Confirmation of presumptive Legionella colonies on culture media according to ISO 11731:2017: Principles, problems and practice

Confirmation of presumptive *Legionella* colonies on culture media according to ISO 11731:2017: principles, problems and practice

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Running headline: *Legionella* colonies confirmation.

Key words: *Legionella*, sensitivity and specificity, validation, method, culture, water samples, Polymerase Chain Reaction, ISO 11731:2017.

Abstract

Aims: The ISO 11731 norm, published in 2017, describes a method to identify and enumerate *Legionella* based exclusively on the confirmation of presumptive colonies by subculturing them on BCYE and BCYE-cys agar (BCYE agar without L-cysteine).

Methods and Results: Despite this recommendation, our laboratory has kept confirming all presumptive *Legionella* colonies by combining the subculture method with the latex agglutination and polymerase chain reaction (PCR) assays. Here, we show that the ISO 11731:2017 method adequately performs in our laboratory according ISO 13843:2017. We compared the performance of the ISO method in detecting *Legionella* in typical and atypical colonies (n=7156) from health care facilities (HCFs) water samples to that of our combined protocol and we found a 2.1% false positive rate (FPR), underscoring the importance of combining agglutination test and PCR with subculture to achieve optimal confirmation. Lastly, we estimated the water system disinfection cost for HCFs (n=7), that due to false positive results, would displayed *Legionella* values exceeding the threshold of risk acceptance established by the Italian guidelines.

Conclusions: Overall, this large-scale study indicates that the ISO 11731:2017 confirmation method is error-prone, leading to significant FPRs and higher costs for HCFs due to remedial actions on their water systems.

Significance and Impact of Study: Identifying *Legionella* strains may help assess the risk associated with contaminated environmental reservoirs. Better characterization of the strains that cause the majority of diseases would allow for more targeted intervention measures.

Introduction

Monitoring for *Legionella* is a crucial public health measure to identify contaminated environmental sources (e.g., evaporative cooling towers, hot- and cold-water distribution systems, spa pools, dental units, air conditioning units, etc.) that could pose a risk of legionellosis. The environmental monitoring is also important for validating the efficacy of control measures and ensuring that such measures remain effective over time (World Health Organization. 2007; Ditommaso *et al.* 2010; Ministero della Salute 2015; Wilson *et al.* 2022). To date, 71 *Legionella* species have been identified (https://lpsn.dsmz.de/search?word=legionella). Most *Legionella* species have been isolated from aqueous environments, with at least 30 of them capable of causing infection in humans, mainly in the lower respiratory tract (Cunha, Burillo and Bouza 2016).

The culture method represents the standard technique for the detection and quantification of viable *Legionella* spp. from environmental water samples (Public Health England 2015; International Standard Organisation 2017; Centers for Disease Control and Prevention 2022). In this regard, the ISO 11731:2017 (International Standard Organisation 2017) describes a method for *Legionella* identification and enumeration based on membrane filtration and subsequent confirmation of presumptive colonies as *Legionella* (*i.e.*, colonies of *Legionella* are white-grey in general but can also appear in other colours, they are smooth with entire edge and exhibit a characteristic ground-glass appearance) by subculturing colonies on BCYE and BCYE-cys agar (BCYE agar without L-cysteine) or alternative media (Blood Agar, Nutrient Agar, Tryptone Soy Agar) to demonstrate their growth requirements for L-cysteine and iron (III) (Feeley *et al.* 1978). Specifically, this protocol recommends to "Regard as *Legionella* those colonies which grow on the plate of BCYE agar but fail to grow on the plate of BCYE-cys agar".

ISO 11731:2017 cancels and replaces the first two editions, ISO 11731:1998 and ISO 11731-2:2004 (International Organization for Standardization 1998, 2004), which suggested performing subculture on BCYE and BCYE-cys agar and further confirmation and identification of the species

64 and serogroup by serology of those colonies which grow on the BCYE agar but not on the BCYE-
65 cys.

66 The confirmation procedure proposed in the revised edition has been validated according to
67 the ISO 13843:2017 standard (International Organization for Standardization 2017), which describes
68 how to determine sensitivity, specificity, efficiency, selectivity, false positive rate (FPR) and false
69 negative rate (FNR). Noteworthy, the ISO 13843:2017 norm also states that it is necessary to carry
70 out a secondary test to confirm or reject the results generated by the primary one. In the case of ISO
71 11731:2017, the validation procedure involved culturing colonies on two media with and without
72 cysteine as primary test, while polymerase chain reaction (PCR) as secondary test (ISO 11731:2017,
73 Annex H, Table H. 1).

74 According to the ISO 13843, " when a confirmatory step is included in the method, the
75 identification data can be divided into four categories: i) number of typical colonies confirmed as
76 being the target organism in the primary confirmatory test, the identification of which is supported
77 by secondary identification test (true positives); ii) number of atypical colonies or typical colonies
78 that are negative in the primary confirmatory test identified as being the target organism by the
79 secondary identification test (false negatives); iii) number of typical colonies confirmed as being the
80 target organism by the primary confirmatory test that are subsequently shown not to be the target
81 organism by the secondary identification test (false positives); iv) number of atypical colonies or
82 typical colonies that are negative in the primary confirmatory test which are shown by secondary
83 identification test to not be a target organism (true negative)". The categories must be distributed in
84 a 2x2 grid, and performance characteristics should be calculated.

85 Table 1 shows the performance data obtained in the intralaboratory trial, which took place in
86 the Netherlands (ISO 11731:2017, Annex H, Table H.1).

87 Since 1997, our laboratory has been conducting *Legionella* spp. testing on environmental
88 samples using two types of medium: BCYE and MWY (Ditommaso *et al.* 2011). The lab adopted
89 MWY because its dyes stain the colonies, ensuring better differentiation (Vickers, Brown and Garrity

1981; Edelstein 1982). The combined use of BCYE agar with selective agar for improved *Legionella* detection has been recommended in 2017 by the second edition of ISO 11731.

In this background, the aims of our study were: i) to demonstrate, as a preliminary step, that the ISO 11731:2017 method could adequately perform in our laboratory; ii) to compare the performance of the ISO method in detecting *Legionella* in typical and atypical colonies from routinely analyzed water samples to that of our laboratory protocol, consisting of subculture combined with latex agglutination and polymerase chain reaction (PCR) assays; iii) to estimate hypothetical costs of the two possible alternative options (i.e., confirmation through subculture only versus additional agglutination and PCR), taking into account laboratory diagnostics costs but also, conversely, the economic burden for health care facilities (HCFs) due to water system disinfection after possibly false positive results.

Material and Methods

Legionella spp. isolation

Briefly, one-liter water samples were concentrated 100 times by filtration using 0.22-µm polycarbonate filters (Millipore, Billerica, MA, USA). After filtration, these filters were aseptically placed into one of the bottom corners of a stomacher bag with 10 ml Page solution (pH 6.8) and rubbed for 1 min to detach bacteria. A 0.2-ml volume of the concentrated sample was placed on MWY and BCYE agar plates (Xebios Diagnostics GmbH, Düsseldorf, Germany), which were then incubated at 36° C for 10 days. The plates were checked at days 2, 3, and 5, and at the end of the incubation period. In case of high concentration of interfering microorganisms at the day 2, the concentrated samples, stored at 5±3°C, were plated after dilution, acid treatment and thermal treatment (this step allowed us to identify samples where overgrowth had occurred according to the suggestion of ISO 11731).

Colonies of *Legionella* are in general white-grey with a ground-glass appearance, but they can also appear in other colors. Thus, we examined all the plates (BCYE and MWY) under an ultraviolet lamp in order to recognize autofluorescent brilliant white (e.g., *L. anisa*, *L. bozemanii*, *L. dumoffii*, etc.), red (*L. erythra*, *L. rubrilucens*), or dull green often tinged with yellow (*L. pneumophila*) colonies. The fluorescence color can in fact help differentiate colonies in samples containing different species of *Legionella*.

Confirmation of presumptive colonies by the culture method

Both colonies with typical morphological characteristics and autofluorescence colonies were deemed presumptive *Legionella* colonies. These were confirmed by subculturing them on BCYE agar plates (Xebios Diagnostics GmbH, Düsseldorf, Germany) and blood agar (Thermo Fisher Scientific Inc., Waltham, MA, USA). We carefully compared growth differences between BCYE agar plates and blood agar plates (BA), as suggested by the ISO 11731 recommendation: "some *Legionella* species, *i.e.*, *L. oakridgensis* and *L. spiritensis*, require L-cysteine and iron (III) for primary isolation but sometimes grow weekly in the absence of L-cysteine thereafter".

Confirmation by the latex agglutination assay

Presumptive colonies were subcultured on BCYE agar and blood agar (BCYE agar+ and BA- respectively) and further identified by means of a *Legionella* latex test (Thermo Fisher Scientific Inc., Waltham, MA, USA), which allows rapid detection and differentiation of *L. pneumophila* serogroup 1, *L. pneumophila* serogroup 2-14, and other seven *Legionella* species (*i.e.*, *L. longbeachae* 1 & 2, *L. bozemanii* 1 & 2, *Legionella dumoffii*, *L. gormanii*, *L. jordanis*, *L. micdadei*, and *L. anisa*). Colonies recognized as *L. pneumophila* serogroup 2 to 14 were further tested with single *Legionella* agglutination latex reagents (Pro-Lab Diagnostics, Richmond Hill, Canada) for the identification of

the different *L. pneumophila* serogroups. In this study, we only reported the results relative to the *L. pneumophila* serogroup 1, *L. pneumophila* serogroup 2-14 and non-*pneumophila Legionella* species.

Confirmation by in-house PCR assay

Latex agglutination test-negative colonies were then subjected to in-house PCR assay to detect the *Legionella* genus by using the 16S ribosomal subunit gene (Miyamoto *et al.* 1997; Wilson *et al.* 2007). The assay was specific to legionellae, and the sensitivity was 1 fg of extracted *Legionella* DNA (Miyamoto *et al.* 1997). We used the LEG primers for the 16S rRNA gene for detection of *Legionella* because the rRNA sequence is a fundamental molecular marker in bacterial taxonomy and phylogenetic analysis (Maidak *et al.* 1996) and rRNA-based PCR has higher sensitivity than that of other target genes, as demonstrated in a previous study (Fox and Brown 1989).

Briefly, presumptive colonies were isolated and resuspended in 100 µl of lysis buffer (NaOH 10 mM, Tween-20 0.5%, NP-49 0.5%), heated at 95°C for 4 min, vortexed, heated again at 95°C for 4 min, and finally centrifuged at 12,000 rpm. Two microliters of DNA sample were added to a PCR master mix of total 20 µl with 20 pM of specific primer (LEG 225 and LEG 858) (Miyamoto *et al.* 1997). PCR-amplified DNA fragments (654bp) were separated in a 2% agarose gel and visualized by ethidium bromide staining. This in-house PCR assay does not provide any information on *Legionella* species or serogroups. We did not validate our PCR protocol according to ISO 12869:2019 as we applied PCR for the confirmation of colonies (DNA was extracted from colonies in abundant quantities), therefore it was not necessary to make evaluations of the limit of detection (LOD) and limit of quantification (LOQ). As positive control we used DNA derived from *Legionella anisa* ATCC 35292 and DNA derived from *Legionella pneumophila* sg 1 ATCC 12821.

Expression of results

The plate with the highest number of confirmed colonies was used to estimate the number of *Legionella* spp. in the original sample. *Legionella* spp. concentration was expressed as colony forming units per liter (CFU l^{-1}). According to the concentration procedure, the detection limit of our method was 50 CFU l^{-1}.

Determination of performance characteristics of ISO 11731 method according to ISO 13843:2017

To establish the performance of the ISO 11731 method in our laboratory, we used environmental samples: mainly potable water and water from cooling towers or thermal sources.

Legionella spp. quantification analyses were performed by adopting an internal method based on ISO 11731:2017, as described elsewhere (Ditommaso *et al.* 2011, 2022) Typical and atypical colonies were counted and then identified using the procedure described below. Experimental data were then used to calculate sensitivity, specificity, false negative rate (FNR), false positive rate (FPR), efficiency and selectivity.

Laboratory workflow on routinely analyzed environmental water samples

Presumptive *Legionella* colonies grown on MWY or BCYE agar were confirmed by subculture on BA and BCYE agar plates. First, all presumptive colonies growing on BCYE agar, but not on BA, were subjected to identification through the latex agglutination test. Agglutination test-negative colonies were then subjected to in-house PCR. Our strategy of not confirming the presumptive *Legionella* colonies directly through PCR was due to the need to further identify different L. *pneumophila* serogroups colonies using specific latex agglutination test in order to assess the risk associated with contaminated environmental reservoirs colonised by more virulent strains (data not shown).

Legionella control costs

We collected data on the economic costs related to the management of water system with apparent *Legionella* colonization. Information on the costs of carrying out control measures and management was obtained from the healthcare facilities (HCFs) participating in the study. Total costs did not include the expenditure for health management or other indirect costs incurred by the organizations.

Costs of laboratory workflow for *Legionella* confirmation

The total costs of laboratory testing for *Legionella* confirmation, including post-remediation sampling, in environmental water samples were carefully evaluated. Each single cost provided information on direct costs of laboratory reagents without including the staff resources dedicated to the investigation.

Data analysis

Data were collected and organized using Microsoft Access and Excel 2016 (Microsoft Corporation, Redmond, WA, USA). The performance characteristics of the identification were calculated according ISO 13843:2017. Agreement between the two methods was determined by comparing the culture vs PCR test results on two-by-two contingency tables by using Cohen's kappa ($Po = (a+d)/N$).

Results

Performance characteristics of the ISO 11731 method on strains collected (typical and atypical colonies) according to ISO 13843:2017

Sixteen different samples were selected according ISO 13843 (samples containing 20 to 80 total colony forming units with 10 to 60 target organisms) and were processed according to ISO

11731:2017. A total of 602 colonies were selected including typical *Legionella* spp. and atypical colonies. All colonies were tested for L-cysteine dependence by culturing them on BCYE agar and BA, and subsequently analyzed by latex agglutination and PCR assays. This allowed grouping the colonies into four categories: (i) true positive, (ii) false negative, (iii) false positive, and (iv) true negative (Table 2). The analysis results were used to calculate sensitivity, specificity, false positive rate (FPR), false negative rate (FNR), efficacy and selectivity of the ISO 11731:2017 method.

By means of subculture, 13 of the 338 typical colonies of *Legionella* were identified as non-*Legionella* species, while 3 of the 264 atypical colonies were considered *Legionella* species (Table 2a). The agglutination test failed to confirm 113/338 typical colonies (FPR = 33.4%) for *Legionella*, whereas none of the 264 atypical colonies were found to be *Legionella* species (Table 2b). The high FPR revealed by this analysis was due to the inability to identify all *Legionella* species and serotypes using agglutination sera, which allows rapid detection and differentiation of only *L. pneumophila* serogroups 1-14 and of seven non-*Legionella* species. For all other *Legionella* species, agglutination identification kits are not commercially available. Finally, PCR identified 13 of the 338 typical colonies for *Legionella* to be non-*Legionella* species, whereas none of the colonies from the 264 atypical colonies were found to be *Legionella* species (Table 2c).

The results of this verification (Table 3) indicate that our laboratory was able to obtain performance data similar to those disclosed by the ISO 11731:2017 norm (Table 1) using confirmation by subculturing.

The FPR was the same (3.9%) for both confirmatory methods because no *Legionella* strain growing in the absence of L-cysteine was confirmed by PCR. Lastly, the FNR of confirmation by the subculture method appeared to be quite low.

Performance characteristics of the ISO 11731:2017 method on colonies collected during microbiological monitoring of water supply

Identification through agglutination and PCR was carried out on 7156 colonies of presumptive colonies of *Legionella*, intended as characteristic colonies growing on BCYE or MWY agar, according to the following diagram (Fig. 1).

Overall, 7156 presumptive *Legionella* colonies were subjected to confirmation tests: i) 74.7% (5344/7156) of the colonies requiring L-cysteine (BCYE agar+ and BA-) were subsequently subjected to latex agglutination; ii) 25.3% of the colonies (1812/7156) were not confirmed to be *Legionella*, as their growth did not depend on L-cysteine and iron (BCYE agar+ and BA+: only the colonies that showed plenty of growth on both media were included in this result category); iii) 78% of the colonies requiring L-cysteine (4189/5344) were indeed *Legionella*, whereas 1155 were not identified by agglutination. These latter were thus sent for PCR testing; iii) of these presumptive *Legionella* colonies, 1041 colonies (90.1%) were confirmed to be *Legionella*, while the remaining 114 (9.9%) were not. These results were used to calculate sensitivity, specificity, false-positive rate, false-negative rate, efficacy and selectivity according to ISO 13843 (Table 4).

The outcomes of our laboratory workflow according to ISO 13843 indicate high sensitivity (100%), specificity (94.1%), and efficiency (98.4%) of the method. There was an excellent agreement between confirmation by subculture and agglutination/PCR (agreement =0.984; Cohen's k= 0.959).

A total of 114 colonies morphologically similar to *Legionella* and dependent on L-cysteine, as they did not grow on BA, could not be confirmed by the serial use of the agglutination and PCR tests. Hence, in a laboratory workflow we would have obtained a 2.1% FPR (114/5344) in the absence of a confirmatory PCR test. These 114 strains were isolated from 109 water samples. Negative PCR test result generated a) 74 samples classified as negative samples on the test report (legionella not detected); b) 35 samples with a final legionella count lower than the count estimate by subculturing confirmation (Table 5).

Estimated costs of *Legionella* control

The PCR-negative samples (n=109) had been collected from 41 health care facilities during 87 routine monitoring procedures. Of these 87 environmental samplings, only 67 had a set of ≥ 5

samples, in compliance with Italian guidelines. Among these 67 samplings, the subculture method detected high concentrations and positivity rates of *Legionella* traceable to the hot water supply of 7 HCFs, which by Italian guidelines, in the absence of a PCR confirmatory test, would have had to carry out water supply disinfection (Fig. 2).

According to the Italian guidelines (Ministero della Salute 2015) for HCF settings, if more than 30% of the samples taken are positive at the *Legionella* concentration between 101-1000 CFU l^{-1} or if more than 20% of the samples taken are positive at the *Legionella* concentration between 1001-10000 CFU l^{-1}, the water system must be resampled at least by the same water outlets which tested positive. If the result is confirmed, a review of the risk assessment should be carried out to identify the necessary further corrective measures such as disinfection. Following the requirements of the Italian guidelines, in this study we identified seven HCFs with putative *Legionella*-positive water systems—these samples were not confirmed to be *Legionella* by PCR.

The estimated costs of *Legionella* disinfection through one hyperchlorination amount to approximately €1,000-1,500 per 100-bed HCF or €1,800 if using monochlorammine. This amount includes the cost of dosing the biocide products in the HCF water system and that related to the personnel in charge of the disinfection. Furthermore, the grand total should include the costs of environmental sampling and analysis following disinfection to verify sanitation efficacy and eventually warn the HCF staff about a potential *Legionella* outbreak. Therefore, we estimated an expenditure of approximately €2,000 for each control and management of water system.

Costs of laboratory testing for *Legionella* confirmation

According to our workflow, three different confirmatory tests, whose cost is reported in Table 6, were performed. For verification through secondary tests, a total of €70,459 euros were spent: 2.5 euros for each negative samples at the first confirmation test, 10.5 euros for confirmation with agglutination, and 19 euros for the colonies identified by PCR. The average cost for confirming suspicious *Legionella* colonies was € 9.8 per colony (€70,459/7,156). Therefore, the subculture

289 expenditure amounted to only €17,890, while the secondary agglutination and PCR tests brought an
290 additional cost of €52,569. However, subculture alone would have led to false positive results,
291 causing unnecessary disinfection procedures for around €2,000 per 100-bed HCF (*i.e.*, €14,000 for 7
292 HCFs with *Legionella* values exceeding the limits established by Italian guideline). Agglutination
293 and PCR provided better detection performances than the simple subculture suggested by ISO
294 11731:2017, even though at a slightly higher cost (around €38,500 more).
295

296 ## Discussion

297 Correct *Legionella* detection and enumeration in water samples is of paramount importance
298 for reliable risk assessment. The plate culture method is the gold standard technique to identify
299 *Legionella* and is normally performed in accordance with ISO 11731:2017, an updated norm that
300 replaces both ISO 11731:1998 and ISO 11731-2:2004. This revised edition was validated by a
301 intralaboratory trial which took place at Vitens laboratory (the Netherlands) and validation results are
302 reported in Annex H of ISO 11731:2017. However, each laboratory following the guidelines of the
303 ISO 11731:2017 standard should carry out a secondary confirmatory test to determine its own
304 performance characteristics.

305 In our laboratory, we were able to generate performance data similar to those obtained in the
306 primary characterization, with the exception of the FPR and FNR (Table 1 *vs* Table 2c). These
307 dissimilarities may be due to a different expertise in differentiating typical from atypical *Legionella*
308 colonies. Indeed, owing to the presence of 71 different species, it is possible to come across
309 *Legionella* colonies with very different morphologies from each other (e.g., *L. pneumophila vs L.*
310 non-*pneumophila* species). According to ISO 13843, the "characterization is an exploratory process
311 with the aim of establishing the likely set of performance characteristics of a new, modified or
312 otherwise inadequately characterized method. It should result in numerical and descriptive
313 specifications for the performance and include a detailed and unambiguous description of the target

of interest (such as positive colony, tube or plaque)". Nevertheless, on a closer look, the description of the colonies of *Legionella* provided by ISO 11731 appears to be quite ambiguous. Indeed, the ISO 11731 norm states that the typical *Legionella* colonies are "white-grey in general but can also appear in other colors. They are smooth with an entire edge and exhibit a characteristic ground-glass appearance", even though "new species of *Legionella* might possess characteristics different from those described above", which is what we routinely check during our laboratory practice. Moreover, it should be taken into account that the isolation procedure used by the Vitens laboratory to differentiate typical from atypical colonies growing on the first inoculated medium was different from ours. In this regard, the Vitens laboratory states that the small differences in the identification process (subculture and PCR) were likely caused by target and non-target colonies that were overgrowing each other. This did not occur in our laboratory because we only worked on the pure growth of colonies subcultured on growth media (BCYE and BA), from which we extracted the DNA for PCR confirmation.

To confirm presumptive colonies of *Legionella* 11731:2017 suggests a comparative analysis of *Legionella* colonies growing on BCYE *vs* BCYE-cys agar. Streaking colonies from one medium type to the other without subsequent techniques, such as PCR, is relatively simple and inexpensive. This saves time and money.

Despite the ISO 11731:2017 instructions, our lab has continued to analyze water samples according to the ISO 11731:1998 guidelines, which recommend to confirm by serology the identity of those colonies which grow on BCYE but not on BCYE-cys agar. Accordingly, we checked all presumptive colonies that were negative on medium without cysteine (blood agar) by performing a secondary confirmation procedure (*i.e.*, latex agglutination and PCR test).

Our analysis of routinely analyzed samples (7156 presumptive colonies of *Legionella*) shows that 114 colonies confirmed to be *Legionella* by subculture were in fact negative after agglutination and PCR testing, which corresponds to a 2.1% FPR. This means that PCR identified fewer positive water samples than those detected by culturing specimens on BCYE agar and BA (5271 *vs* 5344),

besides yielding significantly lower *Legionella* counts in 35 water samples. Therefore, in spite of an excellent agreement between the two confirmation methods (concordance = 0.984; Cohen's K = 0.959), the aforementioned small difference in confirmation results has a negative impact on the overall monitoring outcome. This is in good agreement with Eble et al. (Eble *et al.* 2021), who demonstrated that the subculture method yields a higher proportion of false positive results than those generated by the PCR method. This previous study had shown that a variety of bacterial genera can grow on BCYE-cys (Bradyrhizobium spp. Mycobacterium spp., Acinetobacter). Other colleagues have found that sometimes the growth of *Leifsonia shinshuensis* or *Xanthobacter agilis* in the BCYE may require L-cys, thus leading to a false positive result, which could only be corrected by PCR identification (unpublished data).

Correct detection and enumeration of *Legionella* in water samples is crucial to conduct a truthful risk assessment and devise the most appropriate corrective actions on the source of infection (e.g., water systems, cooling towers, dental units etc.). Each national legislation (Conseil National de pilotage des Agences Régionales de Santé. 2010; Ministero della Salute 2015; European Study Group for Legionella Prevention 2017; Centers for Disease Control and Prevention) defines the maximum legal value of CFU l^{-1} of *Legionella* in water samples: values exceeding the limits established by national guidelines/legislation make it mandatory to carry out water system disinfection and repeat *Legionella* analysis after remediation, to demonstrate conformity with the standards required by law. As the aforementioned *Legionella* remediation efforts can be quite expensive, HCF should check that laboratories where legionella analysis are performed use secondary test to adequate identify legionella in water samples. Avoiding unnecessary water system remediation would in fact bring great benefits in terms of time and money savings to HCFs conducting intensive *Legionella* monitoring.

Systemic disinfection of the entire water distribution system may be conducted by thermal eradication (superheat and flush) or with several chemical products, mostly chlorine-based. Chlorine is widely used due to its effectiveness and low cost. The estimated expenditure for a single

366 hyperchlorination amounts to €1,000-1,500 per water system, but some additional costs for
367 environmental sampling, testing following disinfection and possible system breakdown repairing
368 should be also considered for a correct computation. In fact pipe corrosion is a frequently encountered
369 issue when dealing with chlorine-based disinfection systems, any maintenance costs of chlorine-
370 stressed water systems should also be added to the total cost. Finally, for HCFs planning to organize
371 water system disinfection, it is sometimes necessary to install point-of-use filters to prevent HCF-
372 acquired Legionnaires' disease. This filter costs around €40 and must be regularly replaced after 30-
373 60 days depending on the manufacturer.

374 Last but not least, performing unnecessary disinfections may lead to environmental damage
375 due to the discharge of chlorine into the sewage system and consumption of water discharged after
376 chlorination and flushed to rinse the water system.

377 This is the first large-scale study comparing the effectiveness of the ISO 11731:2017
378 subculture method in identifying presumptive *Legionella* colonies to that of a combined approach
379 (*i.e.*, subculture plus agglutination and PCR). The confirmation step based on the failure of *Legionella*
380 presumptive colonies to grow on BCYE-cys agar is error-prone, and our results quantify this error in
381 terms of false positive samples. Even though the ISO 11731:2017 norm discloses this error, also
382 confirmed by the characterization carried out in our laboratory, we propose that any laboratory
383 involved in *Legionella* analysis should declare its own FPR and disclose to the customer the
384 limitations of using a method without proper confirmation. Indeed, the adoption of a protocol not
385 entailing a secondary confirmation step can result in further, indirect costs for the customers (e.g.,
386 unnecessary disinfection of the water systems). Thus, to adopt a strategy aimed at obtaining the best
387 result in terms of diagnostic performance , the identification—by serology and other procedures—of
388 presumptive *Legionella* colonies that grow on BCYE agar but fail to grow on BA, should be routinely
389 implemented in all laboratories conducting *Legionella* monitoring, even though this would mean
390 withstanding higher costs (in our laboratory routine, around € 9.8 per colony altogether).

391 Each laboratory, carrying out the isolation and enumeration of *Legionella* in water samples,
392 in order to overcome the problem of false positive, could decide its own workflow for the
393 confirmation of typical colonies based on the customer's requests: i) culture + agglutination + PCR;
394 ii) culture + PCR; iii) PCR directly from typical colonies - in this case, it would be necessary to use
395 a multiplex PCR that enables to check simultaneously for *Legionella* spp. and *L. pneumophila*.
396 Finding out which particular strain is present in an environment might be at least as important
397 as knowing the legionellae counts (Harrison *et al.* 2009; Ditommaso *et al.* 2014). It is clear that each
398 strain differs in its capacity to cause disease. *L. pneumophila* serogroup 1 is the most common cause
399 of legionellosis, a sporadic and endemic disease that may be acquired from different environmental
400 sources (plumbing system, cooling tower, spa pools, thermal pools, humidifiers, springs and potting
401 mixes) (Borchardt, Helbig and Lück 2008; Chasqueira *et al.* 2009). Identifying *L. pneumophila* sg 1
402 strains may help assess the risk associated with contaminated environmental reservoirs. HCFs
403 colonized by more virulent strains should be advised to increase their case monitoring efforts and
404 implement more effective contamination control strategies. Better characterization of the strains that
405 cause the majority of diseases would allow for more targeted intervention measures (Helbig *et al.*
406 1997; Ditommaso *et al.* 2014).
407 Given the epidemiological frame of *Legionella*, systematic cost-effectiveness analyses are
408 warranted to assess whether benefits from more accurate *Legionella* detection may outweigh
409 remediation costs.
410

411 ## Acknowledgments

412 The authors are grateful to Dr. Carlo Silvestre and D. Fabrizio Bert for providing data relating costs
413 of disinfection procedures of the water systems.

414 ## Ethical approval

415 This article does not contain any studies with human participants or animals by any of the authors.

Conflict of interest

The authors announce no conflict of interest conduct this work.

Funding

This work was supported by University of Turin (DITS_RILO_21_01).

Data availability

The dataset presented in the current study is not publicly available since data contains information regarding health care facility involved in the study; however, it can be available to the editor upon request.

References

Borchardt J, Helbig JH, Lück PC. Occurrence and distribution of sequence types among Legionella pneumophila strains isolated from patients in Germany: common features and differences to other regions of the world. *Eur J Clin Microbiol Infect Dis* 2008;**27**:29–36.

Centers for Disease Control and Prevention. Legionnaires Disease: Laboratory Guidance for Processing Samples | CDC. 2022.

Centers for Disease Control and Prevention. Guidelines for preventing health-care-associated pneumonia, 2003.

Chasqueira MJ, Rodrigues L, Nascimento M *et al.* Sequence-based and monoclonal antibody typing of Legionella pneumophila isolated from patients in Portugal during 1987-2008. *Euro Surveill* 2009;**14**:19271.

Conseil National de pilotage des Agences Régionales de Santé. Circulaire n° DGS/EA4/2010/448 du 21 décembre 2010 relative aux missions des Agences régionales de santé dans la mise en œuvre de l'arrêté du 1er février 2010 relatif à la surveillance des légionelles dans les installations de production, de stockage et de distribution d'eau chaude sanitaire. 2010.

Cunha BA, Burillo A, Bouza E. Legionnaires' disease. *Lancet* 2016;**387**:376–85.

Ditommaso S, Gentile M, Giacomuzzi M *et al.* Recovery of Legionella species from water samples using an internal method based on ISO 11731: suggestions for revision and implementation. *Diagn Microbiol Infect Dis* 2011;**70**:200–6.

Ditommaso S, Giacomuzzi M, Gentile M *et al.* Effective environmental sampling strategies for monitoring Legionella spp contamination in hot water systems. *Am J Infect Control* 2010;**38**:344–9.

Ditommaso S, Giacomuzzi M, Memoli G *et al.* The use of BCYE medium for the detection of Legionella in environmental water samples: an appropriate update to ISO 11731:2017 standard? *Diagn Microbiol Infect Dis* 2022;**102**:115593.

449 Ditommaso S, Giacomuzzi M, Rivera SRA *et al.* Virulence of Legionella pneumophila strains
450 isolated from hospital water system and healthcare-associated Legionnaires' disease in Northern
451 Italy between 2004 and 2009. *BMC Infect Dis* 2014;**14**:483.

452 Eble D, Gehrig V, Schubert-Ullrich P *et al.* Comparison of the culture method with multiplex PCR
453 for the confirmation of Legionella spp. and Legionella pneumophila. *J Appl Microbiol*
454 2021;**131**:2600–9.

455 Edelstein PH. Comparative study of selective media for isolation of Legionella pneumophila from
456 potable water. *J Clin Microbiol* 1982;**16**:697–9.

457 European Study Group for Legionella Prevention. European Technical Guidelines for the
458 Prevention, Control and Investigation, of Infections Caused by Legionella species. 2017.

459 Feeley JC, Gorman GW, Weaver RE *et al.* Primary isolation media for Legionnaires disease
460 bacterium. *J Clin Microbiol* 1978;**8**:320–5.

461 Fox KF, Brown A. Partial Sequence Analysis of the 16S-rRNA of Legionellae: Taxonomic
462 Implications. *Systematic and Applied Microbiology* 1989;**11**:135–9.

463 Harrison TG, Afshar B, Doshi N *et al.* Distribution of Legionella pneumophila serogroups,
464 monoclonal antibody subgroups and DNA sequence types in recent clinical and environmental
465 isolates from England and Wales (2000-2008). *Eur J Clin Microbiol Infect Dis* 2009;**28**:781–91.

466 Helbig JH, Kurtz JB, Pastoris MC *et al.* Antigenic lipopolysaccharide components of Legionella
467 pneumophila recognized by monoclonal antibodies: possibilities and limitations for division of the
468 species into serogroups. *J Clin Microbiol* 1997;**35**:2841–5.

469 International Organization for Standardization. ISO 11731: 1998 Water Quality—Enumeration of
470 Legionella. 1998.

471 International Organization for Standardization. ISO 11731: 2004 Water quality — Detection and
472 enumeration of Legionella — Part 2: Direct membrane filtration method for waters with low
473 bacterial counts. 2004.

474 International Standard Organisation. ISO 11731: 2017 Water Quality—Enumeration of Legionella.
475 2017.

476 International Standard Organization. ISO 12869:2019 Water quality — Detection and quantification
477 of Legionella spp. and/or Legionella pneumophila by concentration and genic amplification by
478 quantitative polymerase chain reaction (qPCR).

479 Maidak BL, Olsen GJ, Larsen N *et al.* The Ribosomal Database Project (RDP). *Nucleic Acids Res*
480 1996;**24**:82–5.

481 Ministero della Salute. Linee guida per la prevenzione ed il controllo della legionellosi. 2015.

482 Miyamoto H, Yamamoto H, Arima K *et al.* Development of a new seminested PCR method for
483 detection of Legionella species and its application to surveillance of legionellae in hospital cooling
484 tower water. *Appl Environ Microbiol* 1997;**63**:2489–94.

485 Public Health England. UK Standards for Microbiology Investigations Identification of Legionella
486 species. 2015.

Vickers RM, Brown A, Garrity GM. Dye-containing buffered charcoal-yeast extract medium for differentiation of members of the family Legionellaceae. *J Clin Microbiol* 1981;**13**:380–2.

Wilson AM, Canter K, Abney SE *et al.* An application for relating Legionella shower water monitoring results to estimated health outcomes. *Water Res* 2022;**221**:118812.

Wilson DA, Reischl U, Hall GS *et al.* Use of partial 16S rRNA gene sequencing for identification of Legionella pneumophila and non-pneumophila Legionella spp. *J Clin Microbiol* 2007;**45**:257–8.

World Health Organization. *Legionella and Prevention of Legionellosis.* WHO Press, 20 Avenue Appia, 1211 Geneva 27, Switzerland., 2007.

Author Contributions: Conceptualization, S.D. and M.G.; methodology, S.D., G.M., and M.G.; data curation, S.D., G.M., M.G. and J.G.; funding acquisition, S.D.; formal analysis, S.D., G.M. and M.G; supervision, S.D. and C.M.Z.; writing original draft, S.D., M.G.; writing review and editing, S.D., M.G., J.G. and C.M.Z. All authors have read and agreed to the published version of the manuscript.

503 **Table 1** Performance characteristics of the intralaboratory trial (Vitens Laboratory).

504

	Confirmation by subculturing on BCYE agar and BCYE-cys	Confirmation by PCR
Sensitivity	99.0%	98.6%
Specificity	95.3%	97.5%
False-positive rate	3.3%	1.8%
False-negative rate	1.4%	2.1%
Selectivity	57.2%	58.1%
Efficiency	97.5%	98.1%

505 Source: ISO 11731:2017 (Table H.1).
506
507

Table 2. Frequency tables of categories identified through different diagnostic procedures and relevant performance measures.

(a) Confirmation by subculture on BCYE agar and BA.

	Presumptive Colonies		
	Typical colonies	***Atypical colonies***	**Total**
Subculture confirmed colonies	325	3	328
Subculture non-confirmed colonies	13	261	274
Total	338	264	602

Sensitivity 99.1%; Specificity 95.3%; FPR 3.9%; FNR 1.1%; Selectivity 54.0%; Efficiency 97.3%.

(b) Confirmation by agglutination test

	Presumptive colonies		
	Typical colonies	***Atypical colonies***	**Total**
Agglutination confirmed colonies	225	0	225
Agglutination non-confirmed colonies	113	264	377
Total	338	264	602

Sensitivity 100%; Specificity 70.0%; FPR 33.4%; FNR n.a.; Selectivity 37.4%; Efficiency 81.2%.

(c) Confirmation by PCR test

	Presumptive colonies		
	Typical colonies	***Atypical colonies***	**Total**
PCR confirmed colonies	325	0	325
PCR non-confirmed colonies	13	264	277
Total	338	264	602

Sensitivity 100%; Specificity 95.3%; FPR 3.9%; FNR n.a; Selectivity 54.0%; Efficiency 97.8%.

517 **Table 3** Performance characteristics in our laboratory verification.

518

	Confirmation by subculturing on BCYE and BA	Confirmation by PCR
Sensitivity	99.1%	100%
Specificity	95.3%	95.3%
False positive rate	3.9%	3.9%
False negative rate	1.1%	0.0%
Selectivity	54.0 %	54.0%
Efficiency	97.3%	97.8%

519

520

521 **Table 4**. Confirmation by culture *vs* PCR

522

	Presumptive Colonies		
	subculture confirmed colonies	***subculture non-confirmed colonies***	**Total**
confirmation by agglutination/PCR positive	**a)** 5230	**b)** 0	5230
confirmation by agglutination/PCR negative	**c)** 114	**d)** 1812*	1926
Total	5344	1812	7156

523 * These colonies were not confirmed by the second confirmatory test but were considered negative in light
524 of the results of BA subculture testing.

525 Sensitivity 100%; Specificity 94.1%; FPR 2.1%; FNR n.a.; Selectivity 73.4%; Efficiency 98.4%.

526 Agreement = 0.984; Cohen's k = 0.959

527

528 **Table 5.** Comparison between *Legionella* counts obtained with subculture and PCR
529 confirmatory testing in 35 samples.
530

	***Legionella* concentration assigned by subculture CFU l^{-1}**	***Legionella* concentration assigned by PCR CFU l^{-1}**
Geometric mean	$1.1 \cdot 10^3$	$4.5 \cdot 10^2$
Median	$1.0 \cdot 10^3$	$3.0 \cdot 10^2$
Interquartile range (Q1 - Q3)	$3.0 \cdot 10^2 – 3.5 \cdot 10^3$	$1.3 \cdot 10^2 – 1.2 \cdot 10^3$
Range (min – max)	$5.0 \cdot 10^1 – 3.3 \cdot 10^4$	$5.0 \cdot 10^1 – 2.5 \cdot 10^4$

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534 **Table 6.** Comparison of two different approaches to *Legionella* investigation (estimated costs
535 expressed in €).
536

Estimated costs of the laboratory investigation (complete identification)				**Estimated costs of the laboratory investigation (confirmation by subculturing), control and management of *Legionella* in HCFs**			
	Unit cost	**N. test**	**Total**		**Unit cost**	**N. test N. HCF**	**Total**
Subculture in BCYE	1,5	10734	10,734	*Subculture in BCYE*	1,5	10734	10734
Subculture on BA	1	7156	7,156	*Subculture on BA*	1	7156	7156
Agglutination	8	5344	42,752	Water system disinfection	2,000	7	14,000
PCR	8.5	1155	9,817				
Total			**70,459**	**Total**			**31,890**

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538

539 **Figure 1**. Laboratory workflow with results obtained during the study.

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541 **Figure 2**. Probable scenario for environmental management of Legionella contamination in HCFs in

542 the absence of confirmatory PCR testing

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