Institute for Hygiene and Public Health
WHO Collaborating Centre for Health Promoting Water Management and Risk Communication

Application of quantitative PCR to monitor *Legionella pneumophila* contamination in drinking water plumbing systems in comparison to the standard culturing method according to German law

Christiane Schreiber, Nicole Zacharias and Thomas Kistemann

IHPH - Institute for Hygiene and Public Health, WHO CC for Health Promoting Water Management and Risk Communication, Medical Geography and Public Health Research Group, University of Bonn, Sigmund-Freud-Straße 25, 53105 Bonn, Germany

Christiane.Schreiber@ukb.uni-bonn.de, Nicole.Zacharias@ukb.uni-bonn.de, Thomas.Kistemann@ukb.uni-bonn.de

Introduction – Monitoring and culturing

Since the amendment of the German drinking water ordinance (German DWO) in 2012, the occurrence of *Legionella* spp. has to be monitored in drinking water plumbing systems (DWPS) of buildings where hot water is stored centrally and offered to the public, or is used commercially. The monitoring has to be done by standard culturing methods and is rated with reference to a technical threshold level (TTL) of 100 CFU/100mL. The culturing method is known to potentially underestimate contamination in a DWPS, because it does not take into account the possibility of *Legionella* occurring in viable-but-nonculturable (VBNC) states. It is known that bacteria are able to leave this VBNC state and regain cultivability and infectiousness. Gene detection by classic real-time PCR enables to find all DNA of a species, even hidden contamination of bacteria in the VBNC state.

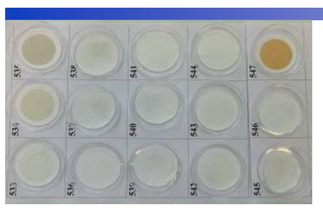

Figure 1: Membrane filters with 1L sample

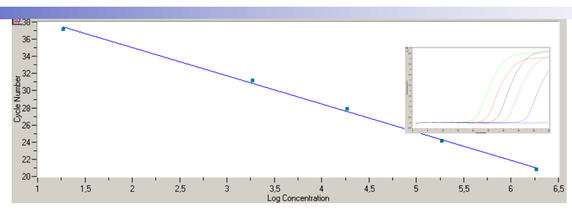

Figure 2: Standard curve used for *L. pneumophila* qPCR (Slope: -3,29)

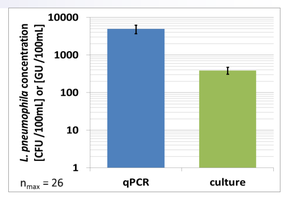

Figure 3: Pre-test experiments

Table 1: Primer and probe sequences as used for qPCR analysis. The sequences as described by Shannon et al. (2007) were modified for more heat stability.

Primer/ Probe	Organism and Target gene	Sequence (5' -> 3') (Shannon et al. 2007, modified)	Melting point [°C]
Forward primer	*L. pneumophila* mip	ACC GAT GCC ACA TCA TTA GCT	54,9
Reverse primer		CCA AAT CGG CAC CAA TGC	52,8
Probe		FAM- CAG ACA AGG ATA+A+G+T TGT CTT-BBQ	49,2

Methods

- ✓ DWPS of nine buildings with known *Legionella* contamination were monitored according to German law and by following DIN EN ISO 19458 (2006) for the analysis of water quality at an outlet in the DWPS (objective b).
- ✓ Monitoring over a period of six month in order to determine long term effects following a fixed sampling schedule.
- ✓ Short term variations of *Legionella* contamination were investigated in one building by sampling every two hours (morning till late afternoon) in summer and winter.
- ✓ Cultivation of *Legionella* spp. in 100mL according to German DWO (2001 and 2012) and DIN EN ISO 11731-2 (2008).
- ✓ Evaluation followed DIN EN ISO 8199 (2008).
- ✓ Determination of isolates using the Latex agglutination test kit (Oxoid) for species and serogroup differentiation (*L. pneumophila* SG 1, *L. pneumophila* SG 2-14, other *Legionella* species).
- ✓ For comparison with the cultivation results and the TTL of 100 CFU/100mL, it was necessary to detect relatively low *L. pneumophila* concentrations using qPCR.
- ✓ One litre water sample filtrated through a cellulose nitrate filter (figure 1).
- ✓ Cell lysis using Proteinase K and chelator Chelex Resin 100, followed by heat incubation (1h 56°C, 10min 95°C) and centrifugation (7,700g) according to Walsh et al. (1991). In pre-tests, this method showed the highest DNA recovery (50%).
- ✓ Reduction of sample volume in a SpeedVac to an end volume of 50µl.
- ✓ Primer and probe sequences for amplification of the *L. pneumophila* by macrophage infectivity potentiator (mip) gene (Shannon et al., 2007) (table 1).
- ✓ Ratio of qPCR reaction components (total volume 25µL) and temperature protocol were determined by the polymerase mixture used (Maxima Probe qPCR Master Mix, Fermentas).
- ✓ The final detection limit was 200 Gene Units *L. pneumophila* DNA in 1L sample, considering the detection limit of the qPCR standard curve as 20 GU/µL DNA-extract (figure 2, using the Light Cycler, Roche), the reduced sample volume (50µL), and the mean recovery rate of the DNA extraction method (50%).

Results

- ✓ Cultivation shows high spatial and temporal variability (up to 4 $\log_{10}$) of *Legionella* concentrations in all buildings over a half year period (n=777, maximum 64,400 CFU/100mL, figure 4) as well as over the course of a day (n=32, figure 5).
- ✓ Only 10 of 105 sampling points showed continuously high *Legionella* concentrations exceeding the TTL during long term monitoring.
- ✓ Positive qPCR results were determined in 303 of 732 (41%) samples. They showed a variation of 3 to 6 $\log_{10}$ within a building, and up to 3 $\log_{10}$ within one tap (figure 4).
- ✓ Only 5.0 to 16.7% of taps within a building show positive *Legionella* results in all samples. Three buildings showed no permanent detectable contamination.
- ✓ Statistically high significant ($p<0.001$), but weak ($r=0.319$) Pearson correlation for all data pairs (n=647) of *L. pneumophila* (cultivation) and *L. pneumophila* (qPCR).

Results

- ✓ Positive qPCR results in combination with no *Legionella* growth on agar plates point to the presence of *Legionella* in VBNC states.
- ✓ Matrix effects, proofed by inhibition of an internal standards, only explain part of the negative qPCR results in case of positive cultivation (table 2).
- ✓ "Real" false-negative qPCR results were often observed when the *Legionella* concentrations were lower than 200 CFU/100mL. This is about 10-fold higher than the detection limit of 200 GU/L (figure 6).
- ✓ The detected GU concentration was often lower than colony growth. This is contrary to results obtained in previously performed experiments, which showed qPCR results being at least one $\log_{10}$ higher than cultivation results (figures 3 and 6).

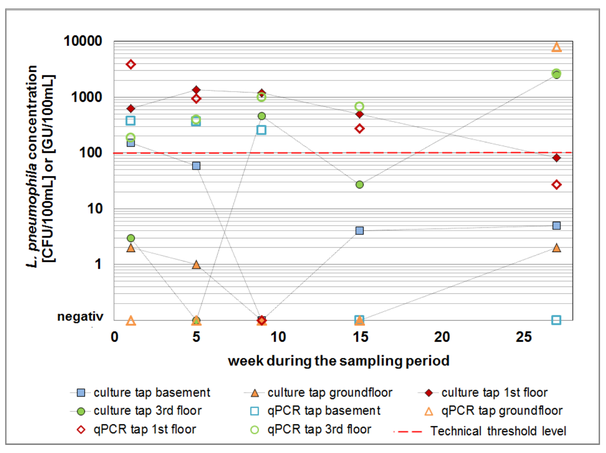

Figure 4: Long-term variations in *Legionella* contamination in building B.

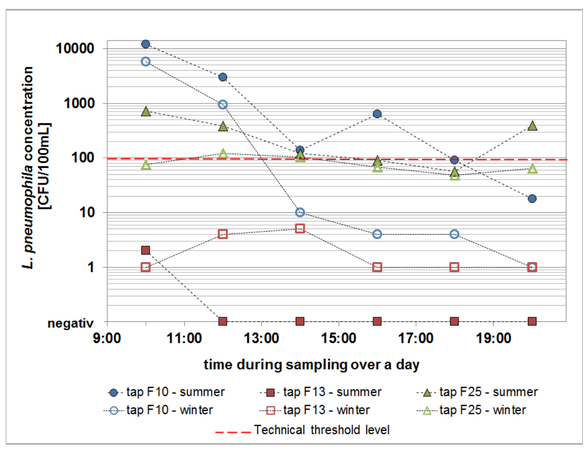

Figure 5: Short-term variations in *Legionella* contamination in building F.

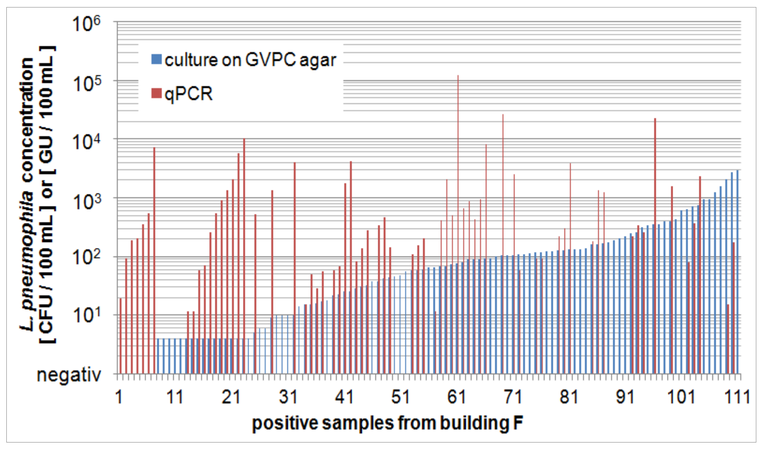

Figure 6: Positive culturing in comparison to qPCR results by example of building F

Table 2:
Inhibition by matrix effects and false-negative qPCR results in building G

n = 106	qPCR positive	qPCR inhibited	qPCR negative
Positive Culturing	24 (23%)	11 (10%)	26 (25%)
Negative Culturing	15 (14%)	11 (10%)	19 (18%)

Conclusions

- ✓ Detected long-term and short-term variations of *Legionella* concentrations in DWPS should result in a re-evaluation of guidelines which propose sampling strategies for monitoring bacterial contamination in DWPS.
- ✓ Exclusion of DNA from dead bacteria cells from detection with qPCR with e.g. propidium monoazide (PMA) to select cells with intact membrane integrity.

Conclusions

- ✓ Due to negative qPCR results in the case of *Legionella* growth on agar plates, qPCR is currently not suitable to be used on its own to monitor drinking water quality in DWPS, but it can be useful in supporting culturing methods.
- ✓ The comparison of both methods showed that the results of qPCR and cultivation were not always consistent with each other..

References

- Shannon, K.E., Lee, D.Y., Trevors, J.T. & Beaudette, L.A. (2007). „Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment". Science of The Total Environment 382 (1): 121–29.
- Walsh, P., Metzger, D. & Higuchi, R. (1991): Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques 10 (4): 506–513.

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