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ORIGINAL ARTICLE

Evaluation of a most probable number method for the enumeration of *Legionella pneumophila* from potable and related water samples

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Significance and Impact of the Study: *Legionella pneumophila* is an opportunistic pathogen of major concern. The current large volume quantitative method employs membrane filtration (MF) and selective culture on GVPC agar followed by confirmation of isolates by serology (ISO 11731-2) We present here the results of a multi-laboratory evaluation of a most probable number (MPN) *in-situ* confirmed method (Legiolert™/Quanti-Tray®). The results indicate that Legiolert/Quanti-Tray yielded on average higher counts of *L. pneumophila* than ISO 11731-2. This development significantly improves and simplifies the enumeration of *L. pneumophila* from potable water samples.

Keywords
drinking water, ISO 11731-2, ISO 17994, Legiolert, *Legionella pneumophila*, MPN enumeration, rapid methods.

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Abstract

This study compared the performance of a novel MPN method (Legiolert/Quanti-Tray) with the ISO 11731-2 membrane filtration method for the enumeration of *Legionella pneumophila* from 100 ml potable water and related samples. Data from a multi-laboratory study analysed according to ISO 17994 showed that Legiolert™/Quanti-Tray® yielded on average higher counts of *L. pneumophila*. The Legiolert medium had a high specificity of 96·4%. The new method represents a significant improvement in the enumeration of *L. pneumophila* from drinking water-related samples.

Introduction

Since its first isolation in 1977 following the 1976 outbreak of Legionnaires' Disease in Philadelphia (Fraser *et al.* 1977), *Legionella pneumophila* has become an opportunistic pathogen of major concern worldwide because of a large number of outbreaks associated with a number of sources including potable, shower and bath water. Germany has one of the highest rates of legionellosis in Europe. In 2009, there were 503 notified cases, of which 49·6% were community-associated (Brodhun and Buchholz 2011) and this rose to 688 cases in 2010 (Beauté *et al.* 2013). In 2011, the German Federal Ministry of Health published amendments to the German drinking water ordinance which included setting a standard for *Legionella* (German Federal Ministry of Health 2011). A technical action value of 100 CFU per 100 ml was established and ISO 11731-2 (2004) was cited as the regulatory method.

The ISO 11731–2 (2004) method is a membrane filtration procedure in which a selected sample volume is filtered through a 0·45 μm pore size membrane filter which

is then placed on a selective agar (either BCYE agar or GVPC agar) and then incubated at 36°C for 10 days. Typical colonies are then subcultured before confirmation of identity by serology. A novel alternative method has been developed based on the MPN determination of *L. pneumophila*. Legiolert™ is presented in as powdered reagent in blister pack format for testing 100 ml water samples and utilizes IDEXX's selective formulation to detect *L. pneumophila*. Legiolert™/Quanti-Tray® is incubated at 39°C ± 0·5°C with humidity for 7 days. When *L. pneumophila* are present in a water sample and tested using Legiolert™/Quanti-Tray®, they produce any combination of brown pigment and turbidity and represent a confirmed detection result. Enumeration is achieved by the most probable number (MPN) technique.

We report here the outcome of a multi-laboratory (four German laboratories) comparison between Legiolert™/Quanti-Tray® and ISO 11731-2 for the enumeration of *L. pneumophila* from naturally contaminated potable and related water samples. Cooling tower and other nonpotable water sources were not analysed for this study.

Results and discussion

Four geographically widespread laboratories in Germany that routinely analyse water samples for *Legionella* (including water company and commercial laboratories) participated in this study. Samples were analysed over the period May to July 2015 and were from premise potable water (cold tap water) and related systems (principally hot tap water and shower water, but also including samples from premise circulation and boiler outlets). The samples were taken from both domestic, public (e.g. hospitals, retirement homes and sports facilities) and industrial systems. One hundred millilitre aliquots were analysed by Legiolert™/Quanti-Tray® and ISO 11731-2 (ISO 2004) and the laboratories submitted data on presumptive and confirmed *L. pneumophila* isolated by the two methods resulting in a valid dataset of 290 paired counts (from an original 1604 samples analysed across the four laboratories each analysing routine samples typical for their laboratory). Counts from Legiolert™/Quanti-Tray® ranged from 0 to 2273 MPN 100 ml^{-1} (mean 132), and from 0 to 368 CFU per 100 ml (mean 26) from ISO 11731-2 membrane filter method. The majority of paired results (183 out of 290, i.e. 63·1%) were in the range of 0–10 CFU by the ISO method but were encompassed within a count range of 0–668 MPN by Legiolert™/Quanti-Tray® (Table 1). This difference in the range of counts was also reflected in other groupings of ISO 11731-2 counts (Table 1). These ranges of counts covered all types of water sample tested. The outcomes of the ISO 17994 mean relative difference analysis of the paired count data for each laboratory and for the combined data are presented in Table 2. The maximum upper and lower values of the "confidence interval" around a zero mean relative difference were set at +10% and −10% according to ISO 17994 (2014). The lower and upper values of the "confidence interval" for laboratories 2 and 3 are both above zero indicating that Legiolert™/Quanti-Tray® yielded significantly higher counts of *L. pneumophila* than the ISO method. For laboratories 1 and 4 the lower and upper values of the "confidence limit" were both outside the set limits resulting in "inconclusive" outcomes. However, for both laboratories, the range of the "confidence limit" were weighted to the upper limit indicating a tendency for higher counts being achieved by Legiolert™/Quanti-Tray®, but more samples would be needed to be analysed by these laboratories to confirm this higher recovery by Legiolert™. ISO 17994 analysis of the combined data from the four laboratories resulted in a value for the mean relative difference of 35·3% with the "confidence limit" being 54·8% and 15·7%. Therefore, it can be concluded from this that Legiolert™/Quanti-Tray® yielded significantly higher counts of *L. pneumophila* than did the ISO 11731-2 MF method. Two aspects of the Legiolert method may explain this. First, we propose that *L. pneumophila* may recover better in broth media compared with the growth on an agar medium as has been seen for some other bacteria (e.g. Ahn *et al.* 2014). Second, the Legiolert™/Quanti-Tray® format allows a substantially greater range of counts (up to 2273 MPN 100 ml^{-1}) compared with a theoretical maximum count by the ISO 11731-2 MF procedure of 100 CFU, although counts up to 400 CFU (depending on size of membrane filter used) were accepted for this study. However, where counts from the ISO 11731-2 method were reported as "too numerous to count" which had a corresponding Legiolert™/Quanti-Tray® count these were excluded from the comparison analysis.

Table 1 Comparative paired counts of *Legionella pneumophila* counts from 100 ml samples by ISO 11731-2 and Legiolert™/Quanti-Tray® based on ranges of counts by ISO 11731-2

Count range ISO 11731-2 CFU 100 ml^{-1}	Number of samples	Comparative count range Legiolert MPN 100 ml^{-1}
0–10	183	0–668
11–20	25	0–53
21–50	40	0–9
51–100	23	7–872
101–00	13	60–297
201–368	6	106–977

One aspect of the two methods is that the MPN counts from Legiolert™/Quanti-Tray® were taken as confirmed counts whereas those from ISO 11731-2 were subject to

Table 2 Outcome of mean relative difference analysis of the paired counts of *Legionella pneumophila* counts from 100 ml samples by Legiolert™/Quanti-Tray® and ISO 11731-2 method for each laboratory and for combined data according to ISO 17994 (2014)

	Number of results	Mean relative difference	Standard deviation	W*	X_L†	X_U‡	Outcome
Lab 1	65	12·4	120·1	29·8	−17·4	42·2	Inconclusive
Lab 2	65	35·5	117·3	29·1	6·4	64·6	Methods different
Lab 3	77	73·1	188·9	43·1	30·1	116·2	Methods different
Lab 4	83	17·8	201·1	44·1	−26·3	62·0	Inconclusive
Combined	290	35·3	166·4	19·6	15·7	54·8	Methods different

*Half-width of the "confidence interval" around the mean relative difference.
†Value of the relative difference at the lower "confidence limit."
‡Value of the relative difference at the upper "confidence limit."

confirmation tests. To test whether there was any potential bias in favor of Legiolert™/Quanti-Tray® through false-positive reactions a total of 1143 positive wells from Legiolert™ from 284 samples covering all sample types used in the study were subcultured to BCYE for confirmation by serology. Of these, 1105 isolates (96·7%) were confirmed as *L. pneumophila*, indicating acceptable specificity for the bacterium. The remaining 38 isolates (3·3%) not confirming as *L. pneumophila* came from 14 of the 284 samples. From these 14 samples, 65 wells were tested (between 1 and 12 wells per sample depending on the total number of positive wells encountered) (Table 3). Most of the false-positive isolates were derived from samples where 1 or 2 wells from 1 to 12 positive wells tested (10% to 100%) were not confirmed as *L. pneumophila*. However, there were three samples where the number of positive wells tested (one with 10 wells and two with seven wells) had all wells failing to have confirmed *L. pneumophila* present when zero counts were recorded by ISO 11731-2. There are, however, rare occurrences. Of the 14 samples with one or more false-positive wells, the water type for seven was reported. These were all hot water samples from school, domestic industrial taps or showers.

This study has investigated a novel MPN method (Legiolert™/Quanti-Tray®) for the enumeration of *L. pneumophila* from potable water-related samples. The method was compared with the MF procedure of ISO 11731-2 using GVPC agar. The Legiolert™/Quanti-Tray® method was found to be superior to the ISO 11731-2 method. This development will significantly add to the reliability of testing for *L. pneumophila* from drinking water and related samples.

Materials and methods

Legiolert™/Quanti-Tray® description and procedure

Legiolert (IDEXX Laboratories, Westbrook, ME) is a commercially available test that consists of three

Table 3 Summary of false-positive *Legionella pneumophila* counts from 100 ml samples by Legiolert™/Quanti-Tray® method compared with the counts obtained by ISO 11731-2

Sample number	Legiolert MPN/100 ml	Number isolates tested	Number confirmed as *L. pneumophila*	Legiolert confirmed MPN/100 ml	ISO 11731-2 *L. pneumophila*/100 ml
36	240	10	0	0	0
59	977	12	10	782	360
60	11	5	3	4	7
206	13	2	0	0	4
209	66	4	3	49	49
518	240	10	9	216	156
1093	463	7	0	0	0
1095	1	1	0	0	0
1096	2	2	0	0	0
1107	196	0	0	0	0
1133	72	7	0	0	0
1163	1	1	0	0	0
1177	1	1	0	0	0
1476	15	3	2	10	13

supplement solutions which were reconstituted to be used in conjunction with the Legiolert™/Quanti-Tray® (IDEXX Laboratories) procedure. The first two supplements (termed "1A" and '1B') were each reconstituted in sterile, deionized water. The final supplement (termed "2") was reconstituted in dimethyl sulfoxide.

The procedure involved determination of the total hardness of each sample using Aquadur® hardness test strips (Macherey-Nagel, Düren, Germany, item#: 91220) as per the manufacturer's protocol. Supplement 1A was then added to all samples with hardness ≤14°days. To all other samples 1 ml of supplement 1B was added. After 1A or 1B were added both the contents of one blister pack of Legiolert reagent and 167 μl of supplement 2 were serially added to all samples. All sample/reagent mixtures were immediately agitated for ≥60 s. The mixture was poured into the Legiolert™/Quanti-Tray® and immediately sealed in a Quanti-Tray® Sealer Model 2X using a modified rubber insert. Sealed Legiolert™/Quanti-Tray® samples were incubated paper side down (wells facing upwards) at 39 ± 0·5°C in a humidified environment. Humidity was generated by the addition of a water reservoir to the lowest shelf of each incubator, but specific humidity level was not measured. Legiolert™/Quanti-Tray® samples were analysed after 7 days for the presence of brown color and/or turbidity.

The specificity of Legiolert™/Quanti-Tray® was analysed by performing secondary confirmations using buffered charcoal yeast extract (BCYE) and trypic soy agar with 5% sheep blood (BA) on 10% of all positive signals (wells of the Legiolert™/Quanti-Tray®) by the following procedure. For each positive well the sampling area on the paper/membrane side of the Legiolert™/Quanti-Tray® was identified and a razor was cleaned using a disposable alcohol wipe. The razor was used to cut a small opening in the paper/membrane above each well to be sampled and 5 μl was transferred from each well to both a BCYE plate and a BA plate. A 3-zone streak was performed for each aliquot on each plate and plates were incubated for 2–4 days at 36 ± 2°C with humidity. Incubation time was variable based on recovery time for individual isolates to yield clear morphology and accurate confirmation. Following incubation isolates were regarded as *L. pneumophila* if they grew on BCYE but failed to grow on BA irrespective of any additional non-*Legionella* isolates present.

ISO 11731-2 procedure

The procedure of ISO 11731-2 (ISO 2004) is well-established and participating laboratories were requested to follow it using GVPC agar as the selective agar. One hundred millilitre samples were filtered through 0·45 μm pore size filters and following acid treatment and washing, these were transferred to GVPC agar plates and incubated at 36°C for 10 days. The sample volume was chosen to be equivalent to that for Legiolert/Quanti-Tray and be consistent with German standards (German Federal Ministry of Health 2011). In addition to the standard protocol outlined in ISO 11731-2 (ISO 2004), presumptive *Legionella* isolates were further screened for fluorescence when exposed to ultraviolet light to determine if isolates were *L. pneumophila* species or non-*pneumophila* species of *Legionella*. Data from fluorescent isolates was filtered from the comparative data analysis to properly compare sensitivity of both methods for *L. pneumophila* isolates. Isolates with ambiguous reactions were further analysed by latex agglutination to confirm speciation.

Study procedure

Participating laboratories were asked to analyse 100 ml aliquots of appropriate water samples that may contain *L. pneumophila* by the ISO 11731-2 membrane filtration method (ISO 2004) and by the Legiolert/Quanti-Tray MPN method. Details of water type were recorded. Presumptive and confirmed counts were also recorded.

Data analysis

The comparative *L. pneumophila* paired count data for each laboratory and the combined data from all laboratories were analysed according to the mean relative difference approach of ISO 17994 (2014). Briefly, the relative difference (x) of each pair of counts was calculated using the equation $x = 100(\ln(a) - \ln(b))$, where $\ln(a)$ is the natural logarithm of the count by the Trial Method (Legiolert) and $\ln(b)$ is the natural logarithm of the count by the Reference Method (ISO 11731-2). Data with a zero count by one method had plus one (i.e. count +1) added to each pair of counts prior to log-transformation. Data with counts that exceeded a method count limit by at least one method or had zero counts by both methods were excluded as required by ISO 17994 (2014). The maximum count limit for Legiolert™/Quanti-Tray® is 2273 MPN 100 ml^{-1} based on all but one well being positive. The theoretical maximum count by the ISO 11731-2 MF procedure according to the standard is 100 CFU, although counts up to 400 CFU (depending on size of membrane filter used) were accepted for this study. Since the objective of the study was to show there was no difference between the Trial Method (Legiolert) with an established Reference Method, it was considered that the "two-sided" comparison according to ISO 17994 (2014) was appropriate. The percentage value of the upper and lower limits was set at +10% and −10% as suggested by ISO 17994 (2014).

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Conflict of Interest

D.P. Sartory was commissioned by IDEXX Laboratories to analyse the data and write the paper for publication.

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