Eur J Clin Microbiol Infect Dis (2015) 34:1387–1393
DOI 10.1007/s10096-015-2363-4

ARTICLE

Design and validation of a qPCR assay for accurate detection and initial serogrouping of *Legionella pneumophila* in clinical specimens by the ESCMID Study Group for Legionella Infections (ESGLI)

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Received: 5 February 2015 / Accepted: 10 March 2015 / Published online: 8 April 2015
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Abstract Prompt detection of *Legionella pneumophila* is essential for rapid investigation of legionellosis. Furthermore, as the majority of *L. pneumophila* infections are caused by serogroup 1 (sg1) strains, rapid identification of such strains can be critical in both routine and outbreak scenarios. The ESCMID Study Group for Legionella Infections (ESGLI) was established in 2012 and immediately identified as a priority the validation of a reliable, easy to perform and interpret, cost-effective qPCR assay to standardise the detection of *L. pneumophila* DNA amongst members. A novel *L. pneumophila* assay targeting the *mip* gene was designed and combined with previously published methodologies amplifying the sg1 marker (*wzm*) and the green fluorescent protein gene (*gfp*) internal process control. The resulting triplex assay was validated internationally on the three qPCR platforms used by the majority of European *Legionella* reference laboratories: ABI 7500 (Life Technologies), LightCycler 480 Instrument II (Roche) and Rotor-Gene Q (Qiagen). Clinical and EQA specimens were tested together with a large panel of strains (251 in total) to validate the assay. The assay proved to be 100 % specific for *L. pneumophila* and sg1 DNA both in silico and in vitro. Efficiency values for *mip* and *wzm* assays ranged between 91.97 and 97.69 %. Limit of detection values estimated with 95 % confidence were adopted for *mip* and *wzm* assays on all three qPCR platforms. Inhibition was not observed. This study describes a robust assay that could be widely implemented to standardise the molecular detection of *L. pneumophila* among ESGLI laboratories and beyond.

Electronic supplementary material The online version of this article (doi:10.1007/s10096-015-2363-4) contains supplementary material, which is available to authorized users.

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Introduction

Microorganisms belonging to the *Legionella* genus can cause a severe life-threatening form of pneumonia known as Legionnaires' disease (LD). The infection is acquired by inhalation of contaminated aerosols and manifests as sporadic cases or outbreaks described worldwide [1]. Due to fastidious growth requirements, LD was not described until 1976 when *L. pneumophila* was isolated for the first time after an outbreak in Philadelphia, PA, USA [2, 3]. To date, more than 50 *Legionella* species have been described; however, *L. pneumophila* still remains the most common cause of LD. Among 16 different serogroups of *L. pneumophila*, serogroup 1 (sg1) alone accounts for approximately 85 % of culture-confirmed cases in Europe and is responsible for almost all outbreaks for which the infecting strain has been isolated and characterised [4].

Isolation of legionellae by culture remains the diagnostic gold standard; however, culture-based LD diagnosis requires experience and dedicated media supplemented with cystine, it

is time-consuming and often hampered by over-growth of fast-growing microorganisms. The detection of *L. pneumophila* antigen in urine is the most commonly used diagnostic method but, as current tests are only reliable for sg1 strains, not all infections can be detected. Molecular techniques are reported to be sensitive and specific in detecting both *L. pneumophila* [5] and sg1 strains [6]. Nevertheless, the detection of *Legionella* DNA is still not considered by the ECDC as confirmed evidence of LD [7]. Following the formation of the ESCMID Study Group for Legionella Infections (ESGLI) in 2012, the design and validation of a qPCR assay was identified as a priority to standardise molecular diagnosis of *L. pneumophila* infections across Europe. During the first ESGLI meeting (Dresden, Germany, September 2012), the preliminary results of a triplex qPCR assay detecting *L. pneumophila* and sg1 DNA, combined with an internal process control (IPC), were presented to delegates coming from *Legionella* reference laboratories in Europe. In November 2012, a survey was distributed among ESGLI members, and 30 laboratories from 25 countries expressed their interest in such a test being developed. ABI 7500 (Life Technologies), LightCycler 480 Instrument II (Roche) and Rotor-Gene Q (Qiagen) were, at the time, used by 23 out of 30 laboratories. Consequently, a multi-centre international validation of the newly developed qPCR method was initiated for these three platforms.

Materials and methods

qPCR design

A new *L. pneumophila* assay targeting the macrophage infectivity potentiator gene (*mip*) was designed for this study. Briefly, the 402-bp sequence of the 59 *mip* alleles available at the time of the study in the *L. pneumophila* Sequence-Based Typing (SBT) database [8] was downloaded and aligned using ClustalW [9]. Oligonucleotides were designed in conserved regions, allowing a maximum of one mismatch in either the primer or probe binding sites using Primer3 [10]. Sequences of subsequently described *mip* alleles were added to the ClustalW alignment; thus, a total of 74 *mip* alleles obtained from 9423 *L. pneumophila* strains were included in this study.

The new *mip* assay was combined with available sg1 and IPC assays, targeting *wzm* [6] and *gfp* [11], respectively. The sequence of primers and probes used in this study is listed in Table 1.

In silico analysis

mip oligonucleotides were tested for *L. pneumophila* specificity against the NCBI database using BLASTn [12]. The presence of mismatches possibly affecting the performance of the qPCR assay was analysed in silico comparing the oligonucleotide sequences to the ClustalW alignment of all 74 *mip* alleles.

The presence of *wzm* was determined in 395 finished or draft *L. pneumophila* genomes (including 372 sg1 and 23 non-sg1 strains) using BLASTn in order to test for sg1 specificity. In the case of the draft genomes, de novo assembles were used which had been constructed using Velvet [13]. The ClustalW alignment of 17 *wzm* alleles characterised from these genomes was analysed to identify mismatches in the oligonucleotide binding sites.

qPCR assays

The final concentration of primers, probes and additional $MgCl_2$, and the amount of *gfp* DNA are listed in Table 2; Taq polymerase was activated at 95 °C for 2 min (ABI 7500) or 3 min (LightCycler 480 Instrument II and Rotor-Gene Q), followed by 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 30 s (LightCycler 480 Instrument II and Rotor-Gene Q) or 1 min (ABI 7500).

PCR reactions were performed with ABI 7500 in a final volume of 50 µL containing 25 µL of Platinum® Quantitative PCR SuperMix-UDG with ROX (Life Technologies) and 5 µL of template using 0.2-µL optical tubes (Life Technologies). The results were analysed with the Auto Threshold function in the green, yellow and red channels using v2.0.6 of the ABI 7500 software.

PCR reactions were performed with LightCycler 480 Instrument II in a final volume of 20 µL containing 10 µL of 2× iQ Multiplex Powermix (Bio-Rad) and 5 µL of template using 0.2-mL thin-wall 8-tube strips (Biozym TC). The results were analysed in the green, yellow and red channels using v1.5.039 of the LightCycler 480 Instrument II software and interpreted by the second derivative maximum method with crossing points automatically calculated. Colour compensation was activated for the green and yellow channels because of an observed crosstalk.

PCR reactions were performed with Rotor-Gene Q in a final volume of 20 µL containing 10 µL of Rotor-Gene Multiplex PCR Kit (Qiagen) and 5 µL of template using 0.1-mL thin-walled tubes (Qiagen). The results were analysed in the green, yellow and red channels using v2.1.0.9 of the Rotor-Gene Q software. The instrument was set to ignore fluorescent signals in the first 15 cycles and to use 0.025 as the threshold value.

L. pneumophila sg1 DNA standards

Two commercially available *L. pneumophila* DNA quantification standards (i.e. Minerva Biolabs, Cat.-No. 52–0101 and LGC Standards, Cat.-No. SRM_LEGDNA_01) prepared from DNA of the Philadelphia-1 strain (NCTC 11192^T) were used in this study.

Table 1 Summary of oligonucleotides used in this study (*FP* forward primer, *RP* reverse primer, *HP* hybridisation probe)

Name	Sequence (5′→3′)	Target	Amplicon	Reference
mip_FP	GAAGCAATGGCTAAAGGCATGC	*mip*	79 bp	This study
mip_RP	GAACGTCTTTCATTTGYTGTTCGG			
mip_HP	**HEX**-CGCTATGAGTGGCGCTCAATTGGCTTTA-**BHQ1**			
wzm_FP	CAAAGGGCGTTACAGTCAAACC	*wzm*	75 bp	[6]
wzm_RP	CAAACACCCCAACCGTAATCA			
wzm_HP	**FAM**-TCTTGGGATTGGGTTGGGTTATTTTAACTCCT-**BHQ1**			
gfp_FP	CCTGTCCTTTTACCAGACAACCA	*gfp*	77 bp	[11]
gfp_RP	GGTCTCTCTTTTCGTTGGGATCT			
gfp_HP	**Cy5**-TACCTGTCCACACAATCTGCCCTTTCG-**BHQ3**			

Efficiency

For each qPCR platform/kit combination, five experiments were performed on separate days. In each run, four dilutions (i.e. 50,000, 5000, 500 and 50 gu/reaction) of *L. pneumophila* DNA standard were tested in triplicate together with a no template control. Efficiency values were obtained from the slope of the standard curve using the qPCR machine software. For each qPCR platform/kit combination, the mean and standard deviation of the efficiency values obtained from the five runs were calculated. Mean values between 90 % (slope=−3.1) and 110 % (slope=−3.6) were considered acceptable [14].

Limit of detection

For each qPCR platform/kit combination, five experiments were performed on separate days. In each run, five dilutions of the *L. pneumophila* DNA standard (namely, 50, 40, 30, 20 and 10 genome units) plus a no template control were tested. The limit of detection (LoD) was determined statistically by Probit analysis [14].

Validation panel

A panel of microorganisms (see the Supplementary Material) consisting of 36*L. pneumophila* reference strains (including all 16 described serogroups), 46*L. pneumophila* isolates representing the 46 *mip* alleles available in the authors' collection, 52 non-*pneumophila Legionella* species (total of 60 strains) and 96 non-*Legionella* species (total of 109 strains) was analysed on the Rotor-Gene Q to test assay specificity. *Legionellae* were cultured on buffered charcoal yeast extract (BCYE) agar (Oxoid) at 37 °C for 48–72 h in a moist atmosphere. Non-*Legionella* strains were grown on Nutrient Agar (Oxoid) at 37 °C for 24 h or specific media/conditions according to growth requirements. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) and quantified with a Qubit® Fluorometer (Life Technologies) and/or a NanoDrop ND-100 spectrophotometer (Thermo Scientific). *L. pneumophila* DNA extracts were tested at a concentration of ca. 10 pg/reaction, while extracts from other species were tested at a concentration of ca. 1 ng/reaction.

Legionella PCR EQA samples

Samples from seven ELDSNet/ESGLI and three QCMD EQA distributions were analysed on the Rotor-Gene Q in parallel to a previously validated *mip* assay [5]. Each distribution included ten samples, and a total of 100 EQA specimens were tested.

Clinical samples

Respiratory samples from 132 consecutive patients with evidence of legionellosis submitted over a 12-month period (16/08/2013 to 14/08/2014) were tested at Public Health England *Legionella* Reference Laboratory (UK) using the Rotor-Gene Q *Legionella* culture and DNA extraction was performed as previously described [5].

Table 2 Final concentrations of forward primers (*FP*), reverse primers (*RP*), hybridisation probes (*HP*) and additional $MgCl_2$, and amount of *gfp* DNA used in this study

	mip			*wzm*			*gfp*			$MgCl_2$	*gfp* DNA
	FP	RP	HP	FP	RP	HP	FP	RP	HP		
ABI 7500	0.5 μM	0.5 μM	0.2 μM	0.5 μM	0.5 μM	0.1 μM	0.05 μM	0.05 μM	0.05 μM	2 mM	0.125 pg
LightCycler 480 Instrument II	0.5 μM	0.5 μM	0.2 μM	0.5 μM	0.5 μM	0.2 μM	0.05 μM	0.05 μM	0.02 μM	0 mM	0.025 pg
Rotor-Gene Q	0.5 μM	0.5 μM	0.2 μM	0.5 μM	0.5 μM	0.2 μM	0.05 μM	0.05 μM	0.1 μM	0 mM	0.5 pg

Table 3 Summary of single nucleotide polymorphisms identified in *mip* and *wzm* oligo binding sites. *mip* analysis was performed on the 74 alleles obtained from 9423 strains deposited in the *Legionella pneumophila* SBT database, while *wzm* analysis was performed on 372 *L. pneumophila* serogroup 1 (sg1) sequenced genomes available to the authors

Target	Oligo	Binding site (5′→3′)	Allele	*L. pneumophila* strains		Tested strain
				n	%	
mip	Forward primer	GAAGCAATGGCTAAAGGCATGC	70 out of 74	9397	99.73	Philadelphia-1 (NCTC 11192)
		GAAGCA**T**TGGCTAAAGGCATGC	3 out of 74 (i.e. mip 18, 35 and 73)	26	0.27	MICU-B (NCTC 12271)
		GAAGC**T**ATGGCTAAAGGCATGC	1 out of 74 (i.e. mip 61)	0	N/A	No strain available (virtual allele)
	Probe	CGCTATGAGTGGCGCTCAATTGGCTTTA	72 out of 74	9369	99.43	Philadelphia-1 (NCTC 11192)
		CGCTATGAGT**A**GCGCTCAATTGGCTTTA	1 out of 74 (i.e. mip 21)	49	0.52	H060760379
		CGCTATGAGTGG**T**GCTCAATTGGCTTTA	1 out of 74 (i.e. mip 22)	5	0.05	R6031
	Reverse primer	GAACGTCTTTCATTTGYTGTTCGG	73 out of 74	9399	99.74	Philadelphia-1 (NCTC 11192)
		GAACGTCTTTCATTTGTTG**C**TCGG	1 out of 74 (i.e. mip 20)	24	0.26	Bloomington (NCTC 11232)
wzm	Forward primer	CAAAGGGCGTTACAGTCAAACC	14 out of 17	367	98.66	Philadelphia-1 (NCTC 11192)
		CAAAGG**A**CGTTACAGTCAAACC	1 out of 17	1	0.27	H091960011
		CAAAGGGCG**A**TACAGTCAAACC	2 out of 17	4	1.08	006/96 (EUL00048)
	Probe	TCTTGGGATTGGGTTGGGTTATTT TAACTCCT	15 out of 17	368	98.92	Philadelphia-1 (NCTC 11192)
		TCTTGGGATTGGGTTGGGTT AT**C**TTAACTCCT	2 out of 17	4	1.08	006/96 (EUL00048)
	Reverse primer	CAAACACCCCAACCGTAATCA	15 out of 17	352	94.62	Philadelphia-1 (NCTC 11192)
		CAAACACCCCAAC**T**GTAATCA	2 out of 17	20	5.38	LD10/94 (EUL00101)

Bold underlined positions are mutated in respect to the oligo nucleotide sequence

Results

mip assay

The new *L. pneumophila* assay was designed in a conserved area of the *mip* allele region with only the reverse primer binding site containing a non-conserved position (A/G), which was taken into account by including a ‘Y’ base (i.e. C/T). Among the 74 alleles, 67 contain no other variation in the oligonucleotide binding sites (Table 3), for a total of 9319 total strains (98.90 % entries) in the SBT database. The seven remaining alleles each contained one mismatch, for a total of five distinct mismatches (Table 2). Nevertheless, *mip* was successfully amplified from representative strains for 4/5 mismatches on all three qPCR platforms. Testing of allele 61 was not possible, as the carrying strain is not internationally available (Table 3).

BLASTn analysis showed the newly designed *mip* oligonucleotides as specific (100 % coverage and homology) for *mip* fragments of three putative non-*pneumophila Legionella* strains, namely, *L. micdadei*, *L. fairfieldensis* and *L. worsleiensis*. These *mip* sequences (AJ496274, U60163 and U60164) were downloaded from the NCBI database and analysed using the ‘*Legionella* species identification’ online tool [15], which uses the *mip* sequence to differentiate between members of the *Legionella* genus [16]. All three sequences were identified as belonging to *L. pneumophila* strains, with homologies between 99.83 and 100 %.

wzm assay

The presence and sequence of *wzm* were analysed in silico on 395 *L. pneumophila* available genomes (data not shown). All sg1 genomes (372) included *wzm*, while this gene was not found in non-sg1 strains. Sequence analysis of *wzm* revealed 17 variants, of which 14 (for a total of 347 sg1 strains) did not show variation in the oligonucleotide binding sites. As per *mip* validation, DNA from a representative strain for each of the four identified mismatches was extracted and successfully tested on each qPCR platform (Table 3).

Table 4 Average crossing points (Cp) and efficiency obtained in this study for the three qPCR platforms

Target	qPCR platform	Average Cp (standard deviation)				Average efficiency (standard deviation)
		50,000 [gu/reaction]	5000 [gu/reaction]	500 [gu/reaction]	50 [gu/reaction]	
mip	ABI 7500	23.08 (0.28)	26.75 (0.27)	30.29 (0.28)	33.61 (0.39)	96.55 % (4.98)
	LightCycler 480 Instrument II	24.57 (1.41)	28.10 (1.37)	31.55 (1.30)	34.90 (0.96)	93.21 % (1.59)
	Rotor-Gene Q	24.65 (0.68)	28.03 (0.54)	31.68 (0.90)	34.86 (2.04)	96.12 % (7.41)
wzm	ABI 7500	23.18 (0.59)	26.86 (0.41)	30.45 (0.44)	33.62 (0.50)	97.69 % (2.19)
	LightCycler 480 Instrument II	24.48 (1.40)	28.02 (1.36)	31.50 (1.29)	34.85 (1.00)	91.97 % (1.40)
	Rotor-Gene Q	25.44 (0.80)	28.84 (0.68)	32.59 (1.52)	35.76 (1.56)	94.80 % (8.45)

Efficiency

Determination of qPCR efficiency is crucial to ensure accurate target quantification. Efficiency should be as close as possible to 100 %, while mean values between 90 and 110 % were considered acceptable [14]. An efficiency value equal to 100 % means that the target sequence is doubling at each cycle during the logarithmic phase of the reaction. A theoretically optimal PCR efficiency gives a 3.3-cycles difference between 10-fold dilutions of template. The triplex qPCR assay generated efficiency values within the required limits on all platforms for both *mip* and *wzm* (Table 4), ensuring reliable target DNA quantification within the standard curve (i.e. between 50,000 and 50 units of *L. pneumophila* sg1 DNA). Efficiency was calculated with Philadelphia-1 strain (NCTC 11192^T) that does not contain mismatches in the *mip* and *wzm* primer binding sites. Less reliable target quantification may occur if mismatches are present.

Limit of detection

Conventionally, the LoD is reported as the "estimate of the detection limit that can be achieved with 95 % confidence" [14] or as "the lowest actual concentration of analyte" (*L. pneumophila* DNA in this instance) "in a specimen that can be consistently detected" (e.g. in 95, 90 or 50 % of specimens tested) "under routine laboratory conditions in a defined type of specimens" [17]. The LoD results are shown in Table 5. Lower LoD 90 % and LoD 95 % values were obtained using

Table 5 Number of *L. pneumophila* genome copies to produce a positive result in 50, 90 and 95 % of replicate samples

Target	qPCR platform	Confidence	Genome copies	Range of genome copies	
				Lower	Upper
mip	ABI 7500	50 %	<0.01	<0.01	11.26
		90 %	36.51	27.26	59.76
		95 %	47.26	35.76	86.01
	LightCycler 480 Instrument II	50 %	3.51	<0.01	5.76
		90 %	8.26	6.76	17.51
		95 %	9.76	7.51	25.76
	Rotor-Gene Q	50 %	21.76	15.51	26.76
		90 %	43.26	37.01	53.76
		95 %	49.26	42.01	62.76
wzm	ABI 7500	50 %	2.51	<0.01	12.76
		90 %	37.01	29.01	56.51
		95 %	47.01	36.76	78.51
	LightCycler 480 Instrument II	50 %	1.26	<0.01	5.26
		90 %	10.26	6.51	42.51
		95 %	12.76	9.26	81.76
	Rotor-Gene Q	50 %	18.76	13.76	22.51
		90 %	34.01	29.51	41.76
		95 %	38.51	33.26	48.01

Lower and *Upper* are the numbers of genome copies corresponding to the lower and upper 95 % confidence limits for positive proportions, respectively

LightCycler 480 Instrument II, but this could be a consequence of the fact that the LGC quantification standard was used, while the Minerva standard was used for Rotor-Gene Q and ABI 7500. In line with Saunders et al. [14], the values estimated with 95 % confidence (LoD 95 %) were adopted as the LoD for *mip* and *wzm* assays on all three qPCR platforms (Table 5).

Validation panel and EQA samples

The triplex assay was 100 % specific for *L. pneumophila* and sg1 DNA on the large panel of type strains (see the Supplementary Material) tested on Rotor-Gene Q. *mip* was successfully amplified form all EQA samples containing *L. pneumophila* DNA. One sample containing PBS thus intended to be negative gave a positive *mip* result. The same happened when the sample was tested with a previously published *mip* assay [5] targetting a different part of the *mip* gene, hence a contaminated DNA extract rather than a specificity issue was considered as the likely cause of the unexpected result. On the same set of samples, *wzm* was successfully amplified whenever DNA from sg1 strains was present.

Clinical samples

Respiratory samples from 132 consecutive patients with evidence of legionellosis were tested for the presence of *L. pneumophila* and sg1 DNA using Rotor-Gene Q. 108/128 (84 %) *L. pneumophila* urinary antigen-positive patients were *L. pneumophila* and sg1 qPCR-positive. *L. pneumophila* sg1 was subsequently grown from 80/108 and a further 17 of the 28 PCR-positive/culture-negative samples yielded DNA sequence typing data directly from the sample by nested SBT [18]. *L. pneumophila* sg1 was isolated from 1/20 PCR-negative samples; however, a second sample from the same patient was both qPCR- and culture-positive. 3/132 samples were submitted as being positive using local *Legionella* spp. PCRs and 1/132 was obtained from an *L. pneumophila* sg6 culture-proven case. All were *L. pneumophila*-positive and sg1-negative in this triplex qPCR. *L. pneumophila* was subsequently isolated from 2/3 samples (sg3 and sg5, respectively) and nested SBT detected ST1775 in the third sample; previously, this ST has only been reported from an *L. pneumophila* sg5 strain.

Inhibition

Under the applied testing conditions, no assay failure due to inhibition issues was observed with any specimen or culture under test. Inhibition is primarily caused by inhibitors present in the DNA template. In this study, methods yielding high-quality DNA extracts were used. Inhibition issues might occur if extraction methods yielding lower quality DNA were used.

Discussion

We describe the design and validation of a triplex qPCR for the simultaneous detection of *L. pneumophila* and *L. pneumophila* sg1 DNA on three real-time PCR platforms to aid international standardisation of molecular detection of *L. pneumophila* amongst ESGLI laboratories.

A rational approach using the *mip* allele sequences available on the *L. pneumophila* SBT database was applied when designing a new *L. pneumophila* assay; a wide range of both in silico and in vitro experiments was then performed to fully validate the newly designed *mip* assay (targeting *L. pneumophila*) and to expand the validation of the *wzm* assay (targeting sg1) previously published [6]. The *gfp* assay used as an IPC was adopted, as previously published [11].

The assay proved 100 % specific for *L. pneumophila* and sg1 DNA on a large panel of type strains, clinical samples and EQA samples tested on Rotor-Gene Q. The efficiency and LoD were calculated for all three qPCR platforms. The LoD was excellent for both *L. pneumophila* and sg1 targets, indicating that this methodology can detect low quantities of target DNA reproducibly. Inhibition issues were not observed. The international validation documented in this study demonstrates that this methodology is applicable for use in laboratories around the globe and can be performed on different qPCR platforms without loss of functionality.

Although the utility of molecular techniques in diagnosing infectious diseases is universally recognised, detection of nucleic acid is still among the laboratory criteria of a probable but not proven LD case according to the ECDC. This is currently a limitation in LD case definition. The validation of this qPCR method by the ESGLI is intended to promote the use of qPCR to detect *L. pneumophila* in LD cases and consideration for the inclusion of *L. pneumophila* DNA detection within laboratory criteria for proven LD cases should be given.

Acknowledgements Preliminary results of this study were presented during the 1st ESGLI meeting in Dresden, Germany, 2012 and at the 8th International Conference on Legionella in Melbourne, Australia, 2013. The authors would like to acknowledge members of the ESCM ID Study Group for Legionella Infections (ESGLI) for help and advice with the validation study, Dr. Ayoub Saei (PHE Statistics) for assistance with the Probit analysis and Dr. Norman Fry for constructive comments on the manuscript.

Conflict of interest The authors declare no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. For this type of study, formal consent is not required.

References

1. Phin N, Parry-Ford F, Harrison T, Stagg HR, Zhang N, Kumar K, Lortholary O, Zumla A, Abubakar I (2014) Epidemiology and clinical management of Legionnaires' disease. Lancet Infect Dis 14: 1011–1021
2. McDade JE, Shepard CC, Fraser DW, Tsai TR, Redus MA, Dowdle WR (1977) Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease. N Engl J Med 297:1197–1203
3. Fraser DW, Tsai TR, Orenstein W, Parkin WE, Beecham HJ, Sharrar RG, Harris J, Mallison GF, Martin SM, McDade JE, Shepard CC, Brachman PS (1977) Legionnaires' disease: description of an epidemic of pneumonia. N Engl J Med 297:1189–1197
4. European Centre for Disease Prevention and Control (ECDC) (2014) Surveillance report: Legionnaires' disease in Europe 2012 (26 March 2014). Available online at: http://ecdc.europa.eu/en/publications/Publications/legionnaires-disease-surveillance-2012.pdf. Accessed 30 Jan 2015
5. Mentasti M, Fry NK, Afshar B, Palepou-Foxley C, Naik FC, Harrison TG (2012) Application of *Legionella pneumophila*-specific quantitative real-time PCR combined with direct amplification and sequence-based typing in the diagnosis and epidemiological investigation of Legionnaires' disease. Eur J Clin Microbiol Infect Dis 31:2017–2028
6. Mérault N, Rusniok C, Jarraud S, Gomez-Valero L, Cazalet C, Marin M, Brachet E, Aegerter P, Gaillard JL, Etienne J, Herrmann JL; DELPH-I Study Group, Lawrence C, Buchrieser C (2011) Specific real-time PCR for simultaneous detection and identification of *Legionella pneumophila* serogroup 1 in water and clinical samples. Appl Environ Microbiol 77:1708–1717
7. European Centre for Disease Prevention and Control (ECDC). Legionnaires' disease: EU case definition. Available online at: http://ecdc.europa.eu/en/activities/surveillance/ELDSNet/Pages/EU%20case%20definition.aspx. Accessed 30 Jan 2015
8. Sequence-based typing (SBT) database for *Legionella pneumophila*. Available online at: http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php. Accessed 30 Jan 2015
9. ClustalW2. Home page at: http://www.ebi.ac.uk/Tools/msa/clustalw2. Accessed 30 Jan 2015
10. Primer3. Home page at: http://frodo.wi.mit.edu. Accessed 30 Jan 2015
11. Murphy NM, McLauchlin J, Ohai C, Grant KA (2007) Construction and evaluation of a microbiological positive process internal control for PCR-based examination of food samples for *Listeria monocytogenes* and *Salmonella enterica*. Int J Food Microbiol 120:110–119
12. BLASTn. Home page at: http://www.ncbi.nlm.nih.gov/BLAST. Accessed 30 Jan 2015
13. Zerbino DR, Birney E (2008) Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res 18:821–829
14. Saunders N, Zambon M, Sharp I, Siddiqui R, Bermingham A, Ellis J, Vipond B, Sails A, Moran-Gilad J, Marsh P, Guiver M, HPA Microbiology Services Division (2013) Guidance on the development and validation of diagnostic tests that depend on nucleic acid amplification and detection. J Clin Virol 56:260–270
15. *Legionella species* identification by *mip* similarity. Available online at: http://www.hpa-bioinformatics.org.uk/cgi-bin/legionella/mip/mip_id.cgi. Accessed 30 Jan 2015
16. Ratcliff RM, Lanser JA, Manning PA, Heuzenroeder MW (1998) Sequence-based classification scheme for the genus *Legionella* targeting the *mip* gene. J Clin Microbiol 36:1560–1567
17. Burd EM (2010) Validation of laboratory-developed molecular assays for infectious diseases. Clin Microbiol Rev 23:550–576
18. Ginevra C, Lopez M, Forey F, Reyrolle M, Meugnier H, Vandenesch F, Etienne J, Jarraud S, Molmeret M (2009) Evaluation of a nested-PCR-derived sequence-based typing method applied directly to respiratory samples from patients with Legionnaires' disease. J Clin Microbiol 47:981–987