rqmicro
rapid quantitative microbiology

LEGIONELLA RAPID TEST INTRODUCED IN BERLIN WATER COMPANY LABORATORY

Reported cases of legionnaire's disease have been on the increase in Switzerland and Germany for years. To get the situation under control, fast, cultivation-independent methods for quantitative identification of Legionella in water samples are indispensable. The laboratory of Berlin Water Company internally validated the rapid test from rqmicro, a Swiss spin-off from the ETH Zurich.

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In 2017, the Swiss Federal Office of Public Health registered around 500 cases of legionnaire's disease – this corresponds to an increase of 35% compared to 2016. At the beginning of 2018, the media reported extensively about it and informed that the government intended to set up a task force to get the situation under control. Germany faces the same problem: according to the Robert Koch Institut, a German federal government agency and research institute responsible for disease control and prevention, there are more fatalities due to this disease than to road accidents. Legionnaire's disease is on the rise in the whole of Europe, as shown in the European «Surveillance and outbreak report» from July 2017 (fig. 1). It concludes that there is a lack of fast and reliable control mechanisms for water systems to prevent outbreaks.

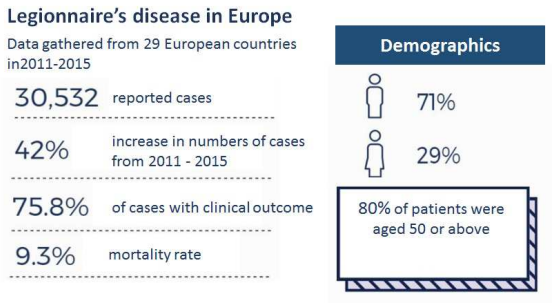

Fig. 1 Increase of legionnaire's disease according to the «Surveillance and outbreak report» from July 2017.

Legionella bacteria were discovered and described in 1976 only. A congress of the American Legion, a veterans' association, took place during which numerous participants contracted a mysterious pneumonia. After an intensive search, doctors and microbiologists identified a bacterium to have triggered this "legionnaire's disease" and called it *Legionella* [2]. Outbreaks are particularly serious in hospitals, old people's homes or hotels. However, there is also a risk in regular residential buildings. In past years, Germany has seen a certain number of cases in newly built domestic installations that were microbiologically contaminated and their rehabilitation proved to be difficult [3, 4].

The challenge when combatting *Legionella* lies first with the fast and reliable detection of the bacteria. The current standard method is ISO 11731 [5], and the analysis can take up to two weeks – which is lengthy in case of an outbreak of legionnaire's disease. To lag two weeks behind an epidemic can have fatal consequences.

Standard detection method takes two weeks

The established standard method (ISO 11731) for nearly every microbiological analysis is based on full or selective bacteria cultivation on agar plates. The cultivation method is labor-intensive, takes 10 to 15 days, and leads to variable results [6-8]. An extensive round robin test in which different American laboratories participated even showed that the *Legionella* concentration could be misjudged considerably when done with cultivation methods [9]. A reason why is that accompanying flora overgrow the agar plates partially or completely, this therefore obstructs or inhibits the growth of *Legionella* [10]. Replicability of measuring results lowers with increasing accompanying flora, as for example with *Legionella* in surface water. Another reason for false negative results is that part of the *Legionella* present in a sample is in fact alive but does not grow on agar plates [11] and the standard methods do not pick it up (fig. 2). Viable but non-culturable (VBNC) cells occur increasingly after stagnation and chemical or

thermal disinfection of water systems. These VBNC cells are potentially still dangerous for human beings [12].

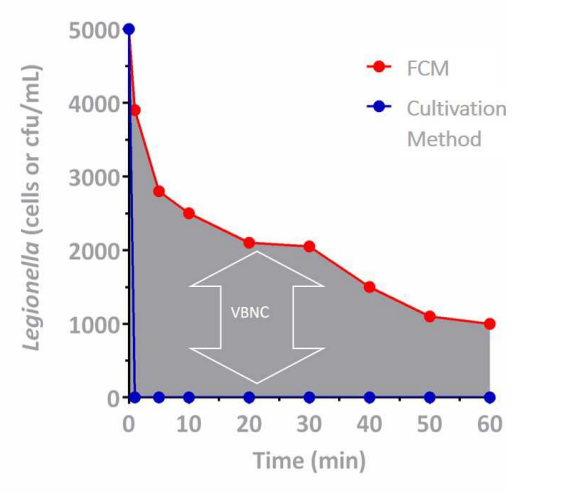

Fig. 2 Real-time surveillance of a disinfection procedure at 70 °C. The rapid test via immuno-magnetic separation and flow cytometry (FCM) shows, VBNC Legionella are still potentially existent after a 60-minute disinfection process. The cultivation method does not detect VBNC Legionella*, as they do not grow on agar plates.*

Rapid analysis enable real-time monitoring

Fast and cultivation-independent processes to show the exact level of microbial contamination are required because of the given reasons. The laboratory of Berlin Water Company (BWB) shares this opinion; it supervises the quality of Berlin's potable and wastewater treatment in all process steps. The space of time between sample taking and findings is too long for an accompanying analysis of possible decontamination procedures after diagnostic findings, Uta Böckelmann, Head of BWB's accredited laboratory, believes. "That is why we were looking for a possibility to identify *Legionella* concentration in the shortest possible time span". Different *Legionella* species exhibit different infectious potential as generally known [1], the BWB's laboratory called for the latest method to detect and quantify the species with the highest potential danger, the *Legionella pneumophila* SG1.

Cultivation independent single cell count in under two hours

The Swiss start-up developed a method to isolate, purify and quantify *Legionella* from complex water samples in a short period. Basis is the immunomagnetic separation (IMS) of the targeted cells with the help of magnetic particles coated with highly specific monoclonal antibodies. Target cells (in this case the *Legionella*) are bound via antibodies to the magnetic particles and are then separated by means of a magnet from the rest of the sample and are thus purified. Fluorescent markers on the antibodies additionally dye the target cells; the flow cytometry (FCM) is therefore able to quantify them after the IMS. It takes approximately two hours from concentration to get the findings (Fig. 3).

Contrary to established methods, the new rapid test records all potentially infectious *Legionella* – including the cells that do not grow on agar plates (VBNC cells). It is even possible to differentiate between living and dead cells thanks to a viability dye (fig. 4), whereupon the VBNC cells can be found in the fraction of living cells.

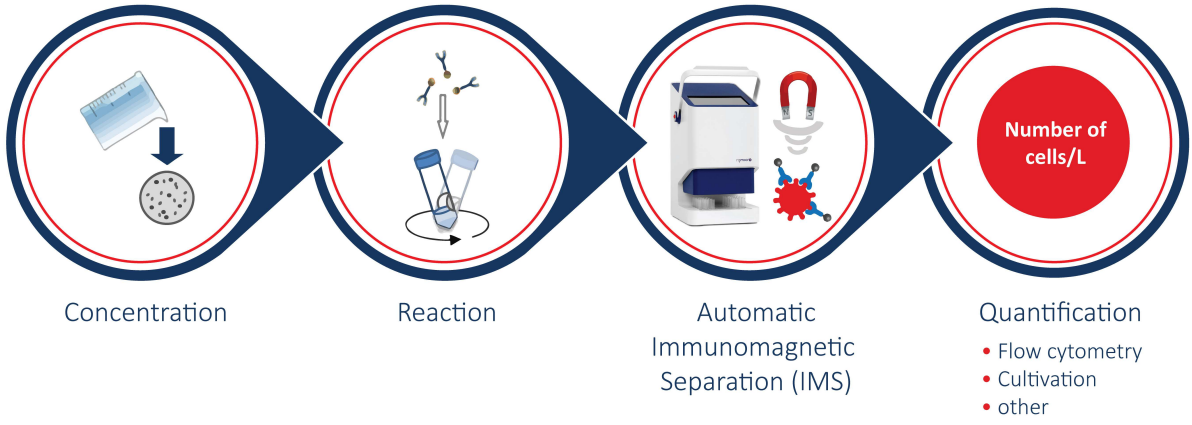

Fig. 3 Legionella *rapid test: quantitative results in under two hours: simple operational procedure from concentration to quantification with flow cytometry.*

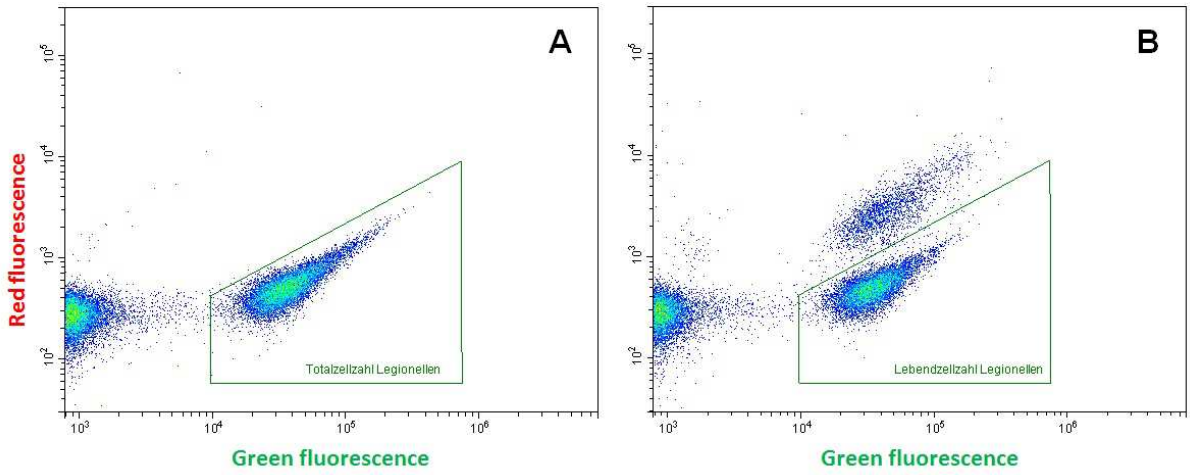

Fig. 4 Legionella pneumophila *SG1 cells spiked in Evian and processed according to the rapid test protocol. (A) Sample stained with dye (green):* L. pneumophila *SG1 total cell number in the green window. (B) Sample with staining dye (green) and viability dye (red):* L. pneumophila *SG1 viable cell count in the green window. The dead cell population is stained red and green and located outside the green window.*

To guarantee a high reproducibility and to eliminate operator bias, rqmicro's inter-disciplinary team developed the CellStream, an instrument for a fully automated IMS. The standardized IMS process using disposable microfluidic cartridges delivers ready-to-use samples for quantification by the flow cytometry or other downstream analytical methods like PCR.

Some water supplies like e.g. in Basel or Geneva as well as companies with cooling units or analytical laboratories have already implemented this new analysis method. BWB's laboratory has successfully deployed the method for six months and plans to test drinking water installations in 200 premises on a regular basis with it. The company is the sector's champion in Germany due to its size and it looks back to 160 years-old tradition. More than 4000 employees deliver annually more than 200 million cubic meters of best drinking water from nine waterworks and clean approximately 245 million cubic meters of wastewater in their six wastewater treatment

plants. Its laboratory tests more than 20.000 drinking water samples per annum.

To ensure quality and to get maximum insight of the state of their waters, BWB's laboratory not only relies on the rapid *Legionella* test but also on other innovative technologies. In the sector relatively unknown is the digital visualizing tool GENOTRAIL, issued from the collaboration between Blue Biolabs and Datalyze Solutions. It gives new insights and correlations between areal and temporal separated events. Lab results, primarily microbiological findings, are put in relation to time and place of sample taking as well as other parameters and then clearly visualized. "This facilitates the causal research enormously", Uta Böckelmann explains.

BWB's laboratory reveals equivalence of *Legionella* rapid test to ISO 11731

To validate the *Legionella* rapid test internally, BWB's laboratory defined its recovery rate and conducted comparisons with the standard method. To establish the recovery rate, it added different cell counts of *Legionella pneumophila* SG1 into phosphate buffered saline to analyze cell suspensions with a combination of IMS and flow cytometry. The average recovery rate amounted to 80% (fig. 5). To compare with ISO 11731, it prepared cell suspension in PBS from a fresh liquid culture and worked on it directly in this matrix. Afterwards, it directly plated this sample material with different volumes following ISO 11731, incubated for 10 days at 36 °C and then counted. It analyzed a second part of the sample material with the combination of IMS and flow cytometry as described above. Results showed that the new rapid test is 10 times more sensitive than the plating process (fig. 6)

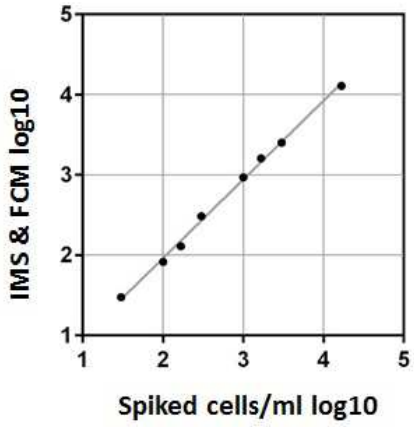

Fig. 5 Recovery rate for performance tests amounted to 80% in average and the correlation to > 99% in a broad scope of application of 30 to 16667 cells/ml. L. pneumophila *SG1 cells in PBS, flow cytometry analysis according to IMS with the CellStream.*

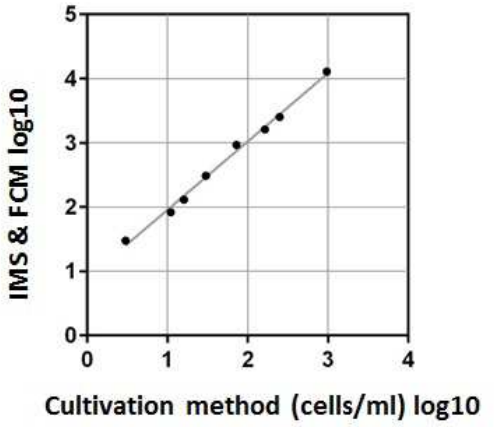

Fig. 6 Analysis by flow cytometry after IMS with the CellStream shows a sensitivity 10 times higher compared to the ISO 11731-method. Depending on the sample type, this factor is subject to change under laboratory conditions.

Practical examples

BWB's laboratory subjected the Swiss rapid test for *Legionella pneumophila* SG1 to a specificity test in the context of a sample taking in buildings. It analyzed *Legionella* concentration in a drinking water installation of building A with the plating method according to ISO 11731. Four sampling points exceeded considerably the technical intervention value of 100 CFU / 100 ml as put down in the German "Drinking Water Ordinance". This intervention value

corresponds to the peak value as decreed by the Swiss Federal Department of Home Affairs in the act about "Drinking Water and Water in Publicly Accessible Baths and Shower Facilities". The laboratory then purified the same samples via CellStream and analyzed via flow cytometry. The results showed no abnormality / the results were negative. DNA sequencing of the identified *Legionella* colonies according to ISO 11731 revealed that none of the colonies was of the *Legionella pneumophila* SG1 type (tab. 1). This emphasizes the high specificity of the antibodies used in filtering and analyzing methods.

Test point	Type of circuit	*L.spp.* after ISO 11731 CFU/100 ml	*L.p.* SG1 via CellStream/FC (cells/100ml)	Identification via qPCR	
				MIP-Gen (GU) specific for *L.spp*	SG1-Gen (GU) specific for *L.p.* SG1
1	cold	2100	0	11	0
2	warm	300	0	0.5	0
3	cold	400	0	0.5	0
4	cold	2200	0	1.5	0

Tab. 1. Methods according to ISO 11731 and the rapid test examined Legionella *concentration in drinking water systems in building A. Detected colonies in the ISO 11731 test were identified by qPCR as* Legionella spp. *The negative results of the rapid test show their specificity as* L. pneumophila *SG1.*

Another example in a sample taking in buildings documents the short time span from sample taking to receipt of quantitative and meaningful results with the rapid test compared to the standard method. The laboratory sampled building B in the course of a routine analysis for *Legionella* in the drinking water system. It then analyzed the samples according to ISO 11731. Three sampling points showed *Legionella* concentration above or around the peak value of 100 CFU/100 ml. It subjected the sampling points that were conspicuous according to the ISO method to a parallel test with both ISO and the new rapid method after thermal disinfection. The rapid test showed a concentration of *Legionella pneumophila* SG1 in the range of 1×105 –2,5×105 cells/100 ml in all samples after a processing time of approximately 3 hours. The differentiation by viability dye however showed that 99% of detected cells were dead material. The cultivated samples confirmed the analysis without result 10 days later (tab. 2). The rapid test detected a small amount of living cells whereas the ISO method showed no *Legionella* in 100 ml. The explanation is that IMS / flow cytometry detects viable but non-culturable *Legionella* (fig. 2). Time savings thanks to the new method means considerable financial savings in case of an emergency, for example after exceeding the intervention value in a re-cooling unit in a big industrial production facility.

Test point	Type of circuit	*L.spp.* after ISO 11731 CFU/100 ml	L.p. SG1 via CellStream/FC (cells/100ml)		
			total	viable	% dead L.p. SG1
x	warm	0	186560	350	99,81%
y	warm	0	149660	150	99,94%
z	warm	0	134340	30	99,98%

Tab. 2 The drinking water system in building B exceeded the intervention value according to ISO 11731. The building was analyzed for Legionella *again via both ISO 11731 as well as the new rapid test after heating / thermal treatment. The rapid test proved the effectiveness of the treatment within three hours; the standard method took ten days to attain the same negative result.*

Accreditation and rapid tests for other pathogens

BWB's laboratory plans to have the rapid test method for *Legionella pneumophila* SG1-15 accredited until the end of the year. It has taken preparatory steps. The ETH start-up rqmicro is meanwhile busy broadening its product range and applying its method to other pathogens from water and food as for example giardien and cryptosporidium, salmonella, E. coli as well as pseudomonad. The new analyzing method is in the process of ISO accreditation. First level of validation is to be achieved this year.

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