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Legionella pneumophila Through the Years; A Look Back and A Step Forward at the Methodologies

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Abstract

Legionella pneumophila was first introduced to the world after an outbreak in 1976 when the attendees of the Philadelphia convention of the American Legion were affected by a kind of pneumonia named Legionnaire's Disease (LD). To date, the problem has not been resolved, and it has been reported many outbreaks over the years. The topic of Legionella outbreaks is one of the most active areas, thus encouraging researchers to focus on how they could effectively monitor the water sources to prevent the risk of an outbreak and high mortality. Research into solving this problem and establishing a state of the art method is already in progress. This paper is an overview of *legionella* detection over the years. What happened to *Legionella* through the past challenging decades. Is there any certified reference method with a functionalized penetration into society for this controversial microorganism or a cutting-edge scenario offering a constructive solution to eliminate the potential risk of the outbreaks, also early identification and source tracking?

Keywords: Waterborne diseases; Microbiology

Introduction

Legionella pneumophila was first introduced to the world after an outbreak in 1976 when the attendees of the Philadelphia convention of the American Legion were affected by a kind of pneumonia named Legionnaire's Disease (LD). To date, the problem has not been resolved, and it has been reported many outbreaks over the years. The topic of *Legionella* outbreaks is one of the most active areas, thus encouraging researchers to focus on how they could effectively monitor the water sources to prevent the risk of an outbreak and high mortality. LD outbreaks are associated with the increased risk of contamination of the cooling towers, drinking water supply systems, spa pools, and decorative fountains [1]. In general, therefore, it becomes a growing public health concern worldwide. *Legionella*, however, is an obligate, intracellular, ubiquitous, and an opportunistic bacterium, causing Legionnaires disease and Pontiac fever. As well, it crawls slowly, colonizes densely, and forms biofilms consequently. This group of bacteria has emerged as an inhabitant of human-made freshwater and aquatic environments; thereby, inhalation of aerosolized droplets containing *Legionella* has caused adverse effects and infection. Once transmission via inhalation, it would easily uptake by its host pulmonary-alveolar macrophages, making a niche for bacterial replication and survival called, Legionella-Containing Vacuole (LCV). Regarding illnesses and clinical manifestations, *L. pneumophila* presents as a cause of nosocomial and community-acquired pneumonia, providing a severe lung disorder. There is no evidence of person-to-person transmission; Nevertheless, one probable case has been reported. Despite the importance of legionellosis, there has been no approach concerning with neglected potential of legionella pathogenesis. Legionellosis is resulting in a case fatality rate of 10%, varying from a mild, benign, and flu-like disease-Pontiac fever- to severe and fatal pneumonia, legionnaire disease. The incubation time and fatality rate are estimated generally 2-14 days and 6%-11%, respectively. Legionnaire disease seems multifactorial with various clinical manifestations such as gastrointestinal and neurological complications and is overlooked in diagnosis. Indeed, Inadequacies of available diagnostic tests, non-specific signs, and symptoms and unknown sources of infection are life-threatening. Humans are frequently the accidental hosts of Legionella; the populations at-risk are predominantly immunocompromised patients, elderly and heavy smokers. Alternatively, co-existing protozoa, including *Acanthamoeba spp., Naegleria spp., or Hartmanella vermiformis*, are the natural hosts, playing a crucial role as a proper shelter; protecting the bacterium from environmental stress conditions. *Legionella* species are fastidious, need high nutritional requirements for growth, and group in terms of hard growing bacteria, conferring vanguard of luxurious microorganisms. Genus of Legionella contains 60 species, while *L. pneumophila* has 16 sero-groups, which sero-group 1 accounts for most European and American clinical isolates. It is also responsible for 70% to 90% of reported human legionellosis. *Legionella's* potential health risk in water sources is about 104 to 105 CFU/L. Since the water is a complex environmental matrix and a consortium of different bacteria, it requires considerable attention to developing a new detection method compared with the conventional routine laboratory tests. There have been different qualitative and quantitative strategies to identify and enumerate *Legionella*, such as culture, immunological and molecular techniques. In recent years, researchers have investigated various biosensors to put a new reliable method providing high sensitivity, portability, simplicity, and potential for real-time and on-site detection. Rapid identification of *Legionella* is excessively significant; hence an entirely advanced method to detect is required. Biosensors are among the widest groups of promising techniques and have been extensively discussed for environmental and clinical diagnosis and food analysis and industry. In our knowledge, One of *Legionella's* main issues is a lack of unique and reference method with a high standard for detection. There is still some controversy surrounding the biosensing systems, yet the final decision has not been made. In the past decades, concerns have arisen regarding *Legionella*. Research into solving this problem and establishing a state of the art method is already in progress. This paper is an overview of *legionella* detection over the years. What happened to

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Legionella through the past challenging decades. Is there any certified reference method with a functionalized penetration into society for this controversial microorganism or a cutting-edge scenario offering a constructive solution to eliminate the potential risk of the outbreaks, also early identification and source tracking? [2]

Conventional Routine Techniques

Microbiological Investigations

Commonly referred to as the gold standard; Culture is still a traditional common method performing by many laboratories. Buffered Charcoal Yeast Extract (BCYE) agar supplemented with an absolute requirement, L-cysteine, and α-ketoglutarate, with or without antibiotics, has been known as a standard medium for the isolation and estimation of *Legionella*. Since L-cysteine is a significant growth enhancer, an organism that grows on BCYE supplemented with L-cysteine is most likely classified as *Legionella spp*. However, culture suffers from a lack of ability to detect Viable But Non Culturable (VBNC) and overgrowth of microbial flora, which are the main obstacles performing culture as a double-edged sword. Likewise, culture is time-consuming, requiring prolonged incubation time about 14 days at 37Co with 5% CO2. Bacterial colonies appear to be gray, glistening with a 3-4 mm diameter. The Culture technique is not able to detect *Legionella* within amoeba [3]. Furthermore, it is not worthwhile for all *Legionella* species because they are not cultivable; it proved inefficient. Another drawback of this technique is that culture is not sensitive enough for the low counts of bacteria and indicates the lowest sensitivity than PCR and UAG test. The sensitivity of culture has been reported by approximately 10%-80%, depending on the samples' nature, technician skills, and antibiotic therapy. Besides, pre-treatment (heat and acid) for water samples before the culture is laborious and contributes to missing *Legionella's* even minimum level. The standard samples for culture are respiratory specimens, BAL and sputum, and water. Apart from the poor survival and yield of *Legionella* in respiratory secretions, most of the patients with Legionnaires' disease are not able to routinely produce a sputum sample. Despite all limitations, culture has been in accordance with the International Organization for Standardization (Water quality-Enumeration of *legionella*-ISO 11731:2017). To make a long story short, the generation of culture-independent techniques are considerably demanding; covering the shortages of classical means, also opening up a new field of research. Due to the proper bioinformatics studies and primer/probe designs in nucleic acid-based methods, the specificity is around 100% and sensitivity is equal or more than culture [4].

PCR (Polymerase Chain Reaction)

The amplification-based assays seem of the utmost value in providing rapid diagnostic tools and are frequent. PCR has been rapidly developed as an alternative method to culture-dependent analysis and is thus recommended as a complement to culture. By comparison with culture, thanks to rapidity, sensitivity, specificity and accuracy, PCR has been gaining importance in recent years. PCR is easy to carry out and all laboratories benefit from this technology to detect the waterborne pathogens and it is able to amplify 109 copies of the target. The main advantages of this technique are the variety of both clinical and environmental samples. Respiratory specimens, urine, and serum obtained from patients with Legionnaire's disease represented 30%-86% sensitivity and 98%-100% specificity [5]. Another point is that the initial diagnosis of Legionnaire's disease would properly increase the sensitivity of PCR. However, despite being successful and not to be tedious, this methodology has a number of weaknesses. First of all, PCR is unqualified to distinguish between live and dead cells and accordingly, extreme caution must be taken when viability assessment is the purpose. Therefore, Ethidium Bromide Monoazide (EMA) and Propidium Mono Azide (PMA) have been broadly used to distinguish dead cells from viable cells. Second, because of the existence of inhibitors in environmental samples, false positive and negative in PCR reaction are inevitable. As for other disadvantages, cross-contamination occurs when handling the samples and DNA extraction; making this approach unreliable. The most popular genes consist of ribosomal subunits (16S, 5S-23S) and/or the macrophage inhibitor potentiator, mip, using to identify the genus of *legionella* and *L. pneumophila* species, respectively. Regarding other candidate genes, it was found that dotA; gyrB, dnaJ, wzm, and wzt are common genetic markers. Besides, Multiplex PCR was accomplished to detect both Legionella genus and species simultaneously [6].

LAMP PCR

Isothermal nucleic acid amplification technologies can be performed at constant reaction temperature and high speed for the amplification. Additionally, heat denaturation is not needed to make single-stranded DNA, obviating the use of thermal cycler. Isothermal amplification is generally classified into four categories; Nucleic Acid Sequence-Based Amplification (NASBA), Self-Sustained Sequence Replication (3SR), Strand Displacement Amplification (SDA) and Loop-Mediated Isothermal Amplification (LAMP). LAMP was first developed in 2000 by Notomi using a DNA and two sets of primers which are able to recognize six distinct regions on the target genes regarding this assay a high specificity around 90% and sensitivity less than 10 copies but costly [7]. Overall, this limit of detection below 10 copies strengthens that it would be interesting to compare strongly LAMP with powerful absolute quantitative real-time PCR, verifying the inconsistent results between Culture and LAMP. However, if applicable, designing appropriate primer pairs with high throughput is a notable difficulty. LAMP has different clinical applications, applying to detect a variety of bacterial and viral pathogens and is less affected by the inhibitory components. Beside, LAMP can amplify RNA sequence by combination with RT (Reverse Transcriptase) is a versatile, economic and valuable method of analysis. The positive results monitored via visual observation, measuring real-time turbidity, using DNA binding dyes, SYBR green, and forming an insoluble complex. One advantage of LAMP is that it avoids the problem of post-amplification procedures and instrumentation requirements. It is interesting to note that, on-site detection, without the need for DNA isolation, is the most surprising aspect of this approach. Moreover, there has been developed different LAMP assays and substantial novel progress has been made to synthesize the lyophilized reagent of LAMP, accelerating the test process [8].

Immunological Experiments

Immunological methods have been succeeded in real-time consideration of microorganisms with a satisfactory short time of analysis, being comparable to that of culture. Urinary Ag Test (UAT) of *Legionella* has revolutionized the laboratory testings and has been a breakthrough point for diagnosis. This test is highlighted for the early diagnosis of LD, almost 2-3 days after the onset of disease, easy-to-obtain samples and emerged as a beneficial tool for outbreaks investigations. Specific urinary Ag is dramatically detectable after antibiotic treatment and persists for days after clearance of infection. As compared to culture, the strength of this assay relates to simplicity, sensitivity (87%), and specificity (97.7%). However, a serious weakness with this assay is

the inability to detect species/serogroups other than *L. pneumophila* serogroup 1. It should be noted that one of the disadvantages is that, it fails to identify other *Legionella* species while detection of serogroup 4 and 6 are vitally important equal to serogroup 1. UAT is commercially available in two Binax Enzyme Immuno Assay (EIA) and Immuno Chromatographic Test (ICT). Since the UAT is particularly useful and has a number of attractive features, further attempts should be undertaken to cover *legionella* species other than serogroup 1. Thus, care is required when interpreting results. Antibody-based approach is of another type of non-invasive method. One downside regarding this methodology is the cross-reactivity between species. Besides, it has a poor performance during the acute phase in serum sample and is rather disappointing as a result of emerging new molecular and standardized culture methods. Direct Fluorescent-Antibody Assay (DFA) is applied for qualitative identification and typing of *Legionella* [9].

Real-time PCR

In the midst of the evolution era in biotechnology, real-time PCR claimed to be a molecular gold standard and represented a leap forward in all fields of research and innovations. It provides a profoundly insight into the kinetics of reactions, also surpassing other PCR models. When compared to other experiments, Quantitative real-time PCR is the method of choice capable of amplifying and quantifying a target sequence simultaneously even for less than five or one copies of a target. Recent studies have revealed some genes of interest regarding qPCR comprising 16s ribosomal RNA, 5S-23S, mip, lolA, sidF, csrA, and dotA. Since rRNA is an indicator of cell metabolism, RT-qPCR targeting genes encoding rRNAs yielded higher information of live bacteria and monitored the metabolic activity of cells. What is important is tackling the severe damages of the infection in an early diagnostic stage with high speed and accurate method. Real-time monitoring of amplification in a closed system by using probe/primers or dyes, reduced-size of amplicon and accurate data analysis in Genome Units (GU/L) are all strikingly pivotal in order to break down the barriers. Also, this technique allows for a relative (gene expression) and absolute quantification (exact copy number) of the amplicon, high melting resolution analysis, and allelic discrimination. As alluded earlier, due to the online monitoring, there is no need to post amplification procedures, being aware of what happened in a single tube reaction by melting curve analysis using SYBR green which is generally employed for gene expression pattern. Indeed, Primer dimers and non-specific amplification can be verified by dissociation curve analysis. Overall, qPCR represents a groundbreaking alternative to PCR and the principal advantages are sensitivity, reproducibility, specificity, but needs particular equipment, as well skilled technician. qPCR (single and/or multiplex) has been designed particularly for environmental samples. As expected, the cell viability was demonstrated utilizing qPCR along with pre-treatments of EMA and PMA, estimating the sanitary risk and quality assessment of water and disinfectants function. Considering all above, qPCR has been extensively approved and has been gained prominence for identification of *L. pneumophila* and *Legionella spp* in environmental investigations. Although culture is the gold standard, qPCR often refers to a rapid supplement, conducting higher positive results compared to Culture. Further, qPCR has the ability to detect dead, VBNC, injured cells and cells inside the amoeba, but it can be affected by the presence of inhibitors [10].

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