ARTICLE

New immunomagnetic separation method to analyze risk factors for Legionella colonization in health care centres

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BACKGROUND: It's pivotal to control the presence of legionella in sanitary structures. So, it's important to determine the risk factors associated with *Legionella* colonization in health care centres. In recent years that is why new diagnostic techniques have been developed.
OBJECTIVE: To evaluate risks factors for *Legionella* colonization using a novel and more sensitive *Legionella* positivity index.
METHODS: A total of 204 one-litre water samples (102 cold water samples and 102 hot water samples), were collected from 68 different sampling sites of the hospital water system and tested for *Legionella* spp. by two laboratories using culture, polymerase chain reaction and a method based on immunomagnetic separation (IMS). A *Legionella* positivity index was defined to evaluate *Legionella* colonization and associated risk factors in the 68 water samples sites. We performed bivariate analyses and then logistic regression analysis with adjustment of potentially confounding variables. We compared the performance of culture and IMS methods using this index as a new gold standard to determine if rapid IMS method is an acceptable alternative to the use of slower culture method.
RESULTS: Based on the new *Legionella* positivity index, no statistically significant differences were found neither between laboratories nor between methods (culture, IMS). Positivity was significantly correlated with ambulatory health assistance ($p = 0.05$) and frequency of use of the terminal points. The logistic regression model revealed that chlorine ($p = 0.009$) and the frequency of use of the terminal points ($p = 0.001$) are predictors of *Legionella* colonization. Regarding this index, the IMS method proved more sensitive (69%) than culture method (65.4%) in hot water samples.
SIGNIFICANCE: We showed that the frequency of use of terminal points should be considered when examining environmental *Legionella* colonization, which can be better evaluated using the provided *Legionella* positivity index. This study has implications for the prevention of Legionnaires' disease in hospital settings.

Keywords: Legionella; Legionnaires' disease; water distribution systems; colonization; rapid techniques; risk factors

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INTRODUCTION

The bacteria in the genus *Legionella* occur naturally in many natural environments and colonizes a variety of engineered systems that sometimes support their proliferation. They grow optimally inside protozoan hosts, such as free-living amoebae associated with biofilms that coat wet surfaces [1–3]. *Legionella* transmitted from environmental sources through contaminated water that is aerosolized and exposing those nearby via inhalation is into the respiratory tract [4]. Patients infected with *Legionella* can develop a milder flu-like condition called Pontiac fever or a pneumonia called Legionnaires' disease (LD); both conditions are referred to as legionellosis. LD can be fatal, with between 3 and 33 per cent of infections leading to death [5, 6]. Those at higher risk for developing LD include the elderly, males, smokers, and especially the immunosuppressed, which case-fatality rate can reach 80% even with proper antibiotic treatment [5].

Even unreported, LD rates have been rising in the United States and Europe over the past 20 years suggesting little progress in decreasing risk for *Legionella* [5, 7, 8]. The overreliance of the urinary antigen test, which only detects *L. pneumophila* serogroup 1, coupled with the low rate of diagnostic testing, contributes to the underestimation of the number of LD cases [2, 9–13]. Although *L. pneumophila* is the most dominant *Legionella* species isolated from patients in North America and Europe [9, 13–16], some other species can lead to disease, including *L. micdadei*, *L. bozemanii*, *L. dumoffi*, and *L. longbeachae* [6, 17]. In the European Economic Area (EEA), the annual notification rate increased from 1.3 per 100,000 in 2014 to 2.2 in 2018. Four countries (France, Germany, Italy and Spain) accounted for 71% of all notified cases in 2018 [18]. In Spain, the cases declared in 2019 add up to a total of 1,408 with a rate of 3.0 per 100,000 inhabitants [19]. Meanwhile, in the United States, the incidence of LD increased by more than six-fold from 2000 to 2018 [20].

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There is great concern about LD acquired in hospitals as settings where sizeable populations at higher risk -due to the user's age and/or health status- may be exposed, which may result in considerable mortality [21]. Previous field studies provide knowledge about key factors associated with *Legionella* contamination in domestic hot water, among others, free chlorine and water temperature [22–24]. However, the inherent complexity in water systems of large buildings such as hospitals make it difficult to pinpoint precise factors that trigger *Legionella* contamination, involving interactive effects of water temperature and flow frequency [25].

The mitigation of *Legionella* colonization and disinfection of water systems used in hospital settings is a key factor for controlling and preventing associated *Legionella* infections [11, 26]. However, environmental monitoring of *Legionella* is also fraught with difficulties in these settings, including what detection methods to use and how to interpret the data. Water systems have traditionally been monitored using culture-based methods as the gold standard, which can take many days to detect growth, making rapid decisions impossible, and can be biased toward *L. pneumophila* and a few other *Legionella* spp. [27]. Furthermore, control strategies (heat treatment, chlorine-based disinfectants, and copper-silver ionization) are known to trigger *L. pneumophila* to enter a viable but non-culturable (VBNC) state [28, 29], which does not form visible colonies on plates but may infect different types of human macrophages and amoebae [21]. These drawbacks also make it difficult to identify sources of LD outbreaks, which are not uncommon despite regulations and guidelines addressing *Legionella* contamination in water systems [30, 31]. Polymerase chain reaction (PCR) methods exist, but their ability to differentiate between viable and nonviable organisms is still evolving [32]. Therefore, it is likely that combinations of culture-based methods with rapid, not growth-based methods will be used in the future to assist in developing risk estimates.

According to the reported studies on the dynamics and phenotypic plasticity of *Legionella* cell surface, the ability of *Legionella* to cause LD hinges predominantly on its cell envelope [33]. These findings highlight the importance of detecting legionellae cells in their environment by considering their cell envelope as an analytical target. Hence, we used an immunomagnetic separation (IMS) technique based on the interaction antigen-antibody at the cell envelope level, thereby making this approach of high diagnostic value for a preventative purpose [34].

In this study, a novel *Legionella* positivity index is proposed based on combining three different techniques (culture, PCR and IMS). Based on this index, different factors associated with *Legionella* colonization in a hospital were evaluated. In particular, the frequency of use of terminals points was examined.

MATERIALS AND METHODS

Study design

The influence of the frequency of use of terminal point on the colonization of the water network by *Legionella* spp. was examined. The variables considered in this study were: (i) pipe length; (ii) chlorine; (iii) temperature; (iv) type of terminal point; (v) period of the year; (vi) type of health assistance (outpatient or hospital); (vii) type of water (hot and cold water), and (viii) the frequency of use of the terminal point. A terminal point was considered as frequent if it is open for at least 5 min every day.

Since *Legionella* grows between 20–50 °C and the effectiveness of maintaining sanitary hot water at a minimum temperature of 55 °C is significantly better than that at 50 °C for *Legionella* environmental control [35–37], three ranges of temperature were considered in this study: less than 25 °C, between 25 and 50 °C, and more than 50 °C).

Sample collection and preparation

A total of 204 water samples were collected at 68 different sampling sites (floors and pavilions) of the University Clinical Hospital (Valencia, Spain), in January, May and October 2017. Of these, 102 (50%) were cold water and 102 (50%) were hot water. Sites sampled included tap cold and hot water, the entrance of potable water into the building, storage tanks, distribution points, and points-of-use (showerheads, bathroom taps) that are close to and far from distribution sites. The water samples were collected in areas where patients could be exposed to contagion, excluding areas such as water reservoirs and accumulators in the basement of the hospital. The sampling sites were randomly selected from those which had *Legionella* colonization in the last three years.

Samples were collected in 3-litre sterile bottles directly from the outlet. Before sampling, a sterile swab was inserted into faucet outlets and rotated against the interior surface two times clockwise and up-and-down two times to dislodge the sediment.

The water collecting was designed to simultaneously provide: (i) water samples that were representative as far as possible of the global state of the water system, including swabbed sediment to compensate at least partly the dilution effect, and (ii) a water sample volume sufficient to apply different techniques for *Legionella* determination on water portions as equivalent as possible for each sampling point. After being concentrated by filtration, these portions were assayed to determine a variety of the analytical targets of legionellae organisms in order to define a new *Legionella* positivity index.

The temperature of each water sample was recorded at the time of sample collection. Each 3-litre sample was divided immediately into equal 1 litre-portions and distributed to laboratory 1 and laboratory 2, both laboratories accredited by the Spanish National Accreditation Body (ENAC) to the UNE-EN ISO/IEC 17025 standard. Portions were distributed at ambient room temperature in sterile 1-litre wide-mouth screw cap polypropylene plastic bottles containing sodium thiosulfate, protected from sunlight, within 4 hours from the time of collection.

Legionella testing was conducted within 24 hours of collection. Each laboratory tested one 1-litre portion for *Legionella* spp. using both standard culture performed according to the recommendations of the International Standard method ISO 11731:1998 (Water quality - Detection and enumeration of *Legionella*) [38], and an immunomagnetic separation-based (IMS) technique (Legipid® *Legionella* Fast Detection Test, Biótica, Spain). Additionally, laboratory 2 tested other 1-litre portion for *Legionella* spp, using the polymerase chain reaction-based (PCR) technique (IELAB, Alicante, Spain) technique.

Culture

Sample treatment and standard culture of *Legionella* were performed according to the recommendations of the International Standard method ISO 11731:1998 (Water quality-–Detection and enumeration of *Legionella*) [38], based on filtration procedure and culture of bacteria on selective media. Briefly, one litre of each sample was filtered through a 0·4-µm-pore-size polycarbonate membrane filter (Millipore, Madrid, Spain); this pore size is convenient to retain *Legionella* cells present in environmental samples and prevents filter clogging. The filter was then removed aseptically and placed in a 100-ml tube containing 15 ml of sterile diluent (Biótica, Castellón, Spain). Bacteria were then resuspended by vortexing for 2 min.

The concentrated samples were directly plated (100 µl) onto BCYEα + GVPC media containing antibiotics (Legionella GVPC agar, code 43032; Biomerieux, France) to enumerate *Legionella* colonies (CFU). All the plates were incubated at 36 ± 2 °C for up to 10 days under aerobic conditions and humidified atmosphere. Colonies were counted after 1, 3, 5, 7, 8, 9, and 10 days. Smooth colonies showing a yellowish or sometimes a yellow-green or greyish-white colour were counted as suspicious legionellae to be confirmed. Up to 5–7 colonies of suspected *Legionella* were subcultured onto BCYE agar (without antibiotics) (Biomerieux), and blood agar (alternatively we can use BCYE agar without L-cysteine) for confirmation (Columbia agar + 5% horse blood, code 43050; Biomerieux, France). The isolated colonies growing only on BCYE agar but not on blood agar were considered to be *Legionella* colonies. The results were expressed as $CFU{\cdot}l^{-1}$, and the quantification limit of the procedure was 50 $CFU{\cdot}l^{-1}$ No further confirmatory tests, namely direct or indirect immunofluorescence and latex agglutination, for cysteine-dependent colonies, were carried out.

IMS

Nine mL of each sample concentrated as described previously was added to a cuvette to be analyzed by the IMS method (Legipid® *Legionella* Fast Detection Test; Biótica, Spain). Briefly, a suspension of magnetic particles that bind to *Legionella* is added. If *Legionella* cells are present in the prepared sample, they will bind to the antibodies immobilized on magnetic particles to form complex bacteria/particle. As these complexes

may be separated by a magnet, they are easily washed and resuspended. The complexes are incubated with an anti-*Legionella* antibody conjugated with an enzyme, to form labelled complexes. After washing the *Legionella*/particle complexes are visualized colourimetrically when the enzyme substrates are added.

PCR

Each 1-litre water sample was mixed by shaking it and filtered through a 0.2 µm-pore diameter polycarbonate membrane filter (Millipore). The membrane filter was then removed and placed in 10 ml of sterile RNase and DNase-free water and 1 ml was used for DNA extraction with a commercially available kit (DNeasy Blood & Tissue Kit, code 69504; Qiagen, Hilden, Germany). DNA was eluted in 200 µl of elution buffer (supplied in the kit). PCR assays were carried out with a commercially available kit (*Legionella* spp. qPCR Quantitative Detection Kit, code 992402; ielab, Alicante, Spain). The commercial mix contained primers specific for *Legionella* spp. TaqMan Universal Master Mix, fluorescent probes, IPC (Internal Positive Control – plasmid DNA). To 15 µl of reaction mix was added: 10 µl of matrix DNA; 10 µl of nuclease-free water (negative control); 10 µl of positive control: 6 successive dilutions of positive control (a strain of Legionella pneumophila) at the initial concentration of 1×10^6 genome units/µl. All samples were tested by 3-fold repetitions. Results were read with the use of standard slope, provided by the producer (slope points were: 100,000, 10,000, 1,000, 100, 10, 1). Amplification comprised: 50 °C, 2 min; 95 °C, 10 min; 42 cycles, each comprising of: 95 °C, 15 sec, and 60 °C, 1 min. The quantification limit was 480 GU·l^{-1}.

Physical and Chemical Analyses

Water temperature and residual free chlorine (DPD method, colourimetric) were determined at the time of sample collection.

Data analysis

A positive-negative *Legionella* index was defined by a consensus of three microbiologists jointly considering the data from all samples tested by culture, PCR and IMS. A water sample was considered as positive for *Legionella* spp. in each of the four following cases: (i) culture count, not less than 100 CFU·L^{-1} reported by at least one laboratory; (ii) IMS result, not less than 300 CFU_{eq}·L^{-1} reported by at least one laboratory; (iii) positive IMS result reported by all laboratories; (iv) positive IMS result reported by at least one laboratory, if PCR is positive; (v). A water sample was considered as negative for *Legionella* spp. in the following two cases; (i) culture count less than 100 CFU·L^{-1} reported by at least one laboratory, even if the PCR was positive; (ii) a culture count less than 100 CFU·L^{-1} reported by all laboratories.

Once the consensus on *Legionella* positivity was achieved, out of the total of 204 water samples examined, 68 that had been analyzed by the three techniques (culture, IMS and PCR), one for each sampling site, were used to examine the effect of the frequency of use on the *Legionella* colonization.

Sensitivity, specificity, positive predictive value (PPV) and negative predictive (NPV) were calculated by comparing IMS method with the culture method (current gold standard). Moreover, these measures were also obtained by comparing both IMS and culture method with the *Legionella* positivity index as a new gold standard.

Statistical analysis

Using the software IBM SPSS Statistics 21.0, a statistical analysis univariate, bivariate and multivariate was carried out. Odds ratios (OR) and 95% confidence intervals (CI) were calculated to assess categorical risk variables associated with Legionella positivity. The bivariate analysis used the Chi-square test and Fisher's exact test for qualitative variables and Student's t-test, considering the analysis of variance according to Levene's test, for continuous variables. Finally, binary logistic regression was applied to conduct multivariate adjustment of the risk factors.

RESULTS

Descriptive data

University Clinical Hospital (Valencia, Spain) is a 587-bed hospital with a 16-bed medical intensive care unit (ICU), consisting of a large structure distributed in 4 pavilions indicated by letters of the alphabet (A, B, C, D).

Table 1. Bivariate analysis of factors associated with *Legionella* contamination.

	n (%)	OR	95% CI		*p*
Date					
Gen	13 (19.1)	1			
May	28 (41.2)	0.857	0.229	3.203	0.819
Oct	27 (39.7)	1.247	0.329	4.731	0.746
Pavilion					
A	18 (26.5)	1			
B	20 (29.4)	0.833	0.229	3.028	0.782
C	20 (29.4)	1.875	0.516	6.813	0.340
D	10 (14.7)	11.250	1.167	108.407	0.036
Type of assistance					
Hospital	54 (74.9)	1.000			
Ambulatory	14 (20.6)	3.949	0.990	15.754	0.052
Floors					
1	3 (4.4)	1			
2	10 (14.7)	0.750	0.050	11.311	0.835
3	8 (11.8)	0.833	0.051	13.633	0.898
4	13 (19.1)	1.125	0.078	16.307	0.931
5	15 (22.1)	0.438	0.032	5.926	0.534
6	5 (7.4)	0.333	0.017	6.654	0.472
7	10 (14.7)	0.333	0.022	5.027	0.427
8	4 (5.9)	0.500	0.023	11.088	0.661
Point of sample					
Washbasin	27 (39.7)	1			
Shower	41 (60.3)	1.953	0.729	5.229	0.183
Frequency of use					
Frequent	48 (70.6)	1			
Not frequent	20 (29.4)	7.933	2.047	30.752	0.003
Type of water					
Hot water	34 (50)	1			
Cold water	34 (50)	0.147	0.050	0.429	0.000
Temperature range (°C)					
<25	28 (41.2)	1			
25–50	24 (35.3)	2.520	0.822	7.729	0.106
>50	16 (23.5)	7.800	1.786	34.070	0.006
Pipe length (m)	1.001	0.985	1.017	0.916	
Chlorine (mg/l)	0.117	0.036	0.377	0.000	

OR Odds Ratio, CI confidence interval, *p* statistical probability (*p*)-value.

Bivariate analysis

Results showed no significant differences neither between the two laboratories nor between culture and IMS method indicating that these methods were equivalent in terms of *Legionella* positivity and also according to the results of the reported validation [39]. Laboratories 1 and 2 reported positivity rates of 33.82 % and 32.35 %, respectively.

The risk factors for *Legionella* colonization were analyzed through logistic regression on dichotomous variables. In our study, the floors and pipe length (distance from the terminal point) were not significantly associated with the risk of *Legionella* colonization. Pipe length was considered as an adjustment variable (Table 1). A water temperature >50 °C was positively associated with a risk for *Legionella* colonization, whereas chlorine (OR = 0.117, 95% CI = 0.036–0.377, $P < 0.05$) was protective. The

Table 2. Multiple logistic regression of factors associated with *Legionella* contamination.

	OR	95% CI		*p*
Date				
Gen	1			
May	0.150	0.014	1.624	0,118
Oct	0.462	0.048	4.407	0,502
Type of health assistance				
Hospital	1			
Ambulatory	13,442	0.374	482.551	0.155
Point of sample				
Washbasin	1			
Shower	8,661	0.932	80.493	0.058
Frequency of use				
Frequent	1			
Unfrequent	11,822	1.386	100.844	0.024
Pipe length (m)	1,006	0.977	1.035	0.696
Temperature range				
<25	1			
25–50	0.472	0.055	4.043	0,493
>50	0.615	0.037	10.100	0,734
Chlorine (mg/l)	0,030	0.002	0.419	0.009

OR Odds Ratio, CI confidence interval, *p* statistical probability (p)-value.

not frequent use of the terminal points supposes a risk for *Legionella* colonization (OR = 7.933, 95% CI = 2.047 to 30.752 P < 0.05) (Table 1). Ambulatory health assistance was significantly associated with an increased risk of *Legionella* colonization (OR = 3.949, 95% CI = 0.990–15.754, P = 0.052).

Multivariate analysis

To address the effects of possible confounding variables, the data were reanalyzed employing multivariate conditional logistic regression models. Date, type of health assistance, terminal point, frequency of use, pipe length, temperature range and chlorine were considered. Chlorine (OR = 0.030, CI = 0.002–0.419, $P < 0.05$) was protective. The study indicated that infrequent use of the terminal point (OR = 11.822, CI = 1.386–100.844, $P < 0.05$) was associated with an increased risk of *Legionella* colonization. Accordingly, shower point (OR = 8.661, CI = 0.932–80.493, $P = 0.058$) was positively associated with this risk. However, ambulatory health assistance (OR = 13.442, CI = 0.374–482.551, $P = 0.155$) was not statistically significant (Table 2).

Multivariate analysis showed that if hot water supplies were not used daily, the risk of *Legionella* colonization was greater than twelve-fold (odds ratio: 11.822, 95% CI = 1.386–100.844). The terminal points not frequently used (NFU) had a greater percentage of water temperatures less than 50 °C than the frequently used (FU) points. None of these NFU points presented a temperature higher than 55 °C, unlike the FU terminal points (Fig. 1). The average temperatures in the not frequently used terminal points was 41.92 °C, while in those of frequent use was 48.06 °C.

Test method comparison

We found sensitivity of IMS compared to culture (current gold standard) to be 68.2%, specificity 73.9%, positive predictive value (PPV) 55.6%, and negative predictive value (NPV) 82.9%. In this study, sensitivity increased in hot water (76.5%) while specificity increased in cold water (86.2%). IMS and culture were compared with the Legionella positivity index as a new gold standard for both hot and cold-water samples (Table 3). Generally, we found similar sensitivity for these two methods, even though higher for IMS method in hot water. In comparison with culture the lower specificity of IMS is probably caused by viable but non culturable *Legionella* bacteria in water. The performance of IMS as a routine method for rapid determination of *Legionella* spp in waters was acceptable.

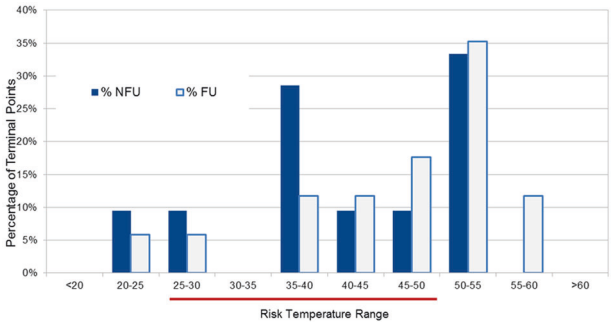

Fig. 1 Hot water temperature range on different terminal points. Percentage of terminal points at a given temperature range (NFU not frequently used, FU frequently used).

DISCUSSION

Highly *Legionella* colonization of point of use which is not daily used has been previously reported [26]. Consistent with this finding, we found that not frequent use of terminal points was positively associated with *Legionella* colonization in hot water. Therefore, the less frequent use of terminal points may play a role in persistent colonization and the development of clinical cases. Our results indicate that all terminal points inside the hospital building such as faucets and showerheads should be run regularly to avoid *Legionella* colonization, probably due to water stagnation and consequential biofilm formation.

In comparison with studies conducted at residential facilities, the complete eradication of *Legionella* spp. seems mostly improbable [9, 40]. Large, old and complex hospital water networks, with dead-end branches and corroded pipelines, may promote the *Legionella* colonization in critical points where the disinfectant cannot be effective against *Legionella* spp. In concordance with previous studies, we found no apparent seasonality in the *Legionella* colonization in hospital over the year [41]. Our observations suggest that *Legionella* colonization is likely consistent throughout the year, indicating the importance of water hygiene in hospital facilities. In this scenario, the frequency of use of terminal points should be considered as one of the most important determinants for *Legionella* colonization in healthcare facilities.

Water disinfection protocols used in hospital include thermal control, chlorination, and *Legionella* sampling. Our findings support the importance of maintaining sanitary hot water at a temperature higher than 50 °C [35, 37] For water temperatures between 20–50 °C, the new index provided more *Legionella* positivity rate (51.56 %) than culture (26.56 %) as well as for temperatures >50 °C (88.00 % vs 54.15% positivity rates). These findings suggest the presence of viable but non-culturable Legionella cells, whose detection using this new index would allow anticipating the need for adequate cleaning and disinfection treatment.

To our knowledge, this study is the first to report hospital-based testing for *Legionella* to examine risk factors associated with *Legionella* colonization by defining a *Legionella* positivity index which combines three different analytical techniques (culture, PCR and IMS). Testing water for the presence of *Legionella* can be an important component of risk management for legionnaires'

Table 3. Comparison of performance indicators for IMS and culture.

Water sample	Test method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV(%)
Total	IMS	61,4	91,1	89,7	65,1
	Culture	60,8	100	100	68,1
Cold	IMS	40,0	93,9	75,0	77,5
	Culture	50,0	100	100	80,7
Hot	IMS	69,0	83,3	93,5	43,5
	Culture	65,4	100,0	100,0	47,1

PPV Positive predictive value, NPV Negative predictive value.

disease (LD) in a hospital [42, 43]. Nevertheless, previous studies reported low sensitivity (59%) and specificity (74%) of a 30% *Legionella* positivity as a metric based on the gold-standard culture method for assessing the risk of health care-acquired LD [44]. That metrics based just on the proportion of *Legionella* positive results by culture may not correspond to the actual risk status of the plumbing systems because stressful conditions for Legionella growth may influence its undetection by culture-based methods. *Legionella* in a viable but non-culturable state should not be neglected when assessing the *Legionella* risk during nosocomial environmental surveillance [45].

We demonstrated that *Legionella* positivity is better estimated by the new *Legionella* positivity index (55.04% positivity rate) than by just culture data (31.09% positivity rate). For any given sampling site, the index is positive if the culture is positive (31.09% of sampling sites) or if it is negative but IMS itself and PCR itself are positive (23.94% of sampling sites). The index was used in helping to solve two shortcomings in the assessment of *Legionella* positivity, namely: (i) underestimation of the presence and concentration of *Legionella* spp. by culture-based method because most *Legionella* cells could remain in a viable but non-culturable (VBNC) state, and (ii) likewise, polymerase chain reaction-based techniques (PCR) cannot differentiate live versus dead (non-viable) cells or free DNA, so the number of Legionellae could be overestimated [46]. Of particular interest is that high percentages of the *Legionella* populations in water systems cannot grow on a conventional culture medium [47].

These findings suggest that the true level of *Legionella* colonization can likely be underestimated by culture and overestimated by qPCR. Furthermore, there is no consensus with regards to the concentration that will cause LD [45]. Therefore, an IMS technique based on the antigen-antibody interaction at the level of the cell envelope was implemented. As the antigens related with virulence mainly resides on the cell envelope, this interaction allows incorporating the effect of envelope integrity, already demonstrated to examine the effect of biocides on *Legionella* in other studies [42].

In agreement with other studies [48], our observations suggest that no-growth based methods should be considered when examining risk factors as determinants to *Legionella* colonization in hospital water to reduce the potential exposure of patients to these bacteria. Our index may help to prevent that many hospitals might fail to mitigate when a true risk is present or might unnecessarily allocate limited resources to deal with negligible risk.

Especially after recognizing *Legionella* as one possible pathogen causing co-infection among COVID-19 patients [49], a more sensitive *Legionella* monitoring and flushing of terminal points should be recommended as a *Legionella* decolonization strategy.

The results obtained suggest that IMS can be used as a routine test, in accordance with previous studies [35]. IMS method focuses on capturing those bacteria that present accessible antigens, many of them virulence related, in the outer envelope [4, 34]. This enables an approach of high diagnostic value because IMS cannot detect dead cell DNA and may detect viable but non-culturable states (VBNC), which could be potentially infectives. Thus, the results from this study and from previous studies [50] suggest that IMS may be useful to prevent cases and outbreaks of Legionnaires' disease.

MAIN CONCLUSIONS

Our study provides a new *Legionella* positivity index to better assess *Legionella* colonization in the water system of a hospital, which is essential to identify relevant risk factors associated with *Legionella* colonization. Our observations suggest that non-culturable methods (IMS, PCR) and frequency of use of terminal points should be considered when examining environmental *Legionella* colonization. In fact, the less frequent use of terminal points may play a role in the proliferation of *Legionella* species and the development of nosocomial cases of Legionnaires' disease (LD). In this way, the index (i) could anticipate the need for a cleaning and disinfection treatment, and (ii) would allow evaluating its effectiveness.

Additionally, given speed of *Legionella* detection, its ability to detect viable but non-culturable forms and its similar sensitivity to culture, even higher in hot water, clinicians are encouraged to consider the use of IMS method in environmental routine testing for *Legionella*. Obtaining results on the same day can be key when applying corrective measures.

Limitations and future research

The criterion used in this study increased the sensitivity of *Legionella* spp. detection in water with respect to culture-based methods, which environmental diagnostic value in prevention is compromised. However, further in-depth studies are recommended to be conducted to define an internationally validated standard in near future.

The study evaluates the risk of colonization by Legionella considering different factors and the use of new diagnostic techniques. More studies are needed to link improvements in the internal validity of diagnostic tests and early detection of this pathogen in water pipes with changes in the effectiveness of corrective practices and with a potential reduction in Legionnaires' cases.

DATA AVAILABILITY

Data are available from the authors upon reasonable request and with permission from the Hospital Clínico Universitario de Valencia.

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COMPETING INTERESTS

The authors declare that they have no conflict of interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

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ADDITIONAL INFORMATION

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