UNIVERSITAT ROVIRA I VIRGILI

# EXPERIMENTAL EVALUATION AND VALIDATION OF PCR AS A DIAGNOSTIC TOOL COMPLEMENTARY TO CULTURE FOR THE DETECTION OF *LEGIONELLA SPP.* IN ENVIRONMENTAL WATER SAMPLES

Final Degree Project

Biochemistry and Molecular Biology

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# INDEX

## ABSTRACT

*Legionella spp.* is a waterborne pathogen capable of causing severe respiratory infections, particularly in vulnerable populations. Its persistence in water systems, biofilms, and protozoa, along with the limitations of the conventional culture method—currently the official standard—highlight the need for faster, more sensitive detection techniques to improve environmental surveillance and prevent outbreaks.

This study aims to evaluate whether the polymerase chain reaction for *Legionella spp.* genome (PCR) technique can be validated as a complementary or alternative method to traditional *Legionella spp.* culture for the detection of this bacteria in water samples, in accordance with current health regulations. To this end, two commercial PCR kits were compared to select the most sensitive one. A sample size of n=30 of water samples from different sources (cooling towers, hot and cold-water systems, spa pools, private pools and ornamental fountains) were analysed using both *Legionella spp.* culture and PCR for *Legionella spp.* genome, applying the same volume (500 mL) for each method. Diagnostic parameters were calculated to compare the results obtained by the two methods. Sensitivity, specificity, positive and negative predictive values were assessed, along with statistical analyses of agreement (to evaluate how consistently the two methods classified the same samples) and association (to determine whether a statistical relationship exists between their results).

The results showed that PCR achieved 100% sensitivity and 100% negative predictive value, which indicates that PCR reliably detected all true positives and correctly ruled out all negatives, while specificity was 63% and the positive predictive value was 61%, compared with the culture results. Statistical analysis revealed a significant association between both methods ($p = 0.0006$) and a moderate Cohen's Kappa index (0.564). Moreover, PCR proved especially useful in highly contaminated samples where culture was limited due to microbial overgrowth.

Overall, the study demonstrates that PCR is an effective, rapid, and sensitive tool for the detection of *Legionella spp.* in water samples, and supports its potential incorporation as a complementary technique in official environmental surveillance programs.

**Keywords:** Culture methods, environmental, *Legionella spp*., Legionnaires' disease, PCR, public health, viable but non-culturable (VBNC), water samples.

## ABBREVIATIONS

°C: Celsius degrees

× g: Relative centrifugal force

BCYE: Yeast extract and activated charcoal agar

CDC: Centres for Disease Control and Prevention

CFU: Colony-forming units

COPD: Chronic obstructive pulmonary disease

Ct: Cycle threshold

DNA: Deoxyribonucleic acid

dNTPs: Deoxynucleotide triphosphates

ECDC: European Centre for Disease Prevention and Control

EWGLI: European Group for the Study of *Legionella* Infections

FISH: Fluorescence in situ hybridization

GU: Genomic units

GVPC: Selective culture medium with Glycine, Vancomycin, Polymyxin B and Cycloheximide

IC: Internal Control

IIF: indirect immunofluorescence

kDa: Kilodalton

LD: Legionnaires' disease

*L. anisa: Legionella anisa*

*L. pneumophila: Legionella pneumophila*

Lp1: *Legionella pneumophila* serogroup 1

*L. micdadei: Legionella micdadei*

MIS: Microbiological Information System

MND: Mandatory Notifiable Diseases

NPV: Negative predictive value

PCR: Polymerase Chain Reaction

PMA: Propidium monoazide

PPV: Positive predictive value

qPCR: Quantitative Polymerase Chain Reaction

RD: Royal Decree

rRNA: Ribosomal ribonucleic acid

RNA: Ribonucleic acid

SL-T01: Buffer solution prepared from monopotassium phosphate and magnesium chloride

UDG: Uracil-DNA Glycosylase

µm: Micrometres

VBNC: Viable but non-culturable

WHO: World Health Organization

# 1. INTRODUCTION

## 1.1. THE PROBLEM OF *Legionella spp.* IN WATER

Access to potable water is fundamental for public health, as well as being a universal human right. However, the availability of quality water is being threatened globally by the scarcity of water resources, the effects of climate change, and increasing pollution. Population growth, along with the increase in food and energy needs that it entails, has led to a considerable rise in the demand for water, surpassing the supply capacity and causing a decrease in the availability of this vital resource. This issue is reflected in current data, which show that 2 billion people worldwide lacked access to safely managed drinking water services in 2022, understood as water from an accessible source for the population and free from faecal and priority chemical contamination (Babuna et al., 2023).

These contamination events occur due to discharges and infiltrations of chemical, biological, or radioactive substances into the drinking water system, as well as deficiencies in water treatment or distribution (Gamage et al., 2016). In this context, microbiological contamination is an especially relevant issue due to its direct impact on the spread of diseases such as cholera, typhoid fever, hepatitis A, and those caused by *Escherichia coli* and *Legionella spp*. Therefore, early detection of these pathogens in water sources is crucial to ensure water security and sustainable development (Zhang et al., 2021).

## 1.2. *LEGIONELLA* GENUS CHARACTERISTICS

The *Legionella* genus belongs to the taxonomic order Legionellales, which includes the families Coxiellaceae and Legionellaceae. Three genera have been proposed within Legionellaceae: *Legionella*, *Fluoribacter*, and *Tatlockia*. However, the latter two have not been widely accepted, so *Legionella* is used as the sole genus to describe all species (Asociación Española de Normalización, 2017).

The Legionellaceae family comprises only the *Legionella* genus, which includes at least 61 described species (*L. pneumophila, L. micdadei, L. anisa*, etc.), with more than 70 serogroups. *L. pneumophila* alone contains at least 15 different serogroups, while nine other species have two distinct serogroups, and the remaining species each contain only one serogroup. The serogroups most frequently associated with disease are serogroups 1, 4, and 6 of *L. pneumophila* and *L. micdadei*.

*Legionellae* are gram-negative bacilli with high growth requirements. They are strict aerobes and range in size from 2 to 20 µm. They possess a polar flagellum that allows them to move. Additionally, they belong to a genus that lacks resistance structures, meaning they do not form spores. They can use proteins rather than carbohydrates as their energy source (Khodr et al., 2016). They are microorganisms able of growing under a wide range of physicochemical conditions, multiplying at temperatures between 20°C and 50°C, although their optimal growth

temperature is between 35°C and 37°C. Their natural ecological niche is surface waters, lakes, and rivers, where they form part of the bacterial flora, without excluding seawater (Boletín Oficial del Estado, 2022).

On the other hand, *L.pneumophila* is a bacterium belonging to the *Legionella* genus. It is a facultative aerobe that can withstand temperatures of 50°C for several hours, although it does not multiply at temperatures below 20°C (Cunha et al., 2016). *L. pneumophila* is an opportunistic pathogen, naturally present in aquatic environments, where it survives as an intracellular parasite of amoebae or protozoa such as *Acanthamoeba castellanii*, species of *Hartmannella*, or species of *Naegleria* (Fields et al., 2002).

*Legionella spp.* can transform into viable but non-culturable (VBNC) and persistent forms in water. Although further studies are required, VBNC *Legionella spp.* has been shown to retain virulence properties and are capable of resuscitation. This is because these cells have thickened outer membranes, have high resistance to environmental and chemical factors, and exert a slow growth rate. It has been demonstrated that *L. pneumophila* enters a VBNC state after a period of starvation in nutrient-free water for 33–40 days (Chang et al., 2007). In addition, they can grow on necrotrophic substrates and survive within protozoa and biofilms, which compromises the effectiveness of control strategies based on chemical, mechanical, and physical disinfection systems. In this context, the prevention of Legionnaires' disease requires a proactive, evidence-based approach that includes both the accurate identification and risk assessment of *Legionella* bacteria in high-risk facilities, as well as the appropriate application of supplemental disinfection treatments (Alleron et al., 2008).

### 1.3. EPIDEMIOLOGY OF LEGIONELLOSIS

Legionellosis refers to a group of infectious diseases caused by bacteria of the *Legionella* genus, which was unknown until the 20th century. It was identified as a pathogenic microorganism after the epidemic outbreak in 1976, which caused severe pulmonary conditions and even deaths among a group of people who attended an American Legion convention in the state of Pennsylvania, a fact that led to its name (WHO, 2022).

As can be seen in the following section of the introduction, *Legionella spp.* infection occurs mainly through the inhalation of contaminated aerosols, which are generated by various sources of contaminated water, such as drinking water systems or air conditioning systems (Donohue et al., 2022).

Once inside the respiratory tract, *Legionella spp.* is phagocytosed by alveolar macrophages, inhibiting the bactericidal activity of phagocytes. Inside the phagosomes, it converts them into a niche for replication, preventing their fusion with lysosomes and their acidification (Fields et al., 2002). At the end of the intracellular phase, a series of changes occur in the bacteria that allow it to adapt to the different stages of the process, which ends after multiplication, acquiring a series of characteristics that will allow it to be released to the outside, induce apoptosis of the

host cells in order to be able to release and reinfect other macrophages (acid resistance, flagella expression and cytotoxicity, among others) (Chauhan & Shames, 2021).

*L. pneumophila* serogroup 1, is the most virulent species and the main cause of legionellosis, being this serogroup the one that produced the epidemic in 1976, and the cause of 65-90% of all cases of legionellosis where microbiological isolation has existed (Mercante & Winchell, 2015). Besides Lp1, the strains most associated with human disease are other *L. pneumophila* serogroups, *L.micdadei*, *Legionella bozemanae*, and *Legionella longbeachae* (Yu et al., 2002). Table 1 shows 25 documented Legionella species other than *L. pneumophila* that cause human infection.

**Table 1.** *Legionella* species associated with human disease.

| | | | |
|---|---|---|---|
| *L. anisa* | *L. erythra* | *L. longbeachae* | *L. pneumophila* |
| *L. birminghamensis* | *L. feeleii* | *L. lytica* | *L. sainthelensi* |
| *L. bozemanii* | *L. gormanii* | *L. maceachernii* | *L. steelei* |
| *L. cardiaca* | *L. hackeliae* | *L. micdadei* | *L. tusconensis* |
| *L. cincinnatensis* | *L. jordanis* | *L. nagasakiensis* | *L. wadsworthii* |
| *L. clemsonensis* | *L. lansingensis* | *L. oakridgensis* | |
| *L. dumoffii* | *L. londiniensis* | *L. parisiensis* | |

Source: (Asociación Española de Normalización, 2017)

The clinical profile of infected humans varies widely, from asymptomatic forms to severe pneumonia with multi-organ failure. However, two clinical forms are classically distinguished: pulmonary infection, *Legionella* pneumonia or Legionnaires' disease, and Pontiac fever or self-limited acute global syndrome (European Centre for Disease Prevention and Control [ECDC], 2022). Legionnaires' disease is the most severe form of legionellosis, presenting as a severe pneumonia affecting the lungs, while Pontiac fever is a milder manifestation (Hamilton et al., 2018). We can see the comparison of the two forms in Table 2.

Pontiac fever presents a feverish illness with joint and muscle pain (arthromyalgia) and general malaise, including fever, cough, chest pain, diarrhoea, and confusion. It is a self-limiting illness with mild symptoms that progress toward recovery. Pontiac fever is therefore an acute, non-pneumonic febrile illness (Mondino et al., 2020).

Legionnaires' disease presents an incidence of 1–5%. The clinical profile can range from atypical pneumonia to a classic form. Other organ involvement is common, such as the kidneys, liver, gastrointestinal tract, and nervous system. The most common symptoms are high fever, cough, muscle pain, chills, headache, chest pain, phlegm, diarrhoea, mental confusion, or altered consciousness. The mortality is 15–20%, although this decreases with early antibiotic treatment (Newton et al., 2010).

**Table 2.** Characteristics of the Clinical Forms Caused by *Legionella spp.* infection in humans.

| | Legionnaires' disease | Pontiac fever |
|---|---|---|
| **Incidence*** | 1–5% | 95% |
| **Incubation Period** | 2–10 days | 1–2 days |
| **Symptoms** | Fever, cough, muscle pain, chills, headache, chest pain, sputum, diarrhoea, confusion, coma | Fever, muscle pain, cough, chills, headache, chest pain, confusion |
| **Effects on Lungs** | Pneumonia | Pleuritis, No pneumonia |
| **Other Organs Affected** | Kidneys, liver, gastrointestinal tract, nervous system | None |
| **Mortality** | 15–20% | 0% |

**TABLE LEGEND:**
***** Incidence within the group of individuals infected by *Legionella spp.* and by year.

Source: Own creation based on (J. Vaqué Rafart & X. Martínez Gómez, 2002)

From water in the form of aerosols, they accidentally reach humans. Most of the exposed people turn out to be asymptomatic, and only a small percentage of them develop pathogenicity, although today there is no evidence of contagion from person to person (Abu Khweek et al., 2013). Affected individuals present risk factors such as respiratory infections, chronic obstructive pulmonary disease (COPD), cardiovascular disease, advanced age, people taking glucocorticoids or immunosuppression, alcoholism, diabetes, malignancy, hepatic or renal failure, chronic obstructive lung disease, a smoking history and patients undergoing dialysis treatment. However, healthy individuals can also suffer from the disease if they have been exposed to sufficiently high concentrations of the infectious agent (Centres for Disease Control and Prevention, 2013).

In Spain, Royal Decree 865/2003 of July 4 establishes the hygiene and health criteria for the prevention and control of legionellosis. This regulation affects all facilities likely to generate aerosols and establishes maintenance strategies and physical, chemical, and microbiological water control for each of them (Boletín Oficial Del Estado, 2003).

Since 1996, it has been included among the Mandatory Notifiable Diseases (MND) of respiratory transmission. When a case is suspected, doctors in both the public and private sectors must report it to the National Epidemiological Surveillance Network (weekly). In the event of an outbreak, it must be urgently and obligatorily reported to the National Epidemiological Surveillance Network. Subsequently, once the outbreak has ended and been brought under control, the affected autonomous community must prepare a final report, reflecting the relevant investigation, and submit it to the National Epidemiology Centre. Furthermore, hospital clinical microbiology laboratories must report any cases they identify to the Microbiological Information System (MIS). These protocols are explained in Table 3. Spain is a member of the European Group for the Study of *Legionella* Infections (EWGLI), which allows us to identify cases among tourists who have contracted the disease in our country and to report cases among Spaniards after a trip abroad (Ministerio de Sanidad, 2017).

**Table 3.** Supplementary information systems and notification sources for legionellosis cases in Spain and Europe.

| | Who reports? | Period of time |
|---|---|---|
| **Mandatory Reportable Diseases (MND)** | Practicing physicians | Weekly |
| **Notification of epidemic situations and outbreaks** | Responsible parties for the study of the Autonomous Community | Quarterly |
| **Microbiological Information System (MIS)** | Clinical microbiology laboratories | To be established by the Autonomous Community |
| **Reporting of legionellosis cases in travellers in Europe** | EWGLI: European group with reciprocal information on cases of legionellosis in travellers | |

Source: Own creation based on (Ministerio de Sanidad, 2017)

## 1.4. SOURCES OF CONTAMINATION

The water distribution systems of healthcare facilities are complex, considering the variation in the size of the building and campus, the objectives of providing healthcare, and the operations of the facilities with variable water demands within the system. All these factors can influence the growth and persistence of plumbing pathogens, sometimes in competitive ways. Additionally, different healthcare environments (e.g., acute care hospitals, long-term care centres) have different infrastructures, operational requirements, and patients. In all these aspects, the proliferation of pathogens such as *Legionella spp.* in water systems can lead to massive infection outbreaks among different individuals (Gamage et al., 2016).

The infection that causes legionellosis diseases, primarily caused by *L. pneumophila*, occurs mainly through the inhalation of contaminated aerosols, as mentioned earlier, which are generated by various sources of contaminated water, such as potable water systems, air conditioning cooling systems, showers, or cooling towers, etc. Although less common, it can also be transmitted through micro aspiration of contaminated water or by direct contact with surgical wounds (Donohue et al., 2022). As shown in the following Table 4, these are the facilities with the greatest capacity for the proliferation and dispersion of *Legionella spp.* (Boletín Oficial Del Estado, 2003).

**Table 4.** Facilities that favour the proliferation and dispersion of *Legionella spp*.

| |
|---|
| **1. Facilities with a higher probability** |
| - Cooling towers and evaporative condensers |
| - Domestic hot water systems with a storage tank and recirculation circuit |
| - Whirlpool baths and swimming pools |
| - Industrial humidification plants |
| **2. Facilities with a lower probability** |
| - Internal installations of cold drinking water (pipes, tanks) and domestic hot water systems with a recirculation circuit |
| - Evaporative cooling equipment that sprays water |
| - Industrial humidifiers |
| - Ornamental fountains |
| - Irrigation systems by sprinkler in urban environments |
| - Water-based fire protection systems |
| - Industrial aerosolization and outdoor irrigation systems |
| - Other devices that accumulate water and can produce aerosols |
| **3. Risk facilities in espiratory therapy** |
| - Respiratory therapy equipment |
| - Respirators |
| - Humidifiers and nebulizers |
| - Other medical equipment in contact with the respiratory tract |

Source: Own creation based on (Boletín Oficial Del Estado, 2003).

From these natural reservoirs, the bacteria can colonize the supply systems, and, through the water distribution network, entering the sanitary water systems (cold or hot) or other systems that require water for their operation, such as cooling towers (Zhang et al., 2021).

The presence of water contaminated with the bacteria in poorly designed, poorly installed, poorly maintained, or inadequately maintained facilities promotes water stagnation and the accumulation of nutrients for the bacteria, such as sludge, organic matter, corrosion products, and amoebas, forming a biofilm. The presence of this biofilm, along with a favourable temperature, explains the multiplication of *Legionella spp.* to infective concentrations for humans (Boletín Oficial del Estado, 2022). If there is an aerosol-generating mechanism in the facility, the bacteria can disperse into the air. The aerosols containing the bacteria can remain suspended in the air and be inhaled into the respiratory system of exposed individuals (Ministerio de Sanidad, 2017).

Due to its ability to persist in water systems and its potential to cause severe respiratory diseases, it is crucial to take preventive and control measures to avoid the spread of *Legionella* (Cunha et al., 2016). For its monitoring, it is important from a public health perspective to identify environmental sources that may constitute a legionellosis risk, such as hotels, hospitals, spas, and others. This is also important for the validation of control measures and to constantly verify their effectiveness (Asociación Española de Normalización, 2017).

### 1.5. DETECTION METHODS OF *Legionella spp.*

Currently, there are several methods for the detection and enumeration of *Legionella spp.,* including: culture, PCR, fluorescence in situ hybridization (FISH), indirect immunofluorescence (IIF), solid-phase cytometry, optical wave light spectroscopy, and enzyme-amplified electrochemical DNA probe detection (Cooper et al., 2009).

The official and current reference method in Spain and other European countries for the detection of *Legionella spp.* is the culture method outlined in the UNE-EN ISO 11731:2017 standard *Water quality – Enumeration of Legionella*. However, the ability of public health officials to respond quickly to an LD outbreak is hindered by the time required for culturing. Faster quantification methods are needed for both public health response and routine sampling (Boletín Oficial del Estado, 2022).

For this reason, other analytical methods than culture may be used for the detection of *Legionella* spp. in the following situations: health risk assessment for the population, investigation of case occurrence, investigation of an outbreak, when deemed necessary by the health authority, and finally, when equipment shows irregular operation or multiple shutdowns and restarts over short periods of time—without prejudice to the performance of the corresponding cultures. In these cases, alternative methods shall be complementary to culture, which remains mandatory at the frequencies established in Part C of Annex V (Boletín Oficial del Estado, 2022).

Other methods, such as the one described in UNE-ISO/TS 12869 *Water quality – Detection and quantification of Legionella spp. and/or Legionella pneumophila* by concentration and polymerase chain reaction (PCR) amplification, may be used as a complement to culture (Boletín Oficial del Estado, 2023). Recently, PCR targeting the 16S rRNA of *Legionella spp.* has been widely applied to overcome the limitations of standard culture methods (Touron-Bodilis et al., 2011).

#### 1.5.1. CULTURE OF *Legionella spp.*

Culture-based methods for the detection of microorganisms are widely used for microbial enumeration. These include dip slides, colorimetric growth methods, viable cell counts by serial dilution, and plate counts. All these procedures provide microorganisms with the necessary nutrients and conditions to form colonies, which can then be visualized, counted, and, in some cases, identified. Their use is widespread in microbiology laboratories, and they represent a low-cost technique (Austin, 2017).

However, culture-based methods require incubation of the plates and waiting for visible growth, which delays the availability of results and means that, at best, the risk is identified 24 hours after the sample has been collected. When using this type of method, it is important to consider that the microbial population in the sample is likely to be significantly underestimated.

Therefore, it is advisable to corroborate negative results with other methodologies that allow the detection of non-culturable microorganisms (Bonnet et al., 2020).

For laboratory culture, *Legionella spp.* requires relatively complex culture media for their multiplication. This necessitates specific additions to standard nutrient media (Pine et al., 1979). There are various agar formulations, with slight differences in selectivity and growth characteristics for different *Legionella* species. Yeast extract and activated charcoal agar (BCYE) is the most used for the growth and general maintenance of *Legionella spp.* It contains 0.1% α-ketoglutarate, L-cysteine, and a variety of supporting amino acids and micronutrients, such as calcium citrate, iron, arginine, isoleucine, leucine, threonine, valine, methionine, phenylalanine, tyrosine, and serine (CDC, 2005).

For their initial growth, they require L-cysteine. Species such as *Legionella oakridgensis* and *Legionella spiritensis* need L-cysteine and iron (III) for primary isolation. In the absence of L-cysteine, they may weakly grow. Clinically significant *Legionella* species grow best at 35°C in humidified air on BCYE agar, taking between 2 and 5 days to develop. However, some species like *Legionella lytica* cannot grow on BCYE agar without co-culturing with amoebae (Asociación Española de Normalización, 2017).

The most significant barriers to the quantitative and reproducible enumeration of *Legionella spp*. by culture arise from different issues: 1) the growth of undesirable microorganisms that hinder identification or inhibition by competing flora, and 2) the presence of viable but non-culturable (*VBNC*) *Legionella spp.,* which can be detected by PCR and 3) the need for selective agars and pre-treatment (thermal or acidic). Besides that, it is worth noting the slowness of the process (observable after 3-10 days), and the loss of bacterial viability. Moreover, selective BCYE may inhibit the recovery of *L.micdadei* or other species due to the antimicrobial agents present in the medium (Nazarian et al., 2008).

In addition, *Legionella spp.* that has replicated intracellularly in amoebae or protozoa are morphologically distinct from other *Legionella spp.* cells (Shih & Lin, 2006). Therefore, the number of *Legionella spp.* cells detected by culture immediately after amoebae host parasitism could be significantly lower than that of *Legionella* at different stages of the life cycle (Chang et al., 2007).

### 1.5.2. POLYMERASE CHAIN REACTION TECHNIQUE

Molecular detection methods are based on the identification and analysis of nucleic acids, primarily DNA and RNA. They offer significant advantages in terms of sensitivity, specificity, and speed compared to traditional culture and staining techniques, and allow for the identification of non-culturable microorganisms (Piémont & Jaulhac, 1995).

In any case, molecular methods do not allow differentiation between viable and non-viable bacteria, especially in the case of DNA-based techniques, due to the high stability of this molecule. Techniques have been developed to overcome this limitation using compounds such

as ethidium monoazide or propidium monoazide (PMA), which can penetrate cells with compromised membranes, bind to DNA, and inhibit its amplification (Nocker et al., 2006).

Quantitative Polymerase chain reaction (qPCR) is an alternative method for the rapid detection of *Legionella spp*. in environmental samples, and currently there are several commercially available PCR chemistries. It simultaneously amplifies and quantifies a target DNA sequence, providing the number of genomic units (GU) per litre. Despite these benefits, there are knowledge gaps regarding the interpretation of PCR GU results compared to those obtained by culture (colony-forming units, CFU). No equivalence has been established between CFU and GU, and the results largely depend on the method used and the composition of the sample (Wellinghausen et al., 2001). Conventional PCR, on the other hand, also allows for the rapid detection of *Legionella spp.* in environmental matrices by amplifying target DNA sequences, but it does not provide quantification. **For this reason, the PCR technique used in this study was the qualitative conventional PCR, aimed at determining the presence or absence of *Legionella spp.*, rather than measuring its concentration.**

The rapid turnaround time and sensitivity of PCR offer advantages over traditional culture methods. Additionally, DNA present in environmental samples can be very stable and persist for long periods of time. The use of PCR as a detection tool can be useful both for ruling out negatives and for quickly identifying positives (Brooks et al., 2004a).

Although PCR is significantly more sensitive than the culture method for detecting lower levels of contamination, it may not be able to detect low levels of *Legionella spp.* contamination in a natural environment (Delgado-Viscogliosi et al., 2009). The main limitations are: 1) the tendency to overestimate due to the amplification of non-viable or dead cells, which can lead to an overestimation of the actual health risk; 2) the high cost; and 3) their dependency on infrastructure, as they require controlled laboratory environments and trained personal (Piémont & Jaulhac, 1995). Another challenge with PCR is the presence of environmental compounds that inhibit the PCR reaction. These inhibitors may include divalent cations, minerals, or other debris that can interfere with the polymerase and reduce amplification efficiency (Brooks et al., 2004b).

## 2. HYPOTHESIS AND OBJECTIVES

In light of the current limitations of culture-based methods and the increasing need for rapid and sensitive detection of *Legionella spp.* in water systems, **the hypothesis of this work was to ascertain if we could consider the conventional PCR technique as a reliable and rapid method for the detection of *Legionella spp*. in water samples, with equal or greater sensitivity than the traditional culture method and therefore be validated as a complementary or alternative tool for official use under current legislation.**

Early and accurate detection of *Legionella spp.* in water systems is essential for the prevention of legionellosis outbreaks. Currently, existing regulations establish culture-based methods as the official technique for its identification, due to their ability to quantify viable bacteria. In contrast, PCR is proposed as an alternative tool, offering faster and more sensitive detection.

The aim of this study is to validate if the PCR technique allows a faster and more sensitive detection of *Legionella spp.* in water samples compared to the traditional culture method, and therefore could be considered a valid tool for inclusion as an official detection method within the current legislative framework. The implementation of this assay could enable rapid screening of negative samples and faster identification of outbreak sources, resulting in significant time and cost savings, as well as substantial benefits for public health.

To this end, 30 water samples will be analysed using both techniques, assessing the presence or absence of bacterial growth in culture and the Ct (cycle threshold) values obtained through PCR. The comparison of the results will allow for the evaluation of the correlation between both techniques, as well as the efficiency and speed of PCR as a potential official diagnostic method in the future.

To achieve this, the following specific objectives have been set:

- To compare the efficacy of different commercial PCR kits in the detection of *Legionella spp.* in water samples, to select the one with the highest sensitivity for subsequent use in the main objective of the study
- To analyse the sensitivity and specificity of PCR compared to traditional culture for detecting *Legionella spp.* in environmental samples.
- To establish a detection limit for PCR determination.
- To evaluate the potential of PCR as a complementary technique to culture, for eliminating negatives and confirming positive samples.
- To compare the response times of each method to assess the speed of detection and its utility in public health emergency situations.

- To evaluate the limitations of traditional culture regarding time, growth conditions, and the risk of false negatives.

- To examine the influence of environmental factors and potential inhibitors on the efficacy of each method.

- To compare the economic and operational viability of PCR and culture, considering costs, equipment, and necessary training.

- To analyse the current regulatory framework and assess potential changes that could allow the incorporation of PCR as a complementary or alternative tool.

# 3. MATERIALS AND METHODS

## 3.1. SAMPLE COLLECTION

As part of this project, 1-litre water samples were collected in accordance with ISO 19458:2007 and submitted to the EMATSA laboratory, accredited under ISO 17025:2017, for routine *Legionella spp.* testing. In addition, several samples were collected exclusively for the purpose of this study. To evaluate both culture-based and PCR methods across various water qualities and sources, a total of 30 water samples were collected between April and May 2025. The sampling included cooling towers (6), hot and cold-water systems (17), spa pools (2), private pools (4), and ornamental fountains (1), as shown in Table 5.

Samples were collected in accordance with the sampling guidelines established in RD 487/2022. They were taken in sterile one-litre polyethylene containers containing 24 mg/L of sodium thiosulphate to neutralize residual disinfectant — sterile bottles for water collection with thiosulphate (Deltalab, Spain). Samples were kept under isothermal conditions during transport to the laboratory and processed within 24 hours of collection. In cases where analysis could not be performed within this time frame, samples were analysed by both culture and PCR methods within a maximum of 48 hours, while being stored at 5°C.

**Table 5.** Supplementary information about sample source, location and type.

| Sample | Source | Location | Type |
|---|---|---|---|
| Sample 1 | Domestic tap | Tarragona | POT |
| Sample 2 | Domestic tap | Tarragona | POT |
| Sample 3 | Domestic tap | Tarragona | POT |
| Sample 4 | Domestic tap | Tarragona | POT |
| Sample 5 | Domestic tap | Tarragona | POT |
| Sample 6 | Domestic tap | Tarragona | POT |
| Sample 7 | Domestic tap | Tarragona | POT |
| Sample 8 | Cooling towers | Tarragona | CNT |
| Sample 9 | Cooling towers | Tarragona | CNT |
| Sample 10 | Cooling towers | Tarragona | CNT |
| Sample 11 | Cooling towers | Tarragona | CNT |
| Sample 12 | Cooling towers | Tarragona | CNT |
| Sample 13 | Cooling towers | Tarragona | CNT |
| Sample 14 | Ornamental fountain | Juncosa | CNT |
| Sample 15 | Spa pool | La Selva del Camp | POT |
| Sample 16 | Spa pool | La Selva del Camp | POT |
| Sample 17 | Community pool | Cambrils | POT |
| Sample 18 | Private pool | Salou | POT |
| Sample 19 | Sports complex shower | Reus | POT |
| Sample 20 | Domestic shower | Reus | POT |
| Sample 21 | Domestic shower | Reus | POT |
| Sample 22 | Sports complex shower | Reus | POT |
| Sample 23 | Sports complex shower | La Selva del Camp | POT |
| Sample 24 | Private pool | Reus | POT |
| Sample 25 | Private pool | Reus | POT |
| Sample 26 | Domestic shower | Tarragona | POT |
| Sample 27 | Domestic shower | Tarragona | POT |
| Sample 28 | Domestic shower | Tarragona | POT |
| Sample 29 | Domestic tap | Reus | POT |
| Sample 30 | Domestic tap | Reus | POT |

**Table Legend:**

**POT**: potable water.

**CNT**: continental water (with a greater risk of contamination)

## 3.2. IDENTIFICATION OF *Legionella spp.* PRESENCE IN SAMPLES BY CULTURE

*Legionella spp.* were isolated and enumerated according to ISO 11731:2017 (Asociación Española de Normalización, 2017). Different culture methods were used depending on the origin and characteristics of each water sample. Samples were either concentrated by membrane filtration, diluted, or directly inoculated onto agar plates.

For this reason, different volumes—with and/or without prior concentration and treatment steps—were inoculated on various plates to account for potentially high *Legionella spp.* concentrations. To reduce the growth of non-target bacteria in samples considered "dirty water" (e.g., cooling tower water, with high levels of interfering microorganisms), portions of the water samples were subjected to heat treatment, acid treatment, or a combination of both, as these treatments helped improve *Legionella spp.* recovery.

As previously mentioned in the introduction, this genus of microorganisms grows on buffered charcoal yeast extract (BCYE) agar, which contains L-cysteine and ferric salts. In addition, the GVPC medium (BCYE supplemented with glycine, vancomycin, polymyxin B, and cycloheximide) was used in cases where heavy microbial interference was expected, as its antibiotic components helped inhibit the growth of non-target organisms.

To ensure consistency and reproducibility, it is recommended to use standardized media supplied by commercial manufacturers. For this reason, the BCYE and GVPC media used were both obtained from (*Legionella* BCYE Agar with L-cysteine (BioMérieux SA, France) and *Legionella* GVPC Agar (BioMérieux SA, France)). On the other hand, the BCYE-cys medium used for confirmation of presumptive colonies was prepared in the laboratory using a dehydrated base medium produced by (*Legionella* agar base (Oxoid Ltd., UK)).

The selection of the appropriate method for *Legionella spp.* enumeration depended on the characteristics of each sample and the objectives of the study. It was necessary to anticipate the expected level of microbial interference based on the origin of the sample. The first step in determining the appropriate method was to identify whether the sample came from water with low or high levels of interfering microorganisms. Based on that, the necessary pre-treatment was selected, and finally, the appropriate culture medium was chosen. Following this process, two culture matrices were defined: matrix A, applied to clean water samples such as drinking water or swimming pools (23 samples), and matrix B, applied to contaminated samples with high microbial content (7 samples).

### 3.2.1. MATRIX A FOR *Legionella spp.* CULTURE

In cases of water with a low concentration of interfering microorganisms, this matrix is applied. This study includes samples from domestic taps, showers and pools (all those identified as POT in Table 5). The following three plating methods were used:

- **Direct inoculation of 0.5 mL onto a GVPC (*Legionella* GVPC Agar (BioMérieux SA, France) agar plate**, where the sample was spread using a sterile Drigalski spreader (Drigalsky spreader I/W (Deltalab, Spain)).
- **Filtration of 10 mL of the sample,** poured into a filtration funnel (Microfil ® filtration funnels (MilliporeSigma-aldrich, Germany)) of 250 mL, fitted with a (Microsart® CN-filter (Sartorius AG, Germany)) membrane filter of 0.45 µm. After filtration, the membrane was placed face-up onto a BCYE agar plate (*Legionella* BCYE Agar with L-cysteine (BioMérieux SA, France)).
- **Filtration of 500 mL of sample through a filter of 0.45 µm (Microsart® CN-filter (Sartorius AG, Germany))**. After filtration, 20 mL of acid solution (prepared from HCl and KCl, following the procedure in Annex A) were added and left for 5 minutes (min). The acid is then filtered, followed by careful washing with 20 mL of SL-T01 buffer solution (prepared from monopotassium phosphate and magnesium chloride, as detailed in Annex B), avoiding contact with the parts of the funnel unaffected by the acid. Finally, the membrane was incubated on a GVPC agar plate.

The use of different types of culture media, depending on the method applied, was because GVPC contains antibiotics, whereas BCYE does not. For this reason, when directly inoculating 0.5 mL of sample, GVPC was used, as no filtration is carried out and therefore cellular debris and interfering microorganisms present in the sample were not removed—these can then be inhibited by the antibiotics in the GVPC medium. The same applies to the 500 mL sample, where GVPC was also used. In this case, a larger volume was inoculated, and therefore a higher concentration of interfering microorganisms may be present, although acid treatment was applied to reduce their growth. Finally, for the 10 mL filtered sample, BCYE was used, as this is a small volume that was already been filtered; theoretically, the number of interfering microorganisms is lower, and the absence of antibiotics in the medium allows for general bacterial growth to be observed.

Once the plates were been inoculated, they were left to stand until the inoculated volume is fully absorbed. Subsequently, they were incubated upside down at a temperature of (36 ± 2) °C for a period of 7 to 10 days in the (Laboratory incubator (Heraeus Holding GmbH, Germany)) incubator.

### 3.2.2. MATRIX B FOR *Legionella spp.* CULTURE

In cases where a high concentration of interfering microorganisms was expected in the water sample, this matrix was applied using filtration and elution.

In this study, samples include cooling towers and ornamental fountains (all those identified as CNT in Table 5). As with Matrix A and PCR processing, the final volume used was 500 mL.

For filtration, a 250 mL funnel (Microfil ® filtration funnels (Millipore Sigma-aldrich, Germany)) and membrane of 0.2 µm (Nuclepore ™ Track-Etch membrane (Whatman™, Cytiva, USA)) were used. The 500 mL water sample was poured into the funnel, and once filtration is complete, the membrane was placed in a tube containing 10 mL of SL-T01 buffer solution. The tube was then vigorously shaken using (Multi Reax (Heidolph Instruments GmbH & Co. KG, Germany)) for 2 min, followed by ultrasonic bath treatment (Ultrasons (J.P.Selecta, Spain)) for 5 min. Once the process was complete, the membrane was removed and discarded using sterile forceps. These two final steps are designed to extract the cells retained on the membrane, which will remain in the buffer solution. This solution was subsequently referred to as the eluate.

The eluate is divided into three portions and subjected to the following analysis procedures:

- **2.5 mL portion of eluate:** 0.5 mL was inoculated onto a GVPC agar plate and spread using a sterile Drigalsky spatula.
- **2.5 mL portion of eluate:** The tube containing this volume was immersed in a (50 ± 1) °C water bath for 30 min. Then, 0.5 mL of the treated eluate was inoculated onto a GVPC plate and spread using a Drigalsky spatula.
- **5 mL portion of eluate:** From this volume, 1 mL was filtered onto a bed of 20 mL SL-T01 buffer solution. After filtration, 20 mL of acid solution was added for 5 min. Once the time has elapsed, the acid was filtered out and the funnel is carefully rinsed with 20 mL of buffer solution, avoiding contact with the inner wall of the funnel. Finally, the membrane was incubated on a GVPC agar plate.

  Additionally, 1 mL of eluate was diluted into 9 mL of acid solution, mixed, and left to stand for 5 min. Then, 0.5 mL of this treated volume was inoculated onto a GVPC plate and spread using a Drigalsky spatula. This procedure was referred to as the **acid treatment (1+9).**

Once the plates were been inoculated, they were left to stand until the inoculated volume was fully absorbed. Subsequently, they were incubated upside down at a temperature of (36 ± 2) °C for a period of 7 to 10 days in the (Laboratory incubator (Heraeus Holding GmbH, Germany)) incubator.

### 3.2.3. CONFIRMATION OF PRESUMPTIVE COLONIES

After incubation, colonies with characteristic morphology (typically white, rounded, or with a slight purple hue) that grow on selective media are considered presumptive *Legionella*. These colonies were confirmed as *Legionella spp.* through sub culturing on BCYE (*Legionella* BCYE Agar with L-cysteine (BioMérieux SA, France)) and BCYE without L-cysteine (BCYE-cys) prepared with yeast extract agar from *Legionella* agar base (Oxoid Ltd., UK). In order to demonstrate their requirement for L-cysteine and ferric iron for growth.

To carry out this procedure, sub culturing was performed from the plates with the highest number of presumptive colonies per volume of water. Both plates were incubated at (36 ± 2) ºC for 2 to 5 days. Presumptive colonies that grow on BCYE but not on BCYE-cys are considered confirmed *Legionella spp*. In contrast, those that grow in both media are considered negative. It will be necessary to select three presumptive colonies and subculture them in both mediums.

### 3.2.4. CONFIRMATION OF *Legionella pneumophila*

The confirmatory test described above was used to differentiate between actual *Legionella spp.* colonies and those that are not. Currently, according to ISO 11731, the species *Legionella pneumophila* is the primary cause of Legionnaires' disease within the *Legionella* genus, due to its high infection rate. For this reason, and as a point of interest, this study includes the identification of *Legionella pneumophila* to assess whether it is the predominant species in the samples analysed—although the main objective is the detection of *Legionella spp.* in various types of samples.

First, a drop of each latex reagent was placed on the edge of the corresponding circle on the test card, as shown in Figure 1 provided by the kit (*Legionella* Latex Test, Oxoid Ltd., UK). One of the reagents targets *L. pneumophila* serogroup 1, another targets *L. pneumophila* serogroups 2–14, and the third is a general *Legionella* species test reagent (for other *Legionella* serogroups). These reagents contain specific antibodies against serogroup 1, serogroups 2–14, and other *Legionella* serogroups, respectively. Next, a drop of buffer suspension was added to each circle, taking care to avoid mixing with the reagents. A confirmed *Legionella spp.* colony (as identified by the previous method) was collected using a sterile loop and carefully emulsified with the buffer and reagent within the circle. The procedure was repeated with the other reagents using colonies with similar appearance. The card was then gently rotated in a circular motion to observe potential agglutination.

A result was considered positive if blue latex particles agglutinate within one min, and no agglutination was observed in the negative control. A positive reaction indicates the presence of antigens from that specific *Legionella* serogroup in the sample.

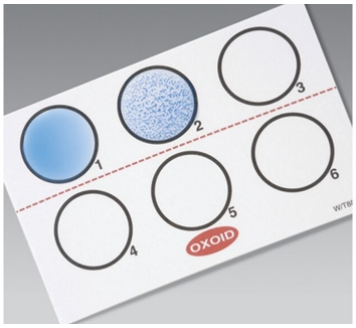


**Figure 1**. Test card of the kit based on agglutination of antigens of different serogroups of *Legionella*.

Obtained from (Oxoid Ltd., 2025).

### 3.3. IDENTIFICATION OF *Legionella spp.* BY PCR

To carry out the identification by PCR, two *Legionella spp.* detection kits were tested—one from Condalab and the other from Ielab. This process was conducted to determine which of the two kits had a lower detection limit.

For this purpose, a dilution series was prepared from a single colony of *Legionella spp.* obtained from a pure culture. First, a colony was collected from the culture using an inoculating loop and introduced into a tube (12 mL tub PS Capped I/B (Deltalab, Spain)) containing 10 mL of SL-T01 buffer solution. From this initial sample, the corresponding dilutions were prepared.

It was considered that a typical bacterial colony is estimated to contain between $10^6$ (one million) and $10^8$ (one hundred million) individual bacterial cells. However, this number can vary significantly depending on the bacterial species, growth conditions, and the age of the colony (Michael T. Madigan et al., 2018). Therefore, the initial sample was considered to contain $10^8$ CFU in 10 mL of buffer solution. Based on this estimate, the necessary dilutions were performed to ultimately obtain a concentration of 10 CFU per tube, to determine whether such a low concentration could still be detected and amplified by PCR. In total, eight serial dilutions were carried out from the initial colony, as we can see in Figure 2.

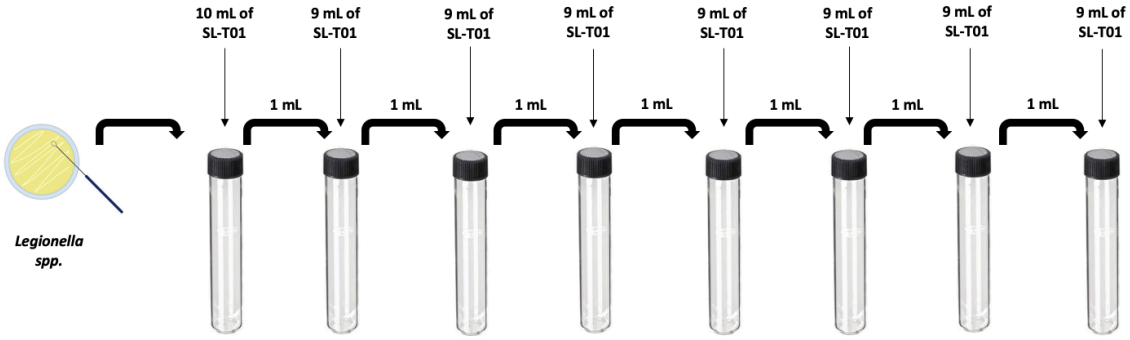


**Figure 2.** General outline of the procedure followed to perform serial dilutions from a pure *Legionella spp.* colony.

Once all the dilutions had been prepared, they were analysed using the two previously mentioned PCR kits to determine which one was more effective at detecting the lowest dilution. To support the estimation that the first tube contained a concentration of $10^8$ CFU, 0.5 mL of each dilution was plated on BCYE agar (without antibiotics), and the number of CFU that grew on each plate was observed.

#### 3.3.1. *Legionella spp.* DNA EXTRACTION AND PCR CONDALAB

The extraction process was carried out using the Condalab *Legionella spp.* DNA extraction kit for water samples, Condagene ® DNA Extraction Rapid 100 rxn (Condalab, Spain), following the manufacturer's protocol. The kit was specially developed and tested for the DNA extraction of *Legionella* bacteria from water samples, in compliance with ISO/TS 12869:2019 requirements.

The kit isolates genomic DNA from water samples after a concentration step such as membrane filtration with a pore size of 0.45µm, Nuclepore ™ Track-Etch membrane (Whatman™, Cytiva, USA). Later, we carefully remove the filter and transfer it into a 2.0 mL microcentrifuge tube ClearLine ® microtube 1.5 mL (Biosigma S.p.A., Italy). After this step, cell lysis was performed in order to release the intracellular DNA, this occurs by the addition of lysis buffer.

To begin, 100 µL of lysis buffer was pipetted into the microcentrifuge tube and the pellet was resuspended by vigorous vortexing. After a brief centrifugation, the tube was incubated for 10–15 min at 95 °C, using ThermoStat C 1.5 mL (Eppendorf AG, Germany). The sample was then centrifuged for 5 minutes, and the clear supernatant was transferred to a new microcentrifuge tube. The extracted DNA was either used immediately or stored at –20 °C for later use. Before reuse, the sample was mixed thoroughly and centrifuged. The procedure with all the steps and details is explained in Annex C.

With the DNA obtained from the extraction, we used *Legionella spp.* PCR (Condalab, Spain). The kit includes an Internal Control (IC). The IC was used for the evaluation of PCR inhibitors in the sample, or for the evaluation of problems that occurred during PCR preparation/amplification.

The kit includes Master mix for the target and IC for the evaluation of the PCR, buffer, dNTPs primers and TaqMan® probes, labelled with non-fluorescent quenchers. The signal for the detection of the targets is detected in the FAM channel, and the one for the IC in the ROX channel. The kit contains an assay mix with Uracil-DNA Glycosylase (UDG), preventing DNA contamination with PCR products, DNA polymerase and storage buffer. Included in the kit is also a positive control (target DNA), allowing an evaluation of the primers and probes used for the detection of the targets and a negative control (nuclease-free water) to confirm the integrity of the kit reagents.

For the PCR, the necessary volume of reaction mix was calculated based on a total volume of 15 µL per sample (comprising 13 µL of master mix and 2 µL of assay mix). In each well of the plate MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems, Thermo Fisher Scientific, USA), 15 µL of this mix were combined with 5 µL of the sample, resulting in a final volume of 20 µL using a micropipette Eppendorf Research Plus (Eppendorf AG, Germany). The same procedure was followed for the positive and negative controls, using 5 µL of each. The PCR was then run following the program set-up specified in the kit protocol in QuantStudio® 3-96-Well 0.2-mL Block (Applied Biosystems, Thermo Fisher Scientific, USA). Thermal reaction conditions were 37ºC for 15 min (initial stage) followed by 95º for 5 min (denaturation) and followed by 45 cycles of 94ºC for 15 seconds, 60ºC for 30 seconds and 72ºC for 15 seconds (repetitive cycles of denaturation, annealing and elongation).

The preparation of the PCR plate was carried out inside a vertical laminar flow cabinet Telstar Mini-V PCR (Telstar, Spain). Once the plate was prepared, it was sealed with adhesive film Optical adhesive covers (Applied Biosystems, Thermo Fisher Scientific, USA) to prevent contamination and evaporation.

It is known that Condagene® kits are designed to comply with the technical specifications outlined in ISO/TS 12869, which suggests that they use highly specific primers for *Legionella spp.* This are JFP (forward; 5'-AGGGTTGATAGGTTAAGAGC-3') and JRP (reverse; 5'-CCAACAGCTAGTTGACATCG-3'). Primers have been used to amplify a region of the 16S rRNA gene of *Legionella spp*. These primers have demonstrated high specificity for *Legionella spp.* and can detect several pathogenic species within the genus, including *L. pneumophila*, *L. micdadei*, *L. longbeachae*, *L. bozemanii*, *L. feeleii*, and *L. dumoffii*.

### 3.3.2. IELAB PCR AMPLIFICATION KIT FOR *Legionella spp.*

The other kit used to determine whether the last prepared dilution could be detected was from the commercial supplier Ielab. For PCR using this kit, the process began with a sample concentration kit, followed by DNA extraction, and finally amplification through the polymerase chain reaction.

#### 3.3.2.1. SAMPLE CONCENTRATION

Sample concentration was carried out using Water Microbial Concentration Kit (Ielab, Spain). This kit has been prepared in accordance with ISO 17034 and ISO/IEC 17025 accreditations, which ensure quality and reliability in the production of reference materials and in laboratory testing.

The kit was been designed for the concentration by filtration of water samples in which the study of the presence of *Legionella spp.* will be performed. Samples were concentrated by filtration in Vacuum pump EZ-stream ™ pump (Millipore Sigma-aldrich, Germany), through 0.45µm, Nuclepore ™ Track-Etch membrane (Whatman™, Cytiva, USA). For highly turbid samples, a preliminary centrifugation step was performed to avoid clogging the membrane during filtration. After filtration, the membrane was placed into a capped tube containing 10 mL of SL-T01 (12 mL tub PS Capped I/B (Deltalab, Spain)). To release the cells from the membrane, the tube was vortexed for 2 min in Multi Reax (Heidolph Instruments GmbH & Co. KG, Germany) and later introduced in Ultrasons (J.P.Selecta, Spain).

The resulting solution, approximately 10 mL, was transferred into the 100,000 kilodalton (kDa) Amicon filter unit provided in the kit. This sample, was further concentrated to a final volume of approximately 250 µL by centrifugation using the AFI LISA Refrigerated Centrifuge (AFI groups, France). The concentrated sample was collected from the upper part of the cartridge using a micropipette Eppendorf Research Plus (Eppendorf AG, Germany), introducing into a 1.5 mL Eppendorf ClearLine ® microtube 1.5 mL (Biosigma S.p.A., Italy). Those samples that did not reach a minimum of 200 µL were brought up to this volume with PCR-grade water, UltraPure™ Destilled water, DNase/RNase free (Invitrogen, Thermo Fisher Scientific, USA). The procedure with all the steps and details is explained in Annex D.

#### 3.3.2.2. *Legionella spp.* DNA EXTRACTION

To allow PCR to be performed on the same 500 mL water sample as culture, and so as not to alter the laboratories accredited methods, DNA was extracted from sample concentrates

following filtration. For clean samples (those that did not contain suspended solids or suspected to contain inhibitors and a reduced microbiota and a low concentration of organic matter were assumed, like drinking water samples), Clean Water DNA Extraction Kit (Ielab, Spain) was used in the concentrated samples obtained from the previous procedure.

To begin, 50 µL of lysis buffer was first added to these samples, and DNA was extracted through three freeze–thaw cycles (−20°C for 10 min and 94°C for 10 min in ThermoStat C 1.5 mL (Eppendorf AG, Germany)). Cellular debris will be collected in a pellet by centrifugation during 1 min at 15,000 relative centrifugal force (× g), in Centrifuge 5415 D (Eppendorf AG, Germany). For PCR, the supernatant will be used. If the samples are not used immediately, they will be stored at −20°C, and prior to later use, they will be spun down again to obtain the supernatant for analysis.

For dirty samples, or samples suspected to contain PCR inhibitors (e.g. cooling towers samples), Dirty water DNA Extraction Kit (Ielab, Spain) was used in the concentrated dirty samples obtained from the previous procedure. It was based on a cell lysis step and the subsequent DNA purification trough a molecular exclusion column.

First, the spin columns were prepared by adding 450 µL of resin to each column to be used, followed it was centrifugated in Centrifuge 5415 D (Eppendorf AG, Germany) to pack the column. The flow-through was discarded. Another 450 µL of resin was added to the same column, centrifuged again, and the flow-through was discarded once more. The spin column was then placed into a new collection tube. Approximately 250 µL of concentrated sample was obtained, to which 50 µL of lysis buffer was added. DNA was extracted through three freeze–thaw cycles (−20°C for 10 min and 94°C for 10 min in ThermoStat C 1.5 mL (Eppendorf AG, Germany)), during which cellular debris was pelleted by centrifugation, 1 min at 15,000 × g.

Next, 60 µL of protein precipitation reagent was added to the supernatant and incubated on ice for 8 min. The sample was then centrifuged for 8 min at 15,000 × g, and the resulting supernatant was transferred to a spin column, which is included in the kit. Finally, the column was centrifuged 2 min at 2,000 × g, and the collected volume in the collection tube was used for further processing. If the samples are not used immediately, they were stored at −20°C.

These two kits were specially developed and tested for the DNA extraction of *Legionella spp.* bacteria from water samples, in compliance with ISO/TS 17034:2017 requirements. And This DNA extraction and purification system from dirty and clean water samples is based on the research and development of LABAQUA, S.A., leading company in environmental diagnostics.

#### 3.3.2.3. PCR AMPLIFICATION OF *Legionella spp.*

As mentioned earlier in the introduction, other analytical methods than culture may be used for the detection of *Legionella spp*. in different situations, like: health risk assessment for the population, investigation of case occurrence, etc. In these cases, alternative methods shall be complementary to culture, which remains mandatory at the frequencies established in Part C of Annex V (Boletín Oficial del Estado, 2022). Other methods, such as the one described in UNE-ISO/TS 12869 *Water quality – Detection and quantification of Legionella spp. and/or Legionella*

*pneumophila* by concentration and polymerase chain reaction (PCR) amplification, may be used as a complement to culture (Boletín Oficial del Estado, 2023).

As stated earlier, the PCR technique employed in this study is qualitative; therefore, the use of external calibration is not required. A standard curve was required to establish the Ct threshold value (number of PCR cycles (denaturation and amplification) required to replicate the DNA copies originally present in the sample), above which samples were considered negative and below which they were considered positive. The Ct threshold for determining the presence of the target organism (*Legionella spp.*) can be established using the corresponding standard curve, derived from the positive control included in the kit (with a known concentration). From the intercept values of the standard curve, an average y-intercept corresponding to 1 genomic unit (Ct 1GU) is determined. A PCR result with a Ct value higher than the y-intercept should be interpreted as negative, whereas a Ct value lower than the y-intercept indicates a positive result (Asociación Española de Normalización, 2021). In the kit it was recommended a standard curve with 6 levels of different concentration, allowing accurate estimation of the average y-intercept corresponding to 1 genomic unit (Ct 1GU), like it is said in ISO/TS 12869:2021.

In this study, a total of eight different concentrations were used to generate the standard curve, starting from an initial concentration of $10^6$GU/10µL (this concentration was derived from the kit, which states that 1 µL contains $10^5$ GU, therefore, since 10 µL of the positive control were ultimately added to the well, this corresponds to a final concentration of $10^6$ GU/10µL). The standard curve was repeated a total of 5 times. From this stock solution, serial 1:10 dilutions were prepared by transferring 10 µL into 90 µL of PCR-grade water, resulting in successive concentrations of $10^5$ GU/10µL, $10^4$ GU/10µL, and so on, until reaching a final concentration of 0 GU/10µL, as we can see in Figure 3. These dilutions were used to establish the standard curve and to determine the average y-intercept corresponding to 1 genomic unit (Ct 1GU).

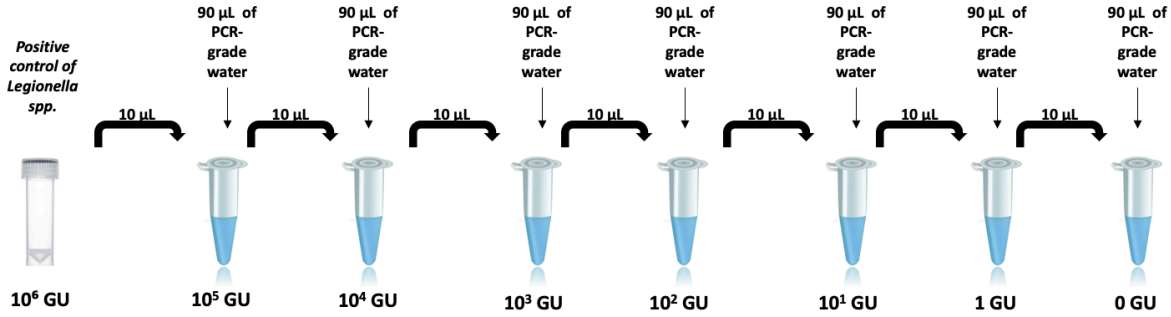


**Figure 3.** General outline of serial 1:10 dilutions of positive control of *Legionella spp.* for standard curve generation.

Once the standard curve has been obtained, PCR will be performed using the samples obtained from the extraction, with *Legionella spp.* Quantitative Detection Kit (Ielab, Spain). The Ielab Kits for the detection of *Legionella spp*. provides the specific primers and probes for a specific *Legionella spp.* gene, the appropriate reagents and the controls needed for the detection and quantification of *Legionella spp.*

The amplification mix consists of a primer set for the specific amplification of the bacterial DNA (specific primers and fluorescent probes), a recombinant DNA that acts as an Internal Control (IC) and that it is amplified with the same primer set that the target fragment from *Legionella spp.*, a TaqMan MGB (Minor Groove Binding) probe for the specific detection of *Legionella spp*., and a TaqMan MGB probe for the IC detection. The kit also provides the appropriate controls, positive (*Legionella spp.* With approx. $10^5$ GU/µL) and negative (nuclease-free water) for performing the analysis.

It employs an MGB probe labelled with FAM for the specific detection of *Legionella spp*. To avoid an inaccurate interpretation of results (false negatives) due to the inhibition of the amplification process, the reaction mix includes an Internal Control (IC). The DNA fragment used as IC consists of a recombinant plasmid that amplifies with the same primers pair than the used to amplify the *Legionella spp*. target, but as it anneals with an MGB probe labelled with VIC it is detected in a different channel.

For the PCR, in each well of the plate MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems, Thermo Fisher Scientific, USA), 15 µL of master mix were combined with 10 µL of the sample, resulting in a final volume of 25 µL, this procedure is shown in Figure 4. The same procedure was followed for the positive and negative controls, using 10 µL of each. The PCR was then run following the program set-up specified in the kit protocol in QuantStudio® 3-96-Well 0.2-mL Block (Applied Biosystems, Thermo Fisher Scientific, USA). Thermal reaction conditions were 50ºC for 2 min (initial stage) followed by 95º for 10 min (denaturation) and followed by 42 cycles of 95ºC for 15 seconds and 60ºC for 1 min (repetitive cycles of denaturation, annealing and elongation).


**Figure 4.** General outline of PCR for *Legionella spp.* amplification procedure.

The preparation of the PCR plate was carried out inside a vertical laminar flow cabinet Telstar Mini-V PCR (Telstar, Spain). Once the plate was prepared, it was sealed with adhesive film Optical adhesive covers (Applied Biosystems, Thermo Fisher Scientific, USA) to prevent contamination and evaporation. It is known that Ielab® kits are designed to comply with the technical specifications outlined in ISO/TS 12869. Primers have been used to amplify a region of the 16S rRNA gene of *Legionella spp*.

### 3.4. STATISTICAL ANALYSIS

To perform the statistical analysis of the results, three technical replicates of each sample were carried out for the PCR in order to assess significant differences between both methods. The PCR results and data were analysed using QuantStudio™ Design & Analysis Software v1.5.1 (Applied Biosystems, Thermo Fisher Scientific, USA). For PCR, sensitivity and specificity were evaluated, for this reason NPV and PPV were performed too, the negative predictive value (NPV)—i.e., the ability of a negative PCR result to predict a negative culture result—and the positive predictive value (PPV)—i.e., the ability of a positive PCR result to predict a positive culture result—were calculated for each sample type. Finally, to evaluate whether there were significant differences between the proportions obtained by culture and PCR, a Fisher's test was performed. This statistical analysis was conducted using Microsoft Excel and R software (version R-4.5.0-x86_64.pkg).

# 4. RESULTS

## 4.1. IDENTIFICATION BY CULTURE OF *Legionella spp.*

### 4.1.1. LIMIT OF DETECTION (LOD) OF THE CULTURE METHOD FOR *Legionella spp.*

The detection limit of the culture method is calculated based on the assumption that it is possible to detect at least one CFU on a plate—that is, the minimum countable value in the event that *Legionella spp.* is present in the sample.

To determine this limit in every sample, the plate inoculated with the highest volume of sample and showing colony growth is selected. In cases where no growth is observed on any plate, the one with the highest inoculated volume is used. For that plate, one CFU is considered the minimum detectable value, which is then divided by the volume of sample inoculated on that plate, multiplied by the total volume of the original sample. The result is expressed in CFU/L. In the case of samples processed using concentration by filtration (such as CNT samples), the dilution factor must also be reversed. The final value obtained represents the detection limit for that specific sample. The detailed calculations for each plate are presented in Table 6.

**Table 6.** Formulas for the detection limit of each plate of culture method.

| Direct plating | Membrane filter on plate |
|---|---|
| $R = \frac{x}{V\,tot} \cdot Vi$<br><br>**R** → Result in CFU/L<br>**x**→ Confirmed CFU on that plate as *Legionella*<br>**Vtot** → Volume analysed on that plate in mL<br>**Vi** → Total volume of the original sample in mL (1000 mL) | $R = \frac{x}{V\,tot} \cdot Vi$<br><br>**R** → Result in CFU/L<br>**x**→ Confirmed CFU on that plate as *Legionella*<br>**Vtot** → Volume analysed on that plate in mL<br>**Vi** → Total volume of the original sample in mL (1000 mL) |
| **Filtration with elution procedure** | **Plating after dilution** |
| $R = \frac{x \cdot Vc}{V \cdot V\,tot} \cdot Vi$<br><br>**R** → Result in CFU/L<br>**x**→ Confirmed CFU on that plate as *Legionella*<br>**Vtot** → Volume analysed on that plate in mL<br>**Vi** → Total volume of the original sample in mL (1000 mL)<br>**Vc** → Concentrated volume of the sample in mL<br>**V** → Volume analysed by filtration in mL | $R = \frac{x \cdot Vi}{Vdil} \cdot Df$<br><br>**R** → Result in CFU/L<br>**x**→ Confirmed CFU on that plate as *Legionella*<br>**Vtot** → Volume analysed on that plate in mL<br>**Vi** → Total volume of the original sample in mL (1000 mL)<br>**Vc** → Concentrated volume of the sample in mL<br>**V** → Volume analysed by filtration in mL<br>**Vdil** → Volume of diluted sample inoculated per plate in mL<br>**Df** → It is the dilution factor |

**Example 1:** In the case of a sample directly inoculated onto a plate with 0.5 mL.

$\frac{1\ CFU}{0.5\ mL} \cdot 1000\ mL$ = 2000 CFU/L

**Example 2:** In the case of a filtered sample of 10 mL.

$\frac{1\ CFU}{10\ mL} \cdot 1000\ mL$ = 100 CFU/L

**Example 3:** In the case of a sample filtered from 500 mL, eluted in 10 mL of buffer, and plated with 0.5 mL.

$\frac{1\ CFU \cdot 10\ mL}{500\ mL \cdot 0.5\ ml} \cdot 1000\ ml$ = 40 CFU/L

**Example 4:** 1 mL of eluate introduced into 9 mL of acid. In this case, it is a 1:10 dilution, and 0.5 mL of the treated sample is inoculated onto the plate.

$\frac{1\ CFU \cdot 1000\ mL}{0.5\ mL} \cdot 1/10$ = 200 CFU/L

### 4.1.2. RESULTS OF THE CULTURE METHOD FOR *Legionella spp.*

As previously mentioned, *Legionella spp.* colonies typically display a whitish, grey, green, or bluish-purple coloration, with a smooth and glassy appearance and well-defined edges. Based on this, for the analysis of results, the plates were examined using a magnifying lens three times during the incubation period (as described in the Materials and Methods section). The first examination was performed after 2 days of incubation, the second on the fifth day, and the final one on the tenth day. The purpose of these observations was to identify samples showing overgrowth.

After these three counts, the number of colonies present on each plate during the final count was considered for the results of every plate, as this is when *Legionella spp.* growth was complete. To express the final results, the confirmed *Legionella spp.* colonies per volume examined on the plate were first counted. To estimate the number of colony-forming units (CFU) of *Legionella spp.* present in the original water sample, the plate with the highest number of **quantifiable confirmed** colonies per initial water volume was selected. This volume is calculated using the formulas shown below. Once the plate with the highest CFU/L is identified, this value is reported as the final result for the sample.

- Direct plating (Matrix A): R = $\frac{x}{V\ tot} \cdot Vi$
- Membrane filter on plate (Matrix A): R = $\frac{x}{V\ tot} \cdot Vi$
- Filtration with elution procedure (Matrix B): R = $\frac{x \cdot Vc}{V \cdot V\ tot} \cdot Vi$
- Plating after dilution (Matrix B): R = $\frac{x \cdot Vi}{Vdil} \cdot Df$

Where

**R** → Result in CFU/L
**x**→ Confirmed CFU on that plate as *Legionella*
**Vtot** → Volume analysed on that plate in mL
**Vi** → Total volume of the original sample in mL
**Vc** → Concentrated volume of the sample in mL
**V** → Volume analysed by filtration in mL
**Vdil** → Volume of diluted sample inoculated per plate in mL
**Df** → It is the dilution factor

The CFU counts from each plate and the confirmation that the colonies are indeed *Legionella spp.* are shown in Table 7. The final result for each sample is shown in Table 8, an example of the final result for samples 1 and 5 is shown below:

Sample 1: Sample 5:

Direct plating: $\frac{5}{0.5\ mL} \cdot 1000\ mL$ = **10,000 CFU/L** Membrane filter on plate: $\frac{23}{500\ mL} \cdot 1000\ mL$ = **46 CFU/L**

**Table 7.** Culture results of every sample for the detection of *Legionella spp.*

| Sample | Matrix A (CFU) | | | Matrix B (CFU) | | | | Confirmation | |
|---|---|---|---|---|---|---|---|---|---|
| | BCYE 10 mL | GVPC 0.5 mL | GVPC 500 mL + A | Untreated eluate | Thermally treated eluate | Acid treated eluate | Acid treated-eluate 1+9 | BCYE | BCYE-cys |
| 1 | 42 | 5 | UNC | | | | | + | - |
| 2 | 28 | NG | 110 | | | | | + | - |
| 3 | 5 | NG | 80 | | | | | + | - |
| 4 | 2 | NG | UND | | | | | + | - |
| 5 | NG | NG | 23 | | | | | + | - |
| 6 | NG | NG | 22 | | | | | + | - |
| 7 | NG | NG | NG | | | | | / | / |
| 8 | | | | 120 | 3 | Fungi | NG | + | + |
| 9 | | | | 60 | 22 | Fungi | 14 | + | + |
| 10 | | | | 20 | 8 | Fungi | 2 | + | + |
| 11 | | | | 10 | 3 | Fungi | NG | + | + |
| 12 | | | | 3 | 1 | NG | NG | + | + |
| 13 | | | | 2 | 7 | 3 | 2 | + | + |
| 14 | | | | 3 | NG | NG | NG | + | + |
| 15 | UND | 60 | 1 | | | | | + | + |
| 16 | 18 | NG | NG | | | | | + | + |
| 17 | 19 | NG | NG | | | | | + | + |
| 18 | 2 | NG | NG | | | | | + | + |
| 19 | 10 | NG | 1 | | | | | + | + |
| 20 | NG | NG | NG | | | | | / | / |
| 21 | NG | NG | NG | | | | | / | / |
| 22 | NG | NG | NG | | | | | / | / |
| 23 | NG | NG | NG | | | | | / | / |
| 24 | NG | NG | NG | | | | | / | / |
| 25 | NG | NG | NG | | | | | / | / |
| 26 | 85 | 23 | UNC | | | | | + | - |
| 27 | 17 | 3 | UNC | | | | | + | - |
| 28 | 4 | NG | UNC | | | | | + | - |
| 29 | 25 | 2 | UNC | | | | | + | - |
| 30 | 1 | NG | NG | | | | | + | - |

**Table legend:**
**(+):** positive in subculture in BCYE or BCYE-cys.
**(-):** negative in subculture in BCYE or BCYE-cys.
**(/):** no confirmatory test was performed, since there was no growth on any plate.
**Fungi:** fungal contamination preventing accurate plate observation.
**NG:** no growth.
**UNC:** uncountable, value could not be determined due to excessive *Legionella spp.* growth covering the entire plate
Underlined in orange corresponds to the plate chosen to express the final result.

**Table 8.** Final culture results of *Legionella spp.* of every sample.

| Sample | Final result |
|---|---|
| | CFU/L |
| 1 | 10,000 |
| 2 | 2800 |
| 3 | 500 |
| 4 | 200 |
| 5 | 46 |
| 6 | 44 |
| 7 | Blod (LOD:2) |
| 8 | Blod (LOD:40) |
| 9 | Blod (LOD:40) |
| 10 | Blod (LOD:40) |
| 11 | Blod (LOD:40) |
| 12 | Blod (LOD:40) |
| 13 | Blod (LOD:40) |
| 14 | Blod (LOD:40) |
| 15 | Blod (LOD:2) |
| 16 | Blod (LOD:100) |
| 17 | Blod (LOD:100) |
| 18 | Blod (LOD:100) |
| 19 | Blod (LOD:2) |
| 20 | Blod (LOD:2) |
| 21 | Blod (LOD:2) |
| 22 | Blod (LOD:2) |
| 23 | Blod (LOD:2) |
| 24 | Blod (LOD:2) |
| 25 | Blod (LOD:2) |
| 26 | 46,000 |
| 27 | 6000 |
| 28 | 400 |
| 29 | 4000 |
| 30 | 100 |

**Table legend:**
**Blod:** Below limit of detection. For those samples that did not test positive for the presence of *Legionella spp.*, the results column specifies that their value is below the detection limit calculated for that sample, as no *Legionella spp*. colonies were detected.
The final result is only calculated in the samples where growth was detected and were positive in the confirmatory tests, that is, those that were positive in BCYE and negative in BCYE-cys.

Regarding the results of *Legionella pneumophila* confirmation, samples are considered positive for this species when agglutination is observed with the reagent for serogroup 1 and/or serogroups 2–14. If agglutination is observed with the third reagent, it corresponds to other *Legionella* species. If no agglutination is observed with any of the three reagents, it suggests the presence of a serogroup that cannot be identified with this kit, as it does not contain antibodies against that specific antigen. This procedure was only carried out on those samples for which the confirmatory test was positive. These results are shown in Table 9.

**Table 9.** Results for *Legionella pneumophila* confirmation in culture method.

| Sample | Latex SG1 | Latex SG 2-14 | Other | *L.pneumophila* |
|---|---|---|---|---|
| 1 | + | - | - | Yes |
| 2 | + | - | - | Yes |
| 3 | + | - | - | Yes |
| 4 | + | - | - | Yes |
| 5 | + | - | - | Yes |
| 6 | + | - | - | Yes |
| 26 | - | - | + | No |
| 27 | - | - | + | No |
| 28 | - | - | + | No |
| 29 | + | - | - | Yes |
| 30 | - | + | - | Yes |

**Table legend:**
**(+):** positive for that serogroup.
**(-):** negative for that serogroup.
**SG1:** serogroup 1.
**SG2-14:** serogroup 2-14.
**Other:** others serogroups of *Legionella*.

Although a confirmation of *Legionella pneumophila* was performed for the *Legionella spp.* positive results, all *Legionella spp.* positive results were ultimately compared with those obtained by PCR, regardless of the identified serogroup, since the PCR primers were designed to target *Legionella spp.*

Among the 30 samples analysed by culture, 4 samples could not be quantified due to interfering flora, even after applying heat and acid treatments. This issue was mainly observed in samples from cooling towers. Based on all these results, it can be stated that 36.66**%** of the samples analysed by culture tested positive, while 63.33**%** tested negative. An example of four different plates showing *Legionella spp.* colony growth can be found in Annex E.

## 4.2. IDENTIFICATION BY PCR OF *Legionella spp.*

Regarding the PCR results, the outcomes obtained with both previously mentioned kits will be presented first, to determine which of them has a lower detection limit. This allows for the identification of samples containing *Legionella spp.* at lower concentration levels.

### 4.2.1. CONDALAB

In relation to the test carried out using the Condalab kit with serial dilutions, the triplicate results for each dilution, which were analysed with QuantStudio ™ Design & Analysis Software v1.5.1, are shown in Table 10. The positive and negative results were determined based on the kit's guidelines, as shown in Table 11.

**Table 10.** PCR amplification results of serial dilution with Condalab kit based on FAM Ct.

| Dilution | PCR (Ct) | | | |
|---|---|---|---|---|
| | Duplicate 1 | Duplicate 2 | Duplicate 3 | Result |
| $10^8$ | 23.23 (+) | 23.31 (+) | 23.12 (+) | **Positive** |
| $10^7$ | 27.04 (+) | 27.65 (+) | 28.01 (+) | **Positive** |
| $10^6$ | 31.34 (+) | 30.39 (+) | 30.29 (+) | **Positive** |
| $10^5$ | 34.87 (+) | 34.65 (+) | 35.02 (+) | **Positive** |
| $10^4$ | 38.89 (+) | 38.76 (+) | 39.87 (+) | **Positive** |
| $10^3$ | UND (+) | UND (+) | UND (+) | **Negative** |
| $10^2$ | UND (+) | UND (+) | UND (+) | **Negative** |
| $10^1$ | UND (+) | UND (+) | UND (+) | **Negative** |

**Table legend:**
**(+):** Internal control detected.

As can be observed, this kit is unable to detect the presence of *Legionella spp.* beyond the fifth sample—that is, the dilution containing $10^4$ CFU, according to the initial calculations, where the first sample was expected to contain $10^8$ CFU.

**Table 11.** Supplementary information on how to interpret the results of Table 9.

| ***Legionella spp.* detection (FAM)** | **IC detection (ROX)** | **Interpretation** |
|---|---|---|
| Positive | Positive | Positive for *Legionella spp.* |
| Negative | Positive | Negative |
| Negative | Negative | Invalid Result * |

**Table legend:**
* Invalid result: Internal control not detected, sample may contain PCR inhibitors or extraction failed.

Additionally, as mentioned in section 3.3 of the Materials and Methods, 0.5 mL of each serial dilution was also cultured on BCYE agar to observe the number of CFUs present in each sample. This allowed for comparison between the PCR-negative samples and their corresponding culture results, to determine whether *Legionella spp.* was truly absent from those dilutions. We can see the results in Table 12. Therefore, according to the results, all dilutions contained *Legionella spp*. In other words, the kit was unable to detect its presence beyond a concentration of $10^4$ cells.

**Table 12.** Results of the serial dilution in culture method.

| Dilution | BCYE 0.5 mL (CFU) | Confirmation | |
|---|---|---|---|
| | | BCYE | BCYE-cys |
| $10^8$ | UNC | + | - |
| $10^7$ | UNC | + | - |
| $10^6$ | UNC | + | - |
| $10^5$ | UNC | + | - |
| $10^4$ | UNC | + | - |
| $10^3$ | 179 | + | - |
| $10^2$ | 57 | + | - |
| $10^1$ | 7 | + | - |

**Table legend:**
**UNC:** uncountable, value could not be determined due to excessive *Legionella spp.* growth covering the entire plate.

### 4.2.2. IELAB

In relation to the test carried out using the Ielab kit with serial dilutions, the triplicate results for each dilution, which were analysed with QuantStudio ™ Design & Analysis Software v1.5.1, are shown in Table 13. The interpretation of the results, as described in the kit protocol, is based on the fluorescence levels emitted by specific probes.

A sample is considered **positive for *Legionella spp*.** when the fluorescence signal emitted by the *Legionella spp.* probe exceeds the established threshold (which is determined by the software algorithm). A **negative result for *Legionella spp.*** is indicated when the fluorescence emitted by the *Legionella* probe remains below the threshold, while the IC probe signal is amplified. This confirms that the PCR reaction worked properly and that no *Legionella spp.* DNA was detected in the sample. Finally, in the case of **inhibited amplification**, neither the *Legionella spp.* probe nor the IC probe generate fluorescence signals above the threshold. This indicates that the reaction is invalid, most likely due to the presence of PCR inhibitors in the sample.

**Table 13.** PCR amplification results of serial dilution with Ielab kit.

| Dilution | PCR (Ct) | | | |
|---|---|---|---|---|
| | Duplicate 1 | Duplicate 2 | Duplicate 3 | Result |
| $10^8$ | 13.85 (+) | 13.55 (+) | 13.89 (+) | **Positive** |
| $10^7$ | 17.43 (+) | 16.98 (+) | 17.97 (+) | **Positive** |
| $10^6$ | 20.70 (+) | 21.03 (+) | 20.53 (+) | **Positive** |
| $10^5$ | 24.30 (+) | 25.24 (+) | 24.79 (+) | **Positive** |
| $10^4$ | 28.80 (+) | 28.97 (+) | 29.32 (+) | **Positive** |
| $10^3$ | 32.37 (+) | 32.37 (+) | 32.65 (+) | **Positive** |
| $10^2$ | 35.77 (+) | 35.06 (+) | 35.98 (+) | **Positive** |
| $10^1$ | 39.81 (+) | 39.85 (+) | 39.34 (+) | **Positive** |

**Table legend:**
**(+):** Internal control detected.

Additionally, as mentioned in section 3.3 of the Materials and Methods, 0.5 mL of each serial dilution was also cultured on BCYE agar to observe the number of CFUs present in each sample, as previously described in the section detailing the procedure with the Condalab kit.

We can see the results in Table 11, the table is the same as the one used in the Condalab results, since the same dilutions were used to test both kits. Therefore, according to these results, all dilutions contained *Legionella spp*. In other words, the kit was able to detect its presence in all the dilutions.

For this reason, the kit the we used to determine the presence/absence of *Legionella spp.* in the real samples for this project was the one provided by Ielab. Another reason for choosing the Ielab kit was that the Condalab kit had not yet been validated at the time. Since, in order to use an alternative method to culture for the detection of *Legionella spp.,* it must have national or international validation of its reliability based on the UNE-EN ISO 16140-2:2016 standard — "Protocol for the validation of alternative methods compared to reference methods" — as stated in (Boletín Oficial del Estado, 2022).

#### 4.2.2.1. LIMIT OF DETECTION (LOD) OF THE PCR TECHNIQUE FOR *Legionella spp.*

A standard curve is required to establish the Ct threshold value. The Ct threshold for determining the presence of the target organism (*Legionella spp.*) can be established using the corresponding standard curve, derived from the positive control included in the kit (with a known concentration). From the intercept values of the standard curve, an average y-intercept corresponding to 1 genomic unit (Ct 1 GU) is determined. A PCR result with a Ct value higher than the y-intercept corresponding to 1 GU should be interpreted as negative, whereas a Ct value lower than the Ct of 1 GU indicates a positive result (Asociación Española de Normalización, 2021).

In this study, a total of eight different concentrations (serial 1:10 dilutions from the positive control) were used to generate the standard curve, starting from an initial concentration of $10^6$ GU/10µL. The standard curve was repeated a total of five times. These dilutions were used to determine the average y-intercept. With this Ct value for 1 GU, the detection limit of the assay is obtained, which represents the minimum number of genomic units that could be detected in the filtered sample volume.

For the preparation of the standard curve, it must be considered that the Ct value is inversely proportional to the base-10 logarithm of the number of genomic units initially present in the reaction mixture. For a valid standard curve, the slope should fall between –4.115 and –2.839, corresponding to an amplification efficiency ranging from 75% to 125%. The results of the five standard curves performed are shown in Table 14, and the corresponding curve graph is presented in Figure 5.

**Table 14.** PCR amplification results of serial dilution from positive control of *Legionella spp.* for standard curve.

| Concentration (GU) | Replicate 1 | Replicate 2 | Replicate 3 | Replicate 4 | Replicate 5 |
|---|---|---|---|---|---|
| $10^6$ | 19.96 (+) | 19.78(+) | 19.06(+) | 19.32(+) | 18.99(+) |
| $10^5$ | 23.62(+) | 23.98(+) | 22.78(+) | 23.67(+) | 23.35(+) |
| $10^4$ | 26.67(+) | 27.03(+) | 26.57(+) | 26.81(+) | 26.5(+) |
| $10^3$ | 30.42(+) | 30.21(+) | 30.69(+) | 30.40(+) | 30.98(+) |
| $10^2$ | 34.04(+) | 34.76(+) | 34.22(+) | 33.68(+) | 34.09(+) |
| $10^1$ | 37.41(+) | 37.37(+) | 37.63(+) | 38.01(+) | 37.87(+) |
| 1 | 40.30(+) | 40.54(+) | 40.22(+) | 40.76(+) | 40.33(+) |

**Table legend:**
**(+):** Internal control detected.

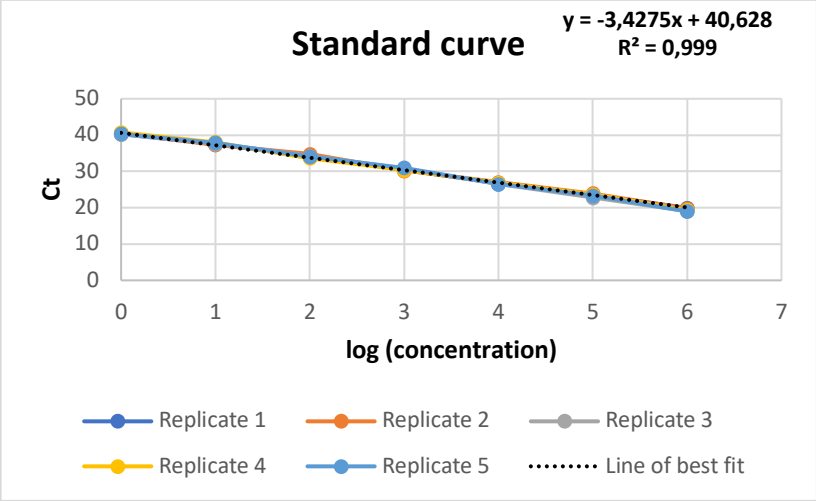


**Figure 5.** Standard curve generated from seven serial dilutions of the positive control of *Legionella spp.,* showing the slope and the $R^2$ value.

To calculate the efficiency (which evaluates the performance of the PCR reaction), the following formula is used according to (Asociación Española de Normalización, 2021):

$$e = \left(10^{-1/a} - 1\right) \cdot 100$$

**a:** slope

$$e = \left(10^{-1/-3.4275} - 1\right) \cdot 100 = 95.77\%$$

The standard curve and its parameters — including the slope, y-intercept, and correlation coefficient ($R^2$) — were automatically calculated using the QuantStudio™ software. The high $R^2$ value (>0.99), good slope and efficiency indicated excellent linearity and reliability of the standard curve across the tested concentration range. Therefore, the limit of detection (LOD) of the assay was determined to be 1 GU with a Ct value of **40.43** in 10 µL (volume of sample added to each well), which corresponds to 100,000 GU/L. Consequently, any sample with a Ct value higher than this threshold will be considered negative.

#### 4.2.2.2. PCR-BASED DETECTION RESULTS OF *Legionella spp.*

Regarding the PCR results (analysed using the QuantStudio ™ Design & Analysis Software v1.5.1) for the real samples in this study (n=30), these are shown in Table 15. To determine the presence or absence of *Legionella spp.* in the samples, the guidelines provided by the *Legionella spp.* Quantitative Detection Kit (Ielab, Spain) kit were followed, as explained in the previous section on PCR result interpretation for the serial dilutions.

Regarding target detection (presence/absence), a result should be considered positive (presence of the target) not only when it is above the threshold, but also when the Ct value is lower than the Ct value corresponding to 1 GU. For this reason, in all samples, the presence or

absence of *Legionella spp.* was determined based on a detection range from 1 GU to $10^6$ GU, established from the standard curve, the results of which are detailed in section 4.2.2.2. This threshold cycle (Ct) represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected.

In addition to analysing the Ct value of the samples, the shape of the amplification curve was also evaluated. Samples should be inspected both in logarithmic and linear scale view and compared with the negative control (Asociación Española de Normalización, 2021). For this reason, an example is shown in Annex F, where the amplification curves of some samples are examined, as well as how the information for these samples was entered into the software plate.

In cases where neither *Legionella spp.* nor the IC are detected by PCR, the result is considered invalid, the most likely cause of the absence of IC amplification is the presence of PCR inhibitors in the sample. Since all three replicates of the inhibited samples showed inhibition, each replicate was diluted at ratios of 1:10, 1:5, and 1:2, with the aim of determining whether *Legionella spp.* could be detected in any of the diluted samples along with amplification of the internal control. This issue was mainly observed in samples from cooling towers, ornamental fountain and pools. The results of the dilutions of the inhibited samples can be seen in Table 16.

**Table 15.** PCR amplification for *Legionella spp*. results per triplicate of every sample.

| Sample | PCR (Ct) | | | Absence/Presence | | |
|---|---|---|---|---|---|---|
| | Duplicate 1 | Duplicate 2 | Duplicate 3 | Duplicate 1 | Duplicate 2 | Duplicate 3 |
| 1 | 28.64 (+) | 28.69 (+) | 28.45(+) | Presence | Presence | Presence |
| 2 | 34.74 (+) | 34.98 (+) | 33.78(+) | Presence | Presence | Presence |
| 3 | 36.53 (+) | 35.78 (+) | 35.88(+) | Presence | Presence | Presence |
| 4 | 34.83 (+) | 35.35 (+) | 34.21(+) | Presence | Presence | Presence |
| 5 | 40.02 (+) | 40.09 (+) | 39.56 (+) | Presence | Presence | Presence |
| 6 | 39.33 (+) | 38.83 (+) | 39.65 (+) | Presence | Presence | Presence |
| 7 | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 8 | UND (-) | UND (-) | UND (-) | / | / | / |
| 9 | 37.65 (+) | 38.36 (+) | 37.24(+) | Presence | Presence | Presence |
| 10 | 34.64 (+) | 35.39 (+) | 34.89(+) | Presence | Presence | Presence |
| 11 | 41.84 (+) | UND (+) | 41.55(+) | Absence | Absence | Absence |
| 12 | 35.71 (+) | 35.59 (+) | 36.01(+) | Presence | Presence | Presence |
| 13 | 32.03 (+) | 31.73 (+) | 32.45(+) | Presence | Presence | Presence |
| 14 | UND (-) | UND (-) | UND (-) | / | / | / |
| 15 | 37.51 (+) | 38.32(+) | 37.05(+) | Presence | Presence | Presence |
| 16 | UND (-) | UND (-) | UND (-) | / | / | / |
| 17 | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 18 | UND (-) | UND (-) | UND (-) | / | / | / |
| 19 | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 20 | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 21 | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 22 | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 23 | 28.94(+) | 29.23(+) | 28.55(+) | Presence | Presence | Presence |
| 24 | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 25 | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 26 | 36.89(+) | 37.19(+) | 37.09(+) | Presence | Presence | Presence |
| 27 | 39.41(+) | 39.18(+) | 39.55(+) | Presence | Presence | Presence |
| 28 | 39.98(+) | 39.56(+) | 38.47(+) | Presence | Presence | Presence |
| 29 | 28.66(+) | 28.75(+) | 29.13(+) | Presence | Presence | Presence |
| 30 | 33.41(+) | 33.74(+) | 33.55(+) | Presence | Presence | Presence |

**Table legend:**
**(+):** Internal control detected.
**(-):** Internal control not detected.
**(/):** It is not possible to determine presence/absence, since the IC did not amplify, and therefore the result is not valid.
**UND:** undetermined, the sample did not amplify.

**Table 16.** PCR amplification results for the dilutions (1:10, 1:5 and 1:2) per triplicate of every inhibited sample.

| Sample | Ct | | | Absence/Presence | | |
|---|---|---|---|---|---|---|
| | Duplicate 1 | Duplicate 2 | Duplicate 3 | Duplicate 1 | Duplicate 2 | Duplicate 3 |
| 8 (Dilution 1:10) | 41.79 (+) | 41.87 (+) | 41.05(+) | Absence | Absence | Absence |
| 8 (Dilution 1:5) | 40.81 (+) | 40.65 (+) | 40.55 (+) | Absence | Absence | Absence |
| 8 (Dilution 1:2) | 39.34 (+) | 39.99 (+) | 39.88(+) | Presence | Presence | Presence |
| 14 (Dilution 1:10) | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 14 (Dilution 1:5) | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 14 (Dilution 1:2) | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 16 (Dilution 1:10) | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 16 (Dilution 1:5) | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 16 (Dilution 1:2) | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 18 (Dilution 1:10) | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 18 (Dilution 1:5) | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |
| 18 (Dilution 1:2) | UND (+) | UND (+) | UND (+) | Absence | Absence | Absence |

**Table legend:**
**(+):** Internal control detected.
**(-):** Internal control not detected.
**UND:** undetermined, the sample did not amplify.

Based on all these results, it can be observed that after the dilutions, only one of the PCR-inhibited samples showed the presence of *Legionella spp.* (sample 8), so it can be stated that 60**%** of the samples analysed by PCR tested positive, while 40**%** tested negative.

## 4.3. COMPARISON OF CULTURE AND PCR RESULTS

In order to perform a statistical analysis and evaluate significant differences between both detection methods, Table 17 is presented below, showing the culture results in comparison with the PCR results for each of the samples.

**Table 17.** Comparison of culture method and PCR amplification results for *Legionella spp.* detection.

| Source | Culture (CFU/L) | PCR (Absence/Presence) |
|---|---|---|
| 1. Domestic tap | 10,000 | Presence |
| 2. Domestic tap | 2800 | Presence |
| 3. Domestic tap | 500 | Presence |
| 4. Domestic tap | 200 | Presence |
| 5. Domestic tap | 46 | Presence |
| 6. Domestic tap | 44 | Presence |
| 7. Domestic tap | ND | Absence |
| 8. Cooling towers | ND | Presence |
| 9. Cooling towers | ND | Presence |
| 10. Cooling towers | ND | Presence |
| 11. Cooling towers | ND | Absence |
| 12. Cooling towers | ND | Presence |
| 13. Cooling towers | ND | Presence |
| 14. Ornamental fountain | ND | Absence |
| 15. Spa pool | ND | Presence |
| 16. Spa pool | ND | Absence |
| 17. Community pool | ND | Absence |
| 18. Private pool | ND | Absence |
| 19. Sports complex shower | ND | Absence |
| 20. Domestic shower | ND | Absence |
| 21. Domestic shower | ND | Absence |
| 22. Sports complex shower | ND | Absence |
| 23. Sports complex shower | ND | Presence |
| 24. Private pool | ND | Absence |
| 25. Private pool | ND | Absence |
| 26. Domestic shower | 46,000 | Presence |
| 27. Domestic shower | 6000 | Presence |
| 28. Domestic shower | 400 | Presence |
| 29. Domestic tap | 4000 | Presence |
| 30. Domestic tap | 200 | Presence |

**Table legend:**
**ND:** Not detected.

To perform a concordance analysis, a contingency table was created (Table 18). For this purpose, **Cohen's kappa index (K)** will be used, which measures the agreement adjusted for chance. It evaluates how much the two methods agree beyond what would be expected by random chance.

**Table 18.** Contingency table of both methods, culture and PCR amplification for *Legionella spp.*

| | Culture | PCR positive | PCR negative |
|---|---|---|---|
| *Legionella spp.* | Positive | 11(a) | 0(b) |
| | Negative | 7(c) | 12(d) |

**Table legend:**
**a:** True Positives → positive by both methods.
**b:** False Negatives → positive by culture, negative by PCR.
**c:** False Positives → negative by culture, positive by PCR.
**d:** True Negatives → negative by both methods.

The Kappa index is calculated according to the following formula:

$$\kappa = \frac{P_o - P_e}{1 - P_e}$$

**$P_o$**: Proportion of observed agreement, that is, the cases in which both methods coincide.

$$P_o = \frac{a+d}{N} = \frac{11+12}{30} = 0.77$$

**$P_e$**: Proportion of agreement expected by chance, calculated from the marginal proportions.

$$P_e = \left(\frac{(a+b)(a+c)}{N^2}\right) + \left(\frac{(c+d)(b+d)}{N^2}\right) = \left(\frac{(11+0)(11+7)}{30^2}\right) + \left(\frac{(7+12)(0+12)}{30^2}\right) = 0.473$$

With our data:

$$\kappa = \frac{0.77 - 0.473}{1 - 0.473} = 0.564$$

The Cohen's kappa coefficient for the agreement between PCR and culture results was 0.564, indicating a moderate level of agreement (since the value falls within the range of 0.41 to 0.60, it indicates a moderate agreement. Higher values, specifically those between 0.61-0.80, and between 0.81-1.00, reflect a substantial and almost perfect agreement, respectively) between the two methods beyond what would be expected by chance alone.

### 4.4. STATISTICAL ANALYSIS

To perform the statistical analysis of the results, as can be seen in section 3.4, three replicates of each sample were carried out for the PCR in order to assess significant differences between both methods.

#### 4.4.1. POSITIVE PREDICTIVE VALUE (PPV) NEGATIVE PREDICTIVE VALUE (NPV)

PPV and NPV were calculated to evaluate the assessment of the diagnostic value and effectiveness of the PCR method in comparison with the culture-based, which is considered the reference standard, since legislation stipulates it as the official method for the detection of *Legionella spp.* **PPV indicates the probability that a sample identified as positive by PCR is truly positive according to the culture method. NPV reflects the probability that a sample identified as negative by PCR is truly negative by culture**.

These parameters provide insight into the reliability of the PCR results, especially in assessing false positives and false negatives, and are particularly useful when the prevalence of the target organism (in this case, *Legionella spp.*) is low or variable.

For the calculation of NPV and PPV, the same data from Table 18 are used. They are calculated according to the following formulas:

$PPV = \frac{a}{a+c}$ and $NPV = \frac{d}{b+d}$

$PPV = \frac{11}{11+7}$ = 0.61

$NPV = \frac{12}{0+12}$ = 1

The PPV of the PCR method was 0.61, indicating that 61% of the samples that tested positive by PCR were also confirmed as positive by culture. In contrast, the NPV was 1.00, meaning that all samples testing negative by PCR were also negative by culture. These values reflect the performance of PCR in correctly identifying the presence or absence of *Legionella spp.* in comparison with the culture method.

### 4.4.2. SPECIFICITY AND SENSITIVITY

We calculated sensitivity and specificity in order to assess the diagnostic performance of the PCR method in detecting *Legionella spp.* compared to the culture method, which is considered the reference standard according to current legislation.

Sensitivity measures the ability of PCR to correctly identify samples that are positive by culture (true positives), while specificity indicates the method's ability to correctly identify negative samples (true negatives). These indicators provide essential information about the reliability of PCR in minimizing false negatives and false positives, which is crucial when evaluating its potential use as an official alternative for rapid *Legionella spp.* detection in water samples.

To evaluate the performance of the PCR method against the culture method, we calculated **sensitivity** and **specificity** using the data from the 2×2 contingency Table 18. The formulas used were as follows:

$Sensitivity = \frac{a}{a+b}$ . This represents the proportion of true positives detected by PCR among all samples that were positive by culture. It reflects the ability of PCR to correctly identify *Legionella spp.* when it is truly present.

$Specificity = \frac{d}{c+d}$ . This represents the proportion of true negatives detected by PCR among all samples that were negative by culture. It reflects the ability of PCR to correctly exclude *Legionella spp.* when it is not present.

$Sensitivity = \frac{11}{11+0}$ = 1

$Specificity = \frac{12}{7+12}$ = 0.6316

The sensitivity of the PCR method was 1.00, indicating that all samples that tested positive by culture were also correctly identified as positive by PCR. This result suggests that the PCR method did not produce any false negatives in this study. The specificity was 0.63, meaning that 63% of the samples that tested negative by culture were also negative by PCR. This value reflects the presence of some false positives in the PCR results when compared to the culture method, which is considered the reference standard.

### 4.4.3. FISHER'S TEST

We applied Fisher's exact test to evaluate the association between the results obtained by PCR and the culture method for detecting *Legionella spp.* in water samples. This test is particularly suitable given our sample size (n=30) and the presence of low cell frequencies in the contingency table, which may limit the accuracy of the chi-square test. Fisher's exact test allows us to determine whether the detection of *Legionella spp.* by PCR is significantly related to the results observed in culture—the current gold standard method according to legislation. By quantifying this relationship, the test provides robust statistical support to assess whether PCR can be considered a reliable and consistent method, reinforcing its potential validation as a rapid and effective alternative for the official detection of *Legionella spp*.

The first step in conducting Fisher's exact test was to establish the null and alternative hypotheses to evaluate the relationship between the PCR results and those obtained by the standard culture method for *Legionella spp.* detection.

- **Null hypothesis ($H_0$):** There is no association between the detection of *Legionella spp.* by PCR and by culture; the two methods are independent.
- **Alternative hypothesis ($H_1$):** There is an association between PCR and culture results; the two methods are not independent.

By testing these hypotheses, the goal was to determine whether the proportion of PCR-positive results significantly differed depending on whether the culture result was positive or negative, thus evaluating the consistency between the two diagnostic methods.

Fisher's test was performed using the statistical software R (version R-4.5.0-x86_64.pkg). This test was chosen due to the small sample size (n = 30) and the presence of low expected frequencies in one of the cells of the 2×2 contingency table (Table 18), which makes it more appropriate and accurate than the chi-square test under these conditions.

This table was coded into R as a matrix using the following script (Figure 6):

```
> # Crear la tabla de contingencia
> tabla <- matrix(c(11, 0, 7, 12), nrow = 2, byrow = TRUE)
> colnames(tabla) <- c("PCR+", "PCR-")
> rownames(tabla) <- c("Cultivo+", "Cultivo-")
>
> # Ver la tabla
> tabla
         PCR+ PCR-
Cultivo+   11    0
Cultivo-    7   12
>
> # Hacer la prueba exacta de Fisher
> fisher.test(tabla)

    Fisher's Exact Test for Count Data

data:  tabla
p-value = 0.0006045
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
 2.921593      Inf
sample estimates:
odds ratio
```

**Figure 6.** Information introduced into the matrix in the R software.

The output of the test provided a **p-value of 0.0006** ($p<0.05$), indicating a statistically significant association between PCR and culture results. Additionally, the estimated odds ratio was greater than 2.9, with a 95% confidence interval from 2.92 to infinity, confirming that the PCR method is strongly associated with the detection results obtained by culture. In relation to the odds ratio, its numerical number is not shown here because there is a 0 in the table, corresponding to b value. These results indicate that samples testing positive by culture are much more likely to also test positive by PCR. Since the confidence interval ranges from 2.92 to infinity and does not include 1, it confirms the statistical significance of the association.

These results support the hypothesis that PCR could be considered a reliable method comparable to culture for detecting *Legionella spp.*, and suggest its potential validity as an official rapid detection technique in water samples.

## 5. DISCUSSION

The detection of *Legionella spp.* through culture yielded positive results in 36.66% of the water samples analysed. These results are consistent with the known limitations of culture as a slow and selective method, which may underestimate bacterial presence—particularly in samples with high microbial diversity. Notably, four out of the thirty samples could not be quantified due to the excessive growth of interfering flora, even after applying thermal and acid treatments. This issue was primarily observed in samples from cooling towers (CNT), which typically exhibit a higher microbial load and more complex microbiota. In such environments, other microorganisms may also find optimal growth conditions on selective media, occasionally overgrowing or masking *Legionella spp.* colonies, thus compromising detection efficiency. In contrast, samples from cleaner water sources (POT), such as domestic taps or showers, showed less microbial interference, allowing for better visual identification of *Legionella spp.* colonies and, consequently, more reliable results using the culture method.

Furthermore, in all culture-positive samples, confirmatory testing was conducted using BCYE and BCYE-cys media, validating the presence of *Legionella spp*. Subsequent serological testing showed that most isolates belonged to *L. pneumophila*, particularly serogroup 1, which is consistent with its well-documented predominance in both clinical and environmental cases of legionellosis.

Despite its limitations, culture remains the only officially recognized method that allows for the quantification of viable bacteria (CFU/L), which is essential for evaluating microbiological risk according to thresholds established by current legislation. However, this method does not detect bacteria in the state of VBNC, a common survival strategy of *Legionella spp.* under unfavourable environmental conditions, such as chemical disinfection or prolonged water stagnation. This limitation may have significant implications in public health contexts, as a negative culture result does not necessarily guarantee the true absence of the pathogen—potentially leading to a false sense of security in high-risk facilities such as hospitals, sports centres, or nursing homes.

Overall, these results highlight the strengths of the culture method in providing viable counts and species-level identification, while also underscoring its limitations in complex or highly contaminated matrices, where its detection capacity may be reduced. These findings emphasize the need to consider more sensitive complementary methods, such as PCR, for more effective and rapid monitoring of *Legionella spp.* in environments with potential health risks.

In this context, the use of PCR in the present study has proven to be a powerful alternative, offering shorter detection times and higher analytical sensitivity compared to the traditional culture method. The two commercial kits evaluated showed notable differences in detection capability. While the Condalab kit was only able to detect *Legionella spp.* down to a concentration of $10^4$ CFU, the Ielab kit successfully identified the presence of *Legionella spp.* across all serial dilutions tested, including the lowest concentration ($10^1$ CFU). These results were

confirmed by parallel culture analysis, where all dilutions, even those undetected by the Condalab PCR kit, contained viable *Legionella spp.* colonies. Therefore, the superior performance of the Ielab kit in terms of detection limit and consistency led to its selection for the analysis of the environmental samples in this study.

Furthermore, the use of a standard curve allowed for precise determination of the Ct threshold corresponding to 1 genomic unit (Ct 1 GU), thus establishing the detection limit of the assay at 100000 GU/L. This enabled a clear distinction between positive and negative samples, with presence defined by Ct values below the established threshold. Notably, PCR detected *Legionella spp.* in a greater number of samples (60%) compared to culture (36.66%), further supporting its higher sensitivity.

Despite these advantages, PCR is not without limitations. A total of 4 samples showed inhibition in the PCR, as indicated by the absence of IC amplification, meaning that the reaction did not occur correctly. The nature of these inhibitors was not investigated in this study, although they are likely to include organic compounds, minerals, or other debris common in complex environmental matrices. Inhibition occurred primarily in samples from cooling towers, ornamental fountains, and pools, which are known to have higher microbial loads and organic content. To address this issue, serial dilutions were applied to reduce inhibitor concentration and enable proper amplification. This strategy was successful in at least one of the previously inhibited samples, demonstrating the usefulness of dilution as a corrective measure for PCR inhibition. In summary, the PCR method demonstrated significant advantages in terms of speed and sensitivity, detecting *Legionella spp.* in multiple samples where culture failed.

The main objective of this study was to evaluate whether the PCR technique could serve as a complementary or even alternative method to the traditional culture-based detection of *Legionella spp.* in water samples. To this end, both techniques were applied to the same set of 30 samples, ensuring methodological consistency by using equal sample volumes (500 mL), and the results were compared both qualitatively and statistically.

The data revealed a higher detection rate for PCR (60%) compared to culture (36.66%), which aligns with the known higher sensitivity of molecular techniques and their ability to detect both culturable and VBNC bacteria. However, to assess whether these differences were statistically significant, various diagnostic parameters and statistical tests were applied.

First, the sensitivity of the PCR method was 1, indicating that all samples testing positive by culture were also detected by PCR, with no false negatives. This is a key result, demonstrating the method's ability to reliably detect *Legionella spp.* when present—an essential feature in public health surveillance. On the other hand, the specificity was 0.63, meaning that 63% of the culture-negative samples were also negative by PCR, while the remaining cases may represent false positives or detection of VBNC forms that cannot be identified by culture.

The PPV was 0.61, suggesting that 61% of PCR-positive results were confirmed by culture. Although this is a moderate value, it is expected given the limitations of culture in detecting

VBNC cells. In contrast, the NPV was 1, confirming that all PCR-negative results were also negative by culture, reinforcing the reliability of PCR in ruling out *Legionella spp.* presence.

These results suggest that the PCR method demonstrates a high capacity to correctly detect both positive and negative samples in comparison to the culture method. The high NPV and moderate PPV indicate that PCR is reliable for identifying true negatives and moderately accurate for detecting true positives. Furthermore, the Cohen's Kappa index, calculated at 0.564, shows a moderate level of agreement beyond chance between the two methods, reinforcing the diagnostic utility of PCR as a complementary tool to the culture reference method.

To support these conclusions, Fisher's exact test was performed to assess the statistical association between the results of both methods. The test yielded a p-value of 0.0006, well below the conventional threshold of 0.05, indicating a strong and statistically significant association between PCR and culture. As a general rule, the lower the p-value, the stronger the evidence that the two methods are not independent, suggesting that PCR and culture are consistently detecting the same target. Moreover, the odds ratio, estimated to be greater than 2.9 with a 95% confidence interval from 2.92 to infinity, further supports that samples testing positive by culture were significantly more likely to also test positive by PCR.

In this study, PCR detected a higher number of positive samples compared to culture. Several discordant samples were identified; these were PCR-positive but culture-negative. In most of these cases, the Ct values were close to the detection limit established for the assay (40.43), which could indicate a low bacterial load not detectable by culture. This reinforces the idea that PCR can identify *Legionella spp.* in samples where culture is limited due to factors such as microbial overgrowth or the presence of VBNC forms and residual DNA from bacteria that were previously present in the sample. Even if viable organisms are not detected by culture, the presence of genetic material implies that the microorganism has been present in the system and may pose a potential risk if conditions once again become favourable for its reactivation and proliferation. Therefore, the use of PCR as a complementary tool may help to reduce false negatives and improve environmental surveillance sensitivity.

Taken together, these results demonstrate that PCR is a reliable, rapid, and sensitive technique that complements the limitations of culture, especially in detecting samples that may be misclassified as negative due to the presence of VBNC bacteria. Its high sensitivity and perfect negative predictive value make it especially suitable for early detection and for confidently ruling out the presence of *Legionella spp*. Although PCR is not currently accepted as a standalone diagnostic method under existing legislation, the findings of this study support its potential validation as an official complementary tool for routine monitoring environmental surveillance and outbreak prevention.

An important consideration arises in cases where culture was negative but PCR was positive. In these situations, it is possible that PCR detected VBNC cells or residual DNA from bacteria that were previously present in the sample. The presence of genetic material indicates that the

microorganism has been present in the system and could pose a potential risk if conditions become favourable again for its reactivation and proliferation. These findings reinforce the value of PCR as a proactive monitoring tool, particularly in high-risk facilities.

The results of this study are consistent with previous research that has evaluated PCR as a complementary tool to culture for the detection of *Legionella spp.* in environmental samples. (Collins et al., 2015) reported that PCR detected more positive samples than culture, with a 100% NPV, as observed in this study. They attributed these differences to the ability of PCR to detect VBNC forms or dead cells, which culture is unable to identify. Similarly, (Collins et al., 2017)observed a weak correlation between the two techniques, noting that many culture-negative but PCR-positive samples came from systems with high microbial contamination, where overgrowth may have inhibited the growth of *Legionella spp.*, resulting in false negatives by culture. This pattern aligns with our own observations, especially in CNT-classified samples, where the most contaminated ones showed greater overgrowth of background flora in culture, while PCR continued to detect *Legionella spp.* DNA. In their conclusions, they supported the idea that PCR could anticipate culture trends and should be used as a complementary method in water surveillance programs.

Taken together, these studies support the main findings of this research, particularly the high sensitivity and strong negative predictive value of PCR, as well as its ability to detect samples that may go unnoticed by culture—especially in complex or highly contaminated matrices. Despite differences in sample volumes or treatment protocols, all studies converge on the same conclusion: PCR is a valuable and reliable tool to complement the standard culture method, particularly when rapid results are needed or when microbial overgrowth interferes with the isolation of *Legionella spp*.

Despite the promising results obtained, some limitations of the PCR method must be acknowledged. One of its main drawbacks is that PCR cannot differentiate between live and dead cells, which may lead to an overestimation of contamination if non-viable *Legionella spp.* DNA is present in the sample. Additionally, the reliability of PCR can be affected by the presence of inhibitors, as observed in several samples throughout this study. Inhibitory substances can interfere with DNA amplification, leading to false-negative or invalid results.

To ensure more robust detection, further studies are needed to identify and characterize the specific inhibitors present in environmental samples, in order to develop optimized pre-treatment protocols that can reliably eliminate them prior to amplification.

The integration of these improvements could enhance the reliability, reproducibility, and legal acceptance of PCR as a complementary or even alternative tool to the culture method for the routine monitoring of *Legionella spp.* in water systems.

## 6. CONCLUSIONS

This study has enabled a comparative evaluation of the effectiveness of the culture method and PCR technique for the detection of *Legionella spp.* in water samples, and has assessed whether PCR could be a valid tool to complement—or potentially replace—culture as the official detection method.

The comparison between different commercial PCR kits allowed for the selection of the one with the highest sensitivity for subsequent application. Then, the analysis of the samples, using both culture and PCR revealed that PCR showed 100% sensitivity and NPV, supporting its reliability in ruling out the presence of the bacterium. Although the PPV was more moderate (0.61) and the specificity was 63%, these results are consistent with the ability of PCR to detect DNA from VBNC or dead cells. The statistical comparison between both methods showed a significant association and a moderate Cohen's Kappa index, indicating an agreement beyond chance.

A detection limit for PCR was also established using standard curves, validating the Ct threshold corresponding to 1 GU. Moreover, PCR was able to detect *Legionella spp.* in samples where culture failed—especially in more contaminated samples, where microbial overgrowth hindered colony isolation—highlighting its value in complex matrices. In these cases, PCR proved capable of overcoming the limitations of culture, detecting bacterial DNA even when viable colonies could not be visualized.

In terms of response time, a clear advantage was observed with PCR, which produced results in under 24 hours, compared to the 10 days of culture. This factor is critical in public health surveillance and in situations requiring urgent corrective measures with a rapid screening. Another specific objective was to assess the technical and economic feasibility of both methods. While PCR requires specialized equipment and trained personnel, its speed and sensitivity often offset the initial investment. Culture, meanwhile, remains the officially recognized method and offers benefits such as the quantification of viable units and serological typing.

The current regulatory framework, which recognizes only culture as a valid method, was also considered. However, the results of this study—supported by prior research—suggest that PCR could be incorporated as an official complementary tool, provided that validated protocols and clear regulatory criteria are established. Finally, regarding the limitations of the study, it is important to note that PCR cannot distinguish between live and dead cells, which may lead to an overestimation of contamination in certain cases. Additionally, the presence of inhibitors in some samples interfered with or prevented amplification.

In conclusion, this study has shown that PCR is a reliable, rapid, and sensitive tool for detecting *Legionella spp.* in water, and it lays a solid foundation for its future incorporation into official monitoring and prevention programs, especially in environments where a fast and trustworthy response is required.

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# 9. ANNEX

## 9.1. ANNEX A: ACID SOLUTION

A 0.2 mol/L hydrochloric acid solution is prepared by adding 17.4 mL of concentrated HCl (density = 1.184 g/mL, minimum content 37%) or 20 mL of concentrated HCl (density = 1.16 g/mL, minimum content 31.5%) to 1 litre of sterile water (Solution A).

A 0.2 mol/L potassium chloride (KCl) solution is prepared by dissolving 14.9 g of KCl in 1 litre of sterile water (Solution B).

To prepare the acidic solution, 3.9 mL of Solution A and 25 mL of Solution B are mixed. The pH is adjusted to 2.2 ± 0.2 by adding a 1 mol/L potassium hydroxide (KOH) solution. The final solution is stored in a suitable glass container, protected from light, at room temperature, for no longer than one month.

## 9.2. ANNEX B: SL-T01 SOLUTION

For the phosphate buffer solution, Solution 1 is prepared first. To do so, 34 grams of monobasic phosphate ($KH_2PO_4$) are dissolved in 500 mL of sterile water. The pH is adjusted to 7.2 ± 0.2 using NaOH at 400 g/L. The solution is then diluted to a final volume of 1000 mL.

For Solution 2, 38 grams of anhydrous magnesium chloride ($MgCl_2$) are dissolved in 1000 mL of sterile water. Alternatively, 81.1 grams of magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) can be dissolved in 1000 mL of sterile water, and both prepared solutions are mixed.

To prepare the final SL-T01 solution, 1.25 mL of Solution 1 and 5 mL of Solution 2 are added, and the volume is adjusted to 1 litre with distilled water. The resulting solution is sterilized in an autoclave at 121 °C for 15 min. The shelf life of these buffered solutions is established at 6 months when stored in the refrigerator.

## 9.3. ANNEX C: EXTRACTION KIT FROM CONDALAB

**PROCEDURE**

Water samples must be collected in sterile containers, following all necessary aseptic precautions. Before DNA isolation, a concentration step is required, such as membrane filtration.

**Filtration Step**

Place a polycarbonate membrane filter with a pore size of 0.45 µm onto the previously decontaminated filtration apparatus. Filter the desired volume of the water sample and record the volume filtered. Carefully remove the membrane from the apparatus using sterile tweezers.

Fold it two or three times into a cone shape and transfer it into a 2.0 mL microcentrifuge tube (not included). Between samples, disinfect the filtration support by rinsing with water, followed by a small amount of alcohol, and briefly flaming for 3 seconds.

**Lysis Step**

To extract the DNA, 100 µL of lysis buffer was pipetted into the microcentrifuge tube and the pellet was resuspended by vigorous vortexing. After a brief centrifugation, the tube was incubated for 10–15 minutes at 95°C. The sample was then centrifuged at more than 10,000 × g for 5 minutes, and the clear supernatant was transferred to a new microcentrifuge tube. The extracted DNA was either used immediately or stored at –20 °C for later use. Before reuse, the sample was mixed thoroughly and centrifuged for 2 minutes at more than 10,000 × g.

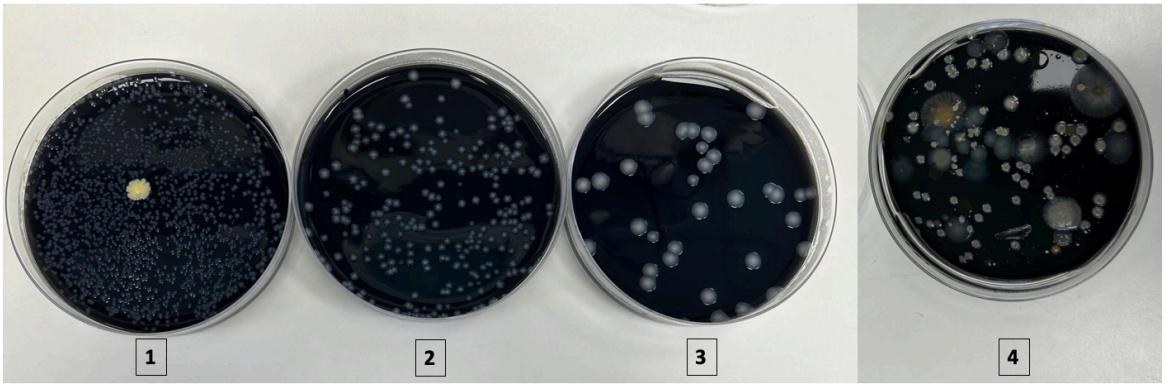
## 9.4. ANNEX D: WATER MICROBIAL CONCENTRATION KIT FROM IELAB

First, concentrate the samples by filtration through 0.2-µm pore size, 47 mm diameter polycarbonate membranes. Place the membrane into a screw-capped tube containing 10 mL of SL-T01. To release the cells from the membrane, vortex the tube for 2 min and later 5 min at the ultrasons.

The 10 mL of solution is further concentrated to 200 µL by centrifugation at 2200 × g for 10 min using the concentrator cartridge supplied. Collect the sample from the upper part of the cartridge by pipetting.

## 9.5. ANNEX E: EXAMPLE OF THE GROWTH OF *Legionella spp.* COLONIES

In these figures, the growth of *Legionella spp.* colonies at different concentrations can be observed. Plate 1 shows overgrowth of *Legionella spp.*, corresponding to the results marked as "UNC" (uncountable) in the culture results table. Plates 2 and 3 display *Legionella spp.* growth that was quantifiable. Finally, Plate 4 corresponds to a sample from a cooling tower, where excessive growth of other microorganisms, such as fungi, was observed.



## 9.6. ANNEX F: EXAMPLE OF A PCR AMPLIFICATION CURVE

These graphs show the amplification curves of different samples from the study. In the first graph, the amplification of the internal control (in red) and *Legionella spp.* (in green) can be seen. In the second graph, only the amplification of *Legionella spp.* is shown, with the corresponding threshold line automatically determined by the software.