Simple method for routine detection of *Legionella* spp. in tap water

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The monitoring of the water quality can be difficult because the microorganisms have to be recovered from the water before detection. At Rigshospitalet the surveillance of *Legionella* spp. from tap water is a part of the routinely surveillance to minimise the risk for hospital acquired infections. The aim was to find the best and simples method by compare different sittings for the analysis of *Legionella* spp.: Water volume, different filters pore size, transferring method, pre-treatment of the water and comparing the recovery of *Legionella* spp. on MWY agar plates from three different companies. The study revealed that there was a significant higher recovery rate of *Legionella* spp. in water samples of 100 ml using filters with a pore size of 0.22 μm that were re-suspension in sterile water before transferring. There were no differences in the recovery rate between the different pre-treatment methods. The MWY agar plates from two companies were superior to the third company. Our recommended for large scale routine investigation of water could be filtration of 100 ml untreated tap water filtered with a pore size of 0.22 μm followed by re-suspension in sterile water before culturing.

Keywords: Water samples; *Legionella* spp.; water volume; filter pore size; pre-treatment of water; Hospital

1. Introduction

Potential hospital environmental reservoir for pathogenic microorganisms has to be monitored so hospital acquired infections (HAI) and epidemic situation can be avoided. To monitor the environment can be difficult because the recovery of pathogenic microorganisms from the surroundings is depending on the chosen detection method and where the samples are taken. Furthermore, the methods used for screening the hospital environment have in some cases the need to be modified depending on the situation.

HAI with *Legionella pneumophilae* from water supply is a common problem worldwide in hospitals, but also HAI caused by other water borne bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter* spp. and *Stenotrophomonas maltophilia* has been reported [1-4]. All these bacteria can colonize the water system by making biofilm which can consists of different bacteria species in pipes, taps, and aerators etc. [5-9]. The bacteria from the biofilm are constant released into the water supply and thereby change the number of bacteria recovered.

A study in 1995 from Statens Serum Institute found *Legionella* spp. in 96 of 116 water samples from 12 Danish hospitals [10]. In Denmark there are 6-8 cases of HAI caused by *Legionella* spp. each year with a mortality rate of 30-50%, which is a decrease of more than 50% compared with the situation 10 years ago [11-15].

Most standards and guidelines recommend the use of 1000 ml water for testing the present of *Legionella* spp.. Furthermore, pre-treatment of the water with heat or acid to break down the amoeba in which the *Legionella* spp. often lives, but also to reduce the number of other bacteria species. [7, 16-19] Water samples of 1000 ml are a heavy volume to carry around when several taps are tested simultaneously, which is the case at Rigshospitalet, and the after-treatment is time consuming. The international standard (ISO 11731-2:2004) and the Danish standard [19] of water quality on detection *Legionella* spp. recommend a minimum water volume of 100 ml but preferably 500 ml or 1000 ml and filter pore size of 0.45 μm.

The aim of this study was to investigate if a simpler approach for testing water samples for *Legionella* spp. could be used for daily routine testing when taken the recommendation from the national and international standards into account. The importance of sample volume (1000 ml vs. 100 ml), filter pore size (0.45 um vs. 0.22 um), transfer/recover method (filter directly on agar plates vs. re-suspension in sterile water), and sample pre-treatment (untreated, head- and acid treated) was tested. The importance of the composition of Modified Wadowsky and Yee (MWY) to recover *Legionella* spp. was investigated with MWY from three different companies

2. Material and methods

30 water samples were collected from taps in kitchens, toilets and patients rooms in different units at Rigshospitalet, where *Legionella* spp. was known to be present. General for all the samples are after filtration the collected *Legionella* spp. on the filter were transferred to MWY agar plates (Oxoid Limited) and incubated at 35^{o}C for 4 and 7 days. On day 4 plates with > 50 colonies of *Legionella* spp. were counted, identified and serotyped by agglutination kit (Oxoid Limited) and on the day 7 the remaining plates were counted.

The statistical significance was calculated using a two-tailed student t-test with a significant level of $p \leq 0.05$. To ensure the data was normal distributed normal score plot was calculated.

2.1 Filter pore size and sample size

100 ml untreated tap water were filtered with a pore size of either 0.45 µm or 0.22 µm (Merck KGaA, Germany) to investigate the influence of pore size on the detection of *Legionella* spp. The impact of the sample size was performed by comparing 100 ml and 1000 ml of untreated tap water which were filtered with a pore size of 0.22 µm (Merck KGaA, Germany). For both the comparisons the filters were placed directly on MWY agar plate (Oxoid Limited) and the filters were removed after one hour.

2.2 Pre-treatment of water

To studying the influence of pre-treatment of tap water, volumes of 1000 ml tap water were used. Before the pre-treatment the tap water were filtered with a pore size of 0.22 µm. The membrane was folded and transferred using sterile tweezers to a sterile tube containing 10 ml milliQ water and the materiel on the membrane was re-suspended by mixing for 30 sec. on vortex mixer (Multi Reax, Heidolph, Germany). After re-suspension 200 µl of water was heat-treated at 50°C for 30 min. and 200 µl of water was acid treated with HCL/KCL buffer pH 2.2 and was incubated for 5 min. at room-temperature. 100 µl of both untreated, heat- and acid treated water was transferred to MWY agar plates for incubation.

2.3 Comparing MWY agar plates from different firms

Commercial MWY agar plates from Oxoid Limited, Liofilchem® SRL and Tritrium Microbiologie B.V were compared with the following conditions: 100 ml of water was filtered with a pore size of 0.22 µm (Merck KGaA, Germany) and the further preparation is as described above with re-suspension.

3. Results

3.1 Transferring method to agar plates

The highest recovery of *Legionella* spp. from tap water was with re-suspension in sterile water (25/30) compared to filter directly placed on agar plates, figure 1. This difference in the transfer method in the recovery of *Legionella* spp. from tap water samples was statistical significant ($p<0.0001$).

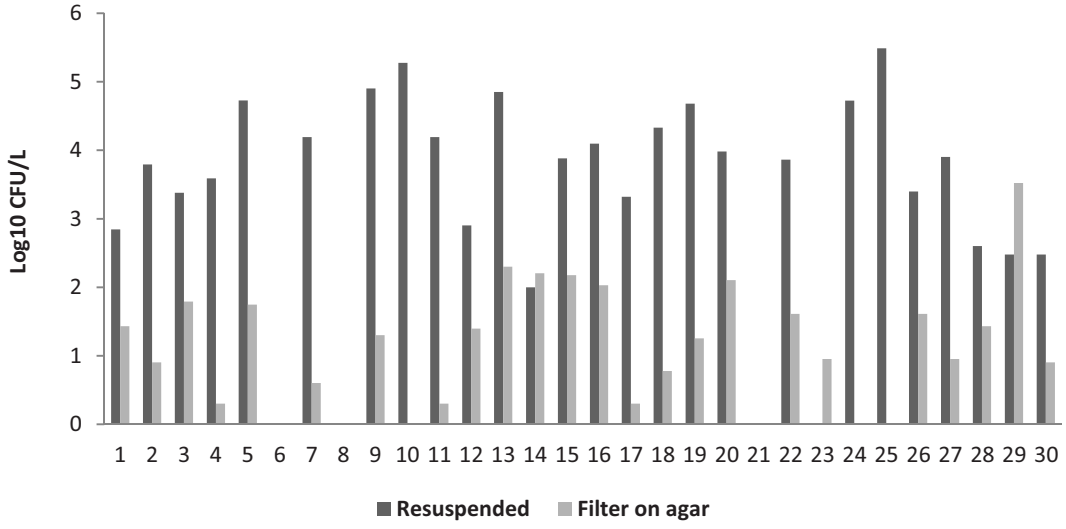

Fig. 1 The recovery of two transferring methods filter directly placed on agar plate for one hour and re-suspension of the material from filters in sterile water. The filter pore size was 0.22 µm in both methods and the tap water was untreated.

3.2 Filter pore size and sample size

The filter with pore size 0.22 µm had a significant ($p=0.0046$) higher recovery of *Legionella* spp. from the tap water samples (18/30) then the tap water samples filtered with pore size 0.45 µm (8/30), figure 2A. Also the recovery of *Legionella* spp. was significant ($p<0.0001$) higher for tap water samples with the volume of 100 ml (25/30) comparing to the sample volume of 1000 ml (2/30), figure 2B. Three tap water samples were without *Legionella* spp. growth.

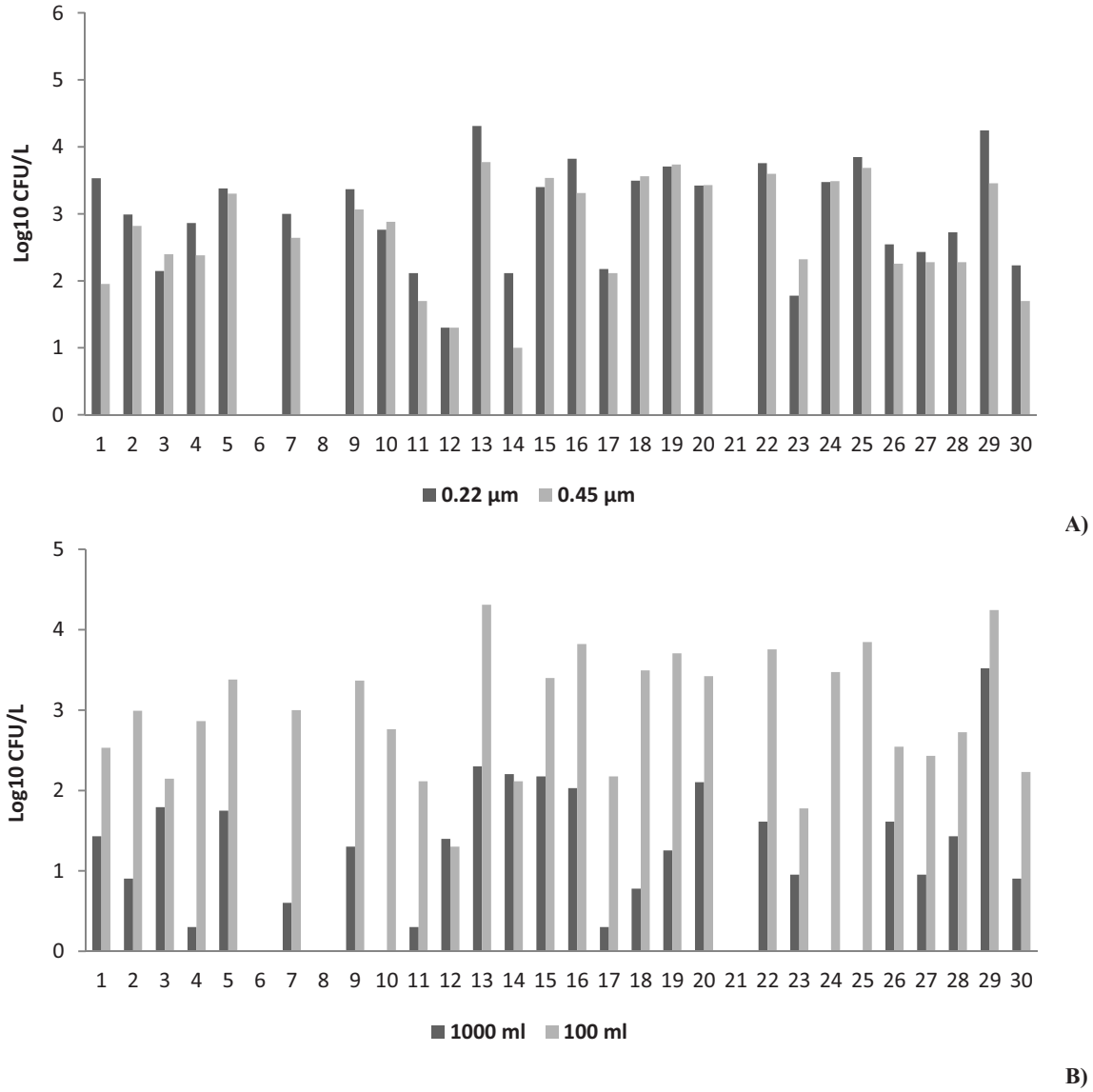

Fig. 2 A) The recovery of *Legionella* spp. in untreated tap water samples using filters with pore size of 0.45 µm and 0.22 µm. The volume of the water samples were 100 ml for both pore sizes. B) The recovery of *Legionella* spp. in untreated tap water samples with the volume of 1000 ml and 100 ml filtered with pore size 0.22 µm

3.3 Pre-treatment of water

Untreated tap water samples (13/30) had the highest recovery of *Legionella* spp. but were not significant to heat treated (5/30, p=0.08) or acid treated (5/30, p=0.96) tap water samples, figure 3. The same tendency is seen in the comparison in recovery of *Legionella* spp. in heat treated and acid treated tap water samples (p=0.22).

3.4 Comparing MWY agar plates from different firms

The MWY plates from Oxoid Limited were superior to the MWY agar plates from Liofilchem® SRL (p<0.0001), but not to the plates from Tritrium Microbiologie B.V. (p=0.09), figure 5. Plates from Tritrium Microbiologie B.V. were significant better than the MWY agar plates from Liofilchem® SRL (p=0.003).

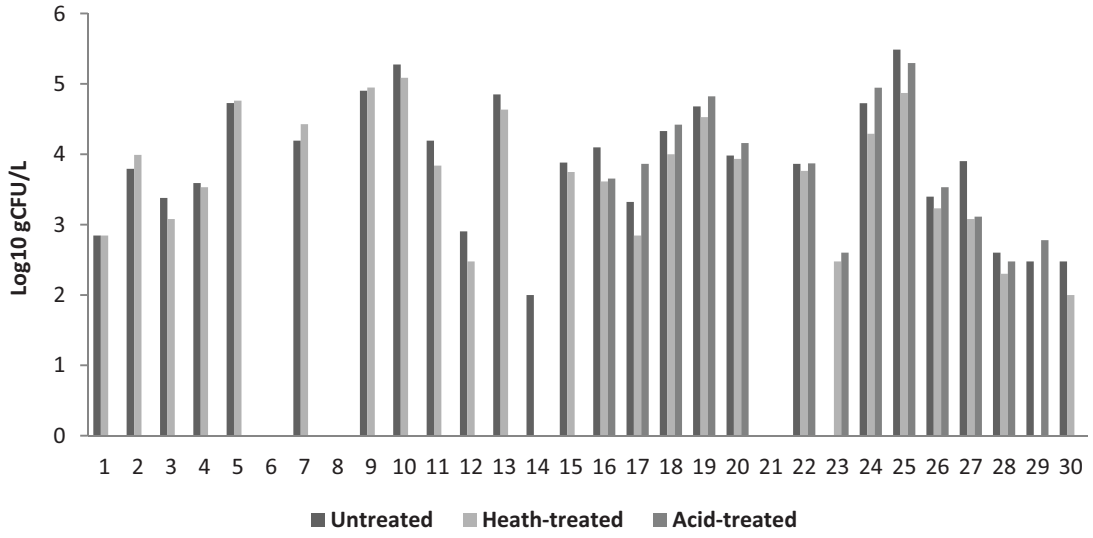

Fig. 3 The ability of heat treated and acid treated tap water samples to recover *Legionella* spp. compared to untreated tap water.

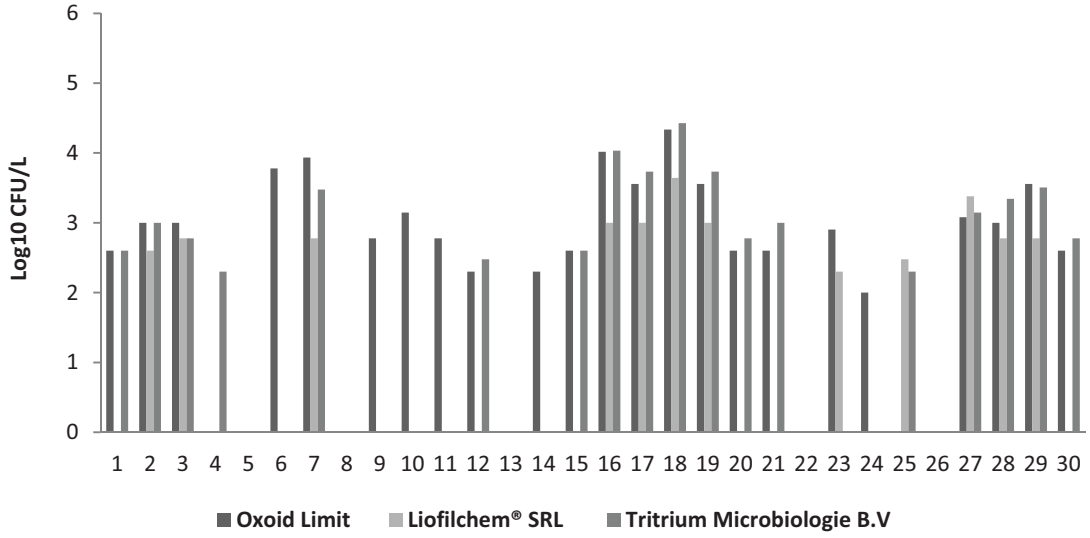

Fig. 4 The recovery ability of *Legionella* spp. from untreated tap water samples on MWY agar plates from three different companies. The tap water volume used is 100 ml and the filter pore size 0.22 μm**.**

3. Discussion

Rigshospitalet is a tertiary hospital with about 1200 beds. Thus, there are several thousand of water taps, water coolers and ice machines in the hospital. Water samples are taken routinely to ensure the bacteriological water quality and to minimize the exposure for immune compromised patients to *Legionella* spp. and other opportunistic bacteria which can live in water. It was therefore of interest to evaluate the method recommended for water surveillance in order to find a method that was practical useful for routine sampling and testing of water, when handling many samples simultaneously. The most laborious part of water surveillance is testing for *Legionella* spp. as large water samples with complex procedures are usually recommended. On that background the focus was on testing for *Legionella* spp. in water samples in this study.

Often when investigating the presence of *Legionella* spp. in water, the volume examination is 1000 ml [7, 16, 17, 20-22]. This is a large sample size when many tap places are tested simultaneously, which is the case for us under routine sampling. A sample size of 100 ml is more reasonable to handle which also is the minimum water volume when testing for *Legionella* spp. according the Danish standard [19] and international standard (ISO 11731-2:2004) Therefore, sample size of 1000 ml water was compared with 100 ml and a significant higher recovery rate of *Legionella* spp. was found when using 100 ml of water. The explanation for this result could be that water contains a high level of organic

and inorganic material which may interfere with the filtration of water samples especially large volumes and the recovery of viable *Legionella* spp. The report regarding organic and inorganic material in the tap water leading to Rigshospitalet shows that allowed limits are kept. Another explanation for the high difference could be that the re-suspension step on the vortex mixer is not long enough for all the material captured by the filter to be released when the sample size is 1000 ml.

A literature search shows that both filters with pore size of 0.45 µm and 0.22 µm are used when filtered water samples are examined for the presented of *Legionella* spp. [7, 16, 17, 20-23]. Our findings show that recovery is higher when filters with pore size 0.22 µm are used. The reason can be that free-living *Legionella* spp. have the physical dimensions 2-20 µm in length and 0.3 - 0.9 µm in width, which may result in some of the *Legionella* spp. getting through the filter when pore size 0.45 µm is used [24]. The recovery of higher number of *Legionella* spp. with a 0.22 µm filter could also indicate that the tap water from Righopitalet contains many free living *Legionella* spp. *Legionella* spp. which often are living inside amoebas will be captured with both filter pore size because the amoebas containing *Legionella* spp. which mainly are *Harmannella vermiformis* and *Naegleria* sp. which have a size of 25-40 µm and 10-15 µm, respectively [18, 23].

The recovery of *Legionella* spp. using different pre-treatment methods which such destroy the amoebas did not have a major influence on the recovery of *Legionella* spp. The almost identical findings between the three pre-treatment methods could indicate that the amoebas are broken doing vortexing. It has been shown that heat treatment is killing some of the *Legionella* spp. and acid treatment to inhibit the growth with 30% [25, 26]. The simplest way to destroy the amoebas to recover the *Legionella* spp. sufficiently must therefore be to re-suspend the material from the filter using a vortex-mixer.

It is common known that the medium have influence on the growth of bacteria and MWY agar plates containing a selective supplement which favor the growth of *Legionella* spp.. The MWY agar plates tested from Oxoid Limit, Liofilchem® SRL and Tritrium Mcrobiologie B.V. revealed large variation in recovery of *Legionella* spp. The difference may be due to composition of the agars used by the companies, but it can also depend on the quality of the raw material used for the production of MWY agar. The raw material used for the MWY agar from Oxoid Limit and Tritrium Mcrobiologie B.V is the same, and this is most likely the reason why there is no significant between these two MWY agar plates.

In conclusion a simple method consisting of 100 ml untreated tap water filtered with a pore size of 0.22 µm filter. Afterwards re-suspension in sterile water and cultured on specific MWY plates could be recommended for large scale routine investigation of water samples. Some of the recommendations may be hospital specific and should be investigated locally.

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