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Distribution and characterization of clinical isolates in Portugal, 1994-2007

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Title: Distribution and Characterization of Listeria monocytogenes clinical isolates in Portugal, 1994-2007

Article Type: Article

Keywords: Listeria monocytogenes; listeriosis; diversity; typing

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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), PCR grouping and by pulsed field gel electrophoresis (PFGE) applying the enzymes AscI and ApaI. 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 AscI and ApaI 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.

Response to Reviewers: Dear Sir or Madam:
Thank you very much for you careful review of this manuscript. In fact it was too repetitive and contained incorrect information mainly concerning surveillance. As you will realize, the paper was shortened and most of the information related to surveillance was omitted, as this is not the main goal of this manuscript. We may not have taken into consideration all your suggestions but we are willing to do them if required.

Reviewers' comments:

Reviewer #1: The manuscript describes the results obtained from typing L. monocytogenes isolates identified in Portugal between 1994 and 2007 (14years). It is generally very useful to report epidemiological data about human listeriosis in the European countries and the information could be of interest for the journal readers. However, the manuscript has to be subjected to major revisions thoroughly before it could meet the standards of EJCMID.
In particular:
Introduction

-Some statements are incorrect. Pag3, lines 15. A disease can be defined underreported only when it is notifiable.

Modified accordingly to: In Portugal, listeriosis is not a notifiable disease and has thus been under-estimated.

-A laboratory surveillance system can contribute to more reliably estimate listeriosis incidence, but a systematical typing - pag. 3, lines 25-26 - per se does not.

Previous lines 19-25 ("The absence of adequate....of this disesase") were omitted.

-Lines 40 and following. There are a number of outbreaks that have been occurring in Europe and, in my opinion, could be more suitable to be cited.

New references were added as suggested.

- Lines 48 and following. Again, typing of L. monocytogenes isolates is a very useful tool to be used in the aim of a better understanding of a complex disease from both clinical and epidemiological points of view, but is not a prerequisite in the implementation of a surveillance system.

Previous lines 48-53 were omitted.

-Traditional serotyping has not been so largely replaced by PCR "grouping or geno-serotyping" as the authors affirm in lines 55 and 56.

Modified to: The characterization of L. monocytogenes strains can be based on its molecular subtyping by PCR grouping or geno-serotyping [9] and by macrorestriction pulsotype analysis of its DNA using the PulseNet protocol [10].

-Geno-serotyping is less discriminatory than traditional serotyping and this observation is confirmed by the data presented in the manuscript.

We agree but it is true that the use of conventional serotyping has been replaced by geno-serotyping in several laboratories.

-Pag. 4, lines 1 to 14. This paragraph has to been rewritten for clarity.

Rewritten as suggested: "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]".

-Pag. 4, line 24. I suggest to replace "hypothetical" with "apparently".
Modified as suggested.

-Pag. 4, lines lines 18 to 28. I would suggest to invert the position of the two paragraphs.

Modified as suggested.

Materials and Methods
-Pag. 4, line 58 and pag.5, lines 1-2. I don't understand how the lack of information could be justified by the non notifiable status of this disease. Unfortunately, It is true that epidemiological information is often difficult or impossible to obtain, but this information should be routinely present in the databases of the diagnostic microbiology laboratories.

We are totally in agreement but this is the Portuguese reality concerning listeriosis. Unfortunately in many institutions the information in not centralized in the diagnostic microbiology laboratories being difficult to gather. Anyway, "in recognition of the fact that this illness is still a non-notifiable disease in Portugal" was omitted.

-Pag. 5, line 60 and pag. 6, lines 1 to 11. The Simpson's index of diversity can be cited only, without further details that should be deleted.

Modified as suggested.

Results
Pag. 6, lines 43 and following. A section entitled "epidemiological data" is inconsistent with the repeated statements about the frequent lack of information. The descriptive data should not be emphasized.

Modified accordingly to: "Data collected from human episodes. "

Lines 49 and following: 90% and 10% are incorrect and have to be recalculated. Moreover, percentages should be expressed with one decimal.

-Corrected

- Pag. 6, line 57. Replace "sex" with "gender"
Modified accordingly

-Pag. 6, line 59. Delete "old"
Modified accordingly

- PFGE results. This is perhaps the most confuse part of the manuscript. Pag. 7, line 54 and following: this is a comment and has to be displaced in the Discussion.

The sentence was omitted.

-There is not an unanimous agreement among the researchers about the combined or hierarchical use of the two enzymes AscI and ApaI (please italicize). Indeed, this is an argument for the discussion. Generally speaking, the entire section is too long, detailed and, unavoidably, boring and dispersive. I would suggest to mark the clusters e.g. with Roman numbers, in the dendrogram and refer to them, instead of listing all pulsotypes.

The old but evergreen suggestion to no duplicate the same information in different forms (text, tables or figures) is to be ever taken into account. Pag. 9, lines 20-28. This statement about the Tenover criteria is a matter of Method, not of Results, and is contradictory withrespect to the previously cited clustering criteria.

The entire section was rewritten.

Discussion
-The Discussion is too long and in some parts repetitive.

The discussion was rewritten in a shorter form.

-The entire paragraph from page 9, line 44 to page 10, line 16 should be rewritten in a more consistent and consequential manner.

Modified accordingly

- Pag. 10, line 36. The authors describe the characteristics of an active surveillance system, but wrongly. Form a conceptual point of view, an active surveillance system is a quite different issue than this one here described. So to speak, an active surveillance system is FoodNet, that is characterized by the active search for epidemiological data that can allow for a reliable estimate of the incidence of some foodborne diseases, including listeriosis. This is a serious misunderstanding that the Authors have to clarify.

We decided not to include this information.

-Pag. 11, lines 1-20. The content of this part of the Discussion has been previously included in the Results.

This information was omitted from the Results

-Pag.11, lines 22-47. It's very difficult and maybe poorly informative to complement the mandatory notification of each case with a questionnaire about food consumption habits. This last should be used during the epidemiological investigation driven by a suspicion about a possible outbreak arising from traditional and molecular epidemiological findings.

It was decided to omit this "information" as we consider that it is not relevant in the context of the aims of the present work.

-Page 11, lines 49 and following. Comparison with French isolates cannot be presented in the Discussion, but also in M&M and Results. Otherwise, the authors could discuss possible similarities between strains or pulsotypes from the two countries with reference to published data.

Modified accordingly:

Methods: "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 as previously described in this section."

Results: "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."

-Pag. 12, lines 4 and following. As I have previously commented, the active surveillance is not a microbiological surveillance. Accordingly, the entire paragraph should be thoroughly revised.

Modified accordingly: "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."

Figure 1. This Figure has to be removed. Indeed, it does not illustrate any finding of this work.

Removed accordingly

Table 1. This table in the present form is not reader-friendly. The Table has to be reorganized in a much more synthetic manner. Maybe less information (e.g. specimen could be removed, age and sex grouped, etc.) and inclusion of molecular subtype clusters instead of pulsotypes could help to lighten the Table.

The table was reorganized. As it is still to long it might be possible to submit it as a "supplementary file"

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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), 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 *ApaI* 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.

Keywords: Listeria monocytogenes, listeriosis, diversity, typing

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 septicaemia, 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 under-estimated. Nevertheless, Almeida and his colleagues in 2006 [2], in a retrospective study, reported an estimated incidence of at least 1.4 cases per million inhabitants in 2003. The result of 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] might give some indication as to the current degree of underestimation in Portugal. Any such programme 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 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 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 hospitalised 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. Only one strain was kept when the pathogen was isolated from both the pregnant woman and her newborn child. If a case does not apply to any of these, it is 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.

These epidemiological characteristics are described in Table 1. Confirmed isolates of *L. monocytogenes* were stored in Tryptic Soy Broth with 30% (v/v) glycerol at -80 ºC 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 were performed in Isosensitest agar plates (ISA, Oxoid, Hampshire, UK), containing 500 µg/ml

sodium arsenite (Merck, Darmstad, 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, Switzerland). PCR was performed in 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 TM imaging system, Bio-Rad Laboratories, Milan, Italy).

Molecular characterisation by pulsed-field gel electrophoresis (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, 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 the 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 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, were denoted, in addition to the capital letters, by lower case 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 as previously described in this section.

Statistical analysis

The statistical package used 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 old with 38 cases (55.9%) equal or up to 60 years. Geographical distribution of listeriosis cases was difficult to define from our collected data in the absence of data about the residence of patient.

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 7 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 serotype 1/2a or 3a), respectively. *D* obtained from these PCR grouping results was 0.449.

Combining 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 macrorestriction 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 (Fig1). 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 combined *Asc*I and *Apa*I.

Comparisons based on such combined patterns are reported in Table 1 and the corresponding dendrogram in Figure 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 to 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 women 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 isolated in the Porto region over a period of one year. 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 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 of

2 cases per million inhabitants [17]. In Portugal's only neighbouring country, Spain, the incidence for 2007 was similar to that described in this study and was also based on 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 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 under-estimates the occurrence of listeriosis in a country, and that the EC requirement for 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 characterisation of isolates for comparisons of clinical and food strains.

The majority of patients with listeriosis in Portugal were people older than 60 years with bacteraemia, as recorded in some other countries including France and United Kingdom [19, 20]. Most of these cases were caused by PCR group IVb strains, 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 characterise 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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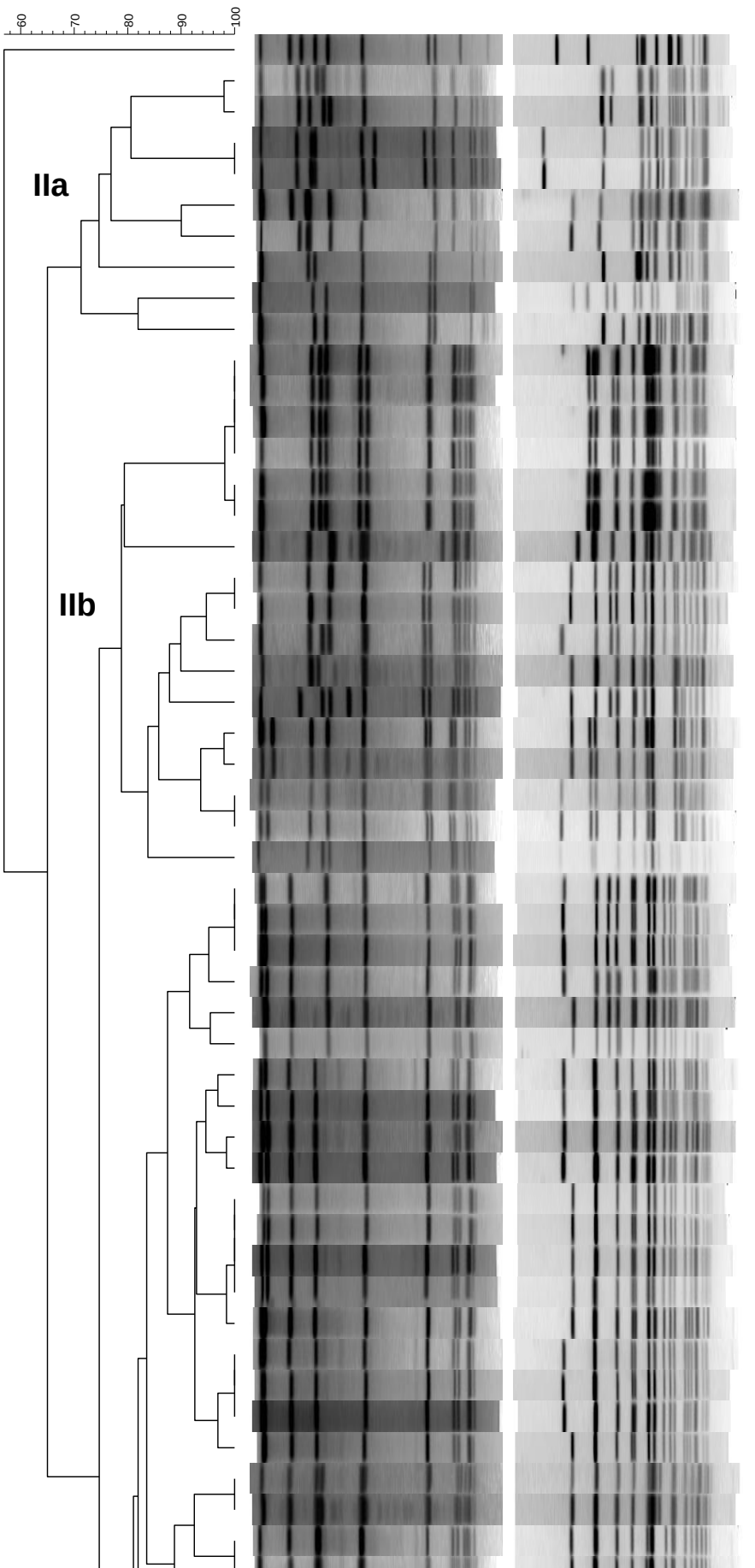
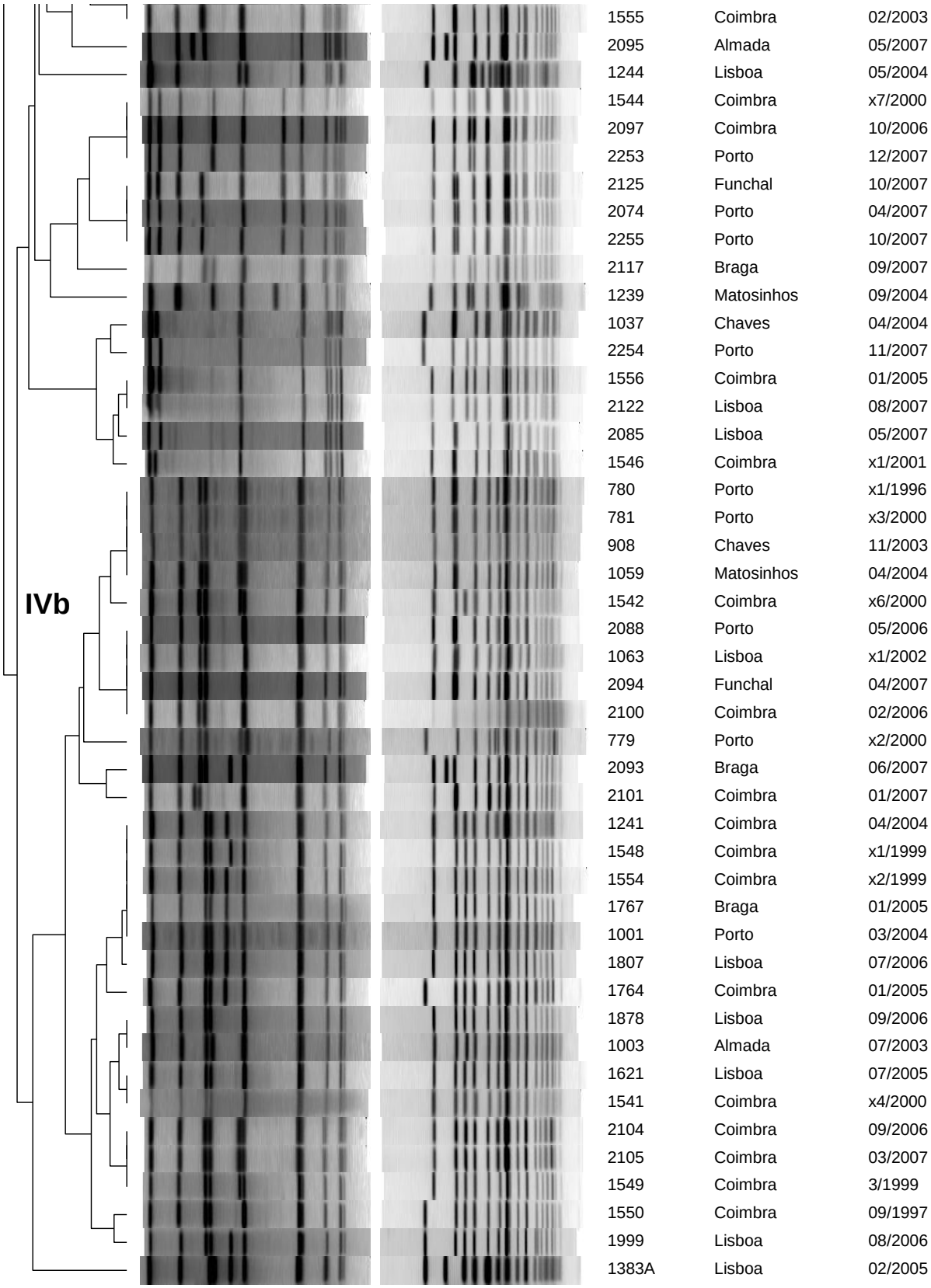
Figure 1. Dendrogram for *Listeria monocytogenes* pulsotypes of all 95 isolates obtained from clinical cases, Portugal, 1994-2007

PFGE-Apal+PFGE-AscI
apa_asc

Figure



Isolate code	Local	Date
2029	Lisboa	01/2007
2103	Lisboa	06/2007
1891	Braga	12/2006
2091	Porto	05/2006
2099	Coimbra	02/2006
1198	Matosinhos	07/2004
2135	Lisboa	11/2007
1880	Porto	09/2006
2086	Matosinhos	05/2007
1761	Coimbra	01/2005
1348	Almada	02/2005
2121	Braga	07/2007
1065	Lisboa	02/2004
1547	Coimbra	x2/2001
1240	Almada	10/2004
1242	Lisboa	08/2004
1002	Almada	x1/1998
1762	Coimbra	01/2005
1796	Lisboa	05/2006
1062	Lisboa	05/2004
866	Matosinhos	11/2003
2098	Coimbra	02/2006
1562	Lisboa	03/2005
999	Coimbra	x1/2000
2076	Lisboa	03/2007
1545	Coimbra	03/2003
2065	Matosinhos	03/2007
2102	Lisboa	06/2007
1792	Lisboa	03/2006
1879	Lisboa	09/2006
1543	Coimbra	x5/2000
856	Chaves	10/2003
1765	Coimbra	01/2005
1552	Coimbra	x3/1997
2087	Lisboa	04/2007
784	Porto	4/1999
2092	Braga	10/2007
1766	Braga	01/2005
1768	Matosinhos	01/2006
2090	Porto	04/2006
2252	Porto	05/2007
1763	Coimbra	01/2005
1553	Coimbra	x2/1998
2064	Matosinhos	12/2006
2096	Porto	05/2005
1803	Matosinhos	07/2006
771	Porto	04/2003
783	Porto	xx/1994
1551	Coimbra	x2/1997
1555	Coimbra	03/2003


IVb
1555 Coimbra 02/2003
2095 Almada 05/2007
1244 Lisboa 05/2004
1544 Coimbra x7/2000
2097 Coimbra 10/2006
2253 Porto 12/2007
2125 Funchal 10/2007
2074 Porto 04/2007
2255 Porto 10/2007
2117 Braga 09/2007
1239 Matosinhos 09/2004
1037 Chaves 04/2004
2254 Porto 11/2007
1556 Coimbra 01/2005
2122 Lisboa 08/2007
2085 Lisboa 05/2007
1546 Coimbra x1/2001
780 Porto x1/1996
781 Porto x3/2000
908 Chaves 11/2003
1059 Matosinhos 04/2004
1542 Coimbra x6/2000
2088 Porto 05/2006
1063 Lisboa x1/2002
2094 Funchal 04/2007
2100 Coimbra 02/2006
779 Porto x2/2000
2093 Braga 06/2007
2101 Coimbra 01/2007
1241 Coimbra 04/2004
1548 Coimbra x1/1999
1554 Coimbra x2/1999
1767 Braga 01/2005
1001 Porto 03/2004
1807 Lisboa 07/2006
1764 Coimbra 01/2005
1878 Lisboa 09/2006
1003 Almada 07/2003
1621 Lisboa 07/2005
1541 Coimbra x4/2000
2104 Coimbra 09/2006
2105 Coimbra 03/2007
1549 Coimbra 3/1999
1550 Coimbra 09/1997
1999 Lisboa 08/2006
1383A Lisboa 02/2005

Table

Table 1. *L. 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	IIa	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
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	IVb	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	IVb	R/S		EE

Strains not similar at more than 98% by PFGE typing with *AscI* 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 *ApaI*. Strains similar at more than 98% by *AscI* but not similar at more than 98% by *ApaI* were denoted, in addition to the capital letters, by lower case 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 *AscI* and *ApaI* were again listed as unique (U). Strains not distinguishable by their PCR grouping or strains similar at equal or more than 98% by PFGE typing with *AscI* and/or *ApaI* were again listed as indistinguishable (I)

a, M: maternoneonatal;NM, non maternoneonatal.

b, Un: Unknown

c, R: Resistant; S: sensitive