Hospital-wide Eradication of a Nosocomial *Legionella pneumophila* Serogroup 1 Outbreak

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**Background.** Two proven nosocomial cases of *Legionella* pneumonia occurred at the Wesley Hospital (Brisbane, Australia) in May 2013. To trace the epidemiology of these cases, whole genome sequence analysis was performed on *Legionella pneumophila* isolates from the infected patients, prospective isolates collected from the hospital water distribution system (WDS), and retrospective patient isolates available from the Wesley Hospital and other local hospitals.

**Methods.** *Legionella pneumophila* serogroup 1 isolates were cultured from patient sputum (n = 3), endobronchial washings (n = 3), pleural fluid (n = 1), and the Wesley Hospital WDS (n = 39). Whole genome sequencing and de novo assembly allowed comparison with the *L. pneumophila* Paris reference strain to infer phylogenetic and epidemiological relationships. Rapid disinfection of the hospital WDS with a chlorinated, alkaline detergent and subsequent superchlorination followed by maintenance of residual free chlorine, combined with removal of redundant plumbing, was instituted.

**Results.** The 2011 and 2013 *L. pneumophila* patient isolates were serogroup 1 and closely related to all 2013 hospital water isolates based on single nucleotide polymorphisms and mobile genetic element profiles, suggesting a single *L. pneumophila* population as the source of nosocomial infection. The *L. pneumophila* population has evolved to comprise 3 clonal variants, each associated with different parts of the hospital WDS.

**Conclusions.** This study provides an exemplar for the use of clinical and genomic epidemiological methods together with a program of rapid, effective remedial biofilm, plumbing and water treatment to characterize and eliminate a *L. pneumophila* population responsible for nosocomial infections.

**Keywords.** bacterial genomics; genomic epidemiology; Legionnaires’ disease; hospital disinfection; transmission pathway.

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*Legionella pneumophila* has a global distribution in freshwater environments and human-made water distribution systems (WDSs). The organism has coevolved with, and replicates within, free-living amoebae. Human infection is by inhalation of aerosol droplets, with infection of pulmonary alveolar macrophages causing Legionnaires’ disease [1]. Nosocomial legionellosis is typically caused by *L. pneumophila* serogroup 1 (LPSG1) infection, although occasional outbreaks due to other *L. pneumophila* serogroups and nonpathogenic *Legionella* species [2] have been reported [3,4]. Nosocomial Legionnaires’ disease outbreaks can be either intermittent or continue for years [5]. Important aerosol sources for hospital-acquired legionellosis include WDS outlets (eg, showers, taps, and cooling towers), therapeutic devices (eg, humidifiers and nebulizer masks) [6, 7], and decorative features such as fountains in common reception areas [8]. While early reports indicate that hospitals at risk of outbreaks have greater than 30% of their potable water outlets culture positive for LPSG1 [5], recent data demonstrate that much lower proportions of culture-positive outlets should also be considered problematic [9].

Many hospital WDS-associated *L. pneumophila* outbreaks have been described worldwide, with patient risk correlating positively with medical comorbidities, therapeutic immunosuppression, and the presence of LPSG1 culture-positive outlets within an institution [10]. An estimated 8000 to 18 000 patients are hospitalized with Legionnaires’ disease annually in the United States alone, with a mortality risk of 10%–50%, a mean inpatient stay of 10.2 days, and a total annual inpatient cost of $434 million [11].

Efforts to prevent transmission to humans revolve around the disinfection of biofilm within air-conditioning cooling towers or the plumbing of hospital WDS by physical (scalding or ultraviolet irradiation) or chemical (application of biocides such as chlorine, chloramine, ozone, or copper–silver ions) methods to sufficiently reduce LPSG1 counts. *Legionella pneumophila* can persist in biofilms within hospital WDSs for many years,
often despite the application of biocides to the system [12]. Only 3 Australian nosocomial outbreaks of Legionnaires’ disease (including the one described here) have been recognized and reported (all due to LPSG1), with the first in 1979 [13, 14]. A recent comprehensive review highlights the need to understand the epidemiology and management of Legionnaires’ disease in the clinical setting [10].

LPSG1 strains demonstrate a high degree of genetic conservation, with more than 80% of the core genome conserved between 4 sequenced reference strains (Paris, Lenz, Corby, and Philadelphia) and strain-specific gene content ranging from 7% to 11% [15]. This high degree of genetic conservation has restricted the ability of classic molecular techniques including ribotyping, amplified fragment length polymorphism, pulsed-field gel electrophoresis, restriction fragment length polymorphism, restriction endonuclease analysis, and arbitrarily primed polymerase chain reaction [15] to monitor the implementation of timely, appropriate outbreak control measures. More recent techniques such as sequence-based typing of multiple housekeeping genes are limited by their inability to discriminate clinical isolates from environmental isolates when 1 clone predominates in the immediate environment [15]. Whole genome sequencing (WGS) facilitated by high-throughput DNA sequencing technologies has increased our understanding of the complexity of nosocomial and community outbreaks by documenting unrecognized transmission events [16, 17], accurately reconstructing transmission pathways [18, 19], disproving apparent clonal outbreaks [20], and enabling the rapid, detailed characterization of pathogens that cause multijurisdictional foodborne outbreaks [21, 22]. WGS also offers the potential advantage of the delivery of “real-time” results to infection control and public health practitioners, with the ability to reconstruct transmission pathways and intervene with precision during the course of an outbreak [16, 19].

Here, we used a detailed WGS approach to investigate Australia’s most recently known nosocomial Legionnaires’ disease outbreak, which occurred at the Wesley Hospital (TWH) in 2013. A preliminary study carried out during the outbreak suggested a link between the 2013 outbreak and a single patient isolate from 2011 at the same hospital [14]. Our WGS analysis of more than 40 isolates shows the full extent of contamination of the hospital plumbing system by an LPSG1 clone, identifying 3 major subclonal populations that correspond to geographically distinct sections of the hospital plumbing system. The resolution afforded by WGS enabled the link between infected patients who shared the same ward in 2011 and 2013 to be unequivocally established and has provided genotyping targets to distinguish the source of future LPSG1 isolates from TWH plumbing. Together, these results highlight the potential of WGS to uncover transmission pathways and provide unparalleled genotyping resolution to distinguish genetically distinct subclones within a long-term clonal outbreak.

MATERIALS AND METHODS

Study Setting

The 2011 legionellosis notification rate in Australia was 1.5 per 100,000 persons [23]. Of the 348 cases, 164 were LPSG1, with 167 of the remaining cases due to L. longbeachae infection [23]. Statutory requirements vary among Australian states with respect to the management of hospital WDSs to prevent nosocomial legionellosis. While strict legislative requirements existed to prevent cooling tower transmission of LPSG1 to humans in Queensland, Australia, there were no statutes or regulations requiring the testing for, or risk-management of, LPSG1 in WDSs before June 2013 [13]. TWH is located in Brisbane, Australia, and has 520 beds, with more than 75,000 admissions per year. The hospital has 3 main wings and 3 co-located medical centers. The municipal reticulated water supplies in Brisbane are treated with monochloramine.

Identification of L. pneumophila Serogroup 1 in Clinical and Environmental Specimens

LPSG1 was cultured from sputum, endobronchial washings, and pleural fluid by plating onto selective media according to standard methods [24] (Supplementary Table 1). LPSG1 antigenuria was detected with the BinaxNOW Legionella kit (Alere, Australia). Standing, first catch, or running potable water samples of 100 mL were collected and cultured for LPSG1 using selective media according to a published Australian standard (AS/NZS 3896:2008) [25]. Isolates were identified to the species level using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [26] (Supplementary Table 1).

Whole Genome Sequence Analyses
Genomic DNA from 46 specimens was sequenced at the Australian Genome Research Facility (Melbourne, Australia) using Illumina HiSeq with 100 bp pair-ended reads. Read mapping against the reference strain L. pneumophila Paris [27] was completed with SHRiMP2 [28] and Nesoni [29] software. Whole genome comparison analyses were performed using a combination of previously reported tools [30]. Phylogenetic trees based on core single nucleotide polymorphism (SNP) alignments of the n-way analysis computed with Nesoni were built using RAxML 7.2.8 [31] with the GTR + GAMMA substitution model and 1000 bootstrap replicates. To investigate the diversity of the plasmid content, we performed plasmid profiling using Seqfindr [32]. Further details of WGS analysis, including recombination inference, can be found in the Supplementary Appendix.

RESULTS

Case Reports

The cases of both outbreak patients (the first from 27 May 2013 and the second from 3 June 2013) and 1 historical case from 2011 are detailed in the Supplementary Appendix.
Response to the *L. pneumophila* Serogroup 1 Outbreak

LPSG1 was cultured from standing and running water samples collected on 29 May 2013 from the shower and hand basin taps of patient 1’s room and from running water specimens collected on 5 June 2013 from the hand basin of patient 2’s room. At baseline, LPSG1 was cultured from 5 of 7 (71.4%) water outlets tested in the east wing, 2 of 12 (16.7%) outlets in the Moorlands wing, 8 of 34 (23.5%) outlets from the main block, and 2 of 32 (6.3%) outlets from the co-located medical centers. In total, 15 of 85 (17.6%) hospital water outlets cultured LPSG1 on initial survey (Supplementary Figure 1). The hospital was closed to all new admissions, and current inpatients were prohibited from using showers, with implementation of alternative hygiene measures on 5 June 2013. The entire hospital WDS was scalded with 60°C water for 10 minutes, with confirmatory testing at the point of use. Five of 89 (5.6%) outlets remained culture-positive 24 hours after scalding. A management decision was made to implement a rapid WDS disinfection strategy to remove the WDS pipe biofilm as conventional biocide applications may require 2–3 months to ensure LPSG1 culture-negativity [12].

Disinfection of the hospital WDS commenced in the east wing on 10 June 2013. The WDS was initially flushed with a chlorinated alkaline detergent (pH = 10.0) to dissolve pipe biofilm, then flushed and superchlorinated with a residual of 10 mg/L free chlorine. Three cycles were required to achieve macroscopically clear water at all outlets without microbial contamination, as determined by the Mycometer Bactiquant method (CETEC, Melbourne, Australia) [33]. An in-line chlorinator system was installed on the water main to the east wing, and free chlorine levels in the water were appropriately maintained at 1–4 mg/L at the point of use. This process was deployed sequentially in the Moorlands wing and then the main block. Water specimens were collected from the beginning, middle, and end of each plumbing circuit in each wing on a daily basis for the first 7 days after the disinfection process for LPSG1 culture. Wings were reopened after 2 consecutive days of negative water cultures for LPSG1. The hospital was reopened, one wing at a time (east on 19 June, Moorlands on 26 June) and was at full operational capacity on 2 July 2013. Weekly culturing of water outlets continued for 6 weeks before the testing frequency was reduced to twice weekly and then monthly sampling. Any water outlets associated with positive LPSG1 cultures had their associated plumbing circuits scalded and, if necessary, the disinfection process repeated. Blind-ending pipes, “dead legs,” were actively sought and removed on discovery. Following WDS disinfection, LPSG1 was only cultured from the following 3 locations: radiology department (4 days post-disinfection), the rehabilitation ward (7 days post-disinfection), and an in vitro fertilisation operating theatre (20 and 63 days post-disinfection). All of these outlets were associated with plumbing dead legs. Removal of these outlets facilitated culture negativity (Figure 1 and Supplementary Figure 1). Following the June 2013 outbreak, no new cases of LPSG1 infection have been reported from TWH. WDS disinfection (chlorine) continues along with systematic, monthly, culture-based testing of the WDS, and no LPSG1 culture-positive samples have been identified since December 2013. Additional details of the intervention can be found in the Supplementary Appendix.

**Genomic Investigation of *L. pneumophila* Serogroup 1 Isolates**

A total of 46 LPSG1 culture isolates from a single colony pick were prepared for genomic investigation (Supplementary Table 1). The collection included all 4 clinical LPSG1 isolates from both patients (LP45–LP48), all 6 potable water isolates from both patients’ rooms (LP01–LP04, LP06, and LP07), 1 bronchoscopic isolate from a TWH patient from 2011 (LP44) that was treated in the same ward as patient 2, 2 historical clinical isolates (1 from each of 2 different hospitals in Queensland

![Figure 1](cidimage.png)

**Figure 1.** Distribution of positive samples collected in all buildings during the initial control phase of the Wesley Hospital Legionella outbreak. The outbreak-specific sampling and control phase are represented as an individual timeline for each building, namely, main block, east wing, and Moorlands wing, covering 17 weeks from the start of the outbreak (29 May 2013). The total number of samples defined as positive, that is, containing *Legionella pneumophila* serogroup 1 to a concentration ≥10 colony-forming unit (CFU)/mL, collected each day is represented by circles drawn to a size proportional to their respective value. Successive interventions relative to scalding (gray) and disinfection (black) procedures for each building are also indicated.
from 2000 [LP42] and 2001 [LP43]), 35 other tap water isolates from TWH (LP05, LP08–LP12, LP14–LP21, LP23–LP39, and LP41), and 1 isolate from a contaminated thermomixing valve (LP40) (Supplementary Table 1).

Collectively, the 2013 hospital outbreak isolates (LP45–LP48) were most similar to the LPSG1 Paris reference strain (2436 SNPs) [27], with 188 532 SNPs identified in comparison to the Philadelphia 1 reference strain [34] (Figure 2A and Supplementary Figure 2). The LPSG1 patient isolate (LP44) contains only 1 SNP difference to a 2013 WDS isolate (LP06). Overall, the 2011 and 2013 patient isolates differ by 193 SNPs (Supplementary Table 2) and a maximum of 54 SNPs, strongly suggesting the hospital WDS as the source of nosocomial infection. Several recombinant regions common to all TWH strains were detected relative to L. pneumophila Paris and LP43, consistent with recombination that occurred prior to divergence of the TWH strains (Supplementary Figures 3 and 4). We also compared these isolates with 2 historical patient isolates obtained from different Queensland locations that were more than 100 km from TWH (Figure 2A). These 2 LPSG1 patient isolates, which were isolated in 2000 (LP42) and 2001 (LP43), clustered distinct from the outbreak isolates.

There is some evidence for geographic microevolution among the LPSG1 outbreak isolates that could be used to resolve epidemiological connections. Phylogenetically, the isolates form a single, clearly defined clade that consists of 3 closely related subclades, with the overall divergence between subclades I and II of 17–36 SNPs, I and III of 24–54 SNPs, and II and III of 31–63 SNPs (Figure 2B). The isolates from patient 1 (LP45 and LP46) are identical and are closely related to environmental isolates from the east wing in subclade III from the same plumbing circuits that supplied the patient’s room (LP40, LP41, LP1–LP4; Figure 2C). Isolates from patient 2 (LP47 and LP48) are most closely related to other subclade II environmental isolates from the plumbing circuits of the main block, both from patient 2’s room (LP06 and LP07) and elsewhere in the main block (LP08 and LP21; Figure 2C). The 2011 isolate LP44 was cultured from a patient who was treated in the same ward that patient 2 was treated in 2013. This isolate also clustered with subclade II and is only 1 SNP different to the 2013 WDS isolate LP06 and approximately 10 SNPs different to LP07, LP47, and LP48 (Figure 2B).

Overall, the 3 TWH subclades possessed distinct mobile genetic element profiles that could provide even greater discrimination between individual isolates in future surveillance efforts (Figure 2B). For example, the integrative conjugative element LpPI-1 [35] was absent from most subclade II strains but present in all other TWH strains (Figure 2B). Plasmid profiling also revealed distinct subclade-specific plasmid types. For example, subclade I-specific plasmid type P1 and its variant P1* shared a high nucleotide sequence identity (>95%) across almost the entire length of L. pneumophila Paris plasmid pLPP (Figure 2B). In contrast, the other strains harbored plasmid variants with a DNA sequence that matched both pLPP and L. pneumophila Lorraine plasmid pLELO, an observation similar to that made in a previous study [36].

**DISCUSSION**

The aging population in developed countries along with medical interventions that may cause immunosuppression have resulted in a fragile in-patient population at high risk of nosocomial Legionnaires’ disease by inhalation of LPSG1-containing aerosols from hospital WDSs [1]. However, tracking the source of Legionella infection, particularly where an isolated case of Legionella pneumonia occurs, is difficult due to a combination of resource constraints, regulatory protocols, and capacity to undertake fine-scale epidemiological discrimination of isolates. Such was the case for the 2011 LPSG1 clinical isolate from a 73-year-old male inpatient with relapsed acute myeloid leukemia who died from pneumonia and complications arising from his leukemia.

The use of detailed, high-throughput WGSs to investigate the genomic epidemiology of infectious disease outbreaks has recently become affordable due to advances in sequencing technologies [16–20]. Indeed, a pilot study using Illumina sequencing of several historical L. pneumophila outbreak isolates has demonstrated the feasibility of WGSs for distinguishing outbreak from nonoutbreak L. pneumophila strains [37]. In this study, we used genomic epidemiology to link LPSG1 isolates from 2 patients from the 2013 outbreak at TWH with a LPSG1 clone that has been present in the hospital water supply since at least 2011. Remedial actions taken following the identification of Legionella in the hospital WDS resolved the source of contamination. The data are most consistent with the primary problem being contamination of the hospital WDS by LPSG1 from municipal mains water prior to the 2011 case, with subsequent amplification within the WDS before dissemination to the points of use, rather than contamination of distinct points of use per se.

The unparalleled resolution of this incident is highlighted by our identification of 3 subclonal populations of LPSG1 that show some level of geographic clustering. The combination of single nucleotide and mobile genetic element profiling used in this study will enable future surveillance efforts at TWH to distinguish new incursions via the municipal water supply from preexisting contamination linked to the 2013 outbreak. The ability to unequivocally establish (or disprove) such epidemiological links with the WGS will enable ongoing evaluation of the region-scale epidemiological discrimination of isolates.

Perhaps unsurprisingly, LPSG1 patient isolates were most closely related to isolates from the patients’ immediate environment (eg, shower head and basin in patients’ rooms), indicative of localized geographic microevolution. However, testament to the remarkable stability of the LPSG1 population that
Figure 2. Epidemiological and geographical characterization of the 2013 Wesley Hospital (TWH) Legionella outbreak. A, Maximum likelihood phylogenetic tree built using 188,602 single nucleotide polymorphisms (SNPs) relative to Legionella pneumophila strain Paris (rooted using L. longbeachae as outgroup; not shown). The red dotted box corresponds to the collapsed branch of all strains from the 2013 TWH outbreak as well as LP44 (2011). The bottom scale indicates branch length in number of SNPs. B, Maximum likelihood phylogenetic tree built using 2,436 SNPs relative to L. pneumophila strain Paris (used as outgroup; not shown). Patient isolates are colored as follows: LP44 (2011), red; LP45 and LP46 from patient 1 (2013), green; LP47 and LP48 from patient 2 (2013), yellow. Three discrete subclades can be delineated and are represented by branches colored blue, yellow, and green representing subclade I, II, and III, respectively. On the right-hand side of the tree, additional metadata relative to the strains are represented as follows: building (gray), floor (dark blue), wing (multicolor), date of isolation (for which the color gradient key is depicted on the left of the tree), and ICE (turquoise corresponds to the presence of the integrative conjugative element LpPI-1 [38]). Plasmid types are indicated in shades of purple, and their corresponding barcodes are shown on the far right of the figure, with black bars indicating sequence identity >95%. C, Map of level 4 of TWH, showing water sampling locations, with negative samples in blue and positive samples in black, along with the respective strains sequenced from them. The patient isolates are also represented according to the location of the patient whom they were collected from: LP44, 2011, red; LP45 and LP46, patient 1, 2013, green; and LP47 and LP48, patient 2, 2013, yellow.
contaminated the hospital WDS is the fact that the 2-year temporal separation of the 2011 LPSG1 patient isolate LP44 revealed only 1 SNP compared with the 2013 WDS isolate LP06, and approximately 10 SNPs compared with patient isolates LP47 and LP48. The culture of virtually identical environmental isolates from differing sites within the hospital, sharing only a common municipal water main, supports this contention. A recent investigation of L. pneumophila strains linked to 13 outbreaks in a single location revealed the major contribution of recombination to strain diversity [38]. Similarly, a 2012 Legionnaires’ outbreak in Edinburgh, United Kingdom, also showed multiple genetic subtypes within an outbreak population, highlighting the necessity of WGS for reliable source attribution [39]. We identified several recombination events that contributed to clonal diversification of the TWH strains from their closest sequenced relative (L. pneumophila Paris). However, nearly all chromosomal differences that distinguished the TWH strains from each other were not due to recombination, supporting a single recent common ancestor.

Interventions to address the source of contamination in the hospital WDS included closure of the hospital to new admissions, implementation of alternative hygiene measures for inpatients, scalding of the WDS with 60°C water for 10 minutes, treatment of the hospital WDS with an alkaline detergent and 10 mg/L free chlorine, installation of in-line chlorinator systems, and intensive monitoring for the presence of LPSG1 in water specimens. The specific cleaning process used, with an initial phase of biofilm removal by alkaline detergent (in addition to dead leg removal) followed by “induction” and then a “maintenance” application of biocide to minimize biofilm recurrence, has not previously been reported in the published medical literature. Copper-silver ion application (without biofilm removal) has an extensive supporting literature and appears to be an approach for many systems [12]. All biocides have reported failures and have generated specific resistant LPSG1 strains, possibly due, in part, to insufficient biofilm removal by these methods [40]. Dead legs appear to have been a source of contamination recalcitrant to intervention, and here we demonstrate that biofilm removal and augmented disinfection with free chlorine pending their removal from the WDS correlated with culture-negativity for LPSG1. Legionella surveillance and risk management had not been a hospital management priority in Queensland, Australia (in either the public or private sectors), before this outbreak. However, state regulatory guidelines that require all hospitals to have Legionella surveillance and risk management strategies [13] were instituted in November 2013 as a consequence of this outbreak.

In conclusion, aggressive intervention and intensive monitoring for LPSG1 was used to manage an outbreak of Legionella pneumonia at TWH in 2013. These approaches, in combination with genomic epidemiological investigation, enabled the contamination of the hospital WDS to be investigated and have provided a rational framework for the management of future Legionella contamination.

**Supplementary Data**

Supplementary materials are available at http://cid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

**Notes**

**Author contributions.** P. B. R. and L. P. coordinated the clinical management of the outbreak. P. B. R., N. L. B. Z., V. G., D. L. P., M. W. S., M. A. S., and S. A. B. designed the study and prepared the manuscript. V. G. developed the disinfection program. V. G. and R. M. managed the disinfection process. K. T. and L. P. carried out sample collection and characterization. G. P., J. R., and T. B. carried out the microbiological analysis. N. L. B. Z., M. S. C., and S. A. B. carried out the genome analysis. All authors contributed to revision of the manuscript.

**Data and materials availability.** Genome sequence data has been deposited at the European Nucleotide Archive under study ERP004963 with accession identifiers ERR440061 to ERR440106.

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