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Review

Determination of viable legionellae in engineered water systems: Do we find what we are looking for?

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ABSTRACT

In developed countries, legionellae are one of the most important water-based bacterial pathogens caused by management failure of engineered water systems. For routine surveillance of legionellae in engineered water systems and outbreak investigations, cultivation-based standard techniques are currently applied. However, in many cases culture-negative results are obtained despite the presence of viable legionellae, and clinical cases of legionellosis cannot be traced back to their respective contaminated water source. Among the various explanations for these discrepancies, the presence of viable but non-culturable (VBNC) *Legionella* cells has received increased attention in recent discussions and scientific literature. Alternative culture-independent methods to detect and quantify legionellae have been proposed in order to complement or even substitute the culture method in the future. Such methods should detect VBNC *Legionella* cells and provide a more comprehensive picture of the presence of legionellae in engineered water systems. However, it is still unclear whether and to what extent these VBNC legionellae are hazardous to human health. Current risk assessment models to predict the risk of legionellosis from *Legionella* concentrations in the investigated water systems contain many uncertainties and are mainly based on culture-based enumeration. If VBNC legionellae should be considered in future standard analysis, quantitative risk assessment models including VBNC legionellae must be proven to result in better estimates of human health risk than models based on cultivation alone. This review critically evaluates current methods to determine legionellae in the VBNC state, their potential to complement the standard culture-based method in the near future, and summarizes current knowledge on the threat that VBNC legionellae may pose to human health.

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1. The importance of legionellae for public health

Legionellae are ubiquitously present at low concentrations in natural aquatic ecosystems (Parthuisot et al., 2010). Due to their sessile mode of life and their preference for temperatures above 25 °C, man-made engineered water systems often select for legionellae, if they are not adequately managed. Among the more than 50 described *Legionella* species, *Legionella pneumophila* is one of the most important water-based bacterial pathogens in developed countries. Legionellae predominantly cause two kinds of respiratory tract infections, the severe and potentially fatal Legionnaires' disease and the mild, non-fatal, influenza-like illness Pontiac Fever (Hornei et al., 2007). Different serogroups (*L. pneumophila* SG 1, 6, 7) and species (*Legionella micdadei*, *Legionella feeleii*, *Legionella anisa*) have been reported to cause Pontiac Fever, but the underlying mechanisms that are responsible for causing either Pontiac fever or Legionnaires' disease have not been elucidated so far (Tossa et al., 2006; Wang et al., 2015). With the exception of Australia, New Zealand and Japan, where *Legionella longbeachae* infections can occur as often as cases of *L. pneumophila* infection (Whiley and Bentham, 2011), the majority (>95%) of all notified cases of Legionnaires' disease has been caused by *L. pneumophila* strains, most of them (>85%) belonging to serogroup 1 (ECDC, 2015). Especially Mab 3/1 positive strains — determined according to the Dresden panel (Lück et al., 1992) — and specific sequence types (ST) — determined according to the seven-gene sequence based typing scheme for *L. pneumophila* (Ratzow et al., 2007) — are more frequently involved in Legionnaires' disease, such as ST1, ST23, ST37, ST47, and ST222. This varies slightly among different countries (Cassier et al., 2015; Harrison et al., 2009; Kozak-Muiznieks et al., 2014). For the European Union and Norway, 5844 notified cases of Legionnaires' disease were recorded in 2013, corresponding to an average notification rate of 1.14 per 100,000 population (ECDC, 2015). Similar rates (1.15 per 100,000 population) have been reported in the United States of America for 2009 (CDC, 2011). An increasing trend in notification rates was observed in many countries until the end of the last decade (CDC, 2011; ECDC, 2015). Potential reasons for this increase were mainly (i) a continuous increase in engineered water systems suitable for *Legionella* growth like cooling facilities, (ii) the ageing society in developed countries as aged people are more vulnerable to *Legionella* infection and (iii) improved diagnosis and reporting. In any case, it was estimated that the number of notified Legionnaires' disease cases is just the tip of the iceberg and that the true incidence rates could be 10× (Parr et al., 2015) to 20× higher (Marston et al., 1997), in addition to the far more underreported Pontiac Fever (Parr et al., 2015).

2. Standard methods to detect and quantify legionellae in water

2.1. Cultivation dependent methods

Standard culture based methods are usually used on a routine basis to detect and quantify legionellae in engineered water systems suspected to harbour high concentrations of these bacterial pathogens (ISO, 1998). Following the standard method, 1000 ml of water (in case of hot water systems) or down to 100 ml (in case of cooling towers) is either concentrated on 0.22 μm or 0.45 μm pore-size polycarbonate or nylon filters, subsequently sonicated and resuspended in 5–25 ml of sterile diluent. Alternatively, 200 ml of sample is centrifuged and the pellet is resuspended in 2–20 ml sterile diluent. Only if the number of *Legionella* is expected to exceed 10^5 per litre, direct plating of liquid is permissive. Aliquots of the concentrate are treated with heat (30 min at 50 °C) and/or acid (buffered 0.2 M HCl for 5 min) in order to reduce background microbiota, in comparison to an untreated control. After seven to ten days of incubation at 36 °C on (GVPC) agar (buffered charcoal yeast extract (BCYE) agar plus supplements glycine, vancomycin, polymyxin B and cycloheximide), representative presumptive colonies (three per plate) are streaked on both BCYE and BCYE agar without cysteine, and checked for growth after 2 days at 36 °C. Alternative to BCYE agar without cysteine, blood-agar or nutrient agar can be used. Those colonies can be regarded as *Legionella* that grow on BCYE but fail to grow on cysteine free medium. Identification of *Legionella* species and serogroups may then be performed by a variety of methods (see ISO 11731:1998). Aside from the fact that this standard method needs up to 14 days for analysis, it often fails to detect legionellae in water samples despite the presence of viable cells (Kirschner et al., 2012; Parthuisot et al., 2011). Moreover, in many instances, clinical cases of legionellosis cannot be traced back to their respective contaminated water sources because no culturable environmental legionellae can be found. In the most recent report on Legionnaires' disease in Europe (ECDC, 2015) it