Distribution and characterization of *Listeria monocytogenes* clinical isolates in Portugal, 1994–2007

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Abstract In recent years, the number of cases of listeriosis has increased worldwide. Ninety-five isolates of *Listeria monocytogenes* recovered from Portuguese human cases of listeriosis have been characterized by biotyping (cadmium and arsenic sensitivity), polymerase chain reaction (PCR) grouping, and by pulsed-field gel electrophoresis (PFGE) applying the enzymes *Asc*I and *Apa*I. Isolates were classified into one of three PCR groups; IVb (71.6%), IIb (17.9%), and IIa (10.5%). Four biotypes were differentiated: sensitive to arsenic/cadmium (48.4%), arsenic-sensitive and cadmium-resistant (25.3%), resistant to arsenic and sensitive to cadmium (18.9%), and resistant to both heavy metals (7.4%). Combined analyses of *Asc*I and *Apa*I patterns yielded a total of 58 PFGE types with five sets (G, Jb, KKa, Me, and U) of Portuguese strains, each of which were indistinguishable by PFGE typing. In the present study, it was demonstrated that there are recurrent pulsotypes and that some were the same pulsotypes linked to outbreaks in France. In addition, there are some pulsotypes spread throughout the country, while others only appear in a restricted region. This study allowed the assembly of a first large pulsotype database of Portuguese clinical strains.

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Introduction

Listeria monocytogenes is recognized as a major, opportunistic, foodborne pathogen in humans. The condition which this species causes, listeriosis, occurs worldwide both sporadically and epidemically, and has a case–fatality rate between 20 and 30%. *L. monocytogenes* has the ability to cross the intestinal, blood–brain, and fetoplacental barriers, and, thus, septicemia, central nervous system (CNS) infections, miscarriages, and stillbirths are risks to immunodeficient or pregnant individuals [1].

In Portugal, listeriosis is not a notifiable disease and has, thus, been underestimated. Nevertheless, Almeida et al. in 2006 [2], in a retrospective study, reported an estimated incidence of at least 1.4 cases per million inhabitants in 2003. The implementation of an active surveillance program in the Netherlands led to a 43% increase in the reported incidence of listeriosis in the first year of operation [3]. This might give some indication of the current degree of underestimation in Portugal. Any such program would certainly omit reporting some sporadic cases and spontaneous miscarriages, as these are often not investigated. Since 2005, several European countries reported an increase of listeriosis and several foodborne outbreaks have been reported [4]. Some examples are the outbreaks which occurred in Switzerland associated with Tome cheese [5], in the United Kingdom associated with sandwich consumption [6], in the Czech Republic associated with soft cheese [7], and a recent multinational outbreak due to the consumption of "Quargel" cheese in Austria and Germany [8].

The characterization of *L. monocytogenes* strains can be based on its molecular subtyping by polymerase chain reaction (PCR) grouping or geno-serotyping [9] and by macrorestriction pulsotype analysis of its DNA using the

PulseNet protocol [10]. The use of subtyping methods to differentiate strains (or subtypes) of *L. monocytogenes* has important epidemiological applications: rapid, precise, and efficient foodborne listeriosis surveillance can minimize outbreaks and track sources of *L. monocytogenes* contamination throughout the food system. Moreover, molecular subtyping in listeriosis surveillance would allow the detection of clusters of cases that could be erroneously considered as sporadic cases.

The association between a particular pulsotype and specific virulence traits is another possibility offered by molecular subtyping [11].

The study presented here was initiated in 2003, with a view to support the establishment of an integrated, food chain surveillance system in Portugal. Ninety-five clinical Portuguese isolates recovered from apparently sporadic cases of listeriosis were collected, identified, biotyped by cadmium and arsenic sensitivity, geno-serotyped, and typed by DNA macrorestriction pulsed-field gel electrophoresis (PFGE). The aim was to obtain epidemiological data on cases of listeriosis in the country and to construct a molecular type database of clinical strains of *L. monocytogenes*.

Materials and methods

Collection of *L. monocytogenes* strains and related data to listeriosis episodes

A total of 95 *L. monocytogenes* strains isolated between 1994 and 2007 were collected from major Portuguese hospitals. The case definition of listeriosis employed was that contained in Commission Decision 2002/253/CE [12]. Thus, on isolation of *L. monocytogenes* from a hospitalized patient with a clinical compatible illness, each strain was considered as a single case of listeriosis. A case was defined as maternal/neonatal (MN) in the following situations: infected pregnant woman, miscarriage, stillbirth, or newborn less than one month of age. Only one strain was kept when the pathogen was isolated from both the pregnant woman and her newborn child. If a case did not apply to any of these, it was considered as non-maternal/neonatal (non-MN). Information regarding gender and age of the patient, the tissue or fluid origin where the bacteria was isolated, and the year of isolation was reported when available.

Confirmed isolates of *L. monocytogenes* were stored in Tryptic Soy Broth with 30% (v/v) glycerol at −80° C in the culture collection of the Escola Superior de Biotecnologia.

Biotyping by arsenic and cadmium sensitivity

The characterization of their differential resistance to arsenic and cadmium was performed in Isosensitest agar plates (ISA, Oxoid, Hampshire, UK) containing 500 μg/ml sodium arsenite (Merck, Darmstadt, Germany) or 75 μg/ml cadmium chloride (Merck) [13, 14].

Geno-serotyping or PCR grouping

Geno-serotyping was determined by PCR grouping with a multiplex PCR as described by Doumith et al. [9] using primers targeting fragments of genes *lmo0737*, *ORF2819*, *ORF 2110*, *lmo1118*, and *prs* (MWG-Biotech, Muenchenstein, Switzerland). PCR was performed in an Eppendorf thermocycler (Eppendorf, Hamburg, Germany) and PCR products were resolved on a 2% agarose gel containing 0.5 μg/ml of ethidium bromide (Eurobio, Courtaboeuf, France) and visualized and photographed under a UV transilluminator (Bio-Rad Gel Doc 2000™ imaging system, Bio-Rad Laboratories, Milan, Italy).

Molecular characterization by PFGE

The DNA macrorestriction was performed at the Centre National de Référence des *Listeria*, Institut Pasteur (Paris, France) with the restriction enzymes *Asc*I (New England BioLabs, Massachusetts, Ipswich, USA) and *Apa*I (MBI Fermentas, Burlington, Canada), as previously described by Graves and Swaminathan [10].

The pulsotypes obtained were scanned and the computerized data were analyzed using BioNumerics software version 5.1 (Applied Maths, Kortrijk, Belgium). Bands automatically assigned by the computer were checked visually and corrected manually when necessary. A position tolerance of 1.5 was selected for each PFGE. Cluster analysis of the individual or combined PFGE pulsotypes was done by the unweighted pair group method with average linkages (UPGMA), using the Dice coefficient to analyze the similarities of the banding pulsotypes. The discriminatory power was determined by calculating the discrimination index (*D*) based on Simpson's index of diversity, as described by Hunter and Gaston [15].

Two PFGE patterns were considered to be indistinguishable when their similarity was higher than 98% [16] and a capital letter was ascribed to *Asc*I pulsotype; when strains were similar at more than 98% by *Asc*I but not similar at more than 98% by *Apa*I, they were denoted, in addition to the capital letters, by lowercase letters.

PFGE pulsotypes of indistinguishable Portuguese strains were compared with PFGE pulsotypes of the French PFGE database of the Centre National de Référence des *Listeria*

using the BioNumerics software package as previously described in this section.

Statistical analysis

The statistical package used for the analyses was Stata v10.0 (StataCorp LP, College Station, TX, USA). The contingency table analysis was based on the Chi-square distribution (Pearson's Chi-square test).

Results

Data collected from human episodes

A total of 95 isolates from cases of listeriosis were collected from major Portuguese hospitals during the study period. From the information available on 81 cases (Table 1), 85.2 and 14.8% corresponded to non-MN infections and MN infections, respectively. For the 69 confirmed non-MN cases, strains were isolated from blood (58.0%), from cerebrospinal fluid (34.8%), and from other specimens (7.2%). The clinical symptoms were not recorded for the majority of cases. The gender ratio (M/F) of confirmed non-MN cases was 2.3. The mean age of non-MN cases with documented age was 60 years, with 38 cases (55.9%) being equal or up to 60 years. The geographical distribution of listeriosis cases was difficult to define from our collected data in the absence of data on the residence of patients.

Biotyping results by arsenic and cadmium sensitivity

Four groupings of sensitivity to heavy metals were differentiated among the 95 isolates (Table 1), the *D*-value for this biotyping method being 0.667. The predominant group—sensitive to both arsenic (As) and cadmium (Cd)—contains 46 isolates (48.4%). Twenty-four isolates were arsenic-sensitive and cadmium-resistant (25.3%), 18 were resistant to arsenic and sensitive to cadmium (18.9%), and seven were resistant to both heavy metals (7.4%).

PCR grouping results

Three PCR groups were identified in the 95 *L. monocytogenes* isolates recovered from sporadic human listeriosis cases in Portugal. Most isolates were of PCR group IVb (including serotypes 4b or 4d or 4e) 71.6% (n=68): 17.9% (n=17) and 10.5% (n=10) of the isolates showed PCR group IIb (including serotypes 1/2b or 3b) and PCR group IIa (including serotypes 1/2a or 3a), respectively. The *D*-value obtained from these PCR grouping results was 0.449.

Combining the results from PCR grouping and sensitivity to heavy metals, it was possible to segregate the 95 isolates into nine groups (D=0.831). Two groups predominate: PCR group IVb, As- and Cd-sensitive (30.5%); PCR group IIb, As-resistant and Cd-sensitive (17.9%).

PFGE results

PFGE typing revealed a total of 39 *Asc*I and 50 *Apa*I macrorestriction types among clinical isolates, distinguished by one or more band differences ranging in molecular size from 50 to 500 kb (Fig. 1). Combined analyses of *Asc*I and *Apa*I PFGE data yielded a total of 58 PFGE pulsotypes, with D=0.966 for *Asc*I, D=0.979 for *Apa*I, and D=0.986 for combined *Asc*I and *Apa*I.

Comparisons based on such combined patterns are reported in Table 1 and the corresponding dendrogram in Fig. 1.

Pulsotypes DD, EE, FF, G, HHb, Jb, KKa, La, LLa, LLb, Me, MM, Pb, S, T, U, V, and Ya contained two or more strains which remained indistinguishable from each other. Only the pulsotypes FF, LLb, MM, Pb, T, U, V, and Ya contained indistinguishable strains by Cd and As sensitivity.

Pulsotypes DD, EE, FF, HHb, Jb, KKa, La, LLa, LLb, Me, MM, Pb, S, and T were constituted of *L. monocytogenes* isolates from geno-serotype IVb, pulsotypes U, V, and Ya of geno-serotype IIb, and pulsotype G of geno-serotype IIa.

While strains of pulsotypes DD, FF, HHb, La, LLa, LLb, MM, Pb, S, T, V, and Ya were recovered from different years and geographical distribution, pulsotypes EE, G, Jb, KKa, Me, and U were related in time or geographical distribution. Two pulsotypes (U and KKa) have six indistinguishable strains. Three pulsotype U strains were isolated in the Lisbon region, respectively in February 2004, August 2004, and October 2004, with the same Cd and As sensitivity. Two pulsotype KKa strains having the same Cd and As sensitivity were isolated in March and in April 2004.

Pulsotype G contained two indistinguishable strains but with distinct Cd and As sensitivity isolated in February and May 2006 in different towns.

Concerning pulsotype EE, two indistinguishable strains but with distinct Cd and As sensitivity were isolated in different hospitals in Porto in 2007 six months apart, from a woman and a newborn child.

The pulsotype Jb contained three indistinguishable strains isolated in Lisbon in March 2006, September 2006, and June 2007. Pulsotype Me is constituted by four indistinguishable strains, three of which were isolated in the Porto region over a period of one year.

Table 1 *Listeria monocytogenes* strains used in this study and collected epidemiological data concerning the cases with which they were associated

Isolate	Hospital localization	Year of isolation	Clinical form[a]	Patient gender/age[b]	PCR group (geno-serotype)	Susceptibility to As/Cd[c]	PFGE combined type	
							U	I
771	Porto	2003	M	Un / < 1 month	IVb	S/S		S
779	Porto	2000	NM	F / 25	IVb	S/R	HHa	
780	Porto	1996	NM	M / Un	IVb	S/S		HHb
781	Porto	2000	M	Un / < 1 month	IVb	S/R		HHb
783	Porto	1994	M	Un / < 1 month	IVb	R/S		S
784	Porto	1999	M	Un / < 1 month	IVb	R/R	Ma	
856	Chaves	2003	NM	M / 50	IVb	R/S	Ka	
866	Matosinhos	2003	NM	M / 67	IVb	S/S	R	
908	Chaves	2003	NM	M / 48	IVb	R/R		HHb
999	Coimbra	2000	NM	M / 48	IIb	S/S	Z	
1001	Porto	2004	NM	M / 65	IVb	R/R		KKa
1002	Almada	1998	NM	M / 54	IIb	S/S	NN	
1003	Almada	2003	NM	F / 85	IVb	S/S		LLa
1037	Chaves	2004	NM	F / 74	IVb	S/S	Pd	
1059	Matosinhos	2004	NM	F / 73	IVb	S/R		HHb
1062	Lisbon	2004	NM	M / 54	IIb	S/S	W	
1063	Lisbon	2002	NM	M / Un	IVb	S/S		FF
1065	Lisbon	2004	NM	M / Un	IIb	S/S		U
1198	Matosinhos	2004	NM	M / 74	IIa	S/S	H	
1239	Matosinhos	2004	M	Un / < 1 month	IVb	R/S	BB	
1240	Almada	2004	M	F / < 1 month	IIb	S/S		U
1241	Coimbra	2004	NM	M / 44	IVb	R/R		KKa
1242	Lisbon	2004	NM	M / 57	IIb	S/S		U
1244	Lisbon	2004	NM	M / 46	IVb	R/S	N	
1348	Almada	2005	NM	F / 77	IIb	S/S		U
1383A	Lisbon	2005	NM	M / 40	IVb	R/R	JJ	
1541	Coimbra	2000	NM	Un / Un	IVb	S/S		LLb
1542	Coimbra	2000	NM	Un / Un	IVb	S/R	HHc	
1543	Coimbra	2000	NM	Un / Un	IVb	S/S	Ja	
1544	Coimbra	2000	NM	Un / Un	IVb	S/S		DD
1545	Coimbra	2003	M	F / 31	IIb	S/S		Ya
1546	Coimbra	2001	NM	M / 41	IVb	S/S	Pc	
1547	Coimbra	2001	NM	M / 85	IIb	S/S		U
1548	Coimbra	1999	NM	Un / Un	IVb	S/S		KKa
1549	Coimbra	1999	NM	Un / Un	IVb	S/R		MM
1550	Coimbra	1997	NM	Un / Un	IVb	S/S		LLc
1551	Coimbra	1997	NM	Un / Un	IVb	S/S		T
1552	Coimbra	1997	NM	Un / Un	IVb	S/S	Md	
1553	Coimbra	1998	NM	Un / Un	IVb	R/S		La
1554	Coimbra	1999	NM	Un / Un	IVb	S/R		KKa
1555	Coimbra	2003	NM	F / 74	IVb	S/S		T
1556	Coimbra	2005	NM	F / 56	IVb	S/S		Pb
1562/1	Lisbon	2005	NM	M / 81	IIb	S/R	Yb	
1621/1	Lisbon	2005	NM	F / 72	IVb	S/S		LLb
1761	Coimbra	2005	NM	M / 57	IIb	S/R	A	
1762	Coimbra	2005	M	F / 33	IIb	S/R		V
1763	Coimbra	2005	NM	M / 51	IVb	R/S	Mf	
1764	Coimbra	2005	M	Un / < 1 month	IVb	S/R	KKc	
1765	Coimbra	2005	M	F / 31	IVb	S/S	Kb	
1766	Braga	2005	NM	F / 4 months	IVb	S/R		Me
1767	Braga	2005	NM	F / 46	IVb	S/R		KKa
1768	Matosinhos	2006	M	F / 31	IVb	R/S		Me
1792	Lisbon	2006	NM	M / 34	IVb	R/S		Jb
1796	Lisbon	2006	NM	F / 80	IIb	S/R		V

Table 1 (continued)

Isolate	Hospital localization	Year of isolation	Clinical form[a]	Patient gender/age[b]	PCR group (geno-serotype)	Susceptibility to As/Cd[c]	PFGE combined type	
							U	I
1803	Matosinhos	2006	NM	M / 77	IVb	R/S	Lb	
1807/1	Lisboan	2006	NM	F / 68	IVb	S/R		KKa
1878	Lisbon	2006	NM	M / 70	IVb	S/S		LLa
1879	Lisbon	2006	NM	M / 58	IVb	R/R		Jb
1880	Porto	2006	NM	M / 78	IIa	S/S	C	
1891	Braga	2006	NM	M / 60	IIa	S/S	F	
1999	Lisbon	2006	NM	M / 70	IVb	S/S		KKb
2029	Lisbon	2007	NM	F / 81	IIa	S/R	D	
2064	Matosinhos	2006	NM	M / 63	IVb	R/S		La
2065	Matosinhos	2007	NM	F / 79	IIb	S/R	X	
2074	Porto	2007	M	Un / < 1 month	IVb	S/S		EE
2076	Lisbon	2007	NM	F / 50	IIb	S/S		Ya
2085	Lisbon	2007	NM	F / 73	IVb	S/S	Pa	
2086	Matosinhos	2007	NM	M / 84	IIa	S/S	B	
2087	Lisbon	2007	NM	M / 50	IVb	S/R	Mc	
2088	Porto	2006	NM	M / 77	IVb	S/S		FF
2090	Porto	2006	NM	M / 54	IVb	S/R		Me
2091	Porto	2006	NM	M / 61	IIa	R/S		G
2092	Braga	2007	NM	M / 78	IVb	R/S	Mb	
2093	Braga	2007	NM	M / 77	IVb	R/R	GG	
2094	Funchal	2007	NM	F / 53	IVb	S/S		FF
2095	Almada	2007	NM	F / 88	IIb	S/S	Q	
2096	Porto	2005	NM	M / 40	IVb	S/S		La
2097	Coimbra	2006	NM	F / 81	IVb	R/S		DD
2098	Coimbra	2006	NM	M / 83	IIb	S/R	AA	
2099	Coimbra	2006	NM	M / 53	IIa	S/S		G
2100	Coimbra	2006	NM	F / 70	IVb	S/S		FF
2101	Coimbra	2007	NM	M / 66	IVb	S/S	II	
2102	Lisbon	2007	NM	M / 74	IVb	R/S	Jb	
2103	Lisbon	2007	NM	M / 19	IIa	S/S	E	
2104	Coimbra	2006	NM	F / 68	IVb	S/R		MM
2105	Coimbra	2007	NM	M / 43	IVb	S/R		MM
2117	Braga	2007	NM	M / 76	IVa	S/S	CC	
2121	Braga	2007	NM	M / 34	IIb	S/S		U
2122	Lisbon	2007	NM	M / 88	IVb	S/S		Pb
2125	Funchal	2007	NM	M / 58	IVb	R/S		EE
2135	Lisbon	2007	NM	M / 52	IIa	S/R	I	
2252	Porto	2007	NM	M / 50	IVb	R/S		Me
2253	Porto	2007	NM	M / 60	IVb	R/S		DD
2254	Porto	2007	NM	M / 69	IVb	S/S	O	
2255	Porto	2007	NM	F / 26	IVa	R/S		EE

Strains not similar at more than 98% by PFGE typing with *Asc*I were addressed as unique, ascribed a capital letter, and in the column U (unique). Strains similar at equal to or more than 98% from at least one other isolate were listed in column I (indistinguishable) and were further typed with *Apa*I. Strains similar at more than 98% by *Asc*I but not similar at more than 98% by *Apa*I were denoted, in addition to the capital letters, by lowercase letters. So, a combined type from PCR grouping as well as from PFGE typing was determined. Strains distinguishable by their PCR grouping or strains not similar at more than 98% by PFGE typing with *Asc*I and *Apa*I were again listed as unique (U). Strains not distinguishable by their PCR grouping or strains similar at equal to or more than 98% by PFGE typing with *Asc*I and/or *Apa*I were again listed as indistinguishable (I)

[a] M: maternal/neonatal; NM, non-maternal/neonatal

[b] Un: unknown

[c] R: resistant; S: sensitive

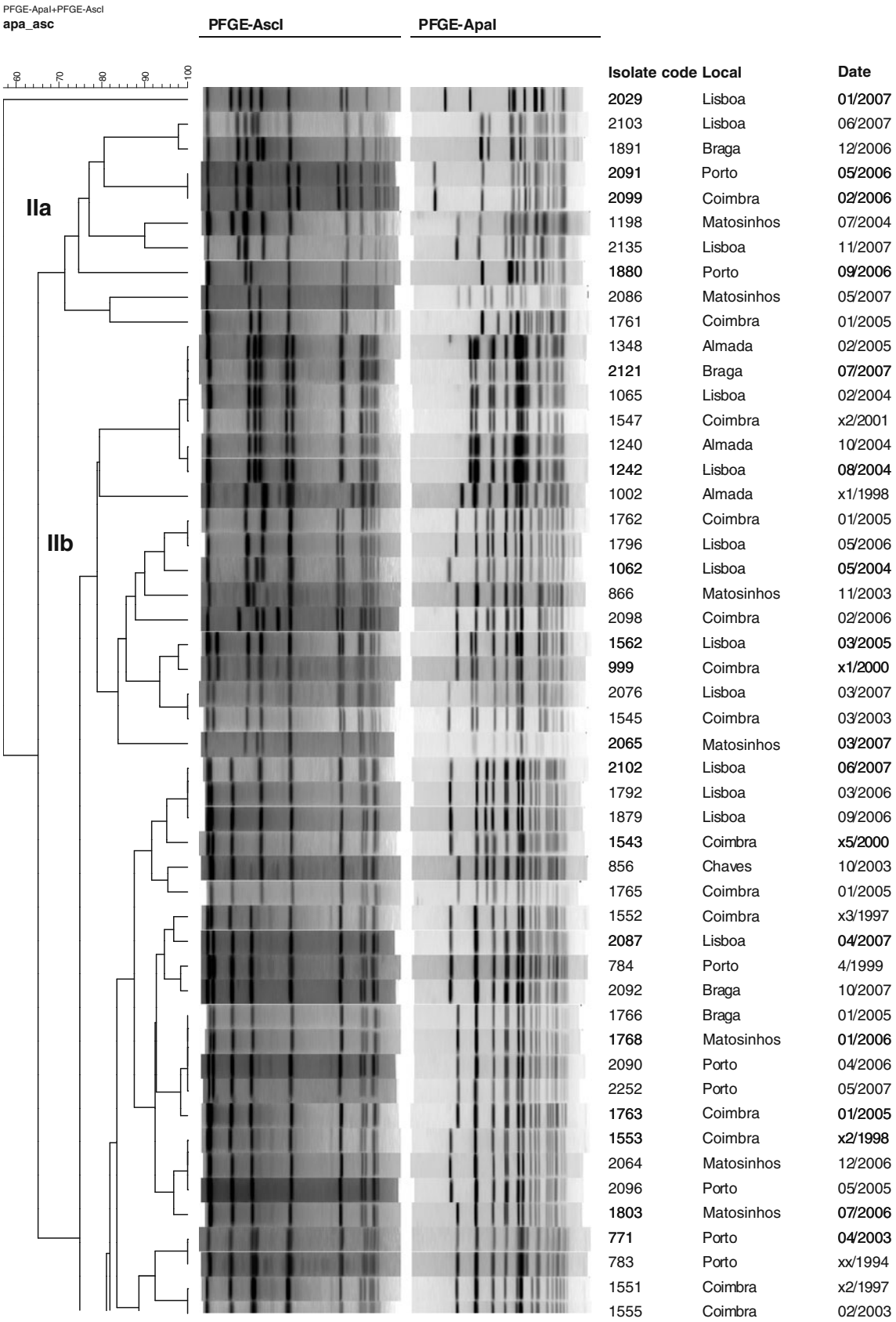

Fig. 1 Dendrogram for *Listeria monocytogenes* pulsotypes of all 95 isolates obtained from clinical cases, Portugal, 1994–2007

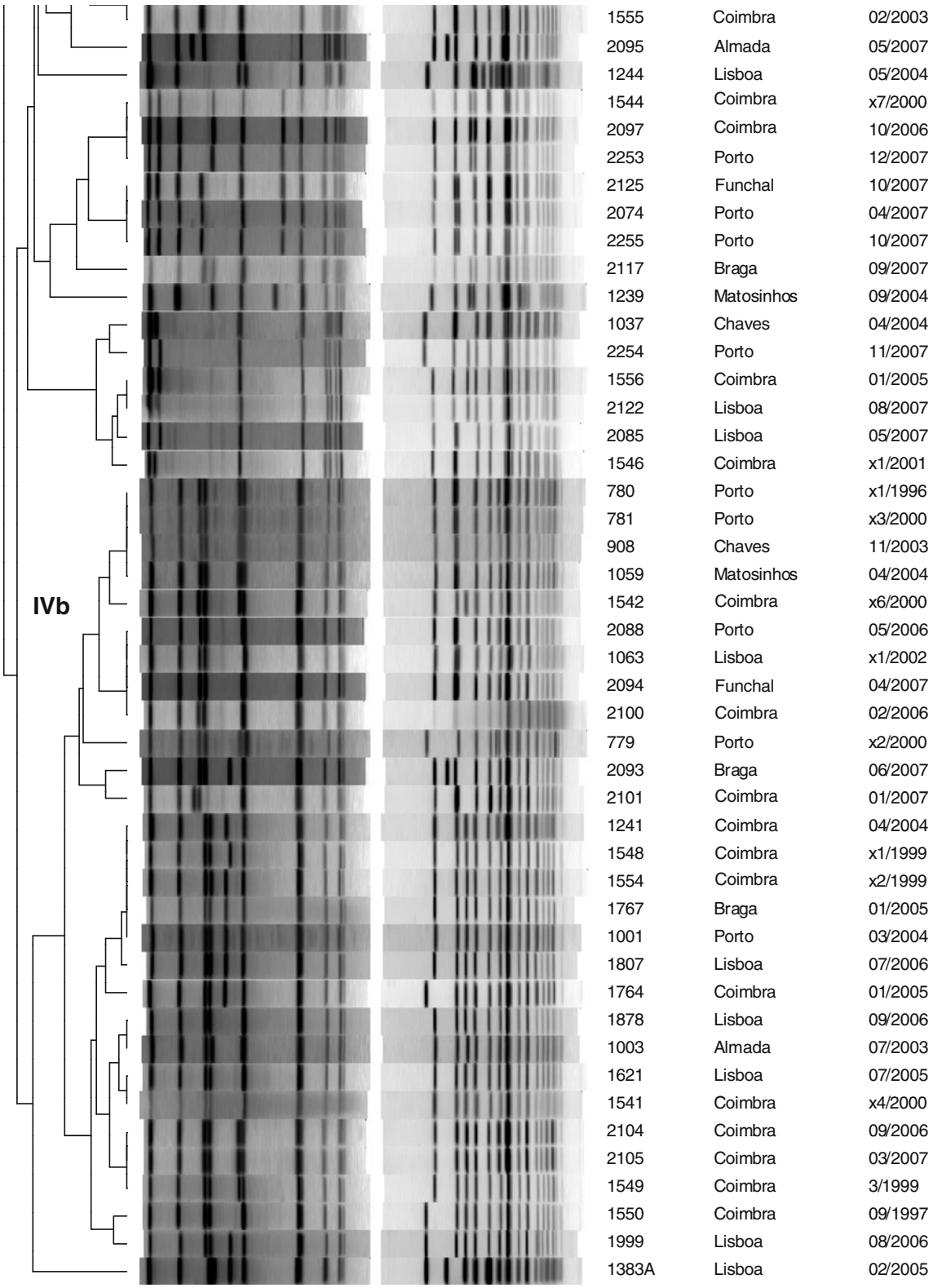

Fig. 1 (continued)

Comparison with the PFGE pulsotypes database of French human strains underlined that pulsotypes Me, Jb, Pb, HHb, FF, MM, and LLb have been previously described in human clusters in France and are part of the main pulsotypes from human strains observed in France. Pulsotypes MM and LLb are indistinguishable from pulsotypes associated with the main outbreak of listeriosis in France in 1992. The main pulsotypes observed in Portugal, U and KKa, have not been recorded for French human cases.

Combination of biotyping, PCR grouping, and PFGE results

The highest *D*-value, 0.994, and, thus, the greatest number of distinguishable types (72) was obtained when the results of multiplex PCR, sensitivity to heavy metals, and analysis of *Asc*I and *Apa*I were combined.

Discussion

The 2007 Annual Community Summary Report by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) established an overall incidence rate of listeriosis in Europe of 3 cases per million inhabitants, with a range of 0–11 cases per million inhabitants. The results presented here estimated an incidence rate of listeriosis in Portugal, based on information from voluntary reporting, of 2.3 cases per million inhabitants for the year 2007, which is comparable to the reported incidence in Austria, Estonia, Latvia, Slovakia, and Slovenia [17]. In Portugal's only neighboring country, Spain, the incidence for 2007 was similar to that described in this study and was also based on the voluntary reporting of cases to the National Reference Laboratory. However, it has been demonstrated that the implementation of an active surveillance system for listeriosis in Navarra, a region of northern Spain, led to an incidence for this region similar to that described in countries with mandatory notification of listeriosis, such as France, Denmark, and the Netherlands [18]. An apparent increase in the numbers of cases of listeriosis in recent years, observed in some other countries, e.g., Denmark, Finland, France, Germany, the Netherlands, Switzerland, and United Kingdom [3, 4], was also observed in Portugal. These results indicate that voluntary reporting underestimates the occurrence of listeriosis in a country, and that the EC requirement for the statutory reporting of listeriosis will probably lead to many more cases being uncovered. A statutory reporting and investigation system in Portugal will inevitably take some time to establish, and it is recommended that, in parallel, a surveillance system for the pathogen in typical foods should be established, with the capability for in-depth characterization of isolates for comparisons of clinical and food strains.

The majority of patients with listeriosis in Portugal were people older than 60 years with bacteremia, as recorded in some other countries, including France and United Kingdom [19, 20]. Most of these cases were caused by PCR group IVb strains, which are generally associated with epidemic clones [21]. As in other countries [18, 20], serogroup 1/2a became more common than serogroup 1/2b in 2006 and 2007.

The results of our study confirm that PCR grouping alone has the lowest discriminatory power but, when combined with PFGE *Asc*I and *Apa*I, it becomes the most powerful of all [22]. Nevertheless, during outbreaks, the screening method of strains based on PCR grouping rather than classical serotyping is useful. Biotyping by As and Cd sensitivity gave additional information data but not enough to consider the use of this method in a routine system of microbiological surveillance. As observed in Belgium [23], an increase in strains resistant to Cd but sensitive to As was verified.

In our study, it was possible to link sporadic cases on six occasions related in time and geographical distributions; thus, a common source of contamination could be suspected. This suspicion underlined the need to characterize food and environmental strains at the same time as human strains at a national level in order to trace sources of contamination and to detect and confirm related cases.

The finding that combined pulsotypes of Portuguese clinical isolates have already been described in human clusters, and even outbreaks, in France can be explained by both human travel and food commerce. It can be argued that there is, at least, a European distribution of clinical strains that necessitates European tracing, such as a PulseNet initiative for the continent. Nevertheless, combined pulsotypes U and KKa have been detected in Portugal but not in France, which suggests that indigenous clones of *L. monocytogenes* may exist in the country which might, at some time, become epidemic clones.

The possible epidemiological links with human strains isolated in other countries underlines the need for an active surveillance system of listeriosis in Portugal harmonized with other national systems. As the French National Surveillance System [20, 24], this should be based on the obligatory notification of human cases of listeriosis and epidemiological data collection, on the basis of European regulation EC 178/2002 (obligatory notification of unsatisfactory food according to microbiological criteria defined by European regulation), and also on the dispatch of isolates to a national reference laboratory for typing.

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