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Addition of *neuA*, the Gene Encoding *N*-Acylneuraminate Cytidylyl Transferase, Increases the Discriminatory Ability of the Consensus Sequence-Based Scheme for Typing *Legionella pneumophila* Serogroup 1 Strains▽

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The standard sequence-based method for the typing of *Legionella pneumophila* serogroup 1 strains was extended by using the *gspA* and *neuA* alleles. The use of *neuA* as a seventh allele for typing significantly increased the index of discrimination calculated for a panel of unrelated strains (from 0.932 to 0.963) and subdivided some known large common complexes (e.g., 1,4,3,1,1,1). This modification to the standard method is proposed as the method of choice in the epidemiological investigation of *L. pneumophila* infections.

Members of the genus *Legionella* are gram-negative bacteria and normally occupy natural aquatic environments, where they survive as intracellular parasites of protozoa. Human infections occur as sporadic or epidemic cases of disease that may be acquired from different environmental sources, such as warm water supplies, cooling towers, and evaporative condensers. They are mainly caused by the species *Legionella pneumophila* and mostly by *L. pneumophila* serogroup (sg) 1 strains (7, 10).

In order to detect the source of the infection as soon as possible, several molecular typing techniques have been used to characterize *L. pneumophila* strains (4, 8, 10, 12). Multilocus sequence typing is a powerful tool that is used to discriminate clonal groups within several bacterial species (14). Recently, a scheme for the sequence-based typing (SBT) of *L. pneumophila* that uses the sequences of six genes was described (9). It is now available through the website of the European Working Group on *Legionella* Infections (EWGLI) (www.ewgli.org). An SBT profile comprises a string of numbers comprising the number of individual alleles of the genes *flaA*, *pilE*, *asd*, *mip*, *mompS*, and *proA* separated by commas. The available SBT data for *L. pneumophila* sg 1 suggest that some of the prevalent SBT profiles, e.g., 1,4,3,1,1,1, are heterogeneous by monoclonal antibody (MAb) subgrouping (9) and/or by pulsed-field gel electrophoresis (2). Thus, it might be speculated that some SBT profiles contain several different types that cannot be distinguished by the standard EWGLI SBT scheme. To test this hypothesis we investigated whether the use of additional genes would enhance the discriminatory power of the standard SBT scheme. The epidemiological concordance (*E*) and stability (*S*) (17) of these new alleles were also examined.

We investigated the panel of 79 unrelated *L. pneumophila* sg 1 strains of the European Union Legionella (EUL) culture collection (8, 9). Additional strains from the collection of one of the authors (P.C.L.) ($n = 16$ unrelated strains) and three reference strains (strains Philadelphia-1^T, Lens, and Paris) were included to explore the potential of subdividing some of the larger six-allele profiles, such as 1,4,3,1,1,1; and strains from the EUL culture collection ($n = 15$ related strains, $n = 6$ strains in the stability panel) were used to assess *E* and *S* (Table 1) (8, 9). MAb typing was performed as described previously (10). SBT was performed according to the EWGLI standard scheme (9). In a similar fashion we amplified and analyzed selected regions of the genes encoding *N*-acylneuraminate cytidylyl transferase (*neuA*) (13) and a general stress protein (*gspA*) (1). Primers *gspA*-up (5′-CCT ATC CGG CCT ATG ACA-3′) and *gsp*A-do (5′-CGT GGT TTC GCT TCT TCC-3′) and primers *neu*A-up (5′-CCG TTC AAT ATG GGG CTT CAG-3′) and *neu*A-do (5′-CGA TGT CGA TGG ATT CAC TAA TAC-3′) were designed by using previously published sequences (1, 13).

For the 79 unrelated EUL strains, the allelic profiles of the six genes were published previously (9). For the strains isolated in Germany as well as other EUL strains, all sequences were determined anew. The allele numbers obtained by the standard protocol and the additional gene, *neuA*, will be available through the website of the EWGLI (www.ewgli.org) (for review purposes, see http://www.hpa-bioinfotools.org.uk/legionella/php/sbt_query1_neu.php). *GspA* allele 16 was taken from the genome sequence of strain Lens (5), and *gspA* allele 17 is from the original description (1).

For the two additional genes, the lengths of the fragments analyzed were 354 bp (nucleotide positions 229 to 583) for *neuA* and 225 bp for *gspA* (nucleotide positions 61 to 286). The numbers of silent and nonsilent mutations and the percentage of nucleotide substitutions were 20.0, 8.0, and 6.6, respectively, for *neuA* and 21.0, 6.0, and 10.7, respectively, for *gspA*. This is in the range of values for other *L. pneumophila* genes (3, 9, 15). Altogether, we determined 15 allele variants for *neuA* and 16 for *gspA*.

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TABLE 1. Unrelated *Legionella pneumophila* sg 1 strains investigated by additional sequencing of *neuA* and *gsp* alleles

Strain	MAb subgroup[b]	Standard EWGLI SBT[c]	No. of alleles	
			neuA	*gsp*
EUL074[a]	Philadelphia	1,4,1,1,14,9	1	13
EUL001, EUL003, EUL037, EUL042, EUL043, EUL060, EUL067, EUL082, EUL084, EUL085, EUL088, EUL093, EUL119, L02-570, Ulm145432, Char6297/2000, W03-685, Paris, L01-127, L04-541	Oxford (2), OLDA (10) Philadelphia (8)	1,4,3,1,1,1	1	10
EUL017, EUL112, L03-610	OLDA (2), Philadelphia	1,4,3,1,1,1	6	10
München 1	Benidorm	1,4,3,1,1,1	9	10
EUL013, EUL014, EUL016	Benidorm (3)	1,4,3,1,1,1	14	10
EUL117	Benidorm	1,4,3,1,1,1	15	10
EUL104, EUL110	Oxford	1,6,3,1,1,1	1	10
EUL053	OLDA	1,6,3,1,14,1	1	13
EUL038	OLDA	1,10,3,1,1,1	1	9
EUL072	Philadelphia	1,10,19,1,9,4	1	7
EUL029	Knoxville	2,2,18,15,2,1	6	10
EUL036	Knoxville	2,3,3,15,2,6	6	1
EUL026	OLDA	2,3,6,10,2,1	6	2
EUL004, EUL008, EUL028, EUL041, L01-403	Allentown (4), Philadelphia	2,3,9,10,2,1	6	1
EUL049	Knoxville	2,3,18,15,2,1	6	6
EUL087	Knoxville	2,4,3,10,9,4	9	7
EUL111	Benidorm	2,6,17,15,12,8	6	4
EUL018	Bellingham	2,6,21,12,12,8	11	4
EUL007	Allentown	2,10,9,13,2,5	6	2
EUL032	Benidorm	2,10,18,10,2,1	9	2
L92-448, L03-518	Philadelphia, Knoxville	3,4,1,1,1,9	1	13
EUL118, Philadelphia-1[T]	Philadelphia	3,4,1,1,14,9	1	13
EUL069, EUL073	Philadelphia (2)	3,4,1,1,14,9	11	13
EUL030	France	3,4,1,14,14,9	11	13
EUL033	France	3,6,1,14,14,9	11	13
EUL020	Benidorm	3,10,1,3,14,9	1	13
HannP9	Knoxville	3,10,1,3,14,9	11	13
L03-572	Knoxville	3,10,1,5,14,9	11	13
EUL019, EUL097, EUL098	Knoxville (3)	3,10,1,3,14,9	11	13
EUL052	Philadelphia	3,10,3,1,14,9	11	10
EUL051	Benidorm	3,10,15,3,21,7	9	7
EUL099	Bellingham	3,13,1,25,14,9	6	13
EUL050	Benidorm	4,6,11,3,11,12	9	13
EUL006	Benidorm	4,7,9,3,11,12	9	9
EUL027, EUL039, EUL075, EUL105, EUL116, EUL120, Vie53	Benidorm (6)	4,7,11,3,11,12	9	9
EUL025	Allentown	4,8,11,10,10,12	2	9
EUL103	OLDA	5,1,22,26,6,10	12	8
EUL068, EUL086	Benidorm (2)	5,1,22,5,6,10	15	8
EUL048	Bellingham	5,2,22,27,6,10	12	8
EUL031, EUL070	Allentown (2)	5,10,22,15,6,2	6	8
EUL055	OLDA	6,4,3,1,1,1	1	10
EUL063	Knoxville	6,4,14,12,2,3	6	10
EUL066	Knoxville	6,10,14,10,2,1	6	7
L01-472	Knoxville	6,10,15,28,9,14	6	7
EUL083	Benidorm	6,10,15,24,17,14	6	3
EUL002	Knoxville	6,10,19,3,19,4	9	13
EUL081, EUL092	Bellingham (2)	6,10,21,12,9,4	11	3
EUL101	Benidorm	7,6,17,3,13,11	9	4
EUL100, EUL102	Bellingham (2)	7,6,17,3,13,11	11	4
EUL054, EUL071	France, Allentown	8,10,3,15,18,1	6	9
EUL091	Bellingham	9,6,3,10,22,15	11	4
EUL040	Philadelphia	11,14,16,1,15,13	6	4
Lens, L03-407	Benidorm (2)	12,9,26,5,26,17	15	16

[a] Strains with the prefix EUL belong to the panel of 79 unrelated strains from the EUL culture collection (8, 9).
[b] The numbers of strains belonging to the MAb subgroup are indicated in parentheses.
[c] From reference 9.

The previously described panel of 79 unrelated *L. pneumophila* sg 1 strains (8) was used to estimate the Hunter-Gaston diversity index (11), with precision expressed as 95% confidence intervals (CIs) by using the V-DICE tool (http://www.hpa-bioinfotools.org.uk/cgi-bin/DICI/DICI.pl). The Dice coefficient (*D*) values for the single genes *neuA* and *gspA* were 0.800 (95% CI, 0.783 to 0.816) and 0.836 (95% CI, 0.814 to 0.859), respectively; these values are approximately in the range of values

TABLE 2. Allelic profiles of *L. pneumophila* sg 1 isolates belonging to epidemiologically related sets and stability panel

Strain set and strain	MAb subgroup	Standard EWGLI SBT[c]	No. of alleles	
			neuA	*gsp*
Epidemiologically related sets[a]				
EUL003, EUL009	Philadelphia	1,4,3,1,1,1	1	10
EUL037, EUL044	Philadelphia	1,4,3,1,1,1	1	10
EUL019, EUL023	Knoxville	3,10,1,3,14,9	11	13
EUL048, EUL056	Bellingham	5,2,22,27,6,10	12	8
EUL073, EUL079	Philadelphia	3,4,1,1,14,9	11	13
EUL097, EUL107	Knoxville	3,10,1,3,14,9	11	13
EUL140, EUL141, EUL142	Knoxville	12,8,11,5,20,12	5	15
Stability panel,[b] strains EUL135, EUL136, EUL137, EUL138, EUL139, EUL147	Knoxville/Denver	6,10,15,28,9,14	6	7

[a] Related patients and environmental isolates from clusters or epidemics of legionellosis.
[b] Variants of strain Corby that differ in their ability to multiply in amoebae and macrophages showed different reactivity patterns with MAbs due to a mutation in a lipopolysaccharide synthesis gene or that were resistant to rifampin (9).
[c] From reference 9.

for other genes (3, 9, 13). The use of the EWGLI standard (six-gene) SBT scheme yielded a *D* value of 0.932 (95% CI, 0.913 to 0.951) for the panel of 79 unrelated strains, but with the addition of the *neuA* gene this increased to 0.963 (95% CI, 0.952 to 0.974), which is above the value recommended for a good epidemiological typing system (17). Although *gspA* was heterogeneous in the strains tested, the additional use of this gene did not enhance the discrimination of the SBT scheme; i.e., the *D* value remained 0.932 (95% CI, 0.913 to 0.951). However, it is noteworthy that MAb subgrouping further increased the *D* value in all cases to 0.974 (95% CI, 0.968 to 0.980) in the standard scheme and to 0.98 (95% CI, 0.974 to 0.985) when *neuA* was included.

The addition of *neuA* to the standard scheme allowed the differentiation of the complex 1,4,3,1,1,1 into five subgroups (Table 1). The fact that the MAb subgrouping appears to correlate to some extent with the subgrouping provided by SBT might be a further indication that the strains within this standard SBT type are really dissimilar, albeit closely related. Thus, within the 1,4,3,1,1,1 complex, *neuA* alleles 1 and 6 were found exclusively in strains of the MAb subgroups OLDA, Oxford, and Philadelphia. The MAb subgroups Philadelphia and OLDA might be considered closely related because the loss of the *lag-1* gene resulted in a switch from the Philadelphia subgroup to the OLDA subgroup (4, 18). In contrast, *neuA* alleles 9, 14, and 15 were found only in strains belonging to MAb subgroup Benidorm within this same complex. The *neuA* allele also further divided two other six-allele complexes, 7,6,17,3,13,11 and 3,10,1,3,14,9, into two further types each, each of which in turn corresponded to a different MAb subgroup (Table 1). Based on the use of the *neuA* allele, complex 3,4,1,1,14,9 determined by SBT could also be divided into two subgroups within the same MAb subgroup, Philadelphia (Table 1). Conversely, strains L03-518 and L92-448 were different by MAb typing but indistinguishable by SBT when up to eight genes were used. Epidemiological concordance ($E = 1$) was demonstrated by using six epidemiologically related sets, and stability was demonstrated ($S = 1$) by using the stability panel comprising Corby strain variants (Table 2).

Recently, sequence typing, which identifies variations in the nucleotide sequences of internal fragments of selected genes, has been established for certain bacterial species (6). In a recently published study (9), the utility of the SBT protocol for epidemiological investigations of *L. pneumophila* sg 1 infections was described. In the present study, we could significantly enhance the discriminatory power of the standard scheme using *neuA* as the seventh target. Similar results were recently published for *Streptococcus pneumoniae* (16). Our data suggest that the extended seven-gene allelic profiles more accurately reflect the diversity between closely related *L. pneumophila* sg 1 strains than the standard scheme does. It remains an open question and it needs to be experimentally proven whether the use of additional genes, e.g., the *icm* and *dot* loci, that have been shown to be highly heterogeneous (15) can further discriminate the presently established SBT types. The seven-gene SBT scheme (including *neuA*) of *L. pneumophila* produces robust, epidemiologically concordant, and highly discriminatory data that can be easily exchanged between laboratories. The authors propose that the standard EWGLI SBT scheme be extended to include the *neuA* allele, and this proposal is now under review by the members of EWGLI. It is anticipated that this revised scheme with seven targets will then be available via the Internet (http://www.ewgli.org). It is not anticipated that the scheme will change again.

Nucleotide sequence accession numbers. The sequences of the alleles of the *gspA* gene have been deposited in the EMBL nucleotide sequence database under accession numbers AM490070 to AM490085.

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REFERENCES

1. **Abu, K., and N. C. Engleberg.** 1994. Cloning and molecular characterization of a *Legionella pneumophila* gene induced by intracellular infection and by various *in-vitro* stress conditions. Mol. Microbiol. **13:**243–251.
2. **Amemura-Maekawa, J., F. Kura, B. Chang, and H. Watanabe.** 2005. *Legionella pneumophila* serogroup 1 isolates from cooling towers in Japan form a distinct genetic cluster. Microbiol. Immunol. **49:**1027–1033.
3. **Aurell, H., P. Farge, H. Meugnier, M. Gouy, F. Forey, G. Lina, F. Vandenesch, J. Etienne, and S. Jarraud.** 2005. Clinical and environmental isolates of *Legionella pneumophila* serogroup 1 cannot be distinguished by sequence analysis of two surface protein genes and three housekeeping genes. Appl. Environ. Microbiol. **71:**282–289.
4. **Bernander, S., K. Jacobson, J. H. Helbig, P. C. Lück, and M. Lundholm.** 2003. A hospital-associated outbreak of Legionnaires' disease caused by *Legionella pneumophila* serogroup 1 is characterized by stable genetic fingerprinting but variable monoclonal antibody patterns. J. Clin. Microbiol. **41:**2503–2508.
5. **Cazalet, C., C. Rusniok, H. Brüggemann, N. Zidane, A. Magnier, L. Ma, M. Tichit, S. Jarraud, C. Bouchier, F. Vandenesch, F. Kunst, J. Etienne, P. Glaser, and C. Buchrieser.** 2004. Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. Nat. Genet. **36:**1165–1173.
6. **Cooper, J. E., and E. J. Feil.** 2004. Multilocus sequence typing—what is resolved. Trends Microbiol. **12:**373–377.
7. **Fields, B. S., R. F. Benson, and R. E. Besser.** 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. Clin. Microbiol. Rev. **15:**506–526.
8. **Fry, N. K., S. Alexiou-Daniel, J. M. Bangsborg, S. Bernander, M. Castellani-Pastoris, J. Etienne, B. Forsblom, V. Gaia, J. H. Helbig, D. Lindsay, P. C. Lück, C. Pelaz, S. A. Uldum, and T. G. Harrison.** 1999. A multicenter evaluation of genotyping methods for the epidemiologic typing of *Legionella pneumophila* serogroup 1: results from a pan-European study. Clin. Microbiol. Infect. **5:**462–477.
9. **Gaia, V., N. K. Fry, B. Afshar, P. C. Lück, H. Meugnier, J. Etienne, R. Peduzzi, and T. G. Harrison.** 2005. Consensus sequence-based scheme for epidemiological typing of clinical and environmental isolates of *Legionella pneumophila*. J. Clin. Microbiol. **43:**2047–2052.
10. **Helbig, J. H., S. Bernander, P. Castellani, J. Etienne, V. Gaia, S. Lauwers, D. Lindsay, P. C. Lück, T. Marques, S. Mentula, M. F. Peeters, C. Pelaz, M. Struelens, S. A. Uldum, G. Wewalka, and T. G. Harrison.** 2002. Pan-European study on culture-proven Legionnaires' disease: distribution of *Legionella pneumophila* serogroups and monoclonal subgroups. Eur. J. Clin. Microbiol. Infect. Dis. **21:**710–716.
11. **Hunter, P. R., and M. A. Gaston.** 1988. Numerical index of discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. **26:**2465–2466.
12. **Lück, P. C., H. M. Wenchel, and J. H. Helbig.** 1998. Nosocomial pneumonia caused by three genetically different strains of *Legionella pneumophila* and detection of these strains in the hospital water supply. J. Clin. Microbiol. **36:**1160–1163.
13. **Lüneberg, E., N. Zetzmann, D. Alber, Y. A. Knirel, O. Kooistra, U. Zähringer, and M. Frosch.** 2000. Cloning and functional characterization of a 30 kb gene locus required for lipopolysaccharide biosynthesis in *Legionella pneumophila*. Int. J. Med. Microbiol. **290:**37–49.
14. **Maiden, M. C. J., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. C. Spratt.** 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. USA **95:**3140–3145.
15. **Morozova, I., X. Qu, S. Shi, G. Asamani, J. E. Greenberg, H. A. Shuman, and J. J. Russo.** 2004. Comparative sequence analysis of the *icm*/*dot* genes in *Legionella*. Plasmid **51:**127–147.
16. **Pettigrew, M., K. P. Fennie, M. P. York, J. Daniels, and F. Ghaffar.** 2006. Variation in the presence of neuraminidase genes among *Streptococcus pneumoniae* isolates with identical sequence types. Infect. Immun. **74:**3360–3365.
17. **Struelens, M. J., and members of the European Study Group on Epidemiological Markers (ESGEM) of the European Society for Clinical Microbiology and Infectious Diseases.** 1996. Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. Clin. Microbiol. Infect. **2:**2–11.
18. **Zou, C. H., Y. A. Knirel, J. H. Helbig, U. Zähringer, and C. S. Mintz.** 1999. Molecular cloning and characterization of a locus responsible for O-acetylation of the O-polysaccharide of *Legionella pneumophila* serogroup 1 lipopolysaccharide. J. Bacteriol. **181:**4137–4141.