Consensus Sequence-Based Scheme for Epidemiological Typing of Clinical and Environmental Isolates of *Legionella pneumophila*

Valeria Gaia,¹ Norman K. Fry,²* Baharak Afshar,² P. Christian Lück,³ Hélène Meugnier,⁴ Jerome Etienne,⁴ Raffaele Peduzzi,¹ and Timothy G. Harrison²

Istituto Cantonale di Microbiologia, Bellinzona, Switzerland; Health Protection Agency, Respiratory and Systemic Infection Laboratory, Centre for Infections, London, United Kingdom; Institute of Clinical Microbiology and Hygiene, Dresden, Germany; and Centre National de Référence des Légionelles, INSERM E0230, Faculté de Médecine Laennec, IFR62, Lyon, France

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A previously described sequence-based epidemiological typing method for clinical and environmental isolates of *Legionella pneumophila* serogroup 1 was extended by the investigation of three additional gene targets and modification of one of the previous targets. Excellent typeability, reproducibility, and epidemiological concordance were determined for isolates belonging to both serogroup 1 and the other serogroups investigated. Gene fragments were amplified from genomic DNA, and PCR amplicons were sequenced by using forward and reverse primers. Consensus sequences are entered into an online database, which allows the assignment of individual allele numbers. The resulting sequence-based type or allelic profile comprises a string of the individual allele numbers separated by commas, e.g., 1,4,3,1,1,1, in a predetermined order, i.e., flaA, pilE, asd, mip, mompS, and proA. The index of discrimination (D) obtained with these six loci was calculated following analysis of a panel of 79 unrelated clinical isolates. A D value of >0.94 was obtained, and this value appears to be sufficient for use in the epidemiological investigation of outbreaks caused by *L. pneumophila*. The D value rose to 0.98 when the results of the analysis were combined with those of monoclonal antibody subgrouping. Sequence-based typing of *L. pneumophila* is epidemiologically concordant and discriminatory, and the data are easily transportable. This consensus method will assist in the epidemiological investigation of *L. pneumophila* infections, especially travel-associated cases, by which it will allow a rapid comparison of isolates obtained in more than one country.

Many phenotypic and genotypic methods have been applied to the epidemiological typing of *Legionella pneumophila* (4, 12, 15, 21, 23, 28, 29, 34–36), the principal cause of the majority of cases of legionellosis (22). Members of The European Working Group for Legionella Infections (EWGLI) have previously evaluated a number of genotypic methods for the epidemiological typing of *L. pneumophila*, which is particularly applicable to cases of travel-associated legionellosis. The aim of such studies has been to achieve a standardized protocol that allows the exchange of typing data rather than strains across countries and borders, the latter of which is increasingly difficult, costly, and time-consuming (8–10). One of these methods, a single-endonuclease, amplified fragment length polymorphism analysis method by which the patterns are resolved by standard agarose electrophoresis (8, 34), was adopted as an international standard (9) and is widely used by the members of EWGLI. However, while this method allows relatively rapid screening of isolates within a single laboratory, interlaboratory results from comparison of the profiles contained in a web-enabled identification library database revealed that a significant proportion of laboratories could not achieve correct identification 100% of the time (8; B. Afshar, N. K. Fry, and T. G. Harrison, Abstr. 19th Annu. Meet. Eur. Working Group Le-

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* Corresponding author. Mailing address: Health Protection Agency, Respiratory and Systemic Infection Laboratory, Centre for Infections, 61 Colindale Avenue, London NW9 5HT, United Kingdom. Phone: 44 (0)20 8327 6776. Fax: 44 (0)20 8205 6528. E-mail: Norman.Fry@HPA.org.uk.
sample was taken. Therefore, confidence intervals were calculated to convey the

MATERIALS AND METHODS

Participants. Four institutes took part in the study. Each of the institutes acts as a national reference laboratory for legionella infections: (i) Istituto Cantonale di Microbiologia, Bellinzona, Switzerland; (ii) Institute of Clinical Microbiology and Hygiene, Dresden, Germany; (iii) Centre National de Référence des Légionelles, INSERM E09230, Faculté de Médecine Laennec, IFREMER, Lyon, France; and (iv) Health Protection Agency, Respiratory and Systemic Infection Laboratory, Centre for Infections, London, United Kingdom. The study was coordinated by the Health Protection Agency, Respiratory and Systemic Infection Laboratory, Centre for Infections.

Bacterial strains. A total of 105 clinical and environmental isolates of L. pneumophila were analyzed, including 96 L. pneumophila sg 1 isolates and 9 L. pneumophila isolates from other serogroups. Ninety-five of the L. pneumophila sg 1 isolates from nine European countries were selected to produce one epidemiologically unrelated panel of 79 clinical isolates (panel 1), one epidemiologically related panel of 16 isolates (panel 2), and one stability panel of five isolates (panel 3). Each of these isolates was obtained from the EWGLI Legionella culture collection and has a unique number (European Union Legionella culture collection number; see http://www.ewgli.org). This collection of isolates was established by members of the EWGLI to facilitate epidemiological typing studies. Each isolate has been previously extensively characterized, and details for these isolates have been described previously (8–11). The epidemiologically related panel (panel 2) comprised five sets of epidemiologically related isolates and two replicates of the same isolate. The stability panel (panel 3) comprised five variants of the same strain.

A number of clinical and environmental non-sg 1 isolates of L. pneumophila recovered during the course of outbreak investigations were also investigated, including some belonging to sg 6 (for flmA, proA, and pilE, and monoclonal antibody (MAb) subgrouping results from previous studies (8, 10, 11). The stability of each gene was assessed by analysis of the five variants of the same strain (panel 3). Sequence-based typing was performed essentially as described previously (11), with the following modifications and additions. Since the previous study, optimization of the amplification and sequencing conditions for the momPS gene target has resulted in improved sequence quality. Therefore, these new conditions were used in this study. Three new gene targets, asd, mip, and pilE, were also investigated.

DNA extraction, PCR amplification, and DNA sequencing. Genomic DNA was extracted as described previously (11) or by emulsifying two colonies of L. pneumophila in 0.5 ml sterile water and heating for 8 min at 100°C. Oligonucleotide primers targeting regions of each of the genes were used to amplify 245- to 648-bp products encompassing regions of variation (Table 1). The same primers were used in this study. Three new gene targets, asd, mip, and pilE, were also investigated.

Sequence analysis. Sequence analyses were performed locally by using the programs described previously by Chromas (Technelysium Pty Ltd., Australia; http://www.technelysium.com.au), Readseq, SeaView (13), Sequencer (Gene Codes Corporation), Autoassembler, or Sequence navigator (Applied Biosystems). For all analyses the data obtained with the forward and reverse sequencing primers were combined and aligned manually to produce a consensus se-
TABLE 2. Sequence variation in genes in *L. pneumophila*

| Gene     | Region used for allele assignment | No. of allele types | No. of variable sites | % Sequence variation |
|----------|----------------------------------|--------------------|----------------------|---------------------|
| asd      | 538–1010                         | 13                 | 35                   | 7.4                 |
| flaA     | 653–749                          | 11                 | 23                   | 12.6                |
| mmpS     | 117–518                          | 13                 | 12                   | 3.0                 |
| pilE     | 103–435                          | 10                 | 46                   | 13.8                |
| proA     | 1134–1230                        | 15                 | 32                   | 7.9                 |

* With respect to the reference sequence (see Table 3).

**TABLE 3. Allelic profiles of *L. pneumophila* sg 1 isolates from epidemiologically related sets**

| EUL no. | Allele no. | pilE | asd | mmpS | proA |
|---------|------------|------|-----|------|------|
| EUL 120 | 4          | 7    | 11  | 3    | 11   |
| EUL 121 | 4          | 7    | 11  | 3    | 11   |
| EUL 73  | 3          | 4    | 1   | 1    | 14   |
| EUL 78  | 3          | 4    | 1   | 1    | 14   |
| EUL 79  | 3          | 4    | 1   | 1    | 14   |
| EUL 71  | 8          | 10   | 3   | 15   | 18   |
| EUL 76  | 8          | 10   | 3   | 15   | 18   |
| EUL 77  | 8          | 10   | 3   | 15   | 18   |
| EUL 48  | 5          | 2    | 22  | 27   | 6    |
| EUL 56  | 5          | 2    | 22  | 27   | 6    |
| EUL 40  | 11         | 14   | 16  | 1    | 15   |
| EUL 47  | 11         | 14   | 16  | 1    | 15   |
| EUL 140 | 12         | 8    | 11  | 5    | 20   |
| EUL 141 | 12         | 8    | 11  | 5    | 20   |
| EUL 142 | 12         | 8    | 11  | 5    | 20   |
| EUL 143 | 12         | 8    | 11  | 5    | 20   |

**RESULTS**

**Typeability.** All isolates included in the study yielded PCR products of the expected size and DNA sequences with primers specific for all genes when they were tested by all four centers. For each isolate, the alleles either could be assigned to a preexisting allele number or could be identified as novel alleles in the course of this study; thus, *T* was equal to 1.0.

**Sequence variation.** The number of nucleotides from each of the gene targets included in the analysis ranged from 182 to 473 bp (Table 2). The numbers of allele types and polymorphic nucleotide sites and the percentage of nucleotide substitutions within each gene locus from the analysis of the panel of 79 strains are shown in Table 2. The highest percentage of polymorphic sites was found in the *mompS* gene (13.8%), and the least was found in the *mip* gene (3.0%).

**Reproducibility.** Data from consensus sequences from six loci (*flaA, pilE, asd, mip, mmpS, and proA*) from all 16 isolates tested by the laboratories by using different methods of DNA extraction, PCR cycling conditions, and DNA sequencing platforms were in complete agreement (*R* = 1.00). By using the cycling conditions described with an annealing temperature of 55°C, primary amplicons suitable for DNA sequence analysis and good-quality sequence data were obtained from all loci.

**Epidemiological concordance.** All six of the sets of isolates included in the epidemiologically related panels of isolates had compelling evidence of epidemiological relatedness (8); and previous analyses revealed concordant MAb subgrouping, restriction fragment length polymorphism analysis, restriction enzyme analysis, amplified fragment length polymorphism analysis, and sequence-based typing results (8, 10). Six genes were sequenced for the 16 isolates representing the six sets of epidemiologically related strains and two replicates of the same strain. The epidemiologic concordance (*E*) was calculated for each of the genes analyzed by using this set of 16 isolates, and for each locus *E* was equal to 1.00. The *mip* gene and the *proA* gene could differentiate only five of the six sets of strains, whereas all of the other targets could distinguish...
of these indices were all above 0.95, indicating that the true index of diversity could be above 0.95 and that the lower 95% confidence limits are slightly above 0.9. When SBT data for all six loci were combined with MAb subgrouping data, the estimated index of diversity was 0.981 and the lower 95% confidence limit was 0.967, indicating that the true index of diversity was unlikely to be below 0.95. If just the combination of flaA, pilE, asd, and MAb types was used, the estimated index of diversity was 0.978 and the lower 95% confidence limit was 0.966, again indicating that the true index of diversity was unlikely to be below 0.95 for this combination.

**Stability.** Analysis of all five variants (EUL 135 to EUL 139) in the stability panel (panel 3) resulted in identical SBTs, i.e., 6,10,15,28,9,14. mip allele 28 was a novel allele and was also found in the reference strain of sg 10 (Table 4).

**DISCUSSION**

Sequence-based typing of *L. pneumophila*, first applied to strains belonging to sg 1 by Gaia and colleagues (11), demonstrated the potential application of this technique to the investigation of outbreaks of legionellosis. In this study we present data validating the method for three additional genes and demonstrating the application of this approach to other serogroups of *L. pneumophila*.

The primary aim of this study was to seek to improve the level of discrimination offered by investigation of the additional genes, asd, mip, and pilE, and to demonstrate the application of this technique to the investigation of outbreaks of legionellosis. In this study we present data validating the method for three additional genes and demonstrating the application of this approach to other serogroups of *L. pneumophila*.

**Indices of discrimination.** Estimates of individual indices of discrimination (*D*) obtained with the six genes, flaA, pilE, asd, mip, mompS, and proA, and various combinations of two or more alleles were determined by using the panel of 79 unrelated clinical isolates (Table 5). Individual-locus *D* values ranged from 0.767 (proA) to 0.848 (mompS), and maximum discrimination (i.e., *D* = 0.943) was achieved by using all six loci. By using the combination of the three loci flaA, pilE, and asd and further combinations obtained by sequential addition of the remaining three loci, the resulting indices were almost identical, i.e., *D* = −0.94. The upper 95% confidence intervals among all six sets of isolates (Table 3). The results for the three additional epidemiologically related sets (LC 202 and LC 206, LC 569 and LC 606, and LC 384 and LC 395), which comprised clinical and environmental isolates belonging to serogroups other than sg 1, were also epidemiologically concordant (*E* = 1.00), suggesting epidemiological linkage, and gave unique profiles for each set (Table 4). The allelic profiles of the unrelated reference strains from sg 1, sg 6, sg 8, and sg 10 are also shown in Table 4 and were distinct from those of the epidemiologically related sets belonging to the same serogroup.

**Table 4. Allelic profiles of *L. pneumophila* isolates belonging to serogroups other than sg 1 from three epidemiologically related sets and four unrelated reference strains**

| Strain type and EUL no. | Serogroup | Allele no. |
|-------------------------|-----------|------------|
| NCTC 11192              | sg 1      | 3 1 1 1 14 9 |
| NCTC 11406              | sg 6      | 3 10 1 28 14 9 |
| NCTC 11985              | sg 8      | 3 10 1 28 14 9 |
| NCTC 12000              | sg 10     | 2 10 3 28 9 4 |

**Table 5. Indices of discrimination (*D*) calculated from the 79 unrelated isolates of *L. pneumophila* sg 1 using combinations of gene loci with and without MAb subgrouping results**

| Target                  | Presence of gene locus | Phenon MAb | D       | Standard error | 95% Confidence interval |
|-------------------------|------------------------|------------|---------|----------------|-------------------------|
|                         | flaA  pilE  asd  mip  mompS  proA |            |         |                |                         |
| Single locus            | X                      |            | 0.767   | 0.0412         | 0.686 to 0.848           |
|                         | X                      |            | 0.791   | 0.0270         | 0.738 to 0.844           |
|                         | X                      |            | 0.794   | 0.0290         | 0.737 to 0.851           |
|                         | X                      |            | 0.825   | 0.0243         | 0.777 to 0.873           |
|                         | X                      |            | 0.827   | 0.0322         | 0.763 to 0.890           |
|                         | X                      |            | 0.848   | 0.0236         | 0.802 to 0.894           |
| Locus combination       | X X                   |            | 0.910   | 0.0205         | 0.870 to 0.950           |
|                         | X X X                 |            | 0.937   | 0.0191         | 0.900 to 0.975           |
|                         | X X X X               |            | 0.939   | 0.0194         | 0.901 to 0.977           |
|                         | X X X X X             |            | 0.940   | 0.0195         | 0.902 to 0.979           |
|                         | X X X X X X           |            | 0.943   | 0.0196         | 0.904 to 0.980           |
|                         | X X X X X X X         |            | 0.981   | 0.0064         | 0.967 to 0.992           |
panel of 79 isolates used to calculate the indices of discrimination was composed entirely of clinical isolates belonging to sg 1, albeit from 10 different European countries, the D values generated probably represent an underestimate of the true discrimination that would be obtained by sampling a larger range of clinical and environmental isolates from different geographical regions and of other serogroups. However, use of the same panel of strains in multicenter studies has facilitated the calculation and comparison of the relative discriminatory powers of different typing systems for the discrimination of L. pneumophila sg 1 isolates.

The use of only three loci, flaA, pilE, andasd, gave an index of discrimination of 0.94; further combinations obtained by the inclusion of mip, mompS, and proA did not appear to offer significantly higher values. However, as the lower 95% confidence limits are above 0.9, this suggests that these combinations are acceptable as the basis of a typing method. Use of the combination of all six loci (flaA, pilE, asd, mip, mompS, and proA) combined with MAb subgrouping yielded a value of 0.981, with a lower 95% confidence limit of 0.967, indicating that the true D value was unlikely to be below 0.95. As such, the approach meets that specified for an “ideal” typing system (32). In order to demonstrate the validity of this sequence-based typing approach for strains of L. pneumophila other than sg 1, a number of additional isolates were also characterized and gave concordant results; i.e., clinical and environmental isolates that were epidemiologically linked gave identical profiles for all six loci. The authors believe that this study demonstrates the validity of the sequence-based typing approach for the typing of L. pneumophila isolates. However, the inclusion of additional well-characterized isolates belonging to serogroups other than sg 1 in such panels would assist with the determination of confidence limits for non-sg 1 strains.

An expanded online SBT database (version 1.5) can now be queried (see www.ewgli.org). The website also proves detailed instructions regarding the submission of putative novel allelic combinations, i.e., submission of consensus sequences together with sequencing results (chromatogram files) from both forward and reverse reactions, to the database curators.

As indicated above, a level of discrimination of about 0.94 was achieved by using only three targets, flaA, pilE, and asd; a level of discrimination of 0.943 was achieved by using all six loci; and a level of discrimination of 0.981 was achieved by using all six loci and MAb subgrouping data. An ideal system for the typing of L. pneumophila should facilitate the ability to determine the relatedness or otherwise of clinical and environmental isolates. In an outbreak situation, depending on the numbers of isolates involved, it is still advisable to first confirm the serogroup and perform MAb subgrouping with sg 1 isolates (subject to the availability of MAb panels). When isolates are not yet available, e.g., in the first 1 to 2 days of an outbreak investigation, it has been shown that the direct amplification of SBT primary amplicons from clinical and environmental samples is possible, for example, with flaA and pilE (i.e., the targets with the smallest primary amplicon size [authors’ unpublished data]), thus enabling the rapid generation of sequence data and, thus, valuable typing data.

The authors propose that epidemiological typing of L. pneumophila isolates be carried out by using sequence-based typing, as described here, with all six loci, at least until a larger data set is established and reviewed. The resulting sequence-based type (or allelic profile) is thus defined by the cumulative allele number description of each locus, in the order flaA, pilE, asd, mip, mompS, and proA. The absence of sequence information for any locus is entered into the profile as a 0.

In conclusion, we have described and evaluated an improved method for the sequence-based epidemiological typing of L. pneumophila. Modifications to the previous online SBT database now allow users to query the database and identify pre-existing allelic profiles for the six target genes described here, and the website provides instructions concerning the submission of novel allele types and profiles.

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ADDENDUM IN PROOF

Analysis of the complete genome sequences of three L. pneumophila strains, Philadelphia-1T, Paris, and Lens, reveals that both the Paris and Lens strains contain two copies of the mompS gene, whereas the type strain (Philadelphia-1) contains only one. The mompS amplification primers described in our study amplify only a single copy of mompS due to sequence variation in the noncoding flanking regions.

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