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Use of Flow Cytometry To Monitor *Legionella* Viability[▽]

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***Legionella* viability was monitored during heat shock treatment at 70°C by a flow cytometric assay (FCA). After 30 min of treatment, for 6 of the 12 strains tested, the FCA still detected 10 to 25% of cells that were viable but nonculturable (VBNC). These VBNC cells were able to produce ATP and to be resuscitated after culture on amoebae.**

Legionellae are widespread in natural and manmade aquatic habitats. Sources of contamination are aerosols from showerheads, air-cooling towers, and other systems distributing water. To prevent outbreaks, surveillance of *Legionella* environmental contamination by use of culture methods has been set up for hot sanitary water systems in collective settings such as hospitals, hotels, and thermal spas (4). However, the findings of environmental surveillance do not always correlate with the occurrence of Legionnaires' disease, since the concentration of *Legionella* bacteria in environmental samples is largely underestimated when culture on GVPC medium (8, 11, 12), the reference method required by current norms (1, 16), is used. The existence of viable but nonculturable (VBNC) bacteria (13, 21, 24), which reduce the sensitivity of culture-based assays, has been demonstrated. Analysis of membrane integrity in order to distinguish between viable and dead cells in various bacterial species has been proposed (2, 14, 17, 19); some of these assays are based on double staining combining Syto 9 and propidium iodide (PI) (6, 9, 26). To date, only three studies dealing with the detection of *Legionella* cells by flow cytometry have been reported (15, 27, 29), and none of them analyzed the presence of VBNC cells, which was undertaken in this study.

The flow cytometric assay (FCA) was performed on a BD FACSCanto II flow cytometer (Becton Dickinson Biosciences, Le Pont-de-Claix, France). A threshold was applied on the FL1 channel to eliminate background noise, and analyses were performed at a low flow rate setting. The concentrations of the two dyes were adjusted for the optimal discrimination of green- and red-fluorescing bacteria. One microliter of 2.3 mM Syto 9 and 5 μl of 1-mg/ml PI (Invitrogen SARL, Cergy Pontoise, France) were used for cell staining. A cytogram was generated for each different *Legionella* strain; unstained cells and double staining of sterilized water were used to define the background noise (data not shown).

During a heat shock treatment from 0 to 60 min at 70°C, the concentration of dead cells increased proportionally with the duration of heat exposure. As shown for *Legionella pneumophila* serogroup 1 (sg 1) in Fig. 1, the bacteria could be segregated within three regions: dead cells stained with PI were located in the P1 region (Fig. 1, dot plot at 60 min); bacteria with intact membranes that were stained with Syto 9 were used to delineate the P2 region (Fig. 1, dot plot at 0 min); and a third population, located in the P3 region and appearing as early as 1 min after the beginning of the heat shock, represented an intermediate physiological state that could correspond to cells that were still alive but exhibited compromised membranes.

A total of 38 experiments using 12 clinical or environmental (isolated from contaminated water circuits) *Legionella* strains (*L. pneumophila* sg 1 [$n = 7$]; *L. pneumophila* sg 4, 6, and 13; *Legionella anisa*; and *Legionella micdadei*) were performed to compare flow cytometry and solid culture for cell quantification. The number of bacteria exhibiting intact membranes, as determined by FCA for the P2 region, was correlated with the colony counts determined either on buffered charcoal yeast extract agar (BCYE) medium (correlation coefficient [r]= 0.83; $P < 0.0001$) or on GVPC medium ($r = 0.76$; $P < 0.0001$). In terms of the ability to recover viable cells, FCA was found to be more sensitive than culture on either medium ($P < 0.05$ by Student's t test in both cases).

Figure 2 illustrates the production of VBNC cells during the shift of the P2 population to the P3 intermediate population when a suspension of *L. pneumophila* sg 1 was heated up to 70°C. The VBNC status of the cells was defined by their capacity (i) to produce ATP, as measured by the Profile-1 system (manufactured by New Horizons and purchased from Microbiodetection, Commercy, France), and (ii) to be resuscitated after passage on amoebae (13). In untreated suspensions (Fig. 2A, 0 min), most of the cells exhibiting intense green fluorescence and clustered in the P2 region were found to be culturable, infectious for amoebae, and able to produce high levels of ATP. After a heat shock of 60 min (Fig. 2A), all the cells clustered in the P1 region; no colonies were detected either after direct culture or after infection of amoebae, and no

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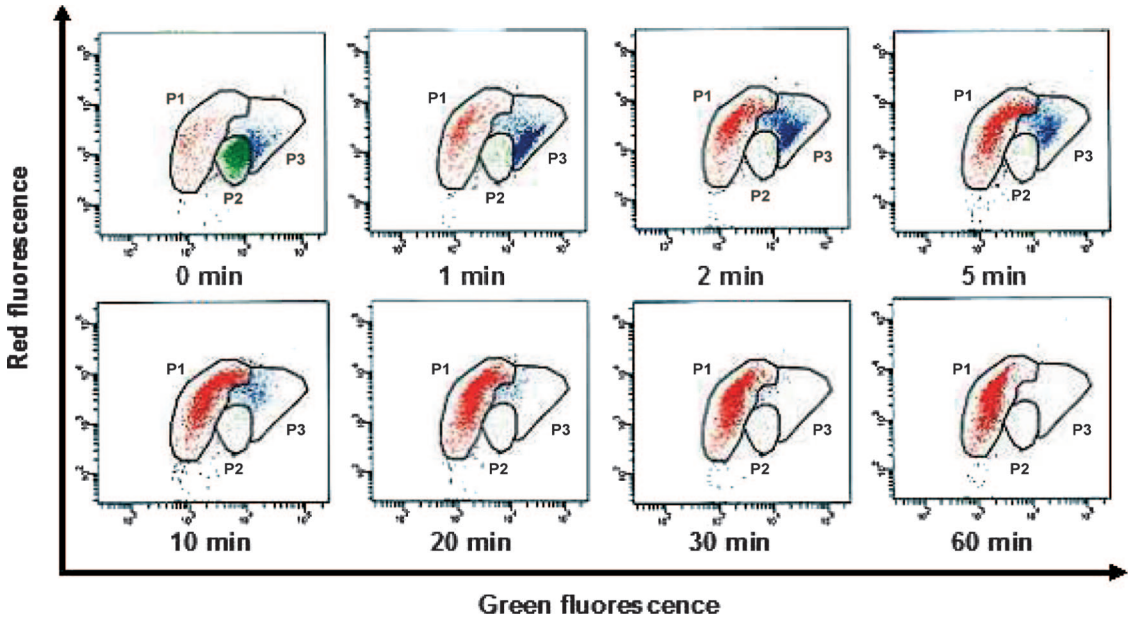

FIG. 1. Kinetic analysis by FCA of *Legionella* extermination after heat shock treatment. A 3-day culture of *L. pneumophila* sg 1 on BCYE with 0.1% α-ketoglutarate was subjected to a heat shock at 70°C from 0 to 60 min. The bacteria were visualized on a dot plot of PI red fluorescence (FL3) versus Syto 9 green fluorescence (FL1). The P1 region corresponds to dead cells. Syto 9-stained bacteria with intact membranes were used to delineate the P2 region (viable, culturable cells). The P3 region (VBNC cells) corresponds to bacteria in an intermediate state with compromised membranes (see the text).

metabolic activity was detected. After a heat shock of 10 min (Fig. 2A), 70% of the bacteria clustered in the P3 region; they were found to be no longer culturable, but some of them were viable, as demonstrated by the detection of metabolic activity and by their ability to be resuscitated after intracellular multiplication in *Acanthamoeba polyphaga* (13). Under various stress conditions, many bacteria are able to enter a state where they are no longer culturable on standard media but still main-

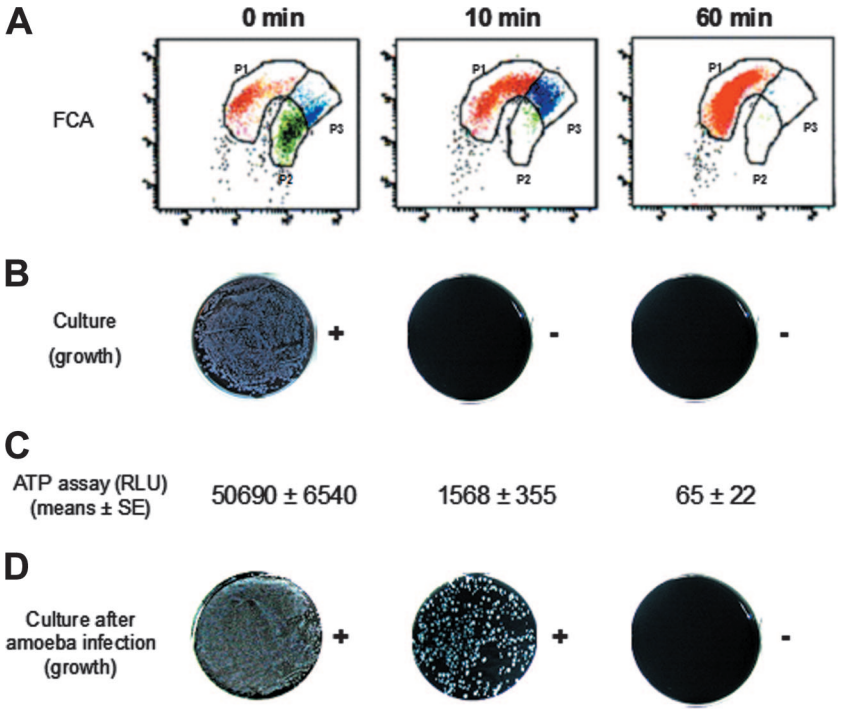

FIG. 2. Evaluation of the characteristics of a suspension of *L. pneumophila* sg 1 PHB before (0 min) and 10 and 60 min after a heat shock treatment at 70°C. Cells were evaluated by an FCA (A), solid culture on BCYE with 0.1% α-ketoglutarate (B), ATP production (C), and solid culture on BCYE with 0.1% α-ketoglutarate after resuscitation on amoebae (D). Representative results (A, B, and D) or means ± standard errors (C) from four independent experiments are shown. RLU, relative light units; SE, standard errors.

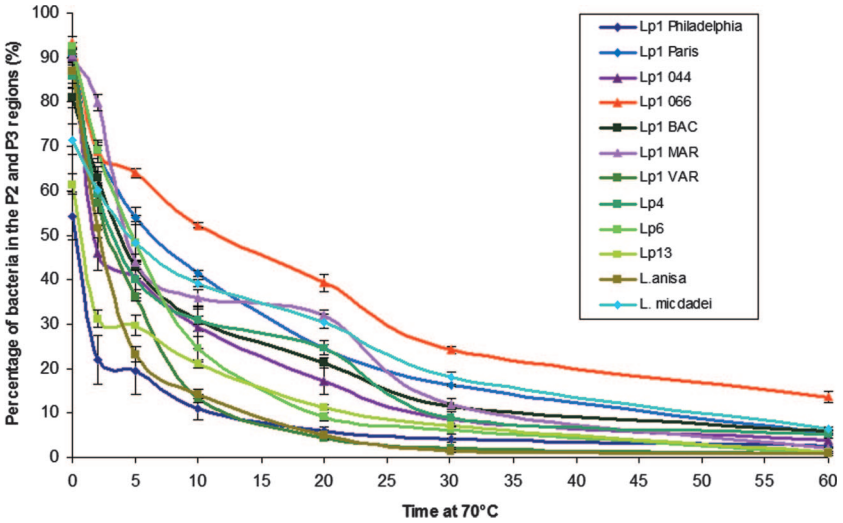

FIG. 3. Resistance patterns of various *Legionella* strains subjected to a heat shock treatment. After a 3-day culture on BCYE with 0.1% α-ketoglutarate, 12 *Legionella* strains were subjected to a heat shock treatment at 70°C from 0 to 60 min, and FCAs were performed. For each strain, the result at each time point shown is the percentage of bacteria detected in both the P2 (viable, culturable cells) and P3 (VBNC cells) regions and is expressed as the mean ± standard error from two to five independent experiments. *Lp*1, *L. pneumophila* sg 1.

tain metabolic activity (3, 5, 7, 18, 24). As previously shown for other bacteria (23), the present results demonstrate for the first time that FCA is a valuable tool for characterizing VBNC cells of the *Legionella* genus.

The 12 *Legionella* strains used in this study exhibited different heat inactivation curves as evaluated by FCA (Fig. 3). *L. pneumophila* sg 1 strain PHB was shown to be the most resistant, with more than 15% of cells exhibiting characteristics of viable bacteria after a heat shock exposure of 1 h at 70°C. At the opposite end, a few strains were totally killed by the heat shock in less than 30 min. Interestingly, for 6 of the 12 strains tested, 10 to 25% of the cells remained viable after a 30-min heat shock at 70°C (Fig. 3), the conditions recommended in France for the decontamination of water systems (10). These results call into question the use of plate culture methods for evaluating the efficacy of preventive or corrective actions against legionellae in the environment and may help to explain the rapid recontamination of some water systems (25, 28), despite the apparently negative results obtained by the reference cell culture protocol (1, 16), after heat shock or chlorination treatments.

In conclusion, flow cytometry opens new perspectives for environmental studies (22) of *Legionella* species. Further work is in progress to evaluate whether FCA could be used in association with an immunomagnetic separation assay for the specific detection of *Legionella* species in complex environmental samples. In combination with cell sorting (20), this assay could also help to better characterize the properties of the VBNC population, notably in terms of virulence.

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