Low genomic diversity of *Legionella pneumophila* within clinical specimens

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**Objectives:** *Legionella pneumophila* is the leading cause of Legionnaires’ disease, a severe form of pneumonia acquired from environmental sources. Investigations of both sporadic cases and outbreaks rely mostly on analysis of a single to a few colony pick(s) isolated from each patient. However, because of the lack of data describing diversity within single patients, the optimal number of picks is unknown. Here, we investigated diversity within individual patients using sequence-based typing (SBT) and whole-genome sequencing (WGS).

**Methods:** Ten isolates of *L. pneumophila* were obtained from each of ten epidemiologically unrelated patients. SBT and WGS were undertaken, and single-nucleotide polymorphisms (SNPs) were identified between isolates from the same patient.

**Results:** The same sequence type (ST) was obtained for each set of ten isolates. Using genomic analysis, zero SNPs were identified between isolates from seven patients, a maximum of one SNP was found between isolates from two patients, and a maximum of two SNPs was found amongst isolates from one patient. Assuming that the full within-host diversity has been captured with ten isolates, statistical analyses showed that, on average, analysis of one isolate would yield a 70% chance of capturing all observed genotypes, and seven isolates would yield a 90% chance.

**Conclusions:** SBT and WGS analyses of multiple colony picks obtained from ten patients showed no, or very low, within-host genomic diversity in *L. pneumophila*, suggesting that analysis of one colony pick per patient will often be sufficient to obtain reliable typing data to aid investigation of cases of Legionnaires’ disease.

Introduction

*Legionella pneumophila* is a Gram-negative bacterium found in fresh-water and soil environments [1]. Human infection with *L. pneumophila* can cause legionellosis, which ranges from a mild, flu-like illness (Pontiac fever) to a severe and potentially fatal pneumonia (Legionnaires’ disease). The usual route of infection is via inhalation of aerosols from a contaminated environmental source [2]. Commonly implicated sources include cooling towers, spa pools, decorative fountains, and water systems of large buildings.

When Legionnaires’ disease cases occur, clinical isolates are usually characterized together with epidemiologically linked environmental isolates to help determine the source of the infection. To date, most clinical microbiological laboratories have relied on analysing a single clinical isolate, or a small number of clinical isolates, from each patient. However, existence of within-host diversity of *L. pneumophila*, which has been poorly studied, would have important implications for the interpretation of molecular typing data. Here, we used sequence-based typing (SBT) [3,4] together with whole-genome sequencing (WGS) to investigate the diversity amongst multiple colony picks recovered from individuals.

**Methods**

Ten colony picks were obtained [5] from single sputum samples of ten epidemiologically unrelated patients with sporadic...
Legionnaires’ disease in England (Table 1). Isolates were stored at −80°C. DNA was extracted after 48–72 hours of incubation on buffered charcoal yeast-extract agar at 37°C using the Wizard kit (Promega UK, Southampton, UK), eluted in 1 x Tris-EDTA buffer (pH 8.0), and quantified using GloMax (Promega, UK). SBT was undertaken as described previously [3,4]. WGS was performed on Illumina X10 with 150-bp paired-end reads. Raw data were submitted to the European Nucleotide Archive (study accession number PRJEB12239/ERP013693). Individual accession numbers are provided in Table 1.

De novo assemblies were generated [6], and MLSTcheck was used to confirm the sequence type (ST) from them [7]—in particular ensuring that at least one of the mompS alleles matched that called by traditional SBT (since this gene is duplicated). Assemblies were annotated using Prokka v1.11 [8].

Single-nucleotide polymorphisms (SNPs) were called for each isolate by mapping to a reference genome of the same ST using the BCFtools was used to call SNPs [11]. Various
tail bias
[43x449]/C21
called by traditional SBT (since this gene is duplicated). Assemblies
were annotated using Prokka v1.11[8].

Table 1

| Number and details of single-nucleotide polymorphisms (SNPs) identified amongst ten isolates recovered from each of ten Legionnaires’ disease patients | Length of reference genome (in bp) | Accession numbers | SNP locations and gene names | SNP locations and gene names |
|---|---|---|---|---|
| Patient age, sex | Isolation date and site | Epidemiological information | ST | Reference genome |
| 1 | 59 M | June 2015 (13) | Admitted to ITU | 42 BL1, 120, 3430, 562 [6] | 3,296,722, 3,298,906, 0 SNPs between all ERR1608296-ERR1608306 |
| 2 | 55 M | May 2015 (6) | Travel-associated | 42 BL1, 120, 3430, 562 [6] | 3,296,722, 3,298,906, 0 SNPs between all ERR1608296-ERR1608306 |
| 3 | 55 M | June 2016 (5) | Severe community-acquired pneumonia | 42 BL1, 120, 3430, 562 [6] | 3,296,722, 3,298,906, 0 SNPs between all ERR1608296-ERR1608306 |
| 4 | 71 M | April 2016 (6) | Travel-associated | 23 BL1, 28, 3,509, 586 [6] | 3,369,271, 3,371,148, 0 SNPs between all ERR1608316-ERR1608317 |
| 5 | 64 M | August 2016 (2) | Travel-associated | 37 BL1, 165, 3,474, 638 [18] | 3,323,524, 3,345,163, 0 SNPs between all ERR1608322-ERR1608316 |
| 6 | 66 F | September 2016 (8) | Travel-associated | 20 De novo assembly (3,560, 463) | 3,344,555, 0 SNPs between all ERR1608326-ERR1608328 |
| 7 | 69 M | July 2016 (5) | Travel-associated | 477 De novo assembly (3,307, 829) | 3,393,086, 0 SNPs between all ERR1608316-ERR1608328 |
| 8 | 69 M | August 2016 (2) | Travel-associated | 1,522 De novo assembly (3,621, 867) | 3,393,086, 0 SNPs between all ERR1608316-ERR1608328 |
| 9 | 75 M | September 2016 (7) | Community-acquired | 2287 De novo assembly (3,307, 829) | 3,393,086, 0 SNPs between all ERR1608316-ERR1608328 |
| 10 | 46 M | July 2016 (15) | Not provided | 1522 De novo assembly (3,621, 867) | 3,393,086, 0 SNPs between all ERR1608316-ERR1608328 |

To estimate the number of isolates that need to be analysed to observe all genotypes identified from a patient, random sampling of between one and ten isolates from each set of ten same-patient isolates was performed 100 times for each number of isolates without replacement.

Roary [12] was used to determine gene content variation between isolates from the same patient. Pairs of assemblies were also aligned and compared using the ‘dnadiff’ tool (v1.3), which is part of the MUMmer package [13].

Public Health England holds approvals to process patient-identifiable information for the purposes of infectious disease surveillance, in accordance with Section 60 of the Health and Social Care Act 2001. Ethical approval was not required for this study. The patient specimens were submitted for Legionella testing, including culture from microbiology laboratories in England. The Legionella data used is collated routinely by the Respiratory and Systemic Bacteria Section, Public Health England (PHE) as part of the national surveillance in England and Wales.

Results

To investigate the within-host diversity of L. pneumophila, we first assessed the diversity of STs (as determined by SBT) amongst each set of ten isolates recovered from ten individual patients with sporadic Legionnaires’ disease. In each patient, all ten isolates had the same ST (Table 1).

The number of SNPs amongst same-patient isolates was then determined. The use of a closely related reference genome ensured that maximum resolution was achieved and that almost all SNP-based diversity amongst same-patient isolates would be captured. In seven out of ten patients, no SNPs were detected. In two patients, a maximum of one SNP was observed, and in one patient there was a maximum of two SNPs (Table 1). In each of these three sets in which diversity was observed, nine out of ten isolates were identical, and only one isolate differed by one or two SNPs.
Assuming that the full within-host SNP diversity in each set of ten same-patient isolates was captured, we performed random sampling of between one and ten isolates, and calculated the number of times that the full diversity was captured with that number of isolates. Since no SNPs were found amongst isolates from seven out of ten patients, the mean probability of capturing the full diversity with only one sample is 70%. This probability rises as the number of samples analysed increases and, on average, seven isolates are required to have a 90% chance of capturing all genotypes (Fig. 1).

Finally, we investigated the extent of gene content variation between isolates from the same patient. We found no evidence of variation in gene content except for small differences introduced by assembly artefacts.

### Discussion

This study provides the most comprehensive analysis of within-host diversity of *L. pneumophila* in Legionnaires’ disease patients to date. The results demonstrate either no or very low within-host diversity in ten patients. We also show that, on average, analysis of one isolate provides a 70% chance of capturing all within-host variation found with ten isolates. Very low within-host diversity has also been observed previously [6,14], albeit with lower numbers of isolates and patients. Others have reported the opposite, including Coscolla et al. [15] who reported mixed infections in several patients based on SBT profiles from uncultured respiratory samples. Another study that used WGS identified two same-patient isolates belonging to distinct ST191 subtypes that differed by approximately 20 SNPs [16]. However, multiple isolates from three other patients in the same study were identical. Thus, while our study suggests that very low within-host diversity is the norm (at least in sporadic infections), greater diversity has occasionally been observed. Indeed, within-host diversity likely depends on several environmental, clinical and epidemiological factors, including the diversity of *L. pneumophila* in environmental sources, variation in infectious dose between patients, and the duration of infection prior to sampling.

A significant limitation to our study is that the use of culturing procedures may favour growth of some strains over others, thereby reducing the observed diversity. Furthermore, because of the collection of isolates from a single time point, as well as the reliance on culture, it is not possible to determine whether the observed diversity was present at the start of the infection, or whether it evolved during the infection or subsequently in culture. We propose that these limitations may be overcome in future studies by the use of metagenomics on multiple samples obtained over time from the same patient.

### Transparency declaration

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