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Rapid investigation of cases and clusters of Legionnaires' disease in England and Wales using direct molecular typing

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Legionella pneumophila is the leading cause of Legionnaires' disease, a severe pneumonia that can occur as sporadic cases or point-source outbreaks affecting multiple patients. The infection is acquired by inhalation of aerosols from contaminated water systems. In order to identify the probable source and prevent further cases, clinical and environmental isolates are compared using phenotypic and genotypic methods. Typically up to 10 days are required to isolate *L. pneumophila* prior to the application of standard typing protocols. A rapid protocol using a real-time PCR specific for *L. pneumophila* and serogroup 1, combined with nested direct molecular typing, was adopted by Public Health England in 2012 to reduce reporting time for preliminary typing results. This rapid protocol was first used to investigate an outbreak that occurred in July/August 2012 and due to the positive feedback from that investigation, it was subsequently applied to other incidents in England and Wales where faster typing results would have aided incident investigation. We present here results from seven incidents that occurred between July 2012 and June 2015 where the use of this rapid approach provided preliminary characterization of the infecting strain in an average 1.58 days (SD 1.01) after sample receipt in contrast to 9.53 days (SD 3.73) when standard protocols were applied.

INTRODUCTION

Legionellae are Gram-negative bacteria, ubiquitously found in natural and man-made water systems, able to survive as free-living cells or infect protozoa and multiply intracellularly (Harrison, 2005). They can also infect humans causing either a self-limiting 'flu-like syndrome known as Pontiac fever, or a severe and potentially lethal pneumonia known as Legionnaires' disease (LD) (Phin *et al.*, 2014). Since the first described LD outbreak that affected 182 people with 29 fatalities in Philadelphia, USA in 1976 (Fraser *et al.*, 1977), several other outbreaks have occurred worldwide with high mortality and public health cost (Bennett *et al.*, 2014; Den Boer *et al.*, 2002; Shivaji *et al.*, 2014).

To date 59 different species of *Legionella* and three subspecies have been described (www.bacterio.net/legionella.html). A single species, *Legionella pneumophila* remains the main cause of LD with serogroup (sg) 1 strains accounting for approximately 85 % of culture proven cases (Yu *et al.*, 2002; ECDC, 2015) and almost all outbreaks (Phin *et al.*, 2014). Prompt detection and characterization of *L. pneumophila* sg1 isolates from clinical and environmental samples is crucial in outbreak situations to identify and disinfect probable sources, preventing further cases.

Legionellae are fastidious and slow growing, often overgrown on culture plates by background flora present in samples. Up to 10 days are required to isolate and purify single colonies which are then further characterized using monoclonal antibodies (mAbs) (Helbig *et al.*, 2002) and sequence-based typing (SBT) (Gaia *et al.*, 2005; Ratzow *et al.*, 2007; Mentasti *et al.*, 2014). Although this approach provides full typing results, when an LD outbreak is possibly occurring, more rapid results could influence the public health response of the incident control team and prevent further cases.

One supplementary table is available with the online Supplementary Material.

Abbreviations: LD, Leigionnaires' disease; ndSBT, nested direct sequence-based-typing; nSBT, nested sequence-based typing; SBT, sequence-based typing; sg, serogroup; ST, Sequence Type; WGS, whole-genome sequencing.

In preparation for the 2012 Olympic Games held in London, UK, Public Health England (PHE) established the capacity to rapidly obtain diagnostic and typing results on a range of potential microbiological threats (Moran-Gilad *et al.*, 2012) with the aim to reduce the turnaround time for reporting of preliminary results in the event of a major microbiological incident. The approach for LD was based on the rapid detection of *L. pneumophila* and sg1 markers from both clinical and environmental samples by PCR followed by direct molecular typing performed on PCR-positive DNA extracts. Nested SBT (nSBT) had already been demonstrated retrospectively to give typing results on *L. pneumophila* PCR-positive clinical samples where the isolation of *L. pneumophila* was not successful using culture (Ginevra *et al.*, 2009; Mentasti *et al.*, 2012). Therefore when typing results are urgently required to aid LD investigations, preliminary data could be obtained using nSBT directly on positive DNA extracts (ndSBT) while waiting for culture results. Full typing results could then be obtained using mAb and SBT on clinical and environmental isolates, if available. This approach was first used in 2012 during the investigation of an LD outbreak (Coetzee *et al.*, 2012) with preliminary typing results available 24 h after samples were received by the National Legionella Reference Laboratory (NLRL) at PHE London. After this investigation, processing of samples related to urgent cases and possible outbreaks was subsequently altered to enable implementation of direct molecular typing when it was considered relevant to aid the LD investigation. This study describes the use and findings of the rapid typing approach on a total of seven LD incidents.

METHODS

Thirty-eight patients involved in seven LD incidents that occurred in England and Wales between July 2012 and June 2015 were included in the study. Twenty-seven respiratory samples and one clinical isolate obtained from 28/38 (73.7 %) patients together with a total of 65 environmental samples were referred to the NLRL. Overall, 93 samples in total were included in this study (Table 1).

Urine and respiratory samples (where available), and *L. pneumophila* isolates were referred by local hospitals to the NLRL for confirmation and further characterization. Urine samples were tested for *L. pneumophila* antigen using in-house and/or commercial assays, while respiratory samples were cultured according to previously described methods (Mentasti *et al.*, 2012).

Environmental samples were cultured according to standard methods based on ISO 11731:1998 (Anonymous, 1998) at regional Food, Water and Environmental (FWE) Laboratories in England and Wales. Swab samples were resuspended in sterile distilled water and processed as water concentrates. Aliquots of water concentrates related to urgent cases and clusters were referred to the NLRL in London for PCR and rapid molecular typing or, from June 2014, tested at the FWE laboratory in PHE Porton by PCR for presence of *L. pneumophila* sg 1 DNA (Collins *et al.*, 2015) with positive DNA extracts referred to the NLRL for rapid molecular typing. At NLRL 1 ml water concentrate was centrifuged (15 000 ***g*** for 10 min), 800 µl supernatant was discarded and DNA extracted from the remaining 200 µl using the MagNA Pure Compact (Roche) and/or Instagene Matrix (Bio-Rad) according to the manufacturer's instructions.

DNA extracts were tested for the presence of *L. pneumophila* and sg1 DNA by a real-time PCR assay targeting the macrophage infectivity potentiator gene (*mip*) of *L. pneumophila* and the sg1 marker *wzm* (Mentasti *et al.*, 2015). Direct typing was immediately performed on *L. pneumophila* PCR-positive DNA extracts by nSBT (Ginevra *et al.*, 2009; Mentasti *et al.*, 2012), a nested modification of the internationally agreed SBT protocol (Gaia *et al.*, 2005; Ratzow *et al.*, 2007; Mentasti *et al.*, 2014), designed to increase sensitivity and obtain typing results on DNA extracted from primary samples. PCR products were sequenced at PHE London sequencing facility with a turnaround time of 6–8 h.

Clinical and environmental isolates (where available) were characterized according to the internationally agreed method using mAb (Helbig *et al.*, 2002) and SBT (Gaia *et al.*, 2005; Ratzow *et al.*, 2007; Mentasti *et al.*, 2014). nSBT, ndSBT and SBT results were analysed online using the *L. pneumophila* Sequence Quality Tool (www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq_assemble_legionella1.cgi) to obtain allelic profiles and sequence types (STs). Alleles and STs that could not be assessed by ndSBT were marked as '0'.

RESULTS

A total of seven LD incidents are included in this study and a brief summary for each incident is presented (Tables 1 and 2).

Incident 1 occurred in July–August 2012 (Coetzee *et al.*, 2012). On 24 July (day 0), the NLRL received sputum samples from six urinary antigen-positive patients for *L. pneumophila* PCR detection and culture. That evening sputum samples from five patients were reported to the outbreak management team as positive for *L. pneumophila* sg1 DNA and ndSBT PCR products were submitted to the PHE London sequencing service for urgent analysis. On 25 July (day 1), two patients were reported as infected by a strain with a novel allelic profile (2,9,2,5,3,17,15) designated as ST1268, while partial allelic profiles consistent with the same ST were obtained for the other three *L. pneumophila* sg1 PCR-positive patients (Table 2). The same approach was applied to environmental samples received on 27 July (day 3). A swab from a spa pool tested positive for *L. pneumophila* sg1 DNA and was analysed by ndSBT. Twenty-four hours later (day 4), a full ST1268 allelic profile was reported from this swab sample. Water concentrates from two cooling towers included in a second batch of environmental samples tested positive for *L. pneumophila* sg1 DNA by PCR (day 3) and were analysed by ndSBT. On 30 July (day 6), partial profiles from the cooling towers, namely 0,0,0,15,18,1,6 and 8,0,0,15,18,1,6, with no allele in common with the profile of the infecting strain were reported. *L. pneumophila* was isolated by culture from both cooling towers and fully characterized as *L. pneumophila* sg1, mAb subgroup 'Allentown/France', ST62 (8,10,3,15,18,1,6). Overall, respiratory samples were available for 11/21 patients involved in the outbreak, nine were PCR-positive for *L. pneumophila* sg1, a full ST1268 profile was obtained for six patients and partial profiles consistent with ST1268 were obtained for the remaining three patients. *L. pneumophila* sg1, mAb subgroup 'Benidorm', ST1268 was isolated by culture from six patients. A total of 41 environmental samples were tested with the rapid protocol, three samples tested positive for *L.*

Table 1. Summary of samples included in this study

Source	Type	No.	Total	
Clinical	Sputum	26	28	93
	Bronchoalveolar lavage (BAL)	1		
	Isolate	1		
Environmental	Water concentrate	43	65	
	Swab	10		
	DNA extract	6		
	Isolate	6		

pneumophila sg1 DNA by PCR and were further analysed by ndSBT. A full ST1268 profile was obtained from a swab collected from the spa pool and although heat and acid treatment were applied, no *L. pneumophila* strain was isolated from the sample due to significant contamination with *Pseudomonas aeruginosa*.

Incident 2 occurred in May 2013. Two patients were admitted to the same hospital in the same week and tested positive for Legionella urinary antigen. Respiratory samples tested positive by NLRL for *L. pneumophila* sg1 DNA using PCR, while ndSBT yielded partial profiles (day 3), namely 5,0,22,15,6,2,6 and 3,0,0,0,0,0,0. *L. pneumophila* sg1, mAb subgroup 'Allentown/France', ST47 was subsequently isolated by culture from one patient but legionellae could not be recovered from the other. Samples from two putative sources, both cooling towers, were positive for *L. pneumophila* sg1 DNA by PCR with ndSBT rapidly yielding a full ST363 profile (7,6,3,3,13,11,11) and a partial profile 7,0,0,3,13,11,11 (day 2), respectively. *L. pneumophila* sg1, mAb subgroup 'Bellingham', ST363 was later isolated from both cooling towers

Incident 3 occurred in July–September 2013. The first two patients were identified 5 weeks apart and investigated as sporadic cases, thus the fast protocol was not deployed. *L. pneumophila* sg1, mAb subgroup 'Philadelphia', ST1534 (3,10,1,1,1,9,11) and *L. pneumophila* sg1, mAb subgroup 'Allentown/France', ST62 (8,10,3,15,18,1,6) were isolated and characterized using mAbs and SBT. When two additional patients in the same area were diagnosed with LD at the end of August and beginning of September, a cluster alert was triggered. *L. pneumophila* sg1 DNA was detected by PCR in both patients and ndSBT yielded a partial allelic profile (5,0,22,27,6,0,12) in patient 3 (day 1) and a full ST46 (5,1,22,5,6,10,15) profile in patient 4 (day 2). *L. pneumophila* sg1, mAb subgroup 'Bellingham', ST48 was later isolated from patient 3 and *L. pneumophila* sg1, mAb subgroup 'Benidorm', ST46 from patient 4.

Incident 4 occurred in August 2013 with four patients diagnosed in the same area by the Legionella urinary antigen test within the same week. Respiratory samples referred to the NLRL were analysed using the rapid protocol. *L. pneumophila* sg1 DNA was detected in all four patients and ndSBT yielded three partial allelic profiles, namely 2,3,0,10,2,1,6 (day 3), 3,0,1,1,0,9,1 (day 1) and 5,0,22,15,6,2,6 (day 1), and a full ST47 (5,10,22,15,6,2,6) profile (day 1). *L. pneumophila* sg1, mAb subgroup 'Allentown/France', ST23 (2,3,9,10,2,1,6) was later isolated from patient 1 and *L. pneumophila* sg1, mAb subgroup 'Allentown/France', ST47 from patients 3 and 4. Isolation of *L. pneumophila* was not successful for patient 2. Ten environmental samples from local potential sources were also referred for rapid testing; *L. pneumophila* sg1 was not detected in any of these specimens or isolated by culture.

Incident 5 occurred in June 2014. *L. pneumophila* sg1, mAb subgroup 'Allentown/France', ST48 was isolated from the bronchoalveolar lavage of a neonate who on enquiry was found to have been born at home using a heated birthing pool (Phin *et al.*, 2014; Collins *et al.*, 2016). Because of the public health implications of this finding an urgent investigation was initiated. Water samples from the domestic water system and swab samples from the birthing pool were tested by PCR at PHE Porton Down (Collins *et al.*, 2015) with four *L. pneumophila* sg1-positive DNA extracts sent to the NLRL for urgent typing using ndSBT. A full ST48 allelic profile was reported for one sample (day 1), while partial profiles consistent with ST48 were reported for the other three samples (Table 2). Despite application of heat and acid treatment, *L. pneumophila* was not isolated from the PCR-positive swab samples due to gross contamination with other microbial flora.

Incident 6 occurred in September 2014. Two urinary antigen-positive patients were found to have visited the same hotel with a swimming pool and spa facility during their incubation period. *L. pneumophila* sg1, mAb subgroup 'Philadelphia', ST1 was isolated from patient 1 while respiratory specimens collected from patient 2 were negative for *L. pneumophila* by both molecular analysis and culture. ndSBT was not undertaken on patient 1 as the isolate was already available at the time of notification of the second case. ndSBT was applied to sg1-positive DNA extracts from environmental specimens (pool and spa) processed at PHE Porton resulting in a full ST1 profile (day 1). Environmental isolates were subsequently obtained by culture and characterized as *L. pneumophila* sg1, mAb subgroup 'Philadelphia', ST1 and *L. pneumophila* sg1, mAb subgroup 'Oxford/Olda', ST1.

Incident 7 occurred in May 2014 when a common exposure was identified between two apparently sporadic cases with LD onset 6 months apart (November 2014–May 2015). Both patients were found to be infected by *L. pneumophila* sg1, mAb subgroup 'Philadelphia', ST37. When two additional cases with the same exposure history were notified in the same area in late May and early June 2015, clinical samples were promptly sent to the NLRL for urgent analysis. *L. pneumophila* sg1 DNA was detected and ndSBT yielded two identical partial allelic profiles (i.e. 3,0,0,1,14,9,11) consistent with ST37 (day 1 and day 5, respectively) strongly supportive of a common infective source among patients.

Table 2. Summary of results obtained from primary samples, DNA extracts and isolates referred to the NLRL

ndSBT was not tested on isolates and non-urgent samples; SBT was not tested where *L. pneumophila* was not isolated. Alleles and STs not obtained by ndSBT are marked as '0'. C, clinical; E, environmental; NT, not tested; NA, not applicable; INH, inhibitory; BAL, bronchoalveolar lavage.

LD incident	Sample			PCR		ndSBT		*L. pneumophila* sg1		SBT	
	Source	ID	Type	*mip*	*wzm*	Allelic profile	ST	Culture	Sub-group	Allelic profile	ST
1	C	1C1	Sputum	+	+	2,9,2,5,3,17,15	1268	+	Benidorm	2,9,2,5,3,17,15	1268
		1C2	Sputum	+	+	2,9,2,5,3,17,15	1268	+	Benidorm	2,9,2,5,3,17,15	1268
		1C3	Sputum	+	+	0,0,2,5,0,17,15	0	+	Benidorm	2,9,2,5,3,17,15	1268
		1C4	Sputum	+	+	2,9,2,5,3,0,15	0	+	Benidorm	2,9,2,5,3,17,15	1268
		1C5	Sputum	–	–	NT	NA	–	NA	NA	NA
		1C6	Sputum	+	+	2,9,2,5,3,17,15	1268	–	NA	NA	NA
		1C7	Sputum	+	+	2,9,2,5,3,17,15	1268	–	NA	NA	NA
		1C8	Sputum	+	+	2,9,2,5,3,17,15	1268	+	Benidorm	2,9,2,5,3,17,15	1268
		1C9	Sputum	+	+	2,0,2,5,3,17,15	0	+	Benidorm	2,9,2,5,3,17,15	1268
		1C10*	Sputum	+	+	2,9,2,5,3,17,15	1268	–	NA	NA	NA
		1C11	Sputum	–	–	NT	NA	–	NA	NA	NA
	E	1E1	Swab	–	–	NT	NA	–	NA	NA	NA
		1E2	Swab	–	–	NT	NA	–	NA	NA	NA
		1E3	Swab	–	–	NT	NA	–	NA	NA	NA
		1E4	Swab	–	–	NT	NA	–	NA	NA	NA
		1E5	Swab	+	+	2,9,2,5,3,17,15	1268	–	NA	NA	NA
		1E6	Water	–	–	NT	NA	–	NA	NA	NA
		1E7	Water	–	–	NT	NA	–	NA	NA	NA
		1E8	Water	–	–	NT	NA	–	NA	NA	NA
		1E9	Water	–	–	NT	NA	–	NA	NA	NA
		1E10	Water	–	–	NT	NA	–	NA	NA	NA
		1E11	Water	–	–	NT	NA	–	NA	NA	NA
		1E12	Water	INH	INH	NT	NA	–	NA	NA	NA
		1E13	Water	–	–	NT	NA	–	NA	NA	NA
		1E14	Water	–	–	NT	NA	–	NA	NA	NA
		1E15	Water	INH	INH	NT	NA	–	NA	NA	NA
		1E16	Water	–	–	NT	NA	–	NA	NA	NA
		1E17	Water	–	–	NT	NA	–	NA	NA	NA
		1E18	Water	–	–	NT	NA	–	NA	NA	NA
		1E19	Water	+	+	0,0,0,15,18,1,6	0	+	Allentown/France	8,10,3,15,18,1,6	62
		1E20	Water	+	+	8,0,0,15,18,1,6	0	+	Allentown/France	8,10,3,15,18,1,6	62
		1E21	Water	–	–	NT	NA	–	NA	NA	NA
		1E22	Water	–	–	NT	NA	–	NA	NA	NA
		1E23	Water	–	–	NT	NA	–	NA	NA	NA
		1E24	Water	–	–	NT	NA	–	NA	NA	NA

Table 2. cont.

LD incident	Sample			PCR		ndSBT		*L. pneumophila* sg1		SBT	
	Source	ID	Type	*mip*	*wzm*	Allelic profile	ST	Culture	Sub-group	Allelic profile	ST
		1E25	Water	–	–	NT	NA	–	NA	NA	NA
		1E26	Swab	–	–	NT	NA	–	NA	NA	NA
		1E27	Swab	–	–	NT	NA	–	NA	NA	NA
		1E28	Water	–	–	NT	NA	–	NA	NA	NA
		1E29	Swab	–	–	NT	NA	–	NA	NA	NA
		1E30	Water	–	–	NT	NA	–	NA	NA	NA
		1E31	Swab	–	–	NT	NA	–	NA	NA	NA
		1E32	Water	–	–	NT	NA	–	NA	NA	NA
		1E33	Water	–	–	NT	NA	–	NA	NA	NA
		1E34	Water	–	–	NT	NA	–	NA	NA	NA
		1E35	Water	–	–	NT	NA	–	NA	NA	NA
		1E36	Water	–	–	NT	NA	–	NA	NA	NA
		1E37	Swab	–	–	NT	NA	–	NA	NA	NA
		1E38	Swab	–	–	NT	NA	–	NA	NA	NA
		1E39	Water	–	–	NT	NA	–	NA	NA	NA
		1E40	Water	–	–	NT	NA	–	NA	NA	NA
		1E41	Water	–	–	NT	NA	–	NA	NA	NA
2	C	2C1	Sputum	+	+	5,0,22,15,6,2,6	0	+	Allentown/France	5,10,22,15,6,2,6	47
		2C2	Sputum	+	+	3,0,0,0,0,0,0	0	–	NA	NA	NA
	E	2E1	Water	+	+	7,6,3,3,13,11,11	363	+	Bellingham	7,6,3,3,13,11,11	363
		2E2	Water	+	+	7,0,0,3,13,11,11	0	+	Bellingham	7,6,3,3,13,11,11	363
3	C	3C1	BAL	+	+	NT	NA	+	Philadelphia	3,10,1,1,1,9,11	1534
		3C2	Sputum	+	+	NT	NA	+	Allentown/France	8,10,3,15,18,1,6	62
		3C3	Sputum	+	+	5,0,22,27,6,0,12	0	+	Bellingham	5,2,22,27,6,10,12	48
		3C4	Sputum	+	+	5,1,22,5,6,10,15	46	+	Benidorm	5,1,22,5,6,10,15	46
4	C	4C1	Sputum	+	+	2,3,0,10,2,1,6	0	+	Allentown/France	2,3,9,10,2,1,6	23
		4C2	Sputum	+	+	3,0,1,1,0,9,1	0	–	NA	NA	NA
		4C3	Sputum	+	+	5,0,22,15,6,2,6	0	+	Allentown/France	5,10,22,15,6,2,6	47
		4C4	Sputum	+	+	5,10,22,15,6,2,6	47	+	Allentown/France	5,10,22,15,6,2,6	47
	E	4E1	Water	–	–	NT	NA	–	NA	NA	NA
		4E2	Water	–	–	NT	NA	–	NA	NA	NA
		4E3	Water	–	–	NT	NA	–	NA	NA	NA
		4E4	Water	–	–	NT	NA	–	NA	NA	NA
		4E5	Water	–	–	NT	NA	–	NA	NA	NA
		4E6	Water	–	–	NT	NA	–	NA	NA	NA
		4E7	Water	–	–	NT	NA	–	NA	NA	NA

Table 2. cont.

LD incident	Sample			PCR		ndSBT		*L. pneumophila* sg1		SBT	
	Source	ID	Type	*mip*	*wzm*	Allelic profile	ST	Culture	Sub-group	Allelic profile	ST
		4E8	Water	–	–	NT	NA	–	NA	NA	NA
		4E9	Water	–	–	NT	NA	–	NA	NA	NA
		4E10	Water	–	–	NT	NA	–	NA	NA	NA
5	C	5C1	Isolate	NT	NT	NT	NA	+	Bellingham	5,2,22,27,6,10,12	48
	E	5E1	DNA	+	+	0,2,22,27,0,10,12	0	NA	NA	NA	NA
		5E2	DNA	+	+	5,2,22,27,6,0,12	0	NA	NA	NA	NA
		5E3	DNA	+	+	0,0,0,0,0,0,012	0	NA	NA	NA	NA
		5E4	DNA	+	+	5,2,22,27,6,10,12	48	NA	NA	NA	NA
6	C	6C1	Sputum	+	+	NT	NA	+	Philadelphia	1,4,3,1,1,1,1	1
		6C2	Sputum	–	–	NT	NA	–	NA	NA	NA
	E	6E1	DNA	+	+	1,0,3,1,1,1,1	0	NA	NA	NA	NA
		6E2	DNA	+	+	1,4,3,1,1,1,1	1	NA	NA	NA	NA
		6E3	Isolate	NT	NT	NT	NA	+	Oxford/Olda	1,4,3,1,1,1,1	1
		6E4	Isolate	NT	NT	NT	NA	+	Philadelphia	1,4,3,1,1,1,1	1
		6E5	Isolate	NT	NT	NT	NA	+	Philadelphia	1,4,3,1,1,1,1	1
		6E6	Isolate	NT	NT	NT	NA	+	Oxford/Olda	1,4,3,1,1,1,1	1
7	C	7C1	Sputum	+	+	NT	NA	+	Philadelphia	3,4,1,1,14,9,11	37
		7C2	Sputum	+	+	NT	NA	+	Philadelphia	3,4,1,1,14,9,11	37
		7C3	Sputum	+	+	3,0,0,1,14,9,11	0	–	NA	NA	NA
		7C4	Sputum	+	+	3,0,0,1,14,9,11	0	–	NA	NA	NA
	E	7E1	Isolate	NT	NT	NT	NA	+	Philadelphia	3,4,1,1,14,9,11	37
		7E2	Isolate	NT	NT	NT	NA	+	Philadelphia	3,4,1,1,14,9,11	37

*Obtained by nSBT thus omitted from Fig. 1 and not taken into account when calculating mean and standard deviation in Table S1.

Legionellae were not isolated from either patient. Control measures were immediately implemented and subsequently environmental isolates from a local cooling tower were isolated and typed as *L. pneumophila* sg1, mAb subgroup 'Philadelphia' ST37.

DISCUSSION

A rapid protocol using a real-time PCR specific for *L. pneumophila* and serogroup 1 combined with direct molecular typing was adopted by PHE in 2012 to reduce reporting time of preliminary typing results in the event of a major incident during the Olympic Games held in London, UK in summer 2012. This approach provided crucial or informative results in seven LD incidents that occurred in England and Wales between July 2012 and June 2015 (Table 2).

Initial diagnosis of LD was established at local hospitals by *L. pneumophila* urinary antigen test for all except one case (Incident 5) where culture was the primary diagnostic method. Respiratory samples were not available for 26.3 % (10/38) of patients. Other studies have shown that during LD investigations respiratory samples are not available for up to 65 % of patients (von Baum *et al.*, 2008), consequently urinary antigen testing remains the main diagnostic method for LD. Unfortunately this method provides limited information regarding the infecting strain, thus the amount of typing data obtainable from available respiratory samples should be maximized.

nSBT was initially developed to obtain typing results on *L. pneumophila* PCR-positive clinical samples where the isolation of *L. pneumophila* by culture was not successful. Here we directly applied nSBT (ndSBT) on clinical and environmental PCR-positive samples prior to *L. pneumophila* culture results to obtain rapid preliminary typing data for urgent LD cases and clusters.

When the rapid protocol was applied, partial to full allelic profiles were obtained for all PCR-positive samples within 1 to 5 days (mean 1.58, SD 1.01) after samples were delivered to the NLRL (Fig. 1, Table S1, available in the online Supplementary Material). ndSBT results were reported within 24 h for 20 out of 29 samples. One sample (1C10) was not immediately tested with the fast approach as it was collected after Incident 1 had already been declared as an outbreak, thus urgent results were no longer required. In this instance a full ST1268 profile was obtained by nSBT only 14 days after sample receipt because *L. pneumophila* sg1 was not isolated. This sample was included in this study to illustrate the utility of molecular typing on culture-negative samples, however it was excluded from Fig. 1 and from the calculation of mean and standard deviation in Table S1.

Five to 20 days (mean 9.53, SD 3.73) were necessary to obtain results when the standard approach was used on positive cultures and referred isolates (Fig. 1, Table S1). In some instances, as useful preliminary data had already been obtained by ndSBT, standard typing protocols were not used on available *L. pneumophila* sg 1 isolates as a matter of urgency, nevertheless this cannot be counted as the single cause of the average 7.95 day delay in obtaining results in respect to the fast direct approach. As referred isolates require minimal processing before being typed with the standard protocol, characterization of the seven *L. pneumophila* sg1 strains (six environmental and one clinical) referred to the NLRL (Table 2) was available in 4 to 7 days (mean 5.71, SD 0.95). However, 7 to 20 days (mean 10.80, SD 3.42) were necessary for the 21 referred samples where *L. pneumophila* sg1 was successfully isolated, increasing the average delay in respect to the fast approach from 7.95 up to 9.22 days.

In this study, all clinical and environmental specimens where *L. pneumophila* sg1 DNA was detected by PCR were later either culture-positive for *L. pneumophila* sg1 or yielded a partial to full allelic profile when tested with ndSBT (Table 2). Where isolates were available, the standard typing protocol always confirmed (100 % concordance) and completed the results obtained by direct approach on DNA extracts, with no discrepant allele found when comparing the two methods (Table 2).

The molecular approach proved effective also on heavily contaminated environmental samples where culture failed to isolate any *L. pneumophila* strain. The PCR assay targeting *mip* and *wzm* was previously demonstrated to be reliable and sensitive when testing clinical samples and 100 % specific for *L. pneumophila* and sg1 DNA using both clinical and environmental strains (Mentasti *et al.*, 2015). The sg1 assay targeting *wzm* was also validated on water samples (Merault *et al.*, 2011). ndSBT was not attempted on PCR-negative samples and *L. pneumophila* was not isolated from any of these samples (Table 2).

The LD incidents described here were caused by *L. pneumophila* sg 1 strains and all patients for whom a urine sample was available tested positive for *L. pneumophila* urinary antigen (data not shown). As urinary antigen tests target *L. pneumophila* sg1 strains, LD diagnosis is biased towards sg1 with non-sg1 strains and other species of *Legionella* estimated to cause ca. 17 % of LD cases (ECDC, 2015). *L. pneumophila* sg1 was isolated from 17 (70.8 %) of the 24 patients whose respiratory samples tested PCR-positive. One more isolate was referred by a local hospital. Sixteen of the clinical strains were shown to react with mAb 3/1 (five sub-grouped as 'Allentown/France', seven as 'Benidorm' and four as 'Philadelphia') which detects an epitope associated with strains more likely to cause LD. The remaining two strains did not react with mAb 3/1 (both sub-grouped as 'Bellingham'), and hence were less likely to cause LD, however the patient involved in Incident 5 was a neonate, thus with immune system not yet fully functioning, while no particular underlying disease was reported for the patient involved in Incident 3. Seven of the infecting STs, namely 1, 23, 37, 46, 47, 48 and 62, are known internationally to cause LD cases and account for 3463 out of the 10 513 (32.9 %, query performed 13 February 2016) entries in the *L. pneumophila* SBT database (www.hpa-bioinformatics.org.uk/legionella/

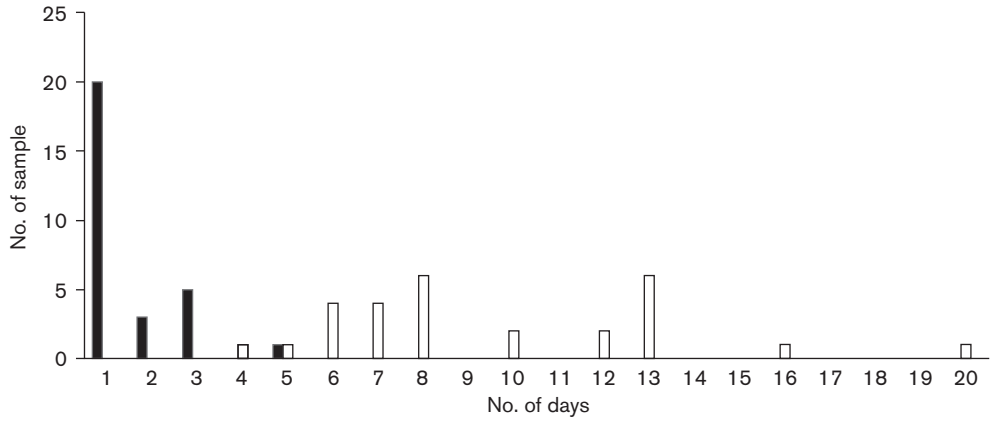

Fig. 1. Comparison between number of days necessary to obtain results when using ndSBT (29 samples; filled bars) and SBT (28 isolates; open bars).

legionella_sbt/php/sbt_homepage.php). ST1268 has never been isolated before or since Incident 1; this is not uncommon as other outbreaks have been caused by unique strain types (Bennett *et al.*, 2014; Shivaji *et al.*, 2014). ST1534 is present on the SBT database with only two isolates characterized in the UK and Switzerland, respectively.

The direct approach was essential in providing typing data especially in culture-negative samples and where growth of other bacteria prevented isolation of *L. pneumophila*. Previous studies demonstrated that successful isolation of *L. pneumophila* from respiratory specimens was only achieved in ca. 65 % of productive patients diagnosed by the Legionella urinary antigen test, and the use of molecular techniques on respiratory specimens increases the amount of typing data that can be obtained (Mentasti *et al.*, 2012). In this study, isolation of *L. pneumophila* was not successful from seven clinical samples, namely, three in Incident 1, one in Incident 2, one in Incident 4 and two in Incident 7 (Table 2). Nevertheless the allelic profiles quickly obtained by ndSBT helped investigators to either confirm a point source outbreak (Incidents 1 and 7) or exclude a possible outbreak (Incidents 2 and 4). No *L. pneumophila* strain was isolated from environmental samples related to Incident 1 and Incident 5: the infecting source would have not been identified and confirmed if the molecular approach had not been used.

During outbreak investigations, environmental site(s) positive with (partial) allelic profiles not matching that of the infecting strain may be incidentally found and other possible sources of LD cases eliminated. Water samples from two cooling towers analysed during the investigation of Incident 1 were found positive for *L. pneumophila* sg1 by PCR, and ndSBT gave partial allelic profiles, (0,0,0,15,18,1,6) and (8,0,0,15,18,1,6), respectively, that when compared to the *L. pneumophila* SBT database (www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php) were found to be most likely consistent with ST62 (comparison performed on 30 July 2012). This particular ST of *L. pneumophila* is presently the fourth most commonly described with 377 out of 10 513 total entries on the SBT database (query made 13 February 2016), and is a well-known cause of both sporadic cases and severe outbreaks: in 2010, 64 patients were infected, with four fatalities in Ulm, Germany (von Baum *et al.*, 2010; Lück *et al.*, 2015), and in 2012, there were 182 cases with 13 fatalities in Quebec City, Canada (Lévesque *et al.*, 2014). When environmental strains were isolated from the two cooling towers analysed in this study, full standard analysis using mAbs and SBT confirmed the strains as *L. pneumophila* sg1, sub-group 'Allentown/France', ST62 (8,10,3,15,18,1,6). Both cooling towers were promptly disinfected and no LD case associated with this strain was subsequently reported.

Isolation of *L. pneumophila* strains from clinical and environmental samples is still necessary to obtain definitive full characterization. Presently, only the sg1 genetic marker (*wzm*) can be identified by molecular analysis (Mérault *et al.*, 2011) and full allelic profiles are not always obtained by ndSBT. Preliminary results obtained with the rapid protocol require confirmation and completion by standard analysis on isolates, where available. A particular ST can be associated with more than one mAb subgroup (e.g. ST1 with Allentown/France, Oxford/Olda or Philadelphia) and one mAb subgroup may contain many STs. mAb sub-grouping is still necessary to further characterize strains of common STs (such as ST1) until new genetic markers able to characterize subgroups of sg1 are identified and assays validated. LD outbreaks can occasionally be caused by non-sg1 strains of *L. pneumophila*, thus identification of molecular markers for rapid characterization of such strains will also be necessary. In the summer of 2007 an LD outbreak occurred on a cruise ship sailing in the Baltic (Sedgwick *et al.*, 2007), the infecting strain was isolated and later characterized as *L. pneumophila* sg5 ST 1327 (11,14,16,31,15,13,210) (Mentasti *et al.*, 2014).

Similarly to SBT, ndSBT amplifies the seven targets of the typing scheme of any *L. pneumophila* strain present in a DNA extract. In the event that samples contained a mixture of different strains, resolving typing data may not be possible due to the overlapping of different signal peaks in the same position in Sanger sequencing results. Although rare, mixed infections have been described (Wewalka *et al.*, 2014) and multiple STs have already been isolated from the same environmental sample during LD investigations (Moran-Gilad *et al.*, 2012). This rapid typing approach would not be suitable for specimens containing more than one ST of *L. pneumophila* or if the infecting strain is not the predominant strain.

Nucleic acid extraction methods validated for clinical samples were found to be less effective when trying to obtain good quality DNA from environmental samples. DNA extracted from the cooling towers during the investigation of Incident 1 were inhibitory and this caused delays in detecting *L. pneumophila* sg1 DNA and obtaining direct molecular typing results as experiments had to be repeated on 1 : 10 dilutions. Testing neat and 1 : 10 dilutions simultaneously can be helpful in some cases, however two further environmental samples in the same investigation, samples 1E12 and 1E15, were inhibitory even after 1 : 10 dilution (Table 2). This highlights the need for more efficient DNA extraction processes for complex environmental samples suspected to contain PCR inhibitors.

In order to obtain fast ndSBT results, a reliable in-house sequencing service operating on an overnight urgent basis is crucial. Reliance on external sequencing services means that PCR products must be shipped, with inevitable delays in obtaining sequencing results. Maintaining an in-house and urgent sequencing service is costly, yet this may be less than the overall public health cost of outbreak management processes and that of treating additional patients when the source of an LD outbreak is not promptly identified.

Whole-genome sequencing (WGS) has progressively become more affordable and accessible, however it still requires a significant amount of pure genomic DNA and several days of work and analysis before results are available. Consequently, where a fastidious and slow growing microorganism such as *L. pneumophila* is involved, WGS cannot yet provide rapid typing results. Until a direct WGS typing protocol for *L. pneumophila* has been validated on clinical samples, PCR combined with ndSBT appears the best available option to generate rapid typing results to improve the investigation of LD urgent cases and clusters.

In conclusion, a fast molecular approach can be used to aid the investigation of urgent LD cases and possible outbreaks. PCR and ndSBT on both clinical and environmental samples provide accurate detection of *L. pneumophila* sg1 and preliminary typing data considerably faster than culture-based identification and SBT on isolates. Confirmatory typing on clinical and environmental isolates, where available, is still required to obtain definitive full characterization, however as isolates are not always available, the direct method can still provide crucial information to assist the Incident Management Team. Where strains are not isolated, due for example to heavy contamination with other microbial flora, this approach can still provide useful typing data to identify the infection source.

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