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# Resistance of *Legionella* and *Acanthamoeba mauritaniensis* to heat treatment as determined by relative and quantitative polymerase chain reactions

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ABSTRACT

*Legionella* and *Acanthamoeba* spp. persist in harvested rainwater pasteurized at high temperatures (> 72 °C) and the interaction mechanisms exhibited between these organisms need to be elucidated. The resistance of two *Legionella* reference strains (*Legionella pneumophila* ATCC 33152 and *Legionella longbeachae* ATCC 33462), three environmental strains [*Legionella longbeachae* (env.), *Legionella norrlandica* (env.) and *Legionella rowbothamii* (env.)] and *Acanthamoeba mauritaniensis* ATCC 50676 to heat treatment (50–90 °C) was determined by monitoring culturability and viability [ethidium monoazide quantitative polymerase chain reaction (EMA-qPCR)]. The expression of metabolic and virulence genes of *L. pneumophila* ATCC 33152 (*lolA, sidF, csrA*) and *L. longbeachae* (env.) (*lolA*) in co-culture with *A. mauritaniensis* ATCC 50676 during heat treatment (50–90 °C) was monitored using relative qPCR. While the culturability (CFU/mL) and viability (gene copies/mL) of the *Legionella* strains reduced significantly ($p < 0.05$) following heat treatment (60–90 °C), *L. longbeachae* (env.) and *L. pneumophila* ATCC 33152 were culturable following heat treatment at 50–60 °C. Metabolically active trophozoites and dormant cysts of *A. mauritaniensis* ATCC 50676 were detected at 50 °C and 60–90 °C, respectively. For *L. pneumophila* ATCC 33152, *lolA* expression remained constant, *sidF* expression increased and the expression of *csrA* decreased during co-culture with *A. mauritaniensis* ATCC 50676. For *L. longbeachae* (env.), while *lolA* was up-regulated at 50–70 °C, expression was not detected at 80–90 °C and in co-culture. In conclusion, while heat treatment may reduce the number of viable *Legionella* spp. in monoculture, results indicate that the presence of *A. mauritaniensis* increases the virulence of *L. pneumophila* during heat treatment. The virulence of *Legionella* spp. in co-culture with *Acanthamoeba* spp. should thus be monitored in water distribution systems where temperature (heat) is utilized for treatment.

## 1. Introduction

In developing and arid countries, where water scarcity is a major problem, the global practice of domestic rainwater harvesting (DRWH) has become a popular alternative to provide households with a decentralized potable and non-potable water source. Domestic rainwater harvesting has thus successfully been employed worldwide in many countries including Australia (Ahmed et al., 2012), Bermuda (Lévesque et al., 2008) and Greece (Sazakli et al., 2007). In South Africa, DRWH tanks have also been implemented by the Department of Water Affairs (DWA) in all nine provinces, as an alternative water supply and for food production (Malema et al., 2016). However, as microbial pathogens such as virulent *Escherichia coli* strains, *Legionella* spp., *Salmonella* spp., and adenovirus have previously been detected in DRWH tanks, health risks are associated with the consumption of this water source and treatment is required before rainwater is utilized as a potable water resource (Ahmed et al., 2012; Dobrowsky et al., 2014, 2015).

Dobrowsky et al. (2015) utilized a closed-coupled solar pasteurization (SOPAS) system to treat harvested rainwater. Results indicated that the level of microbial indicator bacteria (heterotrophic bacteria, *E. coli* and total coliforms) were reduced to below the detection limit at temperatures greater than 72 °C. A follow-up study, aimed at identifying *Legionella* spp. and possible vectors including *Acanthamoeba* spp., *Vermamoeba vermiformis* and *Naegleria fowleri* in the SOPAS system, was then conducted (Dobrowsky et al., 2016). Results indicated that while high pasteurization temperatures were effective in reducing viable *N. fowleri* (5-log; gene copies/mL) and *V. vermiformis* (3-log; gene copies/mL) to below the lower limit of detection at temperatures of 68–93 °C and 74–93 °C, respectively, gene copies of viable *Legionella* and *Acanthamoeba* spp. were detected after pasteurization at 93 °C. Moreover, Reyneke et al. (2016) indicated that *Legionella* spp. may enter a viable but non culturable state during pasteurization, as viable *Legionella* spp.

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were still detected at 95 °C. This is a matter of concern as *L. pneumophila* serotype 1 is the major causative agent of Legionnaires' disease associated with water distribution systems worldwide. Moreover, other *Legionella* spp. commonly associated with disease include *L. micdadei* , *L. bozemanii* , and *L. longbeachae,* amongst others (Yu et al., 2002). While *L. pneumophila* (gene copies/mL) has been detected in harvested rainwater (Hamilton et al., 2016) in the study conducted by Dobrowsky et al. (2016), *L. longbeachae* (29/82) was the species most frequently isolated from unpasteurized harvested rainwater, followed by *L. norrlandica* (22/82) and *L. rowbothamii* (3/82).

Genera of the free-living amoebae, including pathogenic *Acanthamoeba* spp. have been shown to enhance the intracellular growth and proliferation of *Legionella* spp. (Donlan et al., 2005; García et al., 2007; Dupuy et al., 2011) which allows these bacteria to withstand environmental stresses such as heat treatment. *Acanthamoeba* spp. including *A. castellani*, *A. royreba* and *A. mauritaniensis* are the etiological agents of sub-acute to chronic granulomatous amoebal encephalitis (Scheid, 2015). Moreover, *Acanthamoeba* have at least two developmental stages, namely, a metabolically active trophozoite and a dormant cyst form (Rivière et al., 2006). When harsh environmental conditions arise, it is well documented that *Acanthamoeba* cysts have a thick cellulose wall and are able to protect various pathogenic microorganisms, including *Legionella* spp. from certain disinfection procedures including heat treatment (Aksozek et al., 2002; Storey et al., 2004).

Studies have demonstrated that once ingested by amoeba, *Legionella* spp. form a specialized, endoplasmic reticulum (ER)-derived, replicative vacuole known as a *Legionella* containing vacuole within the amoeba (Greub and Raoult, 2004; Isberg et al., 2009). *Legionella* spp. then rely on their Dot/Icm (defective organelle trafficking/intracellular multiplication) type IV secretion system to translocate different effector proteins into host cells, some of which anchor to the *Legionella* containing vacuole by binding to phosphoinositide (PI) lipids (Hilbi et al., 2011; Haneburger and Hilbi, 2013). The effector proteins of *Legionella* spp. manipulate the host cell functions including the protozoan's phagocytic mechanisms and in this manner *Legionella* spp. alter the innate endosomal-lysosomal degradation pathway (Brüggemann et al., 2006) and avoid degradation by the amoeba (Clemens et al., 2000a, 2000b; Brüggemann et al., 2006).

Literature pertaining to the Dot/Icm type IV secretion system of *L. pneumophila* is extensive, with more than 300 Dot/Icm substrates identified (Hubber and Roy, 2010; Lifshitz et al., 2013) and the mechanisms of 15% of these substrates have been characterized (Allombert et al., 2013; Dolinsky et al., 2014). In contrast, while information regarding the formation of *Legionella* containing vacuoles by *L. longbeachae* is limited, studies have indicated that the process appears to differ between the two *Legionella* spp. (Asare and Abu Kwaik, 2007). Although none of the effector proteins have been mechanistically characterized to date, *L. longbeachae* is predicted to produce more than 110 Dot/Icm substrates (Cazalet et al., 2010; Lifshitz et al., 2013). Very few of these effectors are conserved between *L. pneumophila* and *L. longbeachae,* and research has indicated that more than 66 effectors are unique to *L. pneumophila* (Dolinsky et al., 2014). In addition, *L. longbeachae* harbors 50 novel Dot/Icm substrates that have been identified (Dolinsky et al., 2014).

Detailed understanding of how *Legionella* spp. survive heat treatment and manipulate host cell functions on the mechanistic level is still largely unknown. The aim of the current study was thus to determine the heat resistance of five *Legionella* strains and *Acanthamoeba mauritaniensis* ATCC 50676 both in mono- and co-culture. In addition, as *A. mauritaniensis* ATCC 50676 has previously been isolated in South Africa, the ability of *Legionella* spp. to survive in *A. mauritaniensis* ATCC 50676 during heat treatment was determined. For this, five *Legionella* strains including two *Legionella* reference strains (*L. pneumophila* ATCC 33152 and *L. longbeachae* ATCC 33462) and three *Legionella* environmental strains [*L. longbeachae* (env.), *L. norrlandica* (env.) and *L. rowbothamii* (env.)] isolated from harvested rainwater in South Africa (Dobrowsky et al., 2016) and *A. mauritaniensis* ATCC 50676 were selected for heat treatment (50–90 °C). The viability of the respective *Legionella* spp. and *A. mauritaniensis* ATCC 50676 before and after heat treatment (50–90 °C) was confirmed utilizing ethidium monoazide (EMA) quantitative polymerase chain reaction (EMA-qPCR). In addition, *L. pneumophila* ATCC 33152 and *L. longbeachae* (env.) were co-cultured with *A. mauritaniensis* ATCC 50676 and were subsequently heat treated. While the role of genes and effector proteins involved in the formation of the *Legionella* containing vacuole within amoeba have been determined, the expression profiles of these genes have not been investigated. The transcriptional responses of genes associated with metabolism and virulence of *L. pneumophila* ATCC 33152 (*lolA*, *sidF*, *csrA*) and *L. longbeachae* (env.) (*lolA*) during infection and heat treatment (50–90 °C) were determined during the current study by performing relative qPCR on cDNA transcribed from isolated RNA. During absolute quantification the exact copy concentration of the target gene was calculated by relating the quantification cycle ($C_q$) value to a generated standard curve. Relative quantification determined the amount of a target gene in a sample relative to a calibrator sample (a constant ratio of both target and reference genes) (Yu et al., 2005).

## 2. Materials and methods

### 2.1. Enumeration of Legionella spp. before and after heat treatment

*Legionella pneumophila* ATCC 33152 and *L. longbeachae* ATCC 33462 were obtained from Microbiologics® (St. Cloud, Minnesota, USA). Additionally, three environmental *Legionella* spp., previously isolated from harvested rainwater and identified (using sequencing analysis) as *L. longbeachae* (env.), *L. norrlandica* (env.) and *L. rowbothamii* (env.) were utilized in the current study (Dobrowsky et al., 2016). All *Legionella* strains were cultured on buffered charcoal yeast extract (BCYE) [CYE agar base supplemented with ACES buffer/potassium hydroxide (1.0 g/L), ferric pyrophosphate (0.025 g/L), alpha-ketoglutarate (0.10 g/L) and L-cysteine HCL (0.04 g/L)] (Oxoid, Hampshire, England). Cultures were incubated at 35 °C for 72 h.

To obtain liquid cultures for subsequent heat treatment assays, single colonies of the respective *Legionella* strains were inoculated from the BCYE plates into 50 mL Lennox Broth [tryptone (10 g/L); yeast extract (5 g/L); NaCl (5 g/L)] supplemented with ACES buffer/potassium hydroxide (1.0 g/L), ferric pyrophosphate (0.025 g/L), alpha-ketoglutarate (0.10 g/L) and L-cysteine HCL (0.04 g/L) (Oxoid, Hampshire, England) according to Delgado-Viscogliosi et al. (2009). Cultures were grown at 37 °C on an orbital shaker for 5 days. Bacterial cultures were grown to an optical density ($OD_{660}$) of 0.2 [*L. rowbothamii* (env.)] to 0.4 (remaining *Legionella* spp.) which was measured using a T60 UV Visible Spectrophotometer (PG Instruments Limited, Beijing China). Aliquots (6 mL) of the *Legionella* spp. cultures where then pipetted into test tubes which were subsequently heat treated for 30 min at each temperature (50 °C, 60 °C, 70 °C, 80 °C and 90 °C) in a recirculating water bath (FMH instrument, supplied by Labotec, Johannesburg, South Africa). Generally it is recommended that water distribution systems be treated at 70 °C for 30 min (Storey et al., 2004; Allegra et al., 2011) and the heat treatment assays were thus performed for 30 min. Untreated *Legionella* spp. cultures (6 mL) were included in all assays as positive controls and autoclaved (121 °C for 20 min) *Legionella* spp. cultures (6 mL) were included as negative controls in all assays. All heat treatment assays were repeated three times independently.

Following heat treatment, to determine the culturability of the five *Legionella* strains, 1 mL of each treated and autoclaved cell culture was centrifuged (16,000 × *g* for 10 min). The pellet was resuspended in 100 µL phosphate buffer solution (PBS; 1X; pH = 7.0) and spread plated onto BCYE agar. This was performed in duplicate. For all untreated (positive control) *Legionella* cultures, a serial dilution