

# PFGE analysis of *Listeria monocytogenes* isolates of clinical, animal, food and environmental origin from Ireland

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*Listeria monocytogenes* is an important foodborne human pathogen. Human infection is associated with high mortality rates. Epidemiological investigation and molecular subtyping can be useful in linking human illness with specific sources of infection. This retrospective study describes the use of PFGE to examine relationships of 222 isolates from human and non-human sources in Ireland. Human clinical isolates from other countries were also examined. Eight small clusters of human and non-human isolates (mostly serotype 4b) that were indistinguishable from one another were detected, suggesting potential sources for human infection. For non-human isolates, some PFGE types appeared to be exclusively associated with a single source, whereas other PFGE-types appeared to be more widely disseminated. Indistinguishable, or highly related clusters of isolates of Irish and non-Irish origin suggest that some PFGE patterns may be globally distributed.

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## INTRODUCTION

*Listeria monocytogenes* is a Gram-positive foodborne pathogen and the causative agent of listeriosis in humans. Listeriosis is an uncommon condition; however, infection may result in severe illnesses, such as meningitis, meningoencephalitis and septicaemia (Vázquez-Boland *et al.*, 2001). Infants, the immunocompromised and the elderly are groups that typically suffer from the most severe illness. During pregnancy, infection is typically associated with flu-like symptoms in the mother but may spread to the fetus, leading to abortion or premature delivery of a congenitally infected infant (Vázquez-Boland *et al.*, 2001; Klatt *et al.*, 1986). The severity of this illness is reflected by some of the highest mortality rates observed among foodborne infections with crude mortality rates in the USA of 20% and 15.9% as reported by Mead *et al.* (1999) and Scallan *et al.* (2011), respectively. The estimated incidence of listeriosis in the USA was 0.3 cases per 100 000 in 2005 (Center for Infectious Disease Research & Policy, 2011), which is comparable to the reported EU incidence rate of 0.3 per 100 000 (Denny & McLauchlin, 2008). The mortality rate in the EU was estimated to be 16.6% among reported infection cases (~270 deaths), similar to that in the USA (European Food Safety Authority, European Centre for Disease Prevention & Control, 2011).

Listeriosis has been a notifiable disease in Ireland since 2004. Between 2004 and 2009, the number of listeriosis cases in Ireland ranged from 7 to 21 per annum, which equates to a mean annual incidence of 0.3 cases per 100 000. The majority of the 74 cases reported occurred in patients belonging to one of the known risk groups for severe listeriosis: pregnant women ( $n=13$ , 18%), neonates ( $n=6$ , 8%), those over 65 years of age ( $n=32$ , 43%) or younger adults with underlying illnesses that might predispose them to listeriosis ( $n=13$ , 18%). Incomplete risk-group information was available for the remaining 10 cases; however, it is known that they were not pregnancy-associated. During this 6-year period, there was one adult death and two neonatal deaths that were directly attributed to listeriosis, resulting in a case fatality rate of 4%. Furthermore, there were four late miscarriages during this time period due to listeriosis among pregnant women (HSPC, 2010, 2009; Garvey *et al.*, 2008).

The highest annual number of cases of listeriosis in Ireland was in 2007, with a reported increase primarily among pregnant women and neonates (referred to collectively as pregnancy-associated cases), which represented a very significant increase in the proportion of cases that were pregnancy-associated compared with other years. It was notable that the majority of these pregnancy-associated cases were in patients who were non-Irish-born.

The incidence of listeriosis in the EU rose significantly in 2000–2006, emphasizing the need for improved surveillance of *L. monocytogenes* infection and listeriosis (Denny & McLauchlin, 2008). In order to facilitate this, PulseNet Europe was established to perform subtyping on *L. monocytogenes* isolates from across Europe, based on the success of PulseNet USA (Martin *et al.*, 2006). The practicality of PulseNet Europe was assessed by Martin *et al.* (2006); however, at present there is no operational system for routine molecular surveillance of cases of listeriosis in Europe, although some member states, such as France, operate comprehensive surveillance (Goulet *et al.*, 2006).

This study describes the analysis of an *L. monocytogenes* database of isolates from Ireland, which was established to record data from isolates of human, animal, food and environmental origin from Ireland.

## METHODS

**Isolates in this study.** Isolates in the database were gathered from a number of research groups around Ireland and from the National *Salmonella*, *Shigella* and *Listeria* Reference Laboratory (NSSLRL) for human isolates. This study included 222 isolates, each from individual sources, including 14 animal isolates from bovine or poultry faeces; 92 food isolates from meat, vegetables, cheese, or mixed food products (e.g. sandwiches); 73 environmental isolates from food production facilities or farm environments and 43 human isolates. The 43 human isolates of *L. monocytogenes* described in the database were derived from 43 separate cases and represent 58% of all reported listeriosis cases between 2004 and 2009. Eight of the 43 isolates were from pregnant women, four were from neonates and the remaining 31 isolates were from non-pregnancy-associated adult cases, representing 62, 67 and 56% of total Irish cases, respectively, over the period of 2004–2009.

**Species-level identification and serotyping.** Presumptive-positive *L. monocytogenes* isolates were confirmed using biochemical testing as described in the international standard isolation protocols used (McClain & Lee, 1988; United States Department of Agriculture, 2008), or by real-time PCR as described by Fox *et al.* (2009). Serotyping was performed using a combination of commercially available antisera (Denka Seiken), multiplex serotyping PCR (Doumith *et al.*, 2004) and/or ELISA (Palumbo *et al.*, 2003).

**PFGE typing.** PFGE typing was carried out using the most up-to-date International Standard PulseNet protocol in use at the time (PulseNet USA, 2009). Two restriction enzymes, *Apal* and *Ascl*, were used for the analysis. Isolate similarity dendrograms were generated using BioNumerics version 5.10 software (Applied Maths), using the unweighted pair group method with arithmetic mean (UPGMA) and the Dice coefficient with tolerance and optimization settings of 1%.

## RESULTS AND DISCUSSION

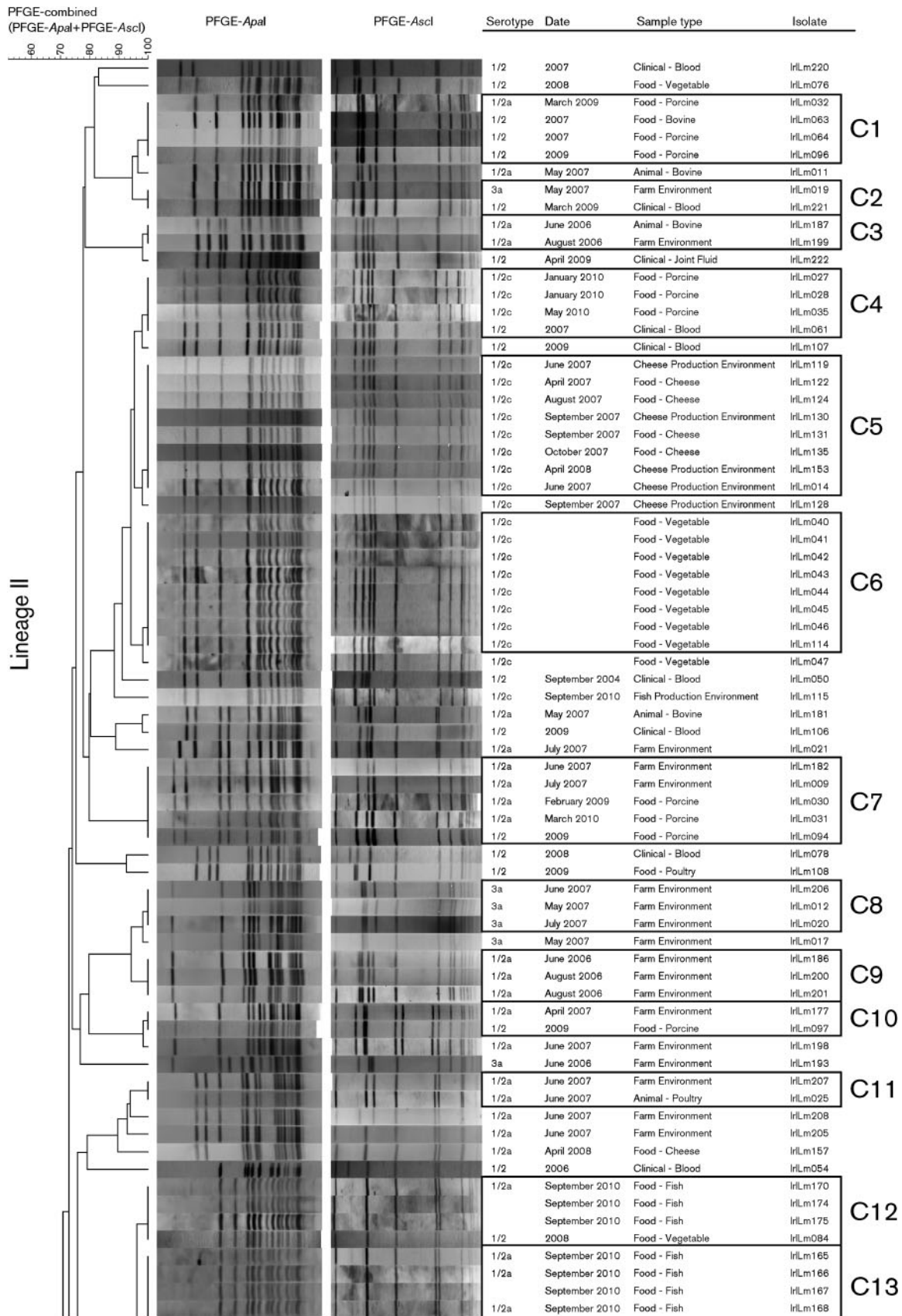
Of the 222 isolates there were 139 of serotype 1/2 (15 human, 70 food, 46 environmental, 8 animal), seven of serotype 3 (6 environmental, 1 animal) and 60 of serotype 4 (28 human, 9 food, 17 environmental, 5 animal). The serotype of 13 isolates was not known and three isolates were untypable. The serotypes most prevalent among the different groups of isolates were: 1/2, for food isolates; 1/2a, for

production and farm environment isolates; and 4b, for human clinical isolates. These results are in agreement with previous observations, which showed a higher prevalence of serovar 1/2 strains among food isolates and 4b as the predominant serovar among human clinical isolates (Boerlin & Piffaretti, 1991; Farber & Peterkin, 1991; Schönberg *et al.*, 1989). The animal isolates were predominantly serovar 1/2a or 4b (1/2a for poultry, 4b for bovine isolates). As in other reports, only two of 13 possible serotypes occurred with any frequency, therefore, serotyping has little practical value in linking cases with sources (Schuchat *et al.*, 1991); however, serotyping may be valuable in prioritization of isolates for molecular typing.

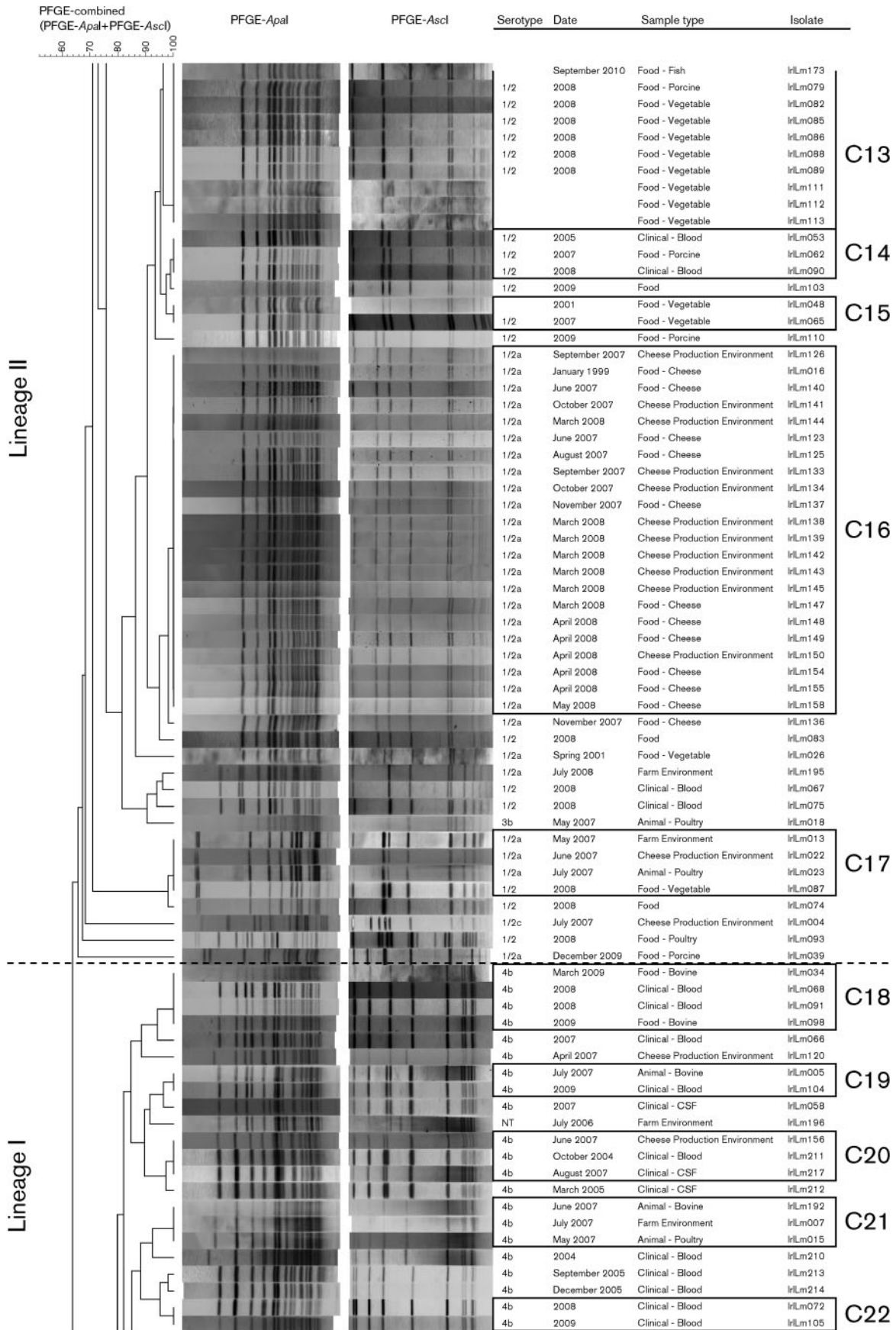
PFGE with *Ascl* alone resulted in 72 different PFGE types, whereas 94 patterns were identified using *Apal*. Combining patterns from both enzymes resulted in 107 distinguishable types. This is consistent with previous reports indicating that two-enzyme PFGE provides a high level of discrimination when applied to *L. monocytogenes* (Fugett *et al.*, 2007).

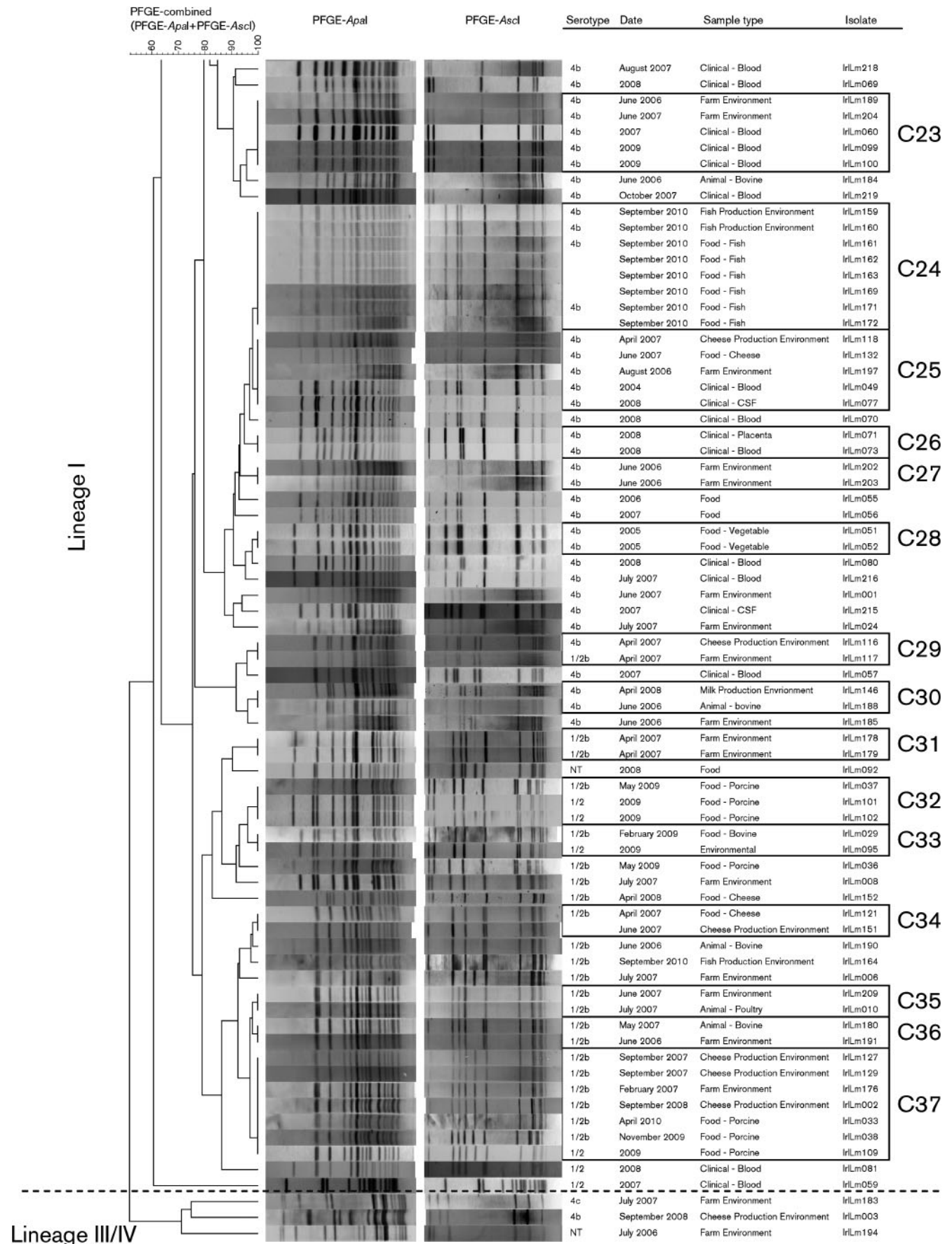
Thirty-seven clusters of isolates (2–22 isolates per cluster) were identified that were indistinguishable or very closely related (>95%) when analysed with both enzymes (Fig. 1, clusters C1–C37). Ten of the clusters included both human and non-human isolates (three of serotype 1/2 and seven of serotype 4b). Indistinguishable PFGE types among isolates of human and non-human origin have been identified in previous studies (Fugett *et al.*, 2007; Gianfranceschi *et al.*, 2009). Although taken in isolation, data such as these cannot determine a source of infection; however, these data are valuable, in combination with epidemiological evidence, in pointing out likely sources of infection and may help identify the source of an outbreak (Fugett *et al.*, 2007). Cluster 24, for example, contained isolates only detected in fish or fish-related materials/environments. If the association between a specific source and genetic cluster is substantiated over time then the detection of such an isolate from a human clinical case may point to a likely suspect food category, which could be taken in to consideration when examining a patient's food history. On the other hand, it appears that there are clusters of isolates that can derive from a variety of food items. For example, cluster 13 includes isolates from fish, pork and vegetables. Likewise, cluster 37 encompasses isolates from different foods. Where a PFGE type is widely distributed among isolates of varying origins, the detection of an isolate in a human case that is indistinguishable from one of these types is of limited value in linking the infection to a specific source.

Only 14 (33%) of the human isolates (4 of serotype 1/2 and 10 of serotype 4b) in this study had PFGE types that were indistinguishable from those of counterparts found in non-human samples. These constituted eight different PFGE types. Only one of these PFGE types was found in multiple sample types, namely, a farm environment, a cheese production environment and a cheese sample. Of the other seven PFGE types, two were derived from isolates from pork meat products, two from farm environment



**Fig. 1.** Dendrogram of PFGE profiles from isolates in this study. The 37 clusters, each containing indistinguishable PFGE profiles from different isolates, are marked C1–C37. NT, not typable. Data relating to individual isolates were included where available.



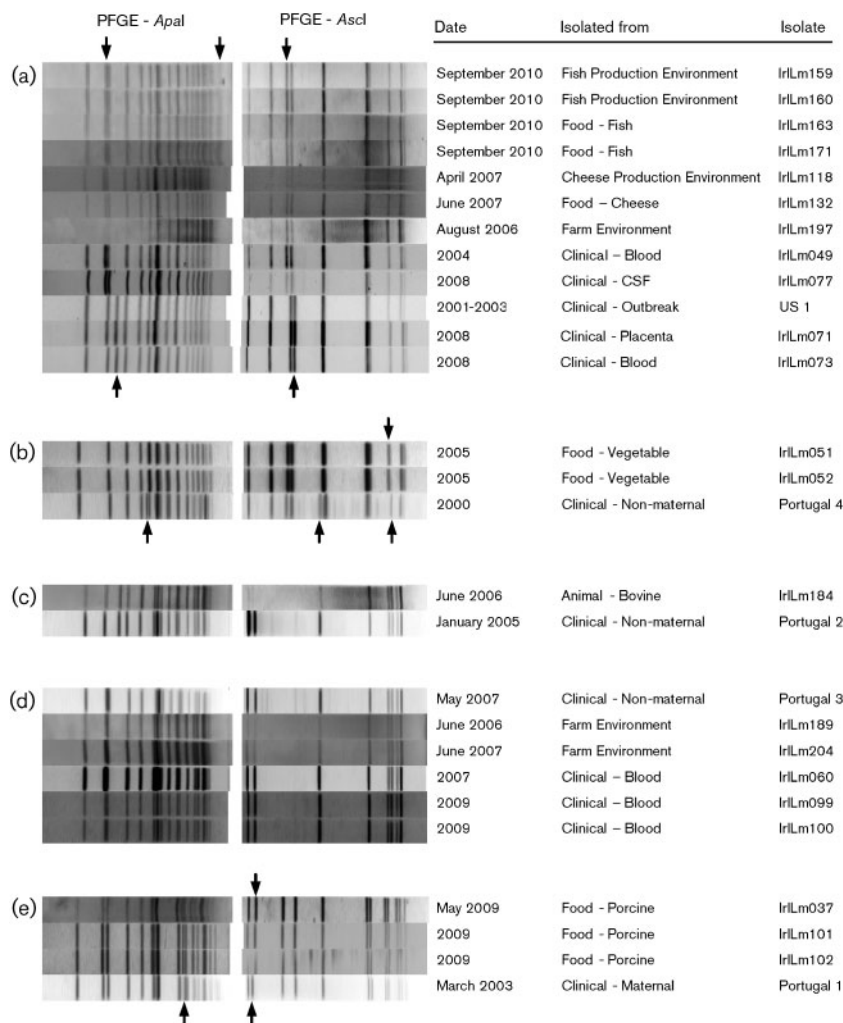


samples, one from beef products, one from a bovine animal sample and one from a cheese production environment. It is not surprising that many human isolates were not matched to isolates entered in to the food and animal databases as the sampling of food and animals for *L. monocytogenes* in Ireland is limited and some cases of infection may be associated with imported food or travel outside Ireland. Serotype 4b constituted a minority (30%) of all isolates typed but yielded a higher density of clusters of related human cases and links to non-human isolates.

Fig. 1 indicates the different genetic lineages of the *L. monocytogenes* isolates used in this study. The isolates are divided into lineage I, lineage II and a third section designated lineage III/IV. As no further analysis was performed on lineage III/IV isolates, it was not possible to identify which of these lineages the three strains in question belonged to. There was only a single discrepancy in this study, regarding isolate IrLm018, with respect to assigning it to a lineage based on PFGE analysis. This isolate clustered with the lineage II strains, although serotype 3b isolates should cluster with lineage I isolates.

Isolates IrLm116 and IrLm117 were serotyped as 4b and 1/2b, respectively, although they had indistinguishable PFGE profiles and had the same genetic lineage. This was also seen with isolates IrLm019 and IrLm221 (serotypes 3a and 1/2, respectively). Other studies have found similar indistinguishable PFGE types among isolates with different serotypes and the same genetic lineage (Gianfranceschi *et al.*, 2009; Chen *et al.*, 2010). The occasional chance finding of indistinguishable PFGE patterns in isolates that are readily distinguishable by other means is not surprising. The banding patterns reflect the number and distribution of specific restriction sites in the chromosome, therefore, PFGE patterns of distantly related isolates may converge and phylogenetically closely related strains may have distinct phenotypes related to altered gene expression or horizontal DNA transfer. Lineage I contained the highest number of human clinical isolates (65%) and lineage II contained the remaining proportion (35%). No human clinical isolates clustered with lineage III/IV.

Using this method of analysis, two serotype 4b human clinical isolates of Irish origin were indistinguishable from



**Fig. 2.** Isolates with PFGE profiles indistinguishable from, or closely related to, clinical isolates from other countries. (a) Includes the serotype 4b outbreak strain responsible for the 1985 Los Angeles and 1983–1987 Switzerland outbreaks, two serotype 4b clinical isolates from Ireland indistinguishable from that strain and other highly related isolates of Irish origin. (b) Highly related clinical isolates from Portugal and vegetable isolates from Ireland. (c) Indistinguishable clinical isolates from Portugal and animal isolate from Ireland. (d) Indistinguishable clinical isolates from Portugal and clinical and environmental isolates from Ireland. (e) Highly related clinical isolate from Portugal and porcine meat food isolates from Ireland. Arrows indicate band differences.

the serotype 4b outbreak strain responsible for outbreaks in California, USA [Centers for Disease Control (CDC), 1985] and Switzerland (Büla *et al.*, 1995). Although no food source was implicated for the human isolates from Ireland, both the USA and Swiss outbreaks were linked to soft cheese. A related pattern was observed in a larger variety of samples, including food, food production and farm samples, as well as human clinical cases. A PFGE profile indistinguishable to this was also present among isolates from human, food and environmental origin as reported by Fugett *et al.* (2007), and one of these isolates (US 1) is included in Fig. 2 (FSL E1-128 in Fugett *et al.*, 2007). This finding may be consistent with the suggestion that there are pandemic clonal groups of *L. monocytogenes* (Fugett *et al.*, 2007). However, as outlined above, similarity of PFGE patterns is not always a reliable indicator of close phylogenetic relationships and validation of this hypothesis with MLST is required. Likewise, some human isolates from Portugal were also indistinguishable from, or were highly related to, PFGE profiles of isolates of Irish origin (Fig. 2b–e).

This study indicates that PFGE analysis using *ApaI* as the restriction enzyme provides useful discrimination between isolates of *L. monocytogenes* in Ireland and that the additional use of *AscI* further enhances discrimination. Routine use of both enzymes is specified in the PulseNet protocol; however, this adds significantly to the costs of molecular typing.

The data show that certain PFGE types are relatively widely disseminated within Ireland and outside of Ireland; therefore, detection of isolates in patients and particular suspect food items with similar, widely disseminated, PFGE patterns is not conclusive evidence of a link between the two, although PFGE may help to support or refute epidemiological evidence. On the other hand, we noted that some PFGE patterns were detected only in association with fish products and detection of such a PFGE pattern in a patient may be a useful clue in determining a likely source of infection. As this study was retrospective, there was no prospect of linking the molecular typing with real-time epidemiological information and food history. Real-time molecular typing linked to epidemiological investigation has been in place in France and the USA for some time. Our data suggest the potential value of a similar system in Ireland. The value of this approach for protection of public health may be greatly enhanced by a single European database for food, animal and human clinical isolates linked to corresponding systems in other regions including the USA.

Given that serotype 4b is the predominant human pathogenic type of *L. monocytogenes* in many countries and that few countries have resources to allow PFGE typing of all non-outbreak-related *L. monocytogenes* isolates from food, we suggest that it may be reasonable to give priority to PFGE analysis of serotype 4b isolates from non-human sources, as the information yielded may be of greater value to public health. However, this suggested pragmatic approach lacks complete coverage and the ideal scenario would involve a more comprehensive approach that would

combine alternative typing methods for non-4b isolates that are less expensive but yield acceptable discriminatory power. Examples of such alternative methods include RAPD analysis (Torrence & Isaacson, 2003) or *actA* typing (Zhou *et al.*, 2005; Bania *et al.*, 2009), both of which are less expensive than PFGE but still provide an acceptable level of differentiation.

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