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Legionella colonisation in hot water systems in care homes from two Danish municipalities

Niss Skov Nilsen [a,*] and Søren Anker Uldum [b]

[a] Danish Building Research Institute (Build), Division of Sustainability, Energy and Indoor Environment in Buildings, Aalborg University, A.C. Meyers Vaenge 15, 2450 Copenhagen SV, Denmark
[b] Department of Bacteria, Parasites and Fungi, Statens Serum Institut (SSI), Artillerivej 5, 2300 Copenhagen S, Denmark
*Corresponding author. E-mail: nbsn@build.aau.dk

NSN, 0000-0002-4705-5143

ABSTRACT

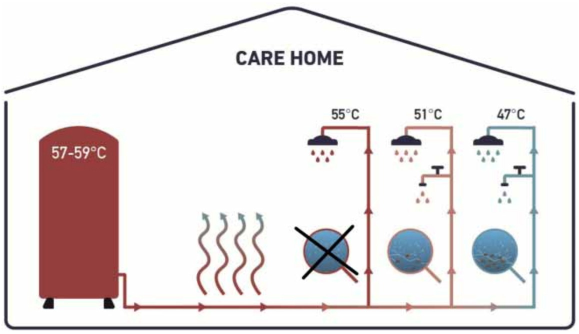
Legionnaires' disease is a serious health risk among the elderly. Water systems in care homes are therefore of particular interest. We investigated the levels of culturable *Legionella* in the hot water systems in care homes in two Danish municipalities. Two hundred and sixty-eight water samples from 98 care homes were evaluated. Contents of culturable *Legionella* counts were calculated, and correlations between temperature and colony-forming units (CFU/L) were analysed. Seventy-seven and 81%, respectively, of the care homes were colonised with *Legionella* in the two municipalities. Most care homes had less than 1,000 CFU/L, but 13 and 16% had more than 10,000 CFU/L. When including first flush samples, 27% of the care homes in Municipality 1 had *Legionella* levels above 10,000 CFU/L. Temperatures of $\geq$50 °C in Municipality 1 and $\geq$55 °C in Municipality 2 correlated with low levels of *Legionella*. The content of *Legionella* colonies was significantly higher in care homes in Municipality 1. However, a significantly higher proportion of taps in Municipality 2 had *Legionella* colonies. In conclusion, temperatures should be raised to 55 °C to avoid high *Legionella* levels. Test procedures should be evaluated, and the regular use of taps and routine testing for *Legionella* should be taken into consideration.

Key words: colonisation rate, elderly, health risk, Legionnaires' disease, test procedures, water temperature

HIGHLIGHTS

- Legionnaires' disease is a serious health risk among the elderly.
- Seventy-seven and 81% of the hot water systems in care homes from two Danish municipalities were colonised with *Legionella*.
- Water temperatures above 55 °C were related to the low contents of *Legionella*.
- Test procedures for the measurement of *Legionella* should be evaluated.
- Routines for the regular use of taps and showers should be implemented at care homes.

GRAPHICAL ABSTRACT



INTRODUCTION

According to the World Health Organization (WHO), *Legionella* (*L.*) *pneumophila* is the waterborne pathogen that poses the highest health burden to humans in Europe (WHO 2017). This bacterium can cause severe life-threatening pneumonia called Legionnaires' disease (LD). Denmark is one of the countries in the European Union with the highest reported incidence of LD (4–5 cases per 100,000 inhabitants). Denmark, along with several other European countries, has seen an increase in LD throughout the last decade (ECDC 2020). Danish cases are characterised by an overall lethality rate of approximately 10%, and the risk of getting LD increases with age (Statens Serum Institut [SSI] 2020). Approximately 5% of cases are associated with care homes where there are high lethality rates of 30–50% (SSI 2019, 2020).

As LD is a serious health risk, especially among the elderly, the quality of water and water systems in care homes is of particular relevance in limiting the risk of acquiring this disease. Danish municipalities are responsible for regulating and controlling such risks in care homes and other public institutions (Danish Building Research Institute [Build] 2019).

The WHO (2017) recommends a national limit of 1,000 *Legionella* colony-forming units per litre (CFU/L), and many European countries have already established similar limits. In contrast, Denmark has not yet announced official limits regarding the acceptable concentration of *Legionella* in potable or hot water systems. The Danish SSI has recommended the limits of 1,000 CFU/L (*L. pneumophilia* Serogroup 1) and 10,000 CFU/L (Serogroups 2–14) for Danish municipalities. Most of them use these limits, even though they are not official governmental limits (SSI 2020).

Furthermore, there are neither national surveillance programmes enacted by the law for testing for the presence of *Legionella* in Danish water systems nor national recommendations or guidelines for municipalities to test their water systems for *Legionella* regularly. Nevertheless, some Danish municipalities conduct the risk assessments of their institutions and regularly test the water systems in these facilities (Build 2019).

Many Danish care homes were built in the 1970s when the average water use per person was twice as high as it is today (Build 1986; Danish Technological Institute [DTI] 2019). Thus, many water systems in these care homes have much higher water capacity than needed (Danva 2018). This contributes to low water flow in the hot water systems of many care homes, which could potentially promote the favourable conditions for biofilm formation and the growth of *Legionella* (Build 2019; Nielsen & Aggerholm 2022). In addition, the hot water systems in many care homes are designed without circulation, which result in long periods of low water temperatures in the water pipes and taps (Build 2019). This also increases the risk of *Legionella* growth and biofilm contamination in the hot water systems (Abdel-Nour *et al.* 2013).

High water temperatures could be effective in controlling the growth of *Legionella*. Of particular interest, water temperatures consistently held over 55 °C seem to be effective (Lee *et al.* 2017), whereas the effect of thermal shock (e.g., raising the water temperature once a week to 70 °C) lacks supporting evidence (Lee *et al.* 2017). Such procedures may cause a selection of thermotolerant bacterial strains if the intermittent thermal shocks are combined with a generally low water temperature the rest of the time. This raises concerns because thermotolerant strains may have a greater significance in terms of harming human health than other strains (Whiley *et al.* 2017).

Finally, aspects of test procedures and techniques are to be discussed. The use of first flush samples from water taps (A-samples) and flush samples (B-samples) at stabile temperature is one aspect. The use of the WHO-recommended culture method and other test procedures to reach the objective of obtaining reliable results from *Legionella* tests is another aspect to be discussed (Uldum *et al.* 2022).

Independent of each other, two large Danish municipalities/cities conducted investigations into the presence of *Legionella* in the water systems of their care homes for the elderly in 2016 and 2017. Both have had relatively few registered cases of LD, with one to two cases per 100,000 inhabitants per year, which is two to four times below the average for the entire country. According to the Danish National LD Surveillance Register at SSI, Denmark, no LD cases were known to be associated with the investigated institutions. This does not necessarily exclude illnesses or deaths associated with *Legionella* infections among residents. Many elderly people acquire pneumonia regularly and may have severe symptoms or even die from it without being investigated for infection with *Legionella* (Build 2019). Numerous cases of pneumonia, as well as some deaths, due to *Legionella* infection are, therefore, expected to occur among elderly residents of care homes without establishing an association with a *Legionella* infection.

The first aim of this study was to determine the presence of culturable *Legionella* in the hot water systems of the care homes in two Danish municipalities. The second aim was to analyse differences in the concentration of *Legionella* in water samples from care homes in the two municipalities. Finally, we calculated the relationship between *Legionella* concentration (CFU/L) in B-samples and water temperature in the hot water systems from the care homes in the two municipalities.

METHODS

Water samples

All of the care homes in this study were supplied with untreated groundwater, and none were treating the water within their premises.

In 2016 and 2017, 1 L of water samples were collected from the hot water systems in all the care homes of two Danish municipalities and analysed for the presence of *Legionella*.

All of the water samples were selected and collected from taps and showers by accredited firms based on the recommended criteria to take samples from taps/showers located most distant from the central boilers in the hot water system. In Municipality 1, an average of 3.6 samples were taken per care home, whereas in Municipality 2, two samples were taken per care home. In a few cases, samples were taken from all the taps/showers in care homes (Table 1).

In Municipality 1, 162 samples were taken from 44 care homes. The samples were divided into 135 A-samples (first flush) from the 44 care homes and 27 B-samples (at constant temperature) from 8 of the care homes. All samples were collected in March and April 2016.

In Municipality 2, 106 flush samples were collected (B-samples) from 54 care homes in September 2017.

Culture method

All the water samples were sent to the SSI in Copenhagen, Denmark, for culture. The samples were processed within 24 h according to the International Organization for Standardization Standard No. 11731, 2017. In short, $2 \times 500\ \mu l$ were plated directly on two glycine vancomycin polymyxin cycloheximide (GVPC) (Oxoid, Thermo Fisher Diagnostics) agar plates, 1 L were filtered through a $0.22\ \mu m$ polyethersulphone membrane filter (MicroFunnel Plus, Pall Life Science) and each filter was vortexed with glass-beads for 4 min with 10 ml of the sample. From each filtrate, 2×100 and $2 \times 500\ \mu l$ were seeded to GVPC agar plates. The plates were incubated at 36 °C in plastic bags for 7 days. The plates were inspected after 2 days and if the growth of interfering bacteria was observed, aliquots of the filtrate (kept at 4–10 °C) were heat- and acid-treated, and $2 \times 100\ \mu l$ of each aliquot were plated on GVPC agar plates and incubated for 7 days. *Legionella* colonies were counted for each plate, and the highest colony count among the three steps was reported as the result and expressed as a concentration in CFU/L. Direct plating was used for enumeration only when $\geq$5 colonies were identified in total on the two plates. The limit of detection by the culture method is 10 CFU/L and the limit of quantification is 100 CFU/L. At least five colonies (if present) from each positive sample were analysed with the Oxoid *Legionella* Latex Test (Thermo Fisher Scientific, Waltham, MA, USA) to separate them into categories of *L. pneumophila* Serogroup 1, Serogroups 2–14 and other *Legionella* species. The representative isolates of Serogroup 1 were further analysed with the Dresden panel of monoclonal antibodies (Helbig *et al.* 2002) to identify the virulence-associated epitope recognised by monoclonal antibody 3/1 (mAb 3/1) for further risk assessments. If *Legionella* was not detected ($<$ 100 CFU/L), 0 was used in the calculations. The culture method is the only accepted method for *Legionella* risk assessment by the Danish Environmental Protection Agency (MST 2015).

Table 1 | Investigation of *Legionella* in hot water systems from care homes in two Danish municipalities

	Number of investigated care homes in municipality	Average number of water samples per care home	Proportion of taps with CFU/L $>$ 0 of all water samples (A and B)	Proportion of care homes with CFU/L $>$ 0 in.water samples (A or B)
Municipality 1 ($n = 162$)	44	3.6	57%	77%
Municipality 2 ($n = 106$)	54	2	75%	81%
Significance (p)			^$p = 0.003$**	^$p = 0.467$ ns

CFU/L, colony-forming units per litre; *, significance level; ns, not significant; n, number of samples; ^, p-value based on the two-sided chi-square test.

Statistical analysis

We used SPSS-v. 27 (IBM USA) to analyse data. Results were considered significant when $p \leq 0.05$. The results did not have a Gaussian distribution pattern; therefore, a non-parametric analysis was used.

The mean counts (CFU/L) of *Legionella* from each care home were used in the analysis (Tables 1–4) to reduce bias-based on differences in the number of samples – as much as possible. In the regression models (Figures 1 and 2), all samples with temperature and *Legionella* measurements (CFU/L) were used.

The contents of *Legionella* in the samples, in the care homes and between the municipalities, were tested with the Mann–Whitney *U* test. The proportions of the samples and the care homes with *Legionella* were tested with the two-sided chi-square test.

The overall proportions of taps and care homes with *Legionella* contents in the two municipalities are shown in Table 1.

In Tables 2 and 3, the highest content of *Legionella* in one sample and the mean counts of *Legionella* in the care homes were calculated. Furthermore, the distribution of care homes with CFU/L levels above 0, between 1,000 and 10,000 and above 10,000 is provided. The median counts were also calculated and compared with the mean counts to illustrate whether the levels and distributions of *Legionella* in the samples were uniform.

A comparison between the A-samples from the 8 care homes (where B-samples were collected) and the A-samples from all 44 care homes in Municipality 1 is detailed in Table 2. The B-samples and the ratio between the results of the B-samples compared to the A-samples from the 8 care homes are also shown in Table 2.

In Table 3, the *Legionella* contents in the B-samples and the distribution of care homes in Municipality 2 with different levels of *Legionella* content are presented. In addition, an approximation of the expected *Legionella* content in the A-samples was calculated based on the ratio between the B-samples and the A-samples from Municipality 1 (Table 2). Finally, the results from B-samples in Municipality 2 are compared with the results from Municipality 1 in Table 3.

In Table 4, the mean and median counts of *Legionella* from the B-samples are compared between the two municipalities for the care homes with central water temperatures below 50, 50–55 and above 55 °C.

Regression models were used to illustrate the general effects of water temperature on the *Legionella* counts (CFU/L) in the hot water systems. In Municipality 1, the *Legionella* counts in the 27 B-samples from the 8 care homes and their water temperatures are illustrated in Figure 1. Likewise, the *Legionella* counts (CFU/L) and the water temperatures based on the 106 B-samples from the 54 care homes in Municipality 2 are depicted in Figure 2.

RESULTS

The measurements of the *Legionella* content in the care homes' water systems in the two municipalities revealed that a large proportion of the hot water installations in the care homes were colonised with *Legionella* (77% in Municipality 1 and 81% in Municipality 2; Table 1). A significantly higher proportion of the taps contained *Legionella* in Municipality 2 (75%) compared to Municipality 1 (57%) (Table 1).

Only *L. pneumophila* was detected in the positive water samples. Most systems were colonised with *L. pneumophila* Serogroups 2–14 only. Serogroup 1 was only found in a few institutions (8% in both municipalities). All investigated Serogroup 1 colonies belonged to the mAb 3/1 negative (less virulent) group in both municipalities (data not shown).

High numbers of *Legionella* units were seen in some samples from Municipality 1. Up to 600,000 CFU/L was seen in an A-sample and 134,000 CFU/L was seen in a B-sample (Table 2). The mean content of *Legionella* was 35,784 CFU/L in the A-samples from the care homes with *Legionella*, and the mean count for all of the A-samples taken from the 44 care homes was in the same order of the magnitude (34,327 CFU/L). The median counts were generally much lower than the mean counts. Furthermore, the mean content in 27% of all the care homes in Municipality 1 exceeded 10,000 CFU/L for A-samples, corresponding to one-third of the care homes with *Legionella*.

The results for the 8 care homes (where B-samples were collected) in Municipality 1 generally demonstrated that the numbers of CFU/L in the A-samples and the distribution of care homes with high, medium and low contents of *Legionella* were in the same magnitude as the results from all 44 care homes (Table 2). Only the content of *Legionella* in all A-samples was significantly lower ($p = 0.023$; equivalent to 10%) for the 8 care homes compared to the result of all 44 care homes. The median count for A-samples from the 8 care homes was 1,000 CFU/L, and the median count for all the samples was 800 CFU/L. This is lower (4,000 CFU/L for all) and higher (150 CFU/L for all) compared to the median counts for all the 44 care homes in Municipality 1.

Table 2 | Investigation of *Legionella* content in hot water systems from care homes in Danish Municipality 1

	Highest number of CFU/L in one water sample	Mean number of CFU/L in care homes with *Legionella* (median)	Mean number of CFU/L in all samples (median)	Proportion of care homes with CFU/L > 0	Proportion of care homes with mean 1,000 < CFUL ≤ 10,000	Proportion of care homes with mean CFU/L > 10,000
A-samples ($n = 135$) from all 44 care homes in Municipality 1	600,000	35,784 (4,000)	34,327 (150)	77%	34%	27%
A-samples ($n = 27$) from 8 care homes in Municipality 1	250,000	34,852 (1,000)	30,983 (800)	85%	19%	31%
B-samples ($n = 27$) from 8 care homes in Municipality 1	134,000	7,962 (2,525)	8,371 (0)	40%	16%	13%
Ratio and significance between B- and A-samples (B/A) from 8 care homes in Municipality 1	0.54	0.23 #$p = 0.014$*	0.27 #$p = 0.003$**	0.47 ^$p = 0.000$***	0.84 ^$p = 0.529$ ns	0.42 ^$p = 0.328$ ns
Ratio and significance between A-samples from 8 care homes compared to all 44 care homes: A(8)/ A(44) in Municipality 1	0.42	0.97 #$p = 0.088$ ns	0.90 #$p = 0.023$*	1.10 ^$p = 0.222$ ns	0.56 ^$p = 0.193$ ns	1.15 ^$p = 0.345$ ns

CFU/L, colony-forming units per litre; *, significance level; ^, p-value based on the two-sided chi-square test; #, p-value for Mann–Whitney U test; ns, not significant; and n, number of samples.

Table 3 | Investigation of *Legionella* content in hot water systems from care homes in Danish Municipality 2 and differences in *Legionella* content between Municipality 1 and Municipality 2

	Highest number of CFU/L in one water sample	Mean number of CFU/L in care homes with *Legionella* (median)	Mean number of CFU/L in all samples (median)	Proportion of care homes with CFU/L > 0	Proportion of care homes with 1,000 < CFUL ≤ 10,000	Proportion of care homes with CFU/L > 10,000
B-samples ($n = 106$) from 54 care homes in Municipality 2	81,000	5,358 (900)	4,380 (350)	81%	24%	16%
Approximated A-samples from 54 care homes in Municipality 2¨	150,000 (app 54%)	23,296 (app 23%)	16,222 (app 27%)			
Ratio and significance between B-samples in Municipality 1 and Municipality 2 (B-Municipality1)/B-Municipality2)	1.65	1.49 #$p = 0.030$*	1.91 #$p = 0.006$**	0.49 ^$p = 0.002$**	0.67 ^$p = 0.560$ ns	0.81 ^$p = 0.743$ ns

*, significance level; ns, not significant; ^, p-value based on the two-sided chi-square test; #, p-value for Mann–Whitney U test; n, number of samples; ¨, approximation of A-mean in Municipality 2 based on B/A gradient from 8 care homes in Municipality 1; CFU, colony-forming units; app, approximately.

Table 4 | Relation between water temperature in categories and contents of *Legionella* in hot water systems from care homes in two Danish municipalities

	Mean water temperature in water systems in care homes < 50 °C (*n*)	Mean CFU/L in B-samples from care homes with water temperature < 50 °C (median)	Mean water temperature in water systems in care homes 50–55 °C (*n*)	Mean CFU/L in B-samples from care homes with water temperature 50–55 °C (median)	Mean water temperature in water systems in care homes > 55 °C (*n*)	Mean CFU/L in B-samples from care homes with water temperature > 55 °C (median)
Municipality 1 ($n = 27$)	47.1 (9)	23,956 (8,000)	52.4 (6)	205 (0)	61.1 (12)	118 (0)
Municipality 2 ($n = 106$)	43.7 (21)	13,683 (6,000)	53.0 (61)	2,933 (100)	56.8 (22)	606 (100)
Significance (p) between Municipality 1/ Municipality 2	#0.981 ns	#0.777 ns	#0.258 ns	#0.034*	#0.000***	#0.008**

CFU/L, colony-forming units per litre; *, significance level; ns, not significant; #, *p*-value for Mann–Whitney *U* test; *n*, number of samples.

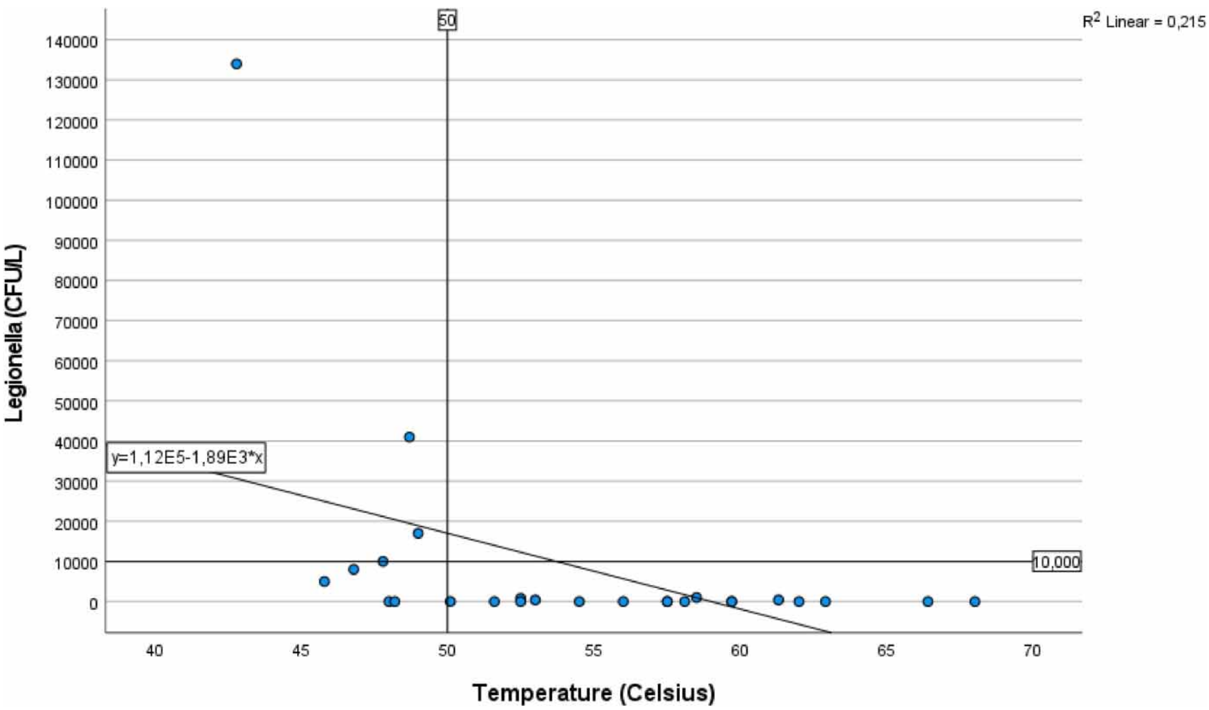


Figure 1 | *Legionella* colony counts (CFU/L) in relation to temperature in hot water systems from 8 care homes (27 samples) in a Danish municipality (Municipality 1); $y = 1.12 \times 10^5 - 1.89 \times 10^{3x}$. $R^2 = 0.215$.

The overall proportion of care homes that were positive for *Legionella* was significantly lower for the B-samples (40%) compared to the A-samples (77 and 85% in the 44 and 8 care homes, respectively) in Municipality 1 (Table 2). The mean number of CFU/L in the B-samples was 7,962 CFU/L in the care homes and 8,371 CFU/L in all samples from the 8 care homes, which corresponds to about a quarter of the mean content of *Legionella* in the A-samples (Table 2). The contents of *Legionella* in all the B-samples ($p = 0.003$) and in the B-samples in the care homes with *Legionella* ($p = 0.014$) were significantly lower compared to the contents in the A-samples.

A significantly higher proportion of the B-samples from the care homes in Municipality 2 contained *Legionella* (81%) compared to Municipality 1 (40%) (Table 3). The content of CFU/L in the care homes with *Legionella* is significantly lower ($p = 0.030$) in Municipality 1, and the contents of *Legionella* in the samples were also significantly lower ($p = 0.006$) in Municipality 1 compared to Municipality 2. The median counts were of the same magnitude in the two municipalities.

The regression models between temperature ('constant' temperatures) and *Legionella* counts in the water samples (Figure 1) showed that the regression line was significant for Municipality 1 ($p = 0.016$), $R^2 = 0.215$, and the regression formula of the line was: $y = 1.12 \times 10^5 - 1.89 \times 10^{3x}$. For Municipality 2 (Figure 2), the regression line was significant ($p = 0.003$), $R^2 = 0.082$, with a regression line of $y = 3.71 \times 10^4 - 6.22 \times 10^{2x}$.

From Figure 1, it is apparent that a temperature of 50 °C or higher is related to low or undetectable levels of *Legionella* in the water samples from Municipality 1. The results from Municipality 2 showed similar results for temperatures of 55 °C or higher. As half of the observations below 50 °C in the water samples showed high levels of *Legionella* (> 10,000 CFU/L) in both municipalities, the odds ratio (OR) of observing high levels of *Legionella* in the water samples (> 10,000 CFU/L) is 0.5 if the hot water temperature is below 50 °C.

Table 4 shows that the *Legionella* content in the B-samples (CFU/L) from the care homes with water temperatures from 50 to 55 °C was significantly higher ($p = 0.034$) in Municipality 2 (2,933 CFU/L) compared to Municipality 1 (205 CFU/L). Furthermore, even though the mean temperature in the care homes with water temperatures > 55 °C was significantly lower in Municipality 2 compared to Municipality 1 ($p = 0.000$), the content of *Legionella* (CFU/L) was significantly higher ($p = 0.008$) in the care homes in Municipality 2 (606 CFU/L) compared to the care homes in Municipality 1 (118 CFU/L).

DISCUSSION

A large proportion of the care homes tested in Municipalities 1 and 2 were colonised with *L. pneumophila* (77 and 81%, respectively). Furthermore, 13% of the care homes in Municipality 1 and 16% of the care homes in Municipality 2 had water samples exceeding 10,000 CFU/L based on the mean counts of the B-samples. While including A-samples, 27% of the care homes in Municipality 1 had samples exceeding 10,000 CFU/L.

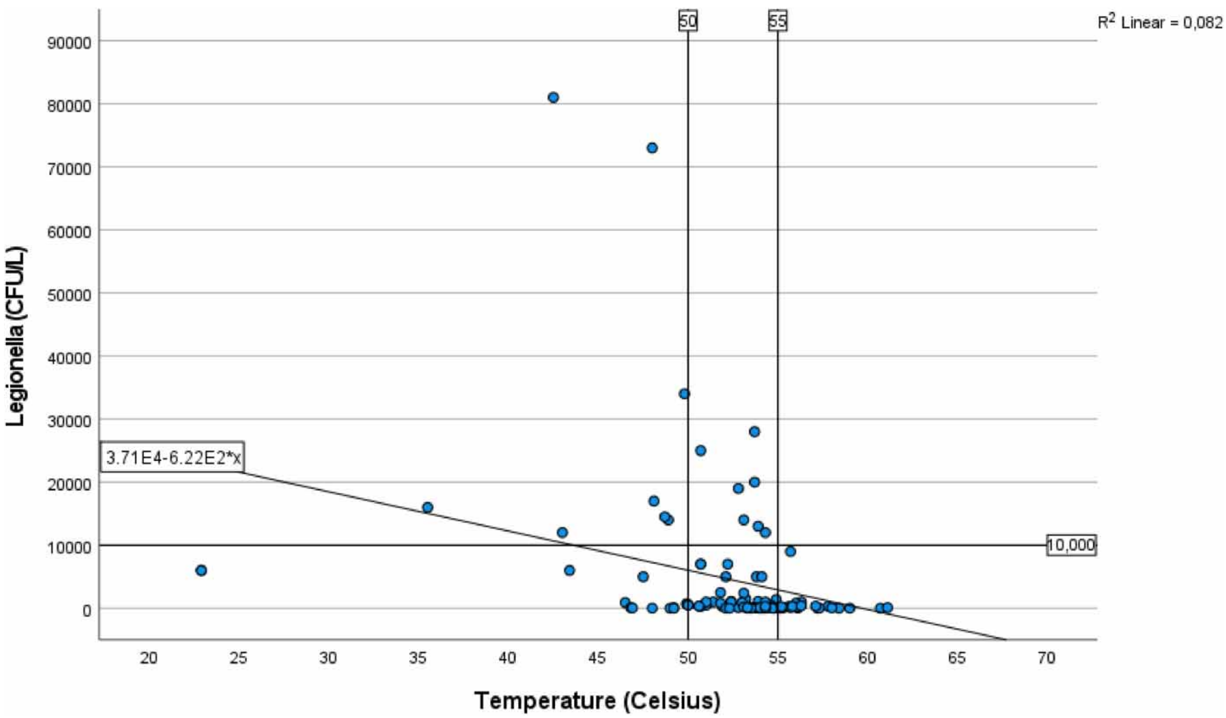


Figure 2 | *Legionella* colony counts (CFU/L) in relation to temperature in hot water systems of 54 care homes (106 samples) in a Danish municipality (Municipality 2); $y = 3.71 \times 10^4 - 6.22 \times 10^{2X}$. $R^2 = 0.082$.

Twice as many care homes in Municipality 2 had *Legionella* in their B-samples compared to those in Municipality 1. On the other hand, the overall content of *Legionella* (CFU/L) in the B-samples from Municipality 1 was significantly higher than the content in Municipality 2. This result is supported by differences in the median counts between the two municipalities.

Based on the samples from the 8 care homes in Municipality 1, the mean number of *Legionella* counts in the A-samples was approximately four times higher than the mean number in the B-samples. Finally, the OR for exceeding 10,000 CFU/L in the B-samples was 0.5 when the water temperature was lower than 50 °C in both municipalities. None of the samples exceeded 10,000 CFU/L in the samples with temperatures of 50 °C or higher in Municipality 1. In Municipality 2, a water temperature of 55 °C or higher was the limit value for none of the samples exceeding 10,000 CFU/L.

First flush samples (A-samples) are expected to yield a higher level of colonies than flush samples collected at constant temperatures (B-samples) (Hirsh *et al.* 2020). A ratio of 2–4 times higher content in the A-samples compared to the B-samples was seen in a similar investigation of *Legionella* content in water samples from apartments in Denmark (SSI 2020, Uldum *et al.* 2022).

As the results for the A-samples in the 8 care homes were of the same magnitude as the results for the 44 care homes in Municipality 1, the results from the 8 care homes can be considered representative of all of the care homes in Municipality 1. The median counts also support the idea that the results from the 8 care homes are representative of the care homes in Municipality 1. The results of the B-samples from the 8 care homes can therefore also be considered representative of the B-sample results from all 44 care homes in Municipality 1. On the other hand, the results from the B-samples demonstrate that only two of the 8 care homes contained *Legionella* in their B-samples. This distribution compared to the generally high counts in all of the A-samples indicates that these care homes have single taps that possess a very high number of colonies in first flush samples but no general *Legionella* content in their hot water systems. Alternatively, the sampling technique used in Municipality 1 may have caused the *Legionella* contents to be flushed out before taking the B-samples at constant temperatures.

This is a source of potential bias, as also illustrated in the diverging results, which shows that the proportion of B-samples with *Legionella* was higher in Municipality 2 but that the mean number of colonies was higher in Municipality 1. We asked the test company about the procedures they used for sampling in Municipality 1. Their procedure was to wait at least 2 min – more if necessary – until the water temperature in the taps was equivalent to the central water temperature. In some cases, it took up to 10 min before the test person approved the temperature as being correct for taking a B-sample. In Municipality 2, the procedure was to wait for 1 min – not more than 2 min – before taking the B-samples (Uldum *et al.* 2022). As the cultivation method is particularly sensitive to increased leaching of colonies after 2 min (SSI 2021), it is likely that the use of different procedures for B-sample testing led to the lower number of care homes with *Legionella* in the B-samples from Municipality 1 compared to Municipality 2. This might also explain why different results were observed between the two municipalities in terms of correlations between water temperature and the *Legionella* counts in the B-samples (Figures 1 and 2). Another indicator is the results in Table 4, which show that even though the mean temperature was higher in the water systems in Municipality 1 compared to Municipality 2, the number of colonies in the B-samples was also higher in Municipality 1 for water systems above 55 °C. Another possibility, of course, is that there are systematically different types of water systems in the two municipalities, which would explain why it took more time before the water reached the necessary temperature. Alternatively, a selection might have taken place, so that there are more temperature-resistant colonies in the water systems in Municipality 1 where the temperature is between 50 and 55 °C. More accurate standards for testing procedures would probably improve the results in the future. For example, a schedule for taking B-samples could be used based on the dimensions (and maybe the age) of the water systems, which have a great influence on how quickly the hot water reaches the taps (Build 2019; Nielsen & Aggerholm 2022).

The non-Gaussian distribution of *Legionella* content related to water temperature for B-sample testing has been observed in other studies as well (Uldum *et al.* 2022). Non-parametric statistics are, therefore, used in this study as well.

The colonisation rates of 77% in Municipality 1 and 81% in Municipality 2 are relatively high compared to the results from similar studies, but a study of colonisation rates in apartments in four Danish cities from 2021 showed similar results (Uldum *et al.* 2022) and the colonisation rates are higher than the results seen in other countries. Studies from Italy have reported colonisation rates of 26% (Totaro *et al.* 2017) and 40% (Leoni *et al.* 2005) in hot water systems. However, *Legionella* was detected in 93.7% of the hospital hot water systems in the later study. It is important to note that while water systems in Italy are chlorinated or otherwise treated, systems in Denmark are generally untreated. In a study from Poland, colonisation rates for the hot water systems were reported at 28.9% (Stojek *et al.* 2012); however, another study from Poland reported that

74.8% of the samples from the hot water systems were positive for viable *Legionella* (Sikora *et al.* 2015). Studies from the USA and Iran showed colonisation rates of 19.8 and 27.3%, respectively (Moore *et al.* 2006; Sikora *et al.* 2015). In Turkey, 69.2% of the hotels in a particular study were colonised with *Legionella* (Khaledi *et al.* 2018).

Although only less virulent types of *L. pneumophila* were detected in the two municipalities in this study, the high levels in some samples and the large proportions of care homes with *Legionella* in the hot water systems nevertheless pose a risk for the elderly and vulnerable residents in care homes. A measurement of culturable *Legionella* colonies of up to 600,000 CFU/L is high in relation to recommended limits of 1,000 or 10,000 CFU/L depending on the serogroup (SSI 2000; WHO 2017). A 60–600 times higher ratio than recommended could be life-threatening for elderly and vulnerable people. A study of 29 Danish LD cases – most of them with links between isolates from hot water systems and patients – showed that the highest numbers of colonies were 485,000 CFU/L in an A-sample and 420,000 CFU/L in a B-sample (Build 2019). In some of the 29 LD cases, there was less than 500 CFU/L, and in other cases, no colonies were detected in either A- or B-samples. The mean values for the 29 cases were 92,000 CFU/L in the A-samples and 49,000 CFU/L in the B-samples. In comparison, the results from the care homes in the two Danish municipalities detected a higher maximum number of colonies in a single A-sample (600,000 CFU/L) but a lower maximum number of colonies in the B-samples (134,000 CFU/L compared to the 420,000 CFU/L). Moreover, the mean value from the A-samples was only one-third – and for the B-samples, it is one-eighth – in the care homes compared to the mean values for the 29 LD cases. However, this discrepancy is not particularly crucial, as the 29 LD cases constitute a selected group, where most of them were linked with detected *Legionella* colonies in their hot water systems. Thus, higher mean values of *Legionella* colonies can be expected for the A- and B-samples in a selected group of LD cases.

No LD cases were known to be associated with the Danish care homes examined in this study, which might be due to the low virulence of most *L. pneumophila* strains. Alternatively, LD cases may have gone undiscovered in the care homes, as pneumonia is a common illness among older or otherwise vulnerable people. Pneumonia is also a cause of death for which an aetiological diagnosis might not always be established (Build 2019; ECDC 2020).

The observed lower levels of the median number of colonies compared to the mean values in both municipalities indicate that some care homes had a tap or a shower with very high numbers of *Legionella*, whereas the rest of the samples had low levels or no detected *Legionella*; this would yield a higher mean count than median count. Such high counts might be due to infrequent usage of taps or showers or from inadequate circulation in the water systems (Build 2019).

As shown in Figures 1 and 2, temperatures below 50 °C increased the odds of having high numbers of *Legionella* ($>$ 10,000 CFU/L) in the system to about 50% or higher. These results suggest that it is preferable to raise water temperatures to 55 °C or higher at the taps (50 °C in Municipality 1) to reduce the viable and culturable *Legionella* content in the water samples to below 1,000 CFU/L or, with one exception, below 10,000 CFU/L (Figure 2).

These findings confirm that temperatures below 55 °C but above 50 °C in hot water systems cannot alone guarantee low levels of *Legionella* in the system (Erdogan & Arslan 2007). A water temperature of around 55 °C preferably 58–59 °C to reduce the content of viable *Legionella* while simultaneously avoiding high calcium precipitation in the water system is preferable (DTI 2019). However, higher water temperatures require care homes to be cautious and introduce behavioural rules to avoid scalding among residents and staff.

Apart from water temperature, other factors also influence *Legionella* content. Disproportionate water system dimensions (e.g., oversized pipelines) may cause low flow rates. Long and poorly insulated pipelines may further result in heat loss along the pipelines (Build 2019; DTI 2019). Such structures increase not only the risk of *Legionella* growth but also biofilm contamination which is expected to protect and facilitate further *Legionella* growth (Abdel-Nour *et al.* 2013). This might also entail unnecessarily high heat consumption if the peripheral water temperature at the taps is to be kept sufficiently high to avoid *Legionella* growth. A compensating elevated hot water tank temperature may then be of 60 °C or higher, which in turn entails the risk of increased calcium precipitation in the water systems (DTI 2019); this again promotes the growth of biofilm and *Legionella* (Abdel-Nour *et al.* 2013).

A complete renovation of the water system is one solution to these problems. A reduction in hot water tank capacity, adding circulation facilities to the water systems and (to some extent) the incorporation of water treatment techniques might be the cheaper solutions to reach the goal of reducing exposure among residents and employees in care homes (Build 2019; Kragh & Buhl 2021; Nielsen & Aggerholm 2022).

In this study, the samples were only investigated by culture. The method is based on ISO 11731 and is the only accepted method for *Legionella* risk assessment in Denmark (MST 2015). By using this technique, only culturable *Legionellae* are detected. Dead and non-culturable *Legionella* can be induced in hot water systems by factors such as high temperatures and starvation. Heat-tolerant viable *Legionella* can be further selected in a system that is regularly being heat-treated at temperatures > 60 °C (Whiley *et al.* 2017). A solution might use the culture technique combined with qPCR for a quicker and an overall more reliable risk assessment of the *Legionella* content in a hot water system (Whiley & Taylor 2016). This combined approach would be particularly useful for water samples collected at high temperatures where *Legionella* can be dead or viable but non-culturable (VBNC). If qPCR is used by itself, it could overestimate what may actually be a lower level of viable *Legionella* in the system. It would also be useful when testing large hot water systems that take several minutes for the hot water to reach the taps or showers because the cultivation method is very sensitive to an increased leaching of colonies after 2 min (SSI 2021).

As the potential risk for infection among the exposed individuals is lower if the source contains dead or VBNC rather than viable *Legionella*, it is particularly important to strengthen the criteria for testing viable colonies using the culture method.

Results at constant temperatures (B-samples) primarily reveal the presence of *Legionella* in central water systems, whereas first flush results (A-samples) primarily reveal the presence in distal tubes and taps. Both measures show different aspects of the presence of *Legionella* in hot water systems as well as different risk aspects that expose individuals to *Legionella*. Both types of tests are, therefore, recommended as test procedures to reveal all aspects of the possible presence of and exposure to *Legionella*. In terms of large and ageing hot water systems, these tests could be supplied by samples from the central boiling tanks, which might improve the accuracy of determining the relationship between temperature and viable *Legionella* content.

Considering the large proportion of Danish care homes from the 1970s that have large hot water systems and the high proportion of care homes with *Legionella*, routine testing should be considered to monitor *Legionella* levels. The extremely high number of viable *Legionella* from the A-samples in a few taps and the low median counts compared to the mean counts of *Legionella* in the care homes' central hot water systems (from the B-samples) further indicate that some taps are seldom used and therefore highly contaminated with *Legionella*. Conversely, the central parts of many care homes' hot water systems have low levels or are uncontaminated. A local recommendation for care homes could include regular-use procedures for all taps.

CONCLUSIONS

In summary, *Legionella* was present in 77 and 81% of the hot water systems from the surveyed care homes in the two Danish municipalities. Even though different procedures were probably used for sampling in the two municipalities, the results support that the care homes in Municipality 1 had a significantly higher content of *Legionella* than the care homes in Municipality 2. More uniform and accurate test procedures are needed, and routine testing for monitoring *Legionella* levels in Danish care homes should be considered. This study also determined that the peripheral temperature in the taps of care home water systems should be raised to a minimum temperature of 55 °C to maintain lower levels of culturable *Legionellae*. In care homes with an overcapacity in the water system and long and poorly insulated pipelines, this may require maintaining a central temperature up to 60 °C or even higher to ensure a high peripheral temperature (above 55 °C). On the other hand, this promotes calcium precipitation and might thereby increase the risk of *Legionella* growth in the water system.

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DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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