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Longitudinal Evaluation of the Efficacy of Heat Treatment Procedures against *Legionella* spp. in Hospital Water Systems by Using a Flow Cytometric Assay[▽]

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***Legionella* spp. are frequently isolated in hospital water systems. Heat shock (30 min at 70°C) is recommended by the World Health Organization to control its multiplication. The aim of the study was to evaluate retrospectively the efficacy of heat treatments by using a flow cytometry assay (FCA) able to identify viable but nonculturable (VBNC) cells. The study included *Legionella* strains (*L. pneumophila* [3 clusters] and *L. anisa* [1 cluster]) isolated from four hot water circuits of different hospital buildings in Saint-Etienne, France, during a 20-year prospective surveillance. The strains recovered from the different circuits were not epidemiologically related, but the strains isolated within a same circuit over time exhibited an identical genotypic profile. After an *in vitro* treatment of 30 min at 70°C, the mean percentage of viable cells and VBNC cells varied from 4.6% to 71.7%. The *in vitro* differences in heat sensitivity were in agreement with the observed efficacy of preventive and corrective heating measures used to control water contamination. These results suggest that *Legionella* strains can become heat resistant after heating treatments for a long time and that flow cytometry could be helpful to check the efficacy of heat treatments on *Legionella* spp. and to optimize the decontamination processes applied to water systems for the control of *Legionella* proliferation.**

Legionella spp. are widespread in natural and human-made aquatic habitats. Approximately one-third of *Legionella* species have been associated with a severe pneumonia in humans, Legionnaires' disease (14, 32). Sources of contamination are aerosols from showerheads, spas, air-cooling towers, or other systems distributing hot water. In order to prevent outbreaks, surveillance of *Legionella* environmental contamination is recommended for hot sanitary water systems of collective settings such as hospitals, hotels, or thermal institutes (4). In France, environmental surveillance of hot water is mandatory for hospitals; to minimize the risk of *Legionella* infection, recommended concentrations of *Legionella pneumophila* must be at least under 1,000 CFU per liter and under the detection threshold (<250 CFU/liter) for immunocompromised patients. A disinfection is required for bacterial loads higher than 10,000 CFU/liter (13, 28). Many disinfection methods have been proposed to control *Legionella* proliferation in hot-water systems, including thermal treatments (delivery of water at 55°C or heat shocks at 70°C) and chemical procedures (continuous or shock chlorination and use of continuous chlorine dioxide, monochloramine, ozone, or aldehydes) (20). However, these disinfection procedures performed with hospital hot water systems (8, 10, 33) often have short-term efficacy, with recolonization occurring after only weeks or months. Indeed, *L. pneumophila* cell populations have been shown to survive as free organisms for long periods by maintaining metabolic activity but temporarily losing culturability under strict environmental conditions and requiring resuscitation by ingestion by amoebas (15, 25, 34). In a previous work using a flow cytometric assay (FCA) (2), we confirmed the existence of viable but not culturable (VBNC) *Legionella* cells (5, 26) in environmental samples.

The aim of this study was to evaluate the added value of FCA for estimating the efficacy of heat treatment procedures used for *Legionella* disinfection in water systems using strains collected prospectively during the environmental surveillance of the water circuits of a university hospital. For each collected strain, FCA profiles were obtained before and after different times of heat shock at 70°C. The results were compared to the decontamination procedures applied to the water circuits from which the strains had been recovered.

MATERIALS AND METHODS

Setting. The University Hospital of Saint-Etienne is composed of several buildings located on two sites and supplied with hot water through independent circuits. From 1992 to 1994, a nosocomial legionellosis outbreak (6, 17) occurred due to water circuit contamination. At that time, there was no mandatory environmental surveillance of hospital hot water systems and no disinfection procedures. In 1995, an environmental surveillance procedure for the whole water system was set up. Sites and frequency of hot water sampling were defined for each building by the staff of the infection control unit and engineers, according to the complexity of the water system and the potential exposure of patients at risk. Hot water samples were collected from showers or hot tap water. The three water circuits of the University Hospital were designated circuits A to C. In 2005, a new hospital located on the same site was opened; its water circuit was designated circuit D. The pipes were made of copper for circuits A to C and mainly of cross-linked polyethylene and polyvinyl chloride for circuit D.

***Legionella* strain recovery.** Thirty-nine *Legionella* strains isolated from 1992 to 2010 and stored at −80°C in Cryobank tubes (Mast Diagnostic, Amiens, France) were used for this study. They were isolated and identified according to French

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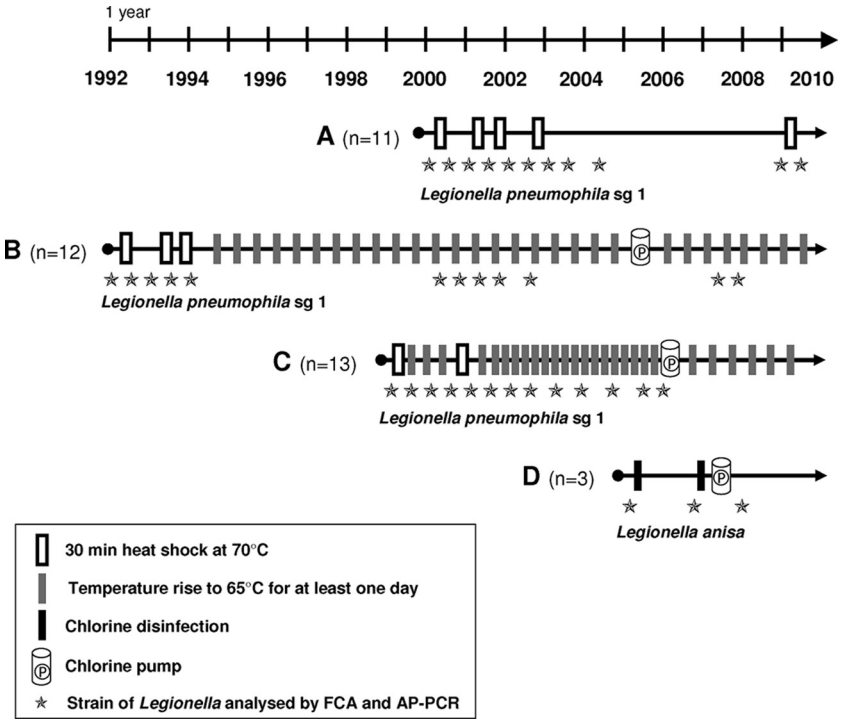

FIG. 1. Chronology of physical treatments applied to four hospital water circuits chronically contaminated with *Legionella*. Rectangles correspond to the different decontamination measures described. Cylinder figures indicate the initiation of a continuous treatment using a chlorine pump. The *Legionella* strains used in the study are represented by stars positioned at the time of their isolation.

Association Française de Normalisation (AFNOR) standard NF T90-431 (3, 19). *Legionella* colonies from frozen samples were recovered by plating onto BCYEα agar medium (buffered activated charcoal yeast extract medium, including *Legionella* charcoal yeast extract agar base and SR0110 supplement; Oxoid, Dardilly, France) and after incubation for 3 days at 36°C ± 2°C before the experiment.

AP-PCR typing. The genomic diversities of the *L. pneumophila* serogroup 1 strains and of *Legionella anisa* were analyzed by arbitrarily primed PCR (AP-PCR) using primer Eric 2 (5′-AAGTAAGTGACTGGGGTGAGC-3′) and primer G (5′-GGTGGTGGCT-3′), as previously described (17).

Preparation of calibrated suspensions of *Legionella* colonies. All suspensions were prepared by suspending *Legionella* colonies into sterile normal saline (0.9% NaCl) to achieve an optical density of 0.2 at 600 nm (Biomate TM3; Avantec, Illkirch, France), which corresponds to a final concentration of 10^8 CFU/ml.

***In vitro* heat shock treatments.** Calibrated suspensions of strains collected were diluted into sterile normal saline to 10^6 CFU/ml and then incubated in a water bath set at 70°C for 0, 10, 30, and 60 min.

Flow cytometric assay. For the FCA, each strain was used at a concentration of 10^5 CFU/ml. FCA profiles were obtained by using a combination of two fluorescent dyes staining nucleic acids, Syto9 for cells with intact membranes and propidium iodide (PI) for cells with damaged membranes, as previously described (2). Flow cytometric measurements were performed by using a BD FACSCalibur instrument (Becton Dickinson Biosciences, Le Pont-de-Claix, France) equipped with an air-cooled argon laser (488-nm emission; 20 mW). The green fluorescent emission from Syto9 was collected in the fluorescence 1 (FL1) channel (500 to 5600 nm), and the red fluorescence from PI was collected in the FL3 channel (>670 nm). A threshold was applied onto the FL1 channel to eliminate background signals. Analyses were performed at a low-flow-rate setting. Results were analyzed with Cell Quest Pro software (Becton Dickinson Biosciences) as previously described (2).

RESULTS

Presentation of the different water circuits contaminated by *Legionella*. Figure 1 illustrates the follow-up of the *Legionella* contamination of the four water circuits taken into consideration in this study and the main control measures that were applied through time. Circuits A to C were found to be contaminated with *Legionella pneumophila* serogroup 1, whereas circuit D was contaminated with *L. anisa*. Circuit A illustrates the efficacy of punctual heat shock treatments leading to the drastic reduction of the *Legionella* load over time. In contrast, circuit C illustrates the inability of a rise of the temperature to control the *Legionella* reservoir, leading to the setup of a chlorine pump in 2006. In comparison to the two above-described circuits, circuit B represents an intermediate situation, with an initial control of *Legionella* growth by a combination of heat shock and temperature rise measures, followed by a resurgence of *Legionella* contamination after 2000, leading in 2005 to the setup of a chlorine pump. Circuit D, contaminated by *L. anisa*, was treated directly by chlorine disinfection because the pipe material, including polyvinyl chloride, did not support high temperatures.

AP-PCR and FCA patterns of *Legionella* strains. *Legionella* strains isolated from the four water circuits described above and illustrated by stars in Fig. 1 were used to study their temperature sensitivity through time by using FCA. First, in order to verify their clonal character, they were all tested by AP-PCR: strains from the same water circuit were shown to share the same profile, whereas each circuit was contaminated by a different clone (Fig. 2). These results demonstrate the persistence over time of the same *Legionella* strain in each independent circuit, even for a long period of time (at least 16 years for circuit B). For each strain displayed in Fig. 1, the respective percentages of viable and culturable (VC), viable but not culturable (VBNC), and dead (D) cells were then evaluated by FCA. The cytograms of representative strains are

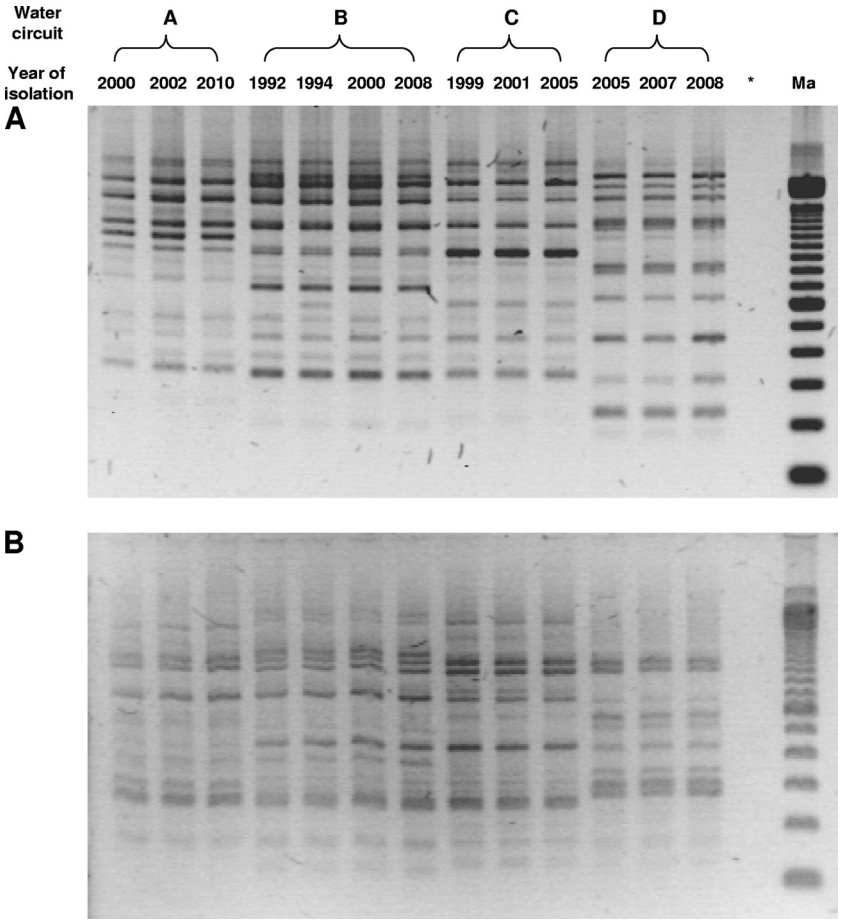

FIG. 2. Representative AP-PCR profiles of strains of *Legionella* isolated from four different water circuits over an 18-year period with primers G (A) and Eric 2 (B). Ma, size marker; ∗, negative control.

displayed in Fig. 3. Interestingly, as shown for the AP-PCR profiles, the distributions among the three cell categories (VC, VBNC, and D) were very similar between strains isolated over time from the same circuit but different from one circuit to another.

FCA analysis of *Legionella* susceptibility to temperature. In order to analyze the temperature susceptibility of the *Legionella* strains shown in Fig. 1, each of them was submitted to a heat treatment at 70°C during 0, 10, 30, and 60 min. The respective percentages of VC, VBNC, and D cells were then determined by FCA. Figure 4 (individual patterns of each strain after 30 min at 70°C) and Fig. 5 (mean kinetics of inactivation at 70°C through time) illustrate the temperature susceptibility of the *L. pneumophila* strains isolated from circuits A to C. Cytometric analyses clearly discriminate two main profiles, a profile of high susceptibility to heating (more than 75% dead cells at 30 min), represented by all strains of circuit A and the first 5 strains of circuit B (cluster B1), and a profile of resistance to heating (less than 50% dead cells at 30 min), represented by all strains of circuit C and the 7 last strains of circuit B (cluster B2). It is worthwhile to note that, despite identical AP-PCR patterns (Fig. 2) and similar FCA profiles before heating (Fig. 3) over the entire study period, the reservoir of *L. pneumophila* from circuit B, which was submitted regularly to heating treatment, evolved from susceptible to resistant to heating over time. As shown in Fig. 4, the change occurred between 1994 and 2000; as no strain was kept frozen in the meantime, it was not possible to determine more precisely the moment at which this evolution took place. Table 1 synthesizes the pooled results for the heat susceptibilities of strains from each circuit before and after treatment at 70°C. As determined by FCA, the mean percentage of viable cells after 30 min at 70°C discriminates the two profiles well and is close to that obtained for viable cells after a heating time of 60 min. *L. anisa* strains recovered from circuit D exhibited a highly susceptible profile to heating (Table 1).

DISCUSSION

This study was conducted on water circuits from two French hospitals that were found to be chronically contaminated with *Legionella* species, from 5 years for circuit D to at least 18 years for circuit B (Fig. 1). The environmental surveillance was started following the occurrence of a nosocomial outbreak (6, 17), a few years before it became recommended by French legislation. The main hygienic measures taken to circumvent the contamination are depicted in Fig. 1. No further cases of hospital-acquired legionellosis have been recorded for a period

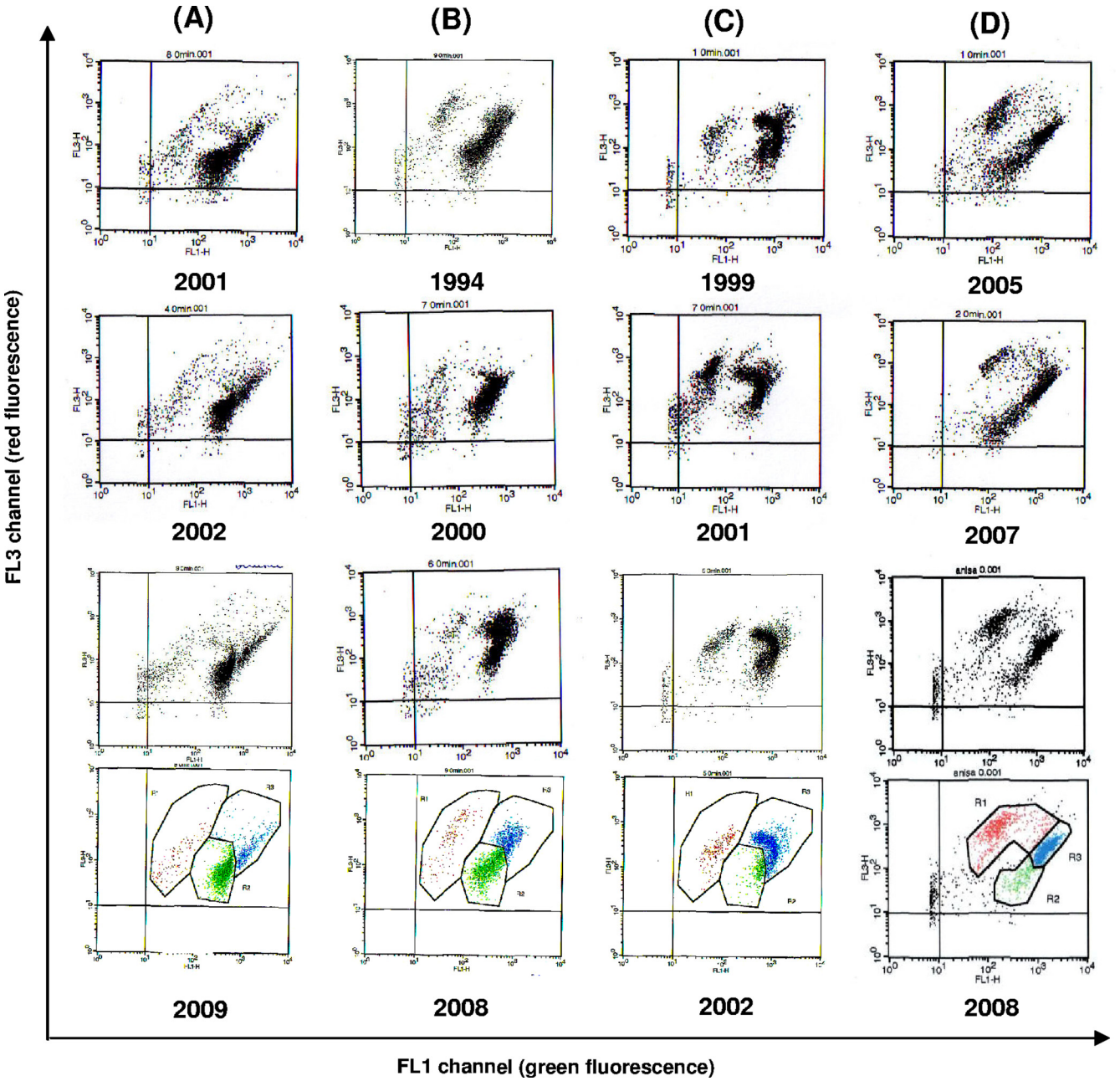

FIG. 3. Representative flow cytometry patterns of *Legionella* strains from circuits A, B, C (*L. pneumophila* sg1), and D (*L. anisa*) after 3 days of culture on BCYE medium. The number under each panel represents the year of isolation of the corresponding strain. Windows allowing determinations of viable (green points), VBNC (blue points), and dead (red points) cell percentages are depicted for strains recovered in 2009, 2008, 2002, and 2008 from circuits A, B, C, and D, respectively.

of 18 years in our setting. These results plead for the usefulness of environmental surveillance, as recommended by several European guidelines (7, 9, 13) and in accordance with the results of the Allegheny County Health Department in the United States (1, 31). Convinced of the efficacy of environmental monitoring, we undertook the present study to evaluate FCA as a refined tool to measure *Legionella* susceptibility to heating in the context of our hospital setting.

FCA was first tested to analyze the relative distribution of cells of different viabilities within the same *Legionella* strain population in the absence of any treatment. It was concluded that FCA profiles were relatively similar for different strains contaminating the same circuit but different for strains isolated from distinct circuits (Fig. 3). Interestingly, these results were in accordance with AP-PCR typing data depicted in Fig. 2, using two different primers. Also, the clonal character of *Legionella* strains contaminating the same circuit was shown by previous works (12, 24, 27, 30).

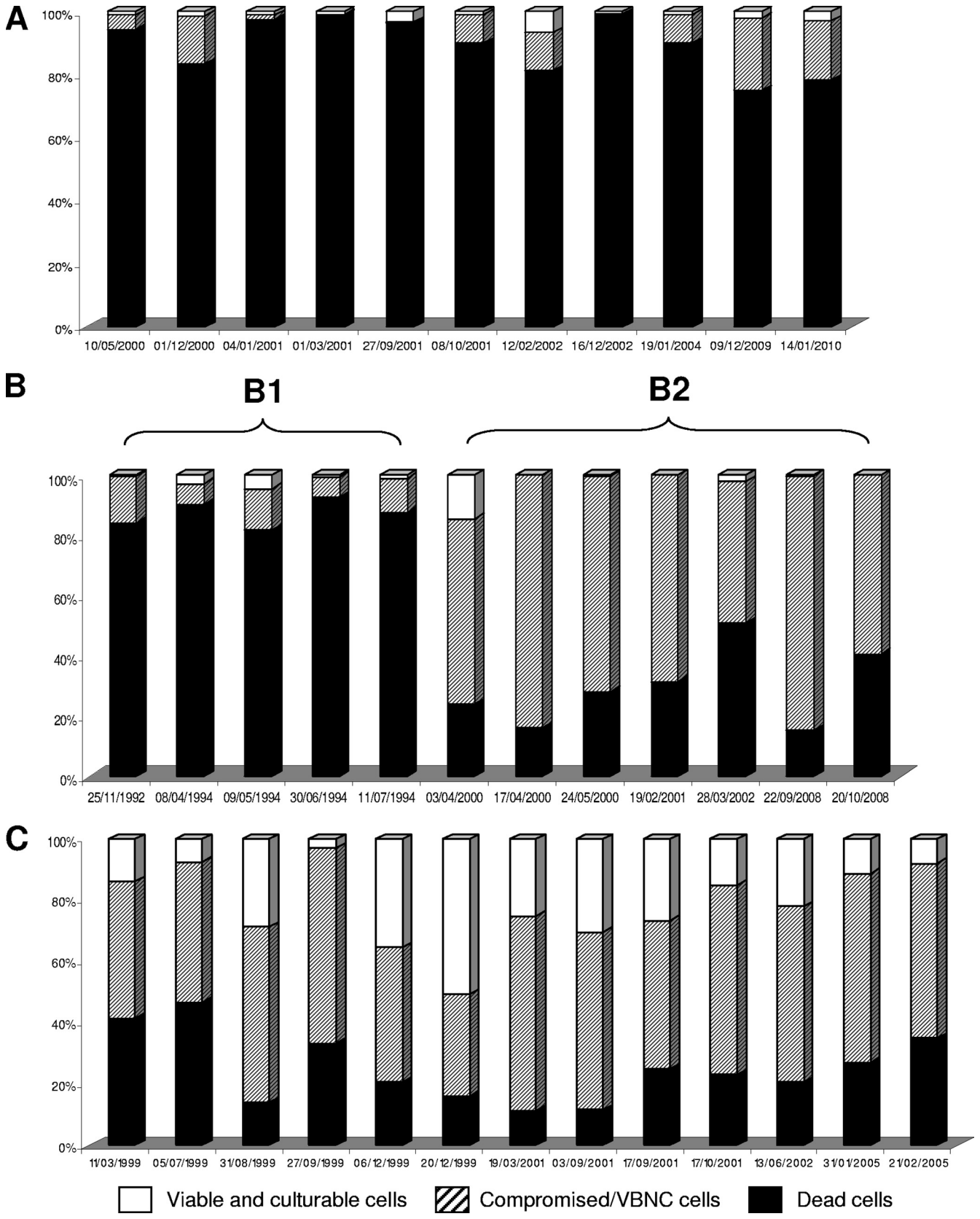

FIG. 4. Resistance of *Legionella* strains to heat shock for 30 min at 70°C. Letters refer to the three water circuits contaminated by strains of *L. pneumophila* sg1. Bars correspond to the different strains presented in Fig. 1. For water circuit B, two successive heat resistance profiles were observed: cluster B1 for strains isolated from 1992 to 1994 and cluster B2 for strains isolated from 2000 to 2008.

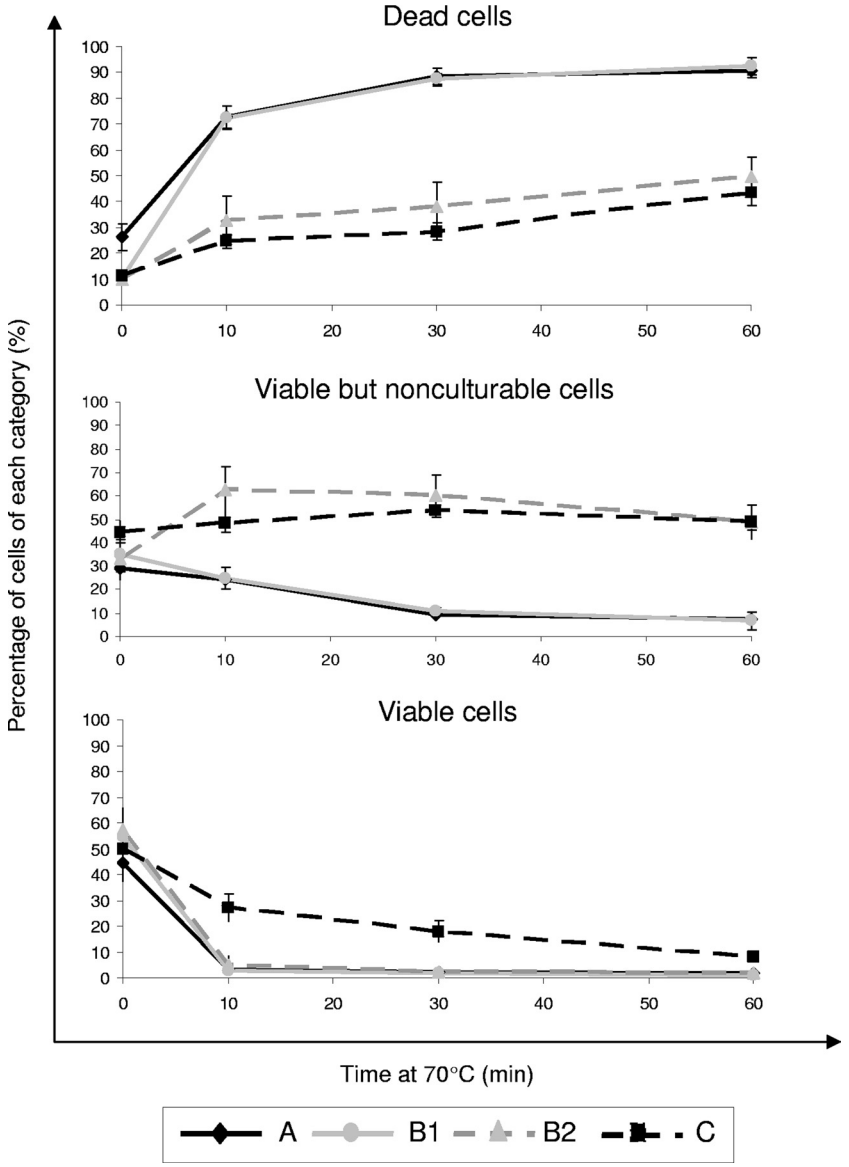

FIG. 5. Viability of *L. pneumophila* sg1 strains obtained by flow cytometry assay after treatment at 70°C for 0, 10, 30, and 60 min. Letters refer to the groups of strains described in the legend of Fig. 4. Results are expressed as means ± standard errors.

The present study documents water circuit contamination by the same strain of *Legionella* for a period of up to 18 years (circuit B).

The main objective of the study was to evaluate the ability of FCA to measure the susceptibility of *Legionella* strains recovered from the environment to heating treatment. The use of FCA was based on the fact that this technique is a performing tool to demonstrate the existence of VBNC in *Legionella* pop-

TABLE 1. Measure of percentages of viable cells (VC and VBNC populations) by flow cytometry assay before and after treatment at 70°C

Group of strains[a] (*Legionella* species)	No. of strains	Mean % of viable cells (range)		
		Before treatment	After 30 min at 70°C	After 60 min at 70°C
A (*L. pneumophila*)	11	73.9 (35.5–92.3)	11.7 (0.8–32.4)	9.1 (0.3–21.6)
B1 (*L. pneumophila*)	5	89.9 (86.3–94.1)	12.7 (7.3–18.4)	7.7 (1.5–20.2)
B2 (*L. pneumophila*)	7	90.2 (80.1–95.0)	70.5 (49.1–84.7)	57.5 (40.5–67.5)
C (*L. pneumophila*)	13	84.4 (42.5–95.1)	71.7 (41.8–88.7)	59.9 (39.4–84.8)
D (*L. anisa*)	3	70.9 (58.8–79.1)	4.6 (1.4–11.8)	2.4 (0.4–5.8)

[a] As defined in the legend of Fig. 4.

ulations (2). By now, increasing the temperature is considered one of the best ways to control the contamination of water circuits by *Legionella* (21, 22, 35) and constitutes the rationale for several guidelines. The minimum temperature for *Legionella* thermal disinfection is 60°C, since the times required to obtain 1-log kill (90% reduction) at 45°C, 50°C, 60°C, and 70°C were 2,500, 380, <5, and < 1 min, respectively (18, 20–22). In contrast, one of the major findings of the present study was that by using FCA, some strains of *Legionella* submitted to superheating in the environment for a long time were shown to develop resistance to high temperatures. This phenomenon was demonstrated by the high proportion of culturable cells and not culturable but viable cells still present after a 30-min treatment at 70°C (Fig. 4 and 5 and Table 1). The percentage of VBNC *Legionella* cells in environmental samples is likely to be associated with variations of biotic or abiotic factors affecting the ecosystem in which *Legionella* agents proliferate (2, 15, 26, 33).

A further factor that fosters the survival and dissemination of *Legionella* in aquatic environments is the biofilm (23, 29). Even if it is not clear whether the pipe material (37) or the type of disinfection influences the development of a biofilm, it was demonstrated previously that the presence of a biofilm reduces the efficacy of disinfection treatments (36). For example, the incorporation of natural noncultivable *L. pneumophila* into potable-water biofilms provides a protective niche against chlorination stress (16). Moreover, if *Legionella* cells are present at a high density, they may communicate in a way that enables them to better survive within a stressful environment (25, 38). In close relation to our study and by using a similar approach, Chang et al. demonstrated recently that starvation enhances significantly the resistance of *Legionella* to superheating or chlorination (11). Those authors postulated that the stabilization of the cell membrane and/or the induction of proteins and other gene products may explain the resistance of starved strains to heat stress (11). Our results obtained by using FCA illustrate for the first time the ability of repetitive heat shocks to generate heat-resistant *Legionella* strains in the environment. The intensity of the heating treatment seems to act as a factor of selection pressure: whereas strains from circuit A or D, submitted to a low frequency of heat shocks (circuit A) or to chlorine treatment due to the material of the pipes (circuit D), were shown to remain susceptible to superheating through time, strains from circuit C, submitted to an intensive program of heating, became rapidly heat resistant. Circuit B offers an intermediate situation, since the same clone, initially highly susceptible to heating, became heat resistant after several years of intensive heat shock procedures (Fig. 1 and 4). Genotypic changes similar to those invoked by Chang et al. (11) may explain this spontaneous evolution. Additional experiments are in progress in our laboratory to document the *in vitro* acquisition of heat resistance by *Legionella* strains submitted to various stresses, including heat shock, chlorine treatment, or starvation in sterile tap water. Further studies should also be undertaken to elucidate the molecular mechanisms involved in the passage from heat susceptibility to heat resistance; actually, genetic analyses comparing clade B1 and B2 strains could help in an understanding of the phenotypic changes mentioned above.

From a practical point of view, this study indicates that FCA could be useful to rapidly evaluate the heat susceptibility of *Legionella* strains in order to optimize the measures used to control their proliferation. Actually, the determination of the proportions of VC and VBNC cells by FCA after a 30-min heating at 70°C (Fig. 4) could help to predict the effectiveness of thermal treatment of a water circuit contaminated by *Legionella*. Many years before the setup of a chlorine pump, this measure could have been a determinant for deciding for which circuits this preventive measure was necessary and the pursuit of repetitive heat shocks was useless. Before FCA may be recommended as an additional tool for the monitoring of the contamination of water circuits by *Legionella*, these observational data need to be confirmed for a larger number of environmental situations.

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