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Rapid Method for Enumeration of Viable *Legionella pneumophila* and Other *Legionella* spp. in Water

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A sensitive and specific method has been developed to enumerate viable *L. pneumophila* and other *Legionella* spp. in water by epifluorescence microscopy in a short period of time (a few hours). This method allows the quantification of *L. pneumophila* or other *Legionella* spp. as well as the discrimination between viable and nonviable *Legionella*. It simultaneously combines the specific detection of *Legionella* cells using antibodies and a bacterial viability marker (ChemChrome V6), the enumeration being achieved by epifluorescence microscopy. The performance of this immunological double-staining (IDS) method was investigated in 38 natural filterable water samples from different aquatic sources, and the viable *Legionella* counts were compared with those obtained by the standard culture method. The recovery rate of the IDS method is similar to, or higher than, that of the conventional culture method. Under our experimental conditions, the limit of detection of the IDS method was <176 *Legionella* cells per liter. The examination of several samples in duplicates for the presence of *L. pneumophila* and other *Legionella* spp. indicated that the IDS method exhibits an excellent intralaboratory reproducibility, better than that of the standard culture method. This immunological approach allows rapid measurements in emergency situations, such as monitoring the efficacy of disinfection shock treatments. Although its field of application is as yet limited to filterable waters, the double-staining method may be an interesting alternative (not equivalent) to the conventional standard culture methods for enumerating viable *Legionella* when rapid detection is required.

Legionella pneumophila, the causative agent of Legionnaires' disease, was first recognized in 1977 following an epidemic of acute pneumonia among veterans of the American Legion in Philadelphia, Pa., and led to the discovery of a new bacterial species and genus (10, 34). Since then, 47 species and more than 60 serogroups have been recognized (7, 21, 24, 29, 43). Although the vast majority of Legionnaires' disease cases are due to *L. pneumophila* (46), 21 other *Legionella* species have been reported as pathogenic in humans (19, 42, 45, 56). Legionnaires' disease is the most severe form of infection, which includes pneumonia, and the fatality rate can approach 50% in immunocompromised patients (59). Over the last few years, the reported incidence of legionellosis has steadily increased. Numerous outbreaks have been documented. One of the worst recorded occurred recently (from November 2003 to January 2004) in the industrial region of Lens in the North of France, resulting in 86 cases of legionellosis and 15 deaths (40).

Outbreaks of Legionnaires' disease have been traced to a wide variety of environmental water sources, including cooling towers, hot tubs, showerheads, whirlpools and spas, and public fountains. These outbreaks have occurred in the home, offices, hotels, hospitals, and cruise ships, among other locations (3, 16, 20, 51, 55). Surveying and monitoring of legionellae in the environment are needed to prevent and control legionellosis, and *Legionella* concentrations in environmental sites may be used as a predictive risk factor (47). When high levels of *Legionella* are detectable in hot water systems, disinfection of water is critical for controlling outbreaks of legionellosis. Disinfection treatments are usually carried out by oxidizing biocides such as chlorine.

The standard culture technique is the most commonly used method for environmental surveillance of *Legionella* (2, 25). This method allows the isolation and the quantification of legionellae from environmental water, but it does have limitations. First, this method requires selective media and prolonged incubation periods (there is an interval of up to 10 days between taking a water sample and getting results). Second, bacterial loss during the concentration stage (centrifugation or filtration) followed by decontamination with heat (50°C for 30 min) or acid (pH 2 for 5 min) leads to a decrease in isolated *Legionella*. Third, the presence of background organisms may interfere with *Legionella* growth, leading to an underestimation of the real number of legionellae present in the sample. Finally, like many other bacteria, legionellae spp. have been detected as noncultivable cells (or PCR-inducing signals) from water samples (22, 23), but their infectivity in these samples has not been demonstrated.

The development of more rapid and sensitive alternative methods for the detection and quantification of viable *Legionella* cells without cultivation is of increasing importance for water monitoring, legionellosis prevention, and reduction in disinfecting treatment costs of water systems. PCR methods appeared as attractive alternatives to the conventional culture

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method for the detection of slow-growing and fastidious bacteria such as *Legionella*. In recent years, different PCR-based methods for the detection and quantification of *Legionella* in water have been described. PCR methodology has been used primarily against the 5S and 16S rRNA genes and against the macrophage infectivity potentiator (*mip*) gene of *L. pneumophila* (6, 13, 28, 30–32, 41, 49, 52, 54, 58). However, all PCR assays lack the ability to discriminate between living and nonliving (noninfectious) *Legionella* cells. Recently, a rapid method based on an immunofluorescence assay combined with detection by solid-phase cytometry (ChemScanRDI detection) has been described (4). This method achieves detection and enumeration of *Legionella pneumophila* in hot water systems within 3 to 4 h. However, like PCR approaches, this method detects viable as well as dead *Legionella* cells, whereas only live or viable bacteria are able to cause infections in human and represent an interest for public health. The development of new and rapid assays that combine both specific detection and viability criteria is essential for monitoring water quality and legionellosis prevention.

A wide array of methods based on the use of fluorescent probes targeting different cellular functions have been described for rapid assessment of microbial viability. Some of them are based on assessment of cell membrane integrity with nucleic acid dye (11) or on the capability of cells to maintain a membrane potential as determined by probe uptake or exclusion (33). Others detected the respiratory activity of bacteria using different tetrazolium salts (8, 26, 48) or the presence of esterase activity and cell membrane integrity using fluorogenic esters (15). Additionally, the capacity of cells to maintain a pH gradient (pH_{in} higher than pH_{out}) may also supply information about viability (12).

The aim of this study was to develop and validate a sensitive and specific method to detect and enumerate viable *Legionella* cells in water by epifluorescence microscopy in a short period of time (a few hours). This method, based on double-staining fluorescent labeling using a bacterial viability marker and specific antibodies, allows simultaneous detection of *L. pneumophila* and other *Legionella* species in water samples as well as the discrimination between viable and nonviable *Legionella* cells. It thus allows the rapid monitoring of the efficiency of disinfection treatments.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study included 14 serogroups of *Legionella pneumophila*, 18 strains of other *Legionella* species, and 15 non-*Legionella* strains (Table 1). All the strains, collected by the Pasteur Institute of Lille (France), were kept in liquid nitrogen. *Legionella* was grown on buffered charcoal yeast extract (BCYE) agar supplemented with L-cysteine and ferric pyrophosphate (OXOID Ltd., Basingstoke, Hampshire, England) (17). Non-*Legionella* strains were grown on LB (Lennox L) agar (Invitrogen, Paisley, Scotland).

Isolation of *Legionella* from natural water samples was performed by culture according to the recommendations of the French Standard method AFNOR-NFT 90-431 (2), which conforms to International Standard method ISO 11731 (24). One-liter samples of water were concentrated by filtration on a 0.4-μm-pore-diameter polycarbonate membrane (Isopore, Millipore, Ireland). After filtration, bacteria collected on the membranes were resuspended in 5 ml of the water to be analyzed by sonication, and 0.1 ml of the suspension was spread on a 90-mm petri dish containing BCYE agar supplemented with vancomycin, polymyxin B, cycloheximide, and glycine (GVPC medium) (OXOID Ltd., Basingstoke, Hampshire, England). Acid and heat treatments for selective inhibition of non-*Legionella* bacteria were performed as described previously (2, 18). Briefly, 2 ml of the suspension was mixed with an equal volume of 0.2 M KCl-HCl buffer (pH 2), and the mixture was allowed to stand at room temperature for 5 min ± 0.5 min before inoculating 0.2 ml of the buffer-treated suspension onto GVPC plates. For heat treatment, 1 ml of the concentrate was treated at 50°C ± 1°C for 30 min ± 1 min before spreading of 0.1 ml onto GVPC plates. The inoculated plates were then incubated for 7 to 10 days at 37°C ± 1°C. Smooth colonies showing a grayish-white or sometimes a grey-blue-purple, yellow, or green color were counted as suspicious legionellae to be confirmed. Up to 5 to 7 colonies of suspected *Legionella* colonies were subcultured onto BCYE agar, BCYE agar without L-cysteine, and blood agar for verification (2, 9). The isolated colonies growing only on BCYE agar were determined to be *Legionella* colonies. Several colonies isolated from each positive sample were used for determining the species and/or serogroups by latex slide agglutination tests with polyclonal antisera against *L. pneumophila* serogroup 1, *L. pneumophila* serogroups 2 to 14, and *Legionella* spp. (OXOID Ltd., Basingstoke, Hampshire, England).

Pure culture of *Legionella* was enumerated by spreading onto BCYE medium (without antibiotics).

TABLE 1. List of *Legionella* and non-*Legionella* strains tested for the specificity of antibodies used for the immunological double-staining method

Strain	Reference no.	Reactivity with antibodies[a]	
		Anti-*L. pneumophila* sg 1-14	Anti-*Legionella* spp.
L. pneumophila sg 1	ATCC 33152	+	−
L. pneumophila sg 2	ATCC 33154	+	−
L. pneumophila sg 3	ATCC 33155	+	−
L. pneumophila sg 4	ATCC 33156	+	−
L. pneumophila sg 5	ATCC 33216	+	−
L. pneumophila sg 6	CIP 103381	+	−
L. pneumophila sg 7	CIP 103382	+	−
L. pneumophila sg 8	CIP 103383	+	−
L. pneumophila sg 9	CIP 103384	+	−
L. pneumophila sg 10	CIP 103385	+	−
L. pneumophila sg 11	CIP 103386	+	−
L. pneumophila sg 12	CIP 103387	+	−
L. pneumophila sg 13	CIP 103388	+	−
L. pneumophila sg 14	NCTC 12174	+	−
L. anisa	CIP 103870T	−	+
L. bozemanii sg 1	ATCC 33217	−	+
L. brunensis	NCTC 12240	−	+
L. cherrii	NCTC 11976	−	−
L. dumoffii sg 1	NCTC 11370	−	+
L. erythra sg 1	NCTC 11977	−	−
L. feeleii sg 1	NCTC 12022	−	+
L. gormanii sg 1	ATCC 33297	−	+
L. hackeliae sg 1	NCTC 11979	−	+
L. jordanis sg 1	ATCC 33623	−	+
L. longbeachae sg 1	ATCC 33462	−	+
L. maceachernii	ATCC 35300	−	+
L. micdadei sg 1	NCTC 33218	−	+
L. oakridgensis sg 1	ATCC 33761	−	+
L. parisiensis sg 1	NCTC 11983	−	+
L. rubrilucens	NCTC 11987	−	−
L. spiritensis	NCTC 11990	−	−
Citrobacter freundii	ATCC 8090	−	−
Enterobacter cloacae	NCTC 13168	−	−
Escherichia coli	NCTC 1677	−	−
Hafnia alvei	CUETM 77/33T	−	−
Klebsiella pneumoniae	CIP 104216	−	−
Klebsiella planticola	ATCC 33531	−	−
Micrococcus luteus	ATCC 9341	−	−
Morganella morganii	NCTC 2818	−	−
Proteus mirabilis	CUETM 85/121T	−	−
Pseudomonas aeruginosa	ATCC 14138	−	−
Pseudomonas fluorescens	CIP 6913T	−	−
Salmonella enterica serovar Typhimurium	CIP 5858	−	−
Serratia marcescens	CIP 104221	−	−
Staphylococcus epidermidis	CIP 6821	−	−
Xanthomonas maltophila	CIP 6077T	−	−

[a] +, positive reaction; −, negative reaction.

Viability staining procedure. Assessment of the viability of *Legionella* cells was analyzed using the ChemChromeV6 (CV6) (Chemunex, Ivry-sur-Seine, France). This marker consists of a nonfluorescent precursor that is internalized and cleaved into a green fluorescent product ($\lambda_{emission}$ = 520 nm) by esterases present in live bacteria. The cells remain fluorescent only if their membranes are intact and the probe is unable to diffuse out. This probe requires both active intracellular enzymes and intact membranes for cells to be counted as viable. Pure cultures of *Legionella* were serially diluted in peptone water (bioMérieux). Samples (1 ml) were filtered through sterile, black polyester membranes (CB04; Chemunex, Ivry-sur-Seine, France). Each membrane was incubated at 30°C for 30 min in the dark on an absorbent pad soaked with CV6 labeling solution according to the manufacturer's instructions. After labeling, cells with esterase activity were enumerated with the ChemScanRDI solid-phase cytometer (Chemunex). The ChemScan laser-scanning device has been described elsewhere previously (38). Essentially, the membrane is placed in the instrument and the entire filter surface is scanned within about 3 min. The system allows enumeration and differentiation between labeled microorganisms and autofluorescent particles present in the sample. A visual validation of all the ChemScanRDI results was made by transferring the membrane to an epifluorescence microscope (Nikon Eclipse E600) fitted with a motorized stage. Under the control of the ScanRDI software, each fluorescing particle was examined to confirm that it was a *Legionella* cell stained with CV6. Formaldehyde-fixed heat-killed *Legionella* cells were used as controls. ChemScanRDI results were compared with the number of CFU detected by culture.

Immunofluorescence labeling techniques. Two commercial polyclonal antibodies (m-TECH, Inc.) were used in this study: an anti-*Legionella pneumophila* (serogroups [sg] 1 to 14) antibody and an anti-*Legionella* species antibody which recognizes 15 species and 19 serogroups of *Legionella* other than *L. pneumophila* associated with diseases (*L. bozemanii* sg 1 and sg 2, *L. dumoffi* sg 1, *L. gormanii* sg 1, *L. micdadei* sg 1, *L. longbeachae* sg 1 and sg 2, *L. jordanis* sg 1, *L. oakridgensis* sg 1, *L. wadsworthii* sg 1, *L. feeleii* sg 1 and sg 2, *L. sainthelensi* sg 1, *L. anisa* sg 1, *L. santicrucis* sg 1, *L. hackeliae* sg 1 and sg 2, *L. maceacherni* sg 1, and *L. parisiensis* sg 1).

The specificity of anti-*Legionella* antibodies was verified by a direct fluorescent antibody test against 18 species of *Legionella*, 14 serogroups of *L. pneumophila*, and 15 non-*Legionella* strains that share the same ecological niche as *Legionella* or are phylogenetically close to the *Legionella* genus (Table 1). Briefly, colonies were dispersed in 0.37% formaldehyde (Sigma-Aldrich, Steinheim, Germany) for 15 min. A drop of each suspension was then placed on multiwell slides, allowed to dry at room temperature, and then heat fixed. Wells were overlaid with the respective fluorescein isothiocyanate-labeled antibody and incubated in a moist chamber at 37°C for 1 h. Unbound antibody was removed by soaking for 10 minutes in phosphate-buffered saline (PBS), pH 7.4, and air dried. Slides were mounted with 50% glycerol in PBS prior to examination by epifluorescence microscopy.

IDS method. To optimize the labeling protocol, pure cultures of *L. pneumophila* sg 1 or of *Legionella* species other than *L. pneumophila* were diluted in peptone water (bioMérieux) in order to obtain about 500 to 1,000 bacteria per membrane after filtration. For natural water samples, different volumes (50 to 100 ml) of water were filtered depending on the contents of particles in suspension naturally present in the samples. Particles can fill in membranes, making the further visualization of stained bacteria difficult and increasing background noise. *L. pneumophila* cells were enumerated by the immunological double-staining (IDS) method using the anti-*L. pneumophila* (serogroups 1 to 14) antibody, whereas the preparation of a cocktail of two antibodies (anti-*Legionella* species and anti-*L. pneumophila* serogroups 1 to 14 antibodies) was necessary for the enumeration of *Legionella*.

Samples were filtered through sterile black polyester membranes (CB04; Chemunex, Ivry-sur-Seine, France) under vacuum, retaining all the bacteria. The membranes were treated with 100 μl of biotin-labeled antibodies diluted in 1% (wt/vol) bovine serum albumin (BSA) (Roche, Mannheim, Germany)–0.1% (vol/vol) Tween 20 (Sigma, St. Louis, Mo.) in PBS (Sigma, St. Louis, Mo.) for 1 h at 37°C. Membranes were then rinsed several times with PBS-Tween-BSA buffer. Streptavidin conjugated to Red670 (Invitrogen SARL, Cergy Pontoise, France) ($\lambda_{emission}$ = 670 nm) diluted in the same washing buffer was applied to the membrane for 30 min at room temperature in order to detect the trapped *Legionella* cells specifically labeled with antibodies. Membranes were rinsed again with PBS-Tween-BSA buffer before viability staining. Staining for bacterial viability was performed using the CV6 protocol established above. The specific fluorescence of stained bacterial cells was observed under an epifluorescent microscope. Viable *Legionella* cells were defined as those bacteria showing both green and red fluorescence. The number of "dead" and "living" *Legionella* cells in each sample was estimated manually from the counts of 100 microscopic fields using a ×50 lens. Enumerations were determined by switching the epifluorescence filters of the microscope. The membrane filter microscope factor (MFMF) was used to calculate *Legionella* concentrations. The MFMF, which depends on the microscope objective lens, was determined by dividing the filter area by the area of the microscope field (determined with a stage micrometer). *Legionella* concentrations were calculated as follows: number of *Legionella* per liter = (average *Legionella* count/microscope field) × (MFMF) × 1,000/volume (ml) filtered. The values were converted to logarithms.

Chlorination treatment. The biocide used for disinfection experiments was chlorine. The chlorine solution was freshly prepared from sodium hypochlorite (9.6°). A volume of 1.1 liter of each natural water sample to be tested was treated with disinfectant (10 mg of free chlorine per liter) for 24 h. Chlorine was neutralized by the addition of sodium thiosulfate (0.01%, final concentration) after treatment. Analyses were performed on these samples for culturable *Legionella* and for total and viable (metabolically active) *Legionella*. One liter of each sample was analyzed by the standard culture method (2) performed as described above, and 100 ml of the same sample was enumerated by IDS method using one or two anti-*Legionella* antibodies. Control experiments of untreated samples were done to determine the initial concentration of culturable and viable *Legionella*. Microscopic enumeration results were obtained from counts of 100 microscopic fields.

RESULTS

In the attempt to detect viable *Legionella* in water samples, we optimized a protocol allowing the detection of *Legionella* cells stained simultaneously with a taxonomic probe (antibodies) and a viability dye (ChemChromeV6). Before establishing a double-staining method, a separate evaluation of the two staining methods was necessary.

Assessment of viability of *Legionella* using ChemChrome V6. Chemchrome V6 was shown to be well adapted to discriminate viable and dead *Legionella* cells. The ability of this fluorogenic ester to assess the viability of *Legionella* cells was first investigated by epifluorescence microscopy. Only esterases of viable bacteria cleave the nonfluorescent substrate, retaining the green fluorescent end product inside the cell. Viable *Legionella* cells were brightly stained with CV6 in contrast to heat-killed *Legionella*, which did not show green fluorescence. To better evaluate the efficiency of the CV6 dye for assessment of viability, samples prepared from pure cultures of different species of *Legionella* were enumerated by cytometry using a laser solid-phase cytometer, ChemScanRDI (Chemunex), and counts were compared with those predicted from culture. Table 2 shows the results obtained from the comparison between CV6-stained cell counts on ChemScanRDI and the counts obtained from BCYE culture counts (CFU counts). For all species of *Legionella* analyzed by solid-phase cytometry, CV6 counts were higher than CFU counts. The ratio of ChemScan counts to culture counts varied from 1.24 to 6.87. Heat-killed *Legionella* cells stained with CV6 were not detectable by solid-phase cytometry.

Specificity of the immunological detection of *Legionella*. For the specific detection of *Legionella*, two commercial polyclonal antibodies were used: an anti-*Legionella pneumophila* serogroups 1 to 14 antibody and an anti-*Legionella* species antibody which recognizes the major *Legionella* species and serogroups other than *L. pneumophila* that are associated with disease. The specificity of the fluorescent antibodies was tested by reactivity to a set of pure cultures of *Legionella* and non-*Legionella* strains with a direct fluorescent antibody test. Fluorescein isothiocyanate-conjugated antibodies were found to be specific for serogroups and species against which they were raised.

TABLE 2. Comparison of viable counts (CV6-stained cells) determined by solid-phase cytometry (ChemScan) and culture counts (BCYE) obtained from independent spiked pure cultures of different *Legionella* species

Strain	No. of $CV6^+$ cells/liter (ChemScan)	No. of CFU/liter (culture)	Ratio of ChemScan/culture
Legionella pneumophila sg 1	4.7×10^5	1.7×10^5	2.76
Legionella gormanii	6.9×10^5	4.5×10^5	1.53
Legionella micdadeii	6.2×10^5	3.1×10^5	2
Legionella anisa	9.5×10^5	2.5×10^5	3.8
Legionella longbeachae	1.1×10^6	1.6×10^5	6.87
Legionella parisiensis	9×10^5	3.2×10^5	2.81
Legionella hackeliae	2.4×10^5	1.5×10^5	1.6
Legionella rubrilucens	6.2×10^5	3.3×10^5	1.87
Legionella pneumophila sg 1	3.9×10^5	5.7×10^4	6.84
Legionella gormanii	1.2×10^6	9.7×10^5	1.24
Legionella micdadeii	3.1×10^5	2.3×10^5	1.34

They were shown not to be reactive with the 15 non-*Legionella* strains tested (Table 1).

Optimization of the IDS protocol on pure cultures of *Legionella*. For double labeling, antibodies were coupled to the fluorochrome Red670. By using selective filter sets, it is possible to distinguish cells labeled for viability from those labeled with antibodies based on the emission spectrum of the two fluorochromes. ChemChrome V6 fluoresces predominantly green and has essentially spectral properties similar to those of fluorescein. Red670-labeled *Legionella* cells are counted based on their red fluorescence. There is no overlap between the emission spectrum of CV6 and that of Red670. Our staining protocol allows the discrimination between viable *Legionella* (green- and red-stained bacteria) from those dead having damaged membranes (red-stained bacteria) (Fig. 1).

Enumeration of viable *Legionella* cells by the IDS method in environmental water samples. It was of interest to test the immunological double-staining method for enumerating *Legionella* spp. in natural water samples as opposed to laboratory-grown pure cultures of *Legionella* cells. In this configuration, the discrimination and the performance of the IDS method could be investigated in the presence of large numbers of other viable bacteria not belonging to the *Legionella* genus.

Natural water samples were analyzed directly using epifluorescence microscopy after the double-labeling procedure. Different background levels were observed depending on the nature of the samples. The presence of particulate material that accumulates during the concentration procedure may sometimes interfere with microscopic analysis.

A total of 38 environmental water samples from different

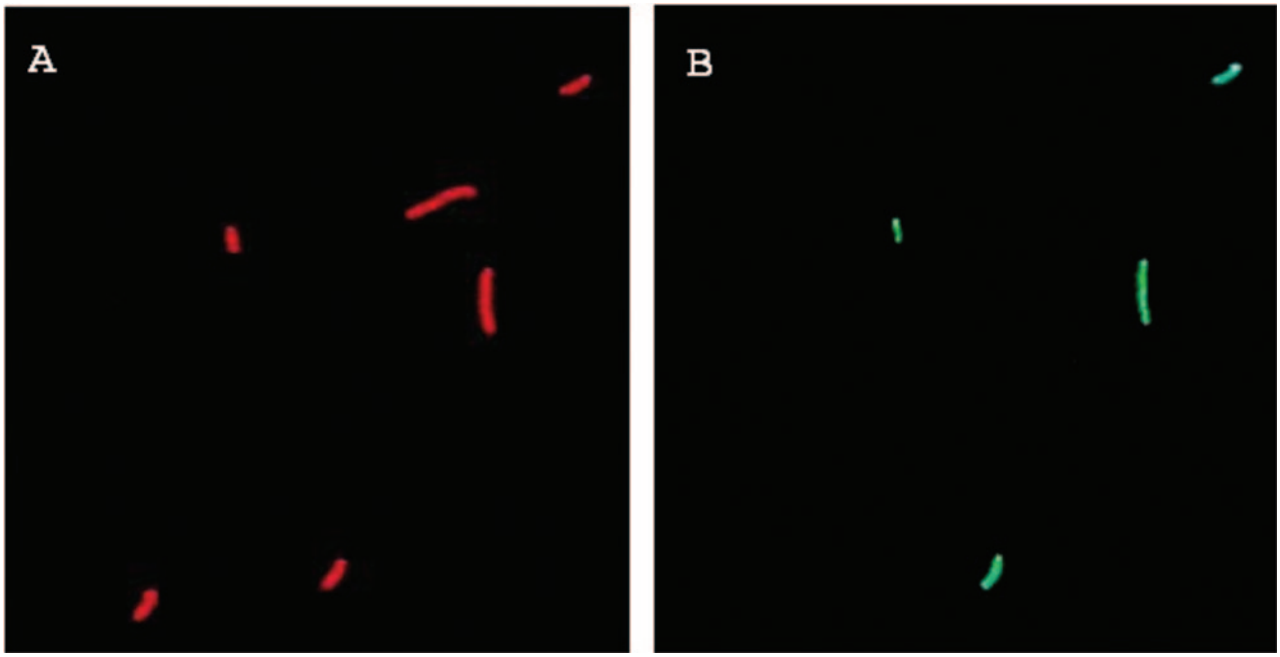

FIG. 1. Epifluorescent micrographs of a *Legionella* population stained by the immunological double-stain method. A and B show the same field visualized using selective filter sets. (A) *Legionella* cells stained with the Red670-coupled anti-*Legionella* antibody. (B) The same field as in panel A showing *Legionella* cells labeled for viability using the CV6 dye. Viable *Legionella* cells are defined as those bacteria showing green and red fluorescence simultaneously.

TABLE 3. Comparison of viable *Legionella* spp. and viable *L. pneumophila* counts in natural water samples obtained by the reference culture method and by the IDS method

Sample	Culture results			IDS results		
	Culture count (log_{10} CFU/liter)		Latex Identification	Antibody	IDS count (log_{10} viable cells/liter)	
	1st replicate	2nd replicate			1st replicate	2nd replicate
1	<1.7	ND[a]	*Legionella*	Lp1-14[b] + Lspp[c]	3.59	ND
2	2	ND	*L. pneumophila*	Lp1-14 + Lspp	4.33	ND
3	<1.7	ND	*Legionella*	Lp1-14 + Lspp	4.5	ND
4	4.88	ND	*Legionella*	Lp1-14 + Lspp	5.04	ND
5	4.88	ND	*Legionella*	Lp1-14 + Lspp	5	ND
6	4.4	ND	*Legionella*	Lp1-14 + Lspp	3.93	ND
7	3.9	ND	*Legionella*	Lp1-14 + Lspp	4.55	ND
8	3.9	ND	*Legionella* spp. and *L. pneumophila*	Lp1-14 + Lspp	4.55	ND
9	5.45	ND	*L. pneumophila*	Lp1-14 + Lspp	5.29	ND
10	<1.7	ND		Lp1-14 + Lspp	3.15	ND
11	<1.7	ND		Lp1-14 + Lspp	4.45	ND
12	1.7	ND	*L. pneumophila*	Lp1-14 + Lspp	3.74	ND
13	4.65	ND	*L. pneumophila*	Lp1-14 + Lspp	4.53	ND
14	4.65	ND	*L. pneumophila*	Lp1-14 + Lspp	4.94	ND
15	4.40	ND	*L. pneumophila*	Lp1-14 + Lspp	4.09	ND
16	4.6	ND	*Legionella*	Lp1-14 + Lspp	4.58	ND
17	<1.7	ND		Lp1-14 + Lspp	<2.38[d]	ND
18	<1.7	ND		Lp1-14 + Lspp	<2.75[d]	ND
19	<1.7	ND		Lp1-14 + Lspp	2.69	ND
20	5.15	4.78	*L. pneumophila*	Lp1-14 + Lspp	5.46	5.52
21	3.24	ND	*Legionella*	Lp1-14 + Lspp	4.12	ND
22	3.84	3.82	*Legionella*	Lp1-14 + Lspp	4.17	4.17
23	<1.7	<1.7		Lp1-14	4.49	4.46
				Lp1-14 + Lspp	4.59	4.59
24	3.04	3.46	*Legionella* spp. and *L. pneumophila*	Lp1-14	4.82	4.79
				Lp1-14 + Lspp	4.91	4.89
25	6.4	5.48	*L. pneumophila*	Lp1-14	5.57	5.6
				Lp1-14 + Lspp	5.74	5.76
26	1.7	2.18	*L. pneumophila*	Lp1-14	4.15	4.14
				Lp1-14 + Lspp	5	ND
27	3.84	3.8	*L. pneumophila* sg 1	Lp1-14	4.53	4.57
				Lp1-14 + Lspp	4.89	4.98
28	4.65	4.6	*L. pneumophila* sg 1	Lp1-14	4.54	4.61
				Lp1-14 + Lspp	5.08	5.1
29	4.40	4.65	*L. pneumophila* sg 1	Lp1-14	5.16	5.15
				Lp1-14 + Lspp	5.31	5.29
30	<1.7	ND		Lp1-14	<2.95[d]	ND
				Lp1-14 + Lspp	<2.95[d]	ND
31	3.55	ND	*L. pneumophila*	Lp1-14	5.47	ND
				Lp1-14 + Lspp	5.61	ND
32	4.40	ND	*L. pneumophila*	Lp1-14	5.07	ND
				Lp1-14 + Lspp	6.06	6.16
33	<1.7	ND		Lp1-14 + Lspp	<2.25[d]	ND
34	<1.7	ND		Lp1-14 + Lspp	<2.25[d]	ND
35	<1.7	ND		Lp1-14 + Lspp	<2.25[d]	ND
36	<1.7	ND		Lp1-14 + Lspp	4.6	ND
37	<1.7	ND		Lp1-14 + Lspp	4.66	ND
38	3.2	ND	*L. pneumophila*	Lp1-14	3.45	ND
				Lp1-14 + Lspp	3.63	ND

[a] ND, not determined.
[b] Lp-1-14, anti-*Legionella pneumophila* serogroups 1 to 14 antibody.
[c] Lssp, anti-*Legionella* species antibody.
[d] The detection limit depends on the number of microscope fields examined and the volume of water filtered.

aquatic sources including water from showerheads, decorative fountains, different hot water systems, and cold and deep well waters were examined for the presence of *L. pneumophila* and/or other *Legionella* spp. by the IDS method. The number of viable *Legionella* cells was counted, and results were compared with those obtained with the traditional culture method (2) (Table 3).

The recovery rate of the IDS procedure was similar to or higher than that with the standard culture method. IDS counts were higher than that based on standard culture in the majority of the samples analyzed. For water samples containing a high concentration of *Legionella* (log_{10} CFU/liter, ≥3), IDS and culture counts were close to the ideal linear relationship. However, for weakly concentrated samples (log_{10} CFU/liter, <3),

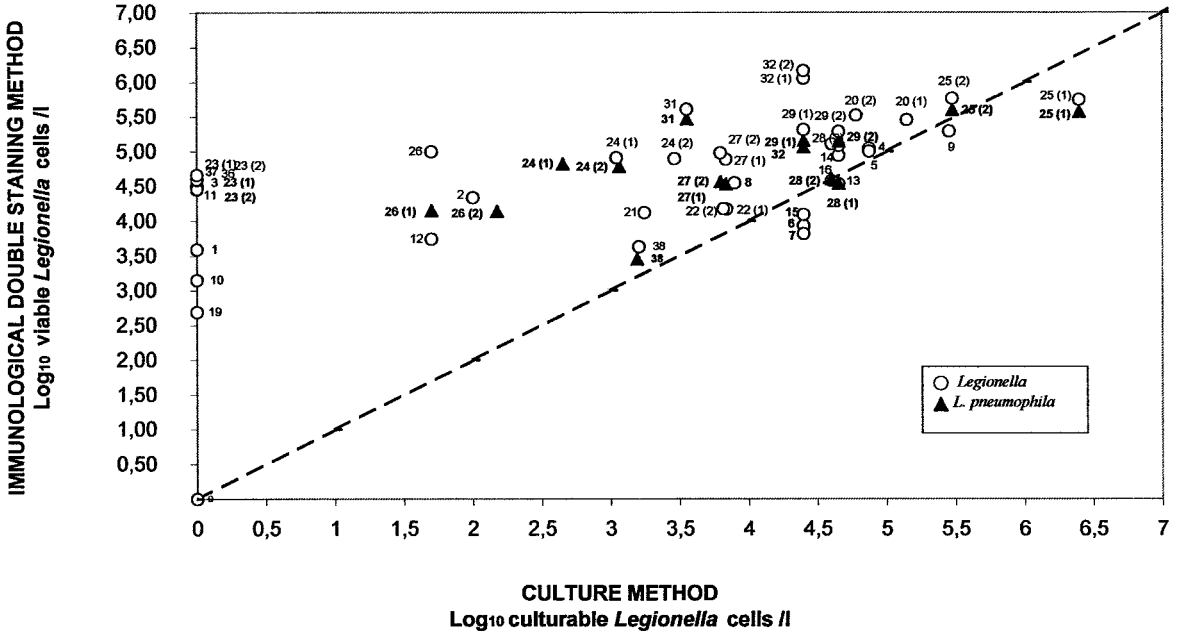

FIG. 2. Graph comparing the logarithms of concentrations of *L. pneumophila* (▲) or *Legionella* spp. (○) per liter in natural water samples determined by IDS method and standard culture method. The straight line represents the theoretical line of equivalence.

no equivalence was found between the two methods (Fig. 2). In fact, for these samples, counts obtained by the IDS method were always higher than those obtained by culture and sometimes exceeded the latter by 2 log or more. Thirty-two samples were positive using the IDS method, and 24 were positive by culture. Samples 17, 18, 30, 33, 34, and 35 were negative by IDS labeling and by culture. These samples correspond to cold domestic waters or well deep waters known to be free of *Legionella*. Eight samples analyzed in singular (samples 1, 3, 10, 11, 19, 36, 37) or in duplicates (sample 23) were positive by the IDS method but negative in culture, whereas the opposite was never found. It is interesting that the sampling temperature of these water samples (excepting samples 1 and 37) exceeded 45°C.

The detection limit of the IDS method is dependent on the volume of water filtered and the number of microscope fields examined. Under our experimental conditions, the detection limit of the IDS method is 176 *Legionella* cells per liter ($\log_{10} = 2.25$) for 100 ml of water analyzed and 100 microscope fields observed. This limit is theoretically reduced to a single *Legionella* cell per filtered volume if the entire membrane is scanned. By comparison, the detection limit of the culture method used in this study is 50 CFU per liter (2). Volumes higher than 100 ml of natural waters are not recommended to be analyzed by the IDS method because of the filtration capacity of the membrane used. Particles present in the natural samples fill in membranes and contribute to increased background noise.

Intralaboratory reproducibility of the IDS method. Ten environmental samples (samples 20, 22 to 29, and 32) were examined for the presence of *L. pneumophila* and/or other *Legionella* spp. in two replicates in order to determine the reproducibility of the IDS method. Each sample was divided into two identical portions, and each portion was analyzed by two different operators using both IDS and culture methods. Table 4 compares *L. pneumophila* and/or other *Legionella* species counts obtained for each replicate with the IDS method and with the standard culture method. Results show that the IDS method exhibits an excellent reproducibility, better than that of the culture method used (Table 4). A maximal difference of 0.1 log was observed between the two replicates of each sample analyzed by the IDS method. Conversely, the difference of two replicates enumerated by the standard culture method can be up to 1 log.

Evaluation of the efficacy of chlorine treatments. In order to determine whether the IDS method could be used to test the efficiency of disinfection shock treatments in emergency situations, six natural water samples were exposed to 10 mg of free chlorine per liter for 24 h. Samples were assayed in singular (sample 32) or in duplicate (samples 24, 25, 27, 28, and 29) in order to determine the effects of this disinfectant on viability (IDS method) and on growth (culture method). Control experiments of untreated samples were done to determine the initial concentration of viable and culturable *Legionella*. After chlorine treatment, surviving *Legionella* cells were enumerated by the IDS method and by the standard culture method (2). Figure 3 compares the results obtained for viable, total (dead plus viable), and culturable *Legionella* concentrations for each sample before and after chlorination using both methods. To determine viable *Legionella* counts, volumes from 5 to 50 ml were analyzed for each sample by the IDS method. In all cases, no viable *Legionella* cells were observed by double staining after 24 h of chlorination. Counts for dead *Legionella* cells were achieved on the basis of their red fluorescence because chlorine inactivation of *Legionella* cells did not destroy their reactivity to antibodies. In contrast to viable counts that

TABLE 4. Comparison of reproducibility of the IDS method with the standard culture method NF T90-431[a]

Sample	*Legionella* spp.								*L. pneumophila*							
	IDS				Culture				IDS				Culture			
	Replicate		Mean	SD (CV)	Replicate		Mean	SD (CV)	Replicate		Mean	SD (CV)	Replicate		Mean	SD (CV)
	I	II			I	II			I	II			I	II		
20	5.46	5.52	5.49	0.04 (0.8)	5.15	4.78	4.96	0.26 (5.3)								
22	4.17	4.17	4.17	0.00	3.84	3.82	3.83	0.01 (0.4)								
23	4.59	4.59	4.59	0.00	<1.70	<1.7			4.49	4.46	4.48	0.02 (0.5)	<1.70	<1.70		
24	4.91	4.89	4.90	0.01 (0.3)	3.04	3.46	3.25	0.3 (9.1)	4.82	4.79	4.81	0.02 (0.4)	3.04	3.46	3.25	0.30 (9.1)
25	5.74	5.76	5.75	0.01 (0.3)	6.40	5.48	5.94	0.65 (11.0)	5.57	5.60	5.59	0.02 (0.4)	6.40	5.48	5.94	0.65 (11.0)
26									4.15	4.14	4.15	0.01 (0.2)	1.70	2.18	1.94	0.34 (17.5)
27	4.89	4.98	4.94	0.06 (1.3)	3.84	3.80	3.82	0.03 (0.7)	4.53	4.57	4.55	0.03 (0.6)	3.84	3.80	3.82	0.03 (0.7)
28	5.08	5.10	5.09	0.01 (0.3)	4.65	4.60	4.62	0.04 (0.8)	4.54	4.61	4.58	0.05 (1.1)	4.65	4.60	4.63	0.04 (0.8)
29	5.31	5.29	5.30	0.01 (0.3)	4.40	4.65	4.52	0.18 (3.9)	5.16	5.15	5.16	0.01 (0.1)	4.40	4.65	4.53	0.18 (3.9)
32	6.06	6.16	6.11	0.07 (1.2)	4.40											

[a] Natural water samples were analyzed in duplicate for the presence of *L. pneumophila* or other *Legionella* spp. Count results are expressed in $\log_{10}$ viable cells/titer for the IDS method and $\log_{10}$ CFU/liter for the culture method. SD, standard deviation. Values in the parentheses represent the coefficient of variation (CV) between two replicates (I and II) expressed in a logarithmic scale.

dropped following the chlorination, the total count (dead plus viable *Legionella* cells) remained constant before and after treatment. Chlorine-treated *Legionella* cells were not detectable by culture. *Legionella* cells quickly lost their capacity to form colonies on BCYE agar plates. Actually, no *Legionella* cells were detected by culture after 5 min of chlorine treatment.

DISCUSSION

We report a sensitive, specific, and rapid (a few hours) method to detect and enumerate viable *L. pneumophila* and other *Legionella* spp. in water using epifluorescence. Our method combines a bacterial viability test and a specific staining using antibodies. Fluorescent stains can be distinguished on the basis of their respective emission wavelengths (green viability stain and red-specific stain). Viable *Legionella* cells are defined as those bacteria showing green and red fluorescence under epifluorescence microscopy.

The performance of the IDS method was investigated in natural filterable water samples. Numbers of viable *Legionella* cells in 38 water samples from different aquatic sources were determined by the IDS method, and results were compared to those obtained with the standard culture method (2). The IDS method appears to be at least as sensitive as this reference culture method. In the majority of water samples analyzed, IDS counts were higher than those obtained by culture. Eight of the 38 samples analyzed were even positive by the IDS method but negative by culture, whereas the contrary was never observed. Aurell et al. (4) obtained similar results using different antibodies directed against *L. pneumophila* and a solid-phase cytometry system. Three of 19 hot water samples tested in their study were shown to be positive by cytometry but negative by culture (ISO 11731 standard method).

Thus, equivalence of the IDS method and the culture method cannot be expected. Cultivability and viability are often considered synonymous terms, but strictly speaking, they measure different properties of the cells. The plate count measures the ability of the bacteria to grow and form a colony on a particular medium. The fluorescent viability probes target different cellular functions and measure the occurrence of metabolism or a particular functional activity in the bacteria. The failure of *Legionella* to form colonies does not necessarily give information about the potential physiological activity of the bacteria. For the samples in which culture method results were negative but IDS method results were positive as well as for those samples in which culture counts were consistently lower than the IDS counts, we may take into account the existence of injured and "viable but nonculturable" (VBNC) cells within stressed populations. *Legionella pneumophila* has been shown to form viable but nonculturable cells which may be responsible for the failure to culture viable *L. pneumophila* from some environmental sources (22, 23, 50). Bacteria exposed to potentially lethal environmental conditions including nutrient restriction, oxidative stress, heat, UV irradiation, osmotic stress, or sublethal concentrations of antibacterial compound undergo physiological or morphological alterations that complicate the detection and accurate enumeration of such stressed bacteria using available culture methods (26, 35). However, these VBNC forms of *Legionella* may be detected by using a dual-labeling method that combines a viability marker for specific anti-*Legionella* antibodies. In fact, nonculturable forms of *Legionella* obtained during extended starvation retain esterase activity for prolonged periods (P. Delgado-Viscogliosi et al., unpublished results). In this study, water temperature could be a factor leading to stressed *Legionella* populations. Actually, the sampling temperature of most of water samples in which no culturable *Legionella* cells were found but the presence of viable *Legionella* were shown by IDS was equal to or higher than 45°C.

The culture-based method may not detect all the viable *Legionella* cells in water samples. It gives a value for CFU; bacterial doublets or chains are counted as only one unit, and those that cannot grow under the prevailing conditions are not counted. In contrast, our method, which is based on an epifluorescence microscopic observation, can distinguish individual cells in *Legionella* clusters. Furthermore, the methodological problems in the isolation of legionellae from environmental water can also contribute to an underestimation of culturable *Legionella* cells. The use of a selective medium and pretreat-

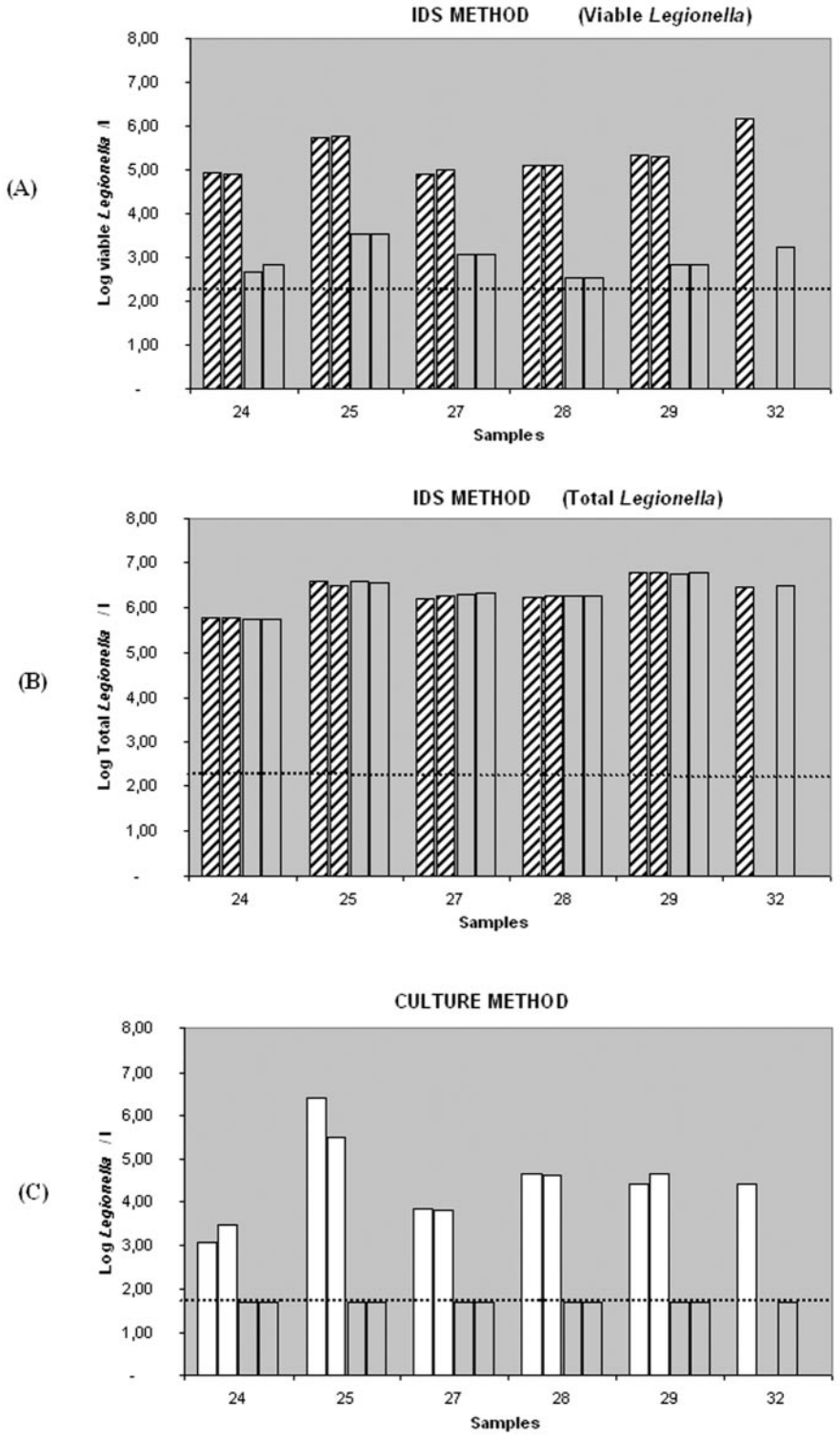

FIG. 3. Evaluation of the efficacy of chlorine treatments by IDS (A and B) and by culture (C). Histograms represent the *Legionella* counts before and after exposure to 10 mg per liter of free chlorine for 24 h in six natural water samples analyzed by IDS method and by culture in a single assay (sample 32) or in duplicate (samples 24, 25, 27, 28, and 29). (A) Results of viable counts determined by the IDS method before chlorination (hatched bars) and after chlorination (transparent bars). (B) Results of total (dead plus viable) counts determined by the IDS method before (hatched bars) and after (transparent bars) chlorination. Note that the total *Legionella* counts remained constant after treatment. (C) Results obtained by the standard culture method before (white bars) and after (transparent bars) chlorine treatment. Dotted lines indicate the limit of detection of each method.

ment by acid or heating reduce the cultivability (58). For all these reasons, it could be concluded that the culture methods underestimate the population of *Legionella*. Nevertheless, the purpose of the analysis is to measure the infection risk, and what should be considered infectious is not clearly known. Colony-forming cells are probably able to colonize a sensitive host, and esterase-negative cells are probably unable to recover, but it is not known if nonculturable, esterase-positive cells are infectious. It will be difficult to test their infectivity because it is impossible to get a population containing only such cells.

It could also be argued that IDS (and a fortiori simple immunostaining methods) as wells as PCR methods overestimate the number of infectious units because they count cells or copies of genes but not propagule. Actually, if several infectious cells are bound in a stable cluster, they will not be able to infect several people. But a multicellular propagule could be more infectious than a single cell and also more resistant to adverse conditions. So, the "true" value for risk evaluation probably lies between the culture and IDS results.

The double-staining approach described in this study allows the detection and enumeration of viable *L. pneumophila* or other *Legionella* spp. in hot water systems within a few hours. Molecular methods have also previously been developed to increase the rapidity of *Legionella* analysis. They are able to achieve a high degree of sensitivity and specificity without the need for a complex cultivation and additional confirmation steps (6, 28, 31, 32, 41, 49, 52, 54, 58). Some of these methods allow the detection of culturable and/or nonculturable *Legionella* within hours instead of the days required with the reference culture method. Recently, a rapid method based on an immunofluorescence assay combined with detection by solid-phase cytometry (ChemScanRDI detection) has been described (4). This method achieves detection and enumeration of *Legionella pneumophila* in hot water systems within 3 to 4 h. Although both approaches measure two different parameters (gene copies and individual cells, respectively), neither of them is able to distinguish between viable and dead *Legionella* cells. Our IDS approach associates the viability criterion to the taxonomic affiliation of detected bacteria. This is one of the main advantages of the IDS method because only alive or viable (maybe culturable) *Legionella* cells cause disease and represent an interest for public health. This method can detect the viable noncultivable forms of *Legionella* which are still metabolically active but unable to form colonies on solid media. It has important public health significance because the VBNC *Legionella* might be a potential source of infection. VBNC *Legionella* forms can be resuscitated by coincubation with amoebae without any loss of virulence (50). Furthermore, VBNC *Legionella* spp. have been shown to be capable of causing pneumonic legionellosis (14), and exposure to high concentrations of them may be an important cause of Pontiac fever (39).

On the other hand, several PCR-based methods (5, 52, 53) as well as the assay described previously by Aurell et al. (4) detect the species *Legionella pneumophila* only. Potentially, any species of *Legionella* may cause the disease. A recent study indicated that *Legionella* species other than *L. pneumophila* may be important in the etiology of community-acquired pneumonia (36) and that their prevalence may have been underestimated due to inadequate diagnostic methods currently in use. Our double-staining approach allows simultaneous detection and discrimination of *L. pneumophila* and other *Legionella* species in water samples depending on the antibody used for analysis.

The detection limit of *Legionella* using the French standard culture method is 50 CFU liter^{-1} ($\log_{10} = 1.7$) (2). Under our experimental conditions, the limit of detection of the IDS method was <176 *Legionella* cells per liter (log = 2.25). This detection limit depends on the number of microscope fields examined and the volume of water filtered.

Legionella spp. appear to represent a health risk to humans when a threshold value estimated at 10^4 to 10^5 CFU liter^{-1} is reached. Epidemiological data show that outbreaks of legionellosis occur at these concentrations (37, 44), and corrective actions such as chlorination must be taken promptly in the case of contaminations. Besides a monitoring of water quality, the IDS method described in this study also allows the evaluation of the efficiency of the disinfection measures as suggested by the chlorination results.

Our approach has two limitations. First, as for any method based on a manual enumeration using a microscope, the IDS assay may cause operator fatigue. It requires trained personnel to identify *Legionella* cells in natural water samples, which are smaller than cultured *Legionella* cells. The automation of the method by the use of solid-phase laser cytometry would ease the enumeration, even if it would not reduce the analysis time by much. Actually, the ChemScanRDI approach also requires a somewhat time-consuming manual confirmation step after laser scanning in order to validate each event selected by the cytometer as a true positive or a false positive. However, it offers the advantage of analyzing the whole membrane and reducing the detection limit of the method. Assays to enumerate double-stained *Legionella* by ChemScanRDI have been conducted in our laboratory. Results indicated that the reading in the red channel still needs some adaptations. Further developments are undertaken to transpose IDS methodology to the ChemScanRDI. Second, the application domain of the IDS method is limited as yet to filterable water. Cooling tower water, frequently cited as a source of infection in outbreaks of Legionnaires' disease (1, 15, 27, 57), cannot be easily analyzed by the IDS procedure due to interferences. The presence of particulate material that accumulates during the concentration procedure interferes with microscopic analysis. Research is under way to apply this method to nonfilterable waters including interfering particles.

In conclusion, the IDS method appears to be suited for rapid surveillance of hot water systems. It is based on an innovative principle combining viability with taxonomic specificity. The main advantage over the standard culture method is its rapidity, which allows results to be obtained within hours versus 10 days for culture. This rapidity may enable better control of water systems because corrective actions in the case of contamination can be taken promptly.

The IDS method may provide the basis for a decision for water system disinfection leading to reduction in treatment costs and enables monitoring of the effectiveness of disinfection treatment. Even if some technical improvements remain to be made, we can conclude that our double-staining approach is an interesting alternative (not equivalent) method to

culture for enumerating viable *Legionella* cells when rapid detection is required, like in emergency situations.

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