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Comparison of the method of quantitative determination of *Legionella pneumophila* acc. to PN-EN ISO 11731:2017 with the Legiolert™/ Quanti-Tray® (IDEXX)

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Abstract

Introduction. *Legionella pneumophila* is the primary etiological agent of Legionnaires' disease. These are opportunistic pathogens causing lung infections by inhalation of contaminated aerosols. Controlling the presence of these bacteria in domestic distribution water systems (mainly hot water systems) is important for reducing the threat they pose to human health. *Legionella* pathogens are detected and quantified during routine testing of water samples according to procedures included in PN-EN ISO 11731:2017. However, these procedures are labour-intensive, and the results are obtained after a relatively long time. Implementing the Legiolert™/Quanti-Tray® test as an alternative method may constitute a good solution: it simplifies the testing procedure and significantly reduces the time necessary to obtain the final result.

Objective.The aim of the study was to compare the relative recovery of *Legionella* from water samples tested according to PN-EN ISO 11731:2017, and the alternative method of the most probable number (MPN) with the Legiolert™/Quanti-Tray® (IDEXX) test, and to assess the suitability of the alternative method for routine testing.

Materials and method. Parallel testing was conducted of 38 hot water samples to detect and determine *Legionella* acc. to PN-EN ISO 11731:2017 and the Legiolert™/Quanti-Tray® test. Statistical analysis of the results was performed according to PN-EN ISO 17994:2014 and the McNemar's test.

Results. The Legiolert™ test was confirmed to be comparable in performance to the reference standardized method in both qualitative and quantitative detection of *L. pneumophila* in hot water samples.

Conclusions. The study confirmed that the Legiolert™ test is specific and easy to use, and may constitute an alternative to standardized procedures used in the quantification of *L. pneumophila* in water.

Key words

drinking water, *Legionella pneumophila*, domestic distribution systems, PN-EN ISO 11731, Legiolert™

INTRODUCTION

Legionnaires' disease, caused by bacteria from the genus *Legionella,* is a type of pneumonia that can have a severe clinical course with accompanying extrapulmonary symptoms [1, 2]. According to epidemiological data, the *Legionella pneumophila* species is responsible for over 90% (some data suggest 95–98%) of cases of Legionnaires' disease recorded in Europe and the USA [1, 3, 4, 5, 6, 7]. The number of species included in the *Legionella* genus is steadily increasing – more than 60 and 80 serogroups have now been identified, with almost half of the known species being associated with human infections [1, 8, 9, 10, 11, 12]. According to the data presented by the European Legionnaires' Disease Surveillance Network (ELDSNet), infections among all registered cases in Europe are most often caused by *L. pneumophila* serogroup 1 (over 80% of cases) [3, 4, 13]. It is estimated that about 2–5% of legionellosis cases are caused by infection with species other than *L. pneumophila,* including: *L. anisa, L. bozemanii, L. longbeachae, L. micdadei* [3, 4, 5, 6, 7]. Many experts, including those at the WHO, have pointed out that in the European Union countries, of all waterborne pathogens, *Legionella* cause one of the greatest health burdens, and the number of registered cases of the disease is steadily increasing [3, 4, 14, 15, 16].

At the same time, the experts indicated that in addition to reservoirs, such as cooling towers and whirlpool tubs, there is an increasing risk of *Legionella* infection associated with domestic distribution water systems (primarily hot water systems) [10, 11, 17, 18, 19]. In this case, infections are often associated by using showers when, through inhalation of contaminated aerosolized water, pathogens can enter the alveoli directly and result in infection [10, 11, 14, 15, 17]. Published data also confirm that hot water systems are an important reservoir from the point of view of risks to human health in which *Legionella* can multiply, especially if the water temperature is too low (<50°C), there is a lack of flow or stagnant water and biofilm is present [10, 14, 15, 16, 17, 19, 20]. The persistence of such conditions in domestic distribution water systems, especially in facilities identified as a priority (e.g., hospitals or hotels), can pose a major threat to the health of the people using such systems [16, 21, 22]. Other factors conducive to the occurrence and proliferation of *Legionella* are oversized water systems, corrosion of construction materials,

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changes in water pressure, inadequate concentration of disinfectants or lack thereof, the presence of blind ends or dead legs, and seasonality of facility operation [10, 11, 14, 17, 19, 23]. Therefore, it is of great importance to ensure and properly supervise the technical and sanitary conditions of water supply systems, to verify the effectiveness of the control measures used, and preventive actions carried out to minimize the risk related to *Legionella* [10, 14, 17, 19, 23, 24]. One of the components of the above measures is regular microbiological testing of water for the determination of these bacteria.

In Poland, tests for the detection and isolation of *Legionella* in hot water samples have been carried out since 2008 acc. to PN-EN ISO 11731–2:2008 (ISO 11731–2:2004) and PN-ISO 11731:2002 (ISO 11731:1998), as required by the Ordinance of the Minister of Health on the quality of water intended for human consumption [25, 26, 27]. As of 2017, according to regulations currently in force, testing is conducted in compliance with procedures provided in PN-EN ISO 11731:2017 (EN ISO 11731:2017) [28,29]. Membrane filtration is commonly used in routine analysis. Typically, 100 ml water samples are filtered, the filter is then treated with an acidic buffer of pH 2.2, and placed on GVPC and BCYE agar media [28]. The presumptive colonies are confirmed as *Legionella* by determining their growth requirements in relation to L-cysteine and iron (III).

The method described is labour-intensive, and the time to obtain the final result is long, usually exceeding 10 days. If it is necessary to identify the species and determine the serogroup, further testing is performed, including latex agglutination tests. In this context, the Legiolert™/Quanti-Tray® (IDEXX) test, based on a chromogenic liquid medium and the most probable number method, may constitute a suitable alternative. This test can determine the number of *L. pneumophila* within 7 days, with high sensitivity and specificity, and requires no further confirmation. The test result is expressed in terms of the most probable number (MPN) in 100 ml of the tested water sample.

OBJECTIVE

The aim of the study was to compare the quantification of *Legionella* by membrane filtration and culture on solid media plates according to PN-EN ISO 11731:2017 procedures, and by the most probable number method and culture on liquid medium using the Legiolert™/Quanti-Tray® (IDEXX) test for the analysis of hot water samples.

MATERIALS AND METHOD

Water samples. Water samples for testing with a volume of 1,000 ml were taken from hot water supply systems in public utility buildings:
- office facility (2 buildings) – 15 samples;
- health care facility (1 hospital) – 4 samples;
- collective housing buildings (5 buildings); 19 samples (3 hotels – 14 samples, 2 student houses – 5 samples).

A total of 38 water samples were tested on the day of sampling. Water samples were collected in sterile polypropylene bottles, transported and stored acc. to PN-EN ISO 19458:2007 [27].

Study methods. Collected water samples were tested in parallel by membrane filtration acc. to PN-EN ISO 11731:2017 and Legiolert™/Quanti-Tray® (IDEXX) test.
- **PN-EN ISO 11731:2017** (reference method) – Matrix A (drinking water), procedure 5 – BCYE, procedure 7 – GVPC. Mixed cellulose esters filters with a pore diameter of 0.45 µm (MILLIPORE) were used for filtration. Water samples were filtered in 1 ml, 10 ml, 100 ml portions, untreated filters were placed on BCYE (OXOID) agar plates, pretreated filters with pH 2.2 buffer were placed on GVPC (OXOID) agar plates. Plates were incubated at 36 ± 2°C for up to 7 days, and readings were taken on days 3, 4, 5, and 7. After incubation, to determine growth requirements in relation to L-cysteine, morphologically characteristic colonies of presumptive *Legionella* were surface streaked on BCYE with and without L-cysteine (BIORAD) in parallel. The plates (BCYE/BCYE-cys) were incubated at 36 ± 2°C for 2–3 days. Identification of *Legionella* for species and serogroup determination was performed with the Legionella Latex Test (OXOID). The number of serogroup-labeled *L. pneumophila* was expressed as cfu/100 ml.
- **Legiolert™/Quanti-Tray® (IDEXX)** (alternative method). Legiolert™/Quanti-Tray® is a commercial test (IDEXX Laboratories, Westbrook, ME, USA). Testing of water samples was conducted according to the protocol for drinking water at a volume of 100 ml. In the collected samples, water hardness was determined using the Aquadur test (Macherey-Nagel); for low water hardness (0–2 fields on the test strip) and for high water hardness (3–4 fields on the test strip), 0.33 ml and 1.0 ml of reagent from the auxiliary kit, respectively, was added. Samples were incubated for 7 days at 39 ± 0.5 °C according to the manufacturer's instructions, ensuring adequate humidity during incubation. The Legiolert™ tray has 6 large wells and 90 small wells. Wells were counted as *L. pneumophila*-positive if they showed turbidity or a brown color change. The bacteria count was determined using IDEXX's Most Probable Number (MPN) Table. The results were expressed as *L. pneumophila* MPN/100 ml.

After reading the results, material was collected for confirmatory testing. From the selected 'positive' and 'negative' wells, material was taken with 10 µl loops and surface streaked onto BCYE. The BCYE plates were incubated at 36 ± 2°C for 2 to 3 days. The grown on BCYE characteristic *Legionella* colonies were surface streaked onto BCYE without cysteine (OXOID) and incubated at 36 ± 2°C for 2 to 3 days. Identification of *Legionella* species and serogroup determination was performed with the Legionella Latex Test (OXOID).

Statistical methods. Equivalence assessment of the methods was performed based on PN-EN ISO-17994:2014 (paired sample t-test of associated results) [28]. The results were also analyzed using the McNemar's test (chi-square test).

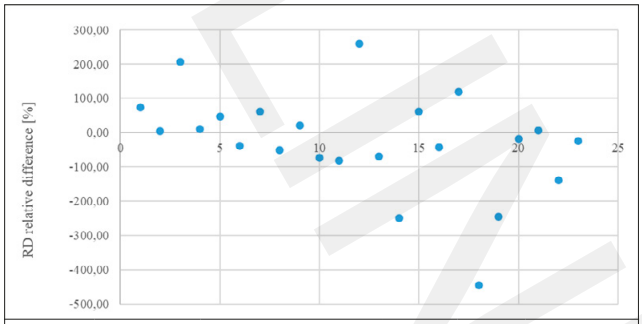
RESULTS

Among the 8 buildings surveyed, bacteria from genus *Legionella* was detected in water samples taken from the domestic water supply systems (hot water) in 7 of them. In only one building – a student house – no bacteria were

detected in any of the water samples. *Legionella* was detected in water samples taken in the office facility (86.7% positive water samples), a hospital (100% positive water samples), hotels (57.1% positive water samples), and one student house (20% positive water samples).

An equivalence assessment of the methods based on PN-EN ISO-17994:2014 (paired sample t-test of associated results). The study compared the paired results of the parallel analyses of 38 hot water samples, from which 15 were excluded from further analysis, including 12 samples in which no *Legionella* were detected, and 3 samples in which excessive contamination of tested material prevented the determination of bacteria count, in tests performed by both the reference method according to PN-EN ISO 11731 (uncountable number of colonies on the plates) and the alternative Legiolert™/Quanti-Tray® method (all wells positive).

The results obtained were analyzed based on the method outlined in PN-EN ISO 17994. Relative difference RD was determined for each pair of confirmed counts which differed from zero, i.e., the difference between 2 results, *a* and *b*, measured on a relative (natural logarithmic) scale. The equation used was x=[(ln(a)-ln(b)]x100%, where ln(a) is the logarithmically transformed test result obtained by the alternative method (Legiolert™/Quanti-Tray®), and ln(b) is the logarithmically transformed test result obtained by the reference method (PN-EN ISO 11731). Since the study involved hot water (drinking water), a limit of 2L = 10% was used to establish a confidence interval.



Standard deviation	Standard uncertainty	Relative difference RD Average value	X_L (95%CI)	X_u (95%CI)	n (number of observations)
148.93	31.05	-26.08	-88.18	36.03	23

Figure 1. Variability summary for RD [%]

Analysis of pairs of associated results, including 3 pairs of results with high uncertainty ≤3 cfu (MPN)/100 ml), showed that the mean variability was not significant i.e. the methods were not statistically different (Fig. 1). For the assumed limit value of 2L=10%, results were consistent with equivalent performance, but the evaluation result was 'inconclusive', more samples were required, indicating that more data were required due to the width of the expanded uncertainty.

Analysis of present/absent results – McNemar's test. Among all water samples tested, 24 samples (63.2%) were determined as positive by the reference method, and 23 samples (60.5%) determined as positive using the Legiolert™ test. To assess the differences between the frequency of positive samples (result: present) and negative samples (result: absent) according to research methods: PN-EN ISO 11731 (reference method) and Legiolert™ (alternative method), the results were analyzed by McNemar's test. This test compares the sensitivity and specificity of 2 methods in the same group of samples. Results of all 38 samples (with low and high uncertainty of measurement) were included.

The presence of *Legionella* was detected in:

- 21 water samples tested by both reference and alternative methods (result: present);
- 3 water samples tested by the reference method (result: present), without confirmation of a positive result by the alternative method (result: absent);
- 2 water samples tested by the alternative method (result: present), without confirmation of a positive result by the reference method (result: absent);
- 12 water samples were negative (absent) for both methods used.

Analysis of the test results using the McNemar's test showed no significant differences, chi-square test statistic = 0 (p>0.999) (Tab. 1).

Table 1. Results of McNemar's test analysis of data for detection of *L. pneumophila* by 100 ml water samples acc. PN-EN ISO 11731: 2017 and Legiolert™/Quanti-Tray®

Study Method		Legiolert™ (IDEXX)		No. of samples
	Result	present	absent	
PN-EN ISO 11731:2017	present	21	3	24
	absent	2	12	14
Number of samples		23	15	38

X^2=0.0; p =1

It was also evaluated whether using the Legiolert™ alternative method had an impact on increasing the chance of detecting *Legionella* in the tested water samples. The odds ratio (OR) for the compared methods was 0.667 (95% Cl confidence interval 0.056–5.820). Since the OR value obtained was <1, this means that in case of the alternative method (compared to the reference method), the chance of detecting the bacteria did not increase.

True and false-positive results. For both the standardized method and the Legiolert™ test, confirmatory testing was performed with the *Legionella* Latex Test. In the case of 19 results out of the 21 (90,5%) obtained according to PN-EN ISO 11731, 100% were confirmed as true positive, in 2 cases out of 21 (9.5%), the confirmation was lower – 87.5%. In the case of the Legiolert™ test, the results were similar to those in the membrane filtration, with 17 results out of 20 (85,5%) obtaining 100% confirmation of the bacterial count, and only in 3 samples out of 20, the material collected from positive wells was not fully confirmed (in 2 cases out of 20 confirmation score – 0.0%; in 1 case out of 20, confirmation score – 66.7%) (Tab. 2).

In 2 cases, significant differences were observed between the number of *Legionella* determined by the standardized method (value expressed as cfu/100 ml) and the Legiolert™ test (value expressed as MPN/100 ml). In the third case, not all wells determined initially as positive were confirmed, with MPN values close to the confirmed cfu values where false-positive results were reported for Legiolert™, while the equivalency of quantification suggests that these may be true positives.

Table 2. Comparison of results of confirmatory tests for *Legionella* detected in water samples acc. PN-EN ISO 11731:2017 and Legiolert™/ Quanti-Tray®

No.	PN-EN ISO 11731:2017				Legiolert™ (IDEXX)			
	Legionella count							
	presumed	confirmed	confirmed [%]	result cfu/100ml	presumed	confirmed	confirmed [%]	result MPN/100ml
1	8	7	87.5	10	5	5	100.0	19
2	14	14	100.0	47	7	0	0.0	31
3	8	7	87.5	94	1	1	100.0	1
4	4	4	100.0	145	5	0	0.0	13
5	6	6	100.0	950	6	4	66.67	1018

DISCUSSION

Methods for detecting and quantifying bacteria in water samples that enable reliable results in the shortest possible time are of great importance in ensuring and controlling water quality. This is of particular importance in the case of *Legionella* testing, as the most commonly used method of culture on solid media requires a long incubation time of at least 7 days, and the need for confirmatory tests. The method of culture on solid media is considered the best practice, but it is not without flaws, which have led researchers to search for and develop alternative methods. Many have emphasized that the culture method on solid media, despite all modifications, is still laborious and time-consuming, and the waiting time for a confirmed result can exceed 10 days [8, 32, 33, 34, 35, 36]. Performing tests according to the procedures described in the methodological standard [28] can pose not only technical problems, but also decision-related problems, among other things, due to the selection of an appropriate procedure, inoculation technique, test depending on the type of water, purpose of the test, and the test meeting the requirements of relevant regulations. Compared to these methods, the Legiolert™ test is simple to perform and obtaining a final result is possible within 7 days, with high sensitivity and specificity [32, 37, 38]. In addition, testing of water samples with this test does not require further confirmation for determination of the *L. pneumophila* species. It is worth noting that for further identification (e.g., for serogroup determination), which can be important in an epidemiological investigation, isolates can be obtained from positive wells of the Legiolert™ test. It is also worth remembering that the Legiolert™ test only detects *L. pneumophila*. Although infections caused by other *Legionella* species are extremely rare, it is important to be aware of the possibility of false-negative results.

The results presented, similar to studies conducted in other European countries, confirmed the usefulness and reliability of the Legiolert™ test for detecting and quantifying *L. pneumophila* in hot water samples. Analysis of the data obtained showed that the methods are not statistically different. Studies conducted by research groups from Europe and the United States have also shown that the methods are statistically equivalent, noting that the Legiolert™ test was characterized by higher sensitivity (i.e., higher number of correctly identified positive samples were obtained) and specificity (i.e., higher number of correctly identified negative samples were obtained), compared to the BCYE/GVPC culture-based method [32, 34, 35, 36, 37]. According to Boczek et al. one of the conditions affecting higher detection rates with the Legiolert™ test may be the temperature of 39°C at which the culture is conducted. This is related to the preference of *Legionella* non-pneumophila spp. for lower temperatures for growth compared to *L. pneumophila* species [32]. Hence, the results of the presented study confirmed the high specificity of the Legiolert™ test which amounted to 96.5% (i.e. 3.5% false positives (9/254) and 0% false negatives (0/82)) [32]. Also, evaluation with the McNemar's test showed no statistical difference, indicating that both methods were equally sensitive in determining the prevalence of *L. pneumophila* [32]. A similar assessment was obtained from an inter-laboratory comparison study conducted in Germany. This showed that in a test sample of 100 ml, a higher number of *L. pneumophila* was determined with the Legiolert™ test than with use of the method based on membrane filtration and GVPC according to ISO 11731–2:2004 [35, 38]. In this case, as a result of analysis of the combined data from 6 laboratories, a mean relative difference value of 89.5% was obtained with a 'confidence limit' of 72.7% and 106.3%. As a result, Legiolert™ yielded significantly higher *L. pneumophila* counts than the ISO 11731–2:2004 method. Furthermore, analysis of the data with the McNemar's test showed that the number of samples positive for *L. pneumophila* with the Legiolert™ test was significantly higher than with the compared ISO method. Similar conclusions regarding the evaluation of the usefulness of the test were also formulated by researchers regarding other types of water samples [35, 36, 39].

SUMMARY

This article presents the results of a comparison between the PN-EN ISO11731:2017 reference method and an alternative method based on the Legiolert™ test for determining the number of *L. pneumophila* in hot water samples. The Legiolert™ method was found to have high sensitivity and specificity, compared to the reference method. Analysis of the results of the conducted studies and data obtained from the literature confirm that the method based on the Legiolert™/ Quanti-Tray® test is very promising for use in testing drinking water [32, 34, 35, 36, 37, 39]. Also, when testing with the Legiolert™/Quanti-Tray® test of other types of water, such as cooling water, process water, or water from spa-type pools, available data indicate its applicability [33, 36]. Technical implementation of the test is simpler and less labour-intensive than the reference method [39].

A limitation of the study may be related to the unusual incubation temperature 39 ± 0.5°C for the tested drinking water samples, however, according to some researchers, this may increase the detection rate of *L. pneumophila* [32, 39]. In comparison, the reference method is based on a temperature of 36 ± 2°C and most microbiological parameters determined in drinking water require incubation at these conditions. The reading of the test, which involves counting the positive wells, is also relatively simple (it involves counting the positive wells), and the result is presented based on statistical tables. However, it has been observed that sometimes it can be difficult to find turbidity and colour change in the small wells, e.g., in the case of high contamination of the water sample.

In conclusion, it can be stated that the Legiolert™ test showed comparable performance to the reference standardized method in both qualitative and quantitative detection of *L. pneumophila* in hot water samples. It is also worth emphasizing that the Legiolert™ test allowed obtaining faster results which, in the context of monitoring water for the presence of bacteria, can significantly speed-up taking appropriate measures, especially in emergency situations and situations posing a serious health risk.

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