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Serotyping, ribotyping, PCR-mediated ribosomal 16S-23S spacer analysis and arbitrarily primed PCR for epidemiological studies on *Legionella pneumophila*

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SUMMARY

Fifty clinical and environmental isolates of *Legionella pneumophila* were typed serologically and by DNA fingerprinting using arbitrarily primed polymerase chain reaction (AP-PCR). Furthermore, variability in and around ribosomal operons was assessed by conventional ribotyping and PCR-mediated amplification of the spacer region separating the 16S and 23S genes. It appears that serotyping suffers from low resolution capabilities, and ribotyping and spacer PCR display intermediate resolving capabilities, whereas AP-PCR is more discriminating. Results from AP-PCR and both forms of ribotyping analysis correlate with epidemiological and environmental data. It is suggested that AP-PCR typing may be the method of choice for rapidly determining clonality among *L. pneumophila* isolates.

Key-words: Ribotyping, PCR, AP-PCR, *Legionella pneumophila;* Epidemiology.

INTRODUCTION

The initial species within the genus *Legionella* was described after the first outbreak of so-called "Legionnaires' disease" in 1977 (Fraser *et al.*, 1977). Since that time not only has the number of species grown at a steady rate, but also a number of subtypes have been identified within the species *L. pneumophila* (Joly *et al.*, 1986). Furthermore, procedures suited for the molecular characterization of *L. pneumophila* have been described. These vary from alloenzyme electrophoresis (Tompkins *et al.*, 1987) to ribotyping (Grimont *et al.*, 1989), fatty acid profiling (Jantzen *et al.*, 1993), pulsed-field gel electrophoresis of DNA macrorestriction fragments (Ott *et al.*, 1991) and DNA fingerprinting protocols employing the polymerase chain reaction (PCR) (Van Belkum *et al.*, 1993). These procedures have been applied to investigations of nosocomial epidemics of mostly waterborne legionellosis (Schoonmaker *et al.*, 1992; Struelens *et al.*,

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1992). This has led to discussions concerning optimal discrimination between epidemiologically linked and sporadic isolates of *L. pneumophila*. To date, no consensus on the preferred typing scheme for nosocomially occurring microbial pathogens in general, and *Legionella* strains in particular, has been realized. In order to evaluate a certain number of these procedures, environmentally and nosocomially acquired isolates of *L. pneumophila* were subjected to serological identification, conventional ribotyping, amplification of the 16S-23S ribosomal spacer region and genotyping by arbitrarily primed (AP) PCR (Gomez-Lus *et al.*, 1993).

MATERIALS AND METHODS

Bacterial strains

L. pneumophila isolates were obtained from various sources in the Netherlands. These sources included water supplies and patients suffering from Legionnaires' disease (see table I). Strains were derived mainly from hospitals. Prior to analysis, strains were grown on two buffered charcoal yeast extract (BCYE) agar plates at 37°C for 36 h until confluency was obtained. One was used for serotyping, the other, for DNA isolation. The same batch of medium was used for all strains, strains were grown in a single incubator under constant atmospheric conditions, and procedures were handled by the same individual.

Serotyping

Serological analysis of the *L. pneumophila* strains was performed with the aid of commercially available *Legionella* immune sera (Seiken, Denka, The Netherlands). A dense bacterial suspension (OD_{600} over 1.5) was heat-treated (60 min, 100°C) and used as the antigen suspension. This suspension was mixed 1:1 with the antiserum solution, and agglutination was observed visually. A detailed description of this serotyping procedure can be retrieved from available literature (Tateyama, 1992).

DNA isolation

For DNA isolation, bacteria were suspended in 2 ml phosphate-buffered saline pH 7.2 (PBS). This stock suspension was stored at −20°C until processing. Aliquots of 200 µl were diluted with 1 ml of a 4 M guanidinium isothiocyanate-containing buffer in order to lyse the cells (Boom *et al.*, 1990). DNA was subsequently purified by affinity chromatography to Celite, washed with 70% ethanol, dried and redissolved in 400 µl of 10 mM Tris-HCl pH 8.0, 1 mM EDTA. On average, 200 µl of the bacterial suspension yielded 4-5 µg of high molecular weight DNA.

Ribotyping

Conventional ribotyping was performed on a subset of the strains (n=38) by methods described previously (Grimont *et al.*, 1990). DNA was digested by a single restriction enzyme (*Eco*RI, Boehringer-Manheim, Germany) according to the manufacturer's instructions. Restriction fragments were subsequently length-separated by electrophoresis through 0.8% agarose gels (2 V/cm for 24 h). Southern-blotted onto "Hybond N" membranes (Amersham Int, UK) and hybridized to a full-length *E. coli* 16S rRNA probe. The probe was equipped with ^{32}P-alpha-dATP using a random-primed labelling protocol (Feinberg and Vogelstein, 1983). After hybridization and washing at 50°C, autoradiography was performed for periods varying between 1 and 24 h (Sambrook *et al.*, 1989).

PCR ribotyping

Amplification of the spacer region between the 16S and 23S rRNA genes was carried out (Kostman *et al.*, 1992, 1994). Employing 16S- and 23S-specific primers sp1 and sp2 (5'-TTGTACACACCGC CCGTCA-3' and 5'-GGTACCTTAGATGTTTCAG TTC-3', respectively) while applying incubation and cycling conditions as described by these authors, amplicons were generated which were subsequently analysed by gel electrophoresis in 2% agarose gels. The length of the amplicons was estimated by comparison with lambda *Hin*dIII molecular weight markers.

AP-PCR = arbitrarily primed PCR.
BCYE = buffered charcoal yeast extract.
PBS = phosphate-buffered saline.
PCR = polymerase chain reaction.
sp = specific primer.
Taq = *Thermus aquaticus*.
TBE = Tris borate EDTA.

AP-PCR

DNA amplification by AP-PCR was performed essentially as described before (Van Belkum *et al.*, 1995). In short, PCR mixtures contained 10 mM Tris-HCl pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01 % gelatin, 0.1 % Triton-X100, 0.2 mM of all deoxyribonucleotide triphosphates, 0.5 units *Taq* DNA polymerase (SuperTaq, Sphaero-Q, The Netherlands), 50 pmoles of oligonucleotide primer(s) and between 50 and 100 ng of template DNA. Primers used were the enterobacterial repetitive intergenic consensus sequences 1 and 2 (ERIC IR-1: 5'-ATGTAAGCT CCTGGGGATTCAC-3'; ERIC 2-I: 5'-AAG TAAGTGACTGGGGTGAGCG-3' (Versalovic *et al.*, 1991)) and the arbitrary primer BG2 (5'-TACATTCGAGGACCCCTAAGTG-3' (Van Belkum *et al.*, 1993)). PCRs were performed using "Biomed model 60" thermocyclers (Biomed, Theres, Germany). Cycling consisted of 40 times 1 min at 94°C, 1 min at 25°C and 2 min at 74°C. PCR products were analysed by electrophoresis in 2% agarose gels, run in 0.5×TBE buffer at a constant voltage of 100 V for 4 hours. Gels were stained with ethidium bromide, and pictures were taken upon UV transillumination with the aid of "Polaroid Polapan 52" films. PCR banding patterns were interpreted by at least two independent investigators who were not aware of the strains' origins.

RESULTS AND DISCUSSION

From table 1, it appears that a large fraction (30%) of the collection of strains remained serologically untypable. One strain could not be analysed for reasons of autoagglutination. Four different serotypes (1, 3, 4 and 6) were identified among the typable strains.

Conventional ribotyping displayed a higher degree of resolution. Among the subset of strains that was analysed, and by application of a single restriction enzyme and a single 16S-rRNA-specific DNA probe, already five clearly different ribotypes were identified. Figure 1 gives a schematic presentation of the diverse banding patterns that were encountered. Clearly, relatively long restriction fragments (the smallest one still being more than 5,000 base pairs in length) hybridized to the probe; data thus collected are summarized in table 1.

Amplification of the ribosomal 16S-23S spacer region led to the detection of a similar but somewhat smaller number of variants (see fig. 1 and table 1). The large majority of strains gave rise to the amplification of two DNA fragments, both approximately 600 nucleotides in length. Besides, an additional number of four banding patterns was encountered, one of which (A' in fig. 1) was very similar to the basic type.

AP-PCR analysis was performed in two different institutions by a single researcher. The results obtained in the two laboratories showed nearly complete concordance with respect to strain grouping; only subtle exceptions were encountered. However, the individual banding patterns that were generated using sequence-identical primers were different when the results from the two laboratories were compared directly. Explanations for this phenomenon are either the differences in the quality of the primers used (Van Belkum, 1994) or the way in which the DNA was prepared. Finally, small differences in electrophoretic conditions have an extensive effect on the reproducibility (unpublished observations). The first series of experiments (PCR type I in table I) revealed the presence of 15 distinct genotypes in the collection of 50 strains that were analysed with both PCR tests (resolution of 30%). When the tests were repeated in another hospital with a more limited set of isolates (n=39; for results see fig. 2), 9 different genotypes were found (resolution 23%), although in this latter case the number of assays was smaller.

It thus appears that the resolution of serotyping and 16S-23S-spacer PCR are inadequate for epidemiological typing of *L. pneumophila* strains. The AP-PCR and ribotyping results reveal a higher degree of strain resolution, AP-PCR providing the largest number of types under the experimental conditions as used in this study. However, increasing the number of enzymes used for ribotyping would probably result in better discrimination. The present study may render conclusions that are weighted in favour of the AP-PCR approach. On the other hand, AP-PCR is technically less

Table I. Survey of experimental, clinical and environmental data of the *L. pneumophila* isolates.

Strain number	Strain code	Institute	Source	PCR type I	PCR type II	Overall PCR type	Sero-type	Spacer PCR	Ribo-type
1	0001/92	hospital A	siphon	Aa	AAA	VIII	nt	A	A
2	0002/92	hospital A	siphon	Bb	ND	XII	nt	B	ND
3	0003/92	hospital A	siphon	Cc	ND	XIII	nt	C	ND
4	0004/92	hospital A	water	Aa	AAA	VIII	4	A	ND
5	0005/92	hospital A	water	Aa	AAA	VIII	4	A	A
6	0006/92	hospital A	water	Aa	AAA	VIII	4	A	A
7	0007/92	hospital A	siphon	Aa	AAA	VIII	nt	A	A
8	0008/92	hospital A	siphon	Aa	AAA	VIII	nt	A	A
9	0009/92	hospital A	water	Aa	AAA	VIII	4	A'	ND
10	0010/92	hospital A	water	Aa	AAA	VIII	4	A	ND
11	0011/92	hospital G	water	Aa	AAA	VIII	auto	A	ND
13	0049/92	hospital A	siphon	Ea	CA'A	IX	nt	A	ND
15	0051/92	hospital A	siphon	Ea	CA'A	IX	nt	A	ND
16	0052/92	hospital A	siphon	Ea	CA'A	IX	4	A	A
17	0053/92	hospital A	siphon	Ea	CA'A	IX	4	A	A
18	0054/92	hospital A	siphon	Ea	CA'A	IX	4	A	A
19	0055/92	hospital A	siphon	Ea	CA'A	IX	4	A	A
20	0056/92	hospital A	siphon	Ea	CA'A	IX	4	A	A
21	0057/92	hospital A	siphon	Fe	ND	IV	3	A	A
22	0058/92	hospital A	boiler	Aa	AA'A	VIIIa	4	A	A
23	0059/92	hospital A	siphon	Aa	AA'A	VIIIa	4	A	ND
24	0014/92	hospital B	water	Dd	BBB	I	1	A	A
25	0060/92	hospital A	siphon	Gf	ND	XV	nt	D	B
26	0062/92	hospital A	siphon	Aa	AA'A	VIIIa	4	A	A
27	0063/92	hospital A	siphon	Aa	AA'A	VIIIa	4	A	ND
28	0064/92	hospital A	siphon	Aa	AA'A	VIIIa	nt	A	A
29	0065/92	hospital A	siphon	A(ND)	ND	VIII	nt	A	A
30	2949/91	hospital F	pat. 1, BAL	Hg	DBC	II	1	A	C
31	2968/91	hospital F	pat. 2, lung	Hg	DBC	II	1	A	C
32	2969/91	hospital F	pat. 2, blood	Hg	DBC	II	1	A	C
33	2988/91	hospital F	pat. 2, BAL	Hg	DBC	II	1	A	C
34	0016/92	hospital F	ventilator	Hg	DBD	IIa	1	A	C
35	0017/92	hospital F	ventilator	Hg	DBC	II	1	A	C
36	0018/92	hospital F	water	Hg	DBC	II	1	A	C
37	0040/92	hospital F	cooling system	Hg	DBC	II	1	A	C
38	0041/92	hospital F	cooling system	Hg	DBC	II	1	A	C
39	0002/93	hospital F	cooling system	Hg	DBC	II	1	A	C
40	0026/92	institute H	water	Ih	ND	V	3	A	B
41	0027/92	institute H	water	Ii	ND	VI	3	A	B
42	0028/92	institute A	water	Ij	ND	X	6	A	B
43	0029/92	institute H	water	J-	ND	VII	3	A	D
44	0032/92	hospital D	laundry room	Kk	ECE	III	1	A	ND
45	0033/92	hospital D	laundry room	Ll	ND	XI	6	ND	ND
46	0001/93	hospital D	laundry room	Ll	ND	XI	6	A	B
47	0024/92	institute C	water instal.	Kk	EBE	IIIa	1	A	C
48	0048/92	institute C	water instal.	Kk	EBE	IIIa	1	A	C
49	0066/92	hospital E	heater	Mm	FDF	XIV	nt	A	E
50	0067/92	hospital E	heater	Mm	FDF	XIV	nt	A	E
51	0068/92	hospital E	heater	Mm	FDF	XIV	nt	A	E
52	0069/92	hospital E	siphon	Mm	FDF	XIV	nt	A	E

Strain number corresponds to lane numbering in figure 2; the strain code represents the identification number. The serotype was determined with the Seiken series of sera. PCR was performed in two institutions. PCR tests involving primer combinations (ERIC1/ERIC2) and (ERIC2/BG2) were performed at the Diagnostic Center SSDZ, Department of Molecular Biology, Delft, The Netherlands. Assays comprising the three following combinations (ERIC1/ERIC2), (ERIC2/BG2) and (BG2) were performed at the Academic Hospital Dijkzigt, Department of Bacteriology, Rotterdam, The Netherlands. The results are presented as PCR types I and II, respectively; the number of letters corresponds to the number of PCR assays, which was 2 or 3 for type I and II, respectively. Lettering (A through M) reflects the variability in the DNA banding patterns. Note that the BG2 code for strain 34 deviates (D instead of C, as for the other strains from the same hospital); this may reflect the acquisition of novel genetic elements or the occurrence of minor genomic rearrangements. nt=non-typable; ND=not done; pat.=patient; BAL=bronchoalveolar lavage; instal.=installation; auto=autoagglutinating strain.

In the case of hospital F, a genuine epidemic was documented. Strains from patients and environment are identical, and multiple isolates from a single patient had the same PCR type. In hospital A, multiple periods involving colonization with genetically different *Legionella* strains have occurred.

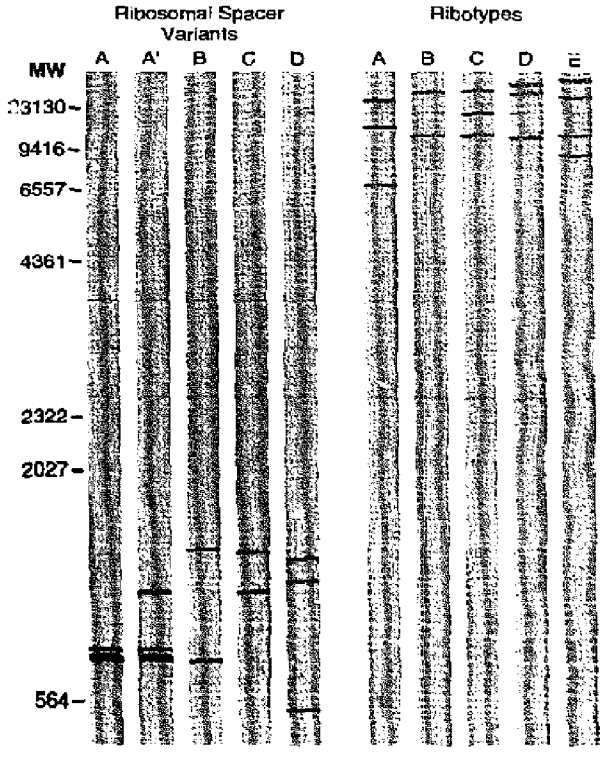


Fig. 1. Schematic representation of the data obtained by amplification of the 16S-23S ribosomal spacer region by PCR (left panel) and by conventional ribotyping (right panel).

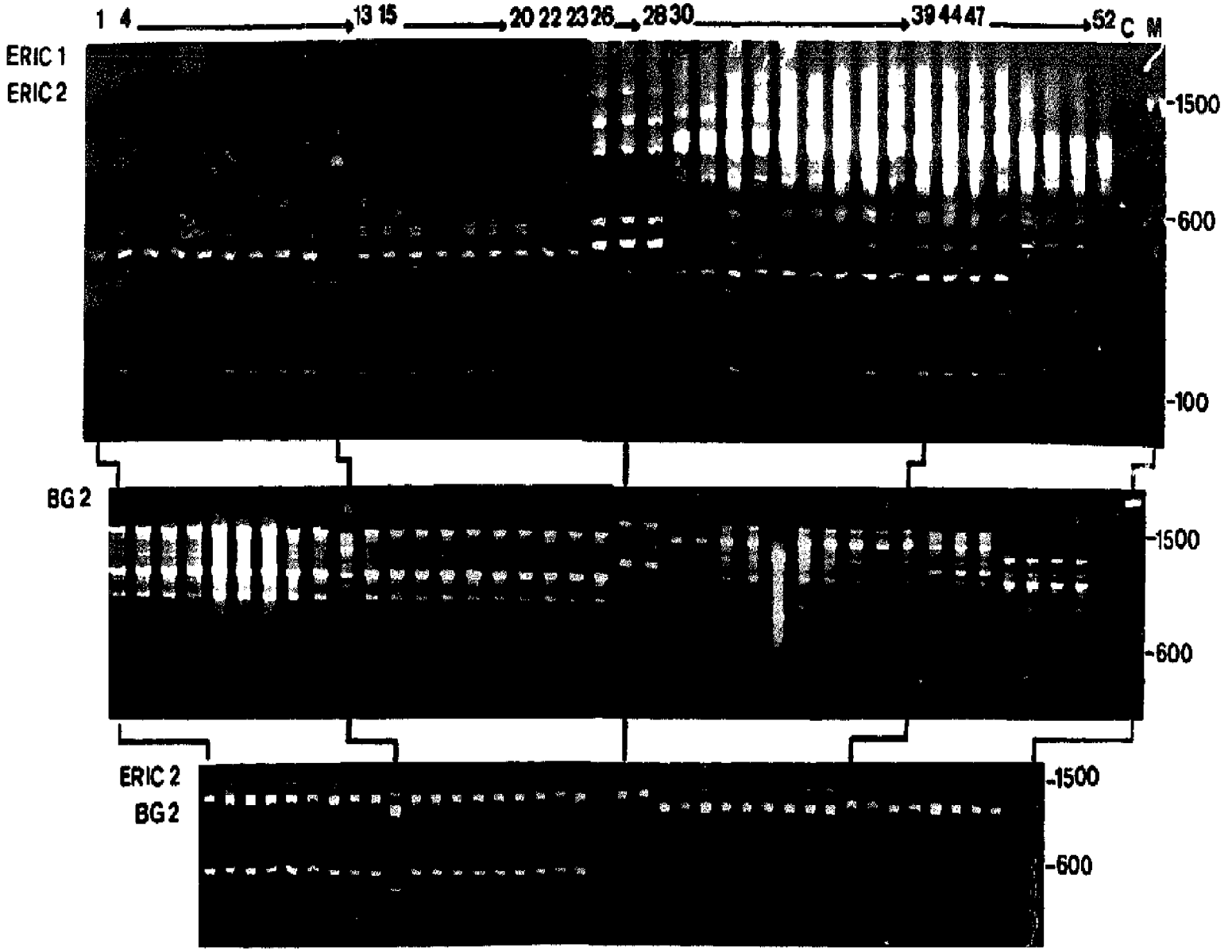
Different types detected by the two techniques are identified by capital letters, as also presented in table I. An apostrophe indicates a subtype. The molecular length markers given on the left are restriction fragments generated by digestion of bacteriophage lambda DNA with *Hin*dIII.

demanding. The genetic profiles are in good agreement with environmental and patient data. It is shown that strains originating from within a single institution are generally genetically homogeneous. Note that, for instance, all strains from hospital E are completely identical, confirmed by all procedures applied. Also, strains isolated from either the environment or from patients within hospital F appear to be identical, although strain 34 displays a slightly deviating overall AP-PCR type (see table I). This demonstrates the potential value of the genotyping approach for unraveling epidemiological features.

There is a clear need for standardization of (molecular) microbial typing methods, especially for those procedures that are used for the analysis of bacterial strains isolated during nosocomial outbreaks. In these circumstances, it is of prime importance to be able to expeditiously determine the genetic relationships among the clinical and/or environmental isolates. The optimal typing system should be fast, highly discriminatory, reproducible, applicable to all strains and versatile with respect to the number of strains to be studied. None of the presently available typing schemes fulfills all of these requirements.

The set of Seiken antisera provides the only instrument for *Legionella* typing that is readily available in the Netherlands. However, comparative analysis of typing results underscores that the discriminatory power of PCR is superior to that of serotyping. All strains identified as Seiken serovar 1, for instance, can be divided into five distinct genotypes: DdBBB, HgDBC, HgDBD, KkEBE and KkEDE. Interestingly, these strains all belong to the spacer type A and ribotype A or C. Moreover, the non-typable strains can all be classified as one out of seven genotypes (designated overall PCR types VIII, VIIIa, IX, XII, XIII, XIV and XV; see table I), whereas these same strains also display variability once assayed with ribotyping or spacer PCR. It is comforting to observe that the strains originating from a single institution generally appear to be genetically related. For instance, all strains encoded Hg are from the same institute. Strains 1-10, derived from hospital A, primarily represent AP-PCR type VIII, although by serotyping, two types (4 and nt) are encountered. The AP-PCR data, however, are supported by the ribotyping, demonstrating concordance between genetic approaches and indicating a lack of reliability for the serotyping assay.

The two assays which aimed at the elucidation of variation within or surrounding ribosomal operons display differing efficacy with respect to typing of *L. pneumophila*. Whereas


1 4
13 15
20 22 23 26
28 30
39 44 47
52
C
M
ERIC 1
ERIC 2
1500
600
100
BG 2
1500
600
ERIC 2
BG 2
1500
600

the 16S-23S spacer PCR has previously been presented as a potentially universally applicable microbial typing procedure (Kostman *et al.*, 1994), in the case of *L. pneumophila*, this applicability seems limited. In comparison with conventional ribotyping, for instance, the resolution seems diminished. Ribotyping gave rise to adequate results, both with respect to resolution and epidemiological concordance. It has to be emphasized that the absolute resolution of this procedure can be greatly improved by increasing the number of restriction enzymes and ribosomal probes used (Grimont *et al.*, 1989).

It appears that the AP-PCR fingerprinting procedure is an efficient means for the initial screening of (large) collections of microorganisms. The procedure is rapid and produces consistent data on genetic relatedness. The usefulness of AP-PCR-mediated typing for *L. pneumophila* was recently confirmed by a study demonstrating the versatility of amplification of regions bordered by repetitive motifs (Georghiou *et al.*, 1994). Assays aiming at the amplification of genomic domains bordered by conserved repetitive extragenic palindrome elements (see Lupski and Weinstock, 1992, for a review) also displayed excellent resolving capacity and epidemiological concordance. A major point of concern, however, is the apparent lack of reproducibility of banding patterns between laboratories. In the present study, it is suggested that the nature of the banding pattern can be influenced by the quality of the DNA preparation or the purity of the oligonucleotide primer batch. It has recently been shown that other factors can also influence the quality of the AP-PCR fingerprints (Gomez-Lus *et al.*, 1993; Meunier and Grimont, 1993; Versalovic *et al.*, 1991). It is our opinion that following PCR analysis, the other genotypic procedures may be effective in establishing interlaboratory consistency. The role of the phenotypically oriented procedures for typing *L. pneumophila* is not immediately clear. Moreover, antisera to all serotypes are not readily available, and a relatively high percentage of strains remains non-typable.

In conclusion, our data indicate that PCR fingerprinting is a valuable typing procedure for *Legionella*. All strains appear to be typable, the resolution can be increased by enlarging the number of primers used, results appear to corroborate epidemiological findings, and the procedure is conveniently fast. However, prior to the initiation of large-scale multicentre comparisons, it may be worthwhile to study the reproducibility of PCR genotyping for *L. pneumophila* in a way similar to that recently presented for (methicillin-resistant) *Staphylococcus aureus* (Tateyama, 1992; Tenover *et al.*, 1994; Van Belkum *et al.*, 1995). In the latter study, a message similar to that presented in the current paper was formulated. Although AP-PCR clustered epidemiologically related strains in an adequate manner, interinstitutional reproducibility clearly needed improvement. Additional studies on the methodological aspects of AP-PCR are mandatory.

Fig. 2. DNA typing of *L. pneumophila* strains by PCR-mediated amplification of randomly selected genomic regions.

Numbering above the lanes identifies *L. pneumophila* strains as in table I. The upper panel displays the results of amplification using a combination of the enterobacterial repetitive intergenic consensus sequences ERIC1 and ERIC2. The panel in the middle shows results obtained with the arbitrary primer BG2, whereas in the lower panel, the results from amplification by primers BG2 and ERIC2 are displayed. On the right, molecular length markers are indicated in multiples of 100 bp. The lane marked C shows results obtained without the addition of extraneous DNA (negative control). Identical lanes are connected between panels. Note that strain number 12 is not included in table I, due to the fact that none of the other typing procedures were applied to this particular isolate.

Etude épidémiologique de *Legionella pneumophila* par sérotypage, AP-PCR, ribotypage et analyse de l'écart ribosomal 16S-23S médiée par la PCR

Cinquante souches isolées de l'environnement ou d'origine clinique de *Legionella pneumophila* ont été typées sur le plan sérologique et par empreinte de l'ADN à l'aide d'amorces arbitraires utilisées pour l'amplification en chaîne de l'ADN (AP-PCR). De plus, la variabilité à l'intérieur et autour des opérons ribosomaux a été évaluée par ribotypage conventionnel et par PCR de la région séparant les gènes 16S et 23S. Il apparaît que le sérotypage souffre de faible capacité de résolution et que le ribotypage et l'analyse d'écart par PCR révèlent des capacités de résolution moyennes, tandis que l'AP-PCR est la technique la plus discriminante. Les résultats de l'AP-PCR et ceux des deux formes de ribotypage sont corrélés avec les données de l'épidémiologie et de l'environnement. Cela suggère que le typage par AP-PCR pourrait être la méthode de choix pour la détermination rapide des clones de *L. pneumophila*.

Mots-clés: Ribotypage, PCR, AP-PCR, *Legionella pneumophila*; Epidémiologie.

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