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MOLECULAR TYPING OF *Legionella pneumophila* STRAINS ISOLATED FROM ENVIRONMENT IN MOROCCO

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Abstract

Legionella pneumophila is a common cause of hospital and community-acquired pneumonia, being transmitted by inhalation of aqueous aerosols. Most legionellosis outbreaks are linked to contaminated hot water systems or cooling towers. The aim of this study was to determine the genetic diversity of (n= 55) environmental strains of *L. pneumophila* recovered from the hot water distribution systems of 16 establishments in seven Moroccan towns during the period 2009–2011. Thirteen chromosomal restriction patterns determined by Pulsed field gel electrophoresis were detected. The strains of *L. pneumophila* serogroup1 exhibited in 6/13 different PFGE patterns, while the strains of *L. pneumophila* serogroups 2-14 showed 7/13 PFGE patterns. The PFGE showed the existence of various patterns in Morocco, The pattern -XI- have tree similar profiles with the endemic *L. pneumophila* Paris's strain. This technique also allowed to conclude that the same pulsotype was found for many strains isolated from different establishments. Moreover, different pulsolypes were found for strains isolated from the same establishment. These results showed that PFGE analysis is a powerful tool to reveal the clonal nature and genetic differences among *L. pneumophila* strains.

Key words: *Legionella pneumophila*, legionellosis, Molecular typing, Pulsed-field gel electrophoresis.

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INTRODUCTION

Legionella is a facultative intracellular pathogen known to cause both community and hospital acquired pneumonia (20, 23). To date, fifty two species of *Legionella* have been described (18) - of which twenty one have been associated with human infection (15). There are more environmental species than pathogenic species (5). It is documented that the species- *Legionella pneumophila* - is responsible for about 90% of cases of legionellosis, and the serogroup 1 accounts for about 84% of cases (24).

L. pneumophila is ubiquitous in the aqueous environment, and most outbreaks are linked to contaminated hot water systems in well-defined areas (e.g., hotels, hospitals, and whirlpool spas) and cooling towers (10). The infection occurs mainly by inhalation of contaminated aerosols generated from hot water distribution systems (21).

In Morocco, during the three last years, the surveillance led by health authorities of water distribution systems has shown that most of the sanitary buildings are contaminated by *L. pneumophila* (22), and this is probably due to several aspects related to the buildings age, the inadequate maintenance of the water distribution systems and the creation of biofilms (2). When a nosocomial or community acquired legionellosis occurs, the identification of the sources of infection is often difficult, even if it required in order to implement more specific preventive control measures (10, 17). However, microbiological typing is a real and useful tool in the epidemiological investigations of infectious diseases, it allows the identification of specific clones among a set of isolates (10).

The adoption of a typing method for an epidemiological investigation is often useful for the definitive identification of the source of infection and the way of transmission. Traditional methods for microbiological typing were based on phenotypic investigations, but genotyping methods rapidly exceeded them. In fact, the latter are more accurate given that are based on the determination of polymorphic sites in the whole bacterial genome. In the last ten years, several studies have demonstrated how genotyping methods can be useful in *Legionella spp* investigations in hospital setting, as well as in other situations (source of contamination in homes, hotels, facilities) (4, 14).

Among the most recent techniques automated ribotyping, amplified fragment length polymorphisms, and pulsed field gel electrophoresis (PFGE) have been applied to Legionella spp typing. These techniques allow fingerprinting of the isolates to be obtained, so that they can be matched in order to establish similarities. At the moment PFGE is the most used method, due to his high discriminatory power, and readily distinguishing epidemic from sporadic isolates (7, 10).

The aims of this study were: (i) The determination of genetic diversity of *L. pneumophila* strains isolated from different Moroccan towns and (ii) Comparison of PFGE patterns of these strains with profiles in the data bank of the French National Reference Center for Legionellosis.

MATERIALS AND METHODS

Bacterial Strains

A total of (n=55) environmental strains were isolated from January 2009 through December 2010, from hot water-systems in large hotels, factories, and gyms in different Moroccan towns (Fig. 1). Strains were isolated as recommended by the French norm for isolation of *Legionella* in water (AFNOR T90-431) (3). Serogrouping was done by the latex agglutination method, which also allowed to distinguish serogroup 1 from serogroups 2–14 (Slidex Legionella- kit, BioMérieux, Lyon, France). However *L. pneumophila* 2-14 was identified by the agglutination technique of latex particles sensitized with monoclonal antibodies (reagent supplied by BioMérieux).

Chromosomal PFGE analysis technique

The genomic profiles of the strains isolated in water samples were determined by PFGE at the French National Reference Center for Legionella. Briefly, legionellae were treated with proteinase K (50 mg/ml) in Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8) for 24 h at 55°C, and DNA was digested with 20 IU of SfiI restriction enzyme (Boehringer Mannheim, Meylan, France) for 16 h at 50°C. Fragments of DNA were separated in a 0.8% agarose gel prepared and run in 0.53 Tris-borate-EDTA buffer (pH 8.3) in a contour-clamped homogeneous field apparatus (CHEF DRII system; Bio-Rad, Ivry sur Seine, France) with a constant voltage of 150 V. Runs were carried out with increasing pulse times (2 to 25 s) at 10°C for 11 h and increasing pulse times (35 to 60 s) at 10°C for 9 h.

The gels were stained for 30 min with ethidium bromide solution. The gels were observed under UV illumination and photographied. Digital images were stocked electronically as TIFF files and the DNA profiles were analyzed by GELCOMPAR II software. (Applied Maths, Sint-Martens-Latem, Belgium) Cluster analysis was done by the UPGMA. DNA relatedness was calculated by using the band-based Dice coefficient with a tolerance setting of 1,5 % band tolerance and 1,5% optimization setting for the whole profile. The profiles are considered as identical when they matched to 100%.

RESULTS

PFGE after SfiI digestion confirmed the presence of 13 chromosomal restriction patterns for *L. pneumophila (Lp)*, the strains of serogroup 1 showed 6/13 different PFGE patterns, while the strains of serogroups 2-14 showed 7/13 PFGE patterns. Four of the thirteen PFGE patterns were shared by several establishments (Table 1).

However, a same pulsotype I, was found for many strains isolated from different establishments such as establishments A, B, C, H and K (Table 1).

In addition, several pulsolypes such as I and XIII corresponding to Lp6 and X and III corresponding to Lp1 were found for strains isolated from the same establishment (for example establishment D).

Patterns I and XIII corresponding to Lp6 have wide geographical distribution, were found in geographically dispersed towns in Morocco (Fig. 1). However, the pattern IV corresponding to Lp3 was found only in Agadir, so the pattern V and VII corresponding respectively to Lp8 and Lp4 were found only in Fes.

The comparison of PFGE patterns for our strains with profiles in the data bank of the FNRCL indicated that the pattern XI including 3 pulsotypes corresponding to *Lp*1 isolated from the same establishment in Ouarzazate, was similar to the Paris's endemic strain (Fig. 2).

DISCUSSION

This study was undertaken to determinate the genetic diversity of *L. pneumophila* isolated in Morocco. To achieve this goal, the PFGE technique that is an important molecular tool having a higher discriminatory potential than molecular typing with PCR-AFLP have been used. PFGE was used to allow the comparison between individual isolates, the determination of a possible clonal relationship and the genetic relatedness between different *Lp* serotypes as well

Towns (*Geographical coordinates*)	Proportion of different *Lp strains* (%)	
Tangier (35° 47'N, 5° 48' W)	*Lp6*	(9)
Rabat (34° 01'N, 6° 50'W)	*Lp6*	(9)
Agadir (30° 24'N, 9° 36'W)	*Lp6*	(9)
	Lp1	(12.5)
	Lp3	(100)
Casablanca (33° 35'N, 7° 36'W)	*Lp6*	(27. 3)
	Lp1	(75)
	Lp4-8	(100)
Fes (34° 03'N, 4° 58'W)	*Lp6*	(9)
	Lp1	(2.5)
	Lp4	(100)
	Lp8	(100)
Marrakech (31° 38'N, 8° 00'W)	*Lp6*	(9)
Ouarzazate (30° 55'N, 6° 55'W)	*Lp6*	(18)
	Lp1	(10)

Figure1. Geographic distribution of Moroccan towns where environment *Lp* strains were isolated and the proportion of each serogroup was found in this towns **(**http://www.tageo.com/index.php?show=search**).**

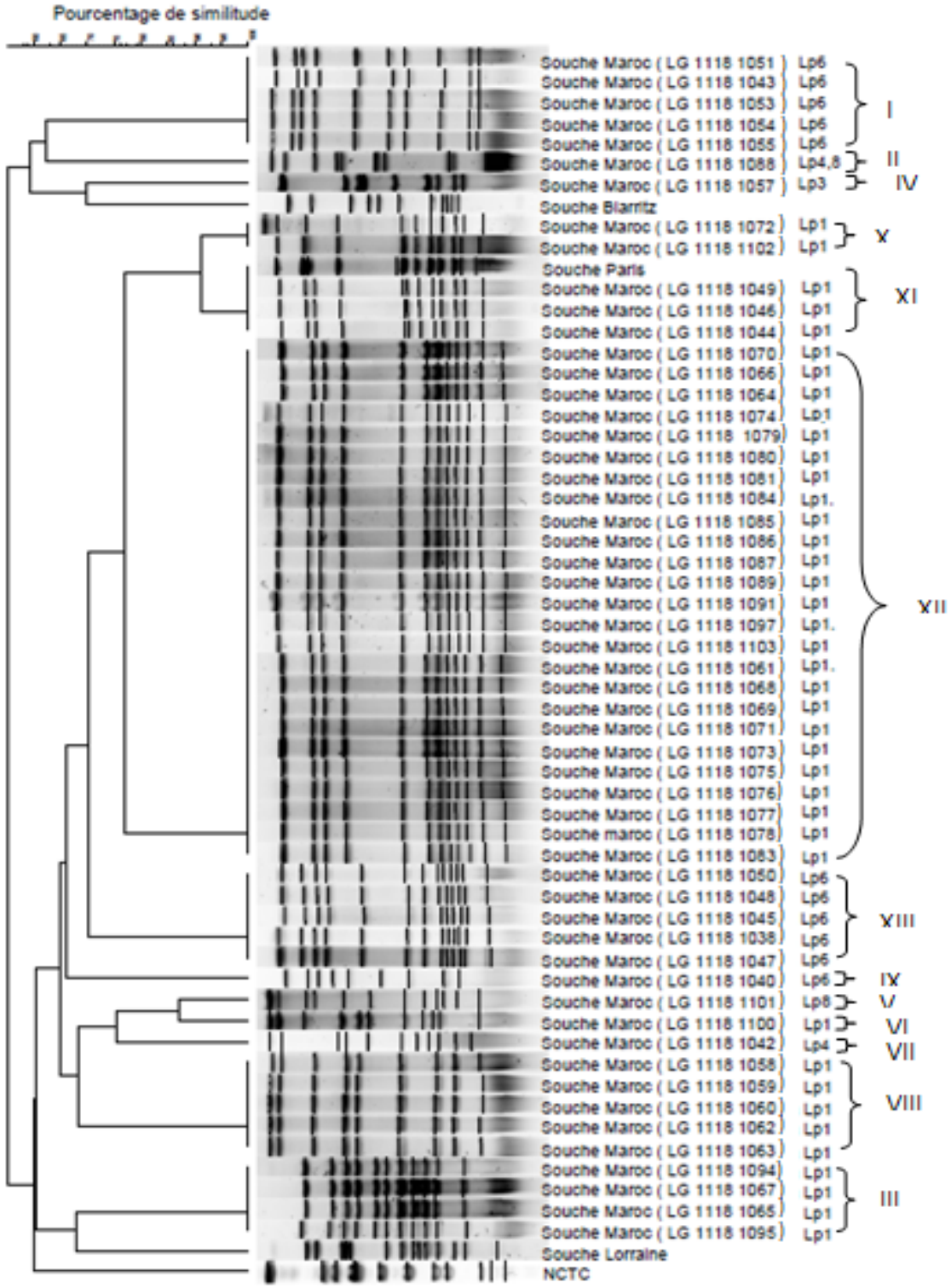

Figure 2. Pulsed-field gel electrophoresis profiles of 55 environmental *Lp* strains isolated from hot water systems and number of pattern founded.

as between strains of the same serogroups. Also, this technique can be employed to differentiate isolates of *Lp* in order to confirm or refute epidemiological associations (6, 9).

Genotyping data obtained for our isolates revealed high genetic heterogeneity. In some establishments concerned, several profiles were found simultaneously; it may be due to the multiple colonization of *Lp*.

In this study the pattern III was isolated in two different establishments in Casablanca (E and F); this result can be explained by the contamination or dissemination of a single clone of *Lp*1 throughout the water distribution network (13). A similar situation was described by Lawrence et al. who reported the presence of same strain in various sites throughout the Paris area over a long period. In addition, Pruckler and colleagues reported that unrelated isolates of *Lp* can display the same PFGE pattern; such isolates can be found at sites separated by up to 60 km (16).

Patterns I and XIII have a wide geographical distribution, they are isolated in towns distanced from each other at about 200 km. These results were confirmed by the observation made by luck et al. who found the same profile for *Lp* strains isolated in remote areas over 750 Km, it was therefore possible that this strain was disseminated in different sites of the country (12).

Our study showed the existence of a profile correspond-

Table 1. Origins of *Lp* strains and PFGE patterns were found in each establishment.

Town	Establishment	No. of strains	Serogroups isolated	Number of patterns	PFGE pattern(s)
Ouarzazate	A	2	Lp6	LG 1118 1051	I
			Lp1	LG 1118 1077	XII
	B	4	Lp1	LG 1118 1049	XI
			Lp1	LG 1118 1046	XI
			Lp1	LG 1118 1044	XI
			Lp6	LG 1118 1045	XIII
Casablanca	C	1	Lp6	LG 1118 1043	I
	D	4	Lp6	LG 1118 1055	I
			Lp6	LG 1118 1050	XIII
			Lp1	LG 1118 1072	X
			Lp1	LG 1118 1102	X
	E	4	Lp1	LG 1118 1067	III
			Lp1	LG 1118 1065	III
			Lp1	LG 1118 1066	XII
			Lp1	LG 1118 1064	XII
	F	20	Lp1	LG 1118 1095	III
			Lp4-8	LG 1118 1088	II
			Lp1	LG 1118 1094	III
			Lp1	LG 1118 1074	XII
			Lp1	LG 1118 1079	XII
			Lp1	LG 1118 1080	XII
			Lp1	LG 1118 1081	XII
			Lp1	LG 1118 1084	XII
			Lp1	LG 1118 1085	XII
			Lp1	LG 1118 1086	XII
			Lp1	LG 1118 1087	XII
			Lp1	LG 1118 1089	XII
			Lp1	LG 1118 1091	XII
			Lp1	LG 1118 1097	XII
			Lp1	LG 1118 1103	XII
			Lp1	LG 1118 1073	XII
			Lp1	LG 1118 1075	XII
			Lp1	LG 1118 1076	XII
			Lp1	LG 1118 1078	XII
			Lp1	LG 1118 1083	XII
	G	5	Lp1	LG 1118 1068	XII
			Lp1	LG 1118 1069	XII
			Lp1	LG 1118 1070	XII
			Lp1	LG 1118 1071	XII
			Lp1	LG 1118 1061	XII
Agadir	H	1	Lp6	LG 1118 1053	I
	I	1	Lp3	LG 1118 1057	IV
	J	5	Lp1	LG 1118 1058	VIII
			Lp1	LG 1118 1059	VIII
			Lp1	LG 1118 1060	VIII
			Lp1	LG 1118 1062	VIII
			Lp1	LG 1118 1063	VIII
Rabat	K	1	Lp6	LG 1118 1054	I
Fes	L	2	Lp8	LG 1118 1101	V
			Lp1	LG 1118 1100	VI
	M	1	Lp4	LG 1118 1040	VII
	N	1	Lp6	LG 1118 1040	IX
Marrakech	O	1	Lp6	LG 1118 1047	XIII
Tanger	P	2	Lp6	LG 1118 1048	XIII
			Lp6	LG 1118 1038	XIII

ing to the endemic *Lp* Paris's strain, which is highly prevalent in France (19), and which was responsible for 12.2% of legionellosis cases (1).

Legionella pneumophila Paris's strain is known to have colonized the entire water distribution network in the Paris area since 1987 (10), suggesting that this clone is well adapted to environmental survival. A review of FNRCL data shows that isolates with the profile of Paris's strain have also been isolated from the water distribution systems of at least ten of the French towns in which clinical strains have been isolated. However, Fry et al, detected the presence of Paris's strain outside France (8). In addition, Aurel et al (1), found the Paris's strain in at least 15 other French towns located up to 900 km from Paris, they have suggested that widespread geographical diffusion of *Legionella* strains may occur through rain and wind transportation (1).

In conclusion, the pulsed-field gel electrophoresis analysis of all *Legionella pneumophila* strains, isolated from hot water systems revealed the existence of different molecular profiles circulating in Morocco, and also allowed to conclude that the same pulsotype was found for many strains isolated from different establishments. Moreover, several pulsolypes were found for strains isolated from the same establishment. This heterogeneity requires the monitoring of warm waters distribution and application of new disinfection strategies to eradicate the disease of legionellosis.

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