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Rapid detection of viable *Legionella pneumophila* in tap water by a qPCR and RT-PCR-based method

R. Boss[1], A. Baumgartner[1], S. Kroos[2], M. Blattner[2], R. Fretz[2] and D. Moor[1]

1 Federal Food Safety and Veterinary Office, Berne, Switzerland
2 Food Safety and Veterinary Office, Canton of Basel-Landschaft, Liestal, Switzerland

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Correspondence
Renate Boss and Dominik Moor, Federal Food Safety and Veterinary Office, Berne, Switzerland.
E-mails: renate.boss@blv.admin.ch, and dominik.moor@blv.admin.ch

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Abstract

Aims: A molecular method for a rapid detection of viable *Legionella pneumophila* of all serogroups in tap water samples was developed as an alternative to the reference method (ISO). Legionellae are responsible for Legionnaires' disease, a severe pneumonia in humans with high lethality.
Methods and Results: The developed method is based on a nutritional stimulation and detection of an increase in precursor 16S rRNA as an indicator for viability. For quantification, DNA was detected by qPCR. This method was compared to the ISO method using water samples obtained from public sports facilities in Switzerland. The sensitivity and specificity were 91 and 97%, respectively, when testing samples for compliance with a microbiological criterion of 1000 cell equivalents per l.
Conclusion: The new method is sensitive and specific for *Leg. pneumophila* and allows results to be obtained within 8 h upon arrival, compared to one week or more by the ISO method.
Significance and Impact of the Study: The method represents a useful tool for a rapid detection of viable *Leg. pneumophila* of all serogroups in water by molecular biology. It can be used as an alternative to the ISO method for official water analysis for legionellae and particularly when a short test time is required.

Introduction

Legionellosis is a bacterial disease with two clinical manifestations in humans: the self-limiting and nonpneumonic Pontiac fever and Legionnaires' disease, characterized by severe respiratory symptoms including pneumonia with high lethality. The infectious agent is *Legionella* spp. and predominantly *Legionella pneumophila* (WHO 2007; Dominguez *et al.* 2009; Parr *et al.* 2015; ECDC 2016). Legionellae naturally occur in environmental water sources and are well adapted to man-made water installations (WHO 2007). They grow in warm water of 25–45°C and are often found in buildings with convoluted water pipelines that are stagnant or rarely flushed parts of the system (Rhoads *et al.* 2015). Predilection sites are shower heads, but also pipes, taps, Jacuzzi tubs, and air conditioning installations (WHO 2007).

Infection follows inhalation of contaminated water aerosols. Elderly or immunocompromised people, smokers and patients suffering from chronic respiratory illnesses are particularly at risk (Lanternier *et al.* 2017). The best preventive method is to adjust temperatures in cold water to below 20°C and to ensure a minimum water temperature of 60°C in boilers and preferably of 55°C in distribution pipes (WHO 2007; Bedard *et al.* 2015; Rhoads *et al.* 2015). To prevent scalding, temperature regulator devices should be installed at points-of-use. Further aspects of prevention are appropriate constructions with minimized water stagnation and materials that do not support microbial growth (Bedard *et al.* 2015).

Legionnaires' disease occurs worldwide (WHO 2007) with an assumed high number of unreported cases (Parr *et al.* 2015). For the European Union and Norway, the incidence of infection was 1·14 and 1·35 per 10^5

inhabitants in 2013 and 2014 respectively (ECDC 2015, 2016). In Switzerland, where it is a reportable disease, it showed a continuous increase to 5·8 infections per 10^5 inhabitants in 2017, compared to an average of 0·8 (0·3–1·1) in the 1990s and 2·1 (0·9–2·8) between 2000 and 2009 respectively (FOPH 1990-2017, 2018). In Europe, approximately 70% of the reported infections are caused by the *Leg. pneumophila* serogroup (SG) 1, 20–30% by *Leg. pneumophila* SGs 2–16, and 5–10% by *Legionella* spp., mainly *Legionella micdadei* (WHO 2007). Nonpneumophila *Legionella* spp. are predominantly involved in nosocomial infection (WHO 2007).

For preventive purposes, microbiological criteria for *Legionella* spp. in public accessible bath and shower water were recently stipulated in Switzerland (FSVO 2017). To test water samples for compliance with these microbiological criteria, an international standard method is specified (ISO 11731) which detects and enumerates living cells of the pathogen by culturing on selective agar plates (ISO 2008). Alternative methods are allowed, when the result's evaluation does not influence the resulting decision. The ISO method is time consuming (up to 10 days) and also has some other disadvantages (Kirschner 2016). For example, legionellae can persist in a viable but not culturable (VBNC) status due to different reasons (Al-Bana *et al.* 2014; Li *et al.* 2015; Kirschner 2016), which may result in false negative results. Under certain conditions, VBNC legionellae are able to regain virulence and infectiousness (Steinert *et al.* 1997; Ducret *et al.* 2014). Molecular-based methods are faster but they usually detect both, viable and dead bacteria. The presence or number of dead cells might be of interest in some situations but for the official control of water samples for legionellae, methods are needed which include only living cells. Methods using DNA intercalating reagents can distinguish between living and dead bacterial cells but they have disadvantages. For example, the presence of biofilms can disturb detection (Taylor *et al.* 2014) and concentration dependent cytotoxic effects of reagents are possible (Yanez *et al.* 2011). Moreover, high cell counts, which usually do not occur in field samples, are required for plausible analyses (Chang *et al.* 2009).

Hence, as an alternative to the ISO method, a molecular detection method of viable *Leg. pneumophila* was developed, based on PCR detection of a precursor 16S rRNA target that is specific for this species and occurs only in living cells. Precursor rRNA represents a significant fraction of the total microbial rRNA and is, because of its higher stability, much easier to detect and handle than mRNA (Cangelosi *et al.* 2010). Precursor rRNA is synthesized by growing bacteria and its leader and tail sequences are subsequently removed during rRNA maturation (Cangelosi and Brabant 1997; Cangelosi *et al.* 2010). Upon stagnancy of growth, precursor rRNA synthesis stops but maturation goes on, draining the precursor rRNA pool. Thus, the presence of precursor rRNA can be used as a molecular indicator for physiological activity and therefore viability of bacterial cells (Stroot and Oerther 2003; Lu *et al.* 2009; Cangelosi *et al.* 2010).

As water is a limited nutritional medium for legionellae, cell populations are barely in an exponential growth phase and the precursor rRNA pool at a low level or even drained off (Al-Bana *et al.* 2014). The transfer of such starved bacteria into a fresh nutritional medium will stimulate them, resulting in a boosted rRNA synthesis. Dead cell is neither activated nor is their rRNA synthesis boosted. Such a stimulation step was included in the newly developed method. It is followed by nucleic acid (NA) extraction and reverse transcription-PCR (RT-PCR) to detect the precursor 16S rRNA. A shift between the cycle threshold (C_T) of an unstimulated and a stimulated sample can be interpreted as the presence of viable *Leg. pneumophila* cells. For quantification, a real-time quantitative PCR (qPCR) was performed using the DNA fractions that were simultaneously extracted from the same samples.

The aim of the study was to develop and evaluate a rapid method to detect viable *Leg. pneumophila* in tap water samples as an alternative to the bacteriological reference method (ISO 11731). The two methods were compared by analysing water samples from public sports facilities in a region of Switzerland with rather high incidence rates of legionellosis, that is, 7·3 and 4·6 infections per 10^5 inhabitants in 2015 and 2016 respectively (FOPH 2018).

Materials and methods

Bacteriological detection of *Leg. pneumophila*

Water samples were analyzed with the reference method ISO 11731-2:2008 (ISO 2008). In brief, 1 l of water was filtered through a 0·2 μm polycarbonate Nuclepore™ membrane (Whatman Inc., Florham Park, NJ) and the bacterial cells then resuspended from the membrane. This suspension was plated on agar plates either directly or after acid or heat pretreatment to minimize accompanying bacterial flora. Plates were evaluated three times during the 10 days incubation period. One presumptive *Legionella* spp. per plate was confirmed by latex agglutination test (Oxoid, Pratteln, Switzerland and Microgen Bioproducts, Camberley, UK) which identify the predominant *Legionella* spp. and allow distinction between *Leg. pneumophila* SG 1 and SGs 2-15.

Molecular detection of *Leg. pneumophila*

A PCR system was designed that detects the precursor region of the 16S rRNA sequence of *Leg. pneumophila*

which occurs threefold within its genome. Three copies of the target were therefore regarded as one cell equivalent (cellEq). The forward primer (5′-CGA GAG CTA GTG CCG GAA T-3′) in this system was located within the precursor region of the 16S rRNA sequence, while probe (5′-FAM-TAG ACA GAT GGC GAG TGG CGA ACG-BHQ1-3′) and reverse primer (5′-CCA AGT TGT CCC CCT CTT C-3′) were located downstream. The amplicon size was 177 bp. Primers and probes were synthesized by Microsynth (Balgach, Switzerland). *In silico* sequence homology searches using the nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov) showed that primers and probes were specific for *Leg. pneumophila*.

Analytical specificity

To determine inclusivity and exclusivity, the following 21 *Legionella* spp. as well as 12 non-*Legionella* strains which can contaminate water, were tested for the presence of the primer's and probe's target sequence: *Leg. pneumophila* subsp. *pneumophila* SG 1 (DSM 7513), 14 strains of *Leg. pneumophila* belonging to the SGs 1-14 (obtained from the National Reference Centre for Legionella, Bellinzona, Switzerland), *Legionella anisa* (DSM 17627), *Legionella feeli* (DSM 17645), *Legionella jordanis* (DSM 19212), *Legionella longbeachae* (DSM 10572), *Legionella oakridgensis* (DSM 21215), and *Leg. micdadei* (wild type), *Aeromonas hydrophila* (wild type), *Enterobacter aerogenes* (DSM 30053), *Escherichia coli* (DSM 1103), *E. coli* (NCTC 13216), *Enterococcus faecalis* (DSM 20478), *Pseudomonas aerugionosa* (DSM 1117), *P. aerugionosa* (DSM 50071), *Salmonella nottingham* (NCTC 7832), *Salmonella thyphimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), *Vibrio cholerae* (NCTC 8042), *Vibrio parahaemolyticus* (DSM 11058).

Legionella spp. were grown on buffered charcoal yeast extract (BCYE) agar with L-cysteine (BioMérieux Suisse S.A., Geneva, Switzerland) at 37°C for a minimum of 48 h. Non-*Legionella* strains were grown on 5% sheep blood agar plates (BioMérieux) at 37°C for 24 h. Total bacterial DNA was extracted by the boiling preparation method using one colony per strain, re-suspended in 100 μl TE Buffer (10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, pH 8·0) and incubated at 95°C for 10 min. DNA extracts were diluted 1 : 100 with PCR grade water prior to qualitatively applied qPCR (see below).

Detection of living *Leg. pneumophila* with RNA stimulation

To detect living cells of *Leg. pneumophila*, an assay was developed which was based on the stimulation of RNA synthesis (Stimulation Based Methodology, SBM) and comprises the following four steps.

Target concentration

A 1 l water sample was filtered through a 0·2 μm polycarbonate membrane filter of 47 mm diameter (Sterlitech, Kent, WA) using an appropriate filtration unit and vacuum pump. The membrane was transferred to a 50 ml tube, rinsed with 3 ml of sterile Page's saline containing 120 mg l^{-1} NaCl, 4 mg l^{-1} $MgSO_4 \times 7H_2O$, 4 mg l^{-1} $CaCl_2 \times 2H_2O$, 142 mg l^{-1} Na_2HPO_4 and 136 mg l^{-1} KH_2PO_4 (according to the ISO method), and then vigorously vortexed for 2 min. As process controls, bottled still mineral water of the brand 'Evian' (Evian-Volvic Suisse S.A., Zurich, Switzerland) was used as a negative control, and freshly sampled warm tap water from a contaminated but not sanitized building was used as a positive control.

Stimulation

The concentrates of the previous step were then used to prepare a unstimulated control (−STIM) and a stimulated sample (+STIM): For −STIMs, a 1 ml aliquot of the concentrate was transferred in a 1·5 ml tube, centrifuged at 16 000 ***g***, 4°C for 5 min, and immediately frozen at −70°C after discarding the supernatant. To prepare +STIMs, another 1 ml aliquot of the concentrate was added to 9 ml sterile filtered and prewarmed stimulation medium, containing 10 g l^{-1} yeast extract (Oxoid) and 10% Legionella BCYE Growth Supplement (Oxoid) in a 50 ml tube. Stimulation occurred during incubation at 37°C, 130 rev min^{-1} for 3 h. After the subsequent centrifugation at 10 000 ***g***, 4°C for 10 min, 9 ml of supernatant was discarded and the residual transferred to a 1·5 ml tube. Following centrifugation at 16 000 ***g***, 4°C for 5 min and removal of the supernatant, the pellet was frozen at −70°C until NA extraction.

NA extraction

Simultaneous extraction of total DNA and RNA fractions from −STIM and +STIM samples was performed using the NucleoSpin RNA Kit complemented by the NucleoSpin RNA/DNA Buffer Set (Macherey-Nagel AG, Oensingen, Switzerland) according to the manufacturer's instructions. An on-column DNase treatment was included during RNA extraction. The final elution volume was 100 μl for the DNA fraction and 60 μl for the RNA fraction. As an extraction control, an aliquot of pelleted and frozen *Leg. pneumophila* were always coprocessed. RNA fractions were analyzed immediately after extraction and afterwards stored at −70°C. DNA fractions were stored at 4°C until analysis and then stored at −20°C.

Molecular detection

DNA fractions were analyzed by qPCR for quantification. RNA fractions were tested by one-step RT-PCR (as

outlined below) to evaluate C_T values of −STIM and +STIM. A shift of >1 cycle (C_T shift) between −STIM and +STIM was considered as a stimulation caused by living cells (i.e. stimulated) of *Leg. pneumophila* in the sample. Shifts <1 cycles may occur due to technical variation in PCR replica. In additional stimulation experiments using heat inactivated cells, C_T shifts <1 cycle or even negative values were observed (data not shown).

QPCR and RT-PCR conditions

DNA samples of bacterial strains and DNA fractions obtained from the SBM were analyzed by qPCR. Reactions were run in a total volume of 25 μl containing 1× Roche LightCycler 480 Probes Master (Roche Diagnostics, Rotkreuz, Switzerland), 400 nmol l^{-1} of both primers, 200 nmol l^{-1} of the probe (see above), and 5 μl of DNA template. QPCRs were run on a LightCycler 480 II (Roche) thermocycler. The run protocol started with an initial step of 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. For quantification, a standard curve of the 16S rRNA precursor region ranging from 10^5 to 10^1 copies per μl was included in each run. Standards were prepared using DNA of *Leg. pneumophila* (DSM 7513): PCR was done using the above-mentioned conditions and the PCR-products were subsequently purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). Concentration of the purified PCR-product was determined by the NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Rockland, Germany) followed by dilutions in 0·2× TE buffer (pH 8.0) to concentrations from 10^5 to 10^1 copies per μl. For data analysis, the second derivative maximum analysis method of the LightCycler 480 Software (release 1.5.1.62) was used. It automatically displayed the standard curve as a linear regression line for higher accuracy of data at the detection limit.

RNA fractions obtained from the SBM were analyzed by RT-PCR immediately after RNA extraction. Reactions were run in a total volume of 25 μl using the QuantiTect Probe RT-PCR Kit (Qiagen, Hombrechtikon, Switzerland) with same primers, probes and their concentration as for qPCR (see above), and 5 μl of RNA template. The same cycler as for QPCR was used with the following protocol: an initial reverse transcription at 50°C for 30 min, and then an initial heat activation at 95°C for 15 min, followed by 45 cycles of 94°C for 15 s and 60°C for 1 min.

For all PCR runs, samples were run in duplicates and a no template control and a positive control were always included. Results were considered positive if both reactions were positive. If only one reaction showed a positive result or the difference of the two C_T values was more than two cycles, the QPCR was repeated. C_T values >40 were omitted.

SBM performance

Assay performance was done according the World Organization for Animal Health (OIE 2013).

The limit of detection (LOD) was determined using artificially starved *Leg. pneumophila*: One colony of *Leg. pneumophila* (DSM 7513) was resuspended in BCYE broth supernatant, that was obtained by centrifugation of BCYE broth at 10 000 ***g*** for 10 min and supplemented with 10% Legionella BCYE Growth Supplement (Oxoid). The culture was incubated at 37°C overnight and the cell count then determined by flow cytometry (Cyflow ML, Sysmex Partec, Horgen, Switzerland). Subsequently, an aliquot containing 10^8 cells was centrifuged at 16 000 ***g*** for 5 min. The pellet was resuspended in 100 ml of sterile filtered Evian water, generating a concentration of 10^6 cells per ml. Starvation of *Leg. pneumophila* occurred during incubation at 37°C, 100 rev min^{-1} for 24 h and lead to drain-out of present precursor 16S rRNA. To find out the LOD, 10-fold serial dilutions were made from the starved culture in sterile filtered Evian water obtaining concentrations of 10^5, 10^4, 10^3, 10^2 and 10^1 cells per ml. From each of these dilutions, 1 ml was taken to spike 1 l of Evian water which was subsequently analyzed with the SBM, as written above. As the SBM method produces two results (a quantification by qPCR and a viability result from RT-PCR), the LOD on the one hand was evaluated with respect to the combined result (including qPCR and RT-PCR) and on the other hand with respect to the quantification by qPCR solely.

The intraassay variability of the complete SBM procedure was determined by simultaneous processing of eight identical samples of tap water (each 1 l) that was naturally contaminated with *Leg. pneumophila*. The interassay variability of the complete SBM procedure was evaluated by processing of eight samples on eight different days. Each day, 1 l Evian water was spiked with 10^8 cells of artificially starved *Leg. pneumophila*, which were prepared as written above and kept at 37°C during the experiment.

Additionally, a stimulation experiment using artificially starved *Leg. pneumophila* was performed for providing a time course over a 4 h stimulation period. Samples were collected after 20, 40, 60, 80, 100, 120, 180 and 240 min of stimulation. The samples underwent the NA extraction as written above and the obtained DNA and RNA fractions were used for qPCR and RT-PCR respectively.

Application of the developed method on field samples

To compare the developed molecular method with the standard method of ISO, 102 tap water samples of 2 l were taken in 51 public sports facilities in the Canton of Basel-Landschaft, Switzerland, between September and

November 2016. In each facility, one sample was taken preferably close to the boiler, another one in the shower, after discarding the first litre of water. One sample was spilled during transport. The samples were divided into two 1-l aliquots in the laboratory. One aliquot was bacteriologically examined the same day in the microbiological laboratory of the Food Safety and Veterinary Office of Canton Basel-Landschaft according to the ISO method. The other aliquot was kept at 4°C, transported to the Federal Food Safety and Veterinary Office the same day, and examined the following day using the SBM procedure. Results of both laboratories were only compared after completing all measurements which allowed a blinded assessment (Greenhalgh 1997).

Comparison of data included quantitative comparison of measured counts (CFU per l for the ISO method and cellEq per l for the SBM) and qualitative comparison (viable *Leg. pneumophila* present/absent) with regard to official microbiological criteria for *Leg. pneumophila*, that are a limit of 1000 CFU per l (MC_{1000}) for water from showers and a limit of 100 CFU per l (MC_{100}) for aerosol-producing installations, for example Jacuzzi tubs (FSVO 2017).

Statistics

The exact McNemar's test was applied using the Statistical Analysis System software (SAS 9.2 analytics software, Cary, NC, USA) to compare the paired nominal data of both methods regarding both criteria, MC_{100} and MC_{1000} respectively. In addition, the Cohen's Kappa coefficient (κ) was calculated as a measure of agreement.

Results

Assay performance

Analytical specificity

Inclusivity and exclusivity of the PCR system were both 100%. All 15 strains of *Leg. pneumophila* belonging to SGs 1-14 tested positive for the precursor 16S rRNA coding sequence. All other tested *Legionella* spp. ($n = 6$) and all bacterial strains of other genera ($n = 12$) tested negative for this target. Using purified precursor 16S rRNA targets, the qPCR showed log-linear results over eight dilutions of a 10-fold dilution series. PCR performance show a high efficiency of 1·93 and a slope of −3·502.

Analytical sensitivity

The LOD of the SBM was determined using water spiked with starved *Leg. pneumophila* serially diluted from 10^5 to 10^1 cells per l. As the SBM comprises two PCRs (qPCR and RT-PCR) a LOD for the combined result and for qPCR alone was evaluated separately. A positive result for both, qPCR (quantification) and RT-PCR (i.e. C_T shift between −STIM and +STIM representing viability), was observed for the three highest concentrations, namely 10^5, 10^4, 10^3 cells per l. Therefore, LOD was 1000 cells per l regarding viability. QPCR solely gave a positive result additionally for 10^2 cells per l, but at this concentration, no C_T shift was observed in the RT-PCR, as only one of the two duplicates was positive or +STIM was negative. Hence, regarding qPCR that was used for quantification, the LOD was 100 cells per l. Additionally, qPCR and RT-PCR were repeated on a Rotor-Gene Q real-time thermal cycler (Qiagen) under the very same conditions, resulting in a LOD of 100 cells per l for both qPCR as well as RT-PCR (see Discussion section).

Repeatability

The intraassay variability of the SBM was determined by a simultaneous processing of eight water samples, naturally contaminated with *Leg. pneumophila*. All samples tested positive for viable *Leg. pneumophila* with cell counts from 5880 to 8455 cellEq per l. The CV was 1·61% ($\log_{10}$ transformed values). C_T shifts between −STIM and +STIM ranged from 2·0 to 5·8 cycles. The interassay variability of the SBM was evaluated by repeated processing of eight water samples, spiked with 10^8 cellEq per l of artificially starved *Leg. pneumophila*, on eight different days. All samples tested positive for viable *Leg. pneumophila* with cell counts from $0{\cdot}47 \times 10^6$ to $1{\cdot}07 \times 10^6$ cellEq per l. The CV was 1·83% ($\log_{10}$ transformed values). C_T shifts between −STIM and +STIM ranged from 3·4 to 8·5 cycles.

Stimulation time course

In a 4 h-stimulation experiment with periodical samplings after 20, 40, 60, 80, 100, 120, 180 and 240 min, RT-PCR showed decreasing C_T values over the whole experiment period. The corresponding C_T shifts, calculated in reference to the C_T value before stimulation (=0 min), were 8.3, 10.0, 11.7, 12.2, 12.9, 13.5, 13.9 and 14.1 cycles respectively (Table 2). The simultaneously extracted DNA showed constant C_T values over time with <1 cycle of variation (Table 2).

Field study

Tap water samples ($n = 101$) from public sports facilities were analyzed, using both the bacteriological reference method according to ISO and the SBM. Results are shown in Table 1.

Bacteriological analysis (ISO method) revealed a positive result in 21 (21%) samples for *Leg. pneumophila*, ranging

Table 1 *Legionella pneumophila* in tap water samples (n = 101) analyzed by the ISO 11731 method (reference) and the newly developed stimulation-based method (SBM). Matches indicate concordant results by both methods

	ISO method		SBM		Matches†	
n	(CFU per l)	SG*	(cellEq per l)	Viability†	MC_{100}	MC_{1000}
ISO positives						
1	87 000	2-15	10 215	Viable	+	+
1	32 000	2-15	76 550	Viable	+	+
1	14 000	2-15	5090	Viable	+	+
1	11 000	1	1335	Viable	+	+
1	10 000	2-15	3755	Viable	+	+
1	7000	1	1121	Viable	+	+
1	5600	2-15	2115	Viable	+	+
1	4000	2-15	5085	Viable	+	+
1	2900	2-15	6130	Viable	+	+
1	2000	2-15	3265	Viable	+	+
1	1200	2-15	361	Viable	+	–
1	800	2-15	628	Viable	+	+
1	800	1	18 650	nv	–	+
1§	600	2-15	1965	Viable	+	–
1	300	2-15	537	nv	–	+
1	200	2-15	703	nv	–	+
1	100	2-15	528	Viable	+	+
1	100	2-15	48 950	nv	–	+
1	100	2-15	–	–	–	+
2	100	1	–	–	–	+
ISO negatives						
13§	–	–	29 750, 4845, 3425, 2585, 1124, 906§, 830, 632, 525, 399, 316, 301, 188	nv	+	+
60¶	–	–	–	–	+	+
2	–	–	2055, 1231	Viable	–	–
5	–	–	956, 740, 227, 159, 145	Viable	–	+
101					87	97

*SG = serogroup of *Leg. pneumophila*, determined by latex agglutination test.

†nv = *Leg. pneumophila* were not viable

‡MC_{100} = microbiological criterion at a limit of 100 CFU per l and 100 cellEq per l, respectively; MC_{1000} = microbiological criterion at a limit of 1000 CFU per l and 1000 cellEq per l, respectively; + = results of ISO method and SBM do match; – = results of ISO method and SBM do not match.

§One sample positive for *Legionella* spp. other than *Leg. pneumophila*.

¶Two samples positive for *Legionella* spp. other than *Leg. pneumophila*.

from 100 to 87 000 CFU per l (mean = 8567 CFU per l, median = 1200 CFU per l). Regarding serogroups, five out of the 21 were *Leg. pneumophila* SG 1, 16 out of 21 were *Leg. pneumophila* SGs 2-15. Evaluation of results under consideration of official microbiological criteria (100 and 1000 CFU per l) showed that 10 of the 21 positive water samples were between MC_{100} and MC_{1000} and 11 were above MC_{1000}. Eighty samples (79%) were negative for *Leg. pneumophila*. Beside this, *Legionella* spp. was found in four samples, one of them in combination with *Leg. pneumophila* SGs 2-15 (Table 1).

The SBM detected viable *Leg. pneumophila* in 21 (21%) of the samples with counts of 145–76 550 cellEq per l (mean = 5888 cellEq per l, median = 1335 cellEq per l). Applying MC_{100} and MC_{1000}, eight out of these 21 positive samples were between MC_{100} and MC_{1000} and 13 were above MC_{1000}. In 17 (17%) of the samples nonviable *Leg. pneumophila* were detected, ranging from 188 to 48 950 cellEq per l (mean = 6745 cellEq per l, median = 830 cellEq per l). Sixty three samples (62%) were negative for *Leg. pneumophila*.

Data comparison of both methods

For comparison of both methods, positive findings of the SBM (viable cells present) and positive results by the ISO method were compared in order to find out whether the new molecular approach was equivalent to the bacteriological standard method used for official testing of water samples for compliance with microbiological criteria. In the comparison, samples with nonviable *Legionella* cells in the SBM and samples with *Legionella* spp. by the ISO method were excluded.

With respect to MC_{100}, 87 samples (86%) gave consistent results (73 negative and 14 positive results). Seven samples of each (7%) did not match and were either positive or negative by one or the other method. The seven samples which did not conform to the results revealed by the ISO method showed 100 to 800 CFU per l, but no viable *Leg. pneumophila* by the SBM. On the other hand, seven other samples that were negative by the ISO method, showed 145–2055 cellEq per l including viable ones tested by the SBM.

For MC_{1000}, the concordance was even higher (96%), with 87 and 10 results consistently negative and positive respectively. Only one out of 11 samples exceeded MC_{1000} when tested by the ISO method (1200 CFU per l) but not by the SBM. On the other hand, three samples were negative or under MC_{1000} by the ISO method, but did not comply when analyzed by SBM showing 1231, 1965 and 2055 cellEq per l.

Diagnostic specificity and sensitivity of the SBM at MC_{100} were 91 and 67%, respectively, at MC_{1000} they were 97 and 91%, respectively, taking the ISO method as the reference and gold standard. Statistically, the two methods did not differ significantly in the frequency of not matching results for both criteria, for MC_{100} ($P = 1.000$) and for MC_{1000} ($P = 0.625$) respectively. Moreover, the agreement of both methods are substantial for MC_{100} ($\kappa = 0.579$) and almost perfect for MC_{1000} ($\kappa = 0.811$) respectively.

Discussion

Application range of the compared test systems

The developed SBM detects *Leg. pneumophila* but no other species of the *Legionella* genus. However, *Leg. pneumophila* is the most important species to cause legionellosis and is responsible for 90–100% of the reported cases (WHO 2007). The SGs 1–14 of *Leg. pneumophila* all tested positive, which is an advantage of the SBM. For the lacking SGs 15 and 16, that were not tested in our study, the assay is expected to work also, as the DNA relatedness within one species of *Legionella* is very high (WHO 2007). *Legionella* spp. other than *Leg. pneumophila* that are not detected by the SBM, are by far less frequently isolated from patients (5–10% of cases) and are predominantly involved in nosocomial infections (WHO 2007). For these species, a PCR system for the precursor 16S rRNA region could easily be developed and implemented in the SBM.

For official control of water samples for *Leg. pneumophila*, the ISO method serves as the reference method. This procedure might not highlight the true status of a water sample (Greenhalgh 1997). For example, VBNC legionellae are known to occur and could lead to false negative results (Kirschner 2016). Furthermore, false positive results could happen as shown in our analysis of field samples, where a presumptive *Legionella* colony was confirmed as *Leg. pneumophila* SGs 2-15 by the latex test, but turned out to be *Acinetobacter tjernbergiae* by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). This false positive result could have been avoided earlier using a blood agar plate. Further cross-reactions with other bacteria have been reported for latex tests (Fields *et al.* 2002). In addition, false negative results are possible because some *Legionella* spp. cannot be cultivated as easily as *Leg. pneumophila* on agar plates used in the ISO method, as they seem to be more sensitive to the acid or heat treatment in the ISO procedure.

Methodology

For quantification, the −STIM was used. Theoretically, −STIM and +STIM should both reveal the same results. No relevant increase in cell counts occur during the stimulation time that is shorter than the doubling time of *Leg. pneumophila* that is 6 h (Warren and Miller 1979) or more (Mauchline *et al.* 1994; Mampel *et al.* 2006). In reality, slightly lower counts for the +STIM were often seen, probably due to the additional centrifugation step for +STIM and sample transfer to another tube, leading to a loss of material. For these reasons, the −STIMs were more suitable for quantification.

In multiple RNA extractions from stimulation experiments, it was shown that contaminating DNA within the RNA fraction was on a low level. Moreover, the same amount of residual background DNA is expected in both −STIM and +STIM, so that it does not influence the possible C_T shift that is based on the RNA. Furthermore 10-fold dilutions of water spiked with starved *Leg. pneumophila* ranging from 4×10^4 to 4×10^1 cells per l were processed by SBM to test C_T shift at varying levels of bacteria concentration. Results showed comparable C_T shifts of 2.3, 4.0, 2.2 and 3.0, respectively, over this wide range of dilutions.

The broth used for stimulation was a yeast extract enriched with commercial growth supplement for legionellae. Basically, this is BCYE broth without activated charcoal, whose benefit is to bind toxic free radicals generated under light exposure or by autoclaving. We omitted the addition of activated charcoal, as we sterile filtered the medium instead of autoclaving and incubation took place in the dark. Moreover, this approach dispels concerns of clogging spin columns by charcoal during NA extraction.

A stimulation time of 3 h seemed to be reasonable since under laboratory conditions, as starved cells already showed C_T shifts after less than 1 h of stimulation (Table 2). As it was not known, how naturally grown legionellae behave and induce their rRNA synthesis under stimulation, the duration was chosen as long as possible to measure the maximum effect, but shorter than the doubling time of legionellae.

Pro and cons of methods

The advantages of the SBM clearly outweigh the disadvantages. Advantages include the speed of the SBM, allowing results to be obtained within 8 h upon arrival of water samples in the laboratory, compared to 1 week or more for the ISO method. In the context of clinical cases or outbreaks, rapid testing of water as a potential source of infection is especially a huge advantage. Also during sanitation of water supply networks in affected buildings, a short test time to monitor failure or success of the applied measures is required. The economic consequences of closed facilities, such as public baths, are directly

Table 2 Cycle threshold values (C_T) for DNA and precursor rRNA samples that were periodically extracted during a 4 h stimulation experiment with *Legionella pneumophila*

	DNA*	Precuror rRNA†	
Time (min)	C_T	C_T	C_T shift
0	20.5	26.8	0
20	20.8	18.5	8.3
40	21.0	16.8	10.0
60	21.3	15.1	11.7
80	20.8	14.6	12.2
100	20.8	13.9	12.9
120	20.6	13.3	13.5
180	20.6	12.9	13.9
240	20.7	12.7	14.1

*DNA quantification was performed by qPCR.
†Precursor 16S rRNA was detected by reverse transcription-PCR. C_T shifts in reference to 0 min (before stimulation) were used as an indicator for viability.

linked to the duration of closure until sanitation is carried out.

In contrast to common molecular methods, the SBM is able to distinguish between viable and nonviable cells. This is based on induction and detection of precursor 16S rRNA indicating viability (Cangelosi and Brabant 1997; Weigel *et al.* 2013), as leader and tail sequences are removed immediately after synthesis. Besides RT-PCR, qPCR was done for quantification of DNA which was simultaneously extracted with RNA. On possible disadvantage of the SBM might be that total DNA is quantified by this procedure, and not only DNA of viable *Leg. pneumophila*, leading to overestimation of cell counts. Therefore, this method alone cannot be applied to test water samples for compliance of microbiological criteria as these criteria include only living cells. High DNA amounts can still be present after sanitation of water supply networks, although the number of viable legionellae is low. However, if no viable legionellae are detected, eventual present DNA is out of concern and interest. Therefore, it is important to interpret quantification data always in combination with the RT-PCR results. In contrast, the ISO method probably underestimates the legionellae count in water, as VBNC bacteria are not readily cultivable.

Furthermore, the SBM implicates a higher manual workload and costs compared to the ISO method. The filtration step is comprised in both methods, but the SBM additionally comprises a NA extraction and PCR detection. NA extraction is especially a labour-intensive step but it could be automatized for a higher throughput. In addition, NA extraction is relatively expensive and linked to appropriate equipment.

In reference to the ISO method, specificity and sensitivity of the SBM were quite high, especially for the MC_{1000} with values of 97 and 91% respectively. For MC_{100} they were 91 and 67% respectively. Both methods have advantages and disadvantages but they might be used for different occasions: environment assessment in the context of clinical cases or outbreaks, water analysis by the SBM has clear advantages as mentioned above, compared to the ISO method that is more preferable for screening purposes.

Improvements and further research

The performance of the developed method is satisfactory but there is still potential for improvement. Among others, the applied simultaneous extraction of DNA and RNA fractions is reasonable and was chosen for better comparison, but this procedure lead to a higher loss of both fractions, compared to separate extraction of DNA and RNA from two aliquots of an identical sample (data not shown). Simultaneous *vs* separated extraction, as well as its costs, should be evaluated prior to future studies or applications of the method. Furthermore, the use of the Rotor-Gene PCR cycler instead of LightCycler not only showed lower C_T values in general for the very same sample, but also showed less variation between duplicates and larger C_T shifts in RT-PCRs. Moreover, the LOD was one $\log_{10}$ lower (100 cells per l) using the Rotor-Gene for the very same samples and PCR conditions compared to the LOD revealed by the LightCycler (1000 cells per l) with regard to the combined result (qPCR plus RT-PCR).

The VBNC status that legionellae achieve in water is a poorly characterized condition (Al-Bana *et al.* 2014). This study did not answer the question, whether VBNC legionellae can be stimulated or not. Further investigations with confirmed VBNC *Legionella* cells are required to better understand RNA synthesis' stimulation compared to growth. In fact, 7% of the study samples were positive for viable *Leg. pneumophila* by the SBM but did not show growth on agar plates. They might have been able to activate their RNA synthesis but were not able to grow on plates. Otherwise, cell counts by the SBM were not considerably higher compared to the ISO method, even though DNA of dead bacteria was included.

In conclusion, the method is a useful and reliable tool for a rapid detection of viable *Leg. pneumophila* of all serogroups in tap water samples with high diagnostic sensitivity and specificity and can be used as an alternative to the ISO method for the official water analysis for *Leg. pneumophila* and particularly when a short test time is required.

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Conflict of Interest

The authors declare no conflict of interest.

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