Direct Sequencing of *Legionella pneumophila* from Respiratory Samples for Sequence-Based Typing Analysis[†]

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We have developed a procedure to test the efficiency and reliability of sequencing of *Legionella pneumophila* genes directly from respiratory samples and have compared the results with those derived from cultured isolates. We tried to obtain the nucleotide sequences of six protein-coding loci included in the sequence-based typing scheme for *Legionella pneumophila* and three intergenic regions from 132 samples corresponding to 106 patients positive for urine antigen. A seminested PCR approach was used to amplify and sequence these nine loci directly from respiratory samples. Nucleotide sequences were directly obtained for 23 *Legionella* isolates and also for 66 respiratory secretions from a total of 69 patients. The efficiency of sequencing from respiratory secretions was higher than that of sequencing after the isolation of the *Legionella* isolates. Moreover, the perfect match between the sequences obtained by both approaches when respiratory samples and cultured isolates from the same patient were available corroborates the suitability of the direct sequencing approach for the identification of *Legionella* species and molecular epidemiology studies with *Legionella* species.

Legionella pneumophila is the etiological agent of Legionnaires' disease (LD) and Pontiac fever and is also one of the causes of community-acquired pneumonia (CAP) (1, 12, 18) and severe pneumonia (22). Therefore, the rapid and sensitive diagnosis of infection with *Legionella* species is essential not only for treatment but also for the implementation of prevention procedures. The standard adopted by the European Working Group for *Legionella* Infections (EWGLI) for the typing and characterization of isolates related to outbreaks and sporadic cases of LD is based on the allelic profile obtained from the nucleotide sequences of seven protein-coding loci (7, 17). For clinical samples, the profiles are usually derived from sputum cultures. However, the efficiency of isolation of *Legionella* species from respiratory samples rarely reaches more than 20%, even when highly experienced laboratory personnel perform the isolation procedure. Molecular methods for the diagnosis of LD have focused on the extraction and specific PCR amplification of *Legionella* DNA from respiratory samples (10, 16). However, epidemiological investigations seldom depend only on the confirmation of infection by *Legionella* species, and more detailed characterization is usually necessary (11, 13). Due to the difficulties in culturing *Legionella* from sputum samples from patients with LD, it is desirable to test the suitability of obtaining nucleotide sequences directly from respiratory secretions from patients for whom a *Legionella* isolate is not available. Hence, our goal has been to obtain *Legionella* sequence information directly from sputum samples, thus avoiding dependence on the isolation of *Legionella*

from cultures, and to compare the accuracy of multilocus sequence typing directly from lower respiratory secretions (bronchoalveolar fluid aspirated secretions [BAS] and sputum) to the accuracy of typing with cultured isolates.

A large outbreak of *Legionella* affecting more than 200 people occurred in 1999 and 2000 in Alcoi, Comunidad Valenciana (CV), Spain (6). Public health authorities conducted an extensive epidemiological investigation, and strict regulations on the cleansing and maintenance of aerosol-producing devices were implemented. However, from 2001 to 2005 other minor outbreaks were detected in that area. The rate of legionellosis in the Alcoi area has been higher than that in the rest of Spain since 1999, with the only exception being 2001. The reasons for this high rate in this specific locality, despite public health authorities' efforts to control and prevent further infections with *Legionella* species, remain unknown.

MATERIALS AND METHODS

Respiratory samples and, when possible, the corresponding cultured isolates were obtained from 106 patients from several hospitals in CV diagnosed with LD by a positive result for urine antigen. Samples from 56 patients were epidemiologically related during a period with a high incidence of legionellosis around the locality of Alcoi from 1999 to 2005 (indicated with an asterisk in the table in the supplemental material). The remaining samples were not related epidemiologically and were derived from sporadic cases of CAP from different localities in CV from 2003 to 2005.

We tried to amplify and sequence up to nine loci from 132 samples derived from 106 *L. pneumophila*-infected patients. Most samples ($n = 83$) were obtained from patients for whom only a respiratory secretion was available, since the corresponding cultures were negative. Forty samples were from 20 patients for whom a respiratory sample and the cultured isolate were available. The remaining nine samples were from three patients for whom sputum, BAS, and culture were available (see the table in the supplemental material). When feasible, we compared the sequences obtained from different samples from the same patient, and after assessing their identity, we kept only one for further analyses.

DNA was extracted from the respiratory isolates by using an UltraClean BloodSpin kit (Mobio Laboratories, Inc). DNA was extracted from the cultured *Legionella* isolates as described previously (4). Briefly, bacterial colonies from pure cultures were resuspended in 200 μ l of 20% Chelex 100 resin (Bio-Rad

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TABLE 1. Internal primer sequences used for seminested amplification and sequencing of *Legionella pneumophila* samples

Locus	Primer ^a	Primer sequence (5' → 3')	Annealing temp (°C)
<i>asd</i>	asd-Fi	GCTCTAAAGCCAATCTATGA	60
<i>fliC</i>	fliC-Ri	TTTGGCATAGGCAGACGTAG	60
L2 ^b	L2-Fi	TCATTGACTGCGTTGATTAG	56
L6 ^b	L6-Fi	ATTCTATCTGTGGTTTGA	62
		CTGC	
L14 ^b	L14-Fi	TGTAGAACCAAGAAGAG	56
		ATTT	
<i>mip</i>	mip-Fi	ATCATTAGCTACAGACAAGG	56
<i>mompS</i>	mompS-R	CAGAAGCTGCGAAATCAG	54
<i>pilE</i>	pilE-Ri	GCGCACTCGGTATCTTTGG	60
<i>proA</i>	proA-Ri	TCGCCAATGCAATTAGTTAT	54

^a The suffixes in the primer names indicate their direction (F, forward; R, reverse) and, if necessary, that they are internal (i) in the seminested reaction.

^b The locations of intergenic loci L2, L6, and L14 are provided by Coscollá and González-Candelas (4).

Laboratories, Richmond, CA). The DNA was then extracted by three freeze-thaw cycles (−75°C for 10 min and 94°C for 10 min), and the cellular debris was removed by pelleting by centrifugation at 10,000 × g for 1 min. The quantity of genomic DNA was measured by spectrophotometry at 260 nm in triplicate, and the purity of the DNA was checked by use of the A_{260}/A_{280} ratio. The purified DNA was stored at −20°C until it was used.

For the cultured isolates, the six loci in the EWGLI typing scheme were amplified as described by Gaia et al. (7); and the three intergenic regions, L2, L6, and L14, were amplified as described by Coscollá and González-Candelas (4). PCRs were carried out with a reaction volume of 50 µl containing 20 ng of genomic DNA, 1 U of *Taq* DNA polymerase, 200 µM of each deoxynucleoside triphosphate, 2.5 mM of Mg-free buffer, 2.5 mM of MgCl₂, and 0.2 µM of each pair of primers. We adopted the gene notation used for the first *L. pneumophila* genome published (2), and, consequently, the *fliC* locus corresponds to the *flaA* locus used in the EWGLI typing scheme (8).

DNAs from respiratory samples were amplified by use of a seminested approach. For the first PCR, we used the same primers and conditions detailed above. In the second PCR we used the internal primers described in Table 1 and 2 µl of the products from the first PCR as the template. The amplification conditions and profiles were also the same as those used for the primary PCR, except that the annealing temperatures were as detailed in Table 1. The PCR products were purified with a High Pure PCR product purification kit (Roche Diagnostics).

Sequencing of the PCR products from isolates was performed as described by Coscollá and González-Candelas (4). The purified DNA was directly sequenced by the dideoxy method with a BigDye Terminator (version 3.0) ready reaction cycle sequencing kit and analyzed in an ABI Prism 3700 sequencer (Applied Biosystems, Foster City, CA). Sequencing of the PCR products from respiratory samples differed only in the use of the same internal primers used in the second PCR of the seminested approach (Table 1). Sequence chromatogram files were analyzed by using the Staden package (19).

The allelic profiles for the first six genes proposed for the sequence-based typing of *L. pneumophila* (7) were obtained from EWGLI (<http://www.ewgli.org>) and were aligned and compared with the sequences derived in this study. Multiple-sequence alignments were obtained by using the CLUSTALX program (21) and were further refined by visual inspection. Identical sequences in the multiple alignments were identified by use of the DAMBE program (23).

RESULTS

The efficiency of sequencing of *Legionella* species after the culture of the respiratory secretions was higher than the efficiency of direct sequencing of *Legionella* genes from respiratory samples, but the latter approach allowed the analysis of many more samples that could not be cultured. Although all cultured samples yielded clear sequences for the nine loci analyzed, only 22% of the samples from patients were success-

TABLE 2. Number of loci sequenced by sample origin

Sample type	No. of samples for which the following loci were sequenced:				
	0	1–3	4–5	6–8	9
BAS	3	0	0	3	2
Culture	0	0	0	0	23
Sputum	40	24	3	16	18

fully cultured (23 cultures of samples from 106 patients). At least one locus was sequenced from 66 respiratory samples (Table 2; see also the table in the supplemental material), which corresponds to 60% of the 109 samples (for three patients there were 2 respiratory samples). In total, respiratory samples from 83 infected patients could not be cultured, but we obtained at least one locus sequence from 46 of them (55.4%). On the other hand, of the 23 samples for which the culturing procedure was positive, all but 4 (17.4%) also yielded positive sequences by the direct approach. The remaining uncultivable samples from 37 patients did not yield any sequence. Hence, confirmation of *Legionella* infection by sequence analysis was possible for more than half of the uncultured samples.

In all the cases ($n = 19$) in which sequences were obtained from the respiratory sample(s) and from a cultured isolate, the corresponding nucleotide sequences for both samples were identical for all the loci sequenced (Table 3). Of the 20 possible comparisons (one patient had both a culture sample and two respiratory samples that yielded at least one sequence), amplification and sequencing from the respiratory sample were possible in nine cases, for seven cases the procedure yielded positive sequences for six to eight loci, and the four remaining cases yielded fewer than six loci. Hence, this result corroborates the suitability of the direct approach for the study of the genetic profiles used in epidemiological analyses.

No significant differences in the rates of successful sequencing from respiratory samples were observed between samples from patients epidemiologically related to the outbreaks in Alcoi and the remaining samples from sporadic cases of CAP (36/57 for patients in Alcoi and 30/52 for the rest of the patients). Similar proportions were observed when only uncultured respiratory samples were considered (23/43 and 20/40 for samples from the outbreaks in Alcoi and samples from patients with CAP, respectively).

Among the coding loci, *fliC* was the least successfully amplified and sequenced and *asd* and *mip* were the most successfully amplified and sequenced. Noncoding loci were less effectively amplified and sequenced than the six genes included in the EWGLI scheme, as most of the negative results for respiratory samples were for these loci, especially locus L14 (see the table in the supplemental material). The high degree of nucleotide diversity of this locus (4) could facilitate the existence of polymorphisms in the primer region that would result in mispriming and negative PCR results. On the other hand, this high degree of genetic variability offers more discriminatory power. For example, the nucleotide sequences from the sample from patient 9056 was identical to the majority of the nucleotide sequences from epidemiologically related samples, according to the EWGLI typing scheme, but differed from them

TABLE 3. Comparison of allelic profiles obtained from cultured isolates and the corresponding respiratory samples^a

Patient identifier ^b	Sample type	No. of loci	Allele no.								
			<i>flhC</i>	<i>pilE</i>	<i>asd</i>	<i>mip</i>	<i>mompS</i>	<i>proA</i>	L2	L6	L14
675	Sputum	9	3	10	1	12	14	9	5	4	2
675	Isolate	9	3	10	1	12	14	9	5	4	2
1864	Sputum	9	1	4	3	1	1	1	3	3	4
1864	Isolate	9	1	4	3	1	1	1	3	3	4
1889	BAS	7	2		9	10	2	1	2		5
1889	Isolate	9	2	3	9	10	2	1	2	2	5
2049	Sputum	5		10	1	12		9	5		
2049	Isolate	9	3	10	1	12	14	9	5	4	32
5532	Sputum	9	6	10	15	28	4	9	5	1	29
5532	Isolate	9	6	10	15	28	4	9	5	1	29
5876	Sputum	5		4		5	18	5		15	
5876	Isolate	9	2	4	5	5	18	5	16	15	5
5876	BAS	8	2	4	5	5	18	5	16		5
8529	Sputum	2	1			1					
8529	Isolate	9	1	4	3	1	1	1	3	3	4
8819	Sputum	8	21	14	28	15	15	29		13	27
8819	Isolate	9	21	14	28	15	15	29	7	13	27
31687	BAS	9	4	7	11	3	11	12	3	1	1
31687	Isolate	9	4	7	11	3	11	12	3	1	1
699*	Sputum	7	6	10	15	13	9	14		1	
699*	Isolate	9	6	10	15	13	9	14	5	1	14
1130*	Sputum	3	6		15					1	
1130*	Isolate	9	6	10	15	3	9	14	5	1	30
1380*	Isolate	9	6	10	15	13	9	14	5	1	14
1380*	Sputum	9	6	10	15	13	9	14	5	1	14
1795*	Sputum	9	6	10	15	3	9	14	5	1	30
1795*	Isolate	9	6	10	15	3	9	14	5	1	30
2039*	Sputum	8	6	10	15	3	9	14	5	1	
2039*	Isolate	9	6	10	15	3	9	14	5	1	30
7758*	Sputum	9	6	10	15	13	9	14	5	1	14
7758*	Isolate	9	6	10	15	13	9	14	5	1	14
7895*	Sputum	9	6	10	15	13	9	14	5	1	14
7895*	Isolate	9	6	10	15	13	9	14	5	1	14
8772*	Isolate	9	6	10	15	13	9	14	5	1	14
8772*	Sputum	9	6	10	15	13	9	14	5	1	14
9083*	Sputum	6	6		15	13	9	14		1	
9083*	Isolate	9	6	10	15	13	9	14	5	1	14
9205*	Sputum	8	6	10	15	13	9	14	5	1	
9205*	Isolate	9	6	10	15	13	9	14	5	1	14

^a In all cases perfect matches between the sequences obtained from the same patient were observed.^b Asterisks indicate samples from epidemiologically related patients during a period with a high incidence of legionellosis around the locality of Alcoi.

at 25 nucleotide positions in locus L14, which showed allele 26 instead of allele 14.

Of the three patients from whom two respiratory secretions were available, only one, patient 5876, yielded positive results for both respiratory samples. The other two patients showed

discordant results for the two respiratory samples, while patient 1889 had a positive sequencing result for BAS and a negative sequencing result for sputum; the contrary result was obtained for patient 7758.

By combining the direct sequencing and culture-based se-

quencing approaches, we were able to derive complete EWGLI allelic profiles for 42 patients which corresponded to 18 different sequence types. Additionally, incomplete profiles, with up to three missing alleles, were derived for five additional patients (see the table in the supplemental material). Three new alleles were found for the *proA* locus, one was found for the *pilE* locus, and one was found for the *mip* locus (indicated A to C, respectively, in the table in the supplemental material). These alleles were derived from sputum samples that could not be cultured satisfactorily, and their sequences were not found in the EWGLI database, to which they are being submitted.

According to the EWGLI scheme, the most common sequence type (6, 10, 15, 13, 9, 14) was found in 17 patients related to the main outbreak that occurred in Alcoi in 1999 and 2000 (6). The isolates from only six patients from this epidemiologically related group did not show the same profile but did show a very closely related one. The isolates from two patients (patients 8945* and 1128*) differed only in the sequence of locus *proA* (they presented allele B or C instead of allele 14, with 9 and 2 nucleotide differences, respectively). The isolates from three patients (patients 1130*, 1795*, and 2039*) differed at 4 nucleotides in locus *mip*, showing allele 3 instead of allele 13. The isolate from one patient (patient 7810) showed a divergent allele in locus *fliC* (allele 2 replaced allele 6). According to the epidemiological information, none of these patients differed by date or locality of infection from the remaining patients with the most common profile.

The remaining 13 allelic profiles, which diverged from the rest at more than one locus, were found for the isolates from the patients with sporadic cases of CAP who were not related epidemiologically to the patients whose results are discussed above or to each other. The sequence type that is the most common and widespread among clinical and environmental strains worldwide (5) (sequence type 5 consists of alleles 1, 4, 3, 1, 1, and 1 for *fliC*, *pilE*, *asd*, *mip*, *mompS*, and *proA*, respectively) was also identified in four patients and for three of them for the directly sequenced samples.

DISCUSSION

PCR directly with respiratory samples has repeatedly been shown to have sensitivity equal to or greater than that of PCR with isolates from culture (3, 14, 20). This study supports this observation and extends this high degree of sensitivity to the collection of sequences useful for epidemiological investigations. It also shows the reliability of the sequences derived directly from respiratory samples, since all of them turned out to be identical to those derived from the corresponding cultured isolates. In the study of most legionellosis outbreaks, the number of clinical samples included in the molecular characterization of strains is very low due to the low efficiency of retrieval of *Legionella* isolates. As an example, O'Loughin et al. (15) analyzed isolates from only 4 of the 18 patients included in an outbreak because they were the only patients from whom they were able to get *Legionella* isolates.

These results highlight the great specificity and reliability of sequencing analysis directly from respiratory samples and its adequacy at obtaining information for the study of the relatedness of *Legionella* variants of clinical origin even when cultured isolates are not available. The results presented

here show the possibility that both types of samples may be used in the same study and, therefore, that increased numbers of samples may be analyzed, thus improving the accuracy and usefulness of the results derived.

Ginevra et al. (9) have recently reported a similar seminested approach for the amplification and sequencing *Legionella*-infected respiratory samples. They studied sequences derived from seven loci from the EWGLI scheme in samples from 63 patients. In this study, we have analyzed almost twice the number of samples and nine loci, with sequences for at least five loci being obtained for 37.6% (41/109) of the samples. On the contrary, Ginevra et al. (9) reported a success rate of 79.3%. This could be due to the fact that they used only respiratory samples which had been positive for *Legionella* species by real-time PCR, which was not the case in this study. This difference could be explained by the higher rate of false-positive diagnostic results in our study or the higher quality of the respiratory samples in their study. Furthermore, their samples resulted in 35 positive cultures (55% of 63 samples), whereas in our study the success rate for culture was only 21.7% (23 of 106 samples), which again can be explained by the worse quality of our respiratory samples.

The negative results for the amplification and sequencing of respiratory samples could be due to different factors. Among the most likely ones, which deserve to be scrutinized further, are the administration of antibiotic treatment before the respiratory samples are obtained (because a diagnostic assay, the urine antigen test, was performed earlier); the presence of inhibitory compounds in the samples; and the delay in the time to the arrival of the samples to our laboratory (up to 2 months), which could lead to *Legionella* DNA of poor quality. We have analyzed the second of these factors in more detail, and we found that the presence of PCR inhibitors in these samples is not a likely cause of the negative results obtained. Since the procedure for the extraction of DNA from respiratory samples is not specific for bacterial DNA, we were able to amplify and successfully sequence specific human genes from the same samples. Hence, we verified that PCR inhibitors were not responsible for negative *Legionella* amplifications from these samples. Nevertheless, the isolates from four respiratory samples could be cultivated but not sequenced directly. This could be due to the poor quality of the respiratory samples, as mentioned above, as well as the late arrival of the samples to our laboratory.

Similar to the results reported by Ginevra et al. (9), *fliC* was the least successfully amplified and sequenced coding locus in our study. Nevertheless, loci from the EWGLI scheme were more efficiently amplified and sequenced from respiratory samples than intergenic regions. However, these noncoding regions may offer more discriminatory power, as previously reported by Coscollá and González-Candelas (4). This can be explained by the high degree of genetic variability that characterizes these noncoding regions.

The analysis of *L. pneumophila* sequences obtained from 1999 to 2005 from epidemiologically related patients around Alcoi, located in an area of Spain with a very high incidence of legionellosis, has revealed the presence of a particular allelic profile which has not been found either in other samples included in this study or in any other study published so far. This indicates that the particular strain causing these infections is

not widely distributed, and it might have been quite overrepresented in the area during that period. We have not yet been able to find this strain in other clinical or environmental isolates (unpublished results).

As an example of the usefulness of incorporating highly variable loci in the detailed study of legionellosis outbreaks, we have been able to identify among the samples from the Alcoi area a small cluster of isolates (from patients 1130*, 1795*, and 2039*) with a slightly divergent profile which indicates a common source of infection for these patients that is different from the source for the remaining patients in this locality. The epidemiological inquiry did not reveal any reason for this grouping, but identical profiles determined by use of the EWGLI scheme were found for these three patient isolates and an environmental isolate from a cooling tower (unpublished results). Sequences from noncoding locus L14 were able to discriminate between the clinical and environmental samples, because the latter showed allele 15 instead of allele 30, which was common to the three clinical samples. In this case, a noncoding marker allowed us to differentiate at the nucleotide level isolates which otherwise would have been identified as identical by use of only the sequence-based typing approach.

In summary, use of the amplification and direct sequencing approach has allowed us to analyze more samples than we could have analyzed by studying only *Legionella* isolates from culture, corroborating the idea that isolates from patients from an area with a high rate of legionellosis are genetically related and different from other circulating strains from the same region. Furthermore, by incorporating highly variable, noncoding loci to the analysis of samples from these outbreaks, it has been possible to differentiate at the molecular level samples that were otherwise considered to be of the same origin as other epidemiologically related samples. These findings confirm the suitability of the procedure introduced here for studying outbreaks, especially when, as in this case, only a small number of isolates are available.

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