Science of the Total Environment 526 (2015) 271–277

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Automobile windshield washer fluid: A potential source of transmission for *Legionella*

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HIGHLIGHTS

- *L. pneumophila* survival in one brand of washer fluid and sterilized deionized water were similar.
- *Legionella* population was maintained in tap water for several months.
- Culturable *Legionella* were detected in 10 of 12 school bus washer fluid reservoirs sampled.
- *Legionella* concentrations up to 8.1 × 104 CFU/mL were detected in school bus washer fluid.
- L. pneumophila was detected in washer fluid reservoirs and aerosolized washer fluid.

ARTICLE INFO

Article history:
Received 22 January 2015
Received in revised form 27 March 2015
Accepted 28 March 2015

Editor: D. Barcelo

Keywords:
Legionella pneumophila
Washer fluid
Automobile
Exposure
Transmission

ABSTRACT

Epidemiological evidence suggesting driving cars to be a risk factor for legionellosis has prompted public health studies to investigate vehicle windshield washer fluid as a novel transmission source of this disease. The goal of the current study was to investigate whether or not windshield washer fluid could serve as a potential source of transmission for *Legionella*. A wide variation in the survival of *L. pneumophila* was observed when incubated in different washer fluids at 25 and 37 °C, however, one brand tested supported *Legionella* survival similar to or greater than sterilized deionized water. In addition, 1 L of tap water contained in a washer fluid reservoir was able to support population growth and survival of *Legionella* for several months. In a field study examining the windshield washer fluid of 12 elementary school buses, *Legionella* were detected from 84% of samples at a high concentration of 8.1×10^4 CFU/mL. Culturable cells were also detected in aerosolized washer fluid during washer fluid spray. By demonstrating survival in certain windshield washer fluids, growth within washer fluid reservoirs, and the presence of viable cells in bus washer fluid spray, we have provided evidence suggesting the potential for a novel route of *Legionella* exposure.

1. Introduction

Legionella species, particularly *Legionella pneumophila*, are responsible for more drinking water and non-recreational water-borne disease outbreaks in the United States than any other microbes (Brunkard et al., 2011). Incidence of legionellosis has consistently and significantly risen (Hicks et al., 2011) since the discovery of the disease in 1976 (Fraser et al., 1997). The majority of cases of legionellosis are caused by exposure through the respiratory route, however, a range of atypical forms of the disease have been reported (Kilborn et al., 1992; McCabe et al., 1984; Yu, 1993). The two most common forms of illness caused by this pathogen are: Pontiac fever, resulting in flu like symptoms, and Legionnaires' disease, a potentially deadly pneumonia (Cordes and Fraser, 1980).

Public health risk from *Legionella* is often associated with environmental conditions conducive to the growth of the organism (Storey et al., 2004), such as biofilm development, protozoan activity, and high temperatures (Borella et al., 2005). Reported outbreaks of legionellosis are commonly traced to sources with potential for aerosolization of high temperature water, such as cooling towers and spas (Fields et al., 2002), though unheated water systems such as decorative fountains and tap water distribution systems have also been linked to transmission (Hlady et al., 1993). Although well documented routes of exposure may be responsible for the majority of legionellosis outbreaks, sources of transmission are not always identified and unusual or poorly understood reservoirs for these pathogens do exist (Sakamoto et al., 2009).

Epidemiological studies have suggested automobiles to be a possible source of transmission for *Legionella*. Research performed in the

Abbreviations: BYE, Buffered Yeast Extract; BCYE, Buffered Charcoal Yeast Extract; CFU, colony forming unit; BLAST, Basic Local Alignment Search Tool; PCR, polymerase chain reaction; PE, polyethylene; DI, deionized.

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http://dx.doi.org/10.1016/j.scitotenv.2015.03.122
0048-9697/

Netherlands (Den Boer et al, 2006) and Turkey (Polat et al., 2007) has shown an increased risk for Legionnaire's disease in professional drivers, while a study conducted in the United Kingdom (Wallensten et al., 2010) determined a novel risk factor for legionellosis when driving in a car containing water in place of washer fluid. In addition, studies performed in Japan (Sakamoto et al, 2009), Greece (Alexandropolou et al., 2013), and the United Kingdom (Palmer et al., 2012) have detected *Legionella* in car air conditioning systems, car cabin air filters, and windshield washer fluid reservoirs without added washer fluid, respectively. In response to mounting evidence produced by these and similar studies, a series of survival experiments and a corresponding field study were conducted with the goal of assessing the potential for *Legionella* exposure from automobile windshield washer fluid.

2. Materials and methods

2.1. Media and laboratory strain of Legionella

All laboratory experiments were performed using a stock of *L. pneumophila* ATCC strain 33152 (American Type Culture Collection, Manassas, VA, USA) cultured in Buffered Yeast Extract (BYE) medium. Laboratory and environmental water samples were assayed for the detection of *Legionella* using Buffered Charcoal Yeast Extract (BCYE) agar medium (Procedures for the Recovery of *Legionella* from the Environment, 2005). BYE medium contained: 10.0 g yeast extract, 0.25 g ferric pyrophosphate, and 0.4 g L-cysteine HCl per liter of distilled water. BCYE medium consisted of BD BBL Buffered Charcoal Yeast Agar (Diagnostic Systems, Sparks, MD, USA) supplemented with 0.4 g/1000 mL L-cysteine HCl, 0.3% glycine, 100 units/mL polymyxin B, 5 μg/mL vancomycin, and 80 μg/mL cycloheximide. Bacterial stocks were prepared by culturing *Legionella* in BYE medium in a tabletop shaker incubator at 37 °C under atmospheric CO_2 for 72 h before quantification via optical density and enumeration of colony forming units (CFU) via the spread plate technique. Stock cultures were washed via centrifugation and resuspension in sterile deionized (DI) water to remove medium prior to inoculation.

2.2. Environmental sampling and culturing

Environmental water and air samples were collected using previously described methods (Procedures for the Recovery of *Legionella* from the Environment, 2005). *Legionella* concentrations were determined for all liquid samples via the spread plate technique. When necessary, liquid samples were concentrated via membrane filtration using 0.45 μm filter discs, followed by vortexing to elute cells from the filters. Plates were incubated at 37 °C under atmospheric CO_2 between 3 and 7 days and colonies were recorded. Prior to spread plate assays, certain environmental water samples were subjected to a heat treatment at 50 °C for 30 min to reduce the growth of non-*Legionella* organisms (Wullings et al., 2011).

Table 1
Fluids used in *Legionella* survival and field studies.

Fluid	Ingredients
Washer fluid A	~1% methanol and unknown mix of cleaning agents
Washer fluid B	0.03–0.16% 2-butoxy ethanol, methanol, and isopropanol with a combined percentage no higher than 0.18% and an unknown mix of cleaning agents
Washer fluid C	0.25–1.25% isopropanol, 0.025–0.25% ethylene glycol, and an unknown mix of 4 proprietary cleaning agents
Washer fluid D	Unknown mix of proprietary cleaning agents, some hazardous
Washer fluid E	0.67–1% ethanol and 0.33% tetrasodium ethylenediamine tetraacetate
Methanol	10% methanol and 20% methanol diluted in sterilized DI water
DI water	Sterilized deionized water
Tap water	Municipal tap water collected from a laboratory faucet, school bus maintenance yard garage sink faucet, or drinking fountain.

2.3. Survival of Legionella in windshield washer fluid under laboratory conditions

Laboratory experiments were conducted using three brands (labeled fluids A, B, or C) of windshield washer fluid prepared, following the manufacturers' suggested procedures, with sterilized DI water. The components of each fluid according to manufacturers are listed in Table 1. To determine the survival of *Legionella* in the windshield washer fluids, a series of 50 mL polyethylene (PE) tubes were initially filled with fluids A, B, or C at half the manufacturer recommended concentration, or sterilized DI water. The windshield washer fluids were diluted in this initial experiment to maintain washer fluid concentrations feasibly occurring in actively used automobiles. Each PE tube was spiked with 1.5×10^6 CFU/mL of *L. pneumophila* and incubated at 37 °C. This temperature was chosen to simulate the high temperatures potentially reached in washer fluid reservoirs. Duplicate samples of each fluid were initially analyzed after 24 and 48 h of incubation to determine *Legionella* concentrations via culturing. Thereafter, samples were periodically collected and analyzed up to 73 days or until a concentration of <1 CFU/mL was reached.

A second set of 50 mL PE tubes filled with fluid A, fluid B, sterilized DI water, 10% methanol, or 20% methanol was prepared. Each tube was spiked with 1.5×10^6 CFU/mL of *L. pneumophila* and incubated at 25 °C, a temperature previously demonstrated to support long-term survival of *Legionella* in water (Schwake et al., 2012). Duplicate samples were periodically collected and *Legionella* concentrations in all PE tubes were periodically measured via culturing for up to 70 days or until a concentration of <1 CFU/mL was reached.

2.4. Growth of Legionella in windshield washer fluid reservoirs

To measure the growth of *Legionella* in windshield washer fluid reservoirs under laboratory conditions, two 1 L PE reservoirs (Interdynamics, Inc., Tarrytown, NY) were filled with 750 mL of dechlorinated municipal tap water (Tempe, AZ, USA) with no initial detectable *Legionella* colonies in 1 mL. An additional two reservoirs were filled with tap water from a laboratory model drinking water distribution system previously spiked with *L. pneumophila* containing approximately 2.5×10^3 CFU/mL of *Legionella*. Each reservoir was incubated at 25 °C or 37 °C. Samples were collected in duplicate and cultured periodically over the course of 75 days to determine *Legionella* growth. Prior to each sample collection, the water in each reservoir was mixed by gentle pipetting to minimize the disruption of potential biofilm formation. The fluid in the reservoirs was sampled to measure suspended cells potentially able to be aerosolized during windshield washer fluid spray. Biofilms were intentionally undisturbed to support potential biofilm associated growth of *Legionella* in the reservoirs.

2.5. Environmental detection of Legionella in school bus windshield washer fluid

A field study was conducted for the detection, quantification, and identification of *Legionella* in windshield washer fluid from school buses. Three sets of windshield washer fluid samples were collected from a fleet of buses considered actively in operation and belonging to a school district in central Arizona, USA. Duplicate samples were collected from the washer fluid reservoirs of buses parked in the school district's maintenance yard on May 30, July 2, and July 31, 2012 between 11 am and 4 pm. Sample volumes ranging from 50 to 250 mL were collected from a total of 12 school buses. In addition, samples were collected from the stock solution used to prepare the windshield washer fluid for the buses, tap water from a drinking water fountain in the maintenance yard garage, and a sink faucet in the maintenance yard garage. The stock solution for the bus windshield washer fluid reservoirs was reportedly prepared in batches of approximately 10 L at a time (based on demand) and stored in a sealed container in an air conditioned garage.