit could then be compared

to limits which have been established using traditional analytical techniques. In addition, better methods need to be developed to precisely relate the amount of PCR product generated from a sample to the concentration of organisms contained in the sample. Follow-up work might include performing PCR and reverse dot-blot hybridization analysis on serially diluted samples to estimate endpoints of detection.

The utility of the PCR in bioaerosol monitoring is dependent on the availability of DNA sequence information for microorganisms which proliferate or exist routinely in the air and are of some clinical significance, *i.e.* pathogenic. An enormous amount of sequence data is available, making PCR possible for a large number of organisms by using published DNA sequences. (34) However, only a few of the many microorganisms which influence human health have been extensively analyzed at the DNA sequence level. (22) Common airborne bacteria for which DNA sequence information has been obtained are *Legionella, Pseudomonas, Bacillus, Micrococcus, Mycobacterium and Corynebacterium.* Merely characterizing sequences of DNA does not itself warrant the use of the PCR. Future research should focus on the development of oligonucleotide primers unique to specific organisms of interest. An interesting strategy which can be used is to identify the DNA sequence of the particular gene involved in pathogenesis. (41) This gene would correlate to the specific disease and could be used as a genetic marker for the disease. (4) A unique segment of the DNA sequence for this gene could then be used as a potential target for amplification by PCR, with subsequent analysis for positive identification. Protocols must also be developed for DNA extraction and PCR amplification which relate to

specific organisms of interest.

A variation on traditional PCR which may have utility in bioaerosol monitoring is called multiplex PCR. This technique involves amplification of several DNA segments simultaneously in the same PCR reaction mix. (8) Multiple pairs of primers are placed in a single PCR reaction tube. Each primer pair is specified for a different target sequence corresponding to a unique microorganism. Choosing the target sequences for the individual organisms requires extensive forethought. The lengths of each individual target DNA sequence should be relatively close, because large differences will favor the amplification of short target DNA over long. (8) At the same time, if gel electrophoresis is used to analyze the PCR products, the lengths should be sufficiently different so distinct bands will be distinguishable in the gel. In addition, the primers should be designed with relatively close annealing temperatures. (8) This will ensure the primers can bind correctly to the intended flanking regions. Future research could explore the use of multiplex PCR to identify different species collected in a single air sample.

Appendix I

Protocol for preparing Yeast Extract Broth (38)

For 1 (one) liter:

1. Combine the following ingredients in a container of sufficient size to hold 1 liter of solution and mix well:

a. 1000ml distilled water
b. 10.0g Yeast Extract Agar
c. 0.40g L-cysteine
d. 0.25g ferric pyrophosphate

2. Filter sterilize solution using a 0.45um pore size membrane filter.

3. Adjust pH to 6.90 using 1 N NaOH.

4. Perform a second filter sterilization using a 0.45 pore size membrane filter.

5. Transfer the media in 10 equal volumes to sterile 125 ml glass bottles.

6. Store media in sealed bottles at 4° C.

Appendix II

Protocol for preparing Charcoal Yeast Extract plating agar

1. Combine the following ingredients in a container sufficient to hold 500ml of solution:

a. 500ml distilled water
b. 18.5g Yeast Extract Agar

2. Adjust pH to 7.10 to 7.20.

3. Sterilize solution at 15 psi and 121 degrees C for 15 minutes.

4. Cool solution to 40 to 50° C.

5. Add 5ml Agar Enrichment.

6. Adjust pH to 6.85 to 7.0.

7. Pour approximate 20 ml quantities of liquid agar into individual sterile 100 mm petri dishes.

8. Allow plates to cool; store at 4 degrees C.

Appendix III

Protocol for the Acridine Orange Direct Count (AODC) Epifluorescence Microscopic Method (28)

1. At time of sample collection, fill a sterile, autoclaved scintillation vial with 1.1ml of filtered formalin and 20ml of sample water. This preserved sample can be stored up to 3 weeks at 4° C.

2. Perform decimal dilutions of the preserved sample. A dilution of 10^{-1} should be sufficient

3. a. Add 1 ml of filtered 0.1% Acridine Orange stain to 9 ml of sample water; allow to stain for approximately 4 minutes.

b. Place a black, 0.22um pore size, 25 mm diameter polycarbonate filter on the filtration apparatus and add the flamed tower.

c. Filter stained water through the filter with a gentle vacuum.

d. Rinse filter apparatus and filter with 9 ml of particle free water; allow vacuum to pull for a few seconds after all liquid has passed through filter.

e. Place a small drop of Cargille Type A immersion oil on a clean glass slide. Remove the tower and place the filter on the drop of immersion oil. Place the filter on the drop of oil. Add another small drop of oil on top of the filter.

f. Examine the slide under epifluorescent illumination and count cells inside grid.

4. Count at least 5 random fields and average the counts for the fields. Count at least 200 cells. If more than 100 or less than 10 cells per field are found, a higher or lower dilution, respectively, should be used.

5. Calculate the number of cells per ml of sample using the following steps and equations:

a. Average the cell counts for the random fields counted.

b. Given: Diameter of Tower = 16.0 mm
Grid Dimension = 55.5 um
Area of Grid = 0.0038 mm^2
Area of Filter = 201.06 mm^2
x = Average Cells/Field
Y = AODC (cells/ml)

d. Formula to Calculate Y:

$$Y = \frac{x * \text{Area of Filter}}{0.95 * \text{Area of Grid}}$$

Appendix IV

Stock Culture Bacterial Concentration Determined by Plate Count and AODC

Trial	Plate Count Data (CFU / 5 ul)	Count Average (CFU / 5 ul)	Dilution Factor	Bacterial Concentration (CFU per ml)
1	32, 30, 24, 25, 31, 22	27	8.0 E −06	6.75 E +08
2	28, 34, 31, 27, 29, 33	30	8.0 E −06	7.50 E +08
3	36, 28, 34, 29, 31, 26	31	8.0 E −06	7.75 E +08

Trial	AODC Count Data (total organisms)	Count Average (total organisms)	Dilution Factor	Bacterial Concentration (organisms per ml)
1	55, 52, 67, 44, 48, 35, 56, 60	52	2.0 E −03	1.45 E +09
2	55, 52, 67, 44, 48, 35, 56, 60	52	2.0 E −03	1.45 E +09
3	47, 40, 51, 57, 48, 50, 52, 43	49	2.0 E −03	1.35 E +09

Appendix V

Summary of High Volume Pump Calibration Data

Trial	Sampling Method	Pre-Cal (lpm)	Post-Cal (lpm)	Average (lpm)
1	Impinger	12.01, 12.07, 12.04	11.61, 11.63, 11.58	11.82
1	Filter	12.01, 12.05, 12.03	12.29, 12.32, 12.27	12.16
1	Impinger	12.04, 12.05, 12.04	11.48, 11.58, 11.55	11.79
1	Filter	12.06, 12.11, 12.07	12.13, 12.16, 12.13	12.11
2	Impinger	12.05, 12.05, 12.07	12.44, 12.45, 12.44	12.25
2	Filter	12.05, 12.01, 12.03	11.98, 11.92, 11.93	11.99
2	Impinger	12.03, 12.07, 12.05	11.81, 11.80, 11.81	11.93
2	Filter	12.06, 12.02, 12.03	12.16, 12.11, 12.15	12.09
3	Impinger	12.01, 12.04, 12.04	12.29, 12.32, 12.26	12.16
3	Filter	12.08, 12.06, 12.05	12.09, 12.09, 12.10	12.08
3	Impinger	12.02, 12.05, 12.05	11.43, 11.38, 11.45	11.73
3	Filter	12.02, 12.06, 12.03	12.08, 12.05, 12.02	12.04

Appendix VI

Summary of Sampling Parameters

Trial	Sampling Method	Sampling Flowrate (lpm)	Nebulizer Fluid Ratio (stock : diluent)	Total Volume Sampled (liters)
1	Impinger	11.82	1:1	260.04
1	Filter	12.16	1:1	267.52
1	Impinger	11.79	1:1	259.38
1	Filter	12.11	1:1	266.42
2	Impinger	12.25	1:3	269.50
2	Filter	11.99	1:3	263.78
2	Impinger	11.93	1:3	262.46
2	Filter	12.09	1:3	265.98
3	Impinger	12.16	1:7	267.52
3	Filter	12.08	1:7	265.76
3	Impinger	11.73	1:7	258.06
3	Filter	12.04	1:7	264.88

Appendix VII

Equipment List

Air Sampling

1. Manostat Glove Box, part # 41-905-000
2. Clamping Ring, (2 each), part # 41-905-320
3. Economy Sleeve Glove (2 each), part # 41-905-544
4. General Electric High Volume Sampling Pump, Model 5KH10GGR105X, Serial # 1090
5. Collison 3-jet Nebulizer, BGI Inc., Part # CN-24
6. Sartorius 47 mm open face membrane filter holders
7. Costar Corp. 47 mm diameter, 0.40 um pore size polycarbonate membrane filters, part # 111107, lot # 1166
8. Lab Safety Supply Inc., 4" x 4" HEPA filters (2 each), 99.99% efficient for 0.30 um particles, part # 7169, lot # 2197 00000004 and 3020 00000005
9. AGI-30 Impinger, Ace Glass Co., part # 7540-10
10. Gilabrator, Gilabrator Instrument Corp., Sensor Block Serial # 10838-S, Control Unit Serial # 6778-B

AODC Analysis

1. Fisher Scientific Glass Microanalysis Filter Holder Assembly with Frit-Glass Support, Catalog # 09-753E
2. Poretics Corp. 25 mm diameter, 0.20 um pore size, black polycarbonate membrane filters, part # 11021, lot # AG83BK21AV15
3. Leitz Wetzlar Otholux II Fluorescent Microscope, Serial # 2523, NPL Fluortar Oil Immersion Lens, 160/0.17, 100/1.332 - 0.60

PCR Analysis

1. EnviroAmp™ *Legionella* Sample Preparation Kit, part # N808-0088
2. EnviroAmp™ *Legionella* PCR Amplification Kit, part # N808-0089
3. EnviroAmp™ *Legionella* PCR Detection Kit, part # N808-0090

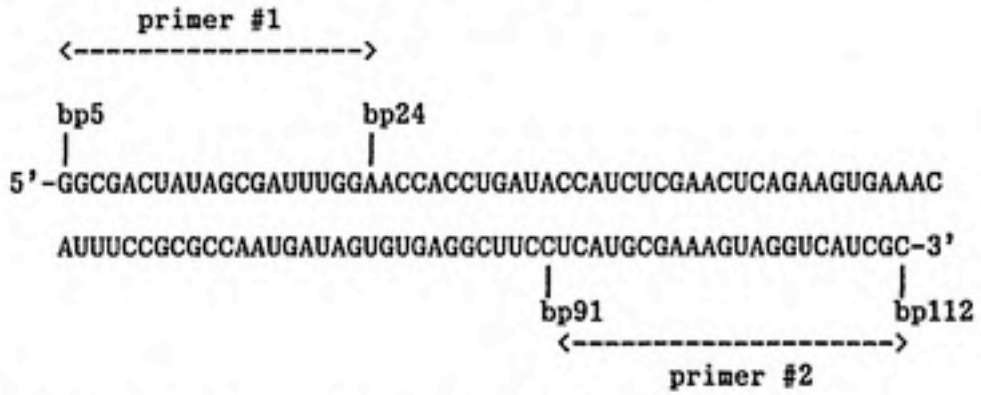
Gel Electrophoresis

1. International Biotechnologies Inc., Multi-purpose Gel Electrophoresis System, Model MPH, Catalog # 5200

Appendix VIII

Sequence and Primer Binding Sites for the 5S rRNA gene of *L. pneumophila* (35,42)

5S rRNA Gene Sequence and Primer Binding Sites



primers bind at bp5 - 24 and bp91 - 112
target sequence: bp25 - 90
PCR product length: 108bp

Sequences for 5S rRNA gene Primers

Four primers, two of which are biotinylated are used in the detection kit.

Primer 1: identical to position 5 to 29 of the 5S rRNA DNA sequence

5' - GGCGACTATAGCGATTTGGAA - 3'

5' - GGCGACTATAGCGGTTTGGAA - 3'

Primer 2: complementary to position 91 to 112 of the 5S rRNA DNA sequence

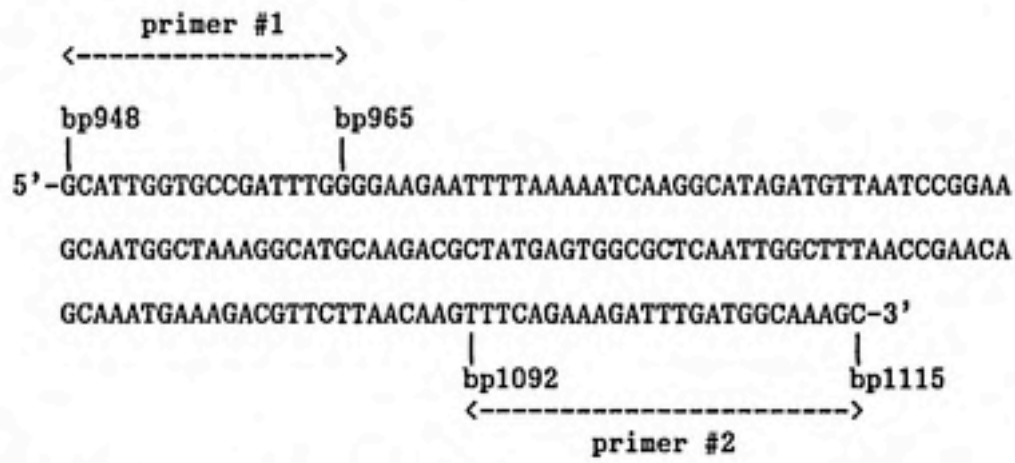
5' - biotin - GCGATGACCTACTTTCGCATGA - 3'

5' - biotin - GCGATGACCTACTTTCACATGA - 3'

Appendix IX

Sequence and Primer Binding Sites for the *mip* genes of *L. pneumophila* (18,35)

mip Gene Sequence



primers bind at bp948 - 965 and bp1092 - 1115
target sequence: bp966 - 1091
PCR product length: 168bp

Primers for the *L. pneumophila mip* gene

Three primers, all of which are biotinylated are used in the detection kit.

Primer 1: identical to position 948 to 965 of the *L. pneumophila mip* sequence

5' - biotin - GCATTGGTGCCGATTTGG - 3'

Primer 2: complementary to position 1092 to 1115 fo the *L. pneumophila mip* sequence

5' - biotin - GCTTTGCCATCAAATCTTTCTGAA - 3'

5' - biotin - GTTTTGCCATCAAATCTTTTTGAA - 3'

Appendix X

Summary of Plate Count Data Determined by Air Sampling

Trial	Sampling Method	Count Data (CFU per 5ul)	Count Average (CFU per 5ul)	Dilution Factor	Bacterial Concentration (CFU per 5ul)
1	Impinger	12, 16, 9, 4, 8, 10	9.83	1.00	1.97 E +03
1	Filter	23, 18, 22, 17, 21, 13	19.00	1.00	3.80 E +03
1	Impinger	0, 4, 2, 2, 2, 3	2.17	1.00	4.33 E +02
1	Filter	1, 3, 4, 3, 5, 4	3.33	1.00	6.67 E +02
2	Impinger	4, 3, 3, 4, 7, 3	4.00	1.00	8.00 E +02
2	Filter	2, 3, 5, 0, 5, 1	2.67	1.00	5.33 E +02
2	Impinger	5, 5, 6, 2, 2, 4	4.00	1.00	8.00 E +02
2	Filter	4, 2, 4, 2, 3, 8	3.83	1.00	7.67 E +02
3	Impinger	0, 2, 0, 2, 0, 0	0.67	1.00	1.33 E +02
3	Filter	4, 6, 2, 3, 1, 1	2.83	1.00	5.67 E +02
3	Impinger	0, 1, 1, 0, 0, 0	0.33	1.00	6.60 E +01
3	Filter	1, 1, 0, 0, 0, 0	0.33	1.00	6.60 E +01

Summary of AODC Data Determined by Air Sampling

Trial	Sampling Method	AODC Count Data (organisms per grid)	Count Average (organisms per grid)	Bacterial Concentration (organisms per ml)
1	Impinger	42, 40, 36, 43, 39, 28, 34, 43	38	2.12 E +06
1	Filter	32, 45, 30, 31, 33, 40, 37, 30	35	1.94 E +06
1	Impinger	31, 34, 30, 31, 31, 47, 47, 39	36	2.02 E +06
1	Filter	27, 31, 22, 29, 27, 40, 41, 22	30	1.66 E +06
2	Impinger	29, 19, 23, 20, 19, 15, 24, 22	21	1.19 E +06
2	Filter	42, 33, 45, 41, 41, 45, 34, 37	40	2.21 E +06
2	Impinger	36, 39, 34, 43, 34, 29, 29, 42	36	1.99 E +06
2	Filter	49, 52, 42, 49, 49, 38, 45, 42	46	2.53 E +06
3	Impinger	31, 33, 21, 28, 27, 32, 37, 25	29	1.63 E +06
3	Filter	23, 18, 24, 28, 18, 25, 25, 19	23	1.26 E +06
3	Impinger	27, 31, 31, 38, 26, 42, 31, 24	31	1.74 E +06
3	Filter	36, 31, 29, 32, 29, 43, 28, 37	33	1.86 E +06

Appendix XI

Protocol for preparation of *L. pneumophila* air samples for PCR amplification (35)

1. Water samples from membrane filter and impinger samples were individually filtered through 25mm diameter, 0.45um pore size Durapore^{tm} HVLP membrane filters to capture bacteria.

2. Each filter containing trapped bacteria was placed into a 8 ml polypropylene tube containing 2 ml of DNA Extraction Reagent

Composition of DNA Extraction Reagent:

a. 20% (w/v) Chelex^{TM} 100 in 10 mM Tris-HCl, pH 8.0
b. 0.10 mM EDTA
c. 0.10% Sodium Azide
d. final volume 100ml

3. Each tube was capped and vortexed for 30 seconds to release bacteria trapped on or in filter matrix.

4. Each capped tube was placed in boiling water bath to allow lysing of cells and release of cellular DNA.

5. Sample was now ready for PCR amplification.

Appendix XII

Protocol for preparing *Legionella* PCR Reaction Tubes (35)

1. Add contents of tube containing Uracil-N-glycosylase (UNG) to *Legionella* PCR Reaction Mix; mix well by swirling.

PCR reaction mix composition:
0.07 units/ul AmpliTaq DNA Polymerase
0.46 mM each of dUTP, dCTP, dATP and dGTP
77 mM Tris-HCl, pH 8.9
77 mM KCl
4 micromolar total of a combination of 7 oligonucleotide primers

Uracil N-Glycosylase composition:
1 unit/microliter of Uracil n-Glycosylase
30 mM Tris-HCl, pH 7.5
150 mM NaCl
1 mM EDTA
1 mM DTT
0.05% Tween^{tm} 20
5% (v/v) glycerol

2. Add 65uL aliquot of *Legionella* PCR Reaction Mix w/ UNG to each of 50 autoclaved 0.50ml thin-walled GeneAmp^{tm} Reaction Tubes.

3. Aliquot entire contents of PCR Reaction Mix w/ UNG at one time.

4. Place 3 drops of mineral oil, using dropper bottle provided, into each tube.

5. Close cap on each tube.

6. Store tubes at -20° C until needed.

Appendix XIII

Protocol for PCR amplification of *L. pneumophila* air samples (35)

1. Individual 20*ul* samples of extracted DNA were added to tubes containing 65*ul* PCR reaction mix with Uracil N-Glycosylase (Components of PCR Reaction Mix and Uracil n-Glycosylase listed in Appendix xx).

2. Positive and negative control tubes were prepared by adding 20*ul* of *Legionella pneumophila* control DNA and distilled water to 2 PCR Reaction tubes, respectively.

3. 15*ul* aliquots of 25mM $MgCl_2$ added to each tube.

4. Tubes capped and placed in a thermal cycler programmed* for the following:

denaturation temperature:	95° C
annealing temperature:	63° C
extension temperature:	72° C

5. Thermal cycler run for 30 cycles.

Appendix XIV

DNA Thermal Cycler Program (35)

1. program 3 linked files

2 a. File XX STEP-CYCLE
Seg 1 Target temp 45° C
Segment time 10 minutes
Seg 2 Target temp 95° C
Segment time 10 minutes
Seg 3 Target temp 0° C
Segment time 0 seconds
Cycle count 1
Auto segment extension = NO
Link to stored File XX + 1

b. File XX + 1 STEP-CYCLE
Seg 1 Target temp 95° C
Segment time 30 sec
Seg 2 Target temp 63° C
Segment time 1 minute
Seg 3 Target temp 0° C
Segment time 0 sec
Cycle count 30
Auto segment extension = NO
Link to stored File XX + 2

c. File 12 STEP-CYCLE
Seg 1 Target temp 72° C
Segment time 7 minutes
Seg 2 Target temp 0° C
Segment time 0 seconds
Cycle count 1
Auto segment extension = NO
Link to stored File X

* note: if samples are not going to be removed immediately, link File XX + 2 to a Soak File at 72 degrees C or store at -20 degrees C

Appendix XV

Probe Sequences for the *Legionella* 5S rRNA Gene (35)

A 1:1 mixture of two probes is used for detection of the 5S rRNA gene. The probes hybridize to positions 66 to 82 of the *Legionella* 5S rRNA DNA sequence.

Probe 1: 5' - (poly dT)* - GCGCCAATGATAGTGTG - 3'

Probe 2 5' - (poly dT)* - GCGCCGATGATAGTGTG - 3'

Probe Sequences for the *L. pneumophila mip* Gene (35)

Probe: hybridizes to position 1012 to 1036 of the *L. pneumophila mip* sequence

5' - (poly dT)* - CATAGCGTCTTGCATGCCTTTAGCC - 3'

*sequences hav a 5' tail of 100 dTs which serves to attach the probe to the nylon membrane while leaving the sequence specific probe region accessible for binding

Appendix XVI

Protocol for preapration of buffers necessary for reverse dot-blot analysis (35)

1. Denaturation Solution

a. Combine 4.80g NaOH and 4.1g Na_2-EDTA (disodium-ethylenediaminetetraacetic acid) with distilled water to a final volume of 100ml.
b. Store at 2 to 8° C.

2. Hybridization Solution

a. Dissolve any crystalline material present in the SSPE Concentrate Buffer and SDS Concentrate by warming to 37 ° C; mix often by swirling.

1. Composition and pH of SSPE Concentrate Buffer
   a. 3.0 M NaCl
   b. 625 mM sodium phosphate
   c. 25 mM EDTA
   d. final volume 160ml
   e. final pH 6.2

2. Composition of SDS Concentrate
   a. 10% (w/v) sodium dodecylsulfate in water
   b. final volume 20ml

b. In a clean, autoclaved glass bottle of sufficient size to contain 160ml, combine the following reagents in order:

1. 32ml SSPE Concentrate Buffer
2. 120ml distilled water
3. 8ml SDS Concentrate

c. Mix well and store at room temperature.

3. Wash Solution

a. Dissolve precipitates in SSPE Concentrate Buffer and SDS Concentrate by warming to 37° C; mix often by swirling to ensure dissolution.

b. In a clean, autoclaved glass bottle of sufficient size to contain 1 liter, combine following reagents in order:

1. 100ml SSPE Concentrate Buffer
2. 890ml distilled water
3. 10ml SDS Concentrate

c. Mix well and store at room temperature.

4. Chromagen Solution:

a. Bring bottle containing 60mg of Chromagen powder (3,3',5,5' tetramethylbenzidine (TMB)) to room temperature.

b. Dissolve powder by slowly adding 30mlof room temperature 100% reagent grade ethanol.

c. Recap bottle and seal with Parafilm^{tm}.

d. Shake the bottle on an orbital shaker for 30 minutes at room temperature to solubilize all of the powder.

e. Store at 2 to 8° C.

Appendix XVII

Protocol for reverse dot-blot analysis (35)

1. Heat a shaking water bath to 55 +/- 1° C; water level should be 1/4 to 1/2 inch above shaking platform.

2. Heat a stationary water bath to 37 +/- 2° C; warm Hybridiza tion and Wash solutions in bath.

3. Add 32ul of Denaturation Solution to each tube of PCR product; use a clean pipet tip for each sample; mix well and microcentri-fuge tubes for 5 seconds.

4. Label one *Legionella* Detection Strip for each sample to be analyzed with a waterproof marking pen; place one labeled strip per well face-up in the Hybridization Tray.

5. Pipet 3ml of pre-warmed Hybridization Solution into each well containing a Detection Strip.

6. Tilt Hybridization Tray slightly so Hybridization Solution flows toward *Legionella* label on strip; do not spill any liquid.

7. Transfer 50ul of sample to the center of each well containing Hybridization Solution.

8. Place the clear plastic lid on the Hybridization Tray; mix carefully by rocking back and forth; place tray in 55° shaking water bath; incubate at 55° for 20 +/- 1 minute.

9. Prepare dilution of Enzyme Conjugate in Wash Solution.

   a. Add 3.1ml of pre-warmed Wash Solution for each strip being analyzed to autoclaved glass flask.
   b. Add 26ul of Enzyme Conjugate (Streptavidin-Horseradish Peroxidase in buffer) for each strip to the same flask.
   c. Mix well by swirling; store at 37° C.
   d. This dilution should be prepared no more than 15 minutes before use.

10. After hybridization, remove tray from bath, tip tray at slight angle and aspirate contents of each well leaving the strip in each well; wipe tray lid with lab wipe.

11. Add 3ml of diluted Enzyme Conjugate in Wash Solution to each well containing a strip; cover with lid and place in 55° C shaking water bath for 12 +/- 1 minute.

12. After incubation is complete, remove tray from bath, remove lid from tray, tip tray slightly and aspirate contents of each well; leave strip in well; wipe lid with lab wipe.

13. Dispense 10ml of pre-warmed Wash Solution into each well; cover with lid; place in 55° C shaking water bath for 10 +/- 2 minutes.

14. Remove the tray from water bath, remove the lid the from tray, and aspirate the solution from each well; leave each strip in its well; wipe lid with lab wipe.

15. Dispense 5ml of pre-warmed Wash Solution into each well; cover with lid and place on orbital shaker at room temperature for 5 minutes at 50 rpm.

16. Remove tray from shaker, remove lid from tray and aspirate wells; wipe lid with lab wipe.

Appendix XVIII

Protocol for color development procedure (35)

1. Dispense 10ml of 100 mM Citrate Buffer, pH 5.0 into each well; cover tray with lid and place tray on an orbital shaker at room temperature for 5 +/- 1 minute at 50 rpm.

2. Prepare Color Development Solution (CDS) by adding the following components sequentially; 5ml required for each strip.

   a. # of strips x 5ml 100 mM Citrate Buffer
   b. # of strips x 5ul 3% Hydrogen Peroxide
   c. # of strips x 0.25ml Chromagen Solution

3. Mix CDS by swirling; do not vortex; cover with aluminum foil to protect from light.

4. Remove Hybridization Tray from orbital shaker; remove lid and aspirate solution from each well.

5. Add 5ml of freshly prepared CDS to each well; dover Hybridization Tray with lid and aluminum foil; develop at room temperature by shaking on an orbital shaker for 30 +/- 2 minutes at 50 rpm.

6. Remove Hybridization Tray from the orbital shaker and aspirate the solution from each well.

7. Stop development by adding 10ml of deionized water to each well; cover Hybridization Tray with lid and aluminum foil; place on orbital shaker at 50 rpm for 5 to 10 minutes.

8. Repeat steps 4 and 5 for a minimum of three washes.

9. Interpret the results on the strips using the guidance in the manual accompanying the EnviroAmp^{tm} *Legionella* PCR Detection Kit.

10. Developed Detection Strips should be photographed wet to provide a permanent record; after air drying Detection Strips can be stored by placing them on filter paper and completely covering them with cellophane tape.

Appendix XIX

Protocol for preparing gel electrophoresis buffers:

To prepare 1 liter of 50x Tris acetate, EDTA (TAE) buffer:

1. In an autoclaved 1 liter Erlenmeyer flask combine:

    a. 121.0g tris base
    b. 61.7g sodium borate
    c. 7.44g Na_2-EDTA

2. Add distilled water to bring the final volume to 1 liter.

3. Store at room temperature until ready for use.

To prepare 1x TAE buffer:

In an autoclaved 1 liter Erlenmeyer flask combine:

    a. 20ml 50x TAE buffer
    b. 980ml distilled water

To prepare 10ml of gel loading buffer:

1. Combine the following in an autoclaved vessel sufficient to hold 10ml

    a. 5ml 100% Glycerol
    b. 200ul 50x TAE buffer
    c. 1ml bromophenol blue

2. Add distilled water to bring the final volume to 10ml.

3. Store at room temperature until ready for use.

Appendix XX

Protocol for preparing DNA marker and PCR product for analysis by gel electrophoresis:

DNA Marker

a. Combine the following in a 1.0ml polypropylene reaction tube:

1. 1.0ul marker DNA
2. 14.0ul 1x TAE buffer
3. 2.20ul loading buffer

PCR Product

a. Combine the following in a 1.0ml polypropylene reaction tube:

1. 15ul PCR Product; avoid transfer of mineral oil
2. 2.20ul loading buffer

Spin all tubes in a microcentrifuge for 5 seconds to thoroughly mix the components.

Appendix XXI

Protocol for analysis by gel electrophoresis (35)

1. A 50x stock solution of Tris acetate, EDTA (TAE) buffer was prepared.

2. A 2% agarose gel was prepared by combining 2g of electrophoresis grade agarose and 100ml of 1x TAE buffer; mixture was then autoclaved for 6 minutes at 121 degrees C and 15 psig.

3. The agarose/TAE buffer mixture was cooled to 50 degrees C (approximately 10 minutes in a 50 degree C water bath).

4. The entire 100ml volume of agarose/TAE buffer mixture was poured into a leveled medium-size gel electrophoresis box; the gel comb was placed into the liquid agarose.

5. The gel was cooled for 20 minutes to allow solidification.

6. Sufficient 1x TAE buffer (approximately 1000ml) was poured into the gel box to cover the entire surface of the gel; the comb was carefully removed.

7. 17.2ul quantities of marker DNA, positive and negative control product, and samples PCR product were added to individual lanes

8. The lane numbers containing individual PCR product samples were recorded.

9. Electrical current (approximately 5.0 mAmps) was supplied to the gel for 90 minutes.

10. After 90 minutes the current was terminated and the gel was removed from the gel box and placed in a solution containing ethidium bromide (concentration xx mg/ml in dH_2) for 6 to 7 minutes.

11. The gel was destained by washing 3 times in distilled water.

12. The gel was viewed using an ultraviolet light to fluoresce the stained PCR product contained in the gel.

13. A Polaroid snapshot was taken of the fluoresced gel to be maintained as a permanent record.

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