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Induction of Viable but Nonculturable (VBNC) state and the effect of multiple subculturing on the survival of *Legionella pneumophila* strains in the presence of monochloramine

Irfan TÜRETGEN*

Istanbul University, Faculty of Science, Department of Biology, 34134 Vezneciler Istanbul, Turkey

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Abstract - The aim of the study was to evaluate the response of two different *Legionella pneumophila* strains with their 20 times subcultured passages, regarding VBNC induction and to test the effect of multiple subculturing on cell vulnerability in the presence of monochloramine. A freshly opened of *L. pneumophila* ATCC 33152, a first subculture of environmental isolate of *L. pneumophila* and the 20th subcultures of both strains were tested for survival in the presence of different doses of monochloramine (24 hours). Besides culture method, live-dead cells were visualised. It was found that multiple subcultured strains were significantly more susceptible to monochloramine and not capable of entering to VBNC state in comparison with freshly opened/isolated strains. Environmental isolate was survived at 2 ppm dose of monochloramine after 24 h, whereas 20th subculture of this strain failed to survive. Multiple subcultured *L. pneumophila* strains were lost their culturability and viability significantly. This phenomenon should be considered while working with laboratory strains. After monochloramine disinfection, *L. pneumophila* bacteria can completely lose their cultivability but do not lose viability, which remains responsible for serious outbreaks worldwide. Even after completely loosing cultivability, it is possible to find live cells in network water in the VBNC state.

Key words: *Legionella pneumophila*, monochloramine, VBNC, multiple subculturing, DAPI-CTC.

INTRODUCTION

Legionella pneumophila has been found in rivers, lakes, hot springs and other bodies of water and soils. It causes a variety of nosocomial, community or travel acquired pulmonary infections, including the Legionnaires' disease, transmitted by inhalation or aspiration of contaminated water (Dennis, 1988; Diederen *et al.,* 2007). In the absence of an adequate disinfectant residual, all water systems are vulnerable to *Legionella* proliferation. Therefore, man-made water systems should be disinfected regularly. Various studies showed that monochloramine could control the bacterial growth efficiently, as well as Legionellae (Kool *et al*., 1999; Turetgen, 2004; Flannery *et al*., 2006; Moore *et al*., 2006; Turetgen and Cotuk, 2006). But, it is known that monochloramine could induce viable but non-culturable (VBNC) bacteria (Alleron *et al*., 2006). *Legionella pneumophila* cells are able to enter a VBNC state, which may account for the fact that *L. pneumophila* often cannot be cultured from the suspected source (Hussong *et al*., 1987; Diederen *et al*., 2007). The VBNC state is defined as a survival mechanism activated by bacteria in response to multiple environmental stresses and allowing microorganisms to conserve their viability despite the loss of their own culturability (Roszak *et al*., 1984). VBNC cells of *L. pneumophila* strains could regain pathogenic potential with reactivation in amoebae and are therefore relevant in the risk assessments of implicated reservoirs (Steinert *et al*., 1997). These VBNC cells are undetectable by standard enumeration methods (Diederen *et al*., 2007); this could lead to an underestimation of the real number of Legionellae present in the sample and an overestimation of the efficacy of disinfectants. The standard culture technique is the most commonly used method for environmental surveillance of genus *Legionella* in aquatic environment (Dennis, 1988). As an alternative counting method, CTC (5-cyano-2,3-ditolyl tetrazolium chloride) redox dye has been introduced for enumeration of active bacteria in water samples. CTC is readily reduced to insoluble, highly fluorescent, and intracellularly accumulated CTC-formazan through bacterial respiration (Rodriguez *et al*., 1992; Søndergaard and Danielsen, 2001).

Standard strains or environmental isolates which were used in laboratories are mainly subcultured too many times. It is known that subculturing could induce phenotypic and genotypic alterations in microorganisms (Fontana *et al*., 1997; Cavalcante *et al*., 2007). Kuchta *et al.* (1985) showed that tap water-maintained *L. pneumophila* is even more resistant to Cl_2 than its already resistant agar medium-passaged counterpart. Because reservoirs of *L. pneumophila* have been implicated in outbreaks of Legionnaires' disease in cooling towers, evaporative condensers, and plumbing systems, the effectiveness of various biocides,

* Corresponding author. Phone: +90 2124555700;
Fax: +90 2125280527; E-mail: turetgen@istanbul.edu.tr

both common and novel, with respect to the prevention of Legionellosis and related respiratory diseases needs to be evaluated (Kool *et al*., 1999; Flannery *et al*., 2006).

The aim of the present study was to evaluate the response of two different *L. pneumophila* strains and with their 20 times subcultured passages, regarding VBNC induction and to test the effect of multiple subculturing on cell vulnerability in the presence of monochloramine.

MATERIALS AND METHODS

Bacterial strains and preparation of disinfectant. Wild *L. pneumophila* serogroup 1 strain was isolated from hot water showerhead, whereas standard strain ATCC 33152 was purchased in lyophilised form. Both strains were subcultured 20 times consecutively with 48 h intervals on BCYE agar media to obtain 20^{th} passage of those strains. *Legionella* bacteria were harvested from plates and carefully suspended in filter-sterilised (0.22 µm pore size) chlorine-free drinking water to determine the cell numbers colorimetrically (McFarland 0.5, approximately 10^8/ml). Then, planktonic cells treated with monochloramine solutions (0.5, 1, 1.5 and 2 ppm doses) for 24 h. The bactericidal action of monochloramine was determined using the qualitative suspension test method by Skaliy *et al.* (1980). Monochloramine stock solution was prepared by mixing 0.11 mg of ammonium chloride with 100 ml of phosphate buffered saline and then slowly adding 1 ml of 5% sodium hypochlorite (commercial bleach) (Donlan *et al*., 2002). Filter-sterilised chlorine-free drinking water used to dilute the stock solution to obtain the desired working concentration. Monochloramine test kit (LaMotte, USA) was used to measure final concentrations. All glassware used was chlorine demand-free.

Epifluorescence microscopy. After exposure to monochloramine, 0.1 ml of samples were spread-plated in triplicate on BCYE agar and incubated at 37 °C for one week and the colonies on the plates were counted by classic culture method. For epifluorescence microscopy, CTC was used in conjunction with DNA-binding DAPI (fluorochrome 4',6-diamidino-2-phenylindole) to differentiate metabolically active cells from inactive cells (Rodriguez *et al*., 1992). Following the manufacturer's instructions, all red cells were considered live while blue cells were considered dead. For live-dead enumeration, triplicate samples of 1 ml were incubated with aliquots of a 50 mM CTC redox dye solution to a final concentration of 5 mM. The samples were incubated in the dark at room temperature for 4 hours. Then, samples were counterstained with 1.0 µg of DAPI per ml. After 30 min, incubations were terminated by vacuum filtration onto black 0.2 µm pore size polycarbonate (Millipore, USA) filters after mixing with filter-sterilised double distilled water (Rodriguez *et al*., 1992; Søndergaard and Danielsen, 2001). The air-dried polycarbonate filters were mounted on a glass microscope slide with paraffin below and above the filter, and with a coverslip on top. A Nikon 80i microscope equipped with 100 W mercury lamp was used with non-fluorescing immersion oil. Microscopical observations were performed with a 0.45 mm dichroic mirror, which allows the simultaneous visualisation of both dyes. The numbers of bacteria were estimated from counts of 20 microscopic fields (at x 1000). An eyepiece with a reticule calibrated was used for all bacterial counting. The number of microorganisms present in a millilitre of sample is calculated by applying the following conversion formula:

$$N = \frac{S \times n}{C \times V} \times D$$

where *N*, number of microorganisms per millilitre; *S*, real area of filtration; *n*, average number of microorganisms per field of vision; *C*, real area of microscopic range; *V*, volume of filtered sample; *D*, sample dilution.

Statistical analysis. Plate counts were $\log_{10}$ transformed and standard errors of the means were calculated. Differences between variables were tested for significance using a t-test; differences were considered significantly different when $P < 0.05$. Statistical analyses were performed using SPSS Version 10.0 program.

RESULTS

After 24 hours of exposure to monochloramine, decrease of cell cultivability significantly began at 1 ppm dosage. Loss of culturability was observed at 1.5 ppm for both strains of their 20^{th} subcultures (Figs. 1 and 2). In the control assay (with no monochloramine) the number of cultivable and viable cells remained constant after 24 hours. No significant decrease of bacterial cells found at 0.5 ppm dose, at 2 ppm dosage the only survivors were come out from the freshly isolated environmental strain. First significant decreases were observed at 1 ppm dose in comparison with initial cell counts. Faster decreases were recorded on strains that were 20 times subcultured. At the beginning (zero time), the viable cell counts were recorded 2 logs higher than cultivable cell counts. This could be explained by the reduced sensitivity of culture method compared to live-dead counting.

Significant decreases for *L. pneumophila* ATCC 33152 were started at 1 ppm. At this dosage, it was remarkable that live count of multiple subcultured standard strain was significantly decreased, in comparison with first cell line of that strain. At 1.5 ppm monochloramine, multiple subcultured standard strain lost its cultivability, as well as multiple subcultured environmental strain, but not viability. At 2 ppm dose of monochloramine, the only survivor was the environmental strain, but not culturable. Standard strain was unable to survive at this dosage of monochloramine.

It is clear that standard strain was significantly more susceptible to monochloramine than new isolated SG1 strain at 1, 1.5 and 2 ppm dosages of monochloramine. The results showed that 20 times subculturing could affect the *L. pneumophila* strains negatively in respect to resistance against monochloramine disinfectant. At certain doses, even in 24 hours, monochloramine induces the *L. pneumophila* cells to enter VBNC state.

DISCUSSION

The VBNC cell is a metabolically active one although cells are incapable of undergoing the sustained cellular division required to form a colony on regular agar media. But, it is still discussed to find the answer, is VBNC phase the way of death or a survival strategy. Furthermore, it was stated

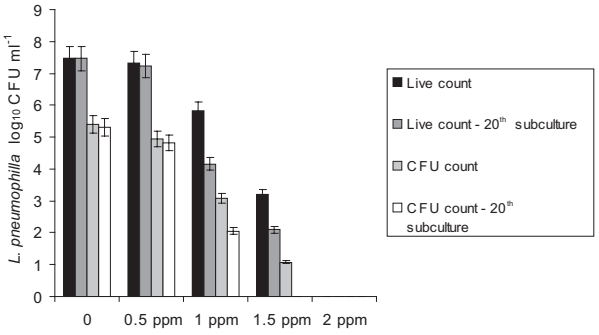

FIG. 1 - Survival rates of *Legionella pneumophila* serogroup 1 standard strain ATCC 33152 in the presence of monochloramine after 24 hours. Error bars represents SD.

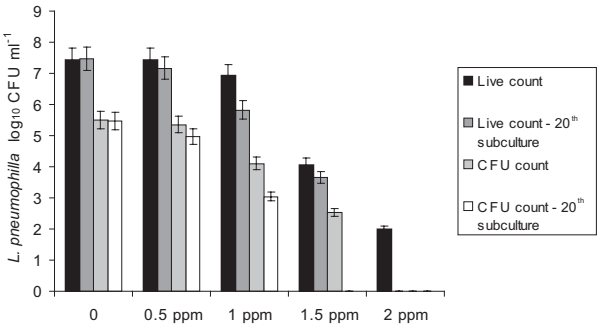

FIG. 2 - Survival rates of *Legionella pneumophila* serogroup 1 environmental isolate in the presence of monochloramine after 24 hours. Error bars represents SD.

that even in 'pure' laboratory cultures, there may be a fraction of cells which are VBNC under conditions normally suited to their cultivation (Bloomfield *et al*., 1998). Like most microorganisms, *L. pneumophila* enter a viable but non-culturable state under adverse conditions. After disinfections, *L. pneumophila* bacteria can completely lose their cultivability but do not lose viability, which remains responsible for serious outbreaks worldwide. Steinert *et al.* (1997) suggested that the VBNC form is a potential source of infection by demonstrating that VBNC *Legionella* cells could be resuscitated by coincubation with amoebae without any loss of virulence. Mcdougald *et al.* (1998) concluded that in the initial stage of VBNC formation, cells lose culturability while maintaining intact membranes and RNA and DNA. And these cells thus maintain the potential for resuscitation. In the later stages of the VBNC state cells gradually experience degradation of nucleic acids and thus lose the potential for resuscitation. Therefore, longer exposures could be tested in future studies. It is also known that disinfectants' *in vitro* activity are less effective in the field. Considering this phenomenon, the biocide dosage and their residuals in real water systems must be evaluated.

Legionella pneumophila grown in a natural environment has been reported to be 6 to 9 times more resistant than cells grown on agar. Kuchta *et al.* (1985) found that after one agar passage their resistance dropped to levels of comparable strains which had not been previously exposed to additional disinfections. Several studies of the effectiveness of biocides for the control of *L. pneumophila* have been published in the past. Some studies were performed using subcultured strains, which could lead the overestimation of the efficacy of disinfecting agent. Our results allow us to consider that in the short term, the culturability of *L. pneumophila* cells were affected to different degrees depending on the number of subculturing. The results demonstrate that even after completely losing cultivability, it is possible to find live cells in drinking water in the VBNC state. Therefore, cultivation-independent techniques, including immunological methods, fluorescent *in situ* hybridisation (FISH), and PCR-based methods are required to detect their presence (Wullings and Kooij, 2006).

Variety of studies has shown that introduction of monochloramine into a municipal water system and cooling towers can reduce *Legionella* colonisation of man-made water systems (Kool *et al*., 1999; Flannery *et al*., 2006; Moore *et al*., 2006; Turetgen and Cotuk, 2006). In public health risk assessments and epidemiological surveys, standard methods should be supplemented by selected growth-independent assay procedures for strategic monitoring of viable but non-culturable microbes. To track down a Legionnaires' disease outbreak, isolation of the responsible bacterium remains essential in order to perform the different typing methods necessary for strain identification and to identify the contamination source. Therefore, using of modern techniques is inevitable to catch the VBNC forms, induced by physical or chemical stressors.

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