Legionella spp. isolation and quantification from greywater

Sara Rodríguez-Martínez a,*, Marina Blanky b, Eran Friedler b, Malka Halpern a,c

a Department of Evolutionary and Environmental Biology, Faculty of Natural Sciences, University of Haifa, Haifa, Israel
b Faculty of Civil and Environmental Engineering, Technion, Haifa, Israel
c Department of Biology and Environment, Faculty of Natural Sciences, University of Haifa, Oranim, Tivon, Israel

ABSTRACT

Legionella, an opportunistic human pathogen whose natural environment is water, is transmitted to humans through inhalation of contaminated aerosols. Legionella has been isolated from a high diversity of water types. Due its importance as a pathogen, two ISO protocols have been developed for its monitoring. However, these two protocols are not suitable for analyzing Legionella in greywater (GW). GW is domestic wastewater excluding the inputs from toilets and kitchen. It can serve as an alternative water source, mainly for toilet flushing and garden irrigation; both producing aerosols that can cause a risk for Legionella infection. Hence, before reuse, GW has to be treated and its quality needs to be monitored. The difficulty of Legionella isolation from GW strives in the very high load of contaminant bacteria. Here we describe a modification of the ISO protocol 11731:1998 that enables the isolation and quantification of Legionella from GW samples. The following modifications were made:

• To enable isolation of Legionella from greywater, a pre-filtration step that removes coarse matter is recommended.

* Corresponding author.
E-mail address: srodriguezmar@gmail.com (S. Rodríguez-Martínez).

http://dx.doi.org/10.1016/j.mex.2015.11.004
2215-0161/© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
Method details

There are several ISO methods for *Legionella* isolation from water [1,2]. However, none of them is suitable for *Legionella* isolation from GW samples due to the fact that GW is contaminated with a very high bacterial load. Thus, almost no data can be found regarding *Legionella* presence in GW.

Here we describe a modification of the ISO 11731:1998 protocol for *Legionella* isolation from GW. Our modified protocol allows the isolation of *Legionella* on GVPC selective *Legionella* medium (glycine–vancomycin–polymyxin–cycloheximide medium, Beckton Dickinson GmbH, Heidelberg, Germany) without the massive bacterial contamination that develops on the media when ISO 11731:1998 is applied.

**Pre-filtration of the greywater sample**

Filter a 100ml GW sample (pre-filtration) to remove coarse matter, using a 100 μm pore size cell strainer (Becton Dickinson, USA) placed in one 50ml tube (two 50ml tubes are needed) (Fig. 1).

**Filtration of the GW sample**

The 100ml pre-filtered GW sample is filtered again through a 0.2 μm cellulose nitrate filter using a vacuum filtration system attached to a 2511 Dry Vacuum Pump (WELCH, Germany) (Fig. 2).

**Bacteria resuspension in PBS**

After filtration, the filter is placed into 10ml phosphate buffered saline (PBS, pH 7.4 ± 0.2; NaCl 0.14M; KCl 2.7 mM; Na2HPO4 10 mM, KH2PO4 1.8 mM) and vortexed for 10 min (Fig. 3).

**Combined acid–thermal treatment**

Each sample is then subjected to a combined acid–thermal treatment as follows: 1 ml of the sample is centrifuged at 6000 × g for 10 min. For the acid treatment, 0.5 ml of the supernatant is replaced with 0.5 ml of acid buffer (HCl 27 mM; KCl 173 mM, pH 2.2). The sample is then vortexed and immediately subjected to thermal treatment for 30 min at 50 °C.

**GVPC *Legionella* media inoculation**

Following the ISO 11731:1998 recommendations, two 0.5ml sub-samples are plated on a GVPC *Legionella* selective media immediately after the thermal treatment. The plates are incubated at 37 °C. Presumptive *Legionella* colonies are counted after 7 and 15 days of incubation.

**Legionella** identification

The presumptive *Legionella* colonies are then identified using a *Legionella* latex test (Oxoid, Basingstoke, UK). This test allows separate identification of *Legionella pneumophila* serogroup 1 and
Fig. 1. (a) A 100 μm pore size cell strainer. (b) Pre-filtration of the greywater samples through the 100 μm pore size cell strainer.
serogroups (2–14) and the detection of seven other Legionella species (L. longbeachae serogroups 1 and 2, L. bozemanii serogroup 1, L. dumoffii, L. gormanii, L. jordanis, L. micdadei, and L. anisa).

Method validation: efficiency of the Legionella isolation protocol and limit of detection (LOD)

We used the method described above to successfully isolate and quantify Legionella along a one year GW monitoring campaign. The results of this study have been already published [3]. Briefly, a total of 16 greywater samples were analyzed. Legionella was isolated from 81% of the samples, with a mean of $1.2 \times 10^5$ cfu/l. Details about the efficiency and the limit of detection of this method can also be found in the mentioned publication. This method is highly aggressive, so the recovery rates of Legionella were very low (2.5%, SD = 1.5%) and the LOD established from this average recovery rate was $4.0 \times 10^3$ cfu/l. Nevertheless, the results were consistent. It should be noted that this modified methods is the only way to isolate Legionella from GW, as using the current ISO protocols does not allow the isolation of this bacteria.
Recommendations

This method is highly aggressive for the sampled bacteria, including Legionella. For that reason, the LOD of the method is high and the efficiency of Legionella isolation is low. We recommend using this method only with problematic samples in which Legionella can’t be isolated using the methods described in the ISO protocols 11731:1998 and 11731-2:2004 [1,2] due to massive contamination with other bacterial species.

Acknowledgments

This study was supported by grants from Zuk Maccabi Aryeh LTD. and The Technion Center of Excellence in Exposure Science and Environmental Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors specially thank O. Yogev, Y. Gilboa, Y. Alfiya, L. Privman and S. Ofer for their technical help in the samplings. MethodsX thanks the reviewers of this article for taking the time to provide valuable feedback.

References

[1] ISO 11731:1998: Water Quality – Detection and Enumeration of Legionella, International Organization for Standardization, 1998.
[2] ISO 11731-2:2004: Detection and Enumeration of Legionella – Part 2: Direct Membrane Filtration Method for Waters with Low Bacterial Counts, International Organization for Standardization, 2004.
[3] M. Blanky, S. Rodríguez-Martínez, M. Halpern, E. Friedler, Legionella pneumophila in greywater – quantification and removal during treatment, Sci. Total Environ. 533 (2015) 557–565.