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ORIGINAL ARTICLE

Identification of *Legionella* spp. in Environmental Water Samples by ScanVIT-Legionella™ Method in Spain

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Abstract Rapid and more sensitive methods for the detection and quantification of viable *Legionella* cells have been developed. In this paper, a comparative analysis of environmental water samples using the ScanVIT-Legionella™ method and the traditional "gold standard" method of culturing is realised indicating the usefulness of the ScanVIT method. The ScanVIT-Legionella™ method was performed on environmental water samples from different locations of Huesca region (Spain). *Legionella* micro-colonies should appear green colour and *Legionella pneumophila* micro-colonies appear red. Twenty-one environmental water samples analysed by standard culture plus five control samples (Two sterile water samples with Legionella as positive controls and three sterile water samples as negative controls). All of them were used to apply ScanVIT-Legionella™ method. From of 21 environmental samples eleven were positive, six negative with both methods and four samples were negative for culture method and positive for ScanVIT-Legionella™ method. The positive control samples were positive and the negative were negative for both methods. A comparative analysis of the results obtained with two methods showed a strong positive determination coefficient ($R^2 = 0.99753$). The results demonstrate the usefulness of the ScanVIT-Legionella™ method as a rapid diagnostic tool in order to provide a diagnosis as quick as possible. ScanVIT-Legionella™ method offers a series of advantages such as quickly diagnosis, higher sensitivity and the possibility to identify *Legionella spp*. and *L. pneumophila* simultaneously.

Keywords Fluorescent probes · Legionella · Rapid diagnosis

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Introduction

The causative agent of Legionnaires′ disease, *L. pneumophila*, was first characterised in 1977 following an epidemic of acute pneumonia among veterans of the American Legion in Philadelphia [1, 2] and since then, 47 species and more than 60 serogroups have been isolated [3–7]. The bacterium is a Gram-negative, aerobic, non-spore-forming, unencapsulated bacillus. Members of *L. pneumophila* have been subdivided into 15 serogroups but approximately 85 % of the human infections are caused by members of serogroups 1, 4 and 6. Human infection is also caused by *L. micdadei, L. bozemanii, L. dumoffii, L. gormanii,* and *L. longbeachae* [8]; infection with these species is usually seen in immunocompromised patients.

The genus *Legionella* includes 52 species and 71 distinct serogroups. Up to now, only 20 species have been associated with human disease, and *Legionella pneumophila* appears responsible for more than 90 % of reported cases of Legionnaires' disease [9–11].

In 1997, the first year in which it arranges of information of the System of Diseases of Obligatory Declaration, there were declared 201 cases of Legionellosis that supposes a rate of 0,51 cases per 100.000 inhabitants in Spain. The same year, hospitals of seven Autonomous Communities (CCAA) declared 114 cases to the SIM (BES, 1998a). Since then the number of cases has presented an increasing incidence until the year 2001.

In 2002 the notification of the disease settle down with 1.461 cases, which supposes a rate of global incidence of 3.60 cases for 100.000 inhabitants. From here the cases started a gradual decrease. Of 1.262 cases and a rate of 3.19 for 100.000 inhabitants in the year 2003, a rate of 2.67 has gone in the year 2004; 2.89 in 2005; 3.07 in 2006; 2.66 in 2007; 2.99 in 2008; 2.90 in 2009 (1.307 cases) and 2.88 in 2010 (1.309 cases).

In 2011 the accumulated notified data of Legionellosis were 62. Up to the date (February, 2012), and belongs to the year 2012, the accumulated notified data of Legionellosis are 24, 15 of them belongs to foreign tourists and are associated with trips to our country [12].

Surveying and monitoring of *Legionella* in the environment is necessary in order to prevent and control Legionellosis, and it has been suggested that *Legionella* concentrations in environmental sites may be used as a predictive risk factor [13]. When high levels of *Legionella* are detectable in hot water systems, disinfection of water with oxidising biocides (e.g. chlorine) is critical for controlling outbreaks of Legionellosis.

The most widely used method for the environmental surveillance of *Legionella* is the "gold standard" culture technique using selective media [14]. Although culturing allows the isolation and quantification of *Legionella*, it has some limitations including the presence of viable cells which can not be cultured, loss of viability of bacteria after collection, difficulties in isolation from bio-contaminated samples, and prolonged incubation periods of seven to 10 days [15]. It has been demonstrated that bacterial loss during the concentration stage (centrifugation or filtration), followed by decontamination with heat or acid, leads to a decrease in isolated *Legionella*; also, other contaminating organisms may interfere with the growth of *Legionella* and this often results in an underestimation of the real number of bacteria present in the sample.

More rapid and more sensitive methods for the detection and quantification of viable *Legionella* cells have been developed. The rapid diagnostic tests used for detection of *Legionella* pneumonia are based on the direct fluorescent antibody (DFA) staining technique, on the polymerase chain reaction (PCR) [16–19] and on fluorescence in situ hybridisation (FISH) of whole cells with 16S rRNA-targeted oligonucleotide probes [20–22].

PCR methodology appeared as attractive method to the conventional culture method for the detection of slow-growing and fastidious bacteria such as *Legionella*. A number of PCR-based assays have been developed for the detection and quantification of *Legionella* in water mainly using the *5S* and *16S rRNA* genes and the macrophage infectivity potentiator (*mip*) gene of *L. pneumophila* [23–31]. However, these assays lack the ability to discriminate between living and dead (non-infectious) *Legionella* cells. Also, because conventional molecular methods require PCR-based amplification followed by hybridisation to a probe, they are labour-intensive and time-consuming [32]; furthermore, the manipulation of the amplification products increases the risk of carry-over contamination resulting in false positives. With the advent of real-time PCR it has become possible to combine the amplification and the detection steps in a single closed reaction [33], thus obviating the need for further manipulation of the specimen, greatly reducing turnaround times, and diminishing the risk of cross-contamination between samples; therefore, it has been argued that such methods offer attractive alternatives to conventional PCR methods in clinical laboratories.

Fluorescence techniques allow the direct detection of microbial cells in environmental samples without any previous isolation step. One such technique is the Scan-VIT- Legionella™ method based on gene probe technology enabling the quantification as well as the simultaneous detection of cultivable *Legionella* and *L. pneumophila* within three days. Detection of the bacterial cells takes place on a filter membrane, which after filtration of a water sample and 72 h of cultivation on GVPC-agar is brought into contact with the fluorescent gene probes.

The Scan-VIT test may offer several advantages for the laboratory. The use of small samples (50 ml vs 1 l) would permit wide-scale sampling with smaller sample volumes that are easier to handle in sample collection, transport and storage. The test results are available in 3 days versus the 10 days needed with the conventional culture method; this could be an additional advantage when rapid test results of water samples are needed, as during an outbreak or to evaluate the efficacy of disinfection interventions.

Given the simplicity of colony identification by fluorescence, the ScanVIT test can also be used in laboratories where staffs are not experienced in identifying typical micro-colonies of *Legionella*.

Here we report a comparative analysis of a number of environmental water samples using the ScanVIT-Legionella™ method and the traditional "gold standard" method of culturing and demonstrate the usefulness of the ScanVIT method.

Materials and Methods

Samples

Samples of environmental water (21) and five control samples (two positive and three negative) were tested for the presence of *Legionella spp*. and *L. pneumophila* using the ScanVIT-Legionella™ method and the "gold standard" method of culturing (ISO 11731). Sterile water samples were used as negative controls and sterile water samples spiked with *Legionella* ATCC 33152 (10^4–10^5 cfu ml^{-1}) were used as positive controls.

The environmental water samples were collected from the piped water supply in different locations in Huesca region (Spain) for six months. All samples were collected by colleagues working in a chemical and microbiological laboratory accredited for the monitoring of food and water quality (Cobrial Laboratory, Huesca, Spain). Briefly environmental water samples were collected from different locations using sterile equipment, kept refrigerated in the dark and analysed. Cultures were performed by the collecting laboratory and the ScanVIT analysis was performed by the Cytogenetics and Molecular Genetics Laboratory of the Veterinary Faculty (University of Zaragoza, Spain) at the same day.

The Culture Method

Briefly, 1 l of water was filtered (0.2 µm pore-size polyamide filter, Millipore, Billerica, MA, USA), resuspended in 10 ml of the original sample water by vortexing for 10 min and 5 ml heat-treated (50 °C for 30 min). Two aliquots of 100 µl of the original and concentrated specimens were plated onto GVPC (Glycine Vancomycin Polymixin Cyclohexamide) selective medium. The plates were incubated at 36 ± 1 °C with CO_2 for 10 days and read from day 4th with a microscope. Presumptive *Legionella* colonies were subcultured on BCYE (with cysteine) and CYE (cysteine-free) media (Oxoid) and incubated at 36 ± 1 °C for 48 h.

A *L. pneumophila* Latex Test Kit (Oxoid, Madrid, Spain) was used according to the manufacturer's recommendations to identify the predominant species cultured; according to the manufacturer's literature, this test allows separate identification of *L. pneumophila* serogroup 1 and serogroups 2–14 and the detection of seven other *Legionella* species: *Legionella longbeachae* 1 and 2, *Legionella bozemanii* 1 and 2, *Legionella dumoffii*, *Legionella gormanii*, *Legionella jordani*, *Legionella micdadei* and *Legionella anisa*.

Results were given according to the best culture procedure able to give the highest number of *Legionellae* and expressed as cfu/l.

The ScanVIT-Legionella™ Method

This method was performed on 50 ml samples using the ScanVIT-Legionella™ kit from Vermicon AG (Munich, Germany; www.vermicon.de) using the manufacturer's recommendations. Inclusion criteria for water samples were that all samples analyzed by Scan-VIT had been analyzed before by culture method with independence the results obtained with last method.

Water samples were filtered through 0.45 µm nitrocellulose filter membranes (Millipore). The filter was treated with acid buffer (0.2 mol/l) for 5 min and incubated at 36 °C in a CO2 environment for 3 days. After incubation, the filter was transferred to a support provided with the kit (ScanVIT Reactor; Vermicon), the detection of *Legionella* spp. takes place on a cultivated filter brought into contact with the gene probes marked with a dye. During the ScanVIT analysis, the marked gene probes enter the bacteria and bind to the matching signatures within the cells. The membrane was then transferred to a slide and examined under a fluorescence microscope. All bacteria that light up green belong to the genus Legionella; all those that light up both green and red belong to the species *L. pneumophila*.

The blue excitation was detected using BP 450-490, FT 510 and LP 515 (e.g. filter set 09 from Zeiss), green excitation was detected using BP 546/12, FT 580 and LP 590 (e.g. filter set 15 from Zeiss), the eyepiece had a magnification of 10× and a visual field number of 23 and the objective had a magnification of 10× (numerical aperture 0.25) suitable for visual field numbers up to 23. Using this procedure, *Legionella* micro-colonies should appear green in colour and *L. pneumophila* micro-colonies should appear red.

The results with this method were expressed in cfu/l and the numbers of *Legionella spp*. and *L. pneumophila* were counted separately.

Statistical Methods

In order to compare the culture versus the Scan-VIT method used in this paper, we have obtained statistical measures of the performance of a classification function, sensitivity, specificity, positive predictive value, negative predictive value, false positive rate, false negative rate, likelihood ratio positive and likelihood ratio negative. In information retrieval positive predictive value is called **precision**, and sensitivity is called **recall**. The F-score can be used as a single measure of performance of the test. The F-score is the harmonic mean of precision and recall.

In order to compare both methods, culture *vs* Scan-VIT, we have used the **coefficient of determination R^2**, most often seen as a number between 0 and 1.0, used to describe how well a regression line fits a set of data. An R^2 near 1.0 indicates that a regression line fits the data well, while an R^2 closer to 0 indicates a regression line does not fit the data very well. It is the proportion of variability in a data set that is accounted for by the statistical model. It provides a measure of how well future outcomes are likely to be predicted by the model.

Results and Discussion

The results obtained by the two methods are summarised in Table 1 that shows the detection of *Legionella* spp. in environmental water samples by the two methods. Both methods were positive for the positive control (*L. pneumophila* ATCC 33152) and negative for the three sterile water controls. Of the test samples, 11 were positive and six were negative with both the culture and the ScanVIT-Legionella™ methods and four were negative with the culture method and positive with the ScanVIT-Legionella™ method. For these last samples the culture method showed concentrations lower than 625 CFU l^{-1} because we obtained 59 bacteria in two cases and 71 in one case. This result could be attributed to the low number of *Legionella* in the samples (20–80 Legionella CFU/1) because they are below the detection limit of the culture method, according to [34] results. These authors described nine samples that were positives with the Scan-VIT test and negatives with the culture.

Statistical measures have been made (2 × 2 table to compute sensitivity and specificity, predictive values and likelihood ratios) and they can be observed in the Table II. Eleven samples (52.4 %) were positive and 6 (28.6 %) were negative by both methods (agreement 81 %). [35] found an agreement of 85.9 % and [34] was 82 %.

As it is described in this Table 2, the sensitivity (S) is 0.733, specificity (E) is 1; the positive predictive value is 1 too and the negative predictive value is 0.60. The F-score can be used as a single measure of performance of the test. The F-score (the harmonic mean of precision and recall) is 0.844.

The comparative analysis of the results obtained with the two methods (Fig. 1) indicates that the determination coefficient (R^2) is 0.99753. The high value of this coefficient evidences that the results obtained by both methods are very close or strongly connected. These results are higher than those obtained by [35] ($R^2 = 0.788$).

With the use of ScanVIT-Legionella™ method, we were able to detect *Legionella spp*. and *L. pneumophila* simultaneously. All Legionellae colonies were seen as green micro-colonies and *L. pneumophila* colonies were visualised as red micro-colonies. The results are shown in Fig. 2.

It is important that routine tests for the environmental monitoring of *Legionella* are rapid and accurate; also, they must be able to detect all living cells including those that can not be cultured. It is claimed by the manufacturer that the commercially available ScanVIT-Legionella™ kit meets all these requirements. In this preliminary study we compared the results obtained with what is considered to be the "gold standard" culture method used routinely for *Legionella* testing with those obtained using the ScanVIT-Legionella™ kit. Other authors used the same method in order to study hospital water [34, 35].

Our results showed that both methods gave identical results for all positive and all negative control samples. In the case of the environmental samples, the two methods were in agreement for 17 samples: 11 positive and six negative with both methods. From these, 6 have higher CFU/l limits when detected by culture than ScanVIT. [34] obtained 48 positive samples (60.76 %) with both methods, the CFU count was

Table 1 - Total number of colonies observed with the culture method and the ScanVIT-Legionella™ kit

Sample	Culture method results	Culture (cfu/l)	ScanVIT (cfu/l)
Positive Control		10^7	3×10^7
Positive Control		10^8	2×10^8
Negative Control		0	0
Negative Control		0	0
Negative Control		0	0
1	Negative	0	71
2	L.p SG 2-14	250	350
3	Negative	0	**0**
4	L.p SG 2-14	2500	1361
5	L.p SG 2-14	17000	11303
6	L.p SG 2-14	5000	4260
7	L.p SG 2-14	3500	3195
8	Negative	0	0
9	Negative	0	0
10	Negative	0	59
11	Negative	0	59
12	L.p SG 2-14	150	59
13	Negative	0	0
14	L.p SG 2-14	17500	12015
15	Negative	0	0
16	Negative	0	625
17	L.p SG 2-14	100	414
18	Negative	0	0
19	L.p SG 2-14	198	819
20	L.p SG2-14	14	59
21	L.p SG2-14	132	546

Table 2 - Statistical measures. 2 × 2 table to compute sensitivity and specificity, predictive values and likelihood ratios

Standard culture	Scan VIT method		
	Positives	Negatives	
Positives	11 (52,4 %) True positives	0 False positives	Positive predictive value
Negatives	4 (19,05 %) False negatives	6 (28,6 %) True negatives	Negative predictive value
	Sensitivity (S)	Specificity (E)	

Sensitivity (S) = Number of true positives/number of true positives + number of false negatives = 11/15 = 0,733

Specificity (E) = number of true negatives/number of true negatives + number of false positives = 6/6 = 1

Positive predictive value = number of true positives/Number of true positives + number of false positives = 11/11 = 1

Negative predictive value = Number of true negative/Number of true negative + number of false negative = 6/10 = 0,60

False positive rate (alpha) = Type I error = 1 − E = 0

False negative rate (Betta) = Type II error = 1 − S = 0,267

Likelihood ratio positive = S/(1 − E) = 0,733

Likelihood ratio negative = (1 − S)/E = 0,267

In information retrieval positive predictive value is called **precision**, and sensitivity is called **recall**

The F-score can be used as a single measure of performance of the test. The F-score is the harmonic mean of precision and recall: F = 2 x (precision x recall/precision + recall)

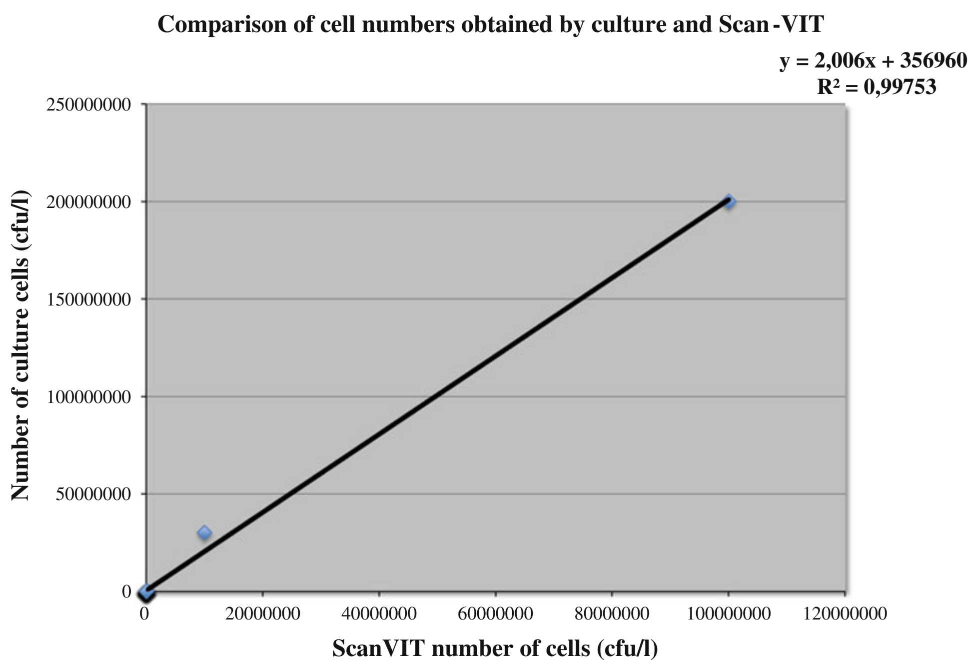

Fig. 1 - Comparative analysis between the results obtained with the culture method and the ScanVIT-Legionella™ method

consistently higher according to the culture method. In our case, in 6 samples the results were higher with culture technique too (54.54 %). These contrasting results between authors could be due to differences in the examined water in terms of higher / lower level of contamination, presence / absence of concomitant microbial flora, supply and structure type, all factors possibly influencing the bacteria detection by the culture method [36].

Other reasons of this difference could be attributed to the different pore size of the filter membranes (0.45 µm for the ScanVIT test and 0.22 µm for culture); decontamination of the filter with acid buffer; growth of only on MWY agar containing antibiotics and antifungals.

On the other hand, 5 of 11 samples that were positive for both methodologies, presented higher concentrations of bacteria with ScanVIT method (45.45 %) than culture method. In the same way that [35], who obtained slightly higher concentrations of *Legionellae* by Scan-VIT method compared to standard culture method.

The ScanVIT-Legionella™ method enables the simultaneous detection of both *Legionella* spp. and *L. pneumophila* since all Legionellae are visualised as green micro-

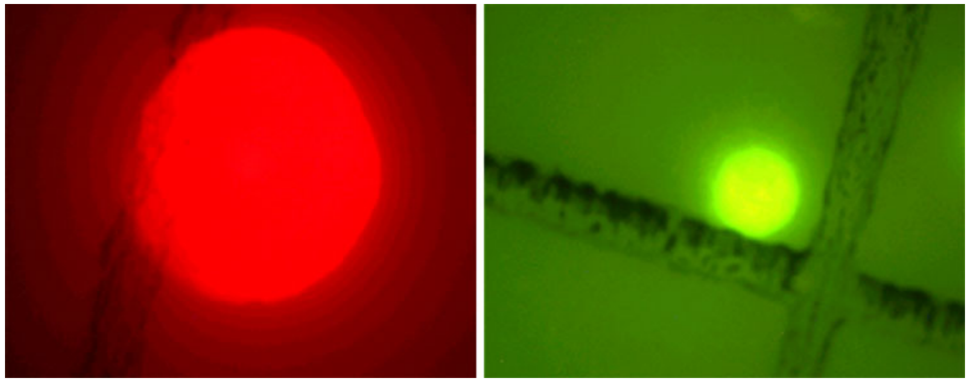

Fig. 2 - A *L. pneumophila* microcolony as seen with the ScanVIT-Legionella^{TM} method. The same microcolony appears as both red and green with different filters

colonies on the filters but only *L. pneumophila* is visualised as red micro-colonies [37].

One of the advantages of the ScanVIT test compared to the standard culture is the reduction in the analysis time (3 vs 10 days), allowing a prompt application of corrective actions aimed at reducing infection risks.

ScanVIT-Legionella^{TM} method offers more advantages such as quickly diagnosis, higher sensitivity and the possibility to identify *Legionella* spp. and *L. pneumophila* simultaneously.

Among the disadvantages of the ScanVIT test is its inability to recover colonies from the filter for typing or biomolecular analysis, which are essential for the epidemiological correlation of human cases and environmental colonization.

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