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Research in Microbiology 166 (2015) 215–219

Research in Microbiology

www.elsevier.com/locate/resmic

Brief note

Monitoring of *Legionella pneumophila* viability after chlorine dioxide treatment using flow cytometry

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Received 6 October 2014; accepted 25 January 2015
Available online 26 February 2015

Abstract

The viability of three *Legionella pneumophila* strains was monitored after chlorine dioxide (ClO_2) treatment using a flow cytometric assay. Suspensions of *L. pneumophila* cells were submitted to increasing concentrations of ClO_2. Culturable cells were still detected when using 4 mg/L, but could no longer be detected after exposure to 6 mg/L of ClO_2, although viable but not culturable (VBNC) cells were found after exposure to 4–5 mg/L of ClO_2. When testing whether these VBNC were infective, two of the strains were resuscitated after co-culture with *Acanthamoeba polyphaga,* but neither of them could infect macrophage-like cells.
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Keywords: Legionella; VBNC; Chlorine dioxide; Flow cytometry

1. Introduction

Legionella pneumophila is an intracellular bacterial pathogen commonly present in natural aquatic environments and sanitary water-distributing systems [1]. It is well established that inhaled *L. pneumophila*-contaminated aerosols can lead to severe and fatal pneumonia, also known as legionellosis or Legionnaire's disease, during which the bacterium multiplies intracellularly after invading alveolar macrophages [2].

Chlorine dioxide (ClO_2) has been widely used as an oxidizing agent for disinfecting man-made water systems such as hospitals, spas and cooling towers, due to its physical and chemical properties that resemble those of chlorine [3]. ClO_2 has been used to control *Legionella* in water supplies [4–6]. However, *Legionella* can resist stressful conditions such as high temperature and chlorine disinfection treatments by entering a viable but not culturable state (VBNC) during which it remains potentially virulent [7–9]. Under suitable conditions, mainly inside amoebae which are their natural hosts, VBNC cells of *Legionella* can be resuscitated and consequently recover their cultivability [10,11] and even their infectivity [12].

The aim of this study was to assess *Legionellae* viability (viable and culturable, viable but not culturable and dead cells) after chlorine dioxide treatment using flow cytometry. In addition, the ability of VBNC cells to be resuscitated after either *Acanthamoeba polyphaga* or macrophage-like cell (HL-60 cells) infection was examined.

2. Materials and methods

Three strains of *L. pneumophila* were used throughout this study. *L. pneumophila* 008 (*Lp1-008*), a fluorescent (green

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http://dx.doi.org/10.1016/j.resmic.2015.01.004
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fluorescent protein) strain was obtained from the National Reference Center of *Legionella* (Lyon, France). *L. pneumophila* RNN (*Lp1-RNN*), an environmental strain, and *L. pneumophila* 044 (*Lp1-044*), a clinical strain, were obtained from the Centre Hospitalier Universitaire de Saint Etienne, France. These strains have shown the capacity to generate VBNC forms after heat shock treatment [12,13].

A pure and highly concentrated chlorine dioxide solution of 3500 mg/L was first prepared using the XzioX 1L test kit (Ximax, France) according to the manufacturer's instructions. Chlorine dioxide treatment was performed for 1 h at room temperature at concentrations ranging from 0.1 to 10 mg/L. Treatment was stopped by addition of sterile sodium thiosulfate (0.1 M).

Upon exposure to ClO_2, and in order to confirm the VBNC state, *L. pneumophila* cultivability was assessed on buffered charcoal yeast extract agar (BCYE) media plates. BCYE is a routine bacteriologic medium commonly used for isolation of *L. pneumophila* [14]. Bacteria in the VBNC state fail to grow on BCYE. However, upon resuscitation, they recover their cultivability and can be plate-counted [15].

Flow cytometric assays (FCAs) were performed on a BD FACS Calibur flow cytometer (Becton Dickinson Biosciences) as previously described by Allegra et al. [13]. For *Lp1-RNN* and *Lp1-044* strains, double staining combining Syto 9 (which stains viable cells) and propidium iodide (PI) (that stains cells with compromised membranes, including VBNC and dead cells) was used. For the *Lp1-008* strain, only PI was used for dead cell staining, as this strain produces a GFP. Unstained bacteria and double staining of sterilized water were used as negative controls to define background noise. Infection experiments of ClO_2-treated or untreated *L. pneumophila* cells with *A. polyphaga* or with HL-60 were performed as previously described [12].

3. Results

Viable and culturable cells were systematically detected after treatment using less than 4 mg/L ClO_2. The switch between the culturable and non-culturable state occurred between a concentration of 4 and 5 mg/L of ClO_2. Above these concentrations, no colonies of any of the three strains were enumerated on BCYE culture media (data not shown).

Fig. 1 shows FCA dot plots patterns generated before and after ClO_2 treatment. VBNC cells were detected (in the R3 region). Percentages of VBNC cells differed from one strain to another (Fig. 1). For *Lp1-RNN* and *Lp1-044* strains, only VBNC and dead cell populations remained after treatment with 4 mg/L of ClO_2. For the *Lp1-008* strain, however, greater concentrations of ClO_2 up to 7 mg/L were necessary to generate VBNC cells, whereas 10 mg/L lysed all bacteria (data not shown). Therefore, cells obtained using 6–7 mg/L of ClO_2 were used in order to evaluate their infectivity against amoeba and macrophage-like cells.

None of the ClO_2 –treated strains was able to multiply inside HL-60 cells. The *Lp1-008* strain treated with ClO_2 at 6 and 7 mg/L recovered its cultivability after passage inside *A. polyphaga*. Likewise, the *Lp1-RNN* strain treated with ClO_2 at 4 mg/L also recovered its cultivability after passage inside *A. polyphaga* (Table 1). Whatever the ClO_2 concentration used, the *Lp1-044* strain never recovered its cultivability. Considering that no resuscitation was observed with 6–7 mg/L, *Lp1-044* and *Lp1-RNN* obtained after treatment with higher concentrations of ClO_2 were not tested for the resuscitation assay.

4. Discussion

Consistent with previous reports, our study confirms the importance of the flow cytometric tool in assessing the viability of bacteria based on outer cell membrane integrity [13,16].

ClO_2 was used in an attempt to eradicate *L. pneumophila* in hospital water systems [5]. In almost all of these studies, the concentration of *L. pneumophila* decreased significantly after ClO_2 introduction into both cold and hot water systems using target concentrations of 0.5–0.7 mg/L. Except for a 17-month period study in a hospital water system where ClO_2 treatment effectively removed *Legionella* species from the network [5,17], eradication of *L. pneumophila* was never obtained. Moreover, concentrations of ClO_2 of 0.2–0.6 mg/L at the water outlets resulted in the occurrence of 12 cases of Legionnaires' disease in a hospital (with persistent levels of *L. pneumophila* of 10^3–10^4 CFU/L) [18].

In the United States, ClO_2 is approved by the Environmental Protection Agency for use as a drinking water disinfectant at a maximum level of 0.8 mg/L. The standard dosage, however, is set at 1 mg/L [19]. In Europe as well, ClO_2 can be used in a continuous disinfection process in sanitary water supplies at a concentration of 1 mg/L [20]. Higher concentrations present a risk for consumers (cancer) and for pipe sections (corrosion).

In our study, ClO_2 treatment at levels of 0.1–1 mg/L were not sufficient to eradicate any of the three strains tested (data not shown). Instead, somewhat higher concentrations were needed to achieve complete eradication of the bacteria, for two main reasons. First, the study was conducted *in vitro* using smaller water volumes that were highly concentrated with bacteria. Therefore, our model cannot directly mimic hospital water chain supplies in which ClO_2 is injected at a constant and continuous debit. Second, the aim of this study was to assess the viability of *L. pneumophil*a after ClO_2 shock treatment. This involves the use of higher concentrations of the disinfectant, which also enables complete eradication of *Legionellae*.

Indeed, Makin et al. showed that eradication of culturable *Legionellae* in a water system was achieved using 3–5 mg/L of ClO_2, but a failure in the ClO_2 injection system resulted in re-emergence of *Legionellae* within 4 days [21] This re-emergence was probably due to the presence of VBNC forms of *Legionellae* in the network.

Our study demonstrates the presence of VBNC forms of *L. pneumophila* following ClO_2 shock treatment. Complete

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