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The *N*-Acylneuraminate Cytidyltransferase Gene, *neuA*, Is Heterogenous in *Legionella pneumophila* Strains but Can Be Used as a Marker for Epidemiological Typing in the Consensus Sequence-Based Typing Scheme▽†

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Sequence-based typing (SBT) is the internationally recognized standard method for genotyping *Legionella pneumophila*. To date all strains of serogroup 1 (SG1) and some of SGs 2 to 14 yield a seven-allele profile and can be assigned a sequence type (ST). However, for some strains belonging to SGs 2 to 14, the targeted region of the *neuA* gene could not be amplified using the published standard primers. We determined the DNA sequence of a *neuA* gene homolog located in the lipopolysaccharide synthesis locus of strain Dallas-1E. By using newly designed degenerate consensus primers based on the *neuA* homolog in strains Dallas-1E, Philadelphia-1, Paris, Lens, and Corby, we were able to obtain DNA sequences for all 48 non-SG1 strains which were untypeable by the standard method. Our data show that the *neuA* gene is present in all *L. pneumophila* strains but differs significantly in some non-SG1 strains at both the DNA and amino acid levels. The new primers can be used to amplify and sequence the *neuA* gene in all strains and can substitute for the standard primers. This offers the possibility of assigning an ST to all strains of *L. pneumophila*.

Legionellae are ubiquitous Gram-negative bacteria which occupy natural and manmade aquatic environments. They are the causative agents of Legionnaires' disease, which occurs as sporadic or epidemic cases of pneumonia acquired by inhalation or aspiration of legionellae from different environmental sources, such as cooling towers and potable water and warm water supplies. Currently, the genus *Legionella* comprises more than 50 species and more than 70 serogroups, with *Legionella pneumophila* serogroup 1 (SG1) causing the majority of human infections (11, 12; J. P. Euzeby, List of Prokaryotic Names with Standing in Nomenclature [http://www.bacterio.cict.fr/l/legionella.html]). Based on DNA homology studies, the species *L. pneumophila* is subdivided into three subspecies, i.e., *L. pneumophila* subsp. *pneumophila*, *L. pneumophila* subsp. *fraseri*, and *L. pneumophila* subsp. *pascullei* (2).

Monoclonal antibody (MAb) subgrouping (12) and sequence-based typing (SBT) (8, 15) are the established epidemiological typing methods for comparison of clinical and environmental isolates of *L. pneumophila.* Typing data showing that linked clinical and environmental strains are indistinguishable by these typing methods can be used to support or refute evidence for establishing the source of infection.

The seven target sequences include the *neuA* gene located within the lipopolysaccharide (LPS) synthesis locus of *L. pneumophila*. This gene has been previously shown to encode an *N*-acylneuraminate cytidylyltransferase, an enzyme involved in the biosynthesis of the LPS in *L. pneumophila* (9). By using the published *neuA* primers (15), standard SBT could be applied in all strains belonging to SG1 and to some other SGs. However, in some non-SG1 *L. pneumophila* strains, the *neuA* gene could not be amplified (1, 11, 16, 20). It was considered likely therefore that this was due to either the heterogeneity of *neuA* DNA sequence preventing primer binding or the absence of the gene in some of these non-SG1 strains, as suggested by microarray analysis (3). The possibility of the absence of a gene used in typing schemes was shown recently for *Haemophilus influenzae.* Due to a deletion of an operon encoding a fuculokinase, which is part of the seven-locus sequence typing scheme (17), a multilocus sequence typing (MLST) scheme could not be applied.

Therefore, the purpose of this study was to determine the degree of heterogeneity, if any, of the *neuA* genes, with the intention of designing new primer sets which could be used to amplify and sequence *neuA* in all *L. pneumophila* strains.

MATERIALS AND METHODS

***Legionella* strains.** Altogether 117 *L. pneumophila* strains were used in this study (Table 1). These strains were originally isolated from patients or water systems and were obtained from the authors' strain collections, the American Type Culture Collection (ATCC), Manassas, VA, and the National Collection of Type Cultures (NCTC), Health Protection Agency, London, United Kingdom (Table 1).

Typing of *Legionella* strains. All strains were typed by using monoclonal antibody subgrouping (12) and sequence-based typing (8, 15). Previous attempts to determine the *neuA* allele using the standard *neuA* primers were unsuccessful for 48 of these strains (indicated in Table 1 by F, for fail).

Determination of the DNA sequence of the LPS locus in strain Dallas-1E. The entire sequence of the LPS locus, including the *neuA* gene, was determined in *L. pneumophila* strain Dallas-1E (ATCC 33216). This strain belongs to *L. pneumophila* subsp. *fraseri*, reacts serologically as SG5, and was originally isolated from

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TABLE 1. Allele numbers for 117 *L. pneumophila* strains isolated from clinical and environmental samples from different sources and regions by using the standard *neuA* primers and the new consensus primers

Strain	Source country[a]	Isolate type[b]	SG	*neuA* allele no.	
				Using standard primers[c]	Using new consensus primers[d]
Dallas-1E ATCC 33216	USA	E	5	F	201
MICU-B ATCC 33735	USA	E	5	F	201
U8W ATCC 33737	USA	E	5	F	201
W227-1	Germany	E	5	F	202
H064160534-4	England	E	8	F	203
Concord 3 ATCC 35096	USA	C	8	F	203
1169-MN-H ATCC 43703	USA	C	14	F	203
W10-219	Germany	E	8	F	203
W10-287	Germany	E	8	F	203
H082960038	England	E	10	F	204
L07-220-1	Germany	C	4	F	204
H064160536-3	England	E	10	F	205
Los Angeles-1 ATCC 33156	USA	C	4	F	206
W10-144	Germany	E	4	F	206
H042740084	England	E	6	F	207
H083580006	England	E	10	F	207
L00-295	Germany	C	2	F	207
L02-135	Germany	C	4	F	207
L08-403	Germany	C	10	F	207
L08-404	Germany	C	10	F	207
LC0395	Belgium	E	10	F	207
LC0569	England	C	8	F	207
LC0606	England	E	8	F	207
LC1132	England	C	10	F	207
LC6813	England	E	10	F	207
LC6817	England	E	10	F	207
ST-247	Germany	E	3	F	207
ST-247-8	Germany	E	3	F	207
W01-1979-4	Germany	E	3	F	207
W03-788	Germany	E	10	F	207
W03-843-1	Germany	E	15	F	207
W04-306-1	Germany	E	2	F	207
W07-1175	Germany	E	10	F	207
W08-259-1	Germany	E	3	F	207
W08-259-2	Germany	E	2	F	207
W08-632	Germany	E	10	F	207
W-1188	Germany	E	3	F	207
W330-21	Germany	E	3	F	207
W-567	Germany	E	2	F	207
W671-1	Germany	E	2	F	207
WS-47-3	Germany	E	3	F	207
W10-341	Germany	E	2	F	207
W08-130	Germany	E	2–14 cross-reacting	F	208
W08-433-1	Germany	E	4	F	208
W10-286	Germany	E	4	F	208
W05-531	Germany	E	13	F	209
W10-960	Germany	E	13	F	210
Chicago 8 ATCC 33823	USA	E	7	F	211
L08-477	Germany	C	1	9	9
Oxford-1 (NCTC 11287)	England	C	6	9	9
H081760005	NK	C	1	9	9
W07-508-2	Germany	E	12	9	9
Charite 7	Germany	E	2	9	9
Togus-1 ATCC 33154	USA	C	2	8	8
Charite 15	Germany	E	2	8	8
W10-285	Germany	E	2	8	8
RR08000504	England	E	4	7	7
Riesa-1	Germany	E	3	7	7
H082680013	England	C	1	6	6
L08-482	Germany	C	1	6	6
L08-532	Germany	C	1	6	6
RV 34-08	France	C	1	6	6

Continued on following page

TABLE 1—*Continued*

Strain	Source country[a]	Isolate type[b]	SG	*neuA* allele no.	
				Using standard primers[c]	Using new consensus primers[d]
EUL 140[e]	Spain	C	1	5	5
4163	Italy	C	1	4	4
NIIB 2481	Japan	C	6	4	4
NMEX 35	Portugal	E		35	35
09.5804	England	E	1	33	33
LG 0919 3016	France	C	1	31	31
HL 0046 3008	France	C	1	30	30
RR09000328	Czech Republic	C	1	3	3
RV 35-08	UK	E	1	3	3
W08-439	Germany	E	10	3	3
W08-882	Germany	E	6	3	3
W08-883	Germany	E	6	3	3
H090520323	NK	C		28	28
LG 0737 1032	France	C	1	26	26
LG 0837 5013	France	C	1	26	26
80519019001	NLD	C	1	25	25
Lansing-3	USA	C	15	24	24
RR07000791	England	E	1	23	23
H094060813	NK	C	1	22	22
H052900085	Greece	C	1	20	20
H080600491	England	E	1	2	2
L07-362	Germany	C	1	2	2
W08-434-1	Germany	E	1	2	2
H074080523	England	C	1	19	19
NIIB 0141	Japan	C	1	18	18
NIIB 2301	Japan	C	1	18	18
EUL 163	Austria	C	2–14 cross-reacting	17	17
EUL 45	Italy	C	1	16	16
H080780061	England	E	1	15	15
L03-346	Germany	E	1	15	15
W10-210	Germany	E	3	15	15
RV 32-08	England	C	1	15	15
RV 36-08	England	E	1	15	15
EUL 13	Scotland	C	1	14	14
H081480590	England	E		13	13
L08-422	Germany	C	3	13	13
H082520166	England	C	1	12	12
W10-995	Germany	E	1	12	12
H083080428	England	E	1	11	11
L08-371	Germany	C	1	11	11
L08-486	Germany	C	1	11	11
W08-394-4	Germany	E	1	11	11
W08-444	Germany	E	1	11	11
W08-446	Germany	E	1	11	11
W08-449-1	Germany	E	1	11	11
W08-449-2	Germany	E	1	11	11
W10-291	Germany	E	3	11	11
RR09000315	Czech Republic	C	1	10	10
Philadelphia-1 ATCC 33152	USA	C	1	1	1
L08-498	Germany	C	1	1	1
L08-555-1	Germany	C	1	1	1
RV 33-08	England	E	1	1	1
W08-445	Germany	E	1	1	1
W08-447	Germany	E	1	1	1
H082940035	Italy	C	1	1	1

[a] NK, not known (country of origin of isolate unknown due to a history of travel).
[b] E, environmental; C, clinical.
[c] Standard primers for *neuA* from the European Working Group for *Legionella* Infections (15). F, failed (no PCR amplification with the standard primers).
[d] This study.
[e] EUL, European Union *Legionella* culture collection (8).

an environmental specimen in the United States. We hypothesized that the *neuA* gene of this strain might be representative for heterogeneity within this region. In order to analyze the region of the Dallas-1E LPS locus between the homolog of *iraB* (lpg0746) and *rmlA* (lpg0760) (Fig. 1), including *neuA*, DNA fragments ranging from 1.5 to 4.0 kbp were amplified with different primer combinations and directly sequenced. Some primers have been previously described (18), while new ones were designed for the Dallas-1E strain (see Table S1 in the supplemental material).

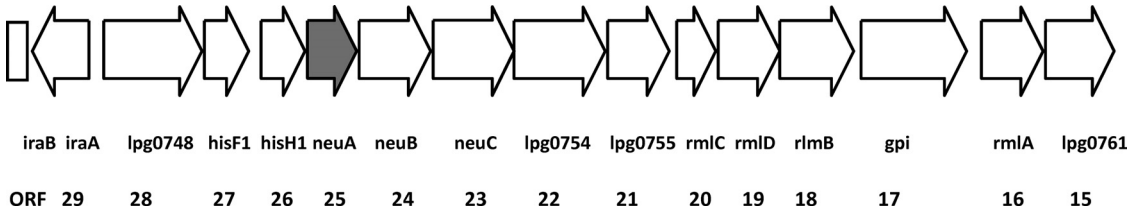

FIG. 1. Schematic representation of the gene organization in the LPS synthesis locus in strain Philadelphia-1 including the *neuA* gene (bp 817030 to 924915 in the genome). Open reading frames (ORFs) are represented as open boxes, with arrowheads indicating the orientation. The design of the open reading frames was based on published sequences (6). The filled box indicates the *neuA* gene.

Design of new *neuA* primers. To prove the presence of the *neuA* gene in all strains, consensus primers *neuA*cons-up (positions 185 to 206; 5′-ATG GDG CYT CWG TDC CHT GG-3′) and *neuA*-cons-do2 (positions 618 to 594; 5′-CTR TYT ARW GCC CAA TCS ATT GG-3′) were designed using published *neuA* sequences of the SG1 strains Philadelphia-1 (6) Lens, Paris (4), and Corby (10) and the newly found sequence from strain Dallas-1E.

Nucleotide sequence accession numbers. The complete sequence of the LPS region of strain Dallas-1E was deposited in the GenBank under accession number FN256429. Partial sequences of the *neuA* gene were deposited under GenBank accession numbers FR750545 to FR750553.

RESULTS

Bioinformatics analysis of the *Legionella* LPS locus in strain Dallas-1E. All single-stranded sequences having phred scores of >20 were aligned, and contig assembly was performed using the computer programs Seqman, version 8.1.2 (DNA STAR, Madison, WI), and BioNumerics, version 5.0 (Applied Maths, Sint-Martens-Latem, Belgium). The complete sequence of the LPS region of strain Dallas-1E was deposited in the GenBank under accession number FN256429. The analysis revealed that a *neuA* homolog of 699 bp (232 amino acids) is present in Dallas-1E. Comparison of this nucleotide sequence with the sequences available from complete genome sequences of *L. pneumophila* using blastn (21; http://blast.ncbi.nlm.nih.gov/Blast.cgi) yielded maximum identification scores of 68% with strains Paris (NC_006368.1), Philadelphia-1 (NC_002942.5), Alcoy (NC_014125.1), Corby (NC_009494.2), and Lens (NC_006369.1). At the amino acid level, analysis using blastp revealed 159/232 (69%) identities and 194/232 (84%) positives (this is the number and fraction of residues for which the alignment scores have positive values) with Philadelphia-1 and 89/229 (39%) identities and 124/229 (54%) positives with NeuA of *Neisseria meningitidis*, the only bacterial *N*-acylneuraminate cytidylyltransferase whose three-dimensional (3D) structure has been characterized to date (14). NeuA proteins of *L. pneumophila* Philadelphia-1, Dallas-1E, and *N. meningitidis* were clearly identified as belonging to the cytidylyltransferase (CTP transf 3) family of proteins when their amino acid sequences were submitted to Pfam (http://pfam.sanger.ac.uk), a database of 12,273 protein families (7).

When the deduced amino acid sequence was aligned by the Lipman-Pearson method using DNA Star software, several regions with identity or high homology could be found (Fig. 2). Interestingly, the homology between the sequenced *neuA* gene of *L. pneumophila* (Philadelphia-1 and Dallas-1E) and of another *Legionella* species, *Legionella longbeachae* (strain NSW150), was only 27% and thus significantly lower than that of non-*Legionella* bacteria. Functionally, the *neuA* gene from *L. pneumophila* could complement a *neuA*-negative *Escherichia coli* mutant (13). Thus, it seems reasonable to suggest that the *neuA* genes of strains Philadelphia-1 and Dallas-1E are functionally equivalent.

Homologies of <70% at both the DNA and amino acid levels suggest that this genomic region may have been acquired by horizontal gene transfer of mobile genetic elements among different *Legionella* strains (5).

Evaluation of new *neuA* primers. Based on these data, we postulated the existence of *neuA* genes in all strains of *L. pneumophila* even though in some non-SG1 strains these *neuA* homolog sequences may have a lower level of similarity. Therefore, the consensus primers were used to amplify an internal fragment of *neuA* in 117 *L. pneumophila* strains (Table 1).

From all strains, the *neuA* gene was successfully amplified with the new consensus primers described above by employing an annealing temperature of 55°C and a primer concentration of 60 pmol per reaction mixture. All strains included in this study yielded readable DNA sequences with the newly designed consensus primers. These sequences were assembled and analyzed manually using BioNumerics, version 5.0 (Applied Maths, St. Martems-Latem, Belgium), for the new *neuA* alleles or using the automated online *Legionella* SBT Sequence Quality Tool (http://www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq_assemble_legionella1.cgi) (19) (Table 1) in the case of known *neuA* alleles.

We further analyzed these internal fragments of the *neuA* genes corresponding to nucleotide positions 229 to 583 of the Philadelphia-1 *neuA* gene. We note that a triplet present at positions 269 to 271 (GAA corresponding to glutamic acid at position 91) in Dallas-1E and MICU-B (*L. pneumophila* subsp. *pascullei* ATCC 33735) is not present in the *neuA* gene homolog obtained from the other non-SG1 strains. Due to this deletion, the analyzed fragment was 354 bp in length for these two strains and 351 bp in the other non-SG1 strains. Altogether, 11 new allele types of *neuA* were determined. They are available under GenBank accession numbers FR750545 to FR750553. For the purposes of this study, they were numbered from 201 to 211 (in order to distinguish them from the standard *neuA* alleles, of which there are currently 39 numbered from 1 to 39). The *neuA* allele type 207 was obtained from the majority of strains which could not be assigned a *neuA* allele using the standard primers (Table 1).

Relatedness of the *neuA* gene in strains belonging to different subspecies and serogroups. In both of our laboratories, the sequence of the 354-bp fragment of the *neuA* gene for the strain Dallas-1E homolog was determined (and 100% identical), demonstrating the robustness of the method. This sequence was designated new *neuA* allele 201. In Fig. 3, a den-

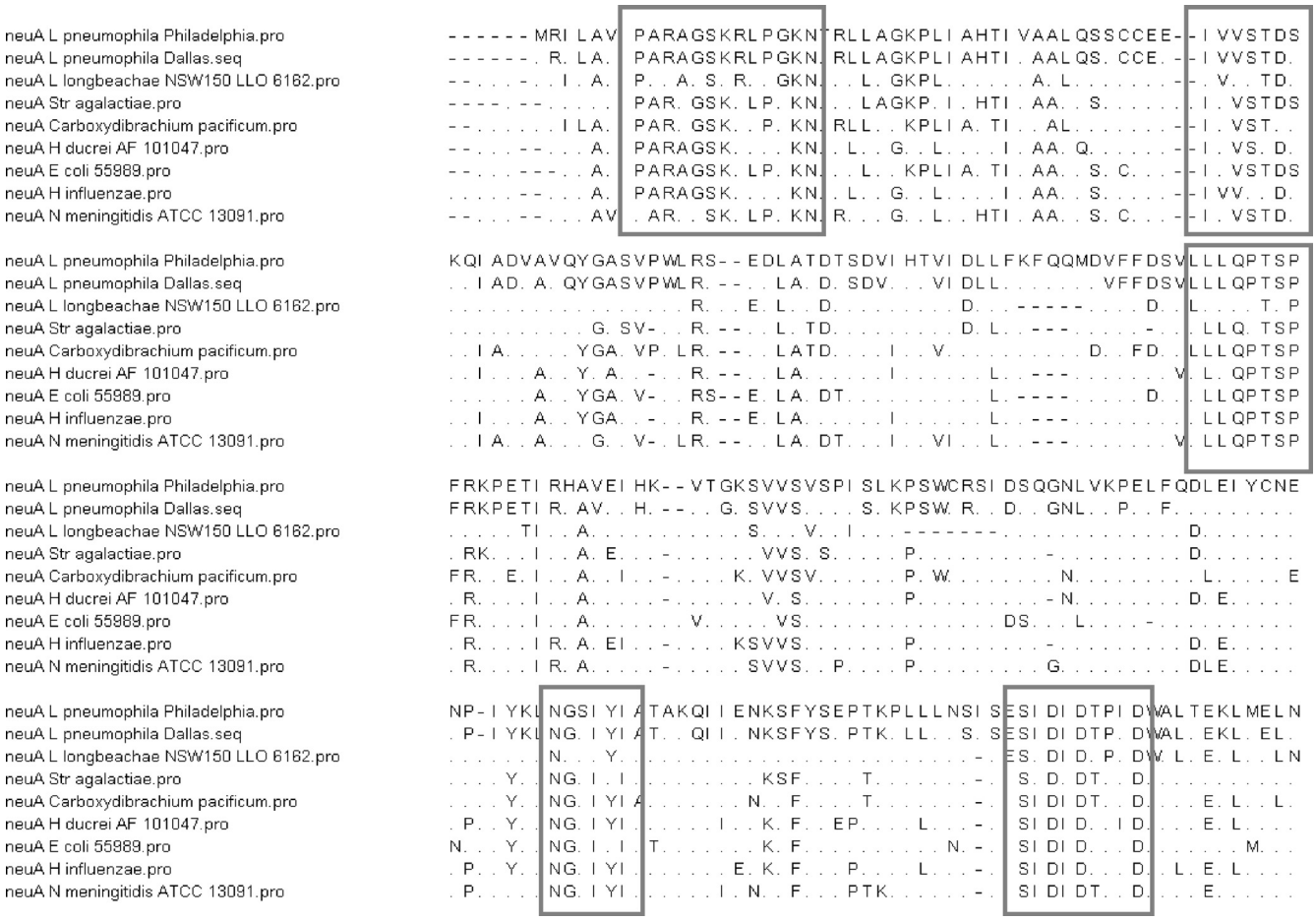

FIG. 2. Amino acid alignment by the Lipmann-Pearson (DNA Star program) method of the *neuA* gene regions of two *L. pneumophila* strains, *L. longbeachae* NSW150, and five other bacterial species showing regions of high similarity (>6 amino acids): *Carboxydibrachium pacificum* DSM 12653 (accession number ABXP01000000; identity, 40%), *Desulfomicrobium baculatum* DSM 4028 (accession number NC_013173; identity, 43%), *Geobacter lovleyi* (accession number NC_01081; identity, 38%), *E. coli* 55989 (accession number NC_011748; identity, 34%), and *N. meningitidis* ATCC 13091 (accession number AEEF01000113; identity, 38%). In the sequences, periods indicate residues that differ from the sequence of Philadelphia-1.

drogram based on the unweighted-pair group method using average linkages (UPGMA) shows the genetic relatedness of the *neuA* sequences. As shown for the complete *neuA* gene, the 354-bp fragments differed markedly between strain Dallas-1E and strain Philadelphia-1. In general, the new *neuA* alleles 201 to 211 showed greater heterogeneity than found among the standard *neuA* alleles, designated 1 to 39 (Fig. 3). It can be demonstrated that among the three subspecies of *L. pneumophila*, strain Dallas-1E, which is representative of *L. pneumophila* subsp. *fraseri*, showed 100% homology to strains MICU-B and U8W (*L. pneumophila* subsp. *pascullei*) (2). In contrast, only 80% homology is present between the *neuA* genes of strains Dallas-1E and Los Angeles-1, both belonging to *L. pneumophila* subsp. *fraseri* (2). Also of interest, the *neuA* gene of strain Lansing 3 serogroup 15 (*L. pneumophila* subsp. *fraseri*) has 98% homology to the corresponding gene in strain Philadelphia-1 (*L. pneumophila* subsp. *pneumophila*) (2). The greatest difference among the strains not having the standard *neuA* gene was found with strain Chicago 8 (SG7; ATCC 33823) (Fig. 3). Our results demonstrated no correlation between the *neuA* sequences in the three subspecies of *L. pneumophila* and the serogroup, and they suggest that this region has not coevolved with the whole genome.

DISCUSSION

Our results demonstrate clearly that the *neuA* gene involved in LPS synthesis is present in all strains but exists with remarkable heterogeneity. These data are consistent with the observation in the published microarray study, which did not find any evidence of the presence of the *neuA* gene in strain Dallas-1E using a threshold defined to a DNA similarity of ≥80% (3).

Previously published data (15) showed the utility of the *neuA* gene in SBT. It substantially increased the discriminatory ability for typing *L. pneumophila* SG1. Based on our new consensus primers, successful amplification and sequencing of the *neuA* gene were achieved with all strains tested. Even though these new *neuA* alleles were heterogenous compared to the original alleles, the use of newly designed primers did allow

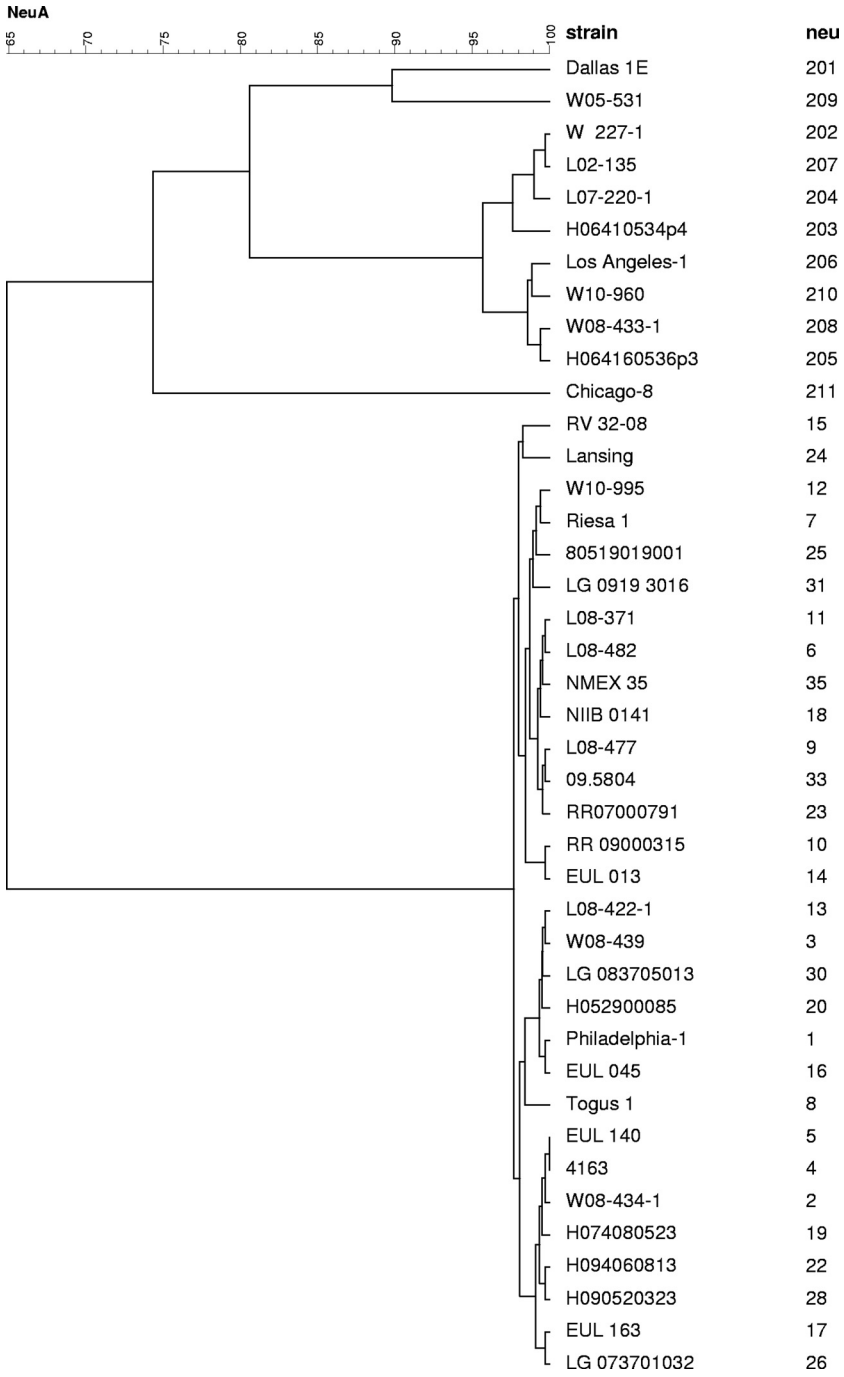

FIG. 3. UPGMA dendrogram showing the alignment of 354/351-bp fragment sequences of the *neuA* alleles for *L. pneumophila* strains by using the software package BioNumerics, version 5.0. For each allele one example is shown.

assignment of new *neuA* allele numbers. This offers the potential of improving both the level of discrimination and the ability to assign a sequence type (ST) for all strains of *L. pneumophila*. The ST, while based on an allelic string, has become a useful and practical shorthand descriptor in epidemiological investigations.

In conclusion, if a novel primer set such as that described here was used to amplify *neuA* genes from those non-SG1 strains where the amplification of the standard *neuA* failed, it could be used to obtain an allele designation for a *neuA* homolog and, thus, an ST where currently this is not possible. This proposal will be discussed among the *Legionella* typing community to see if such changes to the laboratory protocols and online software would be acceptable.

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